ATTENTION BIAS: A NEW TOOL FOR WELFARE ASSESSMENT IN CAPTIVE RHESUS MACAQUES

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Contents

Abbreviations

5-HTTLPR - 5-hydroxytryptamine (serotonin) transporter length polymorphic region

AB – Attention bias

ABDiff – Attention bias difference. Calculated by subtracting the duration of time spent looking at the neutral stimulus from the duration of time spent looking at the threat stimulus.

AVPR1a - Arginine vasopressin receptor 1A gene

CYP17 - C*ytochrome P450 family 17* gene

DRD4 - Dopamine receptor D4

EIA – Enzyme immunoassay

HTR2A - 5-hydroxytryptamine receptor 2A gene

KHCl – Ketamine hydrochloride

MAOA - Monoamine oxidase A gene

MRC-CFM - Medical Research Council Harwell Institute Centre for Macaques

NACWO – Named animal care and welfare officer

Neut – Neutral face stimulus

NHP – Non-human primate

NVS – Named veterinary surgeon

OPRM1 - Opioid receptor mu(μ) 1 gene

OXT - O*xytocin/neurophysin I prepropeptide* gene

OXTR - Oxytocin receptor gene

PCR – Polymerase chain reaction

PRT – Positive reinforcement training

SERPINA6 - Serpin Family A Member 6 gene

SNP – Single nucleotide polymorphism

STin - Serotonin transporter intron 2

THR – Threat face stimulus

TL – Total duration of looking at the threat and neutral face stimuli

TPH2 - Tryptophan 5-hydroxylase 2 gene

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Abstract

Attention bias (AB) describes a tendency to differentially allocate attention towards one of two or more emotional stimuli. In humans these biases reliably map onto physiological and self-reported measures of affect. AB tasks have been shown to detect shifts in emotional state and have been proposed as a novel method of animal welfare assessment. This PhD aimed to determine which factors might influence AB in rhesus macaques (*Macaca mulatta*) through triangulation of these cognitive data with behavioural observations, physiological measures (salivary cortisol) and key genetic polymorphisms related to oxytocin, serotonin, dopamine, and cortisol. Key factors of interest were condition (baseline and post-stressor) sex, age, and time of day. AB trials were conducted with 61 (45 female, 16 male) adult rhesus macaques (*Macaca mulatta*) using an automated computer operated apparatus with threat-neutral conspecific face stimuli presented on screens. Duration of looking at these stimuli was recorded. Two looking time measures were used throughout the thesis: duration looking at the threat face stimulus (THR), total duration looking at the threat and neutral face stimuli (TL). AB trials were conducted before and after the macaques' annual veterinary health check, which is thought to be acutely stressful. A total of 640 AB trials were conducted. The main findings were the relationship between AB measures and the interaction between condition and sex. Female macaques became less attentive to social information from baseline to post-stressor, while male macaques became more attentive. Further, an association between AB measures and time of day was revealed. This thesis demonstrated that the inclusion of pedigree (relatedness) data is vital when conducting genetic analysis to avoid type I errors. Without pedigree data, six genotypes had a significant association with the AB measures; however, with pedigree data only one statistically significant association was found. The cognitive, behavioural, and physiological results suggested that the veterinary health check may be too mild a stressor for use in future AB studies. The use of a more stressful event or procedure may be more informative while the AB measure is studied and developed. This project has shown AB to be a promising tool for welfare assessment, highlights some important influencing variables and should act as a guide for further research.

Declaration

No portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification at Liverpool John Moores University or any other university or institute of learning.

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Chapter 1 – General introduction

1.1 Introduction

In the UK, the use of animals in scientific research is highly regulated (Home Office, 2019). Their continued use remains one of the most controversial issues in biomedical research for many people and the focus of many ethical debates (Festing & Wilkinson, 2007; DeGrazia & Sebo, 2015; Siani, 2019). The use of non-human primates (NHPs; the term NHP is used in experimental settings to distinguish them from humans) is particularly controversial due to their evolutionary proximity to humans, high sentience and cognitive sophistication resulting in similarities in their behavioural and physiological needs and ability to experience pain, distress, and anxiety (Sughrue et al, 2009; APC, 2013; Schönfelder, 2015; Friedman et al, 2017; Walker, 2018). As a result, no great apes have been used in the European Union (EU) since 1999 and there has been a 25% decrease in the use of all NHP species (cynomolgus macaques (*Macaca fascicularis*), rhesus macaques (*Macaca mulatta*), tamarins and marmosets) since 2009 (Department of Health, 2014; Home Office, 2019; Figure 1.1). Despite this decrease, the number of procedures involving NHPs remains significant and NHPs have been identified as a special priority for welfare (NC3Rs, 2015).

In the EU, biomedical procedures involving NHPs are only permitted in specific circumstances in areas of research that are deemed to be essential for the benefit of humans, such as toxicology (Council Directive 2010/63/EU). In 2018, 3,170 procedures involving NHPs were carried out in the UK (Home Office, 2019). Of these, 2,612 were regulatory procedures that included toxicology testing for pharmaceuticals (Home Office, 2019).

All marmosets, tamarins and rhesus macaques used in these biomedical procedures were born in the UK (Home Office, 2019). The rhesus macaques were bred at one of three breeding centres located at Porton Down, Salisbury, Wiltshire, England (MRC, 2019). The largest of the breeding colonies is the Medical Research Council Harwell Institute Centre for Macaques (MRC-CFM), which supplies approximately 30 macaques per year to UK academic institutions for use in biomedical research.

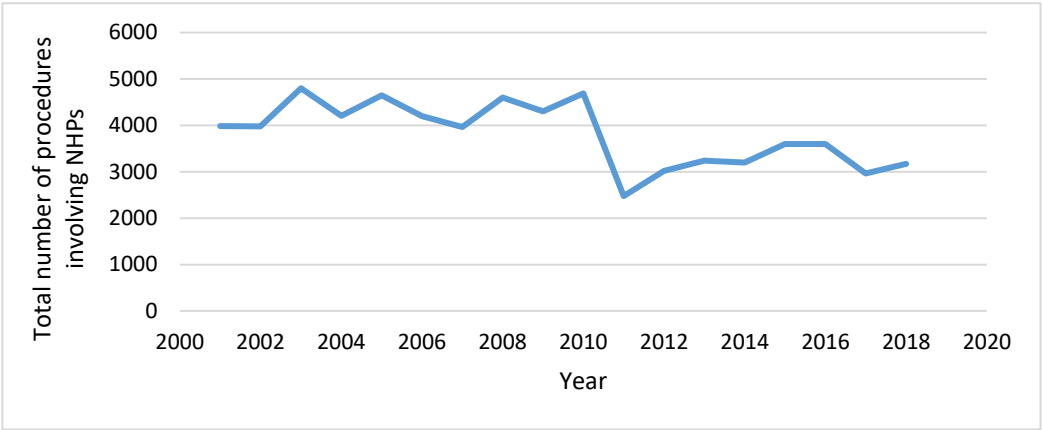

Figure 1.1. Total number of regulated procedures carried out on non-human primates (NHPs) between 2001 and 2018 in the UK (Data from Home Office reports 2002-2019).

NHPs in research laboratories and breeding colonies have different welfare challenges (Hau & Schapiro, 2004; Maple & Perdue, 2013). Breeding colony NHPs are not affected by regulated procedures, which are the primary cause of pain and suffering for laboratory animals (Weatherall, 2006; Carbone, 2011). However, handling and catching practices, socialisation, transport, management, husbandry, and routine veterinary procedures, such as health checks, can compromise welfare and can affect all captive NHPs equally (Hawkins et al, 2001; Reinhardt, 2005; Maple, 2007; Olsson & Westlund, 2007; Elliot et al, 2018). These welfare challenges may also be faced by the large number of NHPs housed in zoos. Over half of the 120 zoological collections accredited by the British and Irish Association of Zoos and Aquariums have at least one primate species (BIAZA, 2019). Zoo NHP welfare may be impacted by the above factors; however, these animals must also contend with zoo visitors, which have been shown to have a negative effect on NHP welfare (Sherwen & Hemsworth, 2019). As a result of these welfare challenges, methods of assessing welfare that are sensitive to the shared as well as diverse challenges of captivity are needed.

Research facilities, breeding centres and zoos are required to minimise animal suffering and promote good welfare (Zoo Licencing Act, 1981; Animals (Scientific Procedures) Act, 1986). In addition to these government policies, there are societal concerns surrounding the ethics of keeping captive wild animals in zoos (Kagan et al, 2018) and public support for the use of animals

in biomedical research is dependent on ensuring that suffering is minimised (Ipsos MORI, 2016). In a UK survey of 987 members of the public, 71% of respondents agreed that they could "accept the use of animals in scientific research as long as there is no unnecessary suffering to the animals and there is no alternative" (Ipsos MORI, 2016).

These ethical and legal concerns may be addressed by the Five Freedoms (FAWC, 1979; Mäekivi, 2018). In brief, the Five Freedoms are a scientific evidenced based framework that was initially developed for farm animals but is now applied to all captive animal management (Webster, 1994; Mäekivi, 2018). The Five Freedoms are:

1. Freedom from hunger and thirst: by ready access to fresh water and a diet to maintain full health and vigour.
2. Freedom from discomfort: by providing an appropriate environment including shelter and a comfortable resting area.
3. Freedom from pain, injury, or disease: by prevention or rapid diagnosis and treatment.
4. Freedom to express normal behaviour: by providing sufficient space, proper facilities, and company of the animal's own kind.
5. Freedom from fear and distress: by ensuring conditions and treatment which avoid mental suffering.

To meet these standards, we must consider an animal's environment, behaviour, physiology, health, and psychological well-being. NHPs bred and housed in captivity need to be free from fear and distress and experience an environment that allows them to display the full range of primate behaviour (NC3Rs, 2018).

At present, minimising suffering for captive NHPs is challenging; welfare assessment is notoriously difficult as there is no single measure of well-being (Wolfensohn & Honess, 2008). On their own, traditional welfare assessment methods such as behavioural, physiological, and physical health indicators, can be misleading and interpretation can be challenging. In this chapter, I set the scene for the thesis and discuss the importance of psychological health in captive NHPs, the more

traditional welfare assessment methods, and the benefit of triangulation of cognitive measures with behaviour, physiology, health, and genetics.

1.2 Psychological health & well-being

Historically, in Western cultures animals were considered unfeeling machines (Harrison, 1964/2013). Descartes (1596-1650) stated "animals are like robots: they cannot reason or feel pain". Aristotle (384 BC - 322 BC) developed the philosophy of hierarchy with humans at the top and all other species below humans and for their benefit (McInerny & O'Callaghan, 2018). This philosophy was adopted by the teachings of St Thomas Aquinas (1225-1274) and Roman Catholic theology, which contributed to religious and geographical diversity for historic concern for animal welfare (McInerny & O'Callaghan, 2018). The development of moral philosophy (utilitarianism and rights) in the 18th century resulted in an emphasis on animal sentiency and led to the first legislation (Bentham, 1780/1982). This change in attitude was encapsulated by Bentham (1748-1832): "the question is not, can they reason? Nor, can they talk? But can they suffer?"

It is now generally accepted that all vertebrate and many invertebrate animals can feel pain, and, although it is not fully understood, there are similarities in pain behaviour and the mechanisms of pain detection and processing between humans and mammals (Smith, 1991; Allen, 2011; Marks, 2012). There is evidence of nociceptors (sensory receptors for noxious stimuli) in many invertebrate species including leeches (*Hirudo medicinalis*; Nicholls & Baylot, 1968), earthworms (Alumets et al, 1979), marine molluscs (*Mytilus edulis*; Kavaliers et al, 1985) and mantis shrimps (*Squilla mantis*; Maldonado & Miralto, 1982). Rollin (2011) suggested that the emotional component of pain may be worse for animals than humans as they are unable to rationalize the pain or understand that it is likely to subside.

Animal welfare science (AWS) has been a "formal discipline" for less than 60 years and is considered a "young science" by many (Millman et al, 2004; BMJ, 2007; Carenzi & Verga, 2009). AWS focuses on improving all aspects of captive animal management from conception to slaughter including transport, husbandry and housing (Mench, 2018) and considers how well an animal is adapted for

its environment through evaluation of measurable parameters (physiological, behavioural, health, cognitive; Brown, 2013). AWS arose as a response to issues of anthropomorphism in the wider study of animal science (Karlsson, 2012). Early welfare assessment focused primarily on the physical health and biological functioning of the animal rather than the emotional component of animal response to stimuli (Mellor, 2012; Proctor et al, 2013) as animal emotions were considered subjective and not reliably measurable (Rose, 2002; Dawkins, 2012; Rose et al, 2012). This resulted in a lack of research focusing on collecting strong scientific evidence of animal emotional experiences (Boissy et al, 2007; Grandin, 2018).

Indeed, there are still those who have suggested that animals may only be acting "as if" they experience the emotion we would attribute to the observed behavioural response, are not truly conscious and that fear responses are automatic "survival circuits" (LeDoux, 2014; LeDoux & Brown, 2017). Further, Dawkins (2017) stated that objective animal welfare science should not be based on assumptions of consciousness and emotional state. She argued that a conscious-free definition would make animal welfare science more accessible, understandable, and irrefutable by people with very different opinions on animals. By contrast, others have argued that an animals' conscious or emotional response is the only thing that matters for welfare (Duncan, 1996, 2004, 2006). The modern consensus is that positive emotions and psychological well-being are key for positive animal welfare (Lawrence et al, 2019).

Psychological and physiological stress contribute to emotional and psychological well-being. Here, stress is defined as "the nonspecific response of the body to any demand, whether it is caused by, or results in, pleasant or unpleasant conditions" (Selye, 1956). Psychological and physiological stress are mediated by different regions of the brain and nervous system (Kogler et al, 2016). Physiological stress results in cessation of non-essential organ functioning while psychological stress results in the shift of attention to the cognitive control of emotion (Kogler et al, 2016). These differences mean that the use of behavioural, physiological or health indicators of welfare alone are insufficient for appropriate and thorough welfare assessment (Wolfensohn & Honess, 2008).

1.3 Current welfare assessment methods

Behaviour

Behavioural analysis is a critical component of animal welfare assessment (Wolfensohn et al, 2018). A change in behaviour from before to after a treatment or event (e.g., transportation, presence of tourists, neuroscience procedures) is often used to determine the impact of a potential stressor on animal welfare (Honess et al, 2003; Maréchal et al, 2016; Descovich et al, 2019). Descovich et al (2019) used 20 minutes of continuous behavioural observation under four conditions (pre-operative, post-operative, pre-analgesia, and post-analgesia) to determine potential behavioural indicators of pain and wellness in rhesus macaques. Key behavioural indicators of wellness included running and arboreal behaviours while lip tightening, leaning the head and body shaking indicated compromised welfare.

There are several established behavioural response paradigms that have been used in NHPs, such as the human intruder test (Raper et al, 2018), novel-object and novel-food tests (Šlipogor et al, 2016; Arnaud et al, 2017), open field test (Larke et al, 2017) and novel predator confrontation models (Barros et al, 2000). In the human intruder test, Raper et al (2018) compared the behavioural response of juvenile rhesus macaques with or without sevoflurane anaesthesia exposure during infancy. Following the mild acute social stressor, which included separation from their social group and nine minutes of each of the following: isolation in an unfamiliar room, the masked human sat in profile and the masked human sat making direct eye contact, macaques that had previously received the anaesthesia had larger increase in the occurrence of self-directed displacement behaviours compared to the control macaques.

However, differences in behaviour can result from personality (Hewson, 2003; Mills, 2010; Konečná et al, 2012), age, sex, and life history (Wolfensohn et al, 2018; Descovich et al, 2019). Behavioural assessment must be completed by an observer familiar with the species and the individual, as individual knowledge will allow subtle differences and difference from normal to be detected (Wolfensohn et al, 2018). This is particularly important in NHPs as many of the behavioural signs of

stress, anxiety and pain are subtle and could easily be missed by an unfamiliar observer (National Research Council, 2009). Behavioural data collection can be time consuming and noisy (Robinson et al, 2017). The development of automated approaches such as accelerometers (Hammond et al, 2016) and data-loggers (Bonk et al, 2013) can provide a quick and simple assessment of, for example, locomotor or resting behaviour; however, accelerometers are unable to assess context-dependent behaviour (Shuert et al, 2018).

The behavioural indicators of good welfare have not received the same attention as the indicators of poor welfare (Lawrence et al, 2018). Yet, over the past 15 years interest in this area has gained momentum (Bracke & Hopster, 2006; Yeates & Main, 2008; Mellor, 2016). Behavioural indicators of positive welfare in NHPs include resting in contact with conspecifics, foraging and grooming (NC3Rs, 2015); however, depending on the context, grooming can also be a displacement activity and a sign of anxiety (Coleman & Pierre, 2014).

Stereotypical behaviours include locomotor behaviours, for example, pacing, bouncing, somersaulting, and rocking, and self-directed behaviours, for example, hair pulling, eye poking and digit sucking (Coleman & Maier, 2010; Pomerantz et al, 2013). These behaviours are often considered an indicator of chronic stress or frustration (Mason & Latham, 2004; Pomerantz et al, 2012a). Stereotypical behaviours may develop in response to early life stress (Lutz et al, 2003, Novak, 2003, Novak et al, 2006, Latham & Mason, 2008). However, recent work with rhesus macaques has shown certain stereotypical behaviours, such as pacing, to be unreliable indicators of stress (Poirier et al, 2019). Following agonistic interaction with conspecifics, the occurrence of stress-related displacement behaviours and agitated locomotion increased but there was no increase in pacing. Poirier et al (2019) suggested that pacing may increase in some stressful situations but not others or that the agitated locomotion had previously been mistaken as pacing. Stereotypies may not be a direct stress response but rather coping behaviour (Pomerantz et al, 2012a) or behaviour that has been dissociated from the underlying emotion (Pomerantz et al, 2012b).

Physiology

Physiology is the study of the internal functioning of the body (Newman, 2017). Physiological indicators of wellbeing and stress can indicate a disruption to the body's homeostatic mechanisms (Modell et al, 2015). Physiological changes in response to stress are controlled by the nervous and endocrine systems. The autonomic (sympathetic and parasympathetic) nervous system and the hypothalamic–pituitary–adrenal (HPA) endocrine axis mediate many of the physiological responses to stress. HPA axis activity is commonly assessed using measures of glucocorticoid (e.g., cortisol) production, while autonomic nervous system activity is determined using either direct measurements of catecholamines (adrenaline, noradrenaline, and acetylcholine) or autonomic changes that occur as a result of changing catecholamine levels (Sneddon et al, 2014). These autonomic changes include body temperature, heart rate, respiratory rate, body weight and blood pressure. For species that show few behavioural responses to pain, such as NHPs, these measures may be useful. However, methods for collecting data on changes in glucocorticoid and catecholamine production can in themselves be stressful.

Cortisol is a validated and widely used measure of physiological arousal (Heintz et al, 2011), which includes both distress and eustress (Selye, 1956). Levels may change response to exercise (Ahmadi et al, 2018), sexual arousal (Hamilton et al, 2008) and because of circadian and ultradian rhythm (Lefcourt et al, 1993; Trifonova et al, 2013). The cause of stress may affect the duration for which cortisol is elevated, for example, plasma cortisol rapidly returns to normal in cows experiencing heat stress despite the maintenance of both rectal temperature and plasma prolactin (Moneva et al, 2011).

Physiological changes can occur due pregnancy (Soma-Pillay et al, 2016), postpartum (Freitas-de-Melo et al, 2017) and aging (Boss & Seegmiller, 1981). The physiological response to increased activity, stress and changes in emotion can be remarkably similar. Ventilation rate, body temperature, skin conductance and heart rate can all increase following activity (Burton et al, 2004)

or a stressor (Skarda & Muir, 2003; Terkelsen, 2005) or change in response to emotions such as surprise, sadness, happiness, anger, disgust, and fear (Purves et al, 2001).

Health

NHPs show few or subtle indicators of compromised welfare (National Research Council, 2009); however, some key health indictors of rhesus macaque welfare exist (Tasker, 2012). Body condition scores (BCS) can be used as a visual aid to evaluate the level of appropriate nutrition (Clingerman & Summers, 2005; Wolfensohn & Honess, 2008). Two validated scoring systems exist for rhesus macaques (Clingerman & Summers, 2005; Wolfensohn & Honess, 2008). These systems can be used to assess sudden weight change (Summers et al, 2012), poor juvenile growth (van Wagenen & Catchpole, 1956; Turnquist & Kessler, 1989; Schapiro & Kessell, 1993) and the impact of fluid and food control protocols (Prescott et al, 2010). BCS must consider the animals' reproductive status and age as these factors can change body morphology, for example, juvenile macaques tend to be leaner than adult macaques so may be scored as underweight if the same classifications are used for both (Clingerman & Summers, 2005). It is recommended that BCS be used in combination with a measure of actual body weight (NC3Rs, 2014a).

A macaques' pelage (hair) can also be used as an indicator of health issues (Novak & Meyer, 2009). Pelage loss (alopecia) is rare in free-ranging NHPs (Honess et al, 2005). In captive macaques, alopecia is frequently caused by nutritional or hormonal imbalances (Novak & Meyer, 2009). Zinc deficiency has been suggested as a cause of nutritional alopecia in talapoin monkeys (*Miopithecus talapoin*; Juan-Salles et al, 2001), marmosets (*Saguinus mysta*; Chadwick et al, 1979), rhesus macaques and bonnet macaques (*Macaca radiata*; Swenerton & Hurley, 1980). Hormonal alopecia can result from seasonal variation (Isbell, 1995), pregnancy and lactation (Davis & Suomi, 2006). Davis & Suomi (2006) reported gestational alopecia in 10 female macaques; hair growth returned to normal within two months of parturition in all 10 NHPs. In addition to the nutritional and hormonal causes of alopecia, alopecia can occur due to parasitic infections (Baker et al, 1971), immunological (Beardi et al, 2007) or genetic conditions (Ratterree & Baskin, 1992) and

psychological factors, including stress (Honess et al, 2005). Stress related alopecia is associated with hair pulling behaviour in NHPs (Reimhardt et al, 1986; Tay et al, 2004) and is more prevalent and severe during periods of poor welfare, for example, barren housing in pigtail macaques (*Macaca nemestrina*; Boccia, 1989) or following alcohol intoxication in cynomolgus macaques (*Macaca fascicularis*; Shively et al, 2002).

Nasal discharge, excessive or insufficient urination and diarrhoea can also indicate health issues (Tasker, 2012; NC3Rs, 2014a). Diarrhoea can be indicative of an underlying illness or stress (NC3Rs, 2014a). In captive populations of NHP, diarrhoea can result in significant levels of mortality and morbidity (Wilk et al, 2007; Prongay et al, 2013; Kanthaswamy et al, 2014).

Food and water consumption can easily be measured and quantified in singularly housed animals (Weary et al, 2006); however, to promote positive welfare, most laboratory animals are not singularly housed (Baker et al, 2012) making distinguishing between group and individual intake difficult. A combination of health indicators and behavioural observations would be required to determine the cause of, for example, the diarrhoea or hair loss and aid in estimating fluid and food intake.

Genetics and behaviour in primates

The genetics of behaviour is complex as so many genes are involved in the hormone and neurotransmitter pathways that underpin various behaviours (O'Connell & Hofmann, 2011; Saez et al, 2014). Most traits, including behaviours, are not controlled by the expression of a single gene or allele. Instead, these polygenic traits are controlled by, or involve, two or more genes (Munafo & Flink, 2004; Plomin & von Stumm, 2018; Sallis et al, 2018; Bordy, 2019). Identifying the genes that are involved in polygenic traits is not straight forward and methods require substantial follow-up work to identify causal genes within the identified regions of DNA (Flint, 2003; Martinez et al, 2016). NHP genetic studies suffer from some of the same issues as early human genetic studies: small sample sizes and large effects of reported genetic variants (Staes et al, 2015; Wilson et al, 2017; von Borell et al, 2019). Authors report sample size concerns with interpretation of NHP behavioural

genetics results (e.g., Adams, 2014; Blomquist & Brent 2014; Brent et al, 2014; Brent & Melin, 2014; Huchard & Pechouskova 2014) and it has been suggested that between 60 (von Borell et al, 2019) and 100 (Brent & Melin, 2014) individuals are needed for robust analysis. Nevertheless, it is possible to dissect the effects of genetic variation on macaque behaviour in some systems (Rogers, 2018; von Borell et al, 2019). For example, in rhesus macaques, gene expression impacts vigilance (Dobson & Brent, 2013) and social behaviour (Chang et al, 2013; Madlon-Kay et al, 2018). Low expression of the *serotonin transporter* gene (*SLC6A4*) has been associated with hypervigilant tendencies compared to individuals who are high expressing homozygotes (Dobson & Brent, 2013). Without genetic data, primatologists are unable to fully answers some of the key fundamental questions such as "What are the physiological and neurobiological mechanisms that underlie the production of behaviors in primates?" (Brent & Melin, 2013).

In both humans and animals, gene-environment interaction is known to affect behaviour (Grandin & Dessing, 2014) and personality (Verhulst et al, 2016). For example, genetic variants at certain loci are strongly linked to an individual's susceptibility to anxiety, depression, and stress related disorders (e.g., Hu et al, 2006; Smoller, 2016; Wingo et al, 2018). A *monoamine oxidase A* gene (*MAOA*) variant in humans predisposes individuals to antisocial and violent behaviour; however, these behaviours are only present in individuals with the variant that were also abused as a child (Ducci et al, 2008).

Genetics and breeding for welfare have received much attention in farm animal species (Rodenburg & Turner, 2012) due to the link with productivity and performance (Ellen et al, 2014). More recently, the genotype of companion animals has been considered (Milne, 2018) as a result of the significant number of serious inherited disorders (e.g., brachycephalic obstructive airway syndrome: Liu et al, 2017; syringomyelia: Cockburn et al, 2018). In a laboratory setting, references to genetic change and welfare are more frequently in the context of concerns for the welfare of genetically modified animals (e.g., Buehr et al, 2003). Human genetic disorders are often studied in animal models (Simmons, 2008), so information about the response of key genes within these experimental

animals is translated to humans and not used for the benefit of animal welfare (e.g., Harding, 2013). Inclusion of genotype with behavioural, physiological, and/or cognitive measures in NHP welfare assessment allows indication of predisposition to certain conditions and the breeding of more robust offspring (Rauw & Gomez-Raya, 2015).

Cognition

Cognitive studies in animals provide an accurate assessment of animal emotion (Mendl et al, 2009). These methods were adapted for use with animals following the idea that cognitive functioning can be a reliable indicator of emotional state in humans (Mendl et al, 2009). It is known that the way humans attend to and interpret information is associated with self-reported feelings of wellbeing and physiological changes (Ardayfio & Kim, 2006; Bar-Haim et al, 2007; Donaldson & Young, 2008). For example, anxious individuals are more pessimistic and likely to negatively judge ambiguous cues compared to non-anxious people (Eysenck et al, 1991, 2006; Richards et al, 2002; Blanchette et al, 2007). Cognitive bias methods provide an accurate assessment of animal affect and there is currently great interest in developing these methods to advance animal welfare science (e.g., Harding et al, 2004; Mendl et al, 2009; Bateson et al, 2011; Baciadonna & McElligott, 2015; Bethell, 2015; Roelofs et al, 2016). Cognitive bias has been categorised as emotional states that arise from interpretation or judgement bias, memory bias and attention bias (Paul et al, 2005; Hertel & Mathews, 2011).

Judgement bias

Animal cognitive bias studies frequently use judgement bias tasks (e.g., Harding et al, 2004; Mendl et al, 2009; Bateson et al, 2011; Baciadonna & McElligott, 2015; Bethell, 2015; Roelofs et al, 2016). Judgement bias tasks involve training an animal to differentiate between abstract cues for reward and non-reward (or punishment) and then tested for optimism and pessimism using intermediate abstract cues. These tasks have demonstrated that animals are more pessimistic about intermediate abstract cues following negative mood manipulation such as pharmacological treatment or a barren environment (pigs: Douglas et al, 2012; rodents: Hales et al, 2014; macaques:

Bethell, 2015; Bethell et al, 2016). Douglas et al (2012) trained pigs to discriminate two auditory cues in a go/no-go task with the positive cue signalling food and the negative cue signalling a mildly aversive experience. The effect of enriched and barren environments on pig response to an intermediate auditory cue was then compared. During the abstract intermediate trials, the enriched pigs were found to be more optimistic than the barren environment pigs. In primates, a judgment bias go/no-go task with visual cues was used to demonstrated that rhesus macaques become more pessimistic following veterinary health checks compared to phases of enrichment (Bethell et al, 2012a). The macaques were trained to touch or ignore lines of different size to receive a reward or avoid an aversive experience. During testing, macaques were presented with lines of intermediate size and following the health check macaques made fewer responses to the ambiguous cues suggesting a negative shift in affective state.

The go/no-go task can result in a false pessimistic interpretation due to a generalised reduction in response (Brilot et al, 2010). Instead, two distinct responses are trained: one to a positive cue and one to a negative or less positive cue. When tested with an ambiguous cue the interpretation of their responses is much clearer (Perdue, 2017). For example, starlings were trained to distinguish between symbols (S+ and S-) on the lids of petri-dishes (Brilot et al, 2010). In the presence of a dark background, the starling received a larger reward (three mealworms) when they chose the S+ petri-dish, while in the presence of a light background they received a smaller reward (one mealworm) if they chose the S- petri-dish. When intermediate background colours were used the starlings' choices of S+ or S- were recorded. The active choice between S+ and S- reduces the potential ambiguity caused by the go/no-go task.

Memory bias

There is extensive evidence for a link between cognition and the storage, consolidation, and retrieval of memories in humans (e.g., Cahill and McGaugh, 1996, 1998; Um et al, 2012; Tyng et al, 2017). For example, depressed humans recall negative experiences more accurately than non-depressed humans (Mineka & Nugent, 1995).

Recent work in neuroscience and psychology has revealed that the emotional and cognitive neural systems are deeply integrated (Dolcos et al, 2011; Okon-Singer et al, 2015) with emotions having a long-term impact on memory through influence on the formation of the hippocampal-dependent memory system (Pessoa, 2008). Depending on the duration and intensity, stress may facilitate and/or impair memory (Vogel & Schwabe, 2016; Tyng et al, 2017). Zoo visitors who were exposed to a stressor (Trier Social Stress Test including a job interview, public speaking and mental arithmetic) were significantly more likely to remember their route though the zoo and events that occurred within 41-65 minutes of the stressor compared to the non-stressed controls (Vogel & Schwabe, 2016). This time dependent stress-enhanced memory formation was related to increased action of the HPA axis and autonomic nervous system (ANS) resulting in stress-induced increases in cortisol and blood pressure (Vogel & Schwabe, 2016).

Mood-congruent biases in memory were only recently established in non-human animals (mice: Takatsu-Coleman et al, 2013; rats: Burman & Mendl, 2018). Burman & Mendl (2018) trained rats to search for specific reward pots containing food in an eight-arm radial maze. Following training, the rats were placed in the maze with access to only one arm and experienced a positive (12 pellets), negative (12 quinine-soaked pellets), or neutral event (1 pellet). Results revealed that regardless of time since the event (rats were tested at 2 hour and 24 hours), they preferred arms where they had experienced the positive event and avoided arms where they had experienced a negative event (Burman & Mendl, 2018). Mice that were exposed to 12-hour social isolation were more avoidant of areas paired with the aversive event in a plus-maze discriminative avoidance task compared to control mice (Takatsu-Coleman et al, 2013). Isolated mice had elevated corticosterone, which the authors suggested was essential for mood-congruent memory in mice.

Evidence in the human literature suggests that memory biases are more strongly influenced by depression rather than by anxiety or acute stress (Mathews & MacLeod, 1994; Mineka et al, 1998; Paul et al, 2005) suggesting this type of cognitive bias is not appropriate for welfare assessment following acute stressors. Both judgment and memory bias tasks are time consuming tools as they

require extensive prior training (Harding et al*,* 2004; Bethell et al, 2012a; Bethell, 2015; Burman & Mendl, 2018). They can be disruptive to management and husbandry routines, costly in terms of both money and time, and study statistical power may be impacted by participant number attrition (Harding et al*,* 2004; Bethell et al, 2012a; Bethell, 2015). Instead, tasks that require less training (such as those that utilise innate attention biases) may be more appropriate and practical in real world settings, as many attention bias (AB) tasks require little or no training (Mendl et al, 2004; Paul et al, 2005; Brilot et al, 2009; Bethell et al, 2012b; Verbeek et al, 2014).

Attention bias

AB describes a tendency to differentially allocate attention towards one of two or more stimuli that vary in emotional content. Fearful or anxious attention biases in humans relate to vigilance towards threatening cues in order to avoid danger and protect the body from harm (Mathews & MacLeod, 1994; Mogg and Bradley, 1998; Lang et al, 2000; Paul et al, 2005). The automatic allocation of attention to treat is an innate mechanism that works to enhance survival (Öhman et al, 1986, 2001a).

In humans, innate AB has been studied in infants (Nelson & Dolgin, 1987; Peltola et al, 2008; LoBue & DeLoache, 2008, 2010). Children aged between eight and 14 months are faster to orientate towards images of angry faces compared to images of happy faces (LoBue & DeLoache, 2010). In addition, seven-month-old children were quicker to disengage with images of positive or neutral facial expression compared to images of fearful faces when presented with a distractor (Peltola et al, 2008). The response of young children to negative facial expressions suggests that social cues of threat are innate responses that could be utilised for measuring AB in non-human animals.

Indeed, AB has been proposed as a novel method of animal welfare assessment (Paul et al, 2005; Bethell et al, 2012b; Crump et al, 2018). AB tasks have previously been shown to be capable of measuring animal emotion in NHPs (Bethell et al, 2012b; Marzouki et al, 2014; Allritz et al, 2016; Boggiani et al, 2018; Morin et al, 2019), birds (Brilot et al, 2009; Brilot & Bateson, 2012; Cussen &

Mench 2014; Campbell et al, 2019a,b), sheep (Verbeek et al, 2014; Vögeli et al, 2014; Lee et al, 2016; McBride & Morton 2018; Monk et al, 2018ab, 2019ab; Raoult & Gygax, 2019), cattle (Lee et al, 2018), pigs (Luo et al, 2019) and rats (Parker et al, 2014).

The above animal studies compared the animals' attention to stimuli following a manipulation of their affective state. In humans, changes in attention can be determined by measuring response time or response slowing in simple tasks with emotional distractors (Fox et al, 2001; Bishop et al, 2004; Mauer & Brokenau, 2007; Mogg et al, 2008; Holmes et al, 2009). In animals, attention has been measured by comparison of latency to detect and orientate towards the stimuli (e.g., Lou et al, 2019), approach an object (e.g., Verbeek et al, 2014) and eat (e.g., Campbell et al, 2019a), reaction time to complete a task (e.g., Allritz et al, 2016), head position duration (e.g., Monk et al, 2018a) and eye gaze (Bethell et al, 2012b). Stimuli were species-relevant and included alarm calls (e.g., Brilot & Bateson, 2012), novel objects (e.g., Verbeek et al, 2014), predators (e.g., Lee et al, 2016) and aggressive conspecific (e.g., Vögeli et al, 2014). Affective state was manipulated using housing conditions (e.g., Parker et al, 2014), veterinary inspection (e.g., Bethell et al, 2012b), food deprivation (e.g., Verbeek et al, 2014) or removal of objects necessary for species-typical behaviour (e.g., Brilot & Bateson, 2012). Affective state without manipulation was also determined using behavioural observation (Marzouki et al, 2014) and personality assessment (Cussen & Mench, 2019).

AB tasks were first developed as a method to assess emotion in humans using paradigms such as the looking time task (Fantz, 1958), the dot-probe task (MacLeod et al, 1986), the visual search task (Green & Anderson, 1956) and emotional Stroop task (Stroop, 1935). These paradigms have since been applied to non-human animals: looking time tasks (e.g., Bethell et al, 2012b), the dot-probe task (e.g., Verbeek et al, 2014), visual search tasks (e.g., Marzouki et al, 2014) and the emotional Stroop task (e.g., Allritz et al, 2016; details of the different paradigms for both humans and animals are included in Chapter 3).

Triangulation of measures to optimise welfare assessment

A triangulation of behaviour, physiology and health indicators has been suggested as one of the current best methods for assessing NHP welfare (Webster, 2008; Jennings & Prescott, 2009; Tasker, 2012). For example, both positive (e.g., exciting) and negative (e.g., distress) events can result in increased heart rate in macaques. The inclusion of simultaneous behavioural observation would allow the observer to determine if the physiological change had resulted from positive or negative experience (NC3Rs, 2012; Tasker, 2012). However, this approach would not fully assess mental state in those instances. Therefore, the development of welfare assessment methods which triangulate these indicators with the animal's mental state are important to ensure holistic welfare assessment.

Welfare assessment frameworks, such as the Five Freedoms (FAWC, 1979), do not fully acknowledge the influence of mental state (Mellor, 2016). As a result of the current emphasis on animal emotions, the Five Domains were developed from the Five Freedoms to include mental state within welfare assessment. The Five Domain Model includes four physical or functional domains (nutrition, environment, health, and behaviour) and one affective experience domain that aligns with what the animal experiences in the functional domains (Mellor, 2016). For example, "constraints on animal-to-animal interactive activity" within the behaviour domain would align with loneliness/isolation, depression, or sexual frustration vs affectionate sociability, maternally rewarded, playfulness, or sexual gratification within the affective experience domain (Mellor, 2016).

Direct measures of conscious emotion are not available (Mendl et al, 2009). Therefore, changes in cognitive functioning measured by new methods including judgement bias (Mendl et al, 2009; Bethell et al, 2012a), memory bias (Burman & Mendl, 2018), or AB (Bethell et al, 2012b; Crump et al, 2018) need to be included within welfare assessment to provide a measure of affective or mental state.

Some studies with NHPs have used a combination of approaches including cognitive measures (e.g., Pomerantz et al, 2012b). Here, emotional state was assessed using a judgement bias task in which capuchins (*Cebus apella*) discriminated between the size of a rectangle. The capuchins were trained to associate the larger shape with a more favourable food reward. During testing the occurrence of pacing behaviour was not significantly correlated with the probability of responding to the ambiguous cue i.e., placing behaviour was unrelated to emotional state (Pomerantz et al, 2012b).

Many traditional animal welfare assessment methods focus on preventing poor or negative welfare rather than promoting positive welfare (Philips, 2008; Yeates & Main, 2008; McCormick, 2012; Lawrence et al, 2018). Considering an animal's emotional or affective state will help to move animal welfare science in a positive direction (Mendl & Paul, 2004). Novel assessment methods, such as AB, that are capable of detecting shifts in emotional state, are quick and require little training, need to be the focus of studies now in order to improve environments and husbandry and management practices to promote positive experiences for captive animals.

1.4 Overview of the thesis

AB tasks have previously been shown to detect shifts in emotional state in humans, with some recent data suggesting they can be adapted for use with animals, including NHPs. In this thesis, I aimed to identify the biological and environmental factors (life history, hormonal, genetic and potentially stress-inducing husbandry procedures) that influence an individual's AB profile and the extent to which this can be used to identify state (e.g., response to veterinary intervention) and trait (e.g., individual differences in personality) affect. These factors should be included in future AB studies and will also highlight which individuals may be more vulnerable following stressful life events. Four looking time measures were used in the analysis in Chapter 3: duration looking at the threat face stimulus (THR), total duration looking at the threat and neutral face stimuli (TL), AB difference score (ABDiff) and ABDiff/TL. AB difference score was calculated by subtracting the duration looking at the neutral face stimulus from the duration looking at the threat face stimulus. In Chapter 4, 5 and 6 only the duration of THR and TL were used.

This project piggybacked onto the macaques' routine annual veterinary health check to ensure further stress was not caused as a result of this research. As part of the annual health screening the macaques were sedated with an intramuscular injection of ketamine hydrochloride (KHCl: 0.1 – 0.2 ml/kg) for blood draw, weighing, a tuberculosis injection in the right eyelid and a rectal swab. For the subsequent two days, the macaques experienced additional rectal swabs; however, although separated from their group and restrained with the crush-back, they were not sedated prior to this procedure.

The thesis is split into a training chapter (Chapter 2) and four methods chapters, which detail the cognitive (Chapter 3), behavioural (Chapter 4), physiological (Chapter 5) and genetic (Chapter 6) components of this project. In Chapter 2, I describe the training required for the rhesus macaques to participate in the research in Chapters 3-6. I describe methods and equipment development, macaque training success and pilot study outcomes. Protocols for station training, desensitisation to the AB apparatus and saliva collection are provided. In Chapter 3, I review the previous AB literature to explain the steps taken to develop the AB methods used here. This chapter contains the detailed AB methods used in each of the other chapters. In Chapters 4 and 5, the AB methods were validated by comparison with traditional welfare assessment methods both before and after a stressor (the macaques' annual veterinary health check). In Chapter 4, AB is correlated with behavioural observations collected using an ethogram of established behavioural indices of stress and anxiety. In Chapter 5, AB was compared with salivary cortisol concentration, which is known to increase in response to stress. In Chapter 6, AB was correlated with genetic polymorphisms in nine key genes relating to serotonin, dopamine, oxytocin, arginine vasopressin and opioids. Finally, in Chapter 7, I summarise the finding of the thesis and discuss these in terms of what I have learned over the course of this PhD.

Chapter 2 – Training & methods development

2.1 Learning theory

Animal training can be challenging, yet it is essential for the effective and safe management of many domesticated and captive species (Laule et al, 2003; Reinhardt, 2004). The two main methods for animal training are classical conditioning (Pavlov, 1927) and operant conditioning (Skinner, 1938). Classical conditioning involves developing an association between an unconditioned stimulus (a stimulus that naturally or automatically triggers a response, e.g., the delivery of food) and a response (e.g., salivation), with a previously neutral new or conditioned stimulus (e.g., the sound of a bell; Pavlov, 1927; Gottleib & Begej, 2014).

Operant conditioning is the development of an association between a behaviour and a consequence (Skinner, 1938). Figure 2.1 shows that there are four main approaches: positive reinforcement, negative reinforcement, positive punishment, and negative punishment (Skinner, 1938; McBride & Montgomery, 2018).

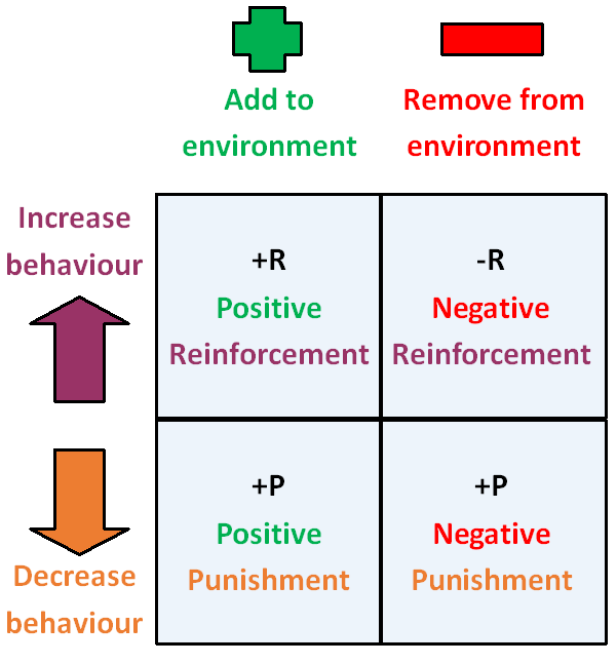

Figure 2.1. Operant conditioning reinforcement theory. All training for this thesis was by positive reinforcement methods only.

Each of these methods involves a different consequence for a given behaviour. The occurrence of a behaviour can be increased using reinforcement:

- **Positive reinforcement (+R)** involves the provision of a reward following the correct behaviour and leads to an increase in the occurrence of that behaviour, for example, using food rewards to train zoo animals to accept oral medications and injections without restraint (Heidenreich, 2015).
- **Negative reinforcement (-R)** involves the removal of an aversive stimulus following the correct behaviour and leads to an increase in the occurrence of that behaviour, for example, the removal of pressure applied by a rider to the side of a horse when the horse begins to move forward (Jones, 2017).

The occurrence of a behaviour can be reduced using punishment:

- **Positive punishment (+P)** involves the application of an aversive stimulus following an incorrect behaviour causing a decrease in the occurrence of that behaviour, for example, the use of electric shock collars (now illegal in the UK) to reduce the incidence of barking in dogs (Schilder & Van der Borg, 2004).
- **Negative punishment (-P)** involves the removal of a stimulus following an incorrect behaviour, for example, the use of timeouts to decrease cocaine-maintained behaviour in experimental rhesus macaques (Nader & Morgan, 2001).

Positive reinforcement training (PRT)

All animal training was conducted using positive reinforcement training (PRT) only. PRT was first described by Skinner (1938) and the method has long been used for training companion and zoo animals (Hagenbeck, 1912; Burch, 2002). PRT is often combined with clicker or whistle training, where the clicker is used as a bridge between good behaviour and presentation of a reward (Laule et al, 2003). PRT in combination with clicker training has been shown to make training less challenging for trainers (Feng et al, 2018), reduce the time required to train complex tasks (Gillis et

al, 2012) and allow a closer working relationship with potentially aggressive animals (Miller & King, 2013).

Pet dogs trained with PRT show fewer signs of stress, have a more relaxed body posture, increased owner directed attentiveness (Deldalle & Gaunet, 2013) and have improved performance in novel training target tasks and response to novel people (Rooney & Cowan, 2011). Parrots trained with PRT show fewer behavioural problems (Martin, 2007) and reptiles, for example, Aldabra tortoises (*Geochelone gigantea*) can be trained to allow venepuncture (Weiss & Wilson, 2003), which can reduce stress during veterinary visits (Reichard et al, 1993). Further, the use of PRT in zoos has been shown to improve keeper-animal relationships in a range of species including black rhinoceros (*Diceros bicornis*), Sulawesi crested black macaques (*Macaca nigra*) and Chapman Zebra (*Equus burchellii*; Ward & Melfi, 2013). In NHPs, PRT results in a significant increase in prosocial and affiliative behaviours and a decrease in stress-related behaviours in zoo-housed chimpanzees (*Pan troglodytes*; Pomerantz & Terkel, 2009) and gorillas (*Gorilla gorilla gorilla*; Carrasco et al, 2009). PRT methods are known to promote improved animal welfare during both the training phase and the research itself (Laule et al, 2003, 2007; Prescott & Buchanan-Smith, 2003; NC3Rs, 2019). Time spent training and rewarding promotes a closer relationship between trainers and the NHPs involved (Buchanan-Smith, 2003; Prescott & Buchanan-Smith, 2003).

PRT has only recently become the standard for training within biomedical facilities, which has since led to evolved training practices for laboratory NHPs (e.g., Perlman et al, 2012; Whittaker & Laule, 2012; Nightingale et al, 2015; Westlund, 2015). PRT had also successfully been used to collect biological samples including blood (Coleman et al, 2008), urine (Smith et al, 2004; Magden, 2017) and saliva (Lutz et al, 2000) from NHPs.

Bloomsmith and colleagues (2015) successfully trained 35 group housed female chimpanzees (*Pan troglodytes*) to provide individual urine samples for a research study. The chimps were trained using PRT, a clicker and the verbal cue “pee” to urinate into PVC pipes used as collection devices. Training was conducted over a two-year period with between two and five training sessions per week. The

authors achieved 100% training success with all chimps learning to urinate on request (median time to urinate was 4.9 minutes) in between eight and 232 training sessions.

McKinley and colleagues (2003) trained 12 pair housed laboratory common marmosets (*Callithrix jacchus*; 6 female, 6 male) to allow home cage weighing and to urinate on request. The authors used the common behaviour of scent marking to train for urine collection. An animal was assessed as having trained if they scent marked 12 times on request during a 10-minute training session. The authors had 100% training success with all marmosets training in between two and 13 training sessions. These trained marmosets were then involved in a further study. Bassett et al (2003) compared the post-stressor (chasing into a nest box, transportation to veterinary room, removal from social group and handling by gloved hand for weighing) behaviour of 24 common marmosets. Twelve of the marmosets had been trained using PRT to provide urine samples on request and the other 12 had not experienced any training. The authors reported a significant difference in the occurrence of self-scratching post-stressor. Self-scratching is associated with anxiety and stress in NHPs (Maestripieri et al, 1992) and the non-trained animals had a significantly higher increase in this behaviour than the trained animals. This suggests that PRT is effective in reducing stress for captive NHPs undergoing procedures.

Rhesus macaques have been successfully trained to provide urine samples on request at the University of Oxford (Rhyanne Dale, PhD Researcher at the University of Oxford, personal communication, July 2017). Trainers at the University of Oxford used PRT, a whistle as a bridge and the verbal cue “pee” to train pair housed male macaques. The samples were collected for a larger researcher project for the analysis of urinary cortisol.

Training aims

The key training requirements for this thesis were:

1. Station train the macaques to the correct location to allow their response to stimuli to be recorded. To record looking times to stimuli for AB trials, macaques were required to sit

still and face the AB apparatus. Training them to sit by a coloured stationing tool was the key first step for AB data collection.

2. Prevent other macaques interfering with the focal macaque's trial. To ensure macaques were not distracted by conspecifics during the AB trials, all macaques in a group were station trained so that they remained stationary during other macaques' trials.
3. Collect individual, non-contaminated saliva samples from animals without separating them from their social group. In order to assess the relationship between salivary cortisol and AB, saliva samples were collected. Macaques needed to be trained to chew on the swabs for long enough to collect a large enough sample for analysis.

2.2 Methods

Ethics

Ethical approval was granted by Liverpool John Moores University (LJMU) in February 2017 (Ethical approval ID. EB_EH/2017-5) and by the Medical Research Council Animal Welfare and Ethical Review Body (AWERB) in November 2017. This project piggybacked onto routine veterinary and husbandry activities that would have occurred whether or not the animals were involved in this study. No regulated procedures were carried out for this study; sample collection for hormone analysis was by non-invasive methods only. Analgesia was not delayed because of any research relating to this PhD. All training was conducted following centre protocol and using PRT. Participation in training, AB trials and sample collection was voluntary, insofar as animals were free to leave the training and testing area (cage room) at any time. Food, water, and social contact with conspecifics were available *ad libitum* throughout training and testing.

Animals & housing

Medical Research Council Centre for Macaques

The rhesus macaques involved in this research were socially housed at the Medical Research Council Harwell Institute Centre for Macaques (MRC-CFM) located at the Defence Science and

Technology Laboratory (dslt) site at Porton Down, Salisbury, Wiltshire, England (https://mrc.ukri.org/research/facilities-and-resources-for-researchers/mrc-centre-for-macaques/). MRC-CFM is home to around 300 macaques and is one of only three rhesus macaque breeding centres in the UK (MRC, 2019). The other two centres are also located within the Porton Down site allowing the easy sharing of information and concentrating macaque veterinary care and welfare expertise. Since its establishment in 2003, it has supplied approximately 30 monkeys per year for use in biomedical research, including neuroscience, ophthalmology, and immunology, at four academic institutions in the UK.

MRC-CFM has capacity for 22 groups over two corridors with additional quarantine space for around 20 adult animals. The 22 groups consisted of 12 breeding groups and 10 weaner groups. To mimic free-ranging conditions, the breeding groups of macaques were housed in matrilineal social groupings with adult males rotated between groups every four to five years. The breeding groups consisted of one adult male, between two and eight adult females and their offspring. Macaques retained for breeding are weaned between 12 and 30-months-old and moved into one of the single sex weaner groups of between seven and 17 individuals where they remain until they are moved on to one of the universities (Dr Claire Witham, Scientific Project Co-ordinator at MRC-CFM, personal communication, June 2017).

Rhesus macaques

Eighty-six group housed adult rhesus macaques housed in 13 social groups were initially trained for participation in the studies presented in this thesis. At the start of training in September 2017, the macaques were 7.69 years old ± 3.42 years with an age range of between 2.75 to 15.42 years. The social groups were 11 breeding groups, one all-female ex-breeding group and one all-male weaner group. The macaques were housed at the Medical Research Council Harwell Institute Centre for Macaques (MRC-CFM), a breeding colony that supplies UK academic institutions with macaques for biomedical research. As a result of their future use in biomedical research, the macaques were bred and held under the Animals (Scientific Procedures) Act 1986 administered by the UK Home Office.

All methods for this thesis were designed to minimise the likelihood of distress and were considered non-regulated procedures by the Home Office inspector, the LJMU ethics committee and the MRC AWERB.

Following desensitisation and training, 61 macaques (45 female, 16 male) were selected for inclusion in the final AB study presented in Chapter 3. Details of the macaques including date of birth, rank, reproductive status, and group composition as well as which animals were involved in each component of this PhD can be found in Table 2.1. Eighteen macaques had been previously station trained using positive reinforcement and clicker training for AB testing (Szott, 2015; Thatcher, 2015; Kemp et al, 2017).

Table 2.1. The social group, sex, rank, age, reproductive status, training success and study inclusion for each monkey involved in the research for this thesis. The previous AB study was conducted by Szott (2015), Thatcher (2015) and Kemp et al (2017). Study 1 included cognitive data collected as AB trials before and after the macaques' annual veterinary health check (stressor) to compare the AB at baseline and following a stressor. Study 2 included cognitive data collected as one AB trial per week over eight weeks to assess the repeatability (Rep) of the AB signal.

Group	Sex	Rank	Monkey ID	Age (months) at the start of training	Repro status	Previous AB study	Station trained	Station with AB apparatus	Trained saliva	Study 1 (stressor)	Study 2 (Rep)
Breeding G01	M	High	Star	109	Breeding male		✓	✓	✓	✓	
	F	High	Zsa-Zsa	33	Cycling		✓			✓	
		Mid	Valentine	78	Cycling		✓		✓	✓	
		Low	Zarita	34	Cycling		✓	✓		✓	
Breeding G03	M	Mid	Utah	87	Breeding male					✓	
	F	High	Saphy	113	Nursing		✓	✓		✓	
			Spice	110	Nursing		✓	✓	✓	✓	
			Yazzoo	49	Nursing		✓	✓		✓	
		Mid	Sugar	111	Nursing		✓	✓	✓	✓	
			Tea	100	Nursing		✓	✓		✓	
		Low	Rupee	126	Nursing		✓	✓		✓	
			Ylang-Ylang	50	Nursing		✓	✓	✓	✓	
Ex-breeding G04	F	High	Linz	185	Implanted	✓	✓	✓			✓
			Venus	72	Nursing	✓	✓	✓			✓
			Wine	60	Cycling		✓	✓	✓	✓	
		Mid	Maj	182	Implanted	✓	✓	✓			✓
			Verity	72	Nursing	✓	✓	✓			✓
		Low	Mindy	174	Implanted						
			Umbrella	89	Implanted						
Breeding G06	M	High	Will.i.am	63	Breeding male		✓	✓			✓
	F	High	Ocelot	144	Nursing	✓	✓	✓			✓
		Mid	Tass	97	Nursing	✓	✓				
			Tes	96	Cycling	✓	✓	✓			✓
		Low	Shirley	109	Cycling	✓	✓				
			Sizzle	109	Cycling		✓	✓			✓
Weaner G07	M	High	Zavier	35	Weaner male		✓	✓			✓
			Zorro	38	Weaner male		✓				

		Mid	Zachariah	38	Weaner male		✓	✓			✓
			Zebedee	36	Weaner male		✓	✓			✓
		Low	Zarson	33	Weaner male		✓	✓			
			Zee	36	Weaner male		✓				
			Zoidberg	37	Weaner male		✓	✓			✓
			Zulu	39	Weaner male		✓	✓			✓
Breeding G09	M	High	Abbott	184	Breeding male		✓	✓			✓
	F	High	Orinoco	145	Implanted	✓	✓	✓			✓
		Mid	Prune	134	Implanted	✓	✓				
Breeding G13	M	High	Plum	133	Breeding male		✓	✓	✓	✓	
	F	Mid	May	173	Cycling		✓				
	F	High	Rach	123	Cycling		✓	✓	✓	✓	✓
	F	Low	Reya	123	Nursing						
	F	Low	Rozanne	124	Nursing		✓	✓	✓	✓	
	F	Low	Yardley	47	Cycling						
	F	Low	Zola	37	Cycling						
Breeding G15	M	High	Thorn	96	Breeding male		✓			✓	
	F	High	Senga	112	Nursing		✓	✓		✓	
			Venice	68	Nursing		✓	✓	✓	✓	
		Mid	Sienna	112	Nursing		✓	✓	✓	✓	
		Low	Tia	102	Nursing						
			Uno	85	Nursing		✓	✓	✓	✓	
			Vixon	77	Nursing						
			Zorilla	38	Cycling						
Breeding G16	M	High	Sequel	106	Breeding male		✓	✓	✓	✓	✓
	F	High	Yibbi	47	Nursing		✓	✓	✓	✓	✓
		Mid	Omelette	143	Nursing	✓	✓				
			Orlanda	140	Implanted	✓	✓	✓			✓
			Pansy	139	Nursing	✓	✓	✓			✓
			Yeva	46	Nursing		✓	✓	✓	✓	✓
		Low	Ruby	127	Nursing	✓	✓				
			Tulip	97	Nursing		✓	✓		✓	
			Wench	59	Cycling						
Breeding G18	M	High	Nodon	159	Breeding male		✓	✓	✓	✓	✓
	F	High	Rene	121	Nursing	✓	✓	✓			✓
			Shallot	106	Cycling	✓	✓	✓			✓
			Yoana	45	Cycling		✓	✓	✓	✓	

		Mid	Razz	122	Cycling	✓	✓	✓			✓
		Low	Rhumba	121	Cycling	✓	✓				
			Robyn	124	Nursing	✓	✓				
Breeding G55	M	High	Vincent	73	Breeding male		✓	✓	✓	✓	✓
	F	High	Versa	74	Nursing		✓	✓		✓	✓
		Mid	Spangle	110	Nursing	✓					
			Tanya	101	Nursing	✓	✓	✓			✓
		Low	Umber	87	Cycling		✓				
			Varsalla	73	Nursing						
Breeding G57	M	High	Sol	185	Breeding male		✓	✓	✓	✓	✓
	F	High	Tallulah	98	Nursing	✓	✓	✓			✓
		Mid	Wanganui	57	Cycling	✓	✓				
			Zena	36	Cycling		✓	✓	✓	✓	✓
		Low	Tilly	97	Nursing		✓	✓	✓	✓	
			V	70	Nursing	✓	✓	✓			✓
			Vanquish	71	Cycling						
			Zumba	35	Cycling		✓	✓	✓	✓	
Breeding G60	M	High	Viktor	74	Breeding male		✓	✓	✓	✓	✓
	F	High	Serena	113	Nursing	✓	✓	✓			✓
			Thyme	99	Nursing	✓	✓	✓			✓
			Yoyo	51	Nursing		✓	✓	✓	✓	✓
		Mid	Tamara	100	Nursing		✓				
			Zelda	36	Cycling		✓	✓	✓	✓	✓
		Low	Sonja	111	Nursing						
			Teal	98	Cycling						

Housing

Each enclosure consisted of a cage room and a free roaming area (Figures 2.2, 2.3 and 2.4). Training and data collection occurred within the cage room area (Figure 2.3). This room consisted of platforms on three levels with access between the levels at either end and four hatches into the free roaming area. The space was designed to allow low ranking macaques to move out of sight and escape during fights. Macaques always had free access to the free roaming area during training and testing. Access between rooms was only restricted outside of data collection phases for husbandry procedures such as cleaning and veterinary treatment. The free roaming areas were furnished with various enchantment items, for example, slides, platforms, climbing frames, swings, buckets, and mirrors. A large proportion of the macaques feed was scattered among the straw on the floor of the free roaming area to promote foraging behaviour. Additional temporary or destructible enrichment was provided in the form of ice-lollies and blocks, dried fruit, bubble machines, water baths, peanut butter, and cardboard boxes.

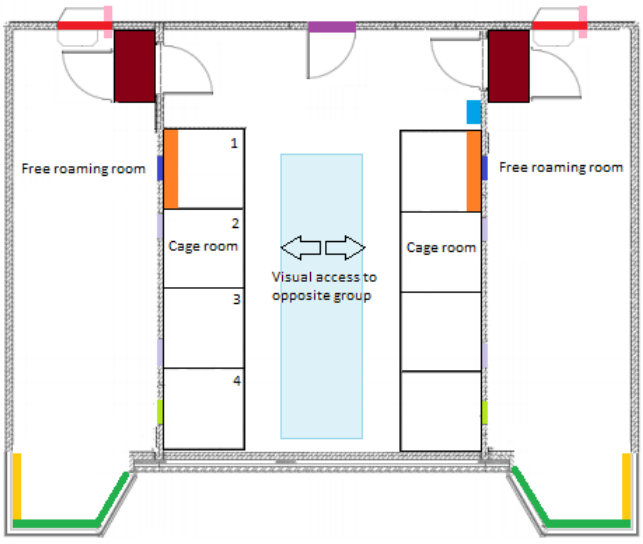

Figure 2.2. Enclosure layout for socially housed rhesus macaques (*Macaca mulatta*) held at the Medical Research Council Harwell Institute Centre for Macaques (MRC-CFM), Porton Down,

Figure 2.3. Social cage room area for breeding groups of rhesus macaques at MRC-CFM. All training and testing occurred within the cage room area. Doors allowed free movement between the cage room and the free roaming area. Photograph: Claire Witham.

Figure 2.4. Two views of the free roaming areas for the breeding groups of rhesus macaques at MRC-CFM. a) Close up view of the free roaming area showing deep bedding and enrichment items. b) Wide view of the free roaming area showing resting platforms, structures for climbing, and a large window to allow in natural light. Photographs: Claire Witham.

The free roaming area had dimensions: 8.04m long x 3.35m wide x 2.8m height while the cage room area had dimensions: 6.12m long x 1.5m wide x 2.8m height. The overall volume was 98.54m^3 and the floor area was 35.19m^2. The enclosures were kept at a consistent humidity of 55-65% and temperature of 18-20°C. Artificial light was provided between 07:00 and 19:30 throughout the year, while large windows in the free roaming area allowed additional natural light into the enclosure. Pressure sensitive water dispensers were positioned in multiple locations in both the cage room and the free roaming area ensuring macaques had constant access to water.

Staff

There were 11 full time staff at MRC-CFM: the Establishment Licence Holder, the Scientific Project Co-ordinator and nine animal technicians including the Named Animal Care and Welfare Office (NACWO). Staff were responsible for feeding, cleaning, behavioural observations, training, record keeping, reporting injury, and administering medications prescribed by the Named Veterinary Surgeon (NVS). Staff recorded signs of injury, illness, aggression, or any other welfare consideration, such as abnormal behaviours, into daybooks that were then entered into a large internal database. The database kept full records of events relating to an individual while at MRC-CFM including their date of birth, mother and father ID, date of weaning, group movements, all offspring including their dates of birth and weaning and dates of all previous health checks, veterinary visits, and medications. This information proved invaluable for collecting life history information about all the macaques involved in this study for inclusion in the statistical analysis. Life history data is presented in Appendix 2b.

Husbandry

Daily feeding occurred between 09:00 – 10:00 within the free roaming area. Feeding occurred later on health check and cleaning days as staff were either busy with the health check in the morning or could not access the macaques to feed during cleaning. The schedule shown in Table 2.2 was designed by the veterinary and management teams to meet all the macaques' nutritional requirements. Diet mixed was fed daily and consisted of specially formulated primate diet pellets mixed with oats, peas, and lentils.

Table 2.2. Feeding schedule for rhesus macaques at MRC-CFM.

Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Bread	Cucumber	Peppers	Eggs	Greens	Pears	Oranges
Diet mix	Diet mix	Diet mix	Diet mix	Diet mix	Diet mix	Diet mix
Apples	Tomatoes	Carrots	Bananas	Melons		

Each enclosure was cleaned over two days once a fortnight. Typically, the cage room was cleaned one day and the free roaming area the next. Macaques were shut into the adjacent room while cleaning occurred. Cleaning consisted of removing all the old straw and sawdust, washing the enclosure with disinfectant, allowing it to soak to soften any remaining dried-on faeces, rinsing the disinfectant and any additional waste away, drying and providing new bedding. The cleaning process took between four and six hours. Cleaning did not occur at the weekend or on bank holidays, but two technicians attended the centre to feed and check the macaques for injuries as well as administer any required medication. Training and behavioural observations did not occur on these days.

A veterinarian from the practice of the NVS visited MRC-CFM every Wednesday to perform routine inspection, prescribe medication, perform surgeries, and provide follow up care. Severe injuries or illnesses, such as fight wounds, were prioritised and ad-hoc visits were performed in emergencies as required.

Health screening

Each year at MRC-CFM, the macaques have a health check overseen by the NVS. As part of the annual health screening the macaques were sedated with an intramuscular injection of Ketamine Hydrochloride (KHCl: 0.1 – 0.2 ml/kg) for blood draw, weighing, a tuberculosis injection in the right eyelid and a rectal swab. For the subsequent two days, the macaques experienced additional rectal swabs; however, although separated from their group and restrained with the crush-back, they were not sedated prior to this procedure.

Research animals must be easily identifiable, therefore, each macaque was tattooed on their chest with an abbreviated three to four letterform of their name, for example, Valentine is VAL. Tattooing was done under sedation by a trained animal technician during the macaques' first annual health check.

All macaques involved in this study had prior experience of the health check and had received their identification tattoo during previous health screenings.

Assessing reproductive status

I recorded the reproductive status of each macaque for each trial. The male macaques were classed as either a breeding male or a weaner male. The breeding males were housed as the only male with between two and eight adult females and their offspring. The weaner males lived in single sex groups of between seven and 17 individuals aged between 12 and 30-months old. The female macaques were classed as cycling, pregnant, nursing or implanted with contraception. Pregnancy was often determined by the veterinarian following palpation of the abdomen during the annual health screening. However, this method was not suitable for the very early stages of pregnancy as the foetus was too small to feel. I determined the likely date of conception by retrospectively backdating from the date of parturition. The average gestation period for rhesus macaques is 163 days (Wolfensohn & Honess, 2005, p. 97); if any macaque gave birth within 163 days of a trial she was assessed as pregnant at the time of the trial.

The typical age at which juvenile rhesus macaques stop suckling and are nutritionally weaned is between 10 and 14 months old (Southwick et al, 1965; Lindburg, 1971; Harvey et al, 1987). Postpartum amenorrhea is an average of 11 months (Nieuwenhuijsen et al, 1985). A macaque was classed as nursing if they had nutritionally dependent offspring aged 11 months or less. All other female macaques who were not pregnant and had no offspring or had offspring older than 11 months were said to be cycling unless fitted with a Nexplanon 68 mg contraceptive implant. Information about contraceptive implantation was collected from the macaques' health records. The reproductive status of each macaque is shown in Table 2.1.

2.3 Training protocols

Establishing a PRT protocol at MRC-CFM

The training protocol is based on Kemp et al (2017) that had previously been developed and implemented at MRC-CFM. Briefly, Kemp and colleagues (2017) established a method of PRT for stationing group housed macaques. The method begins with a period of habituation (desensitisation) to the trainers; during this period, a clicker was established as a bridge. The next step involved training the most dominant animals first to sit by and hold their individual-coloured stationing tools using the verbal cue "hold". Training the male or high-ranking females first allowed later training with low-ranking individuals. Once the macaque was trained to hold the stationing tool for 30 seconds training moved onto the next individual and so on until the whole group was trained. When a macaque held the stationing tool for >30 seconds while the trainer worked with other animals in the group, they were deemed to have reached criterion for training success. Kemp et al (2017) had a success rate of 93.9% with 61 of the 65 individuals initially included successfully training.

The aims of training in the present study were to build a relationship of trust between the trainer and the macaques, establish a consistent signal for rewards (whistle as a bridge), ensure macaques would remain in one location for AB trials (station train) and to be able to collect saliva sample for hormone analysis.

Between September and December 2017, 86 macaques began training to be desensitised to my presence and the AB apparatus, sample collection, the apparatus and were familiarised with the use of a whistle rather than a clicker as a bridge for PRT. To establish the whistle as a bridge during the desensitisation training sessions, I would whistle before presenting the food reward if any macaque in the group approached the front of the enclosure. Desensitisation and familiarisation ensured reduced novelty during testing (Savasta et al, 2003; Samuni et al, 2014) and involved hand feeding with treats such as peanuts and raisins.

During the initial familiarisation and training sessions I collected information on each macaques' cage location (top, middle, or bottom level) and food (nut, raisin, juice, fruit) preference; this ensured that maximum progress was made in subsequent training sessions. I collected information on which monkeys could be stationed together and which had to be kept apart due to aggressive behaviour, for example, chasing. Chasing was particularly a problem with some of the younger breeding males who would chase the female if they saw them getting food rewards during training. It was also important to consider the strict matrilineal social hierarchy within rhesus macaque groups and ensure that lower ranking females were not made to station next to higher ranking, non-compatible individuals. Issues relating to chasing, aggression and dominance have been successfully managed in group housed NHPs using PRT (Schapiro et al, 2001; Veeder et al, 2009), therefore, this knowledge of intra-group relationships allowed the development of appropriate training plans that reduced food related aggression and allowed lower-ranking animals to participate.

To further reduce the risk of in-group aggression and increase the likelihood of lower ranking animals engaging with the training, MRC-CFM provided specially designed boards that could be inserted into the enclosure to provide a visual barrier between the monkeys without preventing them from leaving the testing area (Figure 2.5).

Figure 2.5. A visual barrier board, shown of the left side of the image, used to allow lower ranking macaques at MRC-CFM to engage with training and testing. Photograph: E. Howarth.

As higher-quality footage could be collected when the macaques were on the middle level of the cage room area, having these boards meant that more monkeys were willing to work in this area increasing the amount of high-quality footage and saliva samples that were collected.

Procedure to assess food reward preference

At MRC-CFM, peanuts and raisins were routinely used for training (e.g., Kemp, 2017). Foods with high sugar contents could not be used during data collection as the sugar interacts with salivary cortisol (Schwartz et al, 1998). Both raisins and dehydrated fruit contain between 58 and 65 g of sugar per 100 g of food (USDA, 2019ab). Therefore, cereal such as Kellogg's Rice Krispies, peanuts (USDA, 2019cd) and Robinson's no added sugar black current and apple diluted cordial (Britvic PLC, 2019) were used as rewards during the AB trials and saliva sample collection. Raisins and fruit could be used during training and a preference assessment was conducted to establish the macaques' preferred training and testing rewards.

A food preference test was conducted once for each macaque as previous studies have shown that higher preference food rewards controlled behaviour and improved training success more effectively than less-preferred food rewards (Clay et al, 2009a; Gaalema et al, 2011; Martin et al, 2018). Macaques were stationed and food items (raisins, black current or orange juice, peanuts, dehydrated fruit, for example, apples and bananas and cereals, such as Kellogg's Rice Krispies and Nestle Cookie Crisp) were presented by hand in pairs. The item chosen first was viewed as the higher preference item. Side preference was controlled for by repeating presentations with items being randomly presented on the left or right until the highest preference item was determined. The highest preference items for each macaque were noted in their training record. Generally, the macaques preferred black current and apple juice to orange juice, and peanuts, fruit and Cookie Crisp were found to be the higher preference food rewards.

Initially peanuts were thought to be more premium rewards than raisins and juice but quickly we realised that peanuts hindered training. The groups worked better with reduced competition and aggression and increased engagement with training when only raisins and juice were used. After

the removal of the routine use of high preference food rewards, I noticed a reduction in chasing behaviour and following of the trainer. As a result, premium food items were only used during training to reward specific rare behaviours.

Protocol for station training

Following familiarisation and desensitisation to my presence, the macaques were station trained to allow training for and collection of individual cognitive data and hormone samples from group housed animals. Station training involved encouraging the monkey to not follow the trainer or food but instead remain in one location next to their individual-coloured stationing tool (Kemp et al, 2017). The coloured stationing tools, examples shown in Figure 2.6, consisted of robust coloured objects that could be attached to the outside of the macaques' enclosure by a carabiner.

Figure 2.6. Example coloured stimuli for station training of rhesus macaques (*Macaca mulatta*) for AB testing. Photograph: E. Howarth.

Some of the macaques at MRC-CFM had previously been stationed trained by centre staff following the methods detailed in Kemp et al (2017). I used this to develop a shaping plan (protocol) to station train the remaining untrained monkeys that were involved in this study. The shaping plan broke down the training into smaller achievable steps to prevent the training becoming frustrating for both the humans and animals involved (Clay et al, 2009b; Kemp et al, 2017). Shaping plans have also been shown to improve training outcomes for animals with multiple trainers as they ensure consistency between trainers (Westlund, 2015) and allowed the animal care staff to continue the training when I returned to Liverpool John Moores University to run genetic analysis in January and February 2018. The final station training protocol is shown in Figure 2.7.

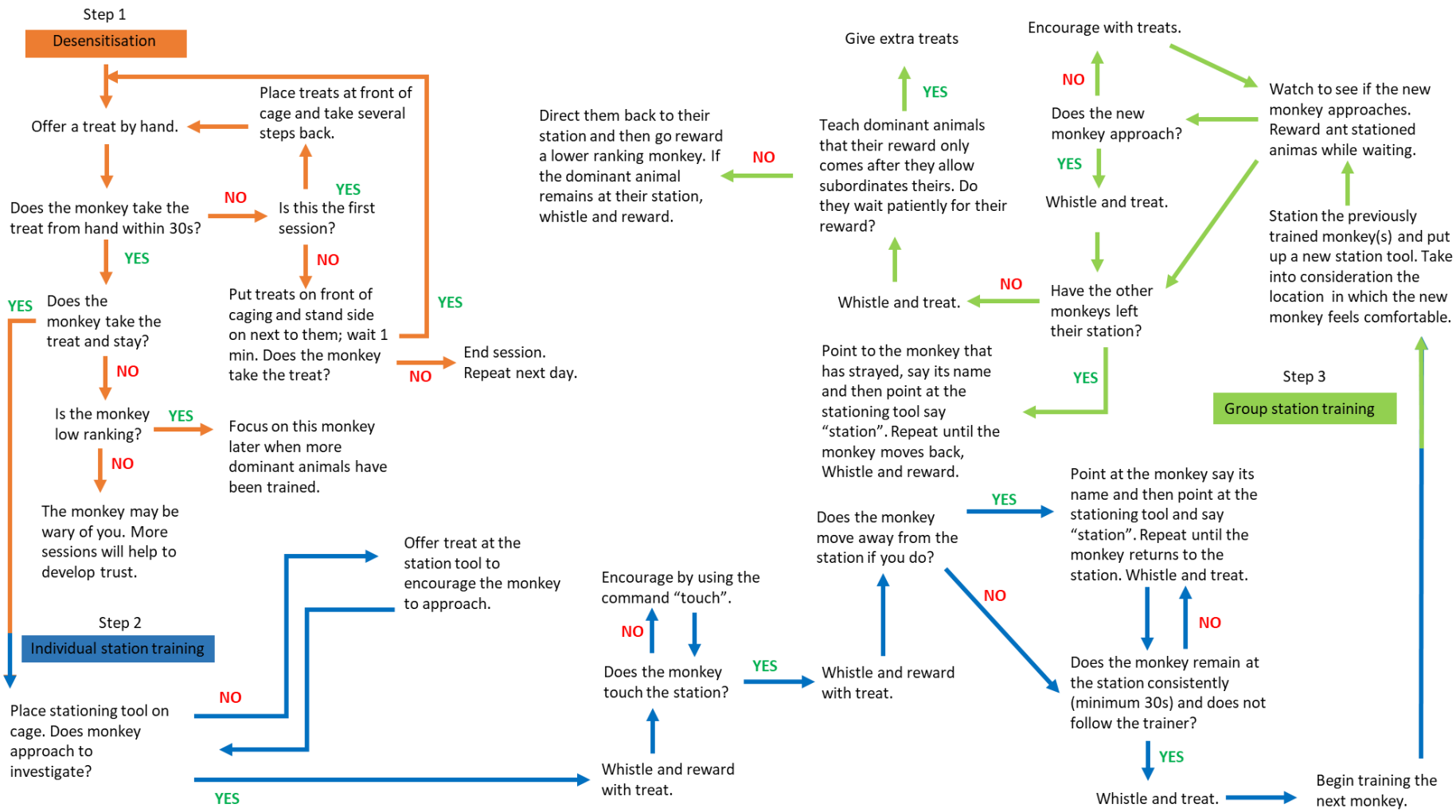

Figure 2.7. Station training protocol for rhesus macaques. Protocol was adapted from Kemp et al (2017).

AB apparatus design

A pilot study using a handheld manual device and stimulus flash cards was unsuccessful (Appendix 2a). Therefore, an automated AB apparatus was developed. Stimuli were digital jpeg files presented on two Eyoyo 8-inch TFT LCD colour video monitor screens. A Sony HC video camera, mounted on a T-bar tripod and positioned equidistant between the monitors, was used to record the macaques' eye movements to the stimuli that appeared simultaneously on the monitor screens (Figure 2.8). The monitors were connected via an HDMI and a UGREEN USB to HDMI external video card to an HP ENVY 15-ah150na laptop computer. Each stimulus on the screen measured 10.2cm x 18cm, thereby taking up 9.72 x 17.06 degrees of visual angle at a 60cm viewing distance. Previous AB trials by Bethell (2012a), Thatcher (2015) and Szott (2015) had relied on apparatus with a slider, which was removed to reveal the stimulus pairs introducing the potential for side bias. Simultaneous presentation of the stimuli on monitor screen should reduce this risk (Bethell et al, 2012b). A MATLAB program was designed by Dr Claire Witham to display the threat-neutral and filler stimuli. The display was designed so that the images filled the 8-inch screens and pictures measured approximately 10.2 cm x 18 cm. Face stimuli were randomly numbered and could be chosen from a drop-down list at the start of each trial. Random numbering of the face stimuli ensured that the researcher conducting the trial was blind to the side of aggressive face presentation, thereby reducing the potential for unintentional cuing effects. The inter-trial interval was kept constant at three seconds for all trials. The face stimuli would appear for three seconds followed by the inter-trial interval (three seconds of a black screen) and then three seconds of the filler stimuli. Filler stimuli included colour images of fruit and vegetables which the macaques are familiar with, find interesting to look at, and are presumably pleasant or neutral (Waitt & Buchanan-Smith, 2006). Following presentation of the filler stimuli, left and right fixation footage was collected using highly coloured attractive stimuli. The stimuli for left and right fixation were presented one of the other and provided a record of a definite right and left look to aid with later coding. The camera and macaque were in the same position as during the trial.

A Bush SP-925 Bluetooth speaker was connected centrally at the top of the apparatus. On presentation and removal of a stimulus pair an audible beep was produced to allow for easier coding of trial footage by identifying the start and finish of each trial on the video. The apparatus included a black screen to prevent macaques seeing the researcher during the trials (Figure 2.9). The camera display was open so that the researcher could, without making eye contact, observe the animal's direction of view and ensure centralised attention prior to commencing the trial. The apparatus was placed at a consistent distance from the bars by lining the feet of the tripod up with the edge of the metal drainage grate that ran the length of the cage room. Most macaques were recorded while on the middle level of the cage room enclosure; however, some lower ranking individuals preferred to station on the top level. For these macaques, the tripod could be adjusted so that the monitors and the camera could be moved up to be in line with their eyes. This flexibility allowed macaques to remain in their preferred location and reduced any stress caused by the trials.

A full AB apparatus desensitisation and training protocol is shown in Figure 2.10. This protocol for the collection of cognitive data in the form of 640 AB trials from 61 macaques.

Figure 2.8. Order of stimulus pair presentation for attention bias training trials with rhesus macaques. Top panel (A): filler (fruit or vegetable) stimuli. Middle panel (B): right fixation trial. Lower panel (C): left fixation trial. Left and right were initially recorded from the coders point of view to prevent accidental errors when coding. The left and right for each trial were later flipped to be relative to the monkey's view. Photograph: E. Howarth.

Figure 2.9. Attention bias apparatus viewed from the macaque's (left) and the researcher's (right) side. Photograph: E. Howarth.

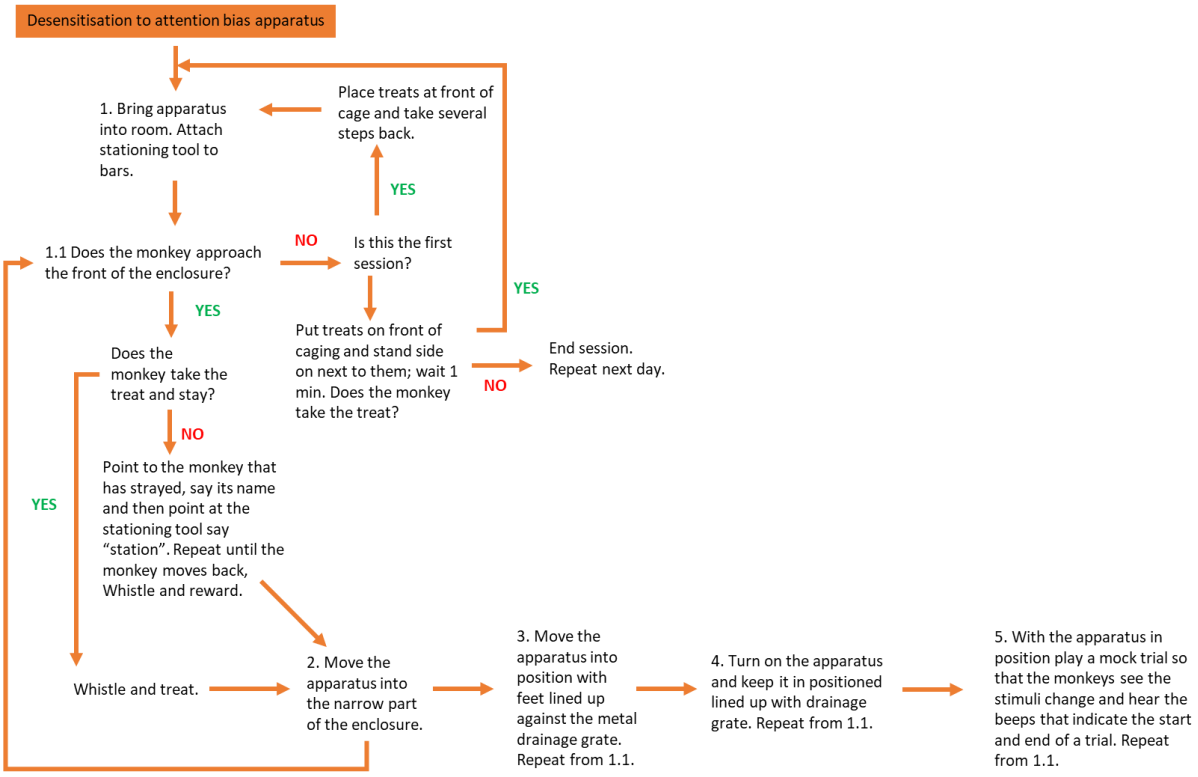

Figure 2.10. Attention bias apparatus desensitisation training steps for rhesus macaques.

Developing a protocol to collect cortisol samples

Saliva has a rapid response of between 20 and 30 minutes (Kirschbaum & Hellhammer, 1989) and the collection method allows for individual sample identification. Salivary cortisol content is highly correlated with serum cortisol levels (Wood, 2009) and saliva has previously been validated as a

suitable, low-stress, non-invasive alternative to serum for cortisol analysis in both NHPs (Boyce et al, 1995; Rapp-Santos et al, 2017) and other mammalian species (e.g., VanBruggen et al, 2011). Saliva was selected as the most appropriate biological substrate for collection of samples to be analysed for cortisol.

Saliva samples were collected using Salimetrics 8 mm polymer SalivaBio Children's Swabs (https://www.salimetrics.com/collection-method/childrens-swab-device/). Swabs are quality controlled, validated for cortisol recoveries, and verified for consistent performance and sample pH (Salimetrics, 2015). Following email advice, to ensure that it would not compromise the swabs, each swab was cut into two pieces of 62.5 mm length (Dr Lindsey Smith, Stratech Scientific Support and Quality Manager, personal communication, 16 November 2017).

A pilot study was conducted in July 2017 to develop the protocol for collecting saliva using a group of six female weaners who were not included in the AB study. I trialled three methods: swab held in hand and presented to the monkey, swab sewn into cotton (e.g., after Higham et al, 2010) and swab clamped in D-shackle. The monkeys were able to steal the swabs when held in a gloved hand and when sewn into cotton, so the swabs were clamped into D-shackles (Figure 2.11). Following the use of the D-shackle, far fewer swabs were stolen. Some determined macaques still managed but this was further reduced with training. Swabs clamped into D-shackles was the most successful method with no swabs being stolen during final sample collection.

The pilot group all chewed the D-shackle clamped swabs for at least 30-seconds, suggesting the macaques involved in the study would quickly familiarise and interact with the swabs. For successful analysis of salivary cortisol, 100 µl of saliva is required per swab from each monkey (Bertrand et al, nd). To achieve this a monkey must preferably chew for 60-seconds (Salimetrics, 2015) but ideally at least 30-seconds. Swabs were introduced to the study animals when sample collection training began in November 2017.

To start, swabs were soaked in black current and apple juice, as it had previously been found to be the preferred juice flavour during the preference test. However, to ensure the juice did not affect

salivary cortisol concentration, after two weeks of desensitisation and familiarisation the swabs were then soaked in a solution of 71.55% boiled tap water and 28.45% granulated sugar for at least three hours and then left to dry completely at room temperature. Newman et al (2007) previously found that a 28.45% granulated sugar solution does not affect salivary cortisol concentration in rhesus macaques.

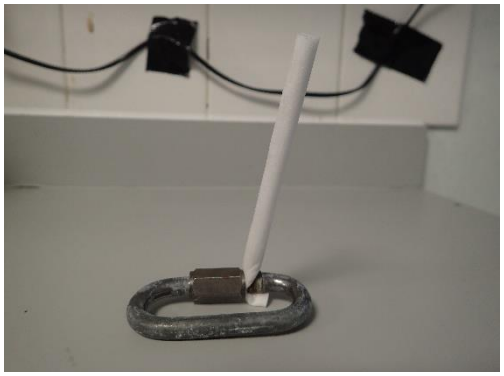

Figure 2.11. Salimetrics Children's Swab clamped into a D-shackle to prevent stealing during saliva sample collection. Photograph: E. Howarth.

The full training protocol for saliva sample collection is shown in Figure 2.12. This method of using D-shackle clamped, sugar-soaked swabs and a protocol of PRT allowed the non-invasive collection of 203 saliva samples from 31 monkeys for cortisol analysis.

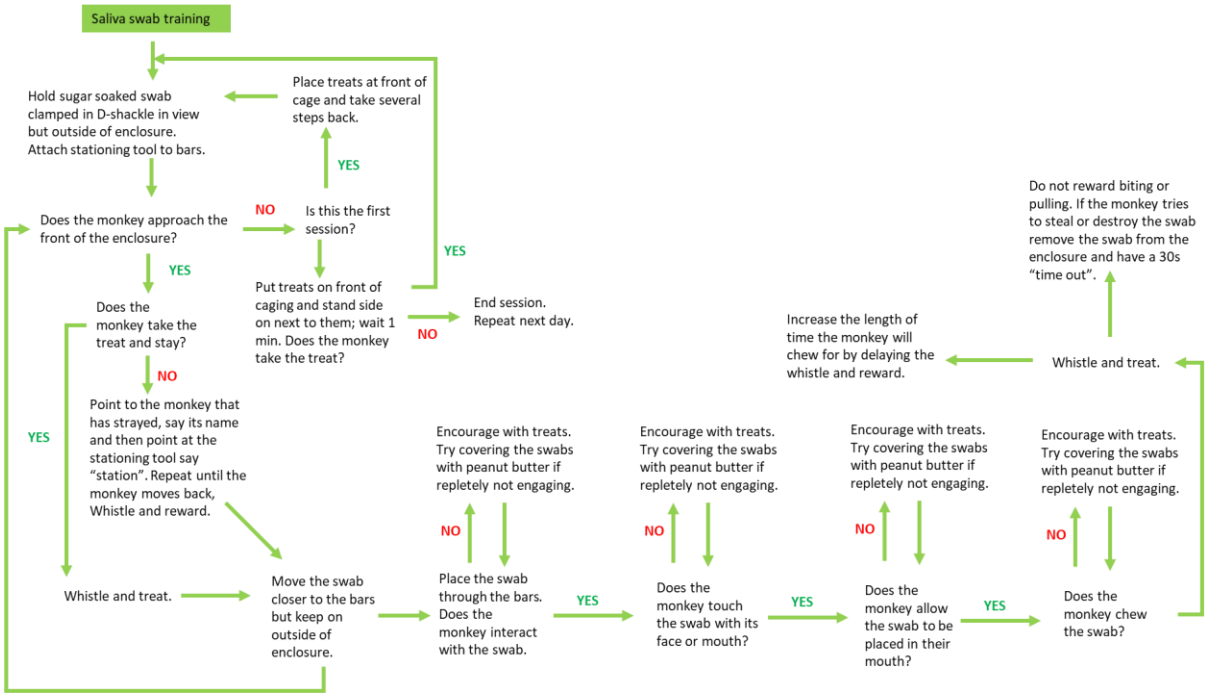

Figure 2.12. Saliva swab collection training steps for rhesus macaques.

The training methods described in this chapter allowed the collection of cognitive data from 61 rhesus macaques and saliva sample collection from 31 macaques. These data were crucial for completing this PhD and therefore the training method learned and developed here were instrumental in success of the project. The following chapters describe the analysis and context of the AB and cortisol data.

Chapter 3 – Validating AB as a measure of affective state: within-individual repeatability and between-individual variability

3.1 Abstract

Attention bias (AB) describes a tendency to differentially allocate attention towards one of two or more emotional stimuli. AB tasks have been shown to detect shifts in emotional state in humans and animals, including non-human primates, and have been proposed as a novel method of animal welfare assessment. Currently, there is little published data on the factors underlying AB in rhesus macaques (*Macaca mulatta*) and previous studies have focused only on males or females. For AB to be a suitable method of welfare assessment we must establish the influence of life-history and stress. Here, I aimed to determine if AB to threat changes following a stressor (Study 1) and if AB shows consistent differences between individuals (Study 2). AB trials were conducted with 61 (45 female, 16 male) adult rhesus macaques using a computer operated apparatus with threat-neutral conspecific face stimuli presented on screens. Duration of looking at these stimuli was recorded. Four measures were used in the analysis: duration looking at the threat face stimulus (THR), total duration looking at the threat and neutral face stimuli (TL), AB difference (ABDiff) score, and ABDiff/TL. ABDiff was calculated by subtracting the duration looking at the neutral face stimulus from the duration looking at THR. In Study 1, AB trials were conducted before and after the macaques' annual veterinary health check to determine whether AB changes with shifts in affective state caused by the presumably stressful veterinary intervention. In Study 2, AB trials were conducted once per week for eight weeks to assess the repeatability of the AB signal for each individual. Factors of interest included condition (baseline and post-stressor), sex, age, and time of day. In Study 1, there was a significant association between TL and time of day. Duration looking at social stimuli was greater at midday and dipped in the afternoon as is also seen in humans. In Study 2, repeatability of the AB signal (TL) was found to be 0.093 ± 0.243, which is within the range of the animal social behaviour and human AB literature. Study 2 revealed a relationship between AB and age in rhesus macaques with younger macaques having a significantly greater THR than older macaques. This study provides the first evidence for the association between AB measures and time of day and age in macaques and highlights that affective state, sex, age, and time of the AB trial should be included in the analysis in future AB studies.

3.2 Introduction

AB describes a tendency to differentially allocate attention towards one of two or more stimuli that vary in emotional content. The automatic allocation of attention to treat is an innate mechanism that works to enhance survival (Öhman et al, 1986, 2001a). For example, in order to avoid danger and protect the body from harm, humans in a fearful or anxious emotional state tend to be more vigilant towards threat (Mathews & MacLeod, 1994; Mogg and Bradley, 1998; Lang et al, 2000; Paul et al, 2005). The amygdala has been implicated in this automatic vigilance for, or AB to, threat through the rapid processing of threat-relevant cues (Anderson & Phelps, 2001; Davis & Whalen, 2001; Öhman, 2002, 2005). This neural mechanism has long been associated with emotion and the processing of aversive information (LeDoux, 1996, 2003). In humans with panic disorder, fMRI scanning has shown AB towards panic related words is associated with enhanced amygdala activity (van den Heuvel et al, 2005). Individuals with a damaged amygdala (bilateral amygdala lesions), do not show AB towards threat (Anderson & Phelps, 2001). The presentation of fearful faces or facial features results in greater activation of the amygdala compared to when happy or neutral face or facial features are shown (Whalen et al, 1998, 2004) indicating that the amygdala responds automatically to fear-relevant information (Cisler & Koster, 2010).

Allocation of attention has been proposed as a form of emotional regulation (Gross, 1998a, 2001, 2007; Koole, 2009). AB to threat may be moderated by emotion regulation strategies (Gross, 1998; Cisler & Kister, 2010). For example, the use of distraction techniques, such as discussion scenarios (Andrews & Shaw, 2010), significantly reduces pain-perception during peripheral venous catheterization compared to local anaesthetic (Balanyuk et al, 2018).

This innate mechanism has been utilised to develop AB tasks to assess emotion in human using paradigms such as the looking time task (Fantz, 1958), the dot-probe task (MacLeod et al, 1986), the visual search task (Green & Anderson, 1956) and emotional Stroop task (Stroop, 1935). Here, I first discuss the human literature for these paradigms and then the adaptation of these tasks for animal emotions and assessing animal welfare.

Looking time task

Looking time tasks are simple tasks that measure participants' direction of and shifts in eye-gaze to different stimuli (Fantz, 1958; Winters et al, 2015). The simultaneous presentation of two stimuli allows comparison of attention to the paired competing images (Desimone & Duncan, 1995).

Due to their simplicity, looking time tasks have been used to assess attention in human infants (e.g., DeNicola et al, 2013; Yeng et al, 2016). Studies have shown that infants (< one year old) are able to recognise the identity (Pascalis et al, 1998) and affect (Cohn & Tronick, 1983; Tronick, 1989) of adult human faces. Human faces are also significantly better at holding infant attention compared to age-appropriate toys but not at orienting attention (DeNicola et al, 2013). DeNicola and colleagues (2013) showed eight pairs of coloured face and toy images to 64 healthy four- to eight-month-old infants and recorded their looking time to each stimulus. The authors reported that the infants looked at the face stimuli for a longer duration than at the toy stimuli; however, there was no association between stimulus type and the direct of first look. This differentiation between attention orienting and holding was first suggested by Cohen (1972, 1976) who proposed that at least two attentional processes are involved in an individuals' attention to visual stimuli. In looking time tasks, both attentional processes can be studied; however, in other AB paradigms, such as the dot-probe task, only the attention-orienting component is assessed.

Dot-probe task

In a dot-probe task, two stimuli, for example threat-neutral words (Mogg et al, 1992) or facial expressions (Bradley et al, 2000; Roberts et al, 2010; Wabnitz et al, 2016), are presented on a screen (MacLeod et al, 1986; Yiend & Mathews, 2005; Van Rooijen et al, 2017). The stimuli are presented for a fixed duration and then disappear; one image is replaced with a target or dot-probe. The time to respond to the dot-probe is measured, with faster reaction times indicating that the participant's attention was already at that location while slower reaction times indicate that the participant's attention was at the other stimulus location. Dot-probe tasks have revealed a stronger attention to

threat in anxious and depressed humans (Reicher et al, 1976; MacLeod et al, 1986; Bradley et al, 1998; Peckham et al, 2010).

A dot-probe task was used by MacLeod and colleagues (1986) to assess the association between anxiety and speed of response in 48 human volunteers (24 clinically anxious). The participants were shown words with social or physical threat connotations or that had no threat connotations. Probes were detected significantly faster when they replaced a threat word compared to a neutral word in anxious individuals. The authors suggested an interference effect and performance deficit resulting from the volunteers' anxiety. This interference effect was greater for physical threat words than social threat words indicating that anxious humans may be particularly concerned with physical dangers and the potential impact on their physical health.

The use of emotional faces as alternatives to emotional words has been suggested as more biologically, ecological, or real-life relevant (Mansell et al, 1999). Mansell and colleagues demonstrated that socially anxious humans show an avoidant AB, away from emotional (negative and positive) faces, compared to non-anxious controls in a dot-probe task. This avoidant bias in attention was only evident following a social threat induction that included giving a speech for which participants had no time to prepare. Salum and colleagues (2017) conducted a dot-probe task with 1,872 irritable and non-irritable children with no known psychiatric or developmental disorders. The children were scored for their irritability using an established Child Behaviour Checklist (CBCL) and parental reports of irritable behaviours such as temper tantrums, sulking and mood swings. Salum et al (2017) reported a significant positive association between irritability and attention towards angry faces compared to neutral or happy faces. The authors concluded that this bias towards threatening information might contribute to chronic irritability (Salum et al, 2017), which has previously been associated with the development of anger and psychiatric disorders in later life (Krieger et al, 2013; Vidal-Ribas et al, 2016).

The dot-probe task measures the time taken to respond following the presentation of two stimuli. The visual search task also measures response time; however, this task involves the presentation of many distracting stimuli.

Visual search task

Visual search tasks can be used as a measure of AB using the time taken to find a target stimulus among an array of distracting stimuli (Dodd et al, 2017). Stimuli include different coloured shapes (Green & Anderson, 1956; Nityananda & Pattrick, 2013), fear relevant stimuli among irrelevant stimuli (Öhman et al, 2001a), abstract shapes (Marzouki et al, 2014) and faces among non-face images (Tomonaga & Imura, 2015). Facial expression and perceived threat affect response time in visual search tasks (Hansen & Hansen, 1988; Öhman et al, 2001b). Humans with specific phobias, for example, agoraphobia or ophidiophobia (fear of snakes), are quicker to find fear relevant stimuli, such as spiders or snakes respectively, among neutral or pleasant irrelevant stimuli (Öhman et al, 2001a).

Lundqvist & Öhman (2005) showed friendly and threatening cartoon face stimuli to humans in a visual search task. The emotional faces were presented on a screen within a grid matrix of identical neutral faces. The participants were faster and more accurate at detecting the location of threatening faces compared to the friendly faces. The authors suggested the emotional expression of the face stimulus could be used to predict attention and response times in visual search tasks. Belopolsky et al (2011) used the same cartoon face stimuli to test a delayed disengagement hypothesis. Participants indicated using eye movements the direction of tilt for each face stimuli. Indications were slower when a threatening face was shown compared to happy and neutral faces. The authors proposed that this delayed indication reflected delayed disengagement from the threatening face.

Some studies have suggested AB may be task specific. Dodd et al (2017) revealed that self-reported anxiety was only associated with AB for angry faces over happy faces in an emotion-irrelevant task. In their study, a visual search task was used to compare the reaction time of anxious and non-

anxious students (n = 42) when identifying the age or emotion of a face stimulus in two tasks: 1) emotion-irrelevant and 2) emotion-relevant. In both tasks, the students were shown old and young, happy, angry, and neutral faces. In the emotion-irrelevant task, they were asked to indicate the age of the target face while in the emotion-relevant task they were asked to indicate if the face was happy or angry. In the emotion relevant task, all participants (regardless of anxiety) were significantly faster to identify the happy faces compared to the angry faces. However, in the emotion-irrelevant task, anxious individuals showed an AB towards angry faces compared to happy faces and quicker reaction times to identify the age of these faces. The authors suggested that task relevance should be considered when making conclusions about the presence of anxiety-linked AB. However, I would also suggest that the order of testing might have influenced the results. The participants always took part in the emotion irrelevant task before the emotion relevant task, which may create an order effect and explain the lack of anxiety related AB within the emotion relevant task. Several human studies have shown there to be no order effect for task or stimuli presentation (e.g., Charash et al, 2006; de Fockert & Cooper, 2014), while others (e.g., Richards et al, 2013) have found a possible priming effect and influence of the order of presentation on response. Previous AB work at LJMU has suggested that the order effect may be an important factor in rhesus macaque studies (Szott, 2015; Thatcher, 2015). These studies found that individuals presented with filler stimuli before conspecific stimuli had reduced shifts in AB compared to those presented with the stimuli in reverse (conspecific then filler). Further, individuals tested post-stressor before baseline showed a different pattern of AB compared to those tested at baseline first.

Emotional Stroop task

The emotional Stroop task involves participants identifying the ink colour of negative and neutral words as accurately and quickly as possible (Stroop, 1935; Williams et al, 1996; Frings et al, 2010; Ben-Haim et al, 2014). It has been suggested that there may be an automatic allocation of attention to emotional stimuli (Frings et al, 2010). The emotional Stroop effect refers to participants'

tendency to be slower and more prone to error when naming the colour of negative or emotional words compared to those of neutral words (Frings et al, 2010).

Several studies have looked at the effect of anxiety on individuals' response to emotional and neutral words (e.g., Mathews, 1997; Williams et al, 1997; MacLeod et al, 1986). MacLeod et al (1986) asked participants to name the text colour of words that had either social or physical threat connotations or had no threat connotations. Clinically anxious individuals were slower to colour name all the words (threat and non-threat) than the non-anxious controls. While the non-anxious controls showed no difference in colour-naming speed between the threat and non-threat words, the anxious individuals were significantly slower to name the threat words compared to the non-threat words.

However, the extent to which emotional Stroop tasks measure attention deficit is unclear. Algom et al (2004) suggested that the performance deficit seen in the emotional Stroop task is a threat-driven generic slowdown. While Zhang et al (2015) reported that the delayed response of anxious students in tests that included emotional distractors (threat or examination related words) was not a universal deficit in attention but a situation-related defect in a single component of attention. The Stroop task has been criticised with some arguing that the fast or non-conscious component has little or no influence on emotional Stroop and slowing occurs due to interference of previously seen negative words on a participant's ability to name colours in the subsequent trial (McKenna & Sharma, 2004; Phaf & Kan, 2007). The task has been further criticised due to large across study variability due an inability to assess disengagement or facilitated attention (MacLeod et al, 1986; Fox, 2004; Ben-Haim et al, 2014).

AB & mood-congruency

Numerous studies that have shown that the allocation of attention to emotional stimuli changes with affective (emotional) state (Mathews & MacLeod, 1985; MacLeod et al, 1986; Fox et al, 2001; Bar-Haim et al, 2007; Mogg et al, 2008; McNally, 2019). AB to threatening stimuli is proposed to be a key component of the development and maintenance of anxiety disorders in humans (Rapee and

Heimberg, 1997; Mogg and Bradley, 1998; Schultz and Heimberg, 2008; Cisler and Koster, 2010; Hirsch & Mathews, 2012). Duschek et al (2014) reported a marked AB for negative words in patients suffering from fibromyalgia syndrome compared to healthy controls. The authors suggested that selective attention for emotional stimuli in patients with painful conditions hinders pain management through a vicious cycle between pain augmentation and negative affective state. Duque & Vazquez (2015) compared the orientation and maintenance of attention of 16 unmediated depressed and 34 never-depressed human participants to emotional faces (happy, angry, and sad). Depressed individuals had a significantly negative AB for sad faces compared to the never-depressed controls. The authors noted that this negative AB was only evident for sad and not angry faces indicating that it was specific to depression-related information.

AB & animal welfare

Non-human animals are used as models of human anxiety, therefore there is a chance they might 'experience' such states (e.g., rodents: Harro, 2018; primates: Coleman & Pierre, 2014). AB has been proposed as a novel method of animal welfare assessment (Paul et al, 2005; Bethell et al, 2012b; Crump et al, 2018). AB tasks have been shown to be capable of measuring animal emotion in NHPs (Bethell et al, 2012b; Marzouki et al, 2014; Allritz et al, 2016; Boggiani et al, 2018; Morin et al, 2019), birds (Brilot et al, 2009; Brilot & Bateson, 2012; Cussen & Mench 2014; Campbell et al, 2019a,b), sheep (Verbeek et al, 2014; Vögeli et al, 2014; Lee et al, 2016; McBride & Morton 2018; Monk et al, 2018ab, 2019ab; Raoult & Gygax, 2019), cattle (Lee et al, 2018), pigs (Luo et al, 2019) and rats (Parker et al, 2014).

The above animal studies compared the animals' attention to stimuli following a manipulation of their affective state. As discussed, in humans, changes in attention can be determined by measuring response time or response slowing in simple tasks with emotional distractors (Fox et al, 2001; Bishop et al, 2004; Mauer & Brokenau, 2007; Mogg et al, 2008; Holmes et al, 2009). In animals, attention has been measured by comparison of latency to detect and orientate towards the stimuli (e.g., Lou et al, 2019), approach an object (e.g., Verbeek et al, 2014) and eat (e.g., Campbell et al,

2019a), reaction time to complete a task (e.g., Allritz et al, 2016), head position duration (e.g., Monk et al, 2018a) and eye gaze (Bethell et al, 2012b). Stimuli were species-relevant and included alarm calls (e.g., Brilot & Bateson, 2012), novel objects (e.g., Verbeek et al, 2014), predators (e.g., Lee et al, 2016) and aggressive conspecific (e.g., Vögeli et al, 2014). Affective state was manipulated using housing conditions (e.g., Parker et al, 2014), veterinary inspection (e.g., Bethell et al, 2012b), food deprivation (e.g., Verbeek et al, 2014) or removal of objects necessary for species-typical behaviour (e.g., Brilot & Bateson, 2012). Affective state without manipulation was also determined using behavioural observation (Marzouki et al, 2014) and personality assessment (Cussen & Mench, 2019).

As with humans, these tasks can be grouped into four main method categories: looking time tasks (e.g., Bethell et al, 2012b), the dot-probe task (e.g., Verbeek et al, 2014), visual search tasks (e.g., Marzouki et al, 2014) and the emotional Stroop task (e.g., Allritz et al, 2016). Each method is discussed below with examples from the NHP literature.

Looking time tasks

Bethell et al (2012b) used emotional faces (threat-neutral male conspecific face pairs) and emotional state manipulation (veterinary inspection and enhanced enrichment) to adapt the looking task for measuring AB in NHPs. The looking time of rhesus macaques to the threat and neutral face stimuli were recorded and AB was calculated by subtracting the duration looking at the neutral face from the duration looking at the threat face. The procedures involved in the veterinary inspection used by Bethell et al (2012b) had previously been shown to be acutely stressful and compromise welfare (Ruys et al, 2004; Heistermann et al, 2006; Bethell et al, 2012a). The enhanced enrichment phase included food enrichment designed to enhance exploratory behaviours. Bethell et al (2012b) demonstrated that negative affect influences looking patterns as, although during both conditions the macaques showed initial vigilance for the threat face stimuli, following the veterinary inspection the initial vigilance was followed by rapid and sustained avoidance. During the period of enrichment, they maintained their gaze towards the threatening stimulus. Macaques

were significantly quicker to look at the threat face first than the neural face first during the period of enrichment but not following the veterinary inspection. The macaques were also quicker to disengage their gaze from the threat face and had a lower total duration of looking at the threat face after the veterinary inspection than during the period of enrichment. The authors suggested that the results indicated a rapid vigilance for threat. Rapid vigilance in humans and non-human animals is evidence of a threat-detection system that is independent of emotional-state and occurs automatically at the early stages of detection and processing (Öhman & Mineka, 2001; Öhman, 2002; Green & Philips, 2004; Holmes et al, 2009). Enhanced threat detection has a selective advantage (Davey, 1995). Following the veterinary inspection, the rapid vigilance was followed by avoidance of the threat face (Bethell et al, 2012b). In macaques, sustained eye contact is a threatening display so, by avoiding eye contact with the threat face the already anxious macaques may have been attempting to deescalate the threatening display and avoid aggression (van Hooff, 1967; Preuschoft, 2000). This avoidance of eye contact led to further study by Thatcher (2015) who compared the AB of rhesus macaques to threat and neutral stimuli with closed and open eyes.

Thatcher (2015) used a similar methodology to Bethell et al (2012b) with face stimuli presented on cards with a sliding door apparatus before and after the macaques' annual veterinary health check. Attention to three face pairs was compared: eyes open neural & aggressive (EO/Ag), eyes closed neutral & aggressive (EC/Ag) and eyes closed neutral & eyes open neutral (EC/EO). Macaques had a significantly greater AB towards EO/Ag than EC/Ag and EC/EO. Within the EO/Ag and EC/Ag combinations, macaques were more attentive to the aggressive face. In EO/EC, macaques were more attentive to the eyes open face than the eyes closed face. The largest differences in AB would be between the aggressive face and the eyes closed neutral face. Further, Kotani et al (2017) revealed a particular attention towards eye regions of face stimuli in common marmosets using an eye-tracking tool. This suggests that the combination of eyes closed neutral and eyes open aggressive would be the most effective for further AB trials with NHPs.

The dot-probe task

The dot-probe task has been used with NHP species including bonobos (*Pan paniscus*; Kret et al, 2016) and macaques (King et al, 2012; Koda et al, 2013; Parr et al, 2013). Kret and colleagues (2016) presented images of bonobos and control animals to four female bonobos. The bonobo images were neutral or showed bonobos in distressing, stressful or positive situations. Bonobos were faster to tap the screen following a picture of an emotional bonobo compared to a neutral bonobo with bonobos' reaction time being correlated with the emotional intensity of the image i.e., the bonobos tapped faster for very emotional images. However, the authors found no significant difference between positive and negative emotional images. This may reflect the equal importance of pro-social and threatening social interaction in bonobo society such as sex (Manson et al, 1997) and grooming (Vervaecke et al, 2000) and highlights the importance of including species-specific emotional stimuli in AB tasks.

In macaques, the presentation of new-born macaque faces in a dot-probe task resulted in no significant difference in attentional capture compared to neutral adult face stimuli (Koda et al, 2013). Koda and colleagues (2013) showed 10 conspecific face images (five adult and five new-born) to two Japanese macaques (*Macaca fuscata*). However, the authors did find that macaques were significantly faster to touch the probe when a visual cue (any conspecific face) was shown compared to when no visual cue (no face) was shown. This suggests that although there is no difference in adult and juvenile faces in terms of attention capture, conspecific faces have a larger attention-orienting effect than a blank screen.

King and colleague (2012) presented threat face stimuli to macaques, which were significantly faster to respond when the dot appeared behind a negative face compared to neutral. This effect was only seen at baseline. The authors administered testosterone to six male rhesus macaques expecting an increase in attention to negative social stimuli; yet this effect was not seen. Following treatment there was no significant difference in reaction time to dots following negative or neutral stimuli. The authors suggested that this might be a habituation effect as all baseline trials were

conducted prior to those with the testosterone treatment. They concluded that repeated exposure during the baseline resulted in habituation to the stimuli mitigating the effects of testosterone. King et al (2012) used a large stimulus set of 144 images including 24 negative and 24 positive images. This is much larger than other studies (e.g., Koda et al, 2013; n = 5 adult, 5 new-born) suggesting that the results presented in King et al (2012) are not the result of a habituation effect.

Visual search tasks

A visual search task has been used in chimpanzees (*Pan troglodytes*; Tomonaga & Imura, 2015). Tomonaga & Imura (2015) showed unfamiliar conspecific faces as well as distractor images such as a house or a car to three adult chimpanzees. The chimps were more accurate and quicker to select the conspecific face than the distracting images and were significantly quicker to detect the front-view faces than faces in profile and inverted and scrambled faces when searching for faces among non-face stimuli. This is congruent with human studies where the perception of emotional facial expressions is affected by horizontal tilt and head orientation (Hess et al, 2007). Images of forward-facing angry expressions had higher signal values than images with left or right orientation.

Marzouki et al (2014) reported that baboons (*Papio papio*) had a slower reaction time in a visual search task following the occurrence of negatively valenced behaviour compared to neutral or positively valenced behaviour. The authors observed the behaviour of six male baboons using instantaneous sampling for three 30-minute observation sessions for eight days (24 sessions, 720 minutes per baboon). The baboons had unrestricted access to the computerised task (touch the T-shaped target stimulus among seven L-shaped distractors). Response times were correlated with the occurrence of positive (e.g., play, allogrooming, lip smack) and negative behaviour (e.g., body shake, fear scream) revealing a significantly slower response time following negative behaviour than positive and neutral behaviour. These data were unbalanced with many of the negatively valenced behaviours having no matched reaction time data. The study included 41 reaction times matched with negative behaviour and 7,335 reaction times matched with neutral or positive

behaviours. This imbalance suggests a type I error (Columb & Atkinson, 2015) in the results of this study and highlights the importance of using balanced categories for meaningful statistical analysis.

The emotional Stroop task

The emotional Stroop task may be modified for use in animals, as the method does not rely on self-report by the participant (Baker & Brandon, 1990; Ben-Haim et al*,* 2014). A modified emotional Stroop task was used by Allritz et al (2016) to assess the relationship between cognition and emotion in chimpanzees. Seven (four female, three male) chimpanzees were trained to select a target stimulus (image with a yellow frame) next to a distractor stimulus (image with a blue frame). The chimpanzees were then shown images of caretaker, stranger and veterinarian humans as the target and distractor stimuli. Response accuracy was lower when veterinarian human stimuli were shown compared to control images. The presentation of veterinary human images also resulted in a longer response latency compared to control, caretaker, and stranger stimuli. The authors reported a strong correlation between response time to veterinary human images and time since last anaesthetisation compared to control and stranger stimuli. Response time and time since last anaesthetisation also correlated with the chimps' behavioural response to the veterinary stimuli. Animals anesthetised in the last six months showed an emotional reaction (vocalisation, refusal of food rewards, backing away and hitting/kicking the screen) compared to animals that had been anesthetised between six and 35 months prior to the study. Allritz et al (2016) concluded that the emotional Stroop task could easily be adapted for NHPs. However, the conclusions of the study (issues with individual variation in response and limitation with interpretation of the effect) highlight the importance of a pre-treatment baseline as animals must be their own controls in these highly variable cognitive tasks. Further, emotional Stroop tasks require a considerable period of training prior to cognitive testing, for example, Allritz et al (2016) used a minimum of 40 training session per chimpanzee indicating that this method may not be suitable for rhesus macaques in the time frame available for the present study.

Repeatability

For AB to be included within welfare assessment the measure needs to meet several criteria including providing high repeatability for multiple readings under identical conditions (Bland & Altman, 1986; Bartlett & Frost, 2008; Kilkenny et al, 2010). Repeatability refers to the variation in repeated measurements made on the same individual using the same method. There is an assumption that the measurements are made under identical conditions by the same researcher over a short period of time (Bartlett & Frost, 2008). The current lack of repeatability data for AB measures in the human or animal literature has been highlighted as a threat to understanding the theory underlying AB (Rodebaugh et al, 2016). An understanding of the extent to which a measure shows within and between individual variability is vital for adapting methods to improve the utility of the measure.

The effect of individual variation on behaviour and cognition is important for the interpretation of study results. A meta-analysis of 759 estimates from 114 studies of animal social behaviour including 98 species reported that the repeatability of social behaviour is significantly greater than zero and that 37% of variation in behaviour between individuals could be attributed to individual differences (Bell et al, 2009). Bell et al (2009) revealed an effect of sex on the repeatability of behaviour with male behaviour being significantly more repeatable than female behaviour across their entire data set (759 estimates, M = 0.5, F = 0.41). However, when mate preference behaviour was excluded, female behaviour was significantly more repeatable than male behaviour (611 estimate, M = 0.40, F = 0.47) suggesting that behavioural domain is important for understanding sex differences. A further meta-analysis has assessed the repeatability of performance in cognitive tasks (e.g., mechanical problem solving, recognition, memory) for 44 studies on 25 species (Cauchoix et al, 2018). The authors reported that there is a consistent repeatability of cognitive measures (0.15 - 0.28).

Repeated cognitive bias trials have revealed no consistency in performance between trials and may be affected by learning (Brilot et al, 2010; Doyle et al, 2010; Carreras et al, 2015). Repeated trials

result in a reduction in latency to respond or rate of response in birds (Brilot et al, 2010) and sheep (Doyle et al, 2010). This suggests rapid learning of the meaning of the ambiguous cue (Perdue 2017). Carreras et al (2015) trained 36 piglets on a go/no-go discrimination task. Buckets were positioned to the left and right of the pen with free access to apples as the rewarded reinforcer and a wire mesh over a bucket of apples as the unrewarded reinforcer. The piglets were then tested once at 10 weeks and again at 15 weeks with a bucket placed centrally as the ambiguous cue. In both trials a high number of piglets were classified as having a positive cognitive bias; however, there was no consistency in performance between trials for which individual piglets had a positive cognitive bias (Carreras et al, 2015). The authors suggested that the piglets remembered the content of the bucket from trial 1 to trial 2.

Unlike cognitive bias measures, the repeatability of the AB measures will not be affected by learning as AB relies on an innate mechanism. In human AB studies, repeatability is between 0.025 and 0.59 (0.45: Bar-Haim et al, 2007; 0.09 – 0.59: Waechter & Stolz, 2015; 0.025 – 0.312: Van Bockstaele et al, 2018). Therefore, I predict the repeatability of the present study to be within a similar rage.

The present study will use a refined method of Bethell et al (2012b; male only) and Thatcher (2015; female only) and automated, computer operated apparatus (compared to a manually operated apparatus used by Bethell et al (2012b) and Thatcher (2015)). Here, male and female macaques were included to assess the effect of sex on AB. This chapter aims to answer two questions:

1. Does AB to threat change following a stressor?

AB trials will be conducted before and after the macaques' annual veterinary health check to determine if the measure is capable of detecting changes in affective state caused by veterinary intervention (Study 1).

2. Does AB show consistent differences between individuals?

AB trials will be conducted once per week for eight weeks to assess the repeatability of the AB signal (Study 2).

3.3 Materials & methods

Ethics

Ethical approval was granted by Liverpool John Moores University (LJMU) in February 2017 (Ethical approval ID. EB_EH/2017-5) and by the Medical Research Council Animal Welfare and Ethical Review Body (AWERB) in November 2017. This project piggybacked onto routine veterinary and husbandry activities that would have occurred whether or not the animals were involved in this study. No regulated procedures were carried out for this study. Analgesia was not delayed because of any research relating to this PhD. All training was conducted following centre protocol and using positive reinforcement methods. Participation in training and AB trials was voluntary, insofar as animals were free to leave the training and testing area (cage room) at any time. Food, water, and social contact with conspecifics were available *ad libitum* throughout training and testing.

Animals & housing

The full description of animals and housing is given in Chapter 2. In brief, 61 (45 female, 16 male), adult (>3 years old) rhesus macaques (*Macaca mulatta*) socially housed at the Medical Research Council Harwell Institute Centre for Macaques (MRC-CFM) were involved in AB trials for this PhD thesis. At the time of testing, macaques selected for inclusion ranged in age from 3.50 to 16.42 years with a mean age of 8.45 ± 3.50 years.

Attention bias experimental design

Apparatus

The AB apparatus consisted of two Eyoyo 8-inch TFT LCD colour video monitors (Figure 3.1). Monitors were connected via an HDMI and a UGREEN USB to HDMI external video card to an HP ENVY 15-ah150na laptop computer. Each stimulus on the screen measured 10.2cm x 18cm, thereby taking up 9.72 x 17.06 degrees of visual angle. The apparatus was positioned so that stimuli were presented at a consistent distance from each macaque (60 cm). A MATLAB (MATLAB 9.3, 2017) program displayed the stimuli. A Sony HD video camera, mounted on a T-bar tripod and positioned

equidistant between the monitors, was used to record the macaques' eye movements to the stimuli that appeared simultaneously on the adjacent monitors. Simultaneous presentation of the stimuli on monitor screen should reduce the potential for side bias compared to previous AB trials where stimuli were revealed using sliding doors (Thatcher, 2015). The movement of the doors may capture initial attention and alter the bias towards the face stimuli, as distracting movement is known to alter attention in a task (Tipper et al, 1998).

Stimuli

Stimuli were digital jpeg files. Macaques were shown pairs of images of unfamiliar male conspecific faces (face stimuli; Figure 3.2) from a stimulus set of seven fix-paired neutral (mouth and eyes closed) and threat (mouth and eyes open) faces (Witham & Bethell, 2019). Images had previously been opportunistically collected at the Caribbean Primate Research Centre, Puerto Rico in 2006 (Bethell, 2009). In an earlier validation study, Thatcher (2015) demonstrated that the combination of eyes closed neutral and threatening stimuli resulted in the largest difference in AB. For each pair of images, a mirror image was created so that the faces would point inwards towards the centre thereby reducing orientation and side bias. Each pair was also duplicated and flipped to allow the threat face to be presented on both the right and the left producing a final stimulus set of 14 images (Figure 3.2). The face stimuli were randomly allocated to each macaque to prevent habituation to the test with even randomisation of the side of threat face presentation. Each face pair was numbered so that the researcher conducting the trial was blind to the side of the threat face thereby reducing the potential for unintentional cuing effects. Following face stimuli presentation, fruit or vegetable images (filler stimuli), for example, bananas, peppers, or peanuts were shown. For each trial, the same filler stimulus was shown on both the left and right. The filler stimuli prevented the macaques developing a negative association with the test, as the final image was a pleasant or neutral image. The full stimulus data set can be found in Witham & Bethell (2019).

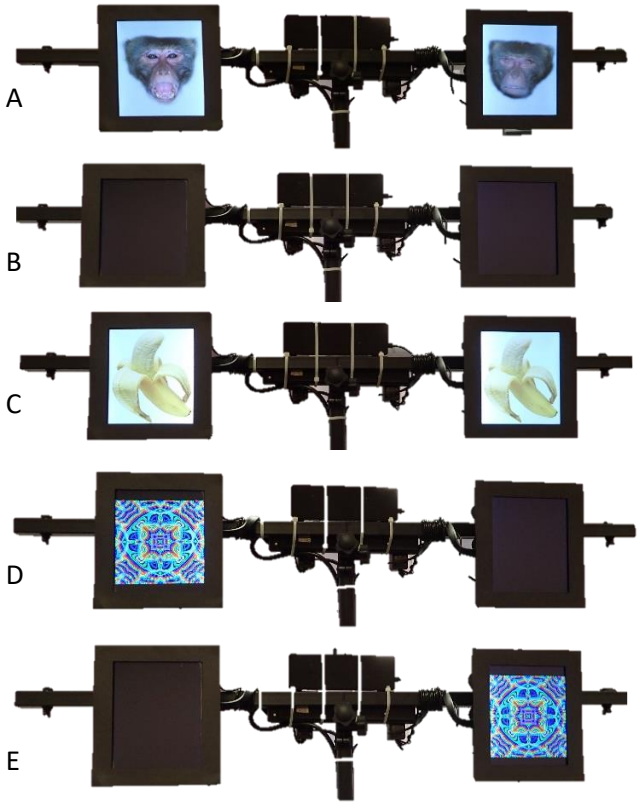

Figure 3.1. Order of stimulus pair presentation for attention bias testing in rhesus macaques. A) Face (threat-neutral conspecific face pair) stimuli, B) inter-trial interval, C) filler (fruit or vegetable) stimuli, D) left fixation, E) right fixation. Each stimulus was presented for 3 seconds. Photograph: E. Howarth.

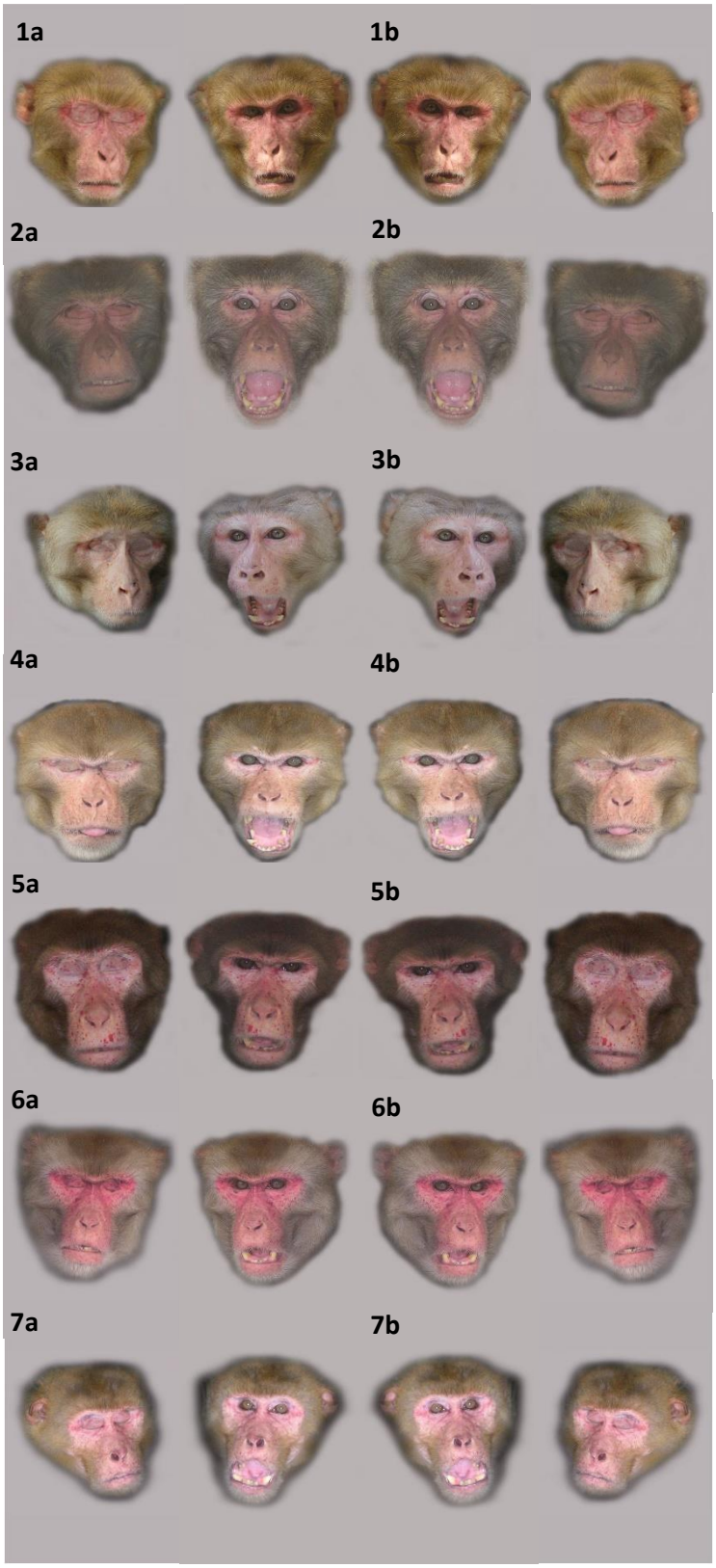

Figure 3.2 Threat-neutral stimuli used in attention bias testing for rhesus macaques (*Macaca mulatta*; Bethell, 2009; Witham & Bethell, 2019). The threat face had eyes and mouth open and the neutral had eyes and mouth closed. The stimuli were numbered 1-7 with a and b for each being the mirror images.

Procedure

At the start of the trial, the researcher selected the predetermined number for that monkey from a drop-down list in MATLAB. A trial was triggered via the MATLAB display on the HP laptop computer (Figure 3.3). The face stimuli appeared simultaneously on the two screens for three seconds (Figure 3.1A) followed by an inter-trial interval (three seconds of black screen; Figure 3.1B) and then a pair of filler stimuli (Figure 3.1C) were automatically presented for three seconds. Following presentation of filler stimuli, left and right fixation footage was collected using highly coloured attractive stimuli (Figure 3.1DE). The camera and macaque were in the same position as during the threat-neutral stimuli trial; this provided a record of a definite right and left look to aid with later coding. A Bush SP-925 Bluetooth speaker positioned centrally at the top of the apparatus made an audible beep at stimulus onset and offset to identify the start and finish of each trial on the video. This allowed easier coding of trial footage by identifying the start and finish of each trial on the video.

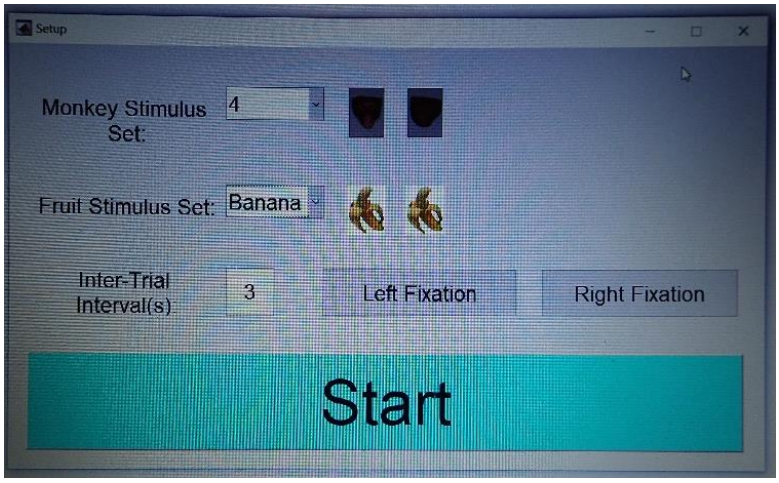

Figure 3.3. MATLAB program user interface for attention bias trials in rhesus macaques. A drop-down list allowed stimuli to be selected for training and test trials. Photograph: E. Howarth.

The apparatus included a black screen to prevent macaques seeing the research during the trials. The camera display was open so that the researcher could observe the animal's direction of view and ensure centralised attention prior to commencing the trial without making eye contact. The apparatus was placed a consistent distance from the bars by lining the feet of the tripod up with

the edge of the metal drainage grate that ran the length of the cage room. Most macaques were recorded while on the middle level of the cage room enclosure; however, some lower ranking individuals preferred to station on the top level. For these macaques, the tripod could be adjusted so that the monitors and the camera could be moved up to be in line with their eyes. This flexibility allowed macaques to remain in their preferred location and reduced any stress caused by the AB trials.

Study 1 - Does AB to threat change following a stressor?

Study 1 aimed to determine if AB is capable of detecting changes in affective state following a stressor.

Animals

Study 1 involved 36 macaques (27 female, nine male, mean age = 7.99 ± 3.04 years, range = 3.5 to 16.17 years). All macaques involved in Study 1 were naïve to AB testing. Macaques were housed in 10 social groups. Thirty-five macaques were from nine breeding groups with one adult male, between three and eight adult females and their offspring. One macaque was from an all-female ex-breeding group with seven adult females and their offspring from previous years. Life history and training information are given in Appendix 2b and 2c, respectively.

Stressor

This study piggybacked onto veterinary and husbandry activities that would have occurred whether or not the animals were involved in this study. The macaques' annual health check was used as the stressor. All macaques underwent a health check, which was overseen by the Named Veterinary Surgeon (NVS). This involved sedation with an intramuscular injection of Ketamine Hydrochloride (KHCl: 0.1 – 0.2 ml/kg) for blood draw, weighing, a tuberculosis injection in the right eyelid and a rectal swab. For the subsequent two days, the macaques received daily rectal swabs for which they were separated from their group and restrained with the crush-back. Restraint with the crush-back has previously been shown to be stressful for NHPs (Sainsbury et al, 1989; Meyer & Hamel, 2014).

All health checks occurred on a Monday with rectal swabs on the subsequent Tuesday and Wednesday.

Procedure

AB trials were run before and after the macaques' health check during baseline and post-stressor conditions, respectively. All baseline trials were conducted before the post-stressor trials. The baseline condition (assumed non-anxious state) was timetabled so that trials occurred in weeks during which there were no activities planned which were deemed potentially stressful i.e., no cleaning, animal removals, or planned veterinary procedures. The post-stressor condition was timetabled so trials occurred on the five working days following the scheduled health check (Tuesday – Monday). AB trials were conducted one per day per macaque on four consecutive weekdays from Tuesday to Friday, and then a fifth trial was conducted on the following Monday.

Baseline trials occurred between 9:33 and 14:54 (mean = 11:37 ± 1hr 33 min, median = 11:17, mode = 10:25). Post-stressor trials occurred between 08:26 and 16:28 (mean = 11:37 ± 1hr 33 mins, median = 11:17, mode = 10:25) with 86.5% of post-stressor trials conducted between 9:33 and 14:54. During the post-stressor condition, trials had to fit around veterinary visits, rectal swabs and enclosure cleaning. Trials conducted before 09:00 (n = 2) and after 16:00 (n = 4) were not included in the statistical analysis.

Study 2 - Does AB show consistent differences between individuals?

Study 2 aimed to determine the repeatability of the AB measures for each macaque.

Animals

Study 2 involved 37 macaques (25 female, 12 male mean age = 9.22 ± 3.82 years, range = 3.75 to 16.42 years). Twelve macaques (seven female, five male) had previously been involved in Study 1 and eight macaques (one female, seven male) were naïve to AB testing. Macaques were housed in 10 social groups. Twenty-eight macaques were from eight breeding groups with one adult male, between three and eight adult females and their offspring. Four macaques were from an all-female

ex-breeding group with seven adult females and their offspring from previous years. Five male macaques were housed in an all-male weaner group. Life history and training information are given in Appendix 2b and 2c respectively.

Procedure

AB trials were run once per week per macaque for eight consecutive weeks to determine the repeatability of AB in a presumed low stress state and check for habituation to the trials. All trials were at least four days apart with the day of testing varying to avoid other veterinary, husbandry or management activities occurring at MRC-CFM. Trials occurred between 09:32 and 15:46 (mean = 11:34 ± 1hr 29 mins, median = 11:16, mode = 10:25) with 93.5% of trials conducted between 09:30 and 15:00. Trials conducted after 15:00 were delayed due to enclosure cleaning and/or the delays with macaque performance in morning trials affecting the afternoon.

Video editing and coding

Trials were edited from the footage using Open Shot Video Editing software (Thomas, 2012). Each trial clip was labelled with a title slide that stated the macaque's name, the date, the trial number, and the stimulus ID, for example, Wine BL3 17.05.18 (s12). Following the title slide was a clip with the researcher stating the same information and then one second of a blank, black screen. The AB trial footage comprising 3 seconds of face stimuli, the inter-trial interval and filler stimuli started immediately after the blank screen. The left and right fixation trials were included at the end of the clip to aid with coding.

For blind coding, the duration of each gaze behaviour from the ethogram of previously defined behaviours (Table 3.1) was continuously coded for the three seconds of face stimuli trial using Behavioral Observation Research Interactive Software (BORIS; Friard & Gamba, 2016). For coding purposes right and left were of the coder not of the monkey as this reduced the likelihood of coding mistakes. Inter-observer reliability tests were conducted in March 2018 between Emmeline Howarth (EH) and Caralyn Kemp, who had previous experience of AB trial coding, with a Cohen's

kappa coefficient score 0.85. The same researcher (EH) coded all AB data presented in this PhD project.

Table 3.1. Gaze ethogram for rhesus macaques (*Macaca mulatta*) for AB testing. The stimuli were presented to the left and right of the camera.

Code	Behaviour	Description
0	First look coder's right	First time the animal looked at the stimulus on the right-hand side of the screen
1	First look coder's left	First time the animal looked at the stimulus on the left-hand side of the screen
A	Away	The animal looks at a point that cannot be classed as any of the other 'away' categories
B	Baby	The animal looks at- and sometimes huddles its baby
C	Central	The animal looks at a point between the two stimuli
D	Away down	The animal looks down towards the apparatus but not at the stimuli
I	Away up right	The animal looks to the top right corner of the room, but not at the stimuli
J	Away up extreme	The animal looks centrally above itself, turning its head up so the chin is facing up
K	Away left	The animal looks away to the left-hand side of the room
L	Look left	The animal looks at the stimulus in the left side of the screen
N	Away down extreme	The animal looks down towards the floor and turning its head down, so the top of the head is facing the camera
O	Out of view	The eyes cannot be seen, and the direction of gaze is not obvious
R	Look right	The animal looks at the stimulus to the right side of the screen
T	Away right	The animal looks away to the right-hand side of the room
U	Away up central	The animal looks upwards, but not at the apparatus or stimuli
Y	Away up left	The animal looks to the top left corner of the room, but not at the stimuli

Data treatment

For each trial, the total duration of each looking behaviour towards the left and right stimuli was entered into a spreadsheet. This left and right looking data were subsequently cross-referenced with the record of whether the threat face stimulus was presented on the right or the left. The duration looking at each of the threat face (THR) and neutral face stimuli were then calculated,

throughout each 3-second trial. Total looking time (TL) was calculated by adding together the duration looking at threat and neutral face stimuli. Attention bias difference (ABDiff) was calculated by subtracting the duration looking at the neutral face stimulus from the duration looking at the threat face stimulus. AB proportion was calculated as ABDiff/TL. These values were used in the analysis.

Baseline trials that may have been impacted by other stressors (injury in the last 48 hours, drug (KHCl) administration in the last 24 hours or any other veterinary treatment in the last 24 hours) were removed from the analysis. The analysis included 175 baseline trials and 157 post-stressor trials.

Predictor variables explanation

Condition under which the AB trials were conducted was either baseline or post-stressor. Trial number was measured 1 – 5 at baseline and 1 – 5 post-stressor so that matching weekdays could be compared, for example, Tuesday for both baseline and post-stressor was always trial 1. Stimulus monkey identity referred to stimuli shown in Figure 3.2. Time was rounded down to the nearest hour, for example, both 14:05 and 14:56 would be rounded to 14:00. Location of the threat face stimulus (AggLoc) was to the left or right of the monkey view. Monkey rank was high, middle, or low; this information was collected following discussion with the animal technicians who knew the individual macaque and group hierarchies through years of experience working with these animals. Age was measured in months and was calculated for each trial to account for the time difference between baseline and post-stressor trials. Sex was male or female. Group size referred to the number of adult macaques housed in the same social group including the macaque taking part in the AB trial. Adult macaques were those over three years old.

Statistical analysis

All statistical analyses were conducted using R version 3.6.0 (R Core Team, 2018). Linear mixed effects models (LMM) were developed and fitted using the function *lmer* of the R-package lme4

(Bates et al, 2015). LMM are used to analyse continuous, hierarchical data and can cope with unequal sample sizes and missing data (Smith, 2012; Gałecki & Burzykowski, 2013).

Animal identity was included as a random effect in all models. To avoid collinearity, all predictor variables were checked for correlations and for those above 0.4, one variable was removed (Crawley, 2007). Criteria for selecting the retained variables was relevance to the study question, for example, in Study 1 condition (baseline or post-stressor) was always retained in the model. In Study 2, age correlated with weight and total number of offspring. In this example, age was retained in the model as it was deemed to be more informative for determining the life-history variables that impact AB measures. Predictor and response variables were also checked for their distribution. Variables that showed non-normal distribution were transformed using Tukey's Ladder of Power (Tukey, 1977). The Tukey transformation provided a λ value that maximised the Shapiro-Wilk W statistic or minimises the Anderson-Darling A statistic (Mangiafico, 2016). The Schapiro-Wilk statistics should be maximised as a significant or small Shapiro-Wilk W statistic indicates that the data is not normally distributed (Oztuna et al, 2006). The Anderson-Darling statistic should be minimised as a smaller Anderson-Darling A statistic indicates that the distribution better fits the data (Lewis, 1961). The Tukey transformation was conducted using the function 'transformTukey' of the R-package rcompanion (Mangiafico, 2019). Variables with a λ of 1.0 were not transformed as this indicated normal distribution. Covariates were z-transformed to a mean of zero and a standard deviation of one to allow easier comparison of estimates and interpretation of interactions from the model output (Schielzeth, 2010). Random slopes are often included in statistical models as they can lower the variance of the estimates; however, it is recommended that there are at least six repeats per individual to prevent erroneous estimates (Wright, 2017). Random slopes were not included due to small sample sizes and a loss of statistical power incurred when random slopes are included (Hofmann, 1997; Mathieu et al, 2012; Matuschek et al, 2017).

For each model, the residuals were plotted against fitted values and qq-plots (scatterplot comprising two sets of quantiles plotted against each other (Ford, 2015)) of the residuals were

visually inspected to check whether the models fulfilled the assumptions of normally distributed and homogeneous residuals (Crawley, 2007). The models were developed by excluding non-significant predictor variables with the greatest *p* values until only those factors with $p<0.05$ were retained in the final model. Factors with non-significant *p* values were retained if they were required for model stability. Models were deemed to be stable if the original value lay between the minimum and maximum values revealed using the function 'summary'. The reduced model estimates were compared with the estimates from the full model and all models were checked for stability using the function 'glmm.model.stab' (Hofner & Hothorn, 2017).

The significance of each model as compared to the null model (comprising only the random effect of animal ID) were established using a likelihood ratio test with the R function ANOVA with argument test set to 'Chisq' (Dobson, 2002; Forstmeier & Schielzeth 2011). Models were fitted using Maximum Likelihood, rather than Restricted Maximum Likelihood, to allow for a likelihood ratio test (Bolker et al, 2008). Likelihood ratio tests comparing the full model with the respective reduced models using the R function 'drop1'. The 'drop1' function provided the *p* values for the individual effects (Barr et al, 2013). Confidence intervals were calculated using the function 'confint.merMod' of the R-package lme4 to calculate the likely range of the sample and allow estimation of the precision of the sample compared to the true population (Bates et al, 2015). To aid interpretation, non-transformed data were used for plotting purposes.

Post hoc analysis was conducted using a least-squares means (estimated marginal means), which obtains estimated marginal means for LMM (Lenth et al, 2020). Analysis was conducted with the function 'emmeans' in the R package emmeans.

Study 1 - Does AB to threat change following a stressor?

The initial models contained key predictor variables relating to stress (condition), AB testing (trial number, stimulus monkey identity, time, location of the threat face stimulus) and the animal (rank, age, and sex). As this is the first macaque study to include both male and female macaques, the initial model included the interaction between condition and sex. The response variables, duration

of looking at the threat face stimulus (THR) and total duration looking at the treat and neutral face stimuli (TL) were square root transformed (λ = 0.5), AB difference (ABDiff) and ABDiff/TL were not transformed (λ = 1).

~ Condition*Sex +
z.Tukey.Trial14 + StimulusID + TimeF + AggLoc +
z.RankR + z.Tukey.AgeMos

where: z = scaled; Tukey = Tukey transformation

The degrees of freedom (df) for the model were 24. The data set contained 343 rows of AB trial data from 36 macaques allowing for ≥10 rows per df (Crawley, 2007). Condition was retained in all reduced models for reporting purposes. Three covariates (age, rank, and trial number) were z-transformed to a mean of zero and a standard deviation of one to allow easier comparison of estimates and interpretation of interactions from the model output (Schielzeth, 2010). A copy of the full R script is shown in Appendix 3a.

Study 2 - Does AB show consistent differences between individuals?

The full models for AB repeatability contained key predictor variables relating to the animal (age in months, group size, rank, sex) and AB testing (location of the threat face stimulus, stimulus monkey identity, time, trial number). The response variables, duration looking at the threat face stimulus (THR) and total duration looking at the treat and neutral face stimuli (TL) were Tukey transformed (λ = 0.55), AB difference (ABDiff) and ABDiff/TL were not transformed (λ = 1).

~ z.Tukey.AgeMos + z.Tukey.GroupSizeAdults + z.RankR + Sex +
AggLoc + StimulusID + z.Tukey.TimeR + z.Tukey.TrialChronological

where: z = scaled; Tukey = Tukey transformation

The degrees of freedom (df) for the model were 15. The data set contained 157 rows of AB trial data from 35 macaques allowing for ≥10 rows per df (Crawley, 2007).

Repeatability of AB was calculated using data from the 'summary' function and the calculation:

$$\frac{\text{Variance for animal ID}}{(\text{Variance for animal ID} + \text{Residual variance})}$$

Significance of the repeatability measure was calculated from the variance components extracted from the final model using the 'repR' package (Stoffel et al, 2017). A full R script is shown in Appendix 3b.

3.4 Results

Study 1 - Does AB to threat change following a stressor?

Thirty-six macaques (27 female, nine male, mean age = 7.99 ± 3.04 years, range = 3.5 to 16.17 years) housed in 10 social groups completed a total of 366 trials. Thirty-four baseline trials were removed from the analysis because a stressor had occurred (five due to injury and 29 due to the vet visiting another monkey in that group; 9.3% of the data were removed). This resulted in 332 trials (175 baseline, 157 post-stressor) for the analyses.

The mean for all the AB measures decreased from baseline and post-stressor, but this change was not significant. Mean total looking time at the threat face stimulus (THR) was 580.70 ± 566.62 ms at baseline and 524.80 ± 420.14 ms post-stressor. Mean total duration looking at the threat and neutral face stimuli (TL) was 1073.47 ± 735.16 ms at baseline and 1017.85 ± 565.72 ms post-stressor. Mean AB difference (ABDiff; total duration looking at the threat face stimulus minus total duration looking at the neutral face stimulus) was 87.94 ± 698.58 ms at baseline and 31.76 ± 635.19 ms post-stressor. Mean ABDiff/TL was 0.03 ± 0.61 at baseline and 0.02 ± 0.61 post-stressor.

Overall, the final model was a significantly better fit than the null model for TL (likelihood ratio test: $\chi^2 = 20.385$, df = 9, $p<0.05$). The final models for THR, ABDiff and ABDiff/TL did not explain the data significantly better than null models (THR: likelihood ratio test: $\chi^2 = 9.916$, df = 7, $p = 0.193$; ABDiff: likelihood ratio test: $\chi^2 = 0$, df = 2, $p = 1$; ABDiff/TL: likelihood ratio test: $\chi^2 = 20.381$, df = 15, $p = 0.158$).

Total duration looking at the treat and neutral face stimuli (TL)

For TL, condition*sex and time were retained in the final model (Table 3.2). There was a significant association between TL and time of day. Generally, the duration of TL was lower in the morning

(Figure 3.4). The shortest durations of TL were at 09:00 (877.81 ± 579.04 ms) and 14:00 (890.25 ± 717.25 ms). The greatest durations of TL were at 12:00 (1314.39 ± 781.26 ms) and 15:00 (1205.06 ± 645.80 ms). Post hoc analysis showed non-significant differences in TL between 09:00 and 12:00 (t = -2.820, *p* = 0.075) and 10:00 and 12:00 (t = -2.724, *p* = 0.096).

The interaction between condition and sex showed a trend with TL that approached significance. However, post hoc analysis revealed no significant difference between the groups.

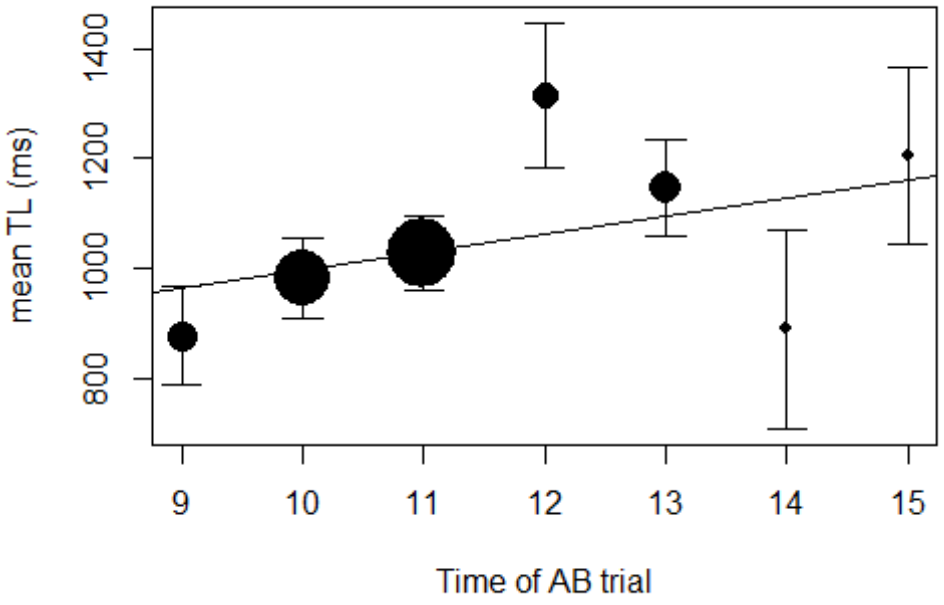

Figure 3.4. The relationship between mean total looking time at the threat and neutral face stimuli (TL) during attention bias testing and time of the AB trial for rhesus macaques at MRC-CFM. Time was rounded to the nearest hour. Dot sizes represent the number of trials conducted at each time point (9:00=42 trials; 10:00=78; 11:00=99; 12:00=36; 13:00=45; 14:00=16; 15:00=16). Error bars represent standard error. Figure was created in R version 3.6.0. See Appendix 3d for example script.

Table 3.2. LMM results for the relationship between stress and life history factors and total duration looking at the threat and neutral face stimuli (TL) during AB testing in rhesus macaques (n = 36). TL was square root transformed (λ = 0.5) for analysis.

Variables in final model		Estimate	Std. Error	t	2.5 %	97.5 %	LRT	*p*
Time (14:00)	09:00	1.512	2.765	0.547	-4.036	6.870	16.989	0.009
	10:00	2.345	2.571	0.912	-3.029	7.139		
	11:00	3.657	2.514	1.455	-1.522	8.407		
	12:00	7.460	2.749	2.714	1.336	12.325		
	13:00	5.470	2.671	2.048	0.451	10.181		
	15:00	7.782	3.223	2.415	1.118	13.825		
Condition*sex		4.200	2.276	1.846	-0.276	8.674	3.385	0.066

Study 2 - Does AB show consistent differences between individuals?

Thirty-seven macaques (25 female, 12 male, mean age = 9.22 ± 3.82 years, range = 3.75 to 16.42 years) housed in 10 social groups completed a total of 291 trials. Thirty-four baseline trials were removed from the analysis because a stressor had occurred (three due to a baby being born in the previous 24 hours, four due to chronic illness, 44 due to the veterinary treatment, 76 due to enclosure cleaning in the previous 24 hours and 14 due to the macaque experiencing the AB trial post-stressor before experiencing the AB trial at baseline; 50% of data were removed). This resulted in 147 trials for the analyses.

For Study 2, mean duration looking at the threat face stimulus (THR) was 675.49 ± 547.43 ms, mean total duration looking at the threat and neutral face stimuli (TL) was 1268.59 ± 656.11 ms, mean AB difference (ABDiff; total duration looking at the threat face stimulus minus total duration looking at the neutral face stimulus) was 82.40 ± 768.11 ms and mean ABDiff/ TL was 0.330 ± 0.560. Overall, the final models were significantly different as compared to the null models for duration of THR (likelihood ratio test: $\chi^2 = 14.646$, df = 2, $p<0.001$), duration of TL (likelihood ratio test: $\chi^2 = 7.763$, df = 1, $p<0.05$), ABDiff (likelihood ratio test: $\chi^2 = 11.58$, df = 1, $p<0.05$) and ABDiff/TL (likelihood ratio test: $\chi^2 = 13.757$, df = 2, $p<0.05$).

Duration looking at the threat face (THR)

For THR, sex and age were retained in the final model (Table 3.3). Both variables were significantly associated with THR. Female macaques had lower THR (599.57 ± 519.61 ms) than male macaques (837.14 ± 572.44 ms; likelihood ratio test: t = 2.268, $p<0.05$; Figure 3.5). Age had a negative relationship with THR. Older macaques had a lower mean THR than younger macaques (likelihood ratio test: t = 2.268, $p<0.05$; Figure 3.6). Eight-year-old (438.29 ± 312.42 ms) and 12-year-old macaques (515.44 ± 428.31 ms) had the shortest duration of THR. Three-year-old (807.30 ± 705.69 ms) and four-year-old (781.97 ± 547.46 ms) macaques had the longest duration of THR.

Table 3.3. LMM results for the relationship of life history and test factors with the duration looking at the threat face stimulus (THR) during repeatability AB testing in rhesus macaques (n = 35). THR was Tukey transformed (λ = 0.55) for analysis.

Variables in final model	Estimate	Std. Error	t	2.5 %	97.5 %	LRT	*p*
Age in months	-3.604	1.302	-2.767	-6.253	-0.959	6.767	0.009
Sex (male)	6.316	2.785	2.268	0.568	11.935	4.565	0.033

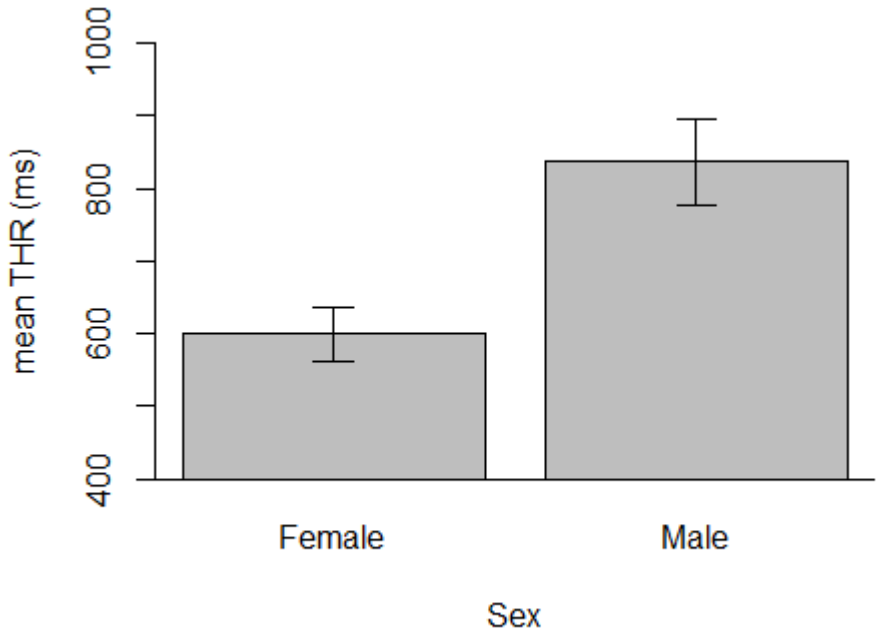

Figure 3.5. The relationship between looking time at the threat face stimulus (THR) during repeatability AB testing and sex for rhesus macaques at MRC-CFM (female=198 trials, male=93 trials). Error bars represent standard error. Figure was created in R version 3.6.0. See Appendix 3e for example script.

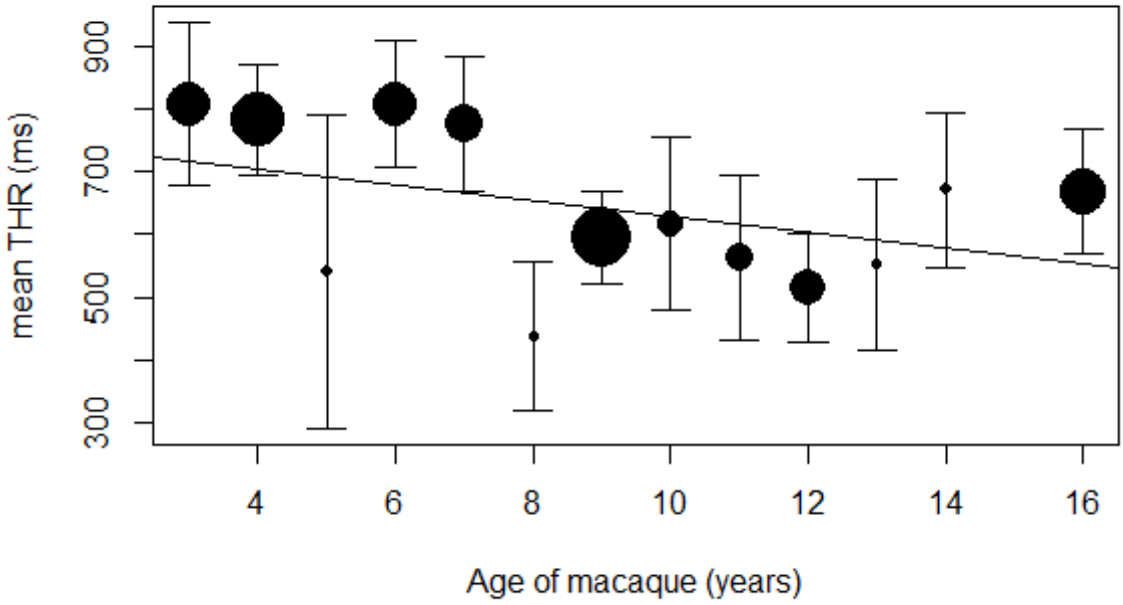

Figure 3.6. The relationship between duration looking at the threat face stimulus (THR) during repeatability AB testing and age for rhesus macaques at MRC-CFM. Age was rounded to the nearest year. Dot sizes represent the number of trials conducted at each time point (3-year-old=30 trials; 4=38; 5=8; 6=30; 7=26; 8=7; 9=42; 10=18; 11=20; 12=25; 13=7; 14=9; 16=32). Error bars represent standard error. Figure was created in R version 3.6.0. See Appendix 3e for example script.

Total duration looking at the threat and neutral face stimuli (TL)

For the total duration of looking at the threat and neutral face stimuli (TL), sex was retained in the final model and was a significant predictor of TL (Table 3.4). Female macaques had a significantly shorter duration of TL (1112.42 ± 570.94 ms) than male macaques (1601.07 ± 703.37 ms; likelihood ratio test: t = 2.985, p<0.05, Figure 3.7).

Table 3.4. LMM results for the relationship of life history and test factors with the total duration looking at the threat and neutral face stimuli (TL) during repeatability AB testing in rhesus macaques (n = 35). TL was Tukey transformed (λ = 0.55) for analysis.

Variables in final model	Estimate	Std. Error	t	2.5 %	97.5 %	LRT	*p*
Sex (male)	8.747	2.931	2.985	2.795	14.644	7.764	0.005

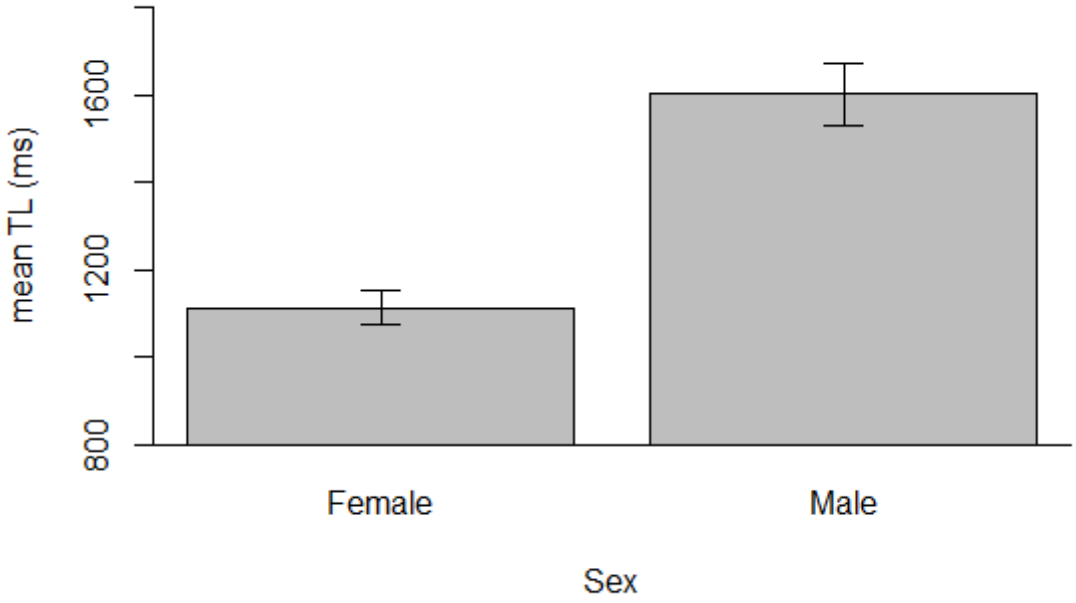

Figure 3.7. The relationship between mean total duration looking at the threat and neutral face stimuli (TL) during repeatability AB testing and sex for rhesus macaques at MRC-CFM (female=198 trials, male=93 trials). Error bars represent standard error. Figure was created in R version 3.6.0. See Appendix 3e for example script.

Attention bias difference (ABDiff)

For ABDiff, rank and age were retained in the final model (Table 3.5). High ranking macaques had the highest ABDiff (121.39 ± 713.54 ms) while low ranking macaques had the lowest ABDiff scores (-21.41 ± 843.51 ms; likelihood ratio test: t = -3.109, p<0.05; Figure 3.8). There was a negative relationship between ABDiff and age. Older macaques had a negative mean ABDiff while younger macaques had a positive mean ABDiff (likelihood ratio test: t = -2.171, p<0.05; Figure 3.9).

Table 3.5. LMM results for the relationship of life history and test factors with AB difference during repeatability (no stressors) AB testing in rhesus macaques (n = 35). AB difference was not transformed (λ = 1.0) for analysis.

Variables in final model	Estimate	Std. Error	t	2.5 %	97.5 %	LRT	*p*
Age in months	-118.47	54.56	-2.171	-226.105	-10.830	4.640	0.031
Rank	-169.62	54.56	-3.109	-277.254	-61.980	9.360	0.002

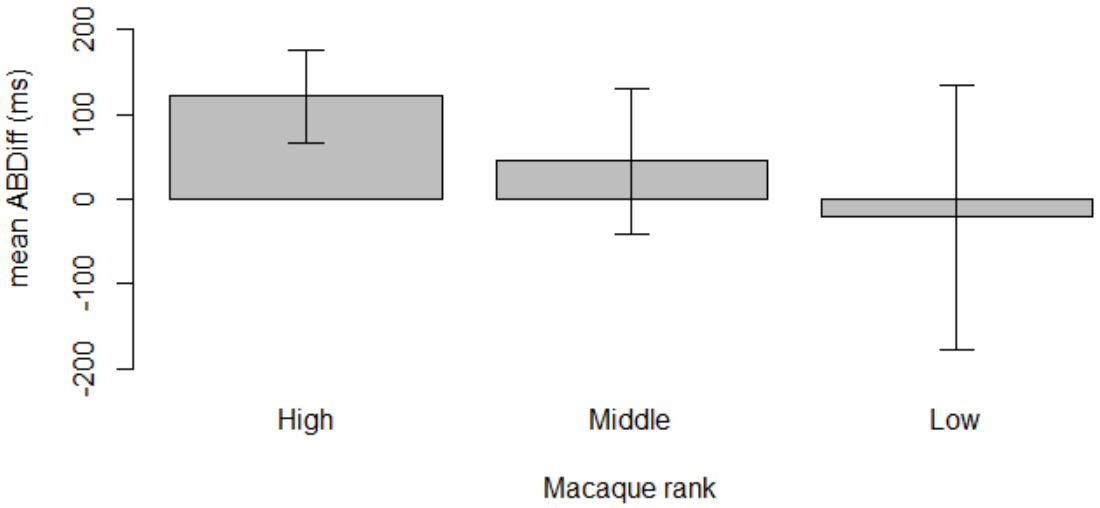

Figure 3.8. The relationship between AB difference (ABDiff) during repeatability AB testing and rank for rhesus macaques at MRC-CFM (high=168 trials, middle=94 trials, low=29 trials). Error bars represent standard error. Figure was created in R version 3.6.0. See Appendix 3e for example script.

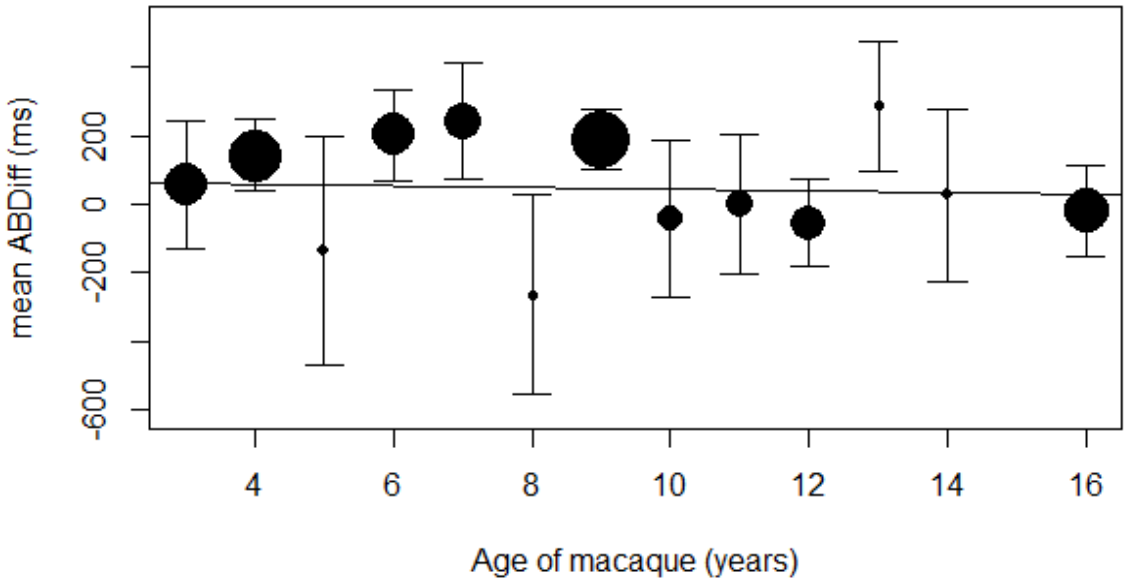

Figure 3.9. The relationship between AB difference (ABDiff) during repeatability AB testing and age for rhesus macaques at MRC-CFM. Age was rounded to the nearest year. Dot sizes represent the number of trials conducted at each time point (3-year-old = 30 trials; 4=38; 5=8; 6=30; 7=26; 8=7; 9=42; 10=18; 11=20; 12=25; 13=7; 14=9; 16=32). Error bars represent standard error. Figure was created in R version 3.6.0. See Appendix 3e for example script.

ABDiff/TL

For the ABDiff/TL, rank and location of the threat face stimulus were retained in the final model (Table 3.6). High ranking macaques had the highest ABDiff/TL (0.091 ± 0.520) while low ranking macaques had the lowest ABDiff/TL (-0.098 ± 0.626; Figure 3.10). The location of threat face stimulus had a significant association with ABDiff/TL. When the threat face stimulus was presented on the left of the monkey's view, they had a lower ABDiff/TL (-0.063 ± 0.598) than when the threat face stimulus was presented on the right of the monkey's view (0.125 ± 0.507; Figure 3.11).

Table 3.6. LMM results for the relationship of life history and test factors with ABDiff/TL during repeatability (no stressors) AB testing in rhesus macaques (n = 35). ABDiff/TL was not transformed (λ = 1.0) for analysis.

Variables in final model	Estimate	Std. Error	t	2.5 %	97.5 %	LRT	*p*
Rank	-0.131	0.043	-3.027	-0.219	-0.045	8.576	0.003
Location of threat face stimulus (right)	0.192	0.086	2.232	0.022	0.363	4.899	0.027

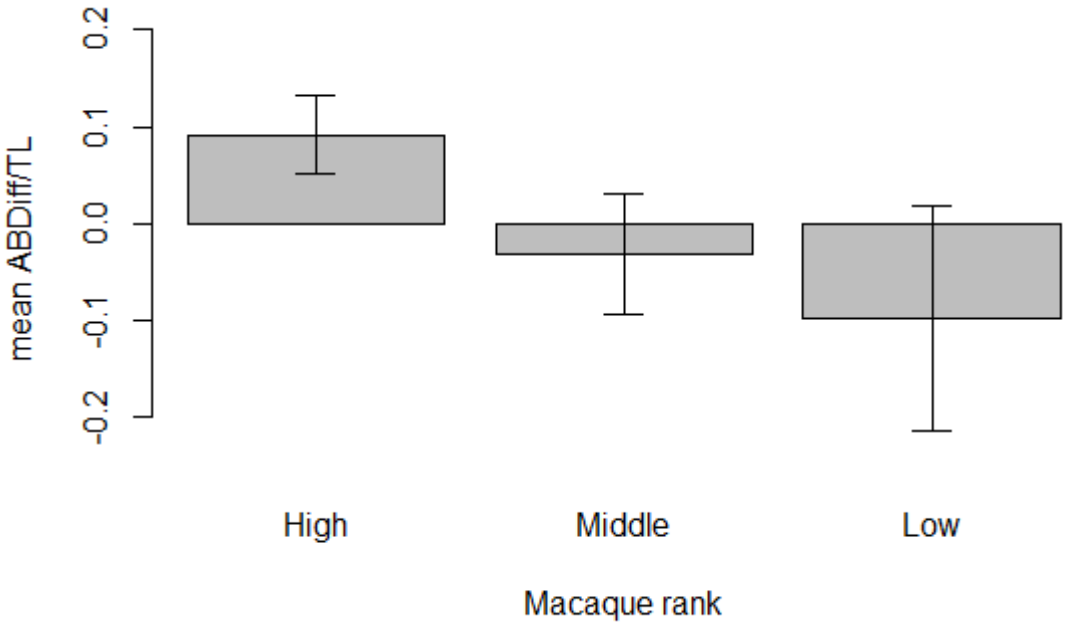

Figure 3.10. The relationship between ABDiff/TL during repeatability AB testing and rank for rhesus macaques at MRC-CFM (high=168 trials, middle=94 trials, low=29 trials). Error bars represent standard error. Figure was created in R version 3.6.0. See Appendix 3e for example script.

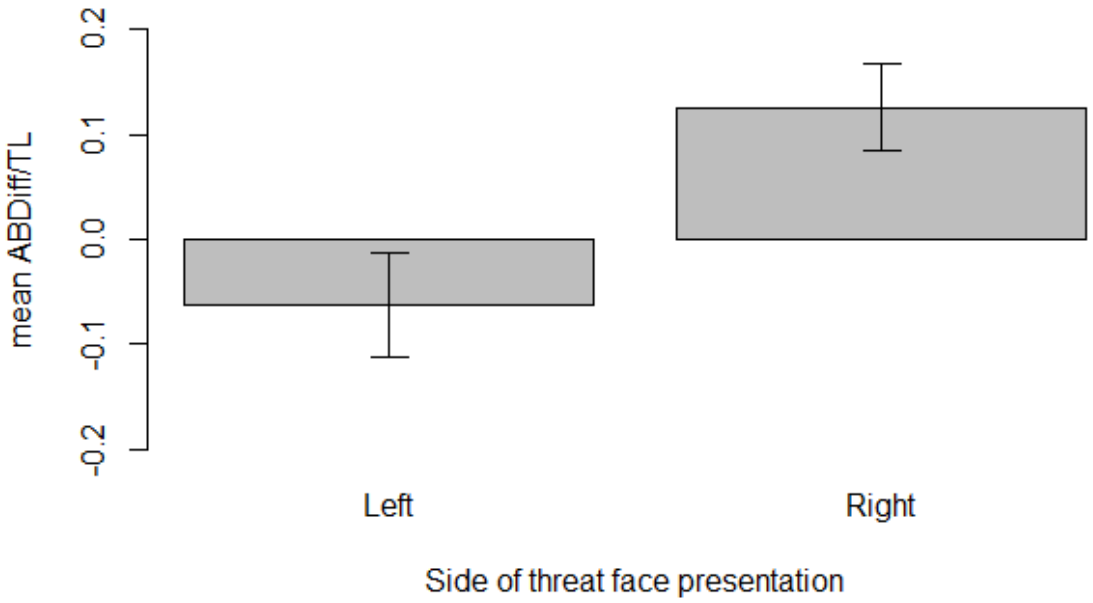

Figure 3.11. The relationship between ABDiff/TL during repeatability AB testing and location of threat face stimulus presentation (left or right relative to the monkeys' view) for rhesus macaques at MRC-CFM (left=143 trials, right=148 trials). Error bars represent standard error. Figure was created in R version 3.6.0. See Appendix 3d for example script.

Repeatability

Repeatability was low and failed to reach significance at $p<0.05$ for all measures. Total duration of looking at the threat and neutral face stimuli (TL) had low repeatability that approached significance (R = 0.093 ± 0.243, CI = 0-0.28, p = 0.071). Repeatability for duration looking at the threat face stimulus (THR) THR was not significant (R = 0.091 ± 0.071, CI = 0-0.19, p = 0.465). ABDiff and ABDiff/TL had a repeatability of 0.00 ± 0.00.

Table 3.7. Repeatability of AB measures (n = 35): duration looking at the threat face stimulus (THR), duration looking at the threat and neutral face stimuli (TL), AB difference (ABDiff) and ABDiff/TL using the variance of animal ID and the residuals.

	Variance of animal ID	Variance of residuals	SD animal ID	SD residuals	Repeatability
THR	21.245	211.466	1.116	14.542	0.091 ± 0.071
TL	18.730	183.13	4.328	13.532	0.093 ± 0.243
ABDiff	0.00	417334	0.00	646	0.00 ± 0.00
ABDiff/TL	0.00	0.2724	0.00	0.522	0.00 ± 0.00

3.5 Discussion

In this chapter, I aimed to determine 1) if AB to threat changes following a stressor (Study 1) and 2) if the AB measures are repeatable (Study 2). I present data for 479 AB trials (Study 1: 332, Study 2: 147) from 61 rhesus macaques. AB trials were conducted using an automated, computer operated apparatus with threat-neutral conspecific face stimuli presented on screens. In Study 1, AB trials were conducted before and after the macaques' annual veterinary health check to determine if the measure is capable of detecting changes in affective state caused by veterinary intervention. Here, there was no evidence for a relationship between affective state and AB. However, there was a significant association between time of the AB trial and the total duration of looking at the threat and neutral face stimuli (TL). In Study 2, AB trials were conducted once per week for eight weeks to assess the repeatability of the AB signal. Here, sex had a significant association with TL and the repeatability of TL approached significance ($R = 0.093 \pm 0.243$; $p = 0.07$).

Study 1 - AB to threat was not associated with condition

In macaques sustained eye contact is a threatening display (van Hooff, 1967; Preuschoft, 2000). Following the stressor, it was anticipated that macaques would become more avoidant of the threatening display to de-escalate and avoid aggression. In Study 1, following the stressor, overall the macaques became more avoidant compared to baseline, indicated by a reduction in the duration of all the AB measures (THR, TL, ABDiff, ABDiff/TL). However, this shift in AB was not significant. Previous human (Mansell et al, 1999) and macaque (Bethell et al, 2012b) AB studies have revealed an avoidant AB, away from negative emotional faces, in anxious individuals. Mansell and colleagues (1999) demonstrated that socially anxious humans show an avoidant AB compared to non-anxious controls in a dot-probe task. However, this avoidant bias in attention was only evident following a social threat induction that included giving a speech for which participants had no time to prepare. A meta-analysis of the human literature revealed AB to be a robust phenomenon in anxious people (Bar-Haim et al, 2007). However, in non-anxious individuals there

is either no AB to threat or a bias only towards very highly threatening stimuli (e.g., Mogg & Bradley, 1999; Wilson & MacLeod, 2003).

In this study the macaques' annual veterinary health check was used as the stressor. This prevented any unnecessary stress for the macaques involved as they all underwent the health check whether or not they were involved in this study. Veterinary interventions are known to be stressful for primates (Weatherall, 2006; Whittaker & Laule, 2012) and the procedures involved in this health check (e.g., KHCl sedation) have been shown to acutely compromise welfare (Ruys et al, 2004; Heistermann et al, 2006; Bethell et al, 2012a).

Bethell et al (2012b) reported that veterinary intervention with similar procedures (KHCl sedation) resulted in rapid vigilance followed by significant avoidance of the threat face compared to an enriched baseline. However, the location of data collection may have influenced the impact of the stressor on macaque welfare and AB measures in this study compared to Bethell et al (2012b). The data collection for this study was conducted in the UK at MRC-CFM with mixed-sex group housed macaques. The data collection for Bethell et al (2012b) was conducted at the Caribbean Primate Research Centre, Puerto Rico with singly housed male macaques. Familiar conspecifics are a source of comfort (Suomi et al, 1973) and there is a selective advantage of living in a stable group (Markham & Gesquiere, 2017). Living alone or in an abnormal social group can be a significant stressor for captive NHPs (Morgan & Tromborg, 2007; DEFRA, 2010). Singly housed macaques are known to have exaggerated fear responses compared to group housed animals (Clay et al, 2009b). These differences in housing and social conditions may have exaggerated the negative impact of veterinary intervention on macaque welfare and resulted in a larger negative shift in AB measures compared to this study. Here, the stressor may only have mildly compromised welfare resulting in no significant shift in AB.

In addition, the stressed condition was compared to a non-stressed baseline, while in Bethell et al (2012b) the stressed condition was compared to a period of enrichment. In the present study, the macaques had access to enrichment in the form of swings, buckets and mirrors at both baseline

and post-stressor. Enrichment for laboratory macaques is associated with a reduction in self-injurious and stereotypic behaviour and cortisol level compared to a non-enriched baseline (Line et al, 1990; Cannon et al, 2016) and is therefore likely to result in a more positive affective state than a non-enriched baseline.

Enrichment can result in neurobiological changes that affect vigilance to threat. The provision of novel environmental enrichment has been shown to reduce submissive behaviour to social stress in mice and affect neuronal activity in the prefrontal cortex (Lehmann & Herkenham, 2011). Heightened amygdala activity is associated with increased vigilance to threat (Anderson & Phelps, 2001; Davis & Whalen, 2001; Öhman, 2002, 2005) and the processing of aversive information (LeDoux, 1996, 2003). The automatic allocation of attention to fear relevant information through heightened activity of the amygdala and possible reduction in social stress caused by the enrichment may have resulted in the macaques displaying less submissive behaviour and therefore they are less likely to show avoidance of social stimuli. Future AB studies should involve macaques involved in neurological or toxicology studies. The severity of the procedure should be included as a factor within the analysis. It is likely that severe procedures would be associated with larger shifts in AB than mild or moderate procedures.

Social attention towards faces was associated with time of day

Time had a significant association with AB measures. The duration of TL generally increased through the day. In humans, time of day is known to influence attention (Knight & Mather, 2013) and performance in attention tasks (Kraemer et al, 2000). Kraemer and colleagues tested 12 human volunteers in a range of tasks including numeracy, visualisation of tangled lines on paper and reaction time to complex target signals (yellow light and sound played simultaneously). Performance in these tasks was low between 07:00 and 09:00, increased from 09:00 to 13:00 and then decreased between 13:00 and 19:00. In the present study, there was a trend suggesting difference in TL between 09:00 (877.81 ± 579.04 ms) and 12:00 (1314.39 ± 781.26 ms). This trough and peak map onto the performance in attention tasks in human studies (Kraemer et al, 2000). As

with some physiological measures such as cortisol (Lefcourt et al, 1993; Trifonova et al, 2013), attention in humans appears to have a circadian rhythm (Valdez et al, 2005). In non-human animals, time of day influences behaviour (e.g., cows: Niu et al, 2014; birds: Ramli & Norazlimi, 2016; primates: Kappeler & Erkert, 2003). Kappeler & Erkert (2003) studied the activity of red-fronted lemurs (*Eulemur rufifrons*) and reported heightened activity between 05:00 and 08:00 followed by a reduction in activity between 08:00 and 14:00 and then an increase between 14:00 and 20:00. This study provides the first evidence of the relationship between time of day and AB measures in rhesus macaques. It may be of benefit to conduct further studies looking specifically at this effect.

The association between TL and the interaction between condition and sex approached significance. The interaction revealed that female macaques become more avoidant from baseline to post-stressor while male macaques become more vigilant. Sex differences in AB are evident in humans, for example, females have a higher variability of AB compared to males (Carlson et al, 2019) and a greater AB for disgust (Kraines et al, 2017) and threat (Montagner et al, 2016) stimuli compared to males. Human studies have reported conflicting results with anxious males being more attentive (Zhang et al, 2017), less attentive (Tan et al, 2011) or having no difference in their attention (Kinney et al, 2017) to threat compared to anxious females. Human females exhibit an own-gender bias in attention to faces, which is not present in men (Lovén et al, 2011; Herlitz & Lovén, 2013). This gender bias in humans is mirrored in primates with female capuchin monkeys showing an AB towards images of female conspecifics over male conspecifics, while male capuchin monkeys showed no preference (Schino et al, 2020).

Further, the difference in response between males and females may be due to the "tend and befriend" alternative stress response pathway, which has been studied in female humans (Taylor et al, 2000). Taylor et al (2000) suggested that a response geared towards aggression might not be adaptive for female animals as it could leave offspring unprotected. Instead, female behaviour is directed at retrieving and protecting offspring while anticipating and avoiding threats to increase the likelihood of offspring survival. MRC-CFM is a breeding colony and 60% of the females included

in this study had offspring < 12 months old and 92% had offspring < two years old. Attentiveness and a drive to ensure offspring survival may have contributed to the female macaques' avoidance of the threatening unfamiliar male face.

Study 2 -AB for faces shows consistent differences between individuals

There was within-individual repeatability that approached significance for total looking time at the threat and neutral face stimuli (TL), but not for THR, ABDiff or ABDiff/TL. This indicates possible detectable individual differences (traits) in duration of looking towards faces. For TL, the repeatability was within the range reported in the animal behaviour and human AB literature (R = 0.37: Bell et al (2009); R = 0.45: Bar-Haim et al, 2007). This suggests that the AB measure, total looking time at the threat and neutral face stimuli, can produce multiple similar readings from the same individual under identical conditions (Bland & Altman, 1986; Bartlett & Frost, 2008; Kilkenny et al, 2010). Greater repeatability of TL compared to the zero repeatability for ABDiff and ABDiff/TL suggests that ABDiff and ABDiff/TL may be more suitable for detecting transient emotional states (Bethell et al, 2012b) or that these calculations that combine variables have introduced additional noise resulting in a less reliable measure. As these measures (ABDiff, ABDiff/TL) have zero repeatability and were not significantly associated with shifts in emotion that may have occurred following the vet check they will not be used within the analysis for the rest of the thesis.

The trials included in the repeatability study were presumed to have been conducted under baseline (low stress) conditions. Trials that had potentially been impacted by stressors including injury in the last 48 hours, drug (KHCl) administration in the last 24 hours, any other veterinary treatment in the last 24 hours or cleaning were removed from the analysis. Sex was significantly associated with THR and TL for these non-stressed trials. Males were more attentive than females for both THR and TL which reflects the findings of Study 1 where females had a lower TL than males at both baseline and post-stressor. In both Study 1 and Study 2 the number of trials from males and females was imbalanced (Study 1: F = 251 trials, M = 51 trials; Study 2: F = 100 trials, M = 47 trials). In Study 1, the males were all high-ranking breeding males. In Study 2, the males were both

breeding males and weaner males (young males housed in single sex groups). Future studies should consider comparing weaner males and females or pair housed males and females, directly. This would remove the effect of breeding-male rank and impact of single vs mixed sex groups on the association between AB measures and sex.

Age was significantly associated with THR. This study is the first research to reveal a relationship between AB and age in macaques, showing shifts in AB with age to work in the opposite direction to that seen in humans. In humans, younger individuals have a larger AB to social stimuli than older individuals (e.g., Carmobna et al, 2015; Namaky et al, 2017). This difference in AB with age has been attributed to better emotional well-being in older adults compared to younger adults (Mather & Carstensen, 2003). Older adults are more focused on emotionally meaningful goals, such as emotionally meaningful relationships, compared to younger adults (Fredrickson & Carstensen, 1990; Fung et al, 1999, 2001). This focus on emotionally meaningful goals is thought to enhance well-being (Carstensen et al, 2003) and numerous studies have revealed an association between aging and increased emotional well-being (e.g., Charles et al, 2001; Mroczek, 2001). For example, Carstensen et al (2001) showed that the duration and frequency of negative emotional experiences decreases with age between 18 and 94 years old. Here, young macaques (≤4 years old) were more attentive to the threat face stimulus than older macaques (≥7 years old) suggesting that younger macaques may have better emotional well-being than older macaques. Evolved training practices in NHP laboratories means younger NHPs are better habituated to humans and experience only PRT methods (e.g., Perlman et al, 2012; Whittaker & Laule, 2012; Nightingale et al, 2015; Westlund, 2015). This shift to PRT promotes improved animal welfare during training (Laule et al, 2003, 2007; Prescott & Buchanan-Smith, 2003; NC3Rs, 2019) and time spent training and rewarding promotes a closer relationship between trainers and the NHPs involved (Buchanan-Smith, 2003; Prescott & Buchanan-Smith, 2003). Older macaques may not have experienced this positive interaction to the same degree or during critical periods of development (five of the older macaques were weaned at less than 12 months old) which may influence their lifelong relationship with keepers and their overall trainability.

Eighty-six macaques started training for this study including 26 that had been involved in a previous AB study. Those that failed to reach criterion for inclusion were generally older animals and only 17 out of the 26 'experienced' animals reached the training criterion and were retained in the study (Chapter 2, Table 2.1). This suggests that prior training is not a reliable indicator for inclusion in a study whereas age of animal may be a better predictor of engagement with younger macaques being generally easier to train and more willing to take part in the AB trials. Since older animals show shorter durations in AB to threat, it is possible that their reduced participation is due to a combination of fear and disinterest.

The effect of age is an important consideration for development of this method for welfare assessment and for macaque welfare generally. At MRC-CFM, macaques retained for breeding are weaned between 12 and 30-months-old and moved into one of the single sex weaner groups where they remain until they are moved on to one of the universities at between four or five-year-old (Dr Claire Witham, Scientific Project Co-ordinator at MRC-CFM, personal communication, June 2017). Once at the university, most macaques spend the first-year training for the experimental protocol (Stuart Mason, Research Assistant at the Experimental Psychology department, University of Oxford, personal communication, August 2017) and do not experience their first procedure (e.g., implantation) until they are around six years old. This means that macaques begin highly stressful, often invasive protocols at an age where they may be more susceptible to stress and anxiety. It may be of benefit to repeat this study with animals that started research protocols at a range of ages to establish if there is a definite relationship between age, anxiety, and attention.

3.6 Conclusion

In the present study there was no evidence of a relationship between condition (baseline and post-stressor) and AB. The duration of all the AB measures decreased from baseline to post-stressor; however, this shift was not significant and may be the result of the veterinary intervention only mildly compromising macaque welfare. Repeating the study with macaques involved in biomedical

protocols involving procedures with known severity ratings is the next step for AB and may reveal significant results.

This chapter revealed a difference in the allocation of attention to threat between males and females following a stressor. This is likely due to differences in the stress response pathway between males and females and the own-gender attentional bias seen in females. Further, this study revealed the first evidence for a relationship between AB and age with younger macaques being more attentive of the threat face compared to older macaques. Understanding the difference between sexes and animals of different ages is crucial if AB tasks are to be used for welfare assessment.

A significant association between TL and time was revealed with shifts in AB throughout the day mapping onto patterns in human cognitive performance. This study provides first evidence for the association between time of day and AB measures in captive rhesus macaques. I recommend further study into the effect of time of day with repeated trials at different times under identical conditions. Establishing the times of day where macaques have more positive or negative affective states may help to develop veterinary and management practices. Welfare could be improved if stressful events can be timed to avoid the more negative affective states.

This chapter included four response variables (THR, TL, ABDiff, ABDiff/TL). Only THR and TL will be included within the analysis for the rest of this thesis as. both ABDiff and ABDiff/TL had a repeatability of 0.

Repeatability of the AB signal (TL) was found to be low, approaching significance, and within the range of the animal social behaviour and human AB literature. This indicates possible detectable individual differences (traits) in duration of looking towards faces. This suggested that the AB signal has some repeatability and that AB trials are suitable for adapting for welfare assessment.

This study highlights that affective state, sex, age, and time of the AB trial should be included in the analysis in future AB studies.

Chapter 4 – Behavioural correlates of attention bias

4.1 Abstract

Stress affects behaviour. In macaques, the occurrence of aggression and displacement behaviours increases in response to stress-inducing contexts. Vigilance, yawing, shaking, grooming, and grimacing are all species-typical behaviours associated with anxiety, stress, and fear in macaques. Here, I aimed to determine 1) to validate the type and extent of stress behaviours exhibited by the study population following a stressor to allow for triangulation with other methods (Study 1) and 2) if the behavioural changes correlated with shifts in AB (Study 2). AB trials were conducted with 36 (27 female, nine male) adult rhesus macaques (*Macaca mulatta*) at baseline and post-stressor (veterinary intervention) using an automated, computer operated apparatus with threat-neutral conspecific face stimuli presented on screens. Duration of looking at these stimuli was recorded. Two looking time measures were used in the analysis: duration looking at the threat face stimulus (THR) and total duration looking at the threat and neutral face stimuli (TL). Following each AB trial, macaques were observed using focal animal continuous observation for five minutes. In Study 1, the duration of anxiety and stress behaviour and inactive behaviour increased from baseline to post-stressor. However, no association between antagonistic and prosocial approach behaviour and condition was seen. This may suggest that veterinary intervention did not significantly compromise macaque welfare to cause a change in social behaviour. Across both conditions (baseline and post-stressor), males displayed a significantly greater duration of anxiety and stress, inactive and antagonistic behaviour and females displayed a significantly greater duration of prosocial approach behaviour. This study further highlights differences in male and female cognition and behaviour and provides additional evidence that the veterinary intervention used as a stressor may have been too mild to result in shifts in social behaviour and attention. In Study 2, evidence for the influence of feeding competition on AB was seen. Macaques with a greater duration looking at the threat face stimulus (THR) spent a shorter duration of time engaged in exploratory behaviour and greater duration of time engaged in inactive behaviour. Feeding competition and the trade-off between vigilance to threat and foraging behaviour may explain the association between exploratory and inactive behaviour and THR.

4.2 Introduction

Stressful events can have a significant effect on the behaviour of humans (Lupin et al, 2009; Clemente-Suárez & Ruisoto-Palomera, 2019) and non-human animals (e.g., NHPs: Worlein, 2014; dogs: Jongman et al, 2018; cats: Amat et al, 2016; rats: Ruvanthika & Manikandan, 2019). When faced with a potential threat, survival may depend on an appropriate behavioural change associated with either the active fight-flight or passive freeze-hide responses (Rupia et al, 2016). This behavioural change is often the most overt, easily observable, and biologically economical response to stress as a threat may be avoided by removing oneself from the stimulation, for example, an animal pursued by a predator will avoid the danger by escaping and a heat-stressed animal may move to find water or shade (Moberg, 2000) preventing the need for energetically costly physiological changes. However, actively avoiding a stressor may not be possible for captive animals as this environment is associated with a range of uncontrollable factors from which the animal cannot escape, for example, inappropriate artificial lighting schedules, temperature variation away from their preferred ranges, space limitations and, sounds and smells of predator species (Morgan & Tromborg, 2007). Captive animals have limited or no opportunities for changing their spatial proximity to conspecifics and caretakers, environmental conditions, and nutrition. Allowing some control over environmental conditions has been shown to enhance the welfare of captive NHPs (Mineka et al, 1986; Line et al, 1991; Buchanan-Smith & Badihi, 2012). Common marmosets (*Callithrix jacchus*) given control over their lighting and heating conditions showed calmer activity patterns compared to those not given control (Buchanan-Smith & Badihi, 2012). The authors suggested that these behavioural changes indicated that control of the environment enhances animal welfare. Uncertainty or a lack of control can lead to heightened chronic stress, compromised welfare (McEwan, 2012; Peters & McEwan, 2015) learned helplessness and depression (Alloy & Abramson, 1982).

Research facilities and zoos are required to minimise animal suffering and promote good welfare (Zoo Licencing Act, 1981; Animals (Scientific Procedures) Act, 1986). Intolerable chronic levels of

stress and prolonged negative emotional states must be avoided and opportunities for dietary, sensory, social, and physical enrichment provided (McPhee & Carlstead, 2010; Sueur & Pelé, 2019). A "one size fits all" approach to the prevention of stress and provision of enrichment does not optimise animal welfare (Wolfensohn et al, 2018; Butler et al, 2019). Individual psychological well-being differs depending on an animal's perception and response to its environment (McPhee & Carlstead, 2010). Previously, behavioural assessment was considered key to determining individual welfare state (Dawkins, 2004, 2006; Wolfensohn et al, 2018); however, with the development of cognitive measures these can now provide a clearer indication of the animal's emotional or psychological state. Behavioural responses, although noisy, have been well studied in a range of contexts.

Behavioural assessment can be conducted via individual behavioural observation. Behavioural observation using an ethogram is a well-established method of welfare assessment for many species (e.g., Dawkins, 2003; Wemelsfelder & Mullan, 2014; NHPs: Lambeth et al, 2013; sheep: Richmond et al, 2017; elephants: Yon et al, 2019). For any behavioural study, a well-defined ethogram of behavioural indices is required to allow accurate measurement and documentation of the observed behaviours (Tinbergen, 1963; Lorenz, 1973; Crews et al, 2002; Stanton et al, 2015: Hall & Heleski, 2017). With an appropriate ethogram, behavioural indices can be easy to measure and may provide information on an animal's response to stressful situations and stimuli (Dawkins, 1990; Würbel et al, 1996; Augustsson & Mayerson, 2004). Ethograms must be taxon or species specific and may need adapted or specialised to fit specific research questions (Brockmann, 1994). Captive animals will have a different behavioural repertoire to their free-ranging counterparts (Veasey et al, 1996). However, free-ranging behaviours should be considered when building an ethogram as they provide a reference of species-typical behaviour (Kagan & Veasey, 2010).

In their natural habitat, macaques (*Macaca sp.*) spend most of their time allogrooming, resting, foraging, and exploring their environment (Hambali et al, 2012; Li et al, 2012; Jaman & Huffman, 2013; Majolo et al, 2013; Zhou et al, 2013; NC3Rs, 2014b). These behaviours should apply to captive

group or pair-housed macaques and a change in the magnitude or frequency of these key species-typical behaviours can be indicative of an underlying issue, such as ill health, pain or stress (Weary et al, 2006).

In macaques, stress and anxiety can manifest as displacement behaviours and aggression (Camus et al, 2013). Camus et al (2013) reported five distinct behavioural profiles when recording spontaneous atypical behaviour in 40 captive cynomolgus macaques. One behavioural profile (E) was associated with a high occurrence of aggressive and displacement behaviours. Displacement behaviours are acts performed by the animal that are irrelevant to the behavioural context (Breed & Moore, 2016). It is thought that these behaviours may occur as a form of energy dissipation or as a conflict between two competing motivations (Anselme, 2008). In NHPs, displacement behaviours include yawning, shaking, auto-grooming, and scratching (review: Coleman & Pierre, 2014).

Facial expressions form a complex suite of behaviour associated with stress and communication. A lip-smack is a characteristic affiliative or appeasement display, while a fear grimace, indicated by bared teeth, is a sign of fear or social submission (Maestripieri & Wallen, 1997). Macaque facial movements have been described using a macaque Facial Action Coding System (MaqFACS; Parr et al, 2010) revealing more similarity in facial expression across species of NHP than previous thought (Vicks et al, 2007; Burrows et al, 2008, 2009; Dobson, 2009; Parr, 2010). However, several species-specific specialisations were also described with macaques having more independent control over their ear movements compared to humans or chimpanzees (Parr et al, 2010). Ear movements are thought to play an important role in macaque social communication as grimace and lip-smack displays include prominent ear movements (van Hooff, 1962, 1967; Partan, 2002).

Enhanced vigilance is a further indicator of anxiety (Coleman & Pierre, 2014) with hypervigilance an indicator of extreme stress in many species (McEwan, 2012; Peters & McEwan, 2015). The function of this behaviour is to detect social and predatory threats and, in response to stress, vigilance behaviour increases (Allan & Hill, 2018). Socially stressed, subordinate macaques engage in vigilant scanning more frequently than dominant animals (Shively & Day, 2015). Vigilance is commonly

accompanied by appeasement (lip smack) or fear (grimace) behaviours (Shively et al, 1997; Shively, 1998). Piloerection and making themselves appear larger are also indicative of fear and anxiety in macaques (Hinde & Rowell, 1962). However, these behaviours are too subtle for inclusion in this study as observations will be conducted at a distance to avoid impacting macaque behaviour.

New assessment methods are often validated by comparison with behavioural data (e.g., Minero et al, 2009; Stockman et al, 2011). Here, behavioural responses will be used to help explain the shifts in AB discussed in Chapter 3. Two AB measures will be included in this chapter: duration looking at the threat face stimulus (THR) and total duration looking at the threat and neutral face stimuli (TL). This chapter aims to answer two questions:

1. Do the type and extent of stress behaviours exhibited by the study population change following a stressor?

Behavioural observation will be conducted before and after the macaques' annual veterinary health check to determine which are the key behaviours that change in response to veterinary intervention (Study 1).

2. Do any of the behavioural changes correlate with shifts in AB?

Behavioural changes will be compared with changes in THR and TL (Study 2).

4.3 Materials & methods

Ethics

Ethical approval was granted by Liverpool John Moores University (LJMU) in February 2017 (Ethical approval ID. EB_EH/2017-5) and by the Medical Research Council Animal Welfare and Ethical Review Body (AWERB) in November 2017. This project piggybacked onto routine veterinary and husbandry activities that would have occurred whether the animals were involved in this study or not. No regulated procedures were carried out for this study. Analgesia was not delayed because of any research relating to this PhD. All training was conducted following centre protocol and using positive reinforcement methods. Participation in training and AB trials was voluntary, insofar as

animals were free to leave the training and testing area (cage room) at any time. Food, water, and social contact with conspecifics were available *ad libitum* throughout training and testing.

Animals

Thirty-six macaques (27 female: mean age = 7.64 ± 2.93 years, range = 3.5 to 11.42 years; nine male: mean age = 9.91 ± 2.35 years, range = 6.67 to 16.17 years) socially housed at the Medical Research Council Harwell Institute Centre for Macaques (MRC-CFM) were involved in this study. The macaques were housed in 10 groups: nine breeding groups comprising one adult male, between three and eight adult females and their offspring and one ex-breeding group with seven adult females and their offspring from previous years.

Stressor

The macaques' annual health check was used as the stressor for this study. All macaques underwent a health check, which was overseen by the Named Veterinary Surgeon (NVS). This involved sedation with an intramuscular injection of Ketamine Hydrochloride (KHCl: 0.1 – 0.2 ml/kg) for blood draw, weighing, a tuberculosis injection in the right eyelid and a rectal swab. For the subsequent two days, the macaques received daily rectal swabs for which they were separated from their group and restrained with the crush-back. Restraint with the crush-back has previously been shown to be stressful for NHPs (Sainsbury et al, 1989; Meyer & Hamel, 2014). All health checks occurred on a Monday with rectal swabs on the subsequent Tuesday and Wednesday.

Attention bias testing

The full protocol is given in Chapter 3. In brief, threat-neutral unfamiliar male conspecific face pair stimuli (Witham & Bethell, 2019) were shown to macaques on a computer monitor screen for three seconds. Duration looking at each stimulus was recorded, and AB score was then calculated by subtracting the duration looking at the neutral face from the duration looking at the threat face.

AB trials were conducted once per day per macaque on four consecutive weekdays from Tuesday to Friday, and then a fifth trial was conducted on the following Monday. The baseline condition

(assumed non-anxious state) was timetabled so that trials occurred in weeks during which there were no activities planned which were deemed to be potentially stressful i.e., no cleaning, animal removals, or planned veterinary procedures. The post-stressor condition was timetabled so trials occurred on the five weekdays following the scheduled health check (Tuesday – Monday).

Ethogram construction

An ethogram of behavioural indices (Table 4.1) was adapted from the behavioural categories used by Szott (2015) and Thatcher (2015). The ethogram was constructed to include key behaviours related to aggression, anxiety, distraction, social behaviour, foraging and inactivity. These behavioural categories were chosen as they have previously been used as indicators of welfare in rhesus macaques (e.g., Maestripieri & Wallen, 1997; Camus et al, 2013; Coleman & Pierre, 2014).

Behavioural observation

Continuous focal animal behavioural observations were completed using Behavioral Observation Research Interactive Software (BORIS: Friard & Gamba, 2016) on a Dell Inspiron 13 700 2-in-1 laptop computer. Behavioural observations were conducted using an ethogram (Table 4.1) of established behavioural indices of stress and anxiety in rhesus macaques (adapted from Szott, 2015; Thatcher, 2015). Observations were conducted following each AB trial with data collected on four consecutive weekdays (Tuesday – Friday) plus the following Monday. Following completion of the AB trials, groups were given 10 minutes to settle without observation before each monkey was observed for five minutes. Observations were conducted within 60 minutes of the AB trials. The duration of each behaviour was recorded. Observations were conducted from the corridor either through the window into the free roaming area or the window in the door into the cage room area. Direct eye contact with and staring at the focal animal were always avoided.

Data treatment

Data were recorded in milliseconds (ms) per five-minute observation. For each monkey, total duration was summed across the five observation sessions per condition and the mean duration

for each behaviour at baseline and post-stressor were calculated for each animal. Individual-specific baseline and post-stressor estimates reduce the confounding effects of variation in the data (Garamszegi, 2016). Due to the zero-bound nature of the data, behaviours were grouped to remove the number of zeros in the data set and improve model stability for statistical analysis. The categories were:

- Anxiety behaviour (abnormal, body shake, groom, sit hunched, vigilance, yawn)
- Exploratory behaviour (foraging, locomotion, manipulate object or cage)
- Inactive behaviour (lying, sit, stand)
- Prosocial approach behaviour (affiliative, allogrooming, lip smack, interaction with baby, sexual)
- Antagonistic and retreat behaviour (aggression, grimace, submissive)

Predictor variables explanation

Condition under which the AB trials were conducted was either baseline or post-stressor. Age was measured in months and was calculated for each trial to account for the time difference between baseline and post-stressor trials. Sex was male or female.

Table 4.1. Ethogram of behaviours and behavioural categories for behavioural observation of captive rhesus macaques.

Group	Behaviour	Description
Anxiety	Abnormal	The behaviour has no obvious function. Includes stereotypic behaviours e.g., pacing, bar biting or head tossing.
	Body shake	Like a dog shake – the animal rapidly moves whole body, usually starting with shaking of the head followed by rest of body.
	Groom	The animal uses their hands or mouth to clean, scratch or manipulate their skin or fur.
	Sitting hunched	The animal is sitting with their back and head curved round so that the head is below slumped shoulder.
	Vigilance	The animal is scanning their environment or looking at a particular thing (may be out of view).
	Yawn	Animal opens its mouth wide (not directed at conspecific)
Exploratory	Foraging	The animal is searching for and / or consuming food or water.
	Manipulate object or cage	The animal uses hands or mouth to investigate and move an inanimate, moveable object in the environment and / or pull or grab parts of the enclosure such as padlocks, sliding adjustable panels and cage dividers.
	Locomotion	Any behaviour (except those otherwise defined) that involves the animal moving from one location to another, for example, quadrupedal and bipedal walking and running, climbing, descending and jumping. The animal must not be engaged in any other activity.
Inactive	Lying	The animal is lying horizontally with the stomach, back or side touching the floor.
	Sit	The animal us sitting upright
	Stand	Weight baring on two or four legs.
Prosocial approach	Affiliation	Friendly interaction between the animal and a conspecific includes huddling, being in physical contact with a conspecific and hugging but not grooming behaviour.
	Allogrooming	Reciprocal grooming. The animal's skin or fur is cleaned, scratched or manipulated by the hands or mouth of a conspecific. The animal uses their hands or mouth to clean, scratch or manipulate the skin or fur of a conspecific.
	Lip smack	Animal opens and closes its lips repeatedly without showing its teeth, occasionally making a smacking sound.
	Interaction with baby	The animal interacts with a baby e.g., grooming, playing, carrying or feeding.
	Sexual behaviour	The animal presents or is presented the hindquarters. The animal is mounted or mounts.
Antagonistic and retreat	Aggression	The animal chases, attacks, threatens, stares at, displaces or lunges towards a conspecific.
	Grimace	The animal's lips are pulled back to expose the teeth.
	Submissive	Animal moves away, flees, is displaced by or ducks away from a conspecific. They may give out high-pitched screams. Animal may also present its hindquarters in a non-sexual context (not followed by mating).
Other	Other	Any behaviour not otherwise defined.
	Out of sight	The animal is not visible to the observer.

Statistical analysis

All statistical analyses were conducted using R version 3.6.0 (R Core Team, 2018). Linear mixed effects models (LMM) were developed and fitted using the function *lmer* of the R-package lme4 (Bates et al, 2015). LMM are used to analyse continuous, hierarchical data and can cope with unequal sample sizes and missing data (Smith, 2012; Gałecki & Burzykowski, 2013).

Animal identity was included as a random effect in all models. To avoid collinearity, all predictor variables were checked for correlations and for those above 0.4, one variable was removed (Crawley, 2007). Criteria for selecting the retained variable was relevance to the study question, for example, in Study 1 condition (baseline or post-stressor) correlated with enclosure cleaning and other veterinary treatment. As Study 1 focused on changes in behaviour from baseline to post-stressor it was important to retain condition in the mode and remove the other variables.

Predictor and response variables were also checked for their distribution. Variables that showed non-normal distribution were transformed using Tukey's Ladder of Power (Tukey, 1977). The Tukey transformation provided a λ value that maximised the Shapiro-Wilk W statistic or minimises the Anderson-Darling A statistic (Mangiafico, 2016). The Schapiro-Wilk statistics should be maximised as a significant or small Shapiro-Wilk W statistic indicates that the data is not normally distributed (Oztuna et al, 2006). The Anderson-Darling statistic should be minimised as a smaller Anderson-Darling A statistic indicates that the distribution better fits the data (Lewis, 1961). The Tukey transformation was conducted using the function 'transformTukey' of the R-package rcompanion (Mangiafico, 2019). Variables with a λ of 1.0 were not transformed as this indicated normal distribution. Covariates were z-transformed to a mean of zero and a standard deviation of one to allow easier comparison of estimates and interpretation of interactions from the model output (Schielzeth, 2010).

For each model, the residuals were plotted against fitted values and qq-plots (scatterplot comprising two sets of quantiles plotted against each other (Ford, 2015)) of the residuals were visually inspected to check whether the models fulfilled the assumptions of normally distributed

and homogeneous residuals (Crawley, 2007). The models were developed by excluding non-significant predictor variables with the greatest *p* values until only those factors with $p<0.05$ were retained in the final model. Factors with non-significant *p* values were retained if they were required for model stability. Models were deemed to be stable if the original value lay between the minimum and maximum values revealed using the function 'summary'. The reduced model estimates were compared with the estimates from the full model and all models were checked for stability using the function 'glmm.model.stab' (Hofner & Hothorn, 2017).

The significance of each model as compared to the null model (comprising only the random effect of animal ID) were established using a likelihood ratio test with the R function ANOVA with argument test set to 'Chisq' (Dobson, 2002; Forstmeier & Schielzeth 2011). Models were fitted using Maximum Likelihood, rather than Restricted Maximum Likelihood, to allow for a likelihood ratio test (Bolker et al, 2008). Likelihood ratio tests comparing the full model with the respective reduced models using the R function 'drop1'. The 'drop1' function provided the *p* values for the individual effects (Barr et al, 2013). Confidence intervals were calculated using the function 'confint.merMod' of the R-package lme4 to calculate the likely range of the sample and allow estimation of the precision of the sample compared to the true population (Bates et al, 2015). To aid interpretation, non-transformed data were used for plotting purposes.

Study 1 - does behaviour change following a stressor?

The initial models contained the key predictor variables revealed in Chapter 3 (condition, sex, and age). The response variables (duration of each behavioural group shown in Table 4.1) were transformed using a Tukey's Ladder of Power (Tukey, 1977; anxiety: $\lambda = 0.25$; exploratory: $\lambda = 0.725$; inactive: $\lambda = 0.375$; prosocial approach: $\lambda = 0.875$; antagonistic: $\lambda = 0.4$).

~ Condition*Sex + z.Tukey.Age

where: z = scaled; Tukey = Tukey transformation

The data set contained 72 rows of behaviour data from 36 macaques. The degrees of freedom (df) for the model were 5 allowing for ≥10 rows per df (Crawley, 2007).

Age was z-transformed to a mean of zero and a standard deviation of one to allow easier comparison of estimates and interpretation of interactions from the model output (Schielzeth, 2010).

Study 2 - are there behavioural correlates of AB?

Behavioural data are typically zero-bound (e.g., Sunday et al, 2015). To reduce the number of zeros in the behavioural data set, behaviours were grouped into categories according to their structural similarities for each monkey under each condition (baseline and post-stressor) for analysis (as is routine in the published behavioural literature e.g., Fahlman et al, 2020; Kim et al, 2020; Pierard et al, 2020). The full model for Study 2 contained the key predictor variables revealed in Chapter 3 (condition, sex, and age) as well as one behavioural category (Table 4.1), for example:

~ z.Tukey.Inactive_behav + Condition*Sex + z.Tukey.Age

where: z = scaled; Tukey = Tukey transformation

The behaviour category was retained in the final model for reporting purposes. The degrees of freedom (df) for the model were 5. The data set contained 72 rows of behaviour data from 36 macaques allowing for ≥10 rows per df (Crawley, 2007). A copy of the full R script is shown in Appendix 4b. Age and the duration of the behaviour category were z-transformed to a mean of zero and a standard deviation of one to allow easier comparison of estimates and interpretation of interactions from the model output (Schielzeth, 2010).

4.4 Results

Study 1 - does behaviour change following a stressor?

Thirty-six macaques (27 female, nine male, mean age = 7.99 ± 3.04 years, range = 3.5 to 16.17 years) housed in 10 social groups completed a total of 366 trials. Thirty-four baseline trials were removed from the analysis because a stressor had occurred (five due to injury and 29 due to the vet visiting another monkey in that group; 9.3% of the data were removed). This resulted in 332 trials (175 baseline, 157 post-stressor). The mean duration of each behaviour and the AB

measures were then averaged for baseline and post-stressor resulting in 72 data points that were used in the analysis.

The mean duration of anxiety, exploratory, inactive, antagonistic behaviour increased from baseline to post-stressor (anxiety baseline: 15464 ± 33763 ms, post-stressor: 27215 ± 41167 ms; exploratory baseline: 90462 ± 119989 ms, post-stressor: 98201 ± 112083 ms; inactive baseline: 55732 ± 86583 ms, post-stressor: 64969 ± 78256 ms; antagonistic baseline: 720 ± 2973 ms, post-stressor: 1074 ± 3949 ms). The mean duration of prosocial approach behaviour decreased from baseline to post-stressor (baseline: 134384 ± 132996 ms, post-stressor: 106273 ± 123226 ms).

The final models were significantly different compared to the null models for anxiety behaviour (likelihood ratio test: χ^2 = 16.288, df = 3, p<0.001), inactive behaviour (likelihood ratio test: χ^2 = 16.775, df = 2, p<0.001), antagonistic (likelihood ratio test: χ^2 = 6.043, df = 2, p = 0.487) and prosocial approach behaviour (likelihood ratio test: χ^2 = 9.760, df = 2, p = 0.008).

The mean duration of anxious behaviour was significantly associated with condition, sex, and age (Table 4.2). The duration of anxiety behaviour significantly increased from baseline to post-stressor (likelihood ratio test: t = 2.787, p<0.05). Males displayed a significantly greater duration of anxiety behaviour compared to females (male: 27576 ± 37571 ms, female: 18905 ± 37769, likelihood ratio test: t = 2.622, p<0.05). Age was negatively correlated with anxiety behaviour (likelihood ratio test: t = -2.937, p<0.05; Figure 4.1). Generally, younger macaques spent a greater duration of time engaged in anxiety behaviour than older macaques (three-year-old: 39740 ± 64299 ms, 16-year-old: 1808 ± 3124). One 14-year-old macaques had a longer duration of anxiety behaviour (108623 ± 50639 ms; Figure 4.2).

The mean duration of inactive behaviour was significantly associated with condition and sex (Table 4.2). The duration of inactive behaviour was significantly greater post-stressor compared to at baseline (post-stressor: 64969 ± 78256 ms, baseline: 55732 ± 86583 ms; likelihood ratio test: t = 2.058, p<0.05). Males displayed a significantly greater duration of inactive behaviour compared to

females (male: 105568 ± 100449 ms, female: 45427 ± 70374 ms; likelihood ratio test: t = 3.915, p<0.001).

The mean duration of antagonistic behaviour was significantly associated with sex. Males displayed a significantly greater duration of antagonistic behaviour compared to females (male: 1520 ± 4263 ms, female: 683 ± 3152; likelihood ratio test: t = 2.352, p<0.05).

The mean duration of prosocial approach behaviour was significantly associated with sex (Table 4.2). Females displayed a significantly greater duration of prosocial approach behaviour compared to males (female: 138416 ± 130716 ms, male: 67404 ± 107902 ms; likelihood ratio test: t = 2.352, p<0.05).

Table 4.2. LMM results for the association between key behaviour groups (anxiety, inactive, antagonistic, and prosocial approach) and condition (baseline & post-stressor), sex and age in rhesus macaques (n = 36).

Response variable	Variables in final model	Estimate	Std. Error	t	2.5 %	97.5 %	LRT	*p*
Anxiety	Condition (post-stressor)	1.182	0.424	2.787	0.332	2.032	7.232	0.007
	Sex (Male)	1.452	0.554	2.622	0.337	2.568	6.295	0.012
	Age	-0.710	0.242	-2.937	-1.196	0.223	7.744	0.005
Inactive	Condition (post-stressor)	7.104	3.452	2.058	0.153	14.055	4.004	0.045
	Sex (Male)	24.448	6.244	3.915	11.876	7.020	12.771	0.0004
Antagonistic	Sex (Male)	4.253	1.808	2.352	0.613	7.893	5.148	0.023
Prosocial approach	Sex (Male)	-3096.5	986.7	-3.138	-5083	-1110	8.705	0.003

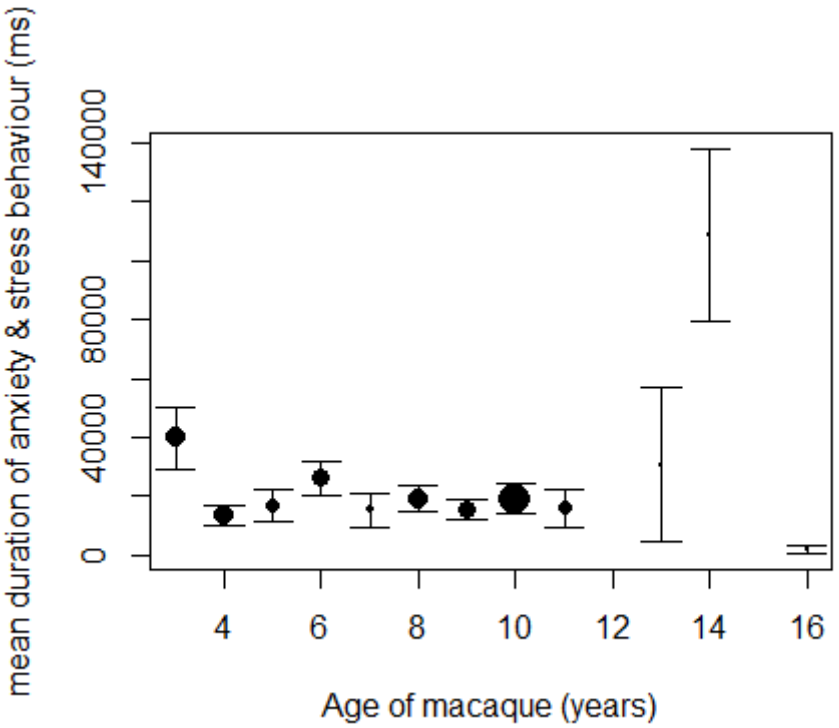

Figure 4.1. The relationship between anxiety behaviour and age for rhesus macaques at MRC-CFM. Age was rounded to the nearest year. Dot sizes represent the number of trials conducted at each time point (n3=38; n4=38; n5=28; n6=34; n7=16; n8=39; n9=33; n10=61; n11=28; n13=5; n14=3; n16=9). Error bars represent standard error. Figure was created in R version 3.6.0.

Study 2 - do any of the behavioural changes correlate with shifts in AB?

Thirty-six macaques (27 female, nine male, mean age = 7.99 ± 3.04 years, range = 3.5 to 16.17 years) housed in 10 social groups completed a total of 366 trials. Thirty-four baseline trials were removed from the analysis because a stressor had occurred (five due to injury and 29 due to the vet visiting another monkey in that group; 9.3% of the data were removed). This resulted in 332 trials (175 baseline, 157 post-stressor). The trials were then averaged for baseline and post-stressor resulting in 72 data points that were used in the analysis.

Total duration looking at the threat face stimulus (THR)

The final models were not significantly different compared to the null models for anxiety behaviour (likelihood ratio test: $\chi^2 = 0.122$, df = 1, $p = 0.727$), antagonistic behaviour (likelihood ratio test: $\chi^2 = 0.049$, df = 1, $p = 0.825$) and prosocial approach behaviour (likelihood ratio test: $\chi^2 = 0.034$, df = 1,

p = 0.854). The final models for exploratory behaviour and inactive behaviour showed a trend that approached significance (exploratory likelihood ratio test: χ^2 = 3.018, df = 1, *p* = 0.082; inactive likelihood ratio test: χ^2 = 3.338, df = 1, *p* = 0.068).

Exploratory behaviour had a negative relationship with THR. Generally, macaques with a greater duration of THR spent a shorter duration of time engaged in exploratory behaviour (likelihood ratio test: t = -1.757, *p* = 0.08; Table 4.3; Figure 4.2). Inactive behaviour had a positive relationship with THR. Macaques with a greater duration of THR spent a greater duration of time engaged in inactive behaviour (likelihood ratio test: t = 1.899, *p* = 0.07; Table 4.3; Figure 4.3).

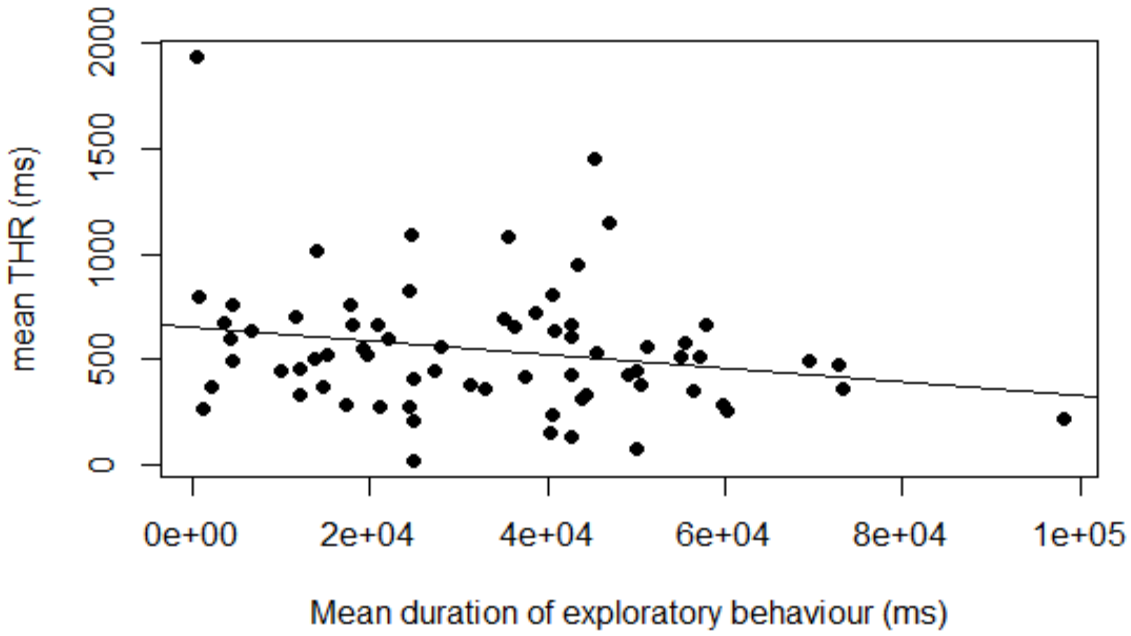

Figure 4.2. The relationship between mean duration of exploratory behaviour and mean duration looking at the threat face stimulus (THR) for rhesus macaques at MRC-CFM. Each point represents behaviour observations and AB trials for one monkey under one condition (baseline or post-stressor). Figure was created in R version 3.6.0.

Table 4.3. LMM results for the association between the duration of looking at the threat face stimulus (THR) and exploratory behaviour in rhesus macaques (n = 36). Each variable was run in a separate model.

Predictor variable	Estimate	Std. Error	t	2.5 %	97.5 %	LRT	p
Exploratory behaviour	-0.608	0.346	-1.757	-1.308	0.080	3.018	0.082
Inactive behaviour	0.754	0.397	1.899	-0.057	1.554	3.338	0.068

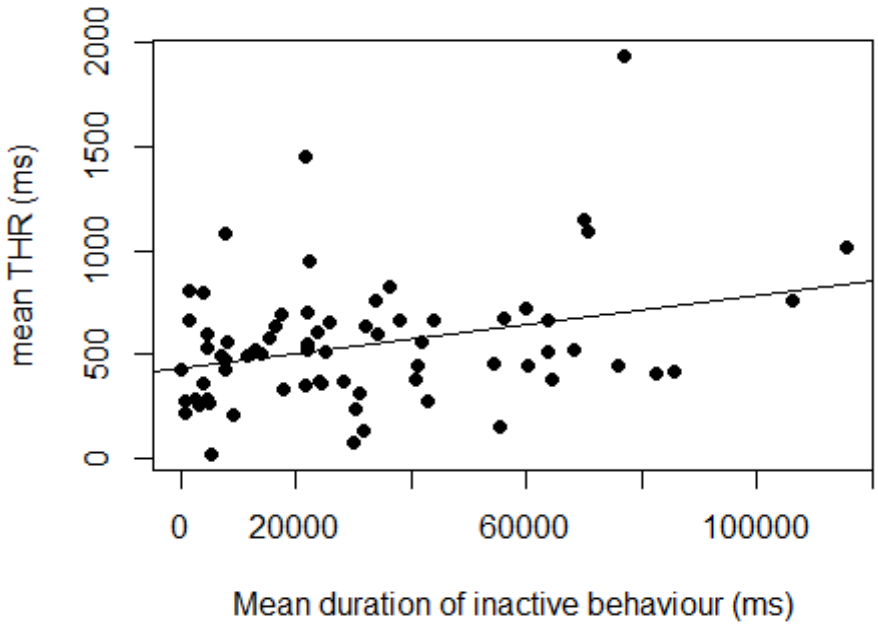

Figure 4.3. The relationship between mean duration of inactive behaviour and mean duration looking at the threat face stimulus (THR) for rhesus macaques at MRC-CFM. Each point represents behaviour observations and AB trials for one monkey under one condition (baseline or post-stressor). Figure was created in R version 3.6.0.

Total duration looking at the threat and neutral face stimuli (TL)

The final models were not significantly different compared to the null models for anxiety behaviour (likelihood ratio test: $\chi^2 = 0.403$, df = 1, $p = 0.526$), exploratory behaviour (likelihood ratio test: $\chi^2 = 0.618$, df = 7, $p = 0.432$), inactive behaviour (likelihood ratio test: $\chi^2 = 0.525$ df = 1, $p = 0.469$), antagonistic behaviour (likelihood ratio test: $\chi^2 = 0$, df = 1, $p = 1$) and prosocial approach behaviour (likelihood ratio test: $\chi^2 = 0$, df = 1, $p = 1$).

4.5 Discussion

In this chapter, I aimed to determine 1) if behaviour changes following a stressor (Study 1) and 2) if any of the behavioural changes correlate with shifts in AB (Study 2). I present data from 332 AB trials and behavioural observations from 36 rhesus macaques.

AB trials were conducted using an automated, computer operated apparatus with threat-neutral conspecific face stimuli presented on screens. Following each AB trial, macaques were observed for five minutes using continuous behavioural observation. AB trials and behavioural observations were conducted before and after the macaques' annual veterinary health check. In Study 1, the behavioural observations were used to determine which behaviours change in response to veterinary intervention. Here, condition was significantly associated with anxiety behaviour and inactive behaviour. The duration of both anxiety behaviour and inactive behaviour was significantly greater post-stressor compared to at baseline. Sex was significantly associated with anxiety, inactive, antagonistic, and prosocial approach behaviour. Males displayed a significantly greater duration of anxiety, inactive and antagonistic behaviour compared to females. Females displayed a significantly greater duration of prosocial approach behaviour compared to males. Age had a negative relationship with anxiety behaviour with younger macaques spending longer engaged in anxiety behaviour than older macaques. In Study 2, mean duration of the AB measures were correlated with mean duration of each behaviour group. Here, exploratory and inactive behaviour were associated with the mean duration looking at the threat face stimulus (THR). Macaques with a greater duration of THR spent a shorter duration of time engaged in exploratory behaviour. Macaques with a greater duration of THR spent a greater duration of time engaged in inactive behaviour.

Study 1 – the duration of anxiety and inactive behaviour change following a stressor

Stress can have a significant impact on the behaviour of NHPs (Coleman & Pierre, 201; Worlein, 2014). In this study, following the stressor, macaques spent longer engaged in anxiety behaviour and inactive behaviour compared to at baseline. The anxiety behaviour group included abnormal

behaviour, body shake, grooming, sitting hunched, vigilance, and yawning. The increase in these behaviours and change in activity level seen in this study is congruent with previous studies. In response to social and veterinary stressors, macaques show an increase in vigilance (Coleman & Pierre, 2014; Shively & Day, 2015) and displacement behaviours, including yawning, shaking, auto-grooming, and scratching (Camus et al, 2013; Coleman & Pierre, 2014; Breed & Moore, 2016). Following relocation stress, macaques have been shown to spend more time engaged in inactivity (Crockett et al, 1995).

Although condition (baseline and post-stressor) had a significant association with anxiety, and inactive behaviour, condition did not have a significant association with either antagonistic or prosocial approach behaviour. Following a stressor, the occurrence of aggression, fear grimace and submissive behaviour has previously been shown to increase (Maestripieri & Wallen, 1997; Camus et al, 2013) and the occurrence of affiliative behaviour decrease (Mallapur et al, 2005; Arnold et al, 2011). For example, in response to the acute stress, caused by the presence of zoo visitors, lion-tailed macaques (*Macaca silenus*) showed a decrease in affiliative behaviour (Mallapur et al, 2005).

In this study the macaques' annual veterinary health check was used as the stressor. Veterinary interventions are known to be stressful for primates (Weatherall, 2006; Whittaker & Laule, 2012) and the procedures involved in this health check (e.g., KHCl sedation) have previously been shown to acutely compromise welfare (Ruys et al, 2004; Heistermann et al, 2006; Bethell et al, 2012a). However, in Chapter 3, although a negative shift in AB was seen, the change was not significant. The lack of change in antagonistic and prosocial approach behaviour, may suggest that, in line with the findings in Chapter 3, the veterinary intervention only mildly compromised macaque welfare. Previous behaviour studies based at MRC-CFM, that used the macaques' annual veterinary health check as a stressor, found no significant changes in the occurrence of antagonistic or prosocial approach behaviour (Howarth, 2016). Howarth (2016) coded 1-minute-long video clips of 15 group housed rhesus macaques at MRC-CFM before and after the annual veterinary health check. The ethogram of established behaviour indices was similar to the behavioural categories used in the

present research. This provides further evidence that this stressor may be too mild for future AB studies.

Sex differences in behaviour are evident in humans (Baron-Cohen et al, 2005; Ellis, 2011) and non-human animals, including NHPs (Keverne, 1993; Wallen & Hassett, 2009; Lonsdorf, 2017). Here, sex was significantly associated with anxiety, inactive, antagonistic, and prosocial approach behaviour. Males spent longer engaged in anxiety, inactive and antagonistic behaviour while females spent longer engaged in prosocial approach behaviour. Male macaques spent a significantly greater duration of time engaged in anxiety behaviour compared to females. Much of the published literature suggests that females tend to exhibit more anxiety behaviour than males. For example, in humans, females are more vulnerable to stress and anxiety-based disorders (Maeng & Milad, 2015) and female vervet monkeys (*Chlorocebus pygerythrus*) have heightened vigilance to snakes compared to males (Isbell & Etting, 2016). In Chapter 3, I highlighted the variation in sex effects on AB measures in humans with anxious males being more attentive (Zhang et al, 2017), less attentive (Tan et al, 2011) or there being no difference in their attention (Kinney et al, 2017) to threat compared to anxious females. Therefore, variation in sex effects on anxiety behaviour is unsurprising. In Chapter 3, males had a higher duration looking at the threat face stimulus (THR) and total duration looking at the threat and neutral face stimuli (TL) than females. This suggests differences in anxiety and stress response between male and female macaques.

Males were more inactive than females. The mean duration of inactive behaviour (lying, sitting, standing) was greater in males than females. Differences in inactivity between the sexes is consisted across other primate species, for example, male West Javan langurs (*Trachypithecus mauritius*) spent significantly more time resting compared to female and juvenile langurs (Asri et al, 2019) and male chimpanzees (*Pan troglodytes schweinfurthii*) spent 39% of their daily activity budget inactive compared to 27% for females (Bates & Byrne, 2009).

Differences in activity could be influenced by dominance rank differences between males and females. Adult male macaques at MRC-CFM are housed as single males with several adult females

and their offspring. Female NHPs in groups with a lower number of males tend to be less dominant over their male conspecifics than females in groups with a larger number of males (Hemelrijk et al, 2008). This difference in dominance behaviour suggests that the male macaques at MRC-CFM would be more dominant than the females. Indeed, all but one of the males included in the study were high ranking (rank was determined following discussion with the animal technicians). Higher ranking macaques did not need to move out of the way for other macaques and could, therefore, spend longer sitting and lying (inactive).

In humans, a study comparing the activity levels of male and female teenagers reported a higher proportion of females were inactive (44%) compared to males (27%; Allison & Adlaf, 1997). This variation highlights yet another difference between human and NHP behaviour and cognition. Applying theory from human literature is a good starting point but to fully understand AB in NHPs more species-specific information is needed.

Male NHPs and humans are known to engage in more aggressive behaviour than females (Kortüm et al, 2013). Female macaques have been shown to engage in affiliative behaviour more frequently and for longer durations than male macaques (Simpson et al, 2016). When male and female infant macaques, raised in controlled homogeneous environments, were compared females displayed more affiliative behaviour and exhibited more social interest than males (Simpson et al, 2016). The sexual dimorphism in aggressive and affiliative behaviour may be due to differences in the stress response pathways between males and females ("tend and befriend"; Taylor et al, 2000) and the evolutionary role of females as caregivers (Buss, 1995; Wood & Eagly, 2002). Further, the social structure of rhesus macaque groups is based on matrilineal hierarchies with stable groups of related females (de Waal & Luttrell, 1985; Jackson and Winnegrad, 1988; Theirry, 2007). Engaging in prosocial approach behaviour, including allogrooming and affiliative behaviour, helps to develop and maintain the sex-specific social hierarchies.

Study 2 – Exploratory and inactive behaviours correlate with shifts in AB

The mean duration looking at the threat face stimulus (THR) was associated with the mean duration of exploratory and inactive behaviour. Macaques with a greater duration of THR spent a shorter duration of time engaged in exploratory behaviour and a greater duration of time engaged in inactive behaviour. Exploratory behaviour included foraging which is considered a key maintenance behaviour.

Disruption to key maintenance behaviours can be an indicator of significant health issues or stress (Weary et al, 2006). Two of the five macaques that displayed no foraging behaviour were in the same group (Valentine and Zsa-Zsa). This group had experienced significant changes in the 2-3 months prior to data collection. The two highest ranking females had been removed which resulted in the daughter of one of the removed females being attacked and killed and the daughter of the other female (Zarita) being bullied by the other macaques. MRC-CFM made the decision to introduce a large adult male (Star) to the group around one month prior to AB baseline data collection.

Familiar conspecifics are a source of comfort (Suomi et al, 1973) and there is a selective advantage of living in a stable group (Markham & Gesquiere, 2017). Macaques at MRC-CFM are housed in matrilineal breeding groups with one adult male, several adult females and their offspring. The above group (Valentine, Zsa-Zsa, Zarita and Star) were an atypical social grouping. The maintenance of abnormal social groups can be a significant stressor in captivity (Morgan & Tromborg, 2007). The macaques' abnormal social situation, group instability and resulting chronic stress (Meyer & Hamel, 2104) may have resulted in a vulnerability that caused a reduction in feeding behaviour in these macaques compared to others. The association between exploratory and inactive behaviour and THR may also be due to feeding competition. Foraging behaviour has previously been shown to have a negative relationship with AB in captive psittacines, (*Amazona amazonica*; Cussen & Mench, 2014). The authors suggested that there is a cost associated with increased vigilance as individuals with AB performed significantly more poorly in a foraging task. Here, monkeys who were more

vigilant towards threat faces spent less time foraging in the home cage. Anxiety in macaques is characterised by increased vigilance (Coleman & Pierre, 2014). Vigilance will increase in response to "threatening social signals" (Ebitz et al, 2013), maternal separation (Reite et al, 1981; Worlein, 2014) and predation (Coleman & Pierre, 2014). However, previous studies have revealed most animals are unable to forage efficiently or explore their environment and remain vigilant to threat (Underwood 1982, Lima 1998; Dalerum et al, 2008). Gregarious species show less individual vigilance behaviour as they rely on communal vigilance while foraging (le Roux et al, 2009).

4.6 Conclusion

In this Chapter, the relationship between AB and behaviour was tested. The key findings were that condition was significantly associated with both anxiety behaviour and inactive behaviour. The mean duration of both behavioural categories was greater at baseline compared to post-stressor, which matches findings from previous macaque behaviour studies (e.g., Bethell et al, 2012b). This would indicate that behavioural response may be a robust measure for validation of AB as AB is considered a measure of anxiety and here, we see shifts in the duration of anxiety related behaviour.

However, in Chapter 3, no significant change in AB was seen following the stressor. Here, no change in antagonistic or prosocial approach behaviour was seen. Changes in both social behaviour and attention to social stimuli may require a more significant stressor, which suggests that the veterinary intervention only mildly compromises macaque welfare. Repeating the study with macaques involved in biomedical protocols involving procedures with known severity ratings is the next step for AB and would likely reveal significant results for AB. However, assessing changes in social behaviour in these macaques may be challenging as they are often pair-housed, which will impact social behaviour even at baseline. Macaques with a greater duration looking at the threat face stimulus (THR) spent a shorter duration of time engaged in exploratory behaviour and greater duration of time engaged in inactive behaviour. Feeding competition and the trade-off between vigilance to threat and foraging behaviour may explain the association between exploratory and

inactive behaviour and THR. Further AB studies involving macaques used in biomedical procedures may wish to consider the impact of feed and fluid controls on AB to threat.

Chapter 5 – Cortisol as a hormonal correlate of attention bias

5.1 Abstract

Many aspects of stress and the stress response, including cortisol concentration, can influence attention and cognitive performance in both humans and macaques. In non-anxious, healthy human volunteers, elevated cortisol is associated with avoidance of potentially threatening stimuli. Here, I aimed to determine 1) if salivary cortisol is associated with stress (veterinary intervention), life history (sex) and test related factors (trial number, location of the stimulus and stimulus ID; Study 1) and 2) if the cortisol changes correlated with shifts in AB (Study 2). AB trials were conducted with 17 (10 female, seven male) adult rhesus macaques (*Macaca mulatta*) at baseline and post-stressor (veterinary intervention) using an automated, computer operated apparatus with threat-neutral conspecific face stimuli presented on screens. Duration of looking at these stimuli was recorded. Two looking time measures were used in the analysis: duration looking at the threat face stimulus (THR) and total duration looking at the threat and neutral face stimuli (TL). Following each AB trial, saliva was collected for cortisol analysis by enzyme immunoassay. In Study 1, none of the stress or life history factors were associated with cortisol concentration; however, a relationship between stimulus ID and AB was revealed. Macaques that had been shown stimulus ID 2 had significantly higher salivary cortisol concentrations than macaques shown other stimuli. In Study 2, no evidence for a relationship between cortisol concentration and AB was seen. Both Study 1 and 2 suggest that the veterinary treatment may not be sufficiently physiological stressful for a change in cortisol concentration to be seen. This further supports the findings in Chapter 3 and 4, where the macaques' annual veterinary check was not associated with the expected changes in cognition or behaviour. This chapter did highlight the considering stimulus ID in study design. Differences in emotional impact of stimuli cause noise in study results and may have a negative physiological and emotional impact on participants.

5.2 Introduction

The stress response is an important evolutionary trait that gives a selective advantage in situations that require action or defence (Selye, 1956; Nesse et al, 2016). When a perceived threat or stressor disrupts homeostasis, the result is physiological and behavioural changes that provide short-term adaptive benefits and elicit one of the following responses: "fight or flight", "freeze-flight-fight" or "tend and befriend" (Sapolsky, 1992; Moberg, 2000; Taylor et al, 2000; Elwood et al, 2009). The "tend and befriend" response involving oxytocin is discussed in Appendix 5a. Incoming sensory cues from potentially threatening stimuli activate structures in the limbic forebrain and brainstem. This triggers a complex cascade of nervous and endocrinological processes in the sympathetic nervous system (SNS) and hypothalamic–pituitary–adrenal (HPA) axis resulting in the physiological and neurobiological changes associated with these responses (Sherman & Guillery, 2001; Ulrich-Lai & Herman, 2009; Ressler, 2011). Changes in the HPA axis and how these relate to cortisol production are explained below.

Hypothalamic-pituitary-adrenal axis

The HPA axis produces cortisol to mobilise energy when needed. In the HPA axis, the action potentials from the forebrain and brainstem systems stimulate hypophysiotropic neurons in the paraventricular nucleus of the hypothalamus to release peptide hormones including arginine vasopressin (AVP), corticotropin releasing hormone (CRH) and oxytocin (Ulrich-Lai & Herman, 2009). These hormones travel via the portal circulation of the median eminence to the anterior pituitary gland where binding triggers the release of adrenocorticotropic hormone (ACTH). ACTH stimulates the adrenal cortices of the adrenal glands to release mineralocorticoid and glucocorticoid hormones.

Cortisol is a key glucocorticoid hormone released from the adrenal gland following stimulation by ACTH. Like the catecholamine hormones, the glucocorticoid hormones, including cortisol, increase blood glucose levels via gluconeogenesis. During the acute stress response, essential tissues, such as the muscle and adipose tissue, must rely on low efficiency energy sources such as fatty acids

from lipolysis and glucose produced via gluconeogenesis as cortisol diverts glucose to the brain by decreasing glucose uptake elsewhere in the body (Heintz et al, 2011). This process suppresses digestion, the immune response and the development of sperm and egg cells. As a result, chronic stress can lead to major physiological changes, for example, infertility and increased susceptibility to disease (Sapolsky, 2002; Wingfield, 2005; Heintz et al, 2011).

Cortisol

In animal studies, cortisol has been measured in blood plasma and serum (Sheriff et al, 2011), saliva (Pearson et al, 2008), urine (Lang & Linnet, 2014), faeces (Touma & Palme, 2005), hair (Cone, 1996), bird feathers (Bortolotti et al, 2009), fish tank water (Scott et al, 2008) and fish, bird and reptile eggs (Auperin & Geslin, 2008; Chin et al, 2009; Warner et al, 2009). Saliva was chosen for the present study as it has previously been validated as a suitable, low-stress, non-invasive alternative to serum for cortisol analysis in human (Hellhammer et al, 2009) and NHPs (Boyce et al, 1995; Rapp-Santos et al, 2017), and other mammalian species (e.g., VanBruggen et al, 2011; Nemeth et al, 2016). Salivary cortisol content is highly correlated with serum cortisol levels (Wood, 2009). Cortisol has a rapid response in saliva of between 20 and 30 minutes (Kirschbaum & Hellhammer, 1989) with only a two to three-minute lag compared to blood cortisol response times (Kirschbaum & Hellhammer, 2000). This rapid response time allows collected cortisol samples to reflect a known period, for example, for the present study an AB trial.

In humans, cortisol concentration has both a circadian (one cycle per 24-hour period) and ultradian (repeated cycles throughout a 24-hour period) rhythm (Lefcourt et al, 1993; Trifonova et al, 2013) coordinated by the suprachiasmatic nuclei of the anterior hypothalamus (Moore & Eicher, 1972; Dorn et al, 2014). Macaques also experience cyclic changes in cortisol. The association between time of day and blood cortisol concentration has been extensively studied; blood cortisol concentration peaks in the morning, around 30-minutes after waking, and steading declines through the day (Plant, 1981; Novak et al, 2013). However, the circadian rhythm of macaque salivary cortisol is less well understood. The available evidence suggested that salivary cortisol

circadian rhythm is far less pronounced in captive macaques than humans, as cortisol levels remain relatively constant across the major activity period of the day for macaques (Pfefferle et al, 2018).

Cortisol & attention bias

Stress can affect attention and cognitive performance (Thayer & Lane, 2000; Maydych, 2019). Socially stressed humans are more likely to avoid negative information (Ellenbogen et al, 2002) and physiologically stressed children will visually disengage attention from footage of distressed conspecifics (Fabes et al, 1993). Bethell and colleagues (2012) showed that following veterinary intervention macaques become more avoidant of potentially threatening stimuli compared to a non-stressed baseline. Morin and colleagues (2019) reported mother-maltreated infant macaques had higher reaction times to social threat than those raised competently.

A major component of the stress response is cortisol, and levels of this hormone can affect attention and cognitive processing. The exogenous application of 40 mg of cortisol acutely reduces anxiety-driven selective attention to threat in men (Putman et al, 2007a). The exogenous application of cortisol acutely reduces selective attention during an unmasked emotional Stroop task in both healthy male volunteers (Putman & Berling, 2011) and those with social anxiety disorder (van Peer et al, 2010). Cortisol has also been shown to differentially regulate spatial working memory for neutral, happy, fearful, and angry facial expressions, reducing the bias for fear compared to a placebo (Putman et al, 2007b). Applehans & Luecken (2006) found that for individuals with higher trait anxiety attentional avoidance predicted decreased cortisol response. Trait anxiety refers to the consistent tendency to attend to, experience, and report negative emotions, for example, anxiety, fears, and worries, across many situations (Gidron, 2013). For individuals with lower trait anxiety attentional avoidance predicted elevated cortisol responses (Applehans & Luecken, 2006).

At the same time, visual stimuli can affect cortisol levels in some individuals, although the direction of effect is unclear. Following the presentation of angry faces to healthy human volunteers, higher salivary cortisol levels have been observed in both attentive individuals (van Honk et al, 2000) and

more avoidant individuals (van Honk et al, 1998). Children with Attention-Deficit/Hyperactivity Disorder (ADHD) have lower cortisol levels than comparable children without ADHD (Isaksson et al, 2012). These studies were conducted in humans and at present little is known about the relationship between cortisol and looking time in rhesus macaques. The available evidence suggested that in NHPs, socially anxious submissive individuals have higher basal cortisol levels and are more avoidant of threatening conspecifics than dominant individuals (Öhman, 1986; Sapolsky, 1990); however, it is not known if this translates to AB.

Cortisol & stress

Although the captive environment does not have same threats that affect free-ranging NHPs, such as predation, food shortages and uncontrolled health challenges (Beehner et al, 2005; Novak et al, 2013; Kamilar & Beaudrot, 2018), the social and spatial constraints of captivity may lead to abnormal social interactions and increased aggression (Novak et al, 2013). This aggression and social stress, along with stress relating to husbandry, management, veterinary and research procedures can make the laboratory a particularly challenging environment for NHPs.

Aggression and social stress can be the result of dominance challenges and overall group instability. Although, rhesus macaque groups have strict matrilineal social hierarchies (de Waal & Luttrell, 1985; Jackson and Winnegrad, 1988; Theirry, 2007), dominance challenges between males can lead to periods of dominance instability (Parga, 2009; Preis et al, 2019). Rank and group stability have been suggested as predictors of cortisol with higher cortisol levels seen in lower ranking subordinate individuals (Sapolsky, 1990; Creel, 2001) and during unstable dominance periods (Preis et al, 2019).

This instability and resulting aggression can lead to fight injuries, which are relatively common in captive rhesus macaques (Springer et al, 2009) with male-male aggression causing the most severe and traumatic injuries (Westergaard et al, 2003). In humans, traumatic brain injury (TBI) is associated with significantly lower concentrations of free and total cortisol (Kusmenkov et al, 2019). Although TBI is a major public health problem for humans, it is extremely rare in macaques unless

part of a research protocol (e.g., Kanda et al, 1981; Antona-Makoshi et al, 2012). Injury to other areas of the body is generally associated with a sustained increase in cortisol concentrations (dogs: Hwang et al, 1988; humans: Delahanty et al, 2003).

An animal's sex can influence stress and resulting cortisol level. Male animals are more likely to be involved in aggressive encounters and dominance challenges and have an increased likelihood of severe injury (Westergaard et al, 2003). In addition, psychological stress results in a twofold higher increase in cortisol levels in human men compared to women (Kirschbaum et al, 1992).

Injury, chronic illness, and the macaques' annual health check all require veterinary attendance. Veterinary treatment can be stressful with many animal species showing increased cortisol concentrations following a vet visit (e.g., Hudec & Griffin, 2019). Common veterinary practices, such as the use of ketamine as a sedative, are also known to increase cortisol concentration in macaques (Crockett et al, 1993, 2000; Winterborn et al, 2008).

Chronic stress can have detrimental effects on wellbeing including behavioural and developmental abnormalities (Clark & Schneider, 1993; Sapolsky, 2002; Pryce et al, 2011). Alopecia (hair loss) is a biomarker for chronic stress in captive rhesus macaques (Novak et al, 2016). Macaques with poor coat condition and lower alopecia scores have higher hair cortisol concentrations than those with higher alopecia scores (Lutz et al, 2016; Novak et al, 2016). The exact cause of alopecia varies, for example, aging, seasonal changes, genetics, nutrition, and hormonal imbalances can all influence the hair cycle and hair loss in macaques (Novak & Meyer, 2009). However, alopecia can also be caused by stress leading to physiological changes that cause hair loss or through self-directed pulling (Novak & Meyer, 2009).

Chronic stress and elevated cortisol levels are also associated with the onset of depression in adult female macaques (Shively et al, 2005; Qin et al, 2016) which negatively affects pre- and postnatal infant development (Clark & Schneider, 1993; Kinsella & Monk, 2009; Pryce et al, 2011). Neurophysiological changes resulting from early life stress include impaired brain development and

increased basal cortisol levels, associated with reduced cognitive performance and poor psychological well-being (Clarke et al, 1994; Lupien et al, 1994; Kubera et al, 2011).

The relationship between key AB testing variables, such as the location of the threat face stimulus and trial number, and cortisol level is currently unclear. Results from previous studies conflict, with some suggesting that the presentation of aggressive or fearful faces increases cortisol concentration (Susta et al, 2008; Hansel & von Kanel, 2012) while others have found similar stimuli presentations to have no effect on cortisol level (Ellenbogen et al, 2006).

This chapter aims to answer two questions:

1. Are stress, life-history and AB test related factors associated with salivary cortisol level?

Cortisol level will be correlated with key stress, life-history, and AB test related factors to determine which of these variables is associated with the largest increase in cortisol (Study 1).

2. Does cortisol concentration correlate with AB?

AB trials and salivary cortisol sample collection will be conducted before and after the macaques' annual veterinary health check. Duration looking at the threat face stimulus (THR) and total duration looking at the threat and neutral face stimuli (TL) will be correlated with cortisol level to determine the relationship between this key stress hormone and AB measures (Study 2).

5.3 Materials & methods

Ethics

Ethical approval was granted by Liverpool John Moores University (LJMU) in February 2017 (Ethical approval ID. EB_EH/2017-5) and by the Medical Research Council Animal Welfare and Ethical Review Body (AWERB) in November 2017. This project piggybacked onto routine veterinary and husbandry activities that would have occurred whether the animals were involved in this study or not. No regulated procedures were carried out for this study. Analgesia was not delayed because of any research relating to this PhD. All training was conducted following centre protocol and using

positive reinforcement methods. Participation in training and AB trials was voluntary, insofar as animals were free to leave the training and testing area (cage room) at any time. Food, water, and social contact with conspecifics were available *ad libitum* throughout training and testing.

Animals

Thirty-six (27 female, nine male), adult (>3 years old) rhesus macaques (*Macaca mulatta*) socially housed at the Medical Research Council Harwell Institute Centre for Macaques (MRC-CFM) were initially trained for this study (mean age = 7.99 ± 3.04 years, range = 3.5 to 16.17 years. The macaques were housed in 10 groups: nine breeding groups with one adult male, between three and eight adult females and their offspring, and one ex-breeding group with seven adult females and their offspring from previous years.

Of the 36 monkeys that started the saliva collection training, eight failed to reach criterion for inclusion. Samples were collected from 28 monkeys (20 female, mean age = 7.77 ± 3.39 years, range = 3.5 to 16.2 years). Samples from all 28 monkey were analysed; however, some samples were removed from the statistical analysis due to very high variation between duplicates (high %CV), low sample volume, contamination, or insufficient repeats for an individual (each macaque had to provide at least two successful samples to be included in the statistical analysis). Successfully collected and analysed samples were from 17 macaques (10 female, 7 male). The reproductive status, date of birth and number of samples collected from each is shown in Table 5.1.

Table 5.1. Reproductive status, age, and number of successfully collected and analysed samples from each macaque included in the analysis for this study. Samples were collected between April and August 2018.

Reproductive status	ID	Age at start of testing (months)	Number of samples
Breeding male	Nodon	166	4
	Plum	140	3
	Sequel	113	5
	Sol	192	3
	Star	116	8
	Viktor	81	8
	Vincent	80	5
Pregnant female	Sienna	119	2
	Uno	92	2
	Venice	75	5
	Yeva	53	3
	Yoana	52	4
	Yoyo	58	3
	Zelda	43	7
	Zena	43	4
Cycling female	Wine	67	7
Nursing female	Yibbi	54	3

Attention bias

The full AB protocol is given in Chapter 3. In brief, threat-neutral unfamiliar male conspecific face pair stimuli (Witham & Bethell, 2019) were shown to macaques on a computer monitor screen for three seconds. Looking time at each stimulus was recorded and AB score was then calculated by subtracting the time spent looking at the neutral face from the time spent looking at the threat face.

AB trials were conducted once per day per macaque on four consecutive weekdays from Tuesday to Friday, and then a fifth trial was conducted on the following Monday. The baseline condition

(assumed non-anxious state) was timetabled so that trials occurred in weeks during which there were no activities planned which were deemed potentially stressful i.e., no cleaning, animal removals, or planned veterinary procedures. The post-stressor condition was timetabled so trials occurred on the five working days following the scheduled health check (Tuesday – Monday).

Saliva sample collection

Saliva samples were collected using Salimetrics 8 mm polymer SalivaBio Children's Swabs (https://www.salimetrics.com/collection-method/childrens-swab-device/). SalivaBio Children's Swabs are quality controlled by Salimetrics, validated for cortisol recoveries, and verified for consistent performance and sample pH (Salimetrics, 2016). To increase the attractiveness of the swabs, the swabs were soaked in a solution of 28.45% granulated sugar and 71.55% boiled tap water for at least three hours and left to dry completely at room temperature. Following Newman et al (2007), swabs were soaked in 28.45% granulated sugar solution, as this does not affect salivary cortisol concentration in rhesus macaques.

Dried swabs were clamped into D-shackled (Figure 5.1) and presented to the test macaque for chewing. Macaques had previously been trained to chew for up to 30 seconds in order to collect > 100 µl saliva required for analysis (Bertrand et al, nd; Chapter 2, 2.3). Saliva samples were taken approximately 20 minutes after each AB trial. Trials conducted before 09:00 or after 15:00 were done so due to enclosure cleaning. Chewed swabs were inspected and any contaminated with blood or food were discarded (Schwartz & Granger, 2004).

Immediately after chewing, swabs were placed into Salimetrics swab storage tubes (https://salimetrics.com/product/swab-storage-tube-sst-50pk/) labelled with monkey ID, condition, and day number (BL1-5, HC1-5) and the date. Tubes were centrifuged for 15 minutes at 4000 rpm in a Heraeus Megafuge 1.0 centrifuge. Centrifuged samples were stored onsite at -20°C (Garde & Hansen, 2005). Centrifugation and freezing occurred within 1 hour of sample collection. Samples remained frozen at MRC-CFM until transportation to Liverpool John Moores University (LJMU). Samples were transported on ice packs in polystyrene freezer boxes and stored at -20°C at

LJMU until defrosted for analysis. Transportation on ice was necessary due to financial restraints; however, it was deemed appropriate as Garde & Hansen (2005) reported that repeated (up to four) freeze thaw cycles had no effect on salivary cortisol concentrations.

Cortisol analysis was by enzyme immunoassay (EIA) at the LJMU Hormone Laboratory. To separate the saliva from the swabs, sample tubes were centrifuged for 15 minutes at 4000 rpm once thawed. Saliva samples for EIA were pipetted from the collection area at the bottom of the storage tube. EIA was a competitive binding assay, which has previously been validated as highly sensitive for salivary cortisol in NHPs, including rhesus macaques (Heintz et al, 2011; Sheriff et al, 2011; Pfefferle et al, 2018). The EIA protocol was adapted from the Palme (2017) method. A full EIA protocol is detailed in Appendix 5b.

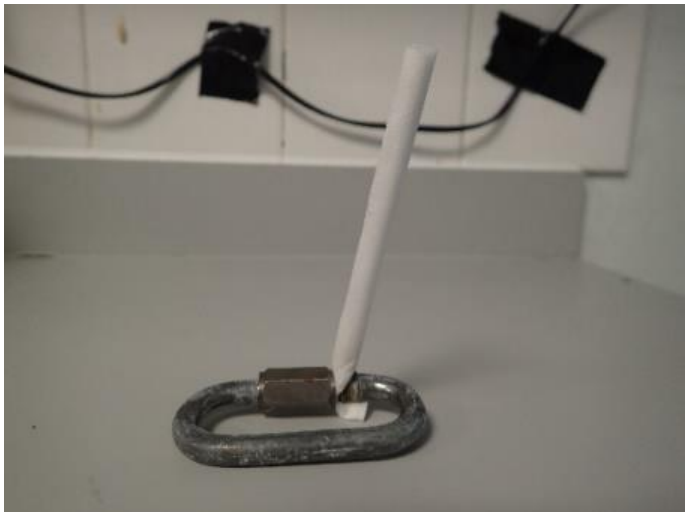

Figure 5.1. Salimetrics Children's swab clamped into a D-shackle for saliva sample collection from rhesus macaques at MRC-CFM. Photograph: E. Howarth.

Data treatment

The absorbances of the samples were first checked for variation. The percentage coefficient of variation (%CV) was calculated as %CV = $\frac{SD}{Mean} * 100$. Ideally the intra- and inter-assay %CVs should be <10% and <15%, respectively (Thomsson et al, 2014). However, using strict cut off points would have resulted in an insufficient sample size for the analysis. Therefore, a more sympathetic intra- and inter-assay %CV of 15% and 20%, respectively, were used. %CV refers to the precision of the

assay results and higher %CVs indicate that there is high variation between duplicates of the same sample. Although studies have used intra- and inter-assay %CVs of 15% and 20% (Reed et al, 2002), the increased risk of error should be considered when interpreting the results. Any samples with a higher %CV were immediately removed from the analysis. High and low cortisol quality controls were included in duplicate twice on each plate. Inter-assay %CV of the quality controls for compared assays was ≤ 19.63%.

Cortisol concentration (pg/ml) was calculated from the duplicate absorbances (nm). Standard curves were produced using MyAssays analysis software (https://www.myassays.com/; Figure 5.2). To calculate the cortisol concentrations (pg/ml) the following steps were taken:

1. Calculate the mean for each of the duplicate absorbances (nm)
2. Calculate the sample bound value (B) for each sample - subtract the mean optical density for the non-specific binding (NSB; no anti-cortisol antibody) wells from all the other absorbance means leaving just the absorbance resulting from the concentration of bound cortisol.
3. Calculate the B/B_0 - Divide each B value by the B_0 value (no cortisol, maximum anti-cortisol antibody bound)
4. Calculate $\%B/B_0$ – multiply B/B_0 by 100
5. Plot the $\%B/B_0$ for the standards against their known cortisol concentrations.
6. Use the calculated $\%B/B_0$ and the equation of the line from the plot to calculate the cortisol concentrations for each sample.

Concentrations of any samples that had been diluted prior to the assay were multiplied by the dilution factor. Samples that fell outside of the range of the standard curve were removed. Animals that had only one successful sample were not included in the statistical analysis.

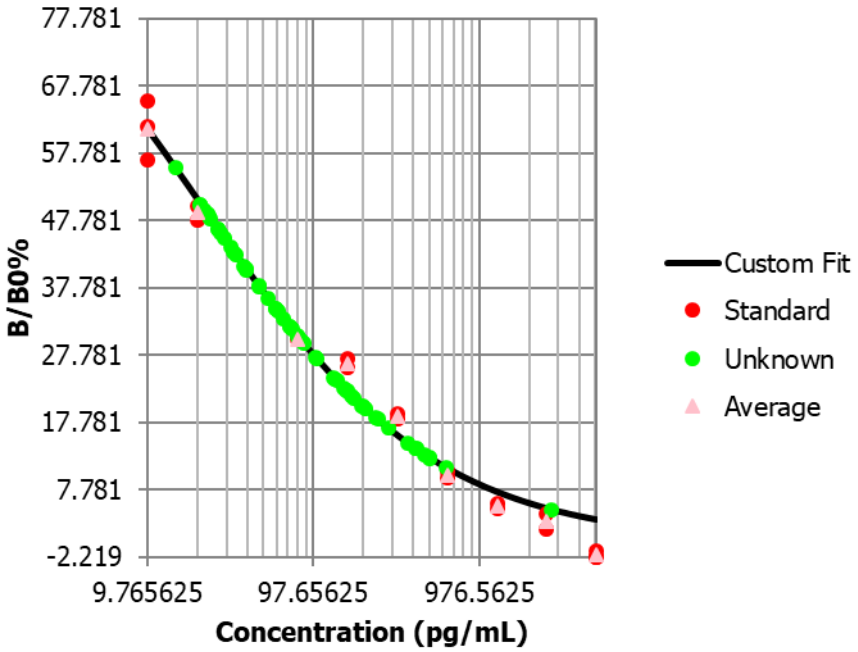

Figure 5.2. Example cortisol assay standard curve and plotted unknown samples. Figure created in MyAssays analysis software (https://www.myassays.com/). %B/B_0 is the ratio of absorbance of a sample or standard well to that of the maximum binding (B_0) well.

Predictor variables explanation

The condition under which the AB trials were conducted was either baseline or post-stressor. Cortisol concentration was salivary cortisol concentration determined from EIA and measured in pg/ml. Trial number was measured 1 – 5 at baseline and 1 – 5 post-stressor so that matching weekdays could be compared, for example, trial 1 was always conducted on Tuesday for both baseline and post-stressor. Stimulus monkey identity referred to stimuli shown in Figure 3.2. Time was rounded down to the nearest hour, for example, both 14:05 and 14:56 would be rounded to 14:00. Location of the threat face stimulus (AggLoc) was to the left or right of the monkey view. Sex was male or female. Disruption in the group referred to if any animal in the group received a veterinary visit in the previous 24 hours.

Statistical analysis

Hormone assay results from monkeys for which I did not have a full set of samples and where duplicates had an intra-sample coefficient of >15% were omitted from analysis. The final number of samples used in the analysis was 77 from 17 monkeys.

All statistical analyses were conducted using R version 3.6.0 (R Core Team, 2018). Linear mixed effects models (LMM) were developed and fitted using the function *lmer* of the R-package lme4 (Bates et al, 2015). LMM are used to analyse continuous, hierarchical data and can cope with unequal sample sizes and missing data (Smith, 2012; Gałecki & Burzykowski, 2013).

Animal identity was included as a random effect in all models. To avoid collinearity, all predictor variables were checked for correlations and for those above 0.4, one variable was removed (Crawley, 2007). Criteria for selecting the retained variable was relevance to the study question, for example, in Study 1 condition (baseline or post-stressor) was always retained in the model.

Predictor and response variables were also checked for their distribution. Variables that showed non-normal distribution were transformed using Tukey's Ladder of Power (Tukey, 1977). The Tukey transformation provided a λ value that maximised the Shapiro-Wilk W statistic or minimises the Anderson-Darling A statistic (Mangiafico, 2016). The Schapiro-Wilk statistics should be maximised as a significant or small Shapiro-Wilk W statistic indicates that the data is not normally distributed (Oztuna et al, 2006). The Anderson-Darling statistic should be minimised as a smaller Anderson-Darling A statistic indicates that the distribution better fits the data (Lewis, 1961). The Tukey transformation was conducted using the function 'transformTukey' of the R-package rcompanion (Mangiafico, 2019). Variables with a λ of 1.0 were not transformed as this indicated normal distribution. Covariates were z-transformed to a mean of zero and a standard deviation of one to allow easier comparison of estimates and interpretation of interactions from the model output (Schielzeth, 2010).

For each model, the residuals were plotted against fitted values and qq-plots (scatterplot comprising two sets of quantiles plotted against each other (Ford, 2015)) of the residuals were visually inspected to check whether the models fulfilled the assumptions of normally distributed and homogeneous residuals (Crawley, 2007). The models were developed by excluding non-significant predictor variables with the greatest *p* values until only those factors with $p<0.05$ were retained in the final model. Factors with non-significant *p* values were retained if they were

required for model stability. Models were deemed to be stable if the original value lay between the minimum and maximum values revealed using the function 'summary'. The reduced model estimates were compared with the estimates from the full model and all models were checked for stability using the function 'glmm.model.stab' (Hofner & Hothorn, 2017).

The significance of each model as compared to the null model (comprising only the random effect of animal ID) were established using a likelihood ratio test with the R function ANOVA with argument test set to 'Chisq' (Dobson, 2002; Forstmeier & Schielzeth 2011). Models were fitted using Maximum Likelihood, rather than Restricted Maximum Likelihood, to allow for a likelihood ratio test (Bolker et al, 2008). Likelihood ratio tests comparing the full model with the respective reduced models using the R function 'drop1'. The 'drop1' function provided the *p* values for the individual effects (Barr et al, 2013). Confidence intervals were calculated using the function 'confint.merMod' of the R-package lme4 to calculate the likely range of the sample and allow estimation of the precision of the sample compared to the true population (Bates et al, 2015). To aid interpretation, non-transformed data were used for plotting purposes.

Study 1 - Are stress, life-history and AB test related factors associated with salivary cortisol level?

Two *lmer* models were fitted with cortisol concentration as the dependent variable. Maximal model 1 contained key predictor variables relating to stress (condition, disruption in the group), sex and time. The degrees of freedom (df) for the model were 6. The data set contained 77 rows of cortisol data from 17 macaques which allowed for >10 rows of data per df (Crawley, 2007).

~Condition + DisruptionInGrpOtherYN + z.Tukey.TimeR + Sex

where: z = scaled; Tukey = Tukey transformation

Maximal model 2 contained key predictor variables relating to AB testing (location of the threat face stimulus, trial number, stimulus ID).

~AggLoc + z.Tukey.Trial14or5InWeekorBlock + StimulusID

where: z = scaled; Tukey = Tukey transformation

The degrees of freedom (df) for the model were 9. The data set contained 77 rows of cortisol data from 17 macaques which did not allow for >10 rows of data per df (Crawley, 2007). This was considered when selecting the final model. A copy of the full R script is shown in Appendix 5d.

Study 2 - Does cortisol concentration correlate with AB?

Two *lmer* models were fitted- one for each of the AB measures (THR, TL, and ABDiff). The initial maximal models contained key predictor variables relating to cortisol concentration (pg/ml), stress (condition: baseline and post-stressor) and AB testing (location of the threat face stimulus, trial number, time). This was a within animal comparison, therefore, factors relating to the animal, for example, sex, age and rank were not included in the model.

~Tukey.CORT + Condition + AggLoc + z.Tukey.Trial14or5InWeekorBlock + z.Tukey.TimeR

where: z = scaled; Tukey = Tukey transformation

The degrees of freedom (df) for the model were 6. The data set contained 77 rows of cortisol data from 17 macaques allowing for >10 rows per df (Crawley, 2007). A copy of the full R script is shown in Appendix 5c.

5.4 Results

Trials for which saliva swabs were collected and successfully analysed occurred between 08:26 and 16:28 (mean = 11:40 ± 1hr 39 mins, median = 11:25, mode = 09:40) with 93.5% of trials conducted between 09:00 and 15:00. Macaque salivary cortisol has been shown to be stable between 09:00 and 15:00 (Pfefferle et al, 2018). The consistency in macaque samples collected between these times suggests that the cortisol circadian rhythm seen in other species (Plant, 1981; Novak et al, 2013) is not as pronounced and, therefore, the samples collected between these times in this study will not have been impacted by this rhythm.

The detectible cortisol values ranged between 112.45 and 13973.40 pg/ml. The mean cortisol concentration was 10189.43 ± 39132.46 pg/ml. Mean total looking time at the threat face stimulus

(THR) was 797.07 ± 651.15 ms and mean total looking time at both the threat and neutral face stimuli (TL) was 1423.62 ± 818.47 ms. The number of samples collected for each level in the factor variable were condition: baseline n = 38, post-stressor n = 39; sex: males n = 36, females n = 41; disruption in the group: no n = 62, yes n = 15; location of the threat face stimulus: left n = 41, right n = 36; stimulus ID: 1 n = 12, 2 n = 26, 3 n = 5, 4 n = 12, 5 n = 10, 6 n = 12, 7 n = 10.

Study 1 - Are stress, life-history and AB test related factors associated with salivary cortisol level?

The final model was not significant as compared to the null model for stress (likelihood ratio test: χ^2 = 1.686, df = 1, p = 0.194). None of the stress related variables influenced salivary cortisol concentration. The final model for AB test factors was different as compared to the null model and that difference approached significance (likelihood ratio test: χ^2 = 12.333, df = 6, p = 0.055).

For the AB test related factors, stimulus monkey identity was retained in the final model (Table 5.2). Monkeys that had been shown stimulus 2 (mean = 25064.1 pg/ml, SD = 62960.71 pg/ml) during AB testing had higher salivary cortisol concentrations than monkeys shown the other stimuli (1, 3-7; Figure 5.3). Monkeys shown stimulus 3 during AB testing had the lowest cortisol concentration (mean = 1188.747 pg/ml, SD = 1839.816 pg/ml).

Table 5.2. Results for the effect of AB test factors on cortisol concentration in rhesus macaques (n = 17). Cortisol was Tukey transformed for analysis (λ = -0.25).

Stimulus ID (compared to 2)	Estimate	Std. Error	t	2.5 %	97.5 %	LRT	*p*
1	-0.035	0.017	-2.032	-0.069	-0.0001	12.333	0.055
3	-0.062	0.023	-2.700	-0.108	-0.016		
4	-0.029	0.017	-1.695	-0.063	0.005		
5	-0.026	0.017	-1.494	-0.061	0.009		
6	-0.002	0.017	-0.148	-0.035	0.031		
7	-0.041	0.018	-2.289	-0.077	-0.005		

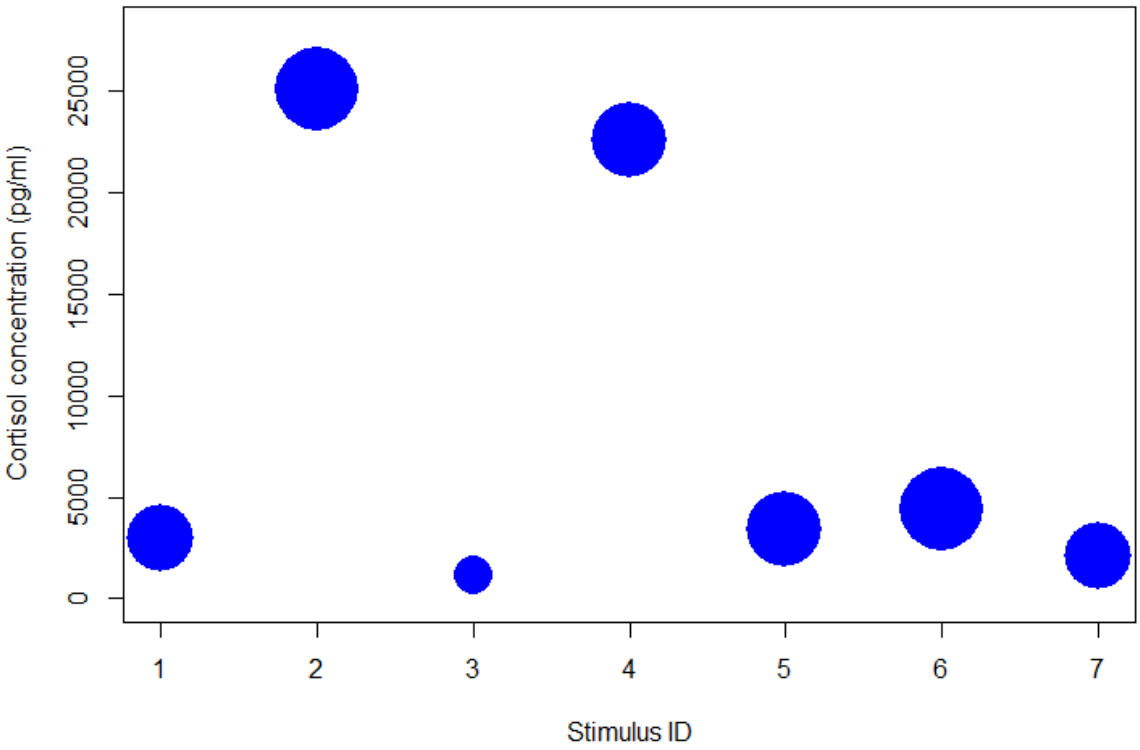

Figure 5.3. The relationship between salivary cortisol concentration (pg/ml) and stimulus monkey identity during AB testing for rhesus macaques. Circle size indicates number of monkeys that saw each stimulus.

Study 2 - Does cortisol concentration correlate with AB?

Overall, the final models were not significantly different as compared to the null models for THR (likelihood ratio test: $\chi^2 = 2.468$, df = 1, $p = 0.116$) and TL (likelihood ratio test: $\chi^2 = 2.218$, df = 1, $p = 0.136$). This suggests that there is no association between cortisol concentration and either of the AB measures in this study.

5.5 Discussion

In this chapter, I aimed to determine 1) if stress, life-history, and AB test related factors are associated with salivary cortisol level (Study 1) and 2) if cortisol concentration correlated with AB (Study 2). I present data from 77 AB trials and salivary cortisol samples from 17 rhesus macaques.

The results show that salivary cortisol does not have a significant association with any of the AB looking time measures. This study also shows that the life history, veterinary and husbandry stressors did not have a significant relationship with salivary cortisol concentration nor did any of

the test related variables except for stimulus ID. Macaques shown stimulus ID 2 had significantly higher cortisol levels than macaques shown other stimuli.

Previous studies with human participants have shown that cortisol significantly increases avoidance and reduces the bias for fearful or angry faces (van Honk et al, 2000; Putman et al, 2007ab; Putman & Berling, 2011); this effect was not seen in rhesus macaques in this study. Due to the evolutionary similarities in behaviour and physiology between humans and macaques (APC, 2013), we expected cortisol to have a similar effect to that seen in humans. However, AB in humans and macaques differ also in other parameters. Humans have enhanced vigilance to threat while anxious or stressed (Bradley et al, 2000) while macaques become more avoidant of negative stimuli following a stressor (Bethell et al, 2012b). This difference may explain the lack of effect seen in this study as humans and macaques respond differently to AB trials more generally.

This study also shows that the life history, veterinary and husbandry stressors did not have a significant effect on salivary cortisol. Key stressors that have previously been shown to affect cortisol concentration, for example, sedation (macaques: Crockett et al, 1993, 2000; Winterborn et al, 2008) and injury (dogs: Hwang et al, 1988; humans: Delahanty et al, 2003) could not be included in the analysis due to insufficient sample sizes and a risk of type I error (Columb & Atkinson, 2015). Ten samples out of the 77 were from macaques that had received sedation in the last 24 hours and only three samples were from macaques that had been injured in the last 48 hours. No macaques included in this study had a chronic illness or a wound and 93.5% had an alopecia score of four or above indicating good hair condition that has not been affected by stress (Lutz et al, 2016; Novak et al, 2016). An effect of rank would be expected as subordinate primates tend to be more stressed than dominant animals displaying physiological and behavioural signs of depression (Michopoulos et al, 2012; Meyer & Hamel, 2014). However, only two samples were from low-ranking macaques meaning a comparison was not possible. As this research piggybacked onto planned routine veterinary and husbandry procedures these low samples sizes could not be controlled. This resulted

in the number of macaques that had experienced these stressors being too low for a statistically significant result to be found.

For this study, larger intra- and inter-assay %CVs than standard were used (15% and 20%, respectively). Although previous studies (e.g., Reed et al, 2002) have used these higher %CVs, the increased risk of error should be considered when interpreting the results. The lack of significant change or association in line with what was expected may be the result of error introduced through analysis or using higher %CVs. Salivary cortisol has previously been validated as a suitable, low-stress, non-invasive alternative to serum for cortisol analysis in humans (Hellhammer et al, 2009) and NHPs (Boyce et al, 1995; Rapp-Santos et al, 2017), and other mammalian species (e.g., VanBruggen et al, 2011; Nemeth et al, 2016). Further, saliva was chosen for this study as cortisol has a rapid response in saliva of between 20 and 30 minutes (Kirschbaum & Hellhammer, 1989) with only a two to three-minute lag compared to blood cortisol response times (Kirschbaum & Hellhammer, 2000). This rapid response time allowed collected cortisol samples to reflect the period in which the AB trial took place.

However, the sensitivity and short lag time of salivary cortisol may also have negatively impacted this study. As cortisol is a measure of physiological arousal (Heintz et al, 2011), which includes both distress and eustress (Selye, 1956), macaque activity prior to the AB trials may have influenced salivary cortisol levels. Cortisol levels may change in response to exercise (Ahmadi et al, 2018), sexual arousal (Hamilton et al, 2008), pregnancy (Soma-Pillay et al, 2016), postpartum (Freitas-de-Melo et al, 2017) and aging (Boss & Seegmiller, 1981). Therefore, the salivary cortisol samples collected for this study may not reflect the macaques' underlying emotional state rather their level of activity and nature of their social interactions prior to testing. It may be beneficial to repeat a similar study using faecal or urinary cortisol, as these materials are more appropriate for assessing the long-term physiological effects of a stressor on macaques, for example, faeces has a cortisol response time of 22 hours in rhesus macaques (Bahr et al, 2000; Palme, 2005; Hodges &

Heistermann, 2011). Faeces was not considered appropriate for this study due to challenges in individual sample identification in group housed animals and the risk of contamination with urine.

Results from Chapter 3 and 4 suggested that the macaques' annual veterinary health check, used as a stressor in this study, may not have been stressful enough to noticeably compromise welfare. Despite the issues with sampling and analysis highlighted above, this lack of significant change in salivary cortisol may reflect the mildness and acute nature of the stressor. Pfefferle et al (2018) reported that the presence of skull-mounted chronic implants had no effect on salivary cortisol and that an increase was only seen in animals with strict fluid control protocols or following cleaning of the chronic head implants that involved being restrained in a primate chair for 45 to 60 minutes while the dead skin was removed, and the area disinfected. In line with the suggestions in the previous chapters, in addition to using faeces, a more severe stressor may be more suitable for studying the relationship between AB and cortisol.

Variation in factors relating to the AB testing (threat face location and trial number) did not have a significant association with cortisol level. This may suggest that repeated exposure to AB trials is not physiologically stressful for rhesus macaques and that the test itself does not compromise welfare. However, macaques that had been shown stimulus ID 2 had significantly higher salivary cortisol concentrations than macaques shown other stimuli. Sustained eye contact is a threatening display in macaques (van Hooff, 1967; McFarland et al, 2013). A Human Intruder Test was used by Hamel and colleagues (2017) to assess the relationship between sustained eye contact, cortisol level and behavioural response. The authors reported that individuals with high cortisol were significantly more aggressive towards the intruder than monkeys with lower cortisol following a period of staring. Stimulus ID 2 (Figure 5.4) had more prominent front facing eyes than the other stimuli used in the present study (see Figure 3.2 for full stimulus set). The prominent eyes may have been perceived as more threatening which may explain the elevated cortisol associated with stimulus ID 2. This highlights the importance of carefully considering differences in the emotional impact of stimuli. This is an important finding as it may explain the conflicting human literature with

some studies finding that stimulus presentation does not result in elevated cortisol levels (Ellenbogen et al, 2006) while others reported an increase in cortisol following exposure (Susta et al, 2008; Hansel & von Kanel, 2012). This indicates the importance piloting a stimulus set prior to conducting research to ensure that stimuli are not creating noise in study results or resulting in physiological and emotional stress for participants.

Figure 5.4. Stimulus ID 2: a threat-neutral unfamiliar conspecific face pair used in attention bias testing with rhesus macaques (*Macaca mulatta*). The threat face is shown on the right (eyes and mouth open) and the neutral on the left (eyes and mouth closed).

5.6 Conclusion

Although cortisol does not influence looking time during AB testing, this study has shown that repeated AB trials are not physiologically stressful for rhesus macaques as there is no significant elevation in salivary cortisol level associated with trial number. This suggests that AB trials do not compromise welfare and, following further testing and validation will be an appropriate method of welfare assessment. Stimulus ID 2 is associated with high cortisol concentrations compared to other stimuli and highlights the importance of trialling stimuli prior to testing to avoid influencing results.

Chapter 6 – Genotypic correlates of attention bias

6.1 Abstract

Genetic variation can affect behaviour and personality in non-human primates with key genetic variants at certain loci resulting in increased susceptibility to anxiety, depression, and stress related disorders. The present study aimed to identify associations between key genetic polymorphisms and attention bias (AB). AB is a measure of anxiety, which has been validated as a non-invasive method of welfare assessment for rhesus macaques (*Macaca mulatta*). Previous studies have revealed that genotype can influence AB in both humans and NHPs. Here, 61 (45 female, 16 male), socially housed, adult (>3 years old) rhesus macaques were genotyped for nine polymorphisms in key candidate genes of the serotonin, dopamine, oxytocin, or cortisol pathways. The relationship between genotype and AB measures (time spent looking at the threat face stimulus, and total looking time) during AB trials was analysed using two different R scripts: without pedigree (measure of relatedness) using the *lmer* function and with pedigree using the *MCMCglmm* function. When pedigree was not included, there was a significant association between looking time and genotype for *tryptophan 5-hydroxylase 2* (*TPH2*), *arginine vasopressin receptor 1A* (*AVPR1a*), *serotonin transporter intron 2* (STin), the *5-hydroxytryptamine receptor 2A* gene (*HTR2A*) and haplotypes for the *oxytocin receptor* (*OXTR*) and *dopamine receptor D4* (*DRD4*). When pedigree was included, there was a significant association between looking time and genotype for *opioid receptor mu(μ) 1* (*OPRM1*) and a relationship that approached significance for 5-HTTLPR and haplotype for *DRD4*. Both with and without pedigree, macaques with the *DRD4* 2-3 haplotype were more attentive and had a higher total looking time than individuals with the 1-1 haplotype. The differing results highlight the importance of including relatedness of the individuals in a genetic study as type I errors can occur when pedigree is not included.

6.2 Introduction

Genetic variation refers to differences in DNA sequences between individuals within populations. In humans, genetic variation can predispose individuals to conditions such as alcoholism, diabetes, and heart disease (e.g., Classen et al, 2012; de Lauzon-Guillain et al, 2019; Schermer, 2019). In both humans and animals, this variation is known to affect both personality (Verhulst et al, 2016) and behaviour (Grandin & Dessing, 2014). For example, there is known to be a strong link between genetic variants at certain loci and an individual's susceptibility to anxiety, depression, and stress related disorders (e.g., Hu et al, 2006; Smoller, 2016; Wingo et al, 2018).

Variation that occurs at a frequency of ≥ 1% is known as a genetic polymorphism (Brookes, 1999). Single nucleotide polymorphisms (SNP) are changes in single base pairs (bp) of DNA (Grandin & Dessing, 2014). SNPs are the most common type of genetic polymorphism in humans with an average of 1 SNP for every 1000 bp, resulting in between 4 and 5 million SNPs in every person's genome (Wang et al, 1998; Guryev et al, 2004; Ma & Gao, 2018). Most SNPs are found between genes, in the non-coding regions, and are presumed to have no effect on protein synthesis; however, some SNPs are found within genes or in the regulatory regions near a gene (Kitts et al, 2013; Genetics Home Reference, 2019a). These within gene SNPs may be synonymous (silent), and have no effect on the amino acid sequence, or nonsynonymous and change the encoded amino acids (Kitts & Sherry, 2002). SNPs may also result in changes to promoter or other regulatory activity and hence influence gene expression (Shastry, 2009; Genetics Home Reference, 2019a). In addition to SNPs, length polymorphisms can also affect gene functioning, for example, rare within gene triplet repeat expansion disorders are associated with a variety of neurological diseases (Budworth & McMurray, 2013). Length variation is caused by insertion, deletion or duplication polymorphisms which result in changes in the number of DNA bp, for example, deletion polymorphisms contain fewer bp and are, therefore, shorter (Rodriguez-Murillo & Salem, 2013; Genetics Home Reference, 2019b). Length polymorphisms can also have significant phenotypic effects if they occur within the introns (non-coding regions of DNA separating the coding exons that are removed (spliced) from the sequence before the RNA is translated into a protein), regulatory regions or promoter regions

(Reinar et al, 2018; see Figure 6.1 for gene structure) particularly in relation to disease (Mirkin, 2007). Like SNPs, length polymorphisms in key genes can also increase susceptibility to anxiety-related disorders. Lesch et al (1996) reported that a length polymorphism in the serotonin reuptake transporter gene accounted for around 3.5% of total variation and 8% of inherited variation in anxiety-related personality traits in a sample of 505 humans. A variable number of tandem repeats (VNTR) length polymorphism and a SNP in the circadian clock-related gene (*PER3*) were associated with higher trait anxiety, sleep and mood disorders and seasonal affective disorder (Liberman et al, 2017). A length polymorphism in the *Aldehyde Dehydrogenase 2* (*ALDH2*) gene was found to alter cognitive functioning in patients with bipolar II disorder and anxiety disorders (Lu et al, 2018).

If a trait is controlled by the expression of a single gene or allele, and a variant occurs within the gene it can result in a monogenic disorder (Richard, 2005). Cystic fibrosis, haemophilia and sickle cell anaemia are all disorders that result from a single defective gene (WHO, 2019). By contrast, polygenic traits are controlled by, or involve, two or more genes (Brody, 2019). Apart from a few behavioural symptoms of specific monogenic disorders, for example, several monogenic disorders associated with epilepsy (Scheffer & Dravet, 2014) or certain rare, syndromic forms of autism (Benger et al, 2018), most behaviours are polygenic. Identifying those loci underpinning such behavioural traits has typically involved treating them as quantitative traits (Flint, 2003). Regions of DNA associated with these traits are then called quantitative trait loci (QTL; Martinez et al, 2016) and specific QTL have been identified relating to avoidance behaviours (Turri et al, 2001), anxiety (Fernandez-Teruel et al, 2002) and aggressive behaviour (Edwards & Mackay, 2009). Typically, QTL are broad, and require substantive follow-up work to identify causal genes within the identified QTL. An alternative way to identify behaviour-associated genes is the genome wide association study (GAWS). GWAS has been utilised to study a range of behavioural conditions (e.g., Coleman et al, 2016; Strawbridge et al, 2018; Tielbeek et al, 2018). After QTL analysis or GWAS, follow-up studies can focus on these specific loci rather than screen the genome.

Criteria for inclusion of candidate genes in a study include a) they play a role in a specific biological pathway related to the trait of interest, b) an allele or alleles of the candidate gene affect overall gene function or c) polymorphisms occur frequently enough to allow statistical interpretation (Wang et al, 2013). An example of a potential candidate gene in human anxiety studies is the *cholinergic receptor nicotinic alpha 4 subunit* (*CHRNA4*) gene, which codes for a subunit of the nicotinic acetylcholine receptor. Humans with the *CHRNA4* cytosine (C/C) variant, which is not present in rhesus macaques (Cunningham et al, 2019), have been observed to have higher harm avoidance than thymine (T)-allele carriers (Ross et al, 2000; Markett et al, 2011, 2016).

Behaviour involves a variety of pathways in the brain and wider nervous system (Cowan et al, 1989; Kim et al, 2013) and any member of these pathways may represent a candidate gene for study of behavioural traits. Here, those candidate genes examined in this chapter are introduced.

Serotonin pathway

A key candidate gene in the study of anxiety and stress-related disorders is the serotonin transporter (5-hydroxytryptamine transporter or 5-HTT) gene (also known as *SLC6A4*). *SLC6A4*

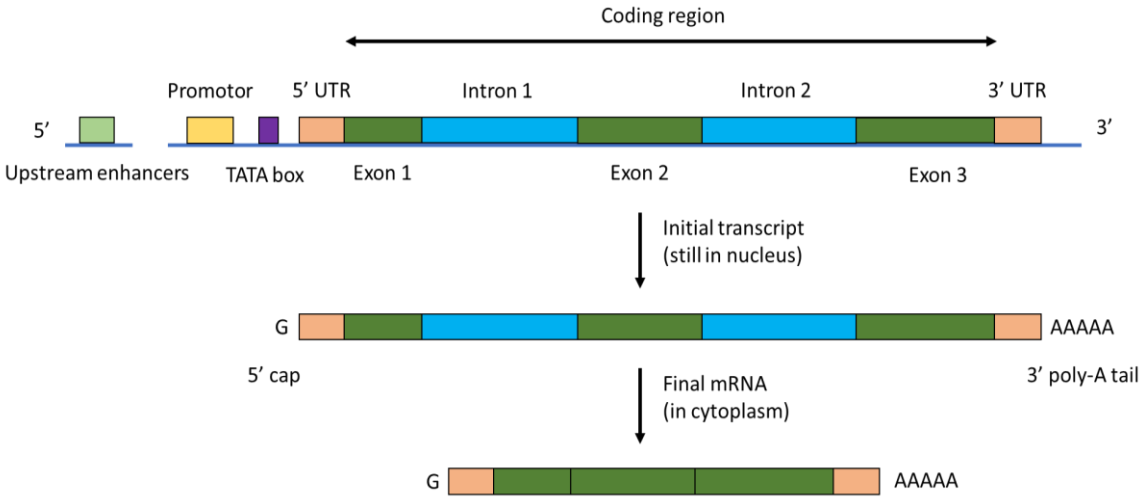

Figure 6.1. Schematic diagram of transcription and RNA processing for translation of a typical eukaryotic gene structure with 5' and 3' untranslated regions (UTR). Transcription, which can be controlled by 5' regulatory regions (e.g., TATA box, promoter and upstream enhancers) produces an initial transcript with a 5' G-cap and 3' poly-A tail. Final processing leads to the final mature mRNA. Polymorphisms in the promoter region or upstream enhancers may affect binding by transcription factors and hence alter expression of the gene. Figure adapted from Walsh (2003).

contains three well-studied polymorphisms in humans: a guanine/thymine (G/T) SNP in a non-coding 3'-untranslated region (3'-UTR), an insertion / deletion polymorphism in the promoter region (the 5-HTT length-polymorphic repeat or 5-HTTLPR) and a 16-17 bp VNTR polymorphism located in intron 2 (STin; de Lima et al, 2012; de Castro et al, 2014).

Serotonin (5-hydroxytryptamine or 5-HT) is a neurotransmitter and 5-HTT regulates the signalling and concentration of synaptic 5-HT (Houwing et al, 2017; Figure 6.2). The neural circuitry controlling temperament and mood relies on 5-HT synapses. Disturbances in this system result in many psychiatric disorders (Andrews et al, 2015; Houwing et al, 2017). Previous studies have focused on 5-HTTLPR as there is an association between allelic variation in 5-HTTLPR, stress reactivity and anxiety (Lesch et al, 1996; McCormack et al, 2009; Qin et al, 2015).

The most widely studied polymorphism is the 5-HTTLPR for which, in humans, the predominant alleles are the 14-repeat short allele (s-allele) and the 16-repeat long allele (l-allele; Peterson et al, 2012; Houwing et al, 2017). The s- and l-alleles differ in their rate of serotonin transcription, with the s-allele having a lower rate of transcriptional efficiency than the l-allele, such that individuals homozygous for the s-allele or who are heterozygous have around a 65% lower 5-HTT mRNA expression level than homozygous l-allele individuals (Lesch et al, 1996; Murphy et al, 2008; Peterson et al, 2012; Wankerl et al, 2014). S-allele carriers thus have a lower availability of the serotonin transporter (5-HTT) for chlorine- and sodium-dependent reuptake of serotonin (5-HT) molecules to the presynaptic terminal from the synaptic cleft (Greenberg et al, 1999; Coleman et al, 2016). A similar length variable allelic system is found in rhesus macaques though here the alleles are a 23-repeat s-allele and a 24-repeat l-allele. S-allele homozygous infant and juvenile monkeys (n = 128) display more anxious and threatened behaviour than l-allele carrier during novel fruit, human intruder, remote-controlled car and free play tests (Bethea et al, 2004).

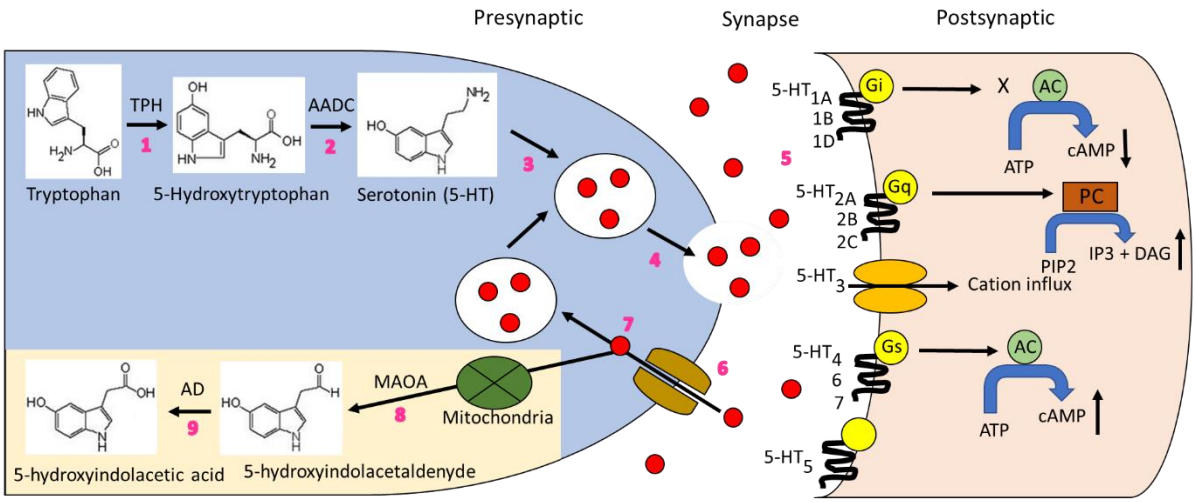

Figure 6.2. Schematic of processes associated with serotonergic neurotransmission and serotonin biogenesis. Serotonin is synthesised by enterochromaffin cells in the gut and in brain serotonergic neurons. **1)** Tryptophan hydroxylase (TPH) catalyses the oxidation of tryptophan to 5-hydroxytryptophan (5-HTP). **2)** Aromatic amino acid decarboxylase (AADC) catalyses the decarboxylation of 5-HTP to serotonin (5-HT). **3)** 5-HT is taken up into storage vesicles mediated by the vascular amine transporter. **4)** 5-HT is released from storage vesicles into the synaptic space after depolarisation of the outer membrane of the presynaptic neuron. **5)** 5-HT can activate subtypes of 5-HT receptor families (1,2,3,4,5,6,7), which couple with their respective system of signal transduction inside the postsynaptic neuron. Binding of 5-HT to autoreceptors on the presynaptic membrane results in inhibition of neurotransmission. **6)** 5-HT is taken up into the presynaptic 5-HT terminal by the serotonin transporter (5-HTT). **7)** 5-HT in the presynaptic terminal is either accumulated and stored in storage vesicles or degraded by monoamine oxidase (MAOA). **8)** Circulating 5-HT is mainly derived for the peripheral tissues and primarily metabolised in the liver by oxidative deamination by monoamide oxidase A (MAOA) to 5-hydroxyindolacetaldenyde. **9)** 5-hydroxyindolacetic acid is produced by oxidation of 5-hydroxyindolacetaldenyde by mitochondrial aldehyde dehydrogenase (AD) and is then excreted in the urine. Figure adapted from Pavlov et al (2012).

5-HTT genotype plays a role in serotonin regulation, which affects hypothalamic-pituitary-adrenal (HPA) axis function (Barr et al, 2004; Reimold et al, 2012). Interactions between genotype and thalamic levels of 5-HTT, and dysregulation of both the HPA axis and serotonergic system, are associated with negative mood states (Caspi et al, 2003; Gotlib et al, 2008; Lanfumey et al, 2008), the control of arousal, depression, and anxiety (Sirvio et al, 1994; Lesch et al, 1996; Nemeroff & Owens, 2009; Goenjian et al, 2012). In a study testing the effect of chronic stress caused by social conflict on 29 female rhesus macaques, 5-HTTLPR genotype was found to influence cortisol

response (Qin et al, 2015). Individuals homozygous for the s-allele had higher hair cortisol compared to heterozygous or l-allele homozygous individuals. As chronic stress and heightened cortisol levels are associated with the onset of depression in adult female macaque (Shively et al, 2005; Qin et al, 2016), Qin et al (2015) suggested that female rhesus macaques may be more susceptible to depression if they have low serotonin transporter efficiency (s-allele) and a history of stress.

By contrast to 5-HTTLPR, the function of the VNTR polymorphism STin is not well known (Yilmaz et al, 2001) and much of the published work focuses on the effect of this polymorphism on tobacco use (e.g., de Castro et al, 2014) and migraine susceptibility (e.g., Joshi et al, 2010). In humans and NHPs, including macaques, there are several known alleles for STin (Yilmaz et al, 2001; Inoue-Murayama et al, 2008; Joshi et al, 2010). In humans, the three most common are STin2.9, STin2.10 and STin2.12, containing 9, 10 and 12 copies of the VNTR element respectively (Yilmaz et al, 2001; Joshi et al, 2010). *In vitro* studies with human embryonic stem cells have shown genotype-dependent reporter gene expression in embryonic stem cells with STin2.12 increasing gene expression 29-fold compared to STin2.10 (Fiskerstrand et al, 1999). The STin2.12 allele has also been associated with obsessive–compulsive disorder (OCD; Baca-Garcia et al, 2007). However, there have been discrepancies and conflicting data published on the effect of the STin alleles on psychological disorders (Lovejoy et al, 2003). For example, Kaiser et al (2001) reported a six- fold increase in the risk of developing a subtype of schizophrenia in human patients that carry the STin2.9 allele, with the allele being significantly associated with an increased risk of unipolar disorder and depression (Ogilvie et al, 1996), while the STin2.10 allele has been reported as a predictor of suicide attempts in female members of the Dubla tribe of Daman (Saha et al, 2014).

The *tryptophan 5-hydroxylase 2* gene (*TPH2*; Reaction 2 in Figure 6.2) is another gene associated with the serotonin pathway. *TPH2* encodes the protein catalyst, tryptophan hydroxylase, for the first and rate-limiting step in the biosynthesis of serotonin (Kim et al, 2002; Walther et al, 2003). In macaques there are several *TPH2* polymorphisms: two mononucleotide repeats, one dinucleotide repeat, 17 SNPs and, a 159 bp insertion polymorphism (TPH2IP) in the 3'-UTR (Chen et al, 2006). In

humans, these polymorphisms have been associated with ADHD (Walitza et al, 2005), bipolar disorder (Lopez et al, 2007) and suicide (Zill et al, 2004) and, in rhesus macaques, with altered HPA axis functioning and aggressive behaviour (Chen et al, 2010). The alleles associated with TPH2IP are either short (s-allele) or long (l-allele) with the l-allele containing the 159 bp insertion and leading to changes in the mRNA secondary structure, which in turn alters the sequence of amino acids and protein synthesis (Chen et al, 2006; Watson et al, 2015). The l-allele is uncommon, with only 20% of captive rhesus macaques being l-allele homozygous or heterozygous (Chen et al, 2006).

The 5-*hydroxytryptamine receptor 2A* gene (*HTR2A*) encodes the 5-HT_{2A} receptor for serotonin, which is expressed mainly in the brain, including in the hippocampus, olfactory tubercle, nucleus accumbens, caudate nucleus and neocortex (Barnes & Sharp, 1999; D'Souza & Craig, 2010). Polymorphisms in *HTR2A* have been associated with neuropsychiatric disorders including impulsive behaviour and schizophrenia (Nomura & Nomura, 2006; D'Souza & Craig, 2008). For example, in humans the carriers of the minor A-allele of the SNP with **r**eference SNP (rs) ID number rs6311 (a G-adenine (A) point mutation ≈2kb upstream of *HTR2A*) experience greater depressive symptoms than individuals who are homozygous for the G-allele (Smith et al, 2013). The rs6311 SNP is only present in humans (Cunningham et al, 2019).

A missense variant results in the production of a different amino acid. In rhesus macaques, the G/C SNP rs80365915 in the protein-coding region of *HTR2A* results in the production of either leucine (GAG) or alanine (GAC). The G-allele is the ancestral form and is found in 100% of Chinese origin macaques and 61% of Indian origin macaques (Cunningham et al, 2019). Leucine is an essential amino acid while alanine is nonessential (NCBI, 2019). A change to the amino acid sequence within a protein-coding region can change the functioning of the pathway and may affect regulation of gene expression (Kimball & Jefferson, 2004).

SNPs within 5'-UTRs can also affect gene expression (Araujo et al, 2012; Dvir et al, 2013). Between 10% and 18% of genes show variation in the 5'-UTR which can act as switches for gene expression. In humans, the expression of both the cancer-related genes *BRCA1* (*breast cancer 1*) and *TGF-β*

(*transforming growth factor β*) are influenced by variants in their 5'-UTRs (Araujo et al, 2012). These variants affect the secondary structure of the promoter, which inhibits the efficiency of translation. The SNPs rs80363349 (A/G) and rs196407124 (A/C) are 5'-UTR variants in *HTR2A* in rhesus macaques. Little is currently known about these SNPs, and like rs80365915, they have not been evaluated for association with anxiety or stress-related disorders. For this study, these three novel SNPs (rs80365915, rs80363349 and rs196407124) within *HTR2A* have been considered.

The monoamine oxidase A enzyme is involved in the degradation of circulating serotonin (Mendelson, 2008). This mitochondrial enzyme catalyses the oxidative deamination of amines, including serotonin, dopamine, noradrenaline, and adrenaline and is encoded by the *monoamine oxidase A* gene (*MAOA*) (Goldman et al, 2013). The *MAOA* linked polymorphic region (LPR) has been found to interact with childhood trauma resulting in a significant increase in antisocial or impulsive behaviour and alcoholism in a sample of 291 women, half of whom had experienced childhood sexual abuse (Ducci et al, 2008). This is mirrored in rhesus macaques where these polymorphisms and early life stress exposure lead to higher levels of impulsive aggression, serotonin dysfunction and increased endocrine and behavioural responses to stress (Barr et al, 2003). A key polymorphism associated with aggressive behaviour in rhesus macaques are 5-, 6- and 7- 18 bp repeat alleles located within the transcriptional control region of the *MAOA* gene (Newman et al, 2005). *MAOA* is an X-linked gene and females can be heterozygous or homozygous while males can only be hemizygous (Goldman et al, 2013). The 5- and 6- repeat heterozygous, homozygous or hemizygous (only one member of the chromosome pair or segment is present rather than two) variants have a significantly higher activity than the 7-repeat resulting in a lower rate of degradation of circulating serotonin in 7-homozygotes (Newman et al, 2005; Kinnally et al, 2010). There is little *in vitro* or *in vivo* evidence of whether 5/7 and 6/7 repeat heterozygous variants have high or low activity, therefore, it is not known whether they result in a higher or lower rate of serotonin degradation (Newman et al, 2005; Kinnally et al, 2010). In the present study, the 5-, 6- and 7- 18 bp repeat alleles have been investigated for their link to anxiety and AB.

Dopamine pathway

The dopamine pathway is shown in Figure 6.3. Dopamine (3, 4-Dihydroxytyramine) is associated with reward-motivated behaviour and memory (Girault & Greengard, 2004). Dopamine is a catecholamine that can act as a hormone, which is released from the hypothalamus, or a neurotransmitter that activates the dopamine receptors (Stott & Ang, 2013; Figure 6.3). There are five known dopamine receptors (Dr1-5) subdivided into two categories: D1-like receptors (Dr1 and Dr5) and D2-like receptors (Dr2-4). The *Dopamine Receptor D4* gene (*DRD4*) encodes the G-protein coupled D4 subtype of the dopamine receptor (Asghari et al, 1994). The receptor activates pertussis toxin-sensitive G-proteins (Kazmi et al, 2000), inhibits adenylyl cyclase activity and mediates dopamine activity in the central nervous system (CNS; Oak et al, 2000). *DRD4* contains several polymorphisms including a 48 bp VNTR in the third exon of the gene (Van Tol et al, 1992). The VNTR is repeated between two and 11 times and the frequency of each allele varies by ethnicity in human populations, for example, the 7-repeat allele is prevalent in 48% of American individuals and only 2% of Asian individuals (Chang et al, 1996). The 7-repeat allele has a lower affinity for dopamine (Ptacek et al, 2011) and is associated with psychiatric disorders including ADHD, bulimia, and alcoholism (Kaplan et al, 2007; Chen et al, 2011). This allele is also associated with anger, aggression, and delinquency in humans (Hohmann et al, 2009; Dmitrieva et al, 2011) and avoidance of mothers and conspecifics in juvenile rhesus macaques (Coyne et al, 2015). Szott (2015) identified four SNPs (rs30041314, rs1079355788, rs290724315, rs301203363) in the *DRD4* gene of rhesus macaques that may be associated with anxiety and found an association between the rs300413141 low expressing T-allele and aggressive behaviour, with homozygous or heterozygous T-allele carriers showing significantly more aggressive behaviour than homozygous A individuals (Szott, 2015). For this study, the four SNPs (rs30041314, rs1079355788, rs290724315, rs301203363) were chosen for further validation of their link with anxiety and stress related behaviours.

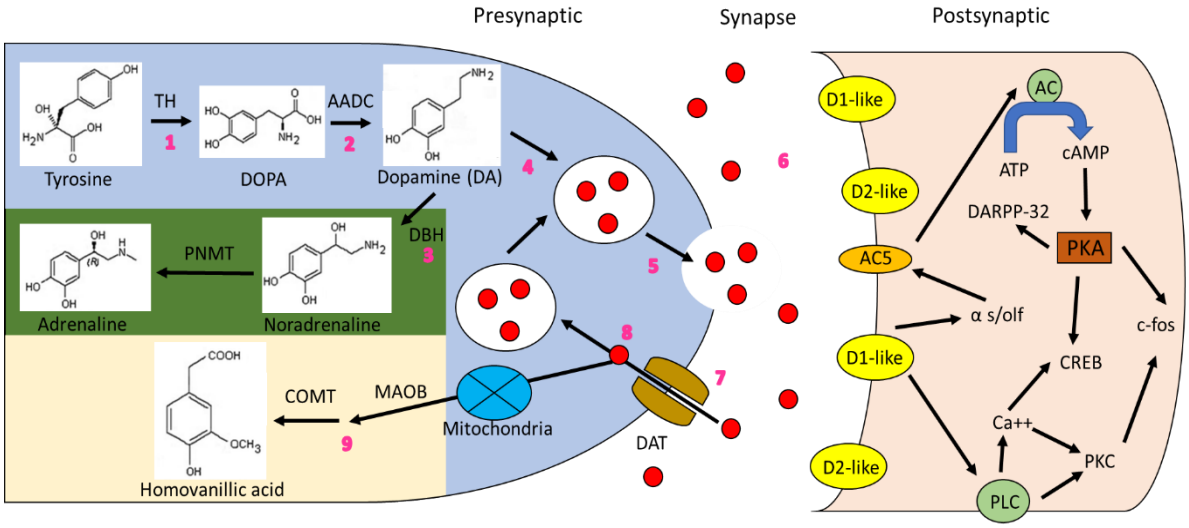

Figure 6.3. Schematic of processes associated with dopaminergic neurotransmission and dopamine biogenesis. Dopamine is mainly produced in the adrenal medulla and nervous tissue. 1) The amino acid L-tyrosine is hydroxylated to L-3,4-dihydroxyphenylalanine (DOPA) by the enzyme tyrosine hydroxylase (TH). **2)** Aromatic amino acid decarboxylase (AADC) catalyses the decarboxylation of DOPA to dopamine. **3)** Noradrenaline and subsequently adrenaline can be produced from dopamine by dopamine-beta-hydroxylase (DBH) and phenylethanolamine-N-methyltransferase (PNMT). **4)** Dopamine is taken up into storage vesicles mediated by the vascular amine transporter. **5)** Dopamine is released from storage vesicles into the synaptic space after depolarisation of the outer membrane of the presynaptic neuron. **6)** Dopamine binds to the postsynaptic dopamine receptors (D1 and D2-like receptors). **7)** Dopamine is taken up into the presynaptic terminal by the dopamine transporter (DAT). **8)** Dopamine in the presynaptic terminal is either accumulated and stored in storage vesicles or degraded by monoamine oxidase (MAOB). 9) Dopamine is inactivated by MAOB and catechol-o-methyltransferase (COMT) to produce homovanillic acid that is excreted in the urea. Figure adapted from Pavlov et al (2012) and Knab & Lightfoot (2010).

Oxytocin & Arginine Vasopressin

Oxytocin is a neuromodulatory posterior pituitary hormone that is associated with increased parasympathetic functioning and plays a counter-regulatory role in the stress and fear responses (Swanson & Sawchenko, 1980, Sawchenko & Swanson, 1982; Dreifuss et al, 1992). The genes *oxytocin/neurophysin I prepropeptide* (*OXT*) and the *oxytocin receptor* (*OXTR*) are potential key candidate genes in the study of oxytocin levels and the downstream effects of these. *OXT* encodes a precursor protein that is required to produce oxytocin. Polymorphisms in this gene are generally

associated with parturition and lactation (e.g., Pauciullo et al, 2012). However, the *OXT* polymorphisms rs4813625 and rs3761248 are associated with schizophrenia in humans (Souza et al, 2010) and rs2770378 is associated with autism-like traits (Hovey et al, 2014). Love et al (2012) reported that female C-allele carriers at the *OXT* SNP rs4813625 showed significantly higher trait-anxiety, attachment anxiety and stress induced dopamine release, and lower emotional well-being compared to G-allele homozygous females.

OXTR encodes G-protein coupled receptor proteins for oxytocin. Polymorphisms in *OXTR*, including the most studied *OXTR* SNPs rs53576 and rs2254298*,* are associated with social behaviour and emotional responsiveness in humans (Feldman et al, 2016), reduced positivity (Saphire-Bernstein et al, 2011), and poor social recognition skills (Skuse et al, 2014). Both rs53576 and rs2254298 are intron variants. These variants can interfere with splice site recognition and impact alternative splicing (Lin et al, 2019). For rhesus macaques, there are no previously characterised SNPs for *OXTR*. For this study, seven intron variants (rs196783445, rs292502465, rs300857875, rs308701533, rs292035217, rs302789768, rs283226059) were identified using Ensembl (Cunningham et al, 2019). They were all within a short intron tractable for PCR and sequencing. Intron variants were chosen to reflect the effect of this type of variant on social behaviour and emotion in humans.

OXTR encodes an oxytocin receptor, a member of a subclass of peptide receptors that also includes the arginine vasopressin receptors (1A, 1B, 2). Arginine vasopressin is a neuropeptide and *the arginine vasopressin receptor 1A* gene (*AVPR1a*) encodes the vasopressin V1a receptor. There are three known length variant polymorphisms in humans: RS1 a $(GATA)_{14}$ tetranucleotide repeat, RS3 a complex $(CT)_4$-TT-$(CT)_8$-$(GT)_{24}$ repeat and STR1 a $(GT)_{25}$ dinucleotide repeat (Thibonnier et al, 2000). RS1 and RS3 are located within two blocks, known as DupA and DupB respectively, of a ~350 bp tandem duplicate region (Staes et al, 2015). Only the DupB region, and therefore RS3, is found in rhesus macaques (Donaldson et al, 2008). RS3 is associated with altruism (Yirmiya et al, 2006; Knafo et al, 2007), pair-bonding behaviour (Young & Wang, 2004), social behaviour including sibling conflict (Bachner-Melman et al, 2005) and reproductive behaviour (Prichard et al, 2007). Walum et

al (2008) reported 11 RS3 alleles in humans (320, 330, 332, 334, 336, 338, 340, 342, 344, 346, 348 bp). The allele 334 was the most common allele in the study (40%) and was associated with marital problems with homozygous 334 individuals having double the risk of a marital crisis compared to 334 non-carriers. In this study, *AVPR1a* RS3 alleles were investigated for their association with anxiety and stress-related behaviours.

Cortisol pathway

During the acute stress response, essential tissues, such as the muscle and adipose tissue must rely on low efficiency energy sources such as fatty acids from lipolysis and glucose produced via gluconeogenesis as cortisol diverts glucose to the brain by decreasing glucose uptake elsewhere in the body (Heintz et al, 2011). The activity of the hypothalamic-pituitary-adrenocortical (HPA) axis is reflected in cortisol levels. Between 80% and 90% of circulating cortisol is bound to corticosteroid-binding globulin (CBG) or albumin and is the biologically inactive form (Lewis et al, 2005). The remaining 10% to 20% is unbound, and this free cortisol (FC) regulates the metabolic and immunological processes (Schwinn et al, 2018). The bound, or inactive form, can be converted to the active form in most body tissues by the enzyme 11-beta-hydroxysteroid dehydrogenase 1 (Ramamoorthy & Cidlowski, 2016). In the pancreas and kidneys active cortisol is converted back to inactive cortisol by 11-beta-hydroxysteroid dehydrogenase 2 (Thau et al, 2020).

The *Serpin Family A Member 6* gene (*SERPINA6*) encodes corticosteroid-binding globulin (CBG; Bolton et al, 2014). In humans, *SERPINA6* is associated with morning cortisol concentrations, for example, the T-allele at rs2749527 was found to be linked to higher total CBG (most cortisol in the blood is bound to CBG; Anderson et al, 2014; Bolton et al, 2014). In pigs, there is a genetic linkage between *SERPINA6* and basal cortisol levels (Sanchez et al, 2011). Lin et al (2012) reported that a SNP in the *SERPINA6* gene which causes a non-synonymous A51V change in CGB results in CBG level that is 50% lower than for individuals homozygous for the wild-type allele. The cytochrome P450 gene family encode cytochrome P450 enzymes that have many functions, including the synthesis of cortisol, testosterone, oestradiol (Li et al, 2012; Pikuleva & Waterman, 2013; Figure 6.4). Here

the *cytochrome P450 family 17* gene (*CYP17*) was studied as it is involved in the production of cortisol (Li et al, 2012).

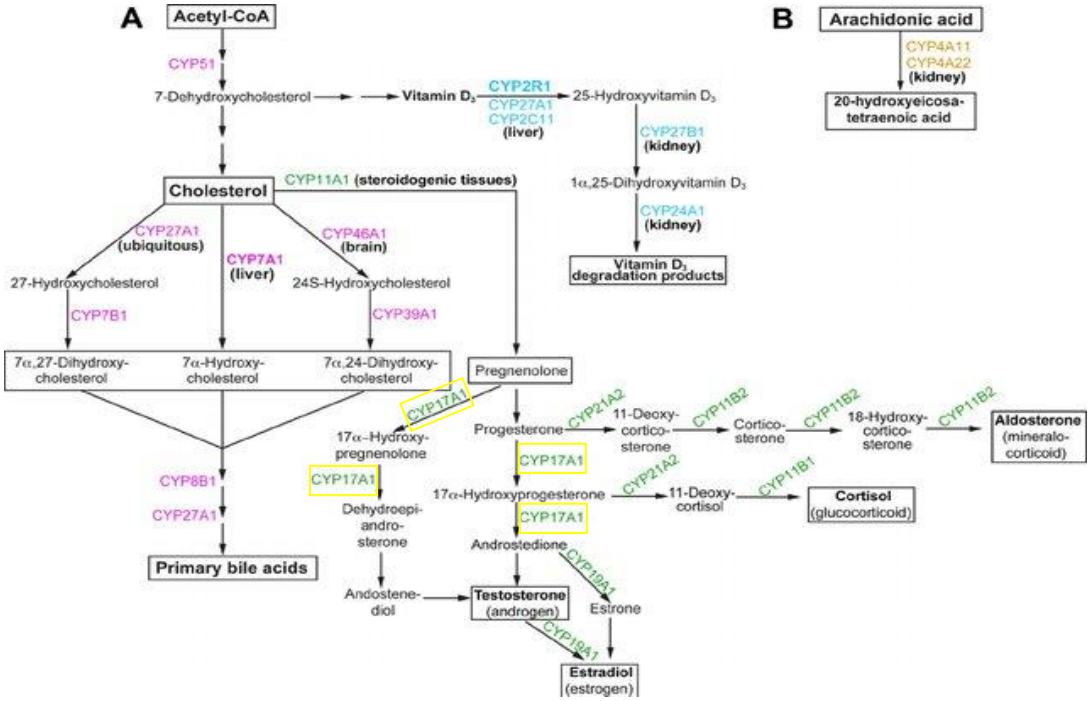

Figure 6.4. A subset of the cytochrome P450 family. Some of the P450s are involved in drug metabolism, and some are needed for endogenous compound synthesis (image from Pikuleva & Waterman, 2013). The cytochrome P450 family 17 gene (*CYP17*) involved in this study is highlighted in yellow.

Opioids

The endogenous opioid system, including the opioid receptors and opioid peptides, is implicated in the process for reinforcement and reward in the brain and nervous system by interaction with specific receptors (Gianoulakis, 2004). The *opioid receptor mu(μ) 1* (*OPRM1*) gene encodes the μ-opioid receptor which is the principal target of endogenous opioid peptides and opioid analgesic agents such as beta-endorphin and enkephalins, which are associated with neurotransmission and pain modulation (McLaughlin, 2013; Veening & Barendregt, 2015). Positive reinforcement is mediated by both indirect (nicotine, cannabinoids, alcohol) and direct (morphine) activation of the μ-opioid receptor (Contet et al, 2004). Mechling et al (2016) demonstrated that the μ-opioid receptor shapes the reward/aversion circuitry in the brain of rats. In humans, G-allele carriers at an A/G SNP in *OPRM1* have a dopamine-mediated lowered response to reward during positive

reinforcement learning (Lee et al, 2011). As a result of the link between reward and reinforcement, the *OPRM1* gene is associated with addiction in humans (e.g., Shi et al, 2002; Riju et al, 2011) whilst a different polymorphism (C77G) is linked to increased levels of alcohol consumption in male macaques (Barr et al, 2007), this polymorphism is also associated with increased aggressive-threat behaviours in rhesus macaques (Miller et al, 2004) with G-allele carriers also showing higher baseline attachment behaviours than CC homozygous individuals (Barr et al, 2008). Here, the relationship between the C77G polymorphism (rs195917455) and anxiety in rhesus macaques was investigated.

Attention bias

AB is associated with anxiety (Bar-Haim et al, 2007; Crump et al, 2018) and previous studies have shown that genotype can affect social attention in both humans (Beevers et al, 2007; Fox et al, 2009) and rhesus macaques (Szott, 2015). However, the extent to which genetic variation influences attention to potentially threatening stimuli is not yet fully understood.

Szott (2015) showed negative-neutral picture pairs including social (e.g., threat-neutral faces) and non-social content (e.g., food and veterinary stimuli) to 29 female rhesus macaques. Individuals with homozygous 5-HTTLPR l-alleles and TPH2 s-alleles were more avoidant of the veterinary stimuli and those with heterozygous or homozygous 5-HTTLPR s-alleles tended to avoid negative social stimuli. This variation in the response to threat suggests that genotype may influence an animal's reaction to stressful life events, for example, veterinary interventions or transportation (Fernström et al, 2008).

This study used a refined method of Szott (2015) to further investigate and validate the link between genotype and AB in adult rhesus macaques. The effect of individual relatedness was included. Additional candidate loci were also investigated.

6.3 Materials & methods

Ethics

Ethical approval was granted by Liverpool John Moores University (LJMU) in February 2017 (Ethical approval ID. EB_EH/2017-5) and by the Medical Research Council Animal Welfare and Ethical Review Body (AWERB) in November 2017. This project piggybacked onto routine veterinary and husbandry activities that would have occurred whether or not the animals were involved in this study. No regulated procedures were carried out for this study. Blood samples were primarily for colony management purposes and would have been collected whether the animals were involved in this study or not. All training was conducted following MRC-CFM protocol and using positive reinforcement methods. Participation in training and AB trials was voluntary, insofar as animals were free to leave the training and testing area (cage room) at any time. Food, water, and social contact with conspecifics were available *ad libitum* throughout training and AB testing.

Animals

Sixty-one (45 female, 16 male), adult (>3 years old) rhesus macaques (*Macaca mulatta*) socially housed at the MRC-CFM were involved in this study. At the time of testing, the mean age of the macaques was 8.45 ± 3.50 years and ranged from 3.50 to 16.42. Six female and two male macaques were weaned early, before 12 months old (6.83 to 16.17 years with a mean age of 9.08 ± 2.70 years).

Nine males and 27 females were involved in Study 1 (mean age = 7.99 years ± 3.04 years, range 3.5 years to 16.17 years). Study 2 included 12 males and 25 females (3.75 to 16.42 years with a mean age of 9.22 ± 3.82 years). For life history information see Appendix 2b.

Attention bias

The full protocol is given in Chapter 3. In brief unfamiliar threat-neutral male conspecific face pair stimuli (Witham & Bethell, 2019) were shown to macaques on a computer monitor screen for three seconds and their looking time to each stimulus recorded and AB score was then calculated by

subtracting the time spent looking at the neutral face from the time spent looking at the threat face.

AB data used in this chapter were collected in two separate studies. In Study 1, AB trials were run before and after the macaques' veterinary health check (stressor). AB trials were conducted once per day per macaque on four consecutive weekdays from Tuesday to Friday, and then a fifth trial was conducted on the following Monday. The baseline condition (assumed non-anxious state) was timetabled so that trials occurred in weeks during which there were no activities planned which were deemed to be potentially stressful i.e., no cleaning, animal removals, or planned veterinary procedures. The post-stressor condition was timetabled so trials occurred on the five working days following the scheduled health check (Tuesday – Monday).

In Study 2, AB trials were run once per week per macaque for eight consecutive weeks to determine the repeatability of AB in a presumed low stress state and check for habituation to the trials. All trials were at least four days apart with the day of testing varying depending on other veterinary, husbandry or management activities occurring at Medical Research Council Harwell Institute Centre for Macaques (MRC-CFM).

DNA collection

Blood samples were collected by the Named Veterinary Surgeon at MRC-CFM during the macaques' annual health screening. Macaques were sedated with KHCl prior to this procedure. Blood samples, which were primarily used for colony management purposes, were collected into EDTA K2 (anticoagulant) tubes, centrifuged, and wrapped in cotton wool prior to transport to B&K Universal Laboratories (Marshall BioResources), Grimston, East Yorkshire, UK. DNA extraction occurred upon arrival at the laboratory using the DNeasy Blood & Tissue Kit from Qiagen (Catalogue no. 69506) and concentrations and purity measured using a Thermo Fisher Scientific Inc. NanoDrop™ Lite Spectrophotometer. The DNA samples were delivered on dry ice to Liverpool John Moores University, James Parsons Building, Liverpool, UK in January 2018 where they were stored at -20°C

for three days. Samples were stored at 4°C during use. COSHH risk assessment forms for DNA extraction and gel electrophoresis can be found in Appendix 6a.

Genotyping

The remaining "Materials and methods" sections describe the genotyping methods in detail. Briefly, I analysed variants in 12 genes (Table 6.1). Four of these were length polymorphisms (5-HTTLPR, *MAOA*, STin, *and TPH2*) and eight were SNPs (*AVPR1a*, *CYP17*, *DRD4*, *HTR2A*, *OPRM1*, *OXT*, *OXTR* and *SERPINA6*). Samples were genotyped for the four length variants using PCR amplification and gel-analysis (Kinnally et al, 2008). SNPs for CYP17, *DRD4*, *HTR2A*, *OXT*, *OXTR* and *SERPINA6* were determined by amplification and sequencing, with haplotypes manually reconstructed from multi-locus genotype data (Okuyama et al, 2000) while SNPs for *AVPR1a* were determined through amplification followed by restriction enzyme digestion and gel-analysis (Roberts, 2005). A TaqMan genotyping assay was carried out for the *OPRM1* C77G polymorphism (Kutyavin et al, 2000). Five of these loci (5-HTTLPR, *MAOA,* STin, *TPH2*, *DRD4*) had previously been screened for polymorphisms of interest for association with AB (Szott, 2015) in a subset of 18 of the animals screened here (Appendix 6b). The primer pairs, gene locations, polymerase chain reaction conditions and gel percentages are shown in Table 6.1. PCR cycle times and temperatures were adapted from established protocols; however, as these protocols were optimised and, therefore, varied from the literature the full cycle protocols used are presented here.

To check for consistency, at least one known sample per genotype from Szott (2015) was repeated and run with the new samples for 5-HTTLPR, *MAOA*, *TPH2*, STin and *AVPR1a*. Any ambiguous samples where results of an assay could not be determined after discussion with my supervisor were run again.

Novel primers

Novel primers were designed for *AVPR1a*, *CYP17A*, *HTR2A*, *SERPINA6*, *OXT* and *OXTR*. Primers are short, single-stranded sequences of DNA used to define the region of DNA that will be amplified

during a polymerase chain reaction (PCR; Loftus, 2019). Primer sequences and chromosome locations are shown in Table 6.1. Primers were chosen so that they would amplify regions of DNA that include many known polymorphisms. For example, *CYP17* includes intron variants, synonymous variants, three missense variants (rs295113573, rs304591167, rs292284528) and one splice region variant (rs300693761), *SERPINA6* includes intron variants, 3'-UTR variants, 5'-UTR variants, synonymous variants and four missense variants (rs283105182, rs294636476, rs307778843, rs284086763) and *OXT* has six known upstream variants (rs1075712153, rs306511402, rs290075921, rs293235370, rs1068370341, rs306824645), three 5' UTR variants (rs288428459, rs301284043, rs308827867) and two synonymous variants located in the protein coding region of *OXT* (rs1069376445, rs287475742).

Table 6.1. Primer pairs, location, and PCR conditions for optimisation of 5-HTTLPR, *AVPR1a*, *CYP17*, DRD4, *HTR2A*, *MAOA, OPRM1, OXT, OXTR, SERPINA6,* STin and *TPH2* amplification. All genomic positions are from Mmul_1 (Gibbs et al, 2007; Cunningham et al, 2019).

Gene	Variant type	Primer	PCR cycles & temperatures
5-HTTLPR	Length	5HTT-F (5'-GCCGCTCTGAATACCAGCAC-3') HTTLPR_intl (5'-CAGGGGAGATCCTGGGAGGG-3') Chromosome 16: 25474060 – 25474477	95°C for 5 mins 40 cycles: 95°C for 30 s, 61°C for 30s, 72°C for 1 min Final extension: 72°C for 7 mins
AVPR1a	SNP	AVPR-F (5'-AAGTCGGGAAGGTGAGCTC-3') AVPR-R (5'-CTTCCCGTAGCAAACACAGG-3') Chromosome 11: 62125571 – 62126181	95°C for 3 mins 35 cycles: 95°C for 30s, 55°C for 30s, 72°C for 1 min Final extension: 72°C for 5 mins
CYP17A	SNP	CYP17-F (5'-GCAGGGAGGAGATAGACACC-3') CYP17-R (5'-CTACTCGTGACCCTCCTGAC-3') Chromosome 19: 46667165-46673172	95°C for 3 mins 35 cycles: 95°C for 30s, 55°C for 30s, 72°C for 1 min Final extension: 72°C for 5 mins
DRD4 haplotype	SNP	DRD4-PROM-SNP-F (5'-CGGGGGCTGAGCACCAGAGGCTGCT-3') DRD4-PROM-SNP-R (5'-GCATCGACGCCAGAGCCATCCTGCC-3')	95° for 1min 35 cycles: 95° for 20s, 72°C for 30s

		Chromosome 14: 482929-486642	final extension: 72°C for 7 mins
HTR2A	SNP	HTR2A-F (5'-GGCATGACAAGGAAACCCAG-3') HTR2A-R (5'-CCAGGACATTTATCTCCCCGA-3') Chromosome 17: 26052789 – 26053509	95°C for 3 mins 35 cycles: 95°C for 30s, 55°C for 30s, 72°C for 1 min Final extension: 72°C for 5 mins
MAOA	Length	MAOA-F(2) (5'-CAGAAACATGAGCACAAACG-3') MAOA-R(2) (5'-TACGAGGTGTCGTCCAAGTT-3') Chromosome X: 43706588-43833584	95°C for 5 mins, 40 cycles: 94°C for 30s, 55°C for 30s, 72°C for 30s Final extension: 72°C for 10 mins
OPRM1	SNP	OPRM1_C77G_F (5'- TGGCGCACTCAAGTTGCT-3') OPRM1_C77G_R (5'- GGGACAAGTTGACCCAGGAA-3') Probes: OPRM1_C77G_VIC (5'-CAGCACGCAGCCC-3') labelled with VIC for detecting G allele OPRM1_C77G_FAM (5'-CAGCACCCAGCCC-3') labelled with 6-FAM for detecting C allele Chromosome 4: 111854397-112105159	95°C for 15mins, 40 cycles: 92°C for 15s, 60°C for 1min
OXT	SNP	OXT-F (5'-GTGAGGGTGAAGACGTTTCC-3')	95°C for 3 mins

		OXT-R (5'-GACTTACCTTGCGCACGTC-3')	35 cycles: 95°C for 30s, 55°C for 30s, 72°C for 1 min
		Chromosome 10: 35900354 – 35901001	Final extension: 72°C for 5 mins
		OXTR- F (5'-CTGGACGCCTTTCTTCTTCG-3')	95°C for 3 mins
OXTR	SNP	OXTR-R (5'-AACTACTAGGGGCTTGGCTG-3')	30 cycles: 95°C for 30s, 62°C for 15s, 72°C for 30s
		Chromosome 2: 57650704 – 57651332	Final extension: 72°C for 5 mins
		SERPIN_A6-F (5'-AGTTGACCAGGACGAGGATG-3')	95°C for 3 mins
SERPINA6	SNP	SERPIN_A6-R (5'-GCCCCATTGACTCAGAGACT-3')	35 cycles: 95°C for 30s, 55°C for 30s, 72°C for 1 min
		Chromosome 7: 156568768 – 156569439	Final extension: 72°C for 5 mins
		STin-F (5'-TGTTCCCAGACTTACACCAGTG-3')	95°C for 3 mins
STin	Length	STin-R (5'-GTCAGTATCACAGGCTGCGAG-3')	35 cycles: 95°C for 30s, 55°C for 30s, 72°C for 1 min
		Chromosome 16: 24286960 – 24287162	Final extension: 72°C for 5 mins
		TPH2-U3F5 (5'-TGTAGGAAACTTCTCATCACAA-3')	95°C for 3 mins
TPH2	Length	TPH2-U3R5 (5'-CAGCATAAAATTCATAGTCCCAAG-3')	35 cycles: 94°C for 30s, 55°C for 30s, 72°C for 30s
		Chromosome 11: 70772942-70863894 forward strand	Final extension: 72°C for 5 mins

Polymerase chain reactions (PCR)

PCR amplifications were carried out on a Bio-Rad T100™ Thermal Cycler. The reaction mixture had a total volume of 25 µl. This included: 12.5 µl Thermo Scientific™ DreamTaq™ Hot Start PCR Master Mix, 0.1 – 1.0 µM of both specific forward and reverse primers, 10 pg – 1 µg of template DNA, and the remainder was sterile, nuclease-free water. The Thermo Scientific™ DreamTaq™ Hot Start PCR Master Mix included DreamTaq™ Hot Start DNA Polymerase, DreamTaq buffer, magnesium, and dNTPs. After mixing on a Heathrow Scientific Sprout® mini vortexer, the PCR tubes were placed in the PCR thermal cycler for DNA amplification. A no template (NT), which included no DNA and an additional 1 µl of sterile water, was included to check for contamination. The thermal cycling conditions, including timing and temperatures for denaturation, annealing and elongation for each gene in this study can be found in Table 6.1.

Gel electrophoresis

Following amplification, PCR products were separated by size via gel electrophoresis in a Bio-Rad Wide Mini-Sub® Cell GT Horizontal Electrophoresis Cell with a Bio-Rad PowerPac™ HC High Current Power Supply. Agarose powder and Tris-borate-EDTA (TBE) buffer were heated in a microwave and mixed by hand-spinning in a conical flask with either <1 µl of GelRed® nucleic acid gel stain or 4 µl of 0.5 µg/ml Ethidium Bromide gel stain to create 1.5 – 3 % Agarose gels (gel percentages are shown in Table 6.2). The Agarose mix was cooled under a running tap, poured into a Bio-Rad Mini Sub-Cell GT Gel Caster and Bio-Rad Sub-Cell GT UV-Transparent Wide Mini-Gel Tray with a 20-well 1.5 mm fixed-height comb and allowed to set for 30 minutes. Once set, 4 µl of each PCR product and the NT were loaded with 1 µl of Gel Loading Dye, Blue (6X). A molecular weight size marker (5 µl of ThermoFisher GeneRuler 100 bp DNA ladder) was loaded into the first or last well for later sizing of the DNA fragments. Gel electrophoresis ran at 80 V for 50 – 60 minutes. After this time, gels were viewed under UV light using a Bio-Rad ChemiDoc MP Imaging System and sized using the DNA ladder. These images were saved, and the gel then returned to the electrophoresis cell for

additional time if further separation was required for adequate visualisation of the genotypes (times for gel electrophoresis for each gene are shown in Table 6.2).

Table 6.2. Gel electrophoresis times and gel percentages for visualisation of 5-HTTLPR, *AVPR1a*, *CYP17*, DRD4, *HTR2A*, *MAOA, OPRM1, OXT, OXTR, SERPINA6,* STin and *TPH2* PCR products.

Gene		Electrophoresis time (minutes)	Gel percentage	Stain
5-HTTLPR		120-150	3.0	Ethidium bromide
AVPR1a	**Without enzyme**	50	1.5	GelRed®
	With restriction enzyme	60	2.0	GelRed®
CYP17		50	1.5	GelRed®
DRD4		50	1.5	GelRed®
HTR2A		50	1.5	GelRed®
MAOA		120-150	3.0	GelRed®
OPRM1		50	1.5	GelRed®
OXT		50	1.5	GelRed®
OXTR		50	1.5	GelRed®
SERPINA6		50	1.5	GelRed®
STin		90 - 120	3.0	GelRed®
TPH2		50	1.5	GelRed®

Sequencing

All products to be sequenced were first visualised by gel electrophoresis on 1.5% agarose gel to ensure failure and contamination had not occurred. Products were prepared using Thermo Scientific™ GeneJET™ PCR Purification Kits, which purifies DNA from PCR reaction mixtures by removing salts, enzymes, unincorporated labelled nucleotides, dNTPs, and primers (Thermo Scientific, 2017). The method for purification followed Protocol A in the manufacturer's product information (Thermo Scientific, 2017). Once purified, 5 µl of product was mixed with 2.5 µl of sterile water and 2.5 µl of the forward primer at 5 µM concentration for that gene and sent for GATC Light Run Sequencing at GATC Biotech. All macaques were genotyped for *DRD4*, *HTR2A*, *and OXTR* by

purification and sequencing. Sequences were manually examined in FinchTV Version 1.4.0 and the genotype at each SNP recorded. Sequences and SNP alleles are shown in Table 6.3.

Table 6.3. Sequences used to search for SNPs for each genotype.

SNP label	dbSNP rs label	Sequence preceding the SNP	Alleles at SNP
DRD4155	rs300413141	AGCCTAAGCTCCGGTCTTCCCGCG	A/T
DRD4201	rs1079355788	GGACGTTTTCCCAGACACCAGGTG	C/G
DRD4226	rs290724315	ACTAGGTGGACGGCCCGAGGGCCG	C/G
DRD4243	rs301203363	AGGGCCGGGACGCACGCAGGGGCC	A/G
HTR2A1	rs80365915	AGGTTGGTTCGATTTTCAGA	C/G
HTR2A2	rs80363349	TTAGGAGAGTCCACGGTTTG	A/G
HTR2A3	rs196407124	ACTTTTAGCATAGAGGTTGC	A/C
OXTR124	rs196783445	AATGTCCAGGGGTCT	G/T
OXTR274	rs292502465	AAGTACCAACTGTCAC	C/T
OXTR288	rs300857875	GGGCATAGGGGCA	C/G
OXTR311	rs308701533	AAAATGCAGTTAAA	A/T
OXTR346	rs292035217	GCTGCATATGGGCTG	C/T
OXTR358	rs302789768	ATGGTTTACTG	C/T
OXTR414	rs283226059	CGTGGTTAGGAGGAG	A/G

An additional three samples of three novel genes (*CYP17*, *SERPINA6* and *OXT*) were also sent for sequencing; however, no variation was seen with the primer pairs used and therefore, no more samples of these genes were analysed.

Restriction enzyme analysis of *AVPR1a*

A Thermo Scientific *Bsp*143I (*Sau*3A1) restriction enzyme with the recognition sequence 5'-GATC-'3 (https://www.thermofisher.com/order/catalog/product/ER0781) was used to genotype a SNP in *AVPR1a* (*arginine vasopressin receptor 1a*; oxytocin & arginine vasopressin pathway). 4 µl of the *AVPR1a* PCR product was added to 1.5 µl buffer, 9.1 µl water and 0.4 µl *Bsp*143I enzyme and incubated at 37°C for 2 hours followed by inactivation at 80°C for 10 mins.

***OPRM* C77G TaqMan assay**

The previously designed TaqMan assay (Szott, 2015) for genotyping the *OPRM1* (*opioid receptor mu(μ) 1*; opioid pathway) *C77G* polymorphism was run at the Liverpool School of Tropical Medicine (LSTM). Dr Craig Wilding designed the TaqMan assay (assay ID AH399N0) in 2015 using the Custom TaqMan Assay Design Tool (https://www.lifetechnologies.com/order/custom-genomic-products/tools/genotyping/).

The Custom TaqMan Assay Design Tool was used to design forward (OPRM1_C77G_F: 5'-TGGCGCACTCAAGTTGCT-3') and reverse (OPRM1_C77G_R: 5'-GGGACAAGTTGACCCAGGAA-3') primers with two minor groove binding probes (Applied Biosystems; OPRM1_C77G_VIC: 5'-CAGCACGCAGCCC-3' and OPRM1_C77G_FAM: 5'-CAGCACCCAGCCC-3'). The two minor groove binding probes were labelled at the 5' end with VIC and 6-FAM for the detection of G and C alleles respectively and at the 3' end with a minor groove binder and a non-fluorescent quencher (see Figure 6.5 for example TaqMan probe). Minor groove binding probes have a higher melting temperature (T_m) and increased sequence specificity and reduced mismatches compared to unmodified DNA (Kutyavin et al, 2000) which increases accuracy for allelic discrimination.

The TaqMan genotyping assay was run on a 96 well plate with optical caps. Into each well 9 μl of the reaction mixture was added to 1 μl of DNA. The reaction mixture consisted of 6.75 μl of sterile water, 0.25 μl of the OPRM1_C77G primer probe and 2 μl of 5x qPCRmix (Solis Biodyne HOT FIREPol® Probe qPCR Mix Plus). Genotyping occurred on a Stratagene MX3005P qPCR system at LSTM, the qPCR system read the fluorescence in the FAM and VIC channels and automatically genotyped based on endpoint fluorescence data. The PCR conditions for *OPRM1* are shown in Table 6.1. Six macaques were repeated from Szott (2015) and consistent genotypes were found (Appendix 6b).

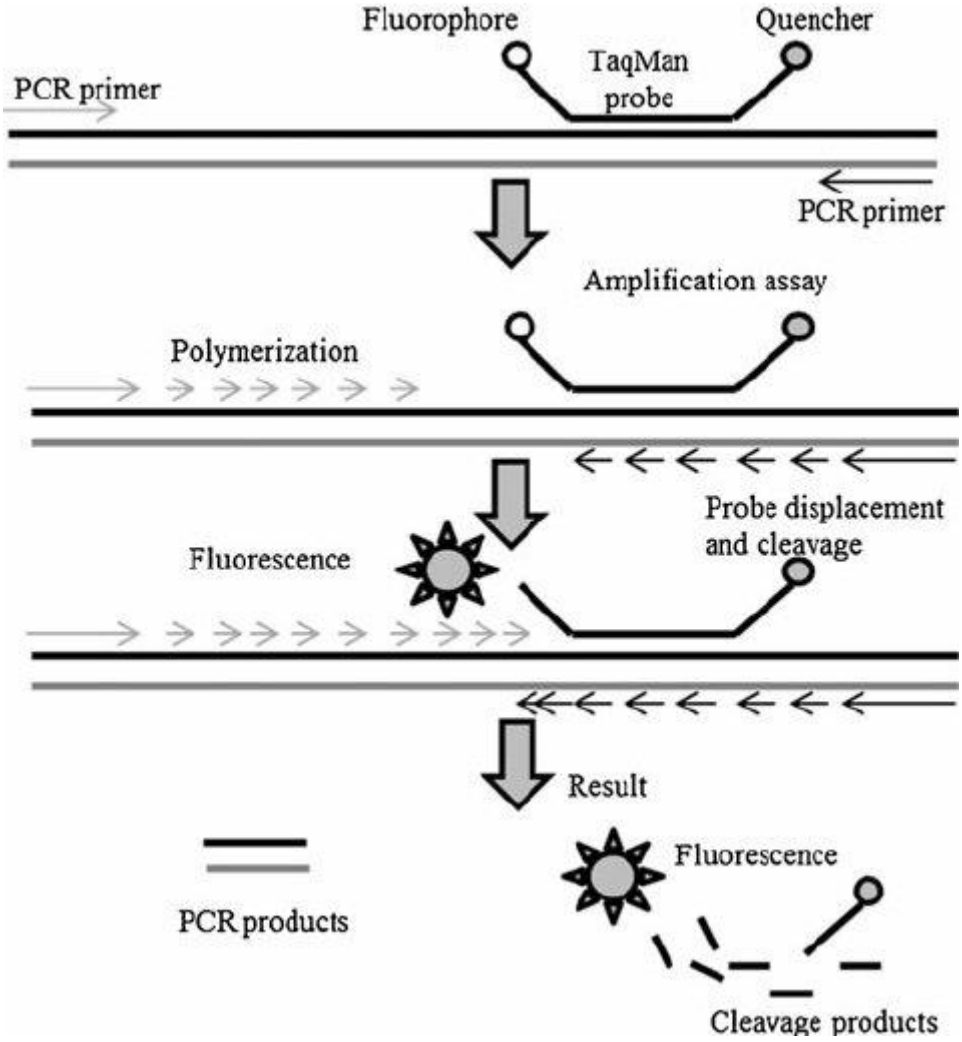

Figure 6.5. Example TaqMan probe consisting of a quencher at the 3'-end and a fluorophore at the 5'-end. When in proximity to the fluorophore, the quencher quenches the fluorescence. The probe anneals to the DNA target. The DNA is amplified by specific primers and the probe is incorporated into the PCR product. The quencher is released and is no longer in proximity to the fluorophore allowing the fluorescence to be detected by the PCR cycler. Probes are allele specific so genotype can be determined using the intensity of fluorescence. Figure from Botes et al (2013).

5-HTTLPR

Genotyping of the 5-HTTLPR polymorphism caused significant problems. Initially, visualisation of the length polymorphisms was attempted on a 3% agarose gel with GelRed stain; however, this method produced very messy gel images when observed under UV light and therefore alternative methods were evaluated. Figure 6.6 was created to show the location of the primers used in published protocols (see also Appendix 6c). Attempts to design new primers based on the most common sequences in the search were undertaken (e.g., Barr et al, 2004; Spinelli et al, 2012).

However, this proved to be no more successful when run on 3% agarose gel with GelRed and concerns were that any results would not be directly comparable with those of Szott (2015). Further investigation showed that GelRed as the visualisation dye caused inconsistencies in accurate sizing through gel electrophoresis. When ethidium bromide was used with the original primers (5HTT-R 5'-GGAGGGATGCAGGGGTTG-3' & HTTLPR_stpr5 5'-GGCGTTGCCGCTCTGAATGC-3') on a 3% agarose gel then this problem was resolved.

Data preparation

The final dataset consisted of genotype variants from nine genes for each monkey. 5-HTTLPR, STin and *TPH2* were length variants and genotypes were either s- or l-allele homozygous or heterozygous. *MAOA* was a length variant with 5-, 6- and 7- 18 bp repeat alleles; macaques could be homozygous or heterozygous for the repeat. SNPs for AVPR1a could be A, B or C homozygous or heterozygous and the OPRM1 SNP could be C or G homozygous or heterozygous. For *DRD4*, *HTR2A* and *OXTR* the SNPs (4, 3 and 7 respectively) were grouped, and these genes were analysed as haplotypes. Haplotypes are groups of genes that are inherited together so the grouping of these SNPs are related and the genotype of one is biologically relevant to another.

It was not possible to calculate Hardy-Weinberg equilibrium as the data did not meet the assumptions of the law, principally the population size is too small and many of the individuals were too closely related (Keats & Sherman, 2013).

Predictor variables explanation

The condition under which the AB trials were conducted was either baseline or post-stressor. Age was measured in months and was calculated for each trial to account for the time difference between baseline and post-stressor trials. Sex was male or female. Time was rounded down to the nearest hour, for example, both 14:05 and 14:56 would be rounded to 14:00. Wean early referred to macaques that were removed from their maternal group before 12 months old.

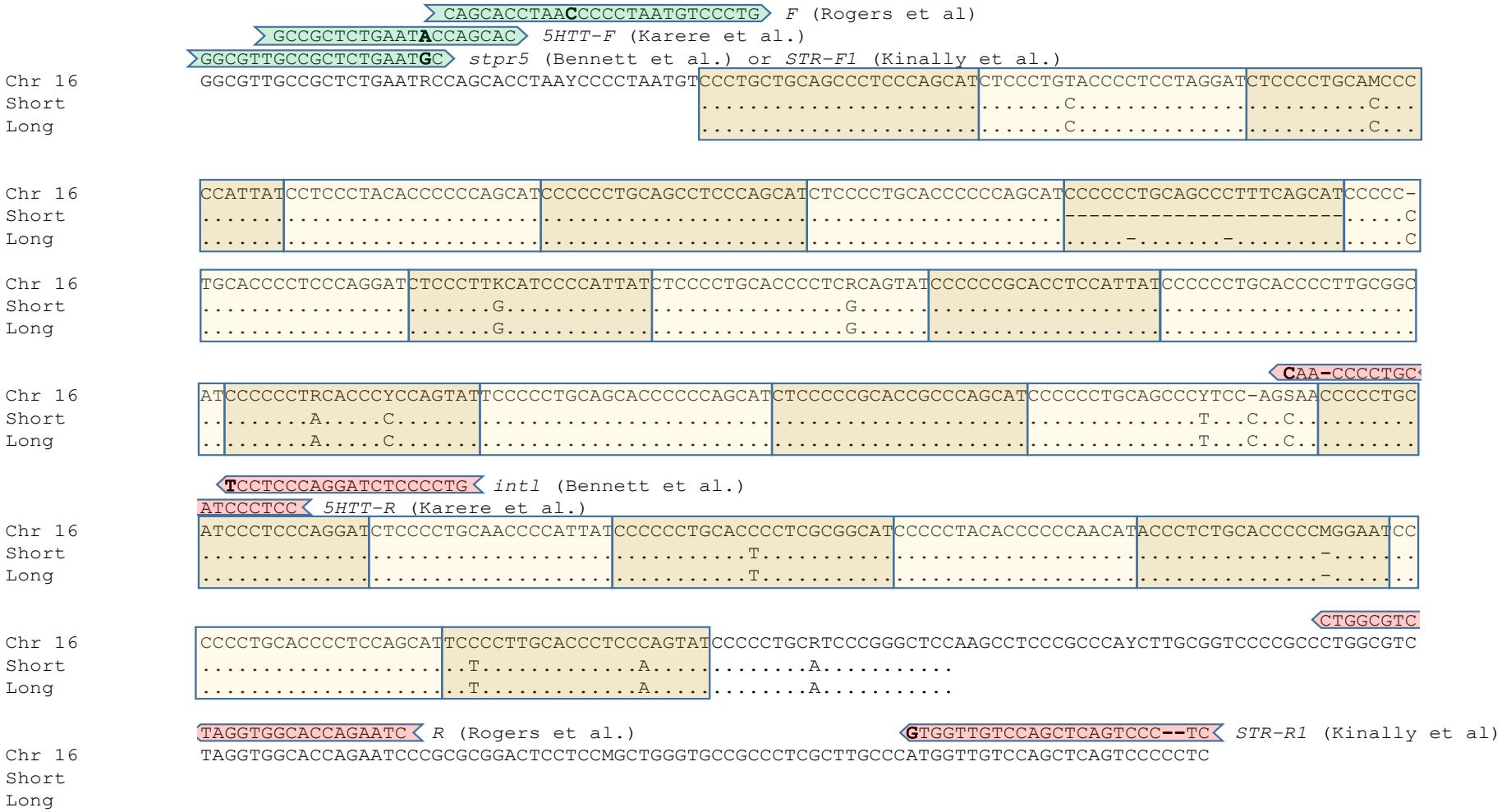

Figure 6.6. Alignment of the region upstream of the *SLC6A4* (serotonin transporter) gene on chromosome 16 (16:25897817-25898497) of macaques with the short and long allele of Lesch et al (1996). Identical nucleotides in the alignment are indicated by a (.). Points of possible variation are indicated with major allele. Variable bases in the *M. mulatta* genome are indicated with nucleotide ambiguity codes. This promoter region displays either 23 (short) or 24 (long) imperfect repeat units of 20-24bp (repeat units are boxed and shaded with alternate shades). Position of primers utilised for genotyping of the *Macaca mulatta* 5HTT-LPR are indicated.

Statistical analysis

All statistical analyses were conducted using R version 3.6.0 (R Core Team, 2018). Linear mixed effects models (LMM) were developed and fitted using the function *lmer* of the R-package lme4 (Bates et al, 2015). LMM are used to analyse continuous, hierarchical data and can cope with unequal sample sizes and missing data (Smith, 2012; Gałecki & Burzykowski, 2013).

Animal identity was included as a random effect in all models. To avoid collinearity, all predictor variables were checked for correlations and for those above 0.4, one variable was removed (Crawley, 2007). Criteria for selecting the retained variable was relevance to the study question, for example, in Study 1 condition (baseline or post-stressor) was always retained in the model.

Predictor and response variables were also checked for their distribution. Variables that showed non-normal distribution were transformed using Tukey's Ladder of Power (Tukey, 1977). The Tukey transformation provided a λ value that maximised the Shapiro-Wilk W statistic or minimises the Anderson-Darling A statistic (Mangiafico, 2016). The Schapiro-Wilk statistics should be maximised as a significant or small Shapiro-Wilk W statistic indicates that the data is not normally distributed (Oztuna et al, 2006). The Anderson-Darling statistic should be minimised as a smaller Anderson-Darling A statistic indicates that the distribution better fits the data (Lewis, 1961). The Tukey transformation was conducted using the function 'transformTukey' of the R-package rcompanion (Mangiafico, 2019). Variables with a λ of 1.0 were not transformed as this indicated normal distribution. Covariates were z-transformed to a mean of zero and a standard deviation of one to allow easier comparison of estimates and interpretation of interactions from the model output (Schielzeth, 2010).

For each model, the residuals were plotted against fitted values and qq-plots (scatterplot comprising two sets of quantiles plotted against each other (Ford, 2015)) of the residuals were visually inspected to check whether the models fulfilled the assumptions of normally distributed and homogeneous residuals (Crawley, 2007). The models were developed by excluding non-significant predictor variables with the greatest *p* values until only those factors with $p<0.05$ were

retained in the final model. Factors with non-significant *p* values were retained if they were required for model stability. Models were deemed to be stable if the original value lay between the minimum and maximum values revealed using the function 'summary'. The reduced model estimates were compared with the estimates from the full model and all models were checked for stability using the function 'glmm.model.stab' (Hofner & Hothorn, 2017).

The significance of each model as compared to the null model (comprising only the random effect of animal ID) was established using a likelihood ratio test with the R function ANOVA with argument test set to 'Chisq' (Dobson, 2002; Forstmeier & Schielzeth 2011). Models were fitted using Maximum Likelihood, rather than Restricted Maximum Likelihood, to allow for a likelihood ratio test (Bolker et al, 2008). Likelihood ratio tests comparing the full model with the respective reduced models using the R function 'drop1'. The 'drop1' function provided the *p* values for the individual effects (Barr et al, 2013). Confidence intervals were calculated using the function 'confint.merMod' of the R-package lme4 to calculate the likely range of the sample and allow estimation of the precision of the sample compared to the true population (Bates et al, 2015). To aid interpretation, non-transformed data were used for plotting purposes.

Linear mixed model analysis

The initial models contained the key predictor variables revealed in Chapter 3 (sex, time, and age), the interaction between condition and early life stress (weaned early) and the genotype variables.

~ Condition*WeanEarlyR +
z.Tukey.AgeMos + z.Tukey.Time + Sex +
HTTLPR + TPH2 + STIN + as.factor(HTR2A) + as.factor(MAOA) + OPRM1 + AVPR +
as.factor(DRD4Haplotype)+ as.factor(OXTRHaplotype)

where: z = scaled; Tukey = Tukey transformation

The degrees of freedom (df) for the model were 50. The data set contained 634 rows of AB trial data from 61 macaques allowing for ≥10 rows per df (Crawley, 2007). Condition was retained in all reduced models for reporting purposes. Two covariates (age and time) were z-transformed to a

mean of zero and a standard deviation of one to allow easier comparison of estimates and interpretation of interactions from the model output (Schielzeth, 2010). A copy of the full R script is shown in Appendix 6d. Post hoc analysis was conducted using a least-squares means (estimated marginal means), which obtains estimated marginal means for LMM (Lenth et al, 2020). Analysis was conducted with the function 'emmeans' in the R package emmeans.

The animal model

Ignoring family structure can increase type I error (false positive) rate (McArdle et al, 2007). To account for relatedness, analyses were repeated using the animal model, in which pedigree is included as a random factor. A Bayesian analysis MCMCglmm was conducted using the MasterBayes function in R (Hadfield, 2010). Bayesian analysis uses observed or "prior" distribution to estimate parameters of an underlying distribution (Gelman et al, 1995). To gain an adequate sample size of 1,000 iterations, the number of iterations was set at 501,000, a burn-in of 1,000 and a thinning interval of 500. For each model, an uninformative prior was specified following de Villemereuil (2012). An uninformative prior was used as the probability distribution was unknown and this type of prior allows for all distributions to be equally likely. Priors are determined based on observed data. As this is a relatively new area of study, there is little prior knowledge that can inform the prior selection. Each model was plotted and visually inspected to check for convergence. Independence of the data points was assessed by checking autocorrelation of <0.1 at the 500-thinning interval using the function autocorr.

6.4 Results

Length variant genotypes

Length variant genotypes were determined from gel electrophoresis images. Example images are shown below (*MAOA* – Figure 6.7, STin – Figure 6.8, *TPH2* - Figure 6.9, and 5-HTTLPR with ethidium bromide – Figure 6.10). The different variants at each point are labelled in the figure caption. *TPH2* and 5-HTTLPR were long or short variants while the variants for *MAOA* were 5-, 6- or 7- 18 bp STin, repeat alleles.

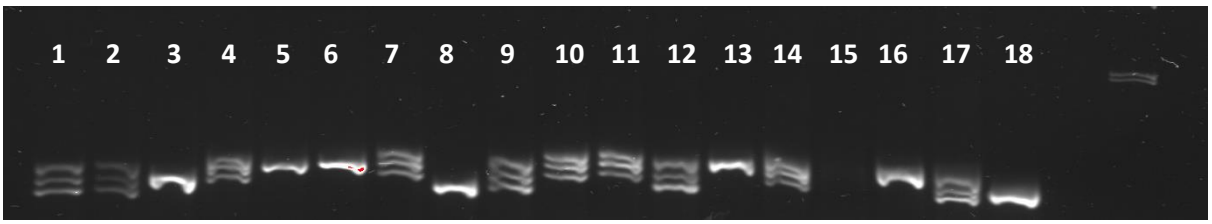

Figure 6.7. PCR products from *MAOA* gel electrophoresis of 18 samples of rhesus macaques. Samples 1 and 17 were 5-6 repeats. Samples 2, 9, 12 and 14 were 5-7 repeats. Samples 3 and 16 were 6-6 repeats. Samples 4, 7, 10 and 11 were 6-7 repeats. Samples 5, 6 and 13 were 7-7 repeats. Samples 8 and 18 were 5-5 repeats. Sample 15 failed.

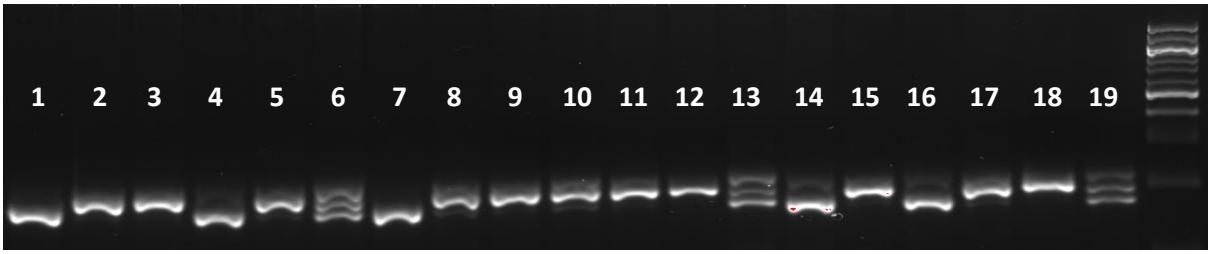

Figure 6.8 PCR products from STin gel electrophoresis of 19 samples of rhesus macaques. Samples 1, 4, 7, 14 and 16 were SS homozygous. Samples 2, 3, 5, 8-12, 15, 17 and 18 were LL homozygous. Samples 6, 13 and 19 were SL heterozygous.

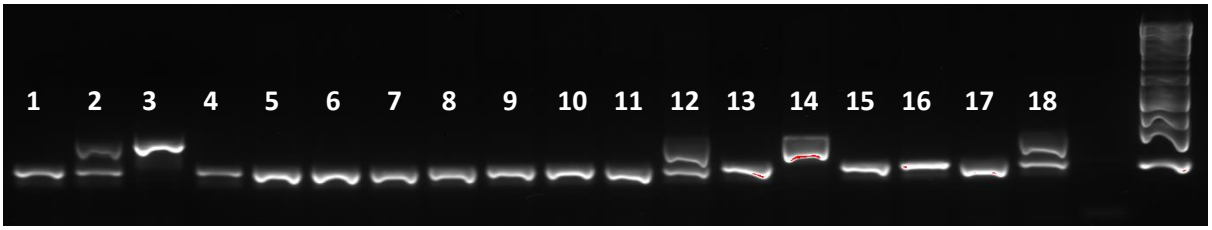

Figure 6.9. PCR products from *TPH2* gel electrophoresis of 18 samples of rhesus macaques. Samples 1, 4-11, 13, 15, 16 and 17 were SS homozygous. Samples 2, 12 and 18 were SL heterozygous. Samples 3 and 14 were LL homozygous.

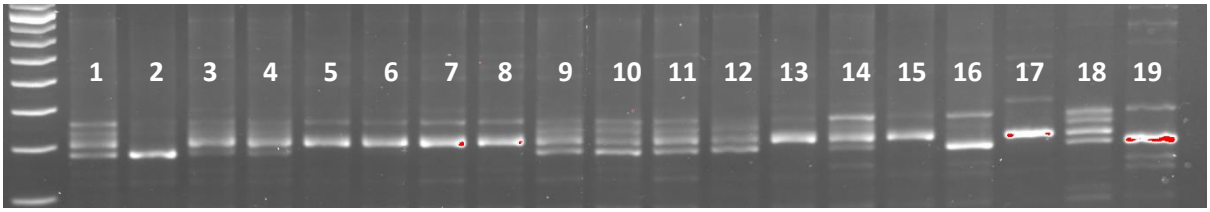

Figure 6.10. PCR products from 5-HTTLPR gel electrophoresis with ethidium bromide of 19 samples of rhesus macaques. Samples 1, 3, 4, 9-12, 14 and 18 were SL heterozygous. Samples 2, 16 and 19 were SS homozygous. Samples 5-8, 13, 15 and 17 were LL homozygous.

SNP genotypes

SNPs for *AVPR1a* were determined from gel electrophoresis images, an example is shown in Figure 6.11. The Taqman assay for the analysis of *OPRM1* yielded clear results and an example output from the real time PCR machine displaying relative fluorescence of samples is shown in Figure 6.12. SNP genotypes for *DRD4*, *HTR2A* and *OXTR* were determined from sequences read using FinchTV. Figure 6.13 shows an example for *HTR2A.*

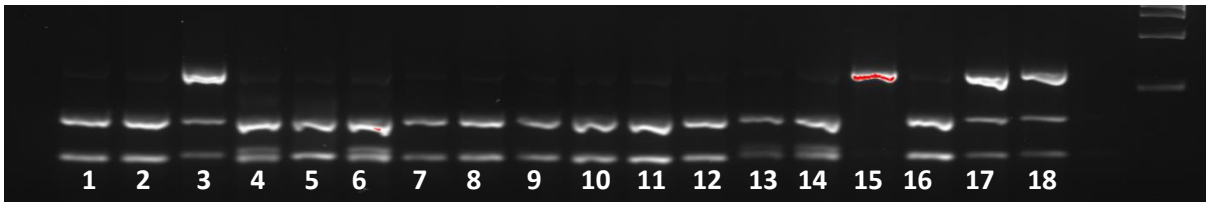

Figure 6.11. PCR products with restriction enzyme from *AVPR1a* gel electrophoresis of 18 samples of rhesus macaques. Samples 1, 2, 5, 7, 8, 9, 10, 11, 12 and 16 were BB. Samples 3, 17 and 18 were AB. Samples 4, 6, 13 and 14 were BC. Sample 15 was AA.

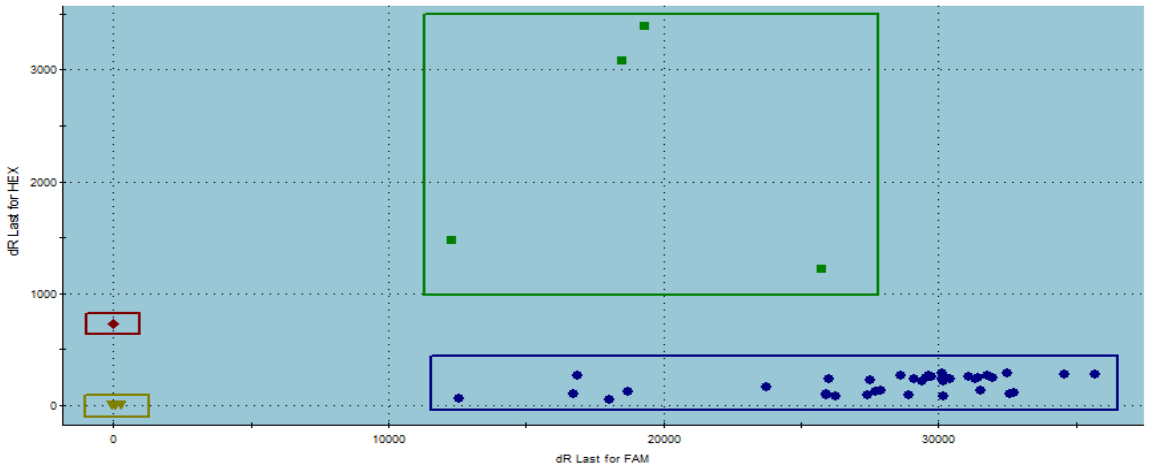

Figure 6.12. Output from the real time PCR machine for *OPRM1* C776 genotype. C-allele homozygous individuals are shown in blue, G-allele homozygotes are shown in red and CG heterozygotes are shown in green. The machine provides an output suggesting a specific genotype for each well and individual.

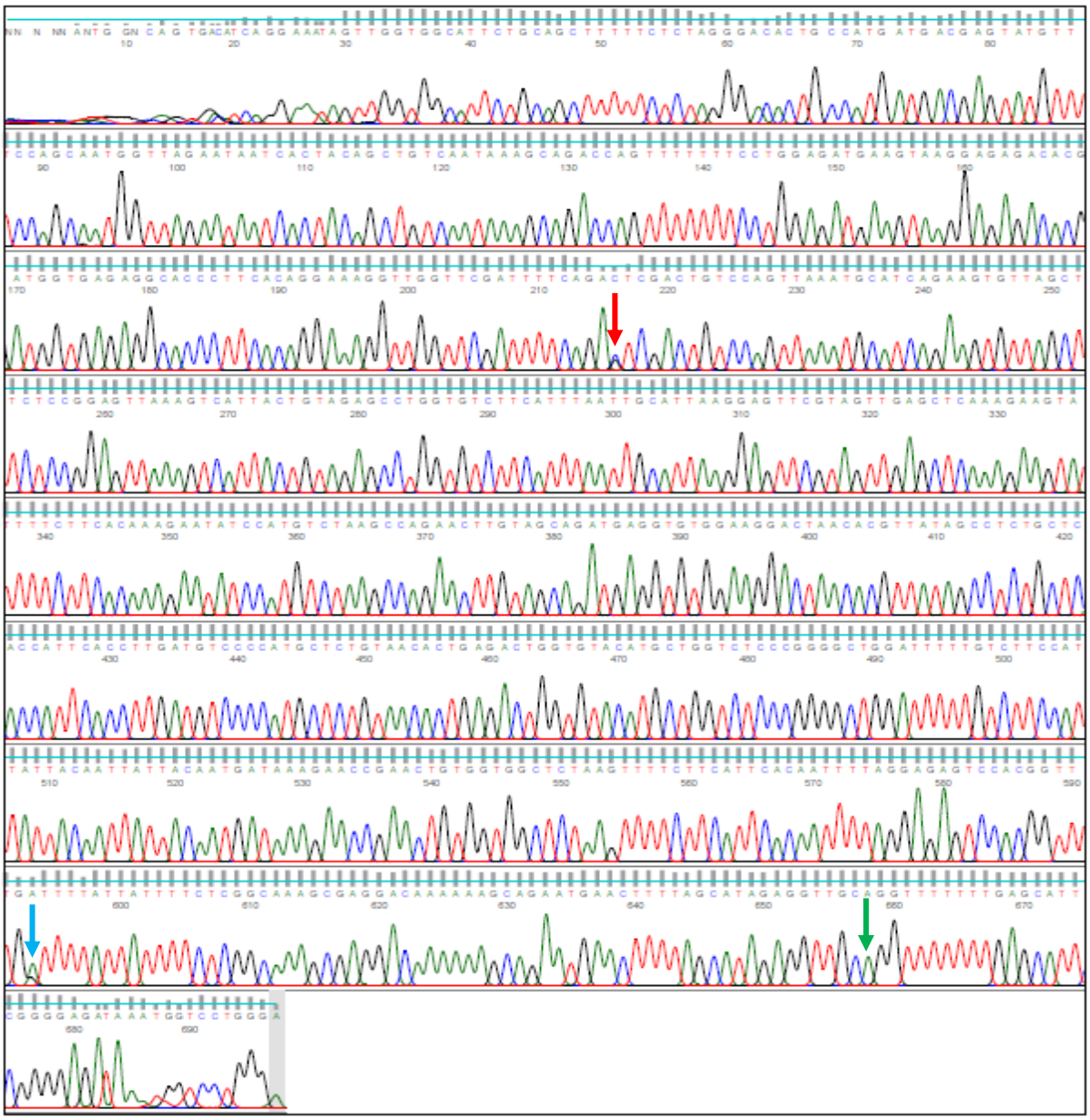

Figure 6.13. Print out of FinchTV showing chromatogram from a female rhesus macaque for 3 SNPs for *HTR2A*. The C>G SNP (rs80365915) is indicated by the red arrow (this individual being a CG heterozygote), the A>G SNP (rs80363349) is indicated by the blue arrow (AG heterozygote) and the A>C SNP (rs196407124) is indicated by the green arrow (this individual is an AA homozygote).

The occurrence and frequency of each genotype or haplotype for the 61 macaques involved in this study are shown in Table 6.4. The occurrence of some genotypes is very low and should be considered when interpreting the results.

Table 6.4. Occurrence and frequency of genotypes for 5-HTTLPR, *AVPR1a*, *DRD4*, *HTR2A*, *MAOA, OPRM1, OXTR,* STin and *TPH2* (n = 61). All frequencies ≥10% are highlighted.

Gene	Genotype	Occurrence	Frequency	Gene	Genotype	Occurrence	Frequency
5-HTTLPR	SS	5	0.08	***MAOA***	5-5	3	0.05
	SL	28	0.46		5-6	7	0.11
	LL	28	0.46		5-7	19	0.31
AVPR	AA	0	0.00		6-6	13	0.21
	AB	16	0.26		6-7	12	0.20
	AC	1	0.02		7-7	6	0.10
	BB	35	0.57	***OPRM1***	CC	49	0.80
	BC	9	0.15		CG	9	0.15
	CC	0	0.00		GG	3	0.05
HTR2A	1-1	14	0.23	***OXTR***	1-1	5	0.08
	1-2	27	0.44		1-2	28	0.46
	1-4	5	0.08		1-3	5	0.08
	2-2	9	0.15		2-2	17	0.28
	2-4	4	0.07		2-3	6	0.10
	3-3	1	0.02	***DRD4***	1-1	13	0.21
	3-4	1	0.02		1-2	26	0.43
STin	SS	9	0.15		1-3	2	0.03
	SL	17	0.28		1-4	1	0.02
	LL	35	0.57		2-2	14	0.23
TPH2	SS	47	0.77		2-3	2	0.03
	SL	13	0.21		2-4	2	0.03
	LL	1	0.02		3-4	1	0.02

Linear mixed model analysis

Sixty-one macaques (45 female, 16 male, mean age = 8.45 ± 3.50 years, range = 3.5 to 16.42 years) housed in 13 social groups completed a total of 634 trials. Overall, the final models were significantly different as compared to the null models for the total duration looking at both face stimuli (TL; likelihood ratio test: $\chi^2 = 61.355$, df = 27, $p<0.001$) but not the duration looking at the threat face stimulus (THR; likelihood ratio test: $\chi^2 = 24.356$, df = 16, $p = 0.082$). A significant relationship was revealed between TL and three genotypes within the serotonin pathway (*TPH2*, STin, *HTR2A*), one genotype within the oxytocin pathway (*AVPR1a*) and haplotypes within the dopamine (*DRD4*) and oxytocin (*OXTR*) pathways (Table 6.5).

Table 6.5. LMM results for the relationship between genotype and total duration looking at the threat and neutral face stimuli (TL) during AB testing in rhesus macaques (n = 61). TL was Tukey transformed (λ = 0.55) for analysis.

Variables in final model		Estimate	Std. Error	t	2.5 %	97.5 %	LRT	*p*
Condition (post-stressor)		-1.749	1.349	-1.297	-4.407	0.905	1.670	0.196
Age (months)		-2.510	0.926	-2.709	-4.353	-0.659	6.862	0.009
Time of day		1.979	0.584	3.387	0.831	3.128	11.352	0.0007
TPH2 (SS, n=47)	LL	-11.426	6.013	-1.900	-23.588	0.639	10.916	0.004
	SL	-6.147	2.108	-2.916	-10.320	-1.922		
STin (SL, n=17)	LL	-9.014	2.353	3.831	-13.713	-4.293	14.021	0.0009
	SS	-9.014	2.939	-3.293	-15.521	-3.797		
HTR2A (1-1, n=14)	1-2	6.143	2.221	2.766	1.742	10.592	20.825	0.0019
	1-4	12.380	3.313	3.737	5.769	18.990		
	2-2	7.446	2.774	2.684	1.963	13.029		
	2-4	10.579	4.361	2.426	1.858	19.311		
	3-3	-3.214	6.846	-0.470	-16.816	10.385		
	3-4	-16.689	6.771	-2.465	-30.198	-3.142		
AVPR1a (BC, n=9)	AB	1.755	2.963	0.592	-4.111	7.708	14.260	0.003
	AC	2.598	6.782	0.383	-10.890	16.051		
	BB	8.161	2.469	3.306	3.233	13.071		
DRD4 (1-1, n=13)	1-2	7.824	2.354	3.324	3.160	12.574	24.592	0.0009
	1-3	12.206	4.886	2.498	2.482	21.949		
	1-4	10.868	7.581	1.434	-4.231	25.926		
	2-2	9.215	2.597	3.549	4.059	14.418		
	2-3	18.368	4.087	4.494	10.160	26.620		
	2-4	12.862	5.067	2.538	2.811	22.966		
	3-4	9.407	8.383	1.122	-7.256	26.135		
OXTR (1-2, n=27)	1-1	2.784	3.667	0.759	-4.514	10.114	9.267	0.055
	1-3	9.012	3.688	2.443	1.691	16.359		
	2-2	4.806	2.025	2.374	0.791	8.861		
	2-3	-2.834	3.442	-0.823	-9.666	4.145		

Serotonin pathway

The *TPH2* s-allele was associated with a greater duration of TL compared to the l-allele (LRT = 10.916, p = 0.004). Individuals that were homozygous for the s-allele had a greater duration of TL (1196 ± 671 ms; n = 487 trials) than l-allele homozygotes (835 ± 676 ms; n = 17 trials, t = -1.90, CI = =23.59 – 0.64). For STin there was a significant effect of length polymorphism on the duration of TL (LRT = 14.02, p = 0.0009). SL heterozygotes spent significantly more time looking at both stimuli (1292 ± 732 ms, n = 170 trials) than l-allele homozygotes (1151 ± 697 ms, n = 365 trials, t = 3.831, p = 0.018, CI = 4.29 – 13.71) and s-allele homozygotes (948 ± 631 ms, n = 99 trials, t = -3.293, p = 0.043, CI = 3.78 – 15.52). There was no evidence for a difference between s-allele homozygotes and l-allele homozygotes (although with only 9 l-allele homozygotes it is possible this analysis was underpowered). *HTR2A* haplotype was significantly associated with the duration of TL (LRT = 20.825, p = 0.0019). Post hoc analysis revealed a difference that approached significance between 1-4 haplotypes (1199 ± 639 ms, n = 52 trails) and 3-4 haplotypes (1051 ± 473 ms, n = 12 trials, t = 2.783, p = 0.088).

Dopamine pathway

The *DRD4* haplotype was associated with the duration of TL (LRT = 24.592, p = 0.0009). Post hoc analysis revealed a significant difference in the duration of TL between individuals with the 1-1 haplotype and individuals with the 2-3 haplotype (t = -3.134, p = 0.044). The 1-1 haplotype was associated with a shorter duration of looking at both stimuli (993 ± 593 n = 124 trials) compared to the 2-3 haplotype (1655 ± 640 ms, n = 34 trials).

Oxytocin pathway

Genotype for *AVPR1a* was significantly associated with the duration of TL (LRT = 14.260, p = 0.003). BC individuals were less attentive to social stimuli (1092 ± 660 ms, n = 97 trials) compared to BB individuals (1191 ± 680 ms, n = 376 trials). Post hoc analysis showed that the difference between the BB and BC genotypes approached significance (t = -2.456, p = 0.0723). Haplotype for *OXTR* was

associated with the duration of TL (LRT = 9.267, p = 0.055). However, post hoc analysis revealed no significant difference between any of the individual haplotypes. The 5-HTTLPR, *OPRM1* and *MAOA* genotypes did not have a significant association with any of the AB measures.

The animal model, a Bayesian analysis controlling for relatedness

The same models were re-run with pedigree included to account for relatedness of the macaques involved in the study. When all the genotypes were included in one model, none of the genotypes had a significant effect on THR or TL. When the variables were grouped by pathway (serotonin: 5-HTTLPR, *HTR2A*, *MAOA,* STin, *TPH2*; dopamine: *DRD4*, *MAOA*; oxytocin & arginine vasopressin: *AVPR1A*, *OXTR*; opioid: *OPRM1*) the analysis revealed significant effects (p<0.05) and non-significant trends (p<0.1) for genotype with both measures of TL and THR (Table 6.6-6.9).

TPH2, STin*, HTR2A* (serotonin pathway), *OXTR* and *AVPR1a* (oxytocin pathway) were all identified as been associated with the duration of TL using the LMM analysis. However, none of these genotypes had a significant relationship with THR or TL (all p>0.1) when pedigree was included.

The association between *DRD4* haplotype and the duration of TL, that was revealed using the LMM analysis, was also identified when pedigree was included. When results with and without pedigree are compared, there is only agreement for *DRD4* for total looking time. Using both methods, individuals with the 2-3 haplotype for *DRD4* were more attentive to social stimuli (1655 ± 640 ms, n = 34 trials) than 1-1 haplotype individuals (993 ± 593 n = 124 trials). With pedigree, *DRD4* haplotype was weakly associated with the duration of THR (CI = -0.430 – 19.864, p = 0.1, Table 6.6), while the association between *DRD4* haplotype and the duration of TL approached significance (CI = -1.286 – 49.294, p = 0.054, Table 6.7).

Both 5-HTTLPR and *OPRM1* were not associated with looking time in the LMM analysis. However, when pedigree was included an association was revealed. For 5-HTTLPR, s-allele carriers had a higher duration of TL than l-allele homozygotes, a trend that approached significance (SL: CI = -0.532 – 11.273, p = 0.078, SS: CI = -0.442 – 18.319, p = 0.09, Table 6.8). OPRM1 was significantly

associated with the duration of TL (Table 6.9). *OPRM1* G-homozygotes were significantly more attentive towards both the threat and neutral stimuli (1876 ± 601 ms) than C-homozygotes (1139 ± 659 ms, CI = 4.701 – 30.250, p = 0.014). The full R script of the statistical analysis and results is shown in Appendix 6d.

Table 6.6. Results for the effect of dopamine pathway related genotypes on time spent looking at the threat face stimuli (THR) during AB testing in rhesus macaques (n = 61) with pedigree using Bayesian analysis. THR was square root transformed (λ = 0.5) for analysis.

Variables in final model		Post mean	Lower 95% CI	Upper 95% CI	*p*
DRD4 (1-1)	1-2	3.078	-1.325	7.033	0.138
	1-3	1.018	-8.277	10.392	0.850
	1-4	2.515	-9.090	16.013	0.692
	2-2	2.123	-3.373	6.625	0.400
	2-3	8.371	-0.430	19.864	0.100
	2-4	10.126	-0.971	23.021	0.112
	3-4	0.355	-14.507	14.438	0.976
MAOA (5-7)	5-5	0.252	-8.302	8.399	0.970
	5-6	3.287	-2.706	9.492	0.282
	6-6	0.799	-4.811	6.027	0.764
	6-7	1.489	-2.928	5.783	0.490
	7-7	0.417	-6.054	7.530	0.882

Table 6.7. Results for the effect of dopamine pathway related genotypes on total looking time (TL) during AB testing in rhesus macaques (n = 61) with pedigree using Bayesian analysis. TL was Tukey transformed (λ = 0.55) for analysis.

Variables in final model		Post mean	Lower 95% CI	Upper 95% CI	*p*
DRD4 (3-4)	1-1	1.544	-21.790	23.155	0.882
	1-2	7.222	-16.262	30.282	0.542
	1-3	4.680	-19.583	26.611	0.708
	1-4	1.959	-26.194	30.318	0.888
	2-2	7.261	-13.625	32.851	0.558
	2-3	23.727	-1.286	49.294	0.054
	2-4	24.809	-1.699	52.528	0.068
MAOA (6-6)	5-5	3.272	-7.103	13.677	0.534
	5-6	4.547	-4.834	15.217	0.342
	5-7	1.073	-6.713	8.954	0.802
	6-7	2.032	-5.929	10.534	0.600
	7-7	0.337	-8.567	9.561	0.948

Table 6.8. Results for the effect of serotonin pathway related genotypes on total looking time (TL) during AB testing in rhesus macaques (n = 61) with pedigree using Bayesian analysis. TL was Tukey transformed (λ = 0.55) for analysis.

Variables in final model		Post mean	Lower 95% CI	Upper 95% CI	*p*
5-HTTLPR (LL)	SL	5.538	-0.532	11.273	0.078
	SS	8.051	-0.442	18.319	0.090
TPH2 (LL)	SL	4.261	-13.819	21.039	0.670
	SS	9.281	-6.490	27.954	0.290
STin (SS)	LL	2.665	-5.168	10.400	0.508
	SL	3.409	-5.399	11.928	0.438
HTR2A (3-4)	1-1	5.827	-16.264	27.506	0.624
	1-2	4.972	-16.105	27.515	0.660
	1-4	9.072	-14.069	31.807	0.462
	2-2	7.761	-14.812	31.978	0.536
	2-4	7.744	-18.115	31.962	0.516
	3-3	3.815	-25.194	34.259	0.802
MAOA (5-5)	5-6	8.386	-6.283	21.039	0.230
	5-7	7.102	-4.902	19.071	0.256
	6-6	6.916	-5.681	18.280	0.282
	6-7	7.899	-4.664	19.875	0.192
	7-7	6.427	-7.878	19.470	0.400

Table 6.9. Results for the effect of opioid pathway related genotype on total looking time (TL) during AB testing in rhesus macaques with pedigree (n = 61). TL was Tukey transformed (λ = 0.55) for analysis.

Variables in final model		Post mean	Lower 95% CI	Upper 95% CI	*p*
OPRM1 (CC)	CG	2.460	-4.010	8.314	0.424
	GG	17.156	4.701	30.250	0.014

6.5 Discussion

In this chapter I successfully genotypes 61 rhesus macaques for variants in nine genes (5-HTTLPR, *AVPR1a*, *DRD4*, *HTR2A MAOA*, *OPRM1*, *OXTR*, STin, *TPH2*) finding evidence of variation in all of them. A further three gene SNPs (*CYP17*, *OXT*, *SERPINA6*) were trialled for a subset of monkeys, but no variation was found.

When relatedness between individuals was not controlled for, I found evidence for an influence of several gene polymorphisms on social attention. However, when I reran the analyses using the animal model in which relatedness was controlled for by including pedigree, there was only

evidence for an association between genotype and social attention for three genes (5-HTTLPR, *DRD4* and *OPRM1*)

For the linear mixed model, which did not control for relatedness, the genotypes for *TPH2*, *AVPR1a, HTR2A* and STin and haplotypes for *DRD4* and *OXTR* had a significant or trend suggesting a relationship that approached significance between genotype (single locus or multi-locus) and macaque looking time. *TPH2*, *OXTR*, *AVPR1*a, *HTR2A* and STin had no association with looking time when pedigree data were included.

The macaques involved in this study were housed in matrilineal breeding groups and many were very closely related (full or half-sisters and daughters). The chance of type I errors in genetic studies including related individuals is higher when individuals in the sample are more closely related (McArdle et al, 2007). Therefore, the results without pedigree, concluding that *TPH2*, *HTR2A, OXTR*, *AVPR1*a and STin are associated with AB measures, are potentially type I errors, and these associations should be considered false positives.

Analysis using the *MCMCglmm* function which included pedigree information did reveal that 5-HTTLPR, *DRD4* and *OPRM1* had either a significant association or showed a trend that approached significance with the AB measures, suggesting that genotypes at these loci is related to looking time.

Individuals carrying the 5-HTTLPR s-allele have a longer duration of total looking time than those homozygous for the l-allele. The 5-HTTLPR low expressing s-allele has been previously shown to be associated with stress sensitivity and depression in humans and rhesus macaques (Bennett et al, 2002; Caspi et al, 2003; Barr et al, 2004; Bethea et al, 2004). The heightened looking time for s-allele carriers in this study may be explained as the allele is associated with heightened vigilance and AB to negative information (Mrazek et al, 2013). However, Szott (2015) previously found that individuals with 5-HTTLPR s-alleles were more avoidant of the threat face. This variation could be the result of different methods used by the two studies. Howarth et al (2021) reported that there was no significant correlation between the screen (data from this thesis) and card (Szott, 2015) methods for AB difference and total looking time when individuals that were involved in both

studies were compared. Furthermore, Watson et al (2009) found an association between vigilance behaviour and genotype with *TPH2* l-allele homozygotes being more vigilant than s-allele carriers. The opposite effects found in this study and the present study may be due to a number of factors: Watson et al (2009) used a sample size of nine rhesus macaques while this study used a sample of 61, the 5-HTTLPR forward primer used was different (Watson: hMUT (5'-TCGACTGGCGTTGCCGCTCTGAATGC-3'); here: 5HTT-F (5'-GCCGCTCTGAATACCAGCAC-3')), and the stimuli used by Watson included known conspecific faces and female perinea rather than threat-neutral unfamiliar conspecific faces. Most significantly, Watson et al (2009) did not genotype any s-allele homozygotes. Therefore, it could be concluded that the data presented in this thesis are more reliable than that published by Watson et al. The results may not be directly comparable; however, as attention related genotype information in rhesus macaques is generally lacking, the inclusion of any published work in this area is important.

Polymorphisms in *DRD4* can result in a lower affinity for dopamine (Ptacek et al, 2011) and are associated with psychiatric disorders including ADHD, bulimia, and alcoholism (Kaplan et al, 2007; Chen et al, 2011), behavioural problems including anger, aggression, and delinquency in humans (Hohmann et al, 2009; Dmitrieva et al, 2011) and avoidance of mothers and conspecifics in juvenile rhesus macaques (Coyne et al, 2015). In the present study, *DRD4* 3-4 haplotype individuals were more avoidant of the threat face and had a shorter duration of total looking time than 2-3 haplotype (THR: 0.1, TL; 0.054) and 2-4 haplotype individuals (THR: 0.11, TL: 0.07). This result was not found by Szott (2015) who reported no association between *DRD4* and AB measures. As these were novel polymorphisms first sequenced by Szott (2015) and the results of these studies differ, additional research is needed to properly understand the effect of these polymorphisms on cognition and behaviour in rhesus macaques.

OPRM1 is associated with reward and aversion behaviour and the C77G polymorphism is linked to aggressive-threat behaviours in rhesus macaques (Miller et al, 2004) with G-allele carriers also showing higher baseline attachment behaviours than C-allele homozygous individuals (Barr et al,

2008). In the present study, the results are as expected as G-allele homozygotes were more attentive to the threat face stimuli than CG heterozygotes and had a significantly longer duration of total looking time than C-homozygotes. However, Szott (2015) did not find this effect, there were only three G-homozygotes involved in this study and one of those individuals (Zebedee) had a rhesus macaque form of undiagnosed Down or Williams syndrome. These syndromes in humans are associated with heightened social interest (Cicchetti & Beeghly, 1990; Javinen et al, 2013) and increased AB to positive emotional faces (Goldman et al, 2017). Therefore, this result is likely due to the adverse effect of this one individual in a small sample size and might be lost if a large sample size could be studied.

Some of the polymorphisms presented here are novel to this study (*AVPR1a*, *HTR2A*, *OXTR*) or have only been analysed once before (*DRD4, MAOA*; Szott, 2015) and therefore, their link to AB and anxiety is unknown. In humans, there is some conflicting evidence as to the effect of polymorphisms in *OXTR* and STin on attention, anxiety, and stress-related disorders. For example, Haddley et al (2012) demonstrated a lack of reproducibility in *STin* study results, Lovejoy et al (2003) demonstrated no difference in enhancer activity between the *STin* polymorphisms and a recent study by Connor et al (2018) suggested that *OXTR* variants are not involved in emotional differences.

A lack of effect of *AVPR1a*, *HTR2A*, *MAOA*, *OXTR*, *TPH2* and STin may be because these genes are not related to, or have any underlying effect on AB. However, no effect may be the result of small sample sizes both here and in previous studies. Genotype studies involving humans typically include many more individuals than included here as larger sample sizes have higher statistical power (Hong & Park, 2012; Button et al, 2013). Although the sample size here (n = 61) is small, it is a significant improvement on other published rhesus macaque studies (e.g., n = 20 - Ferguson et al, 2007; n = 20 – McCormack et al, 2009; n = 9 - Watson et al, 2009; n = 7 – Ebitz et al, 2013; n = 29 – Qin et al, 2015).

For *DRD4* there were only two monkeys with the 2-3 haplotype, two monkeys with the 2-4 haplotype and only one monkey with the 3-4 haplotype. The monkey with the 3-4 haplotype, who was more avoidant of the stimuli, was an older, implanted female while the 2-3 and 2-4 haplotype monkeys were breeding males. Table 6.7 shows that the presence of a contraceptive implant has a negative effect on looking time at the threat face stimulus suggesting this difference may be hormonal rather than genetic.

There was an appropriate sample size for the comparison of s-allele carriers for 5-HTTLPR as, even though only five monkeys were homozygous for the 5-HTTLPR s-allele, there were 28 monkeys that were SL heterozygotes and 28 monkeys that were l-allele homozygotes.

There is a known relationship between ELS, stress reactivity and many of the genes included in this study. However, the study included only nine monkeys that had experienced ELS. The interaction between a condition (baseline and post-stressor) and early weaning (before 12 months; Prescott et al, 2012) was included in the full statistical model for both THR and TL but did not have a significant effect in either model; possibly because of the low sample size. There are known interaction effects between ELS and *AVPR* (Liu et al, 2015), *HTR2A* (Parade et al, 2017), *MAOA* (Enoch et al, 2010), *OXTR* (Unternaehrer et al, 2015) and *TPH2* (Forssman et al, 2013) on behavioural disorders in humans, *OPRM1* (Vassoler et al, 2018) and *AVPR* (Lucas et al, 2011) on behaviour in rats and 5-HTTLPR and behaviour in rhesus macaques (Bennett et al, 2002; 2007; Barr et al, 2004; Bethea et al, 2004). Perhaps if there were more individuals that had experienced ELS then an association between these and AB measures would have been found. However, as laboratories move towards better practices, fewer animals are weaned early (Prescott et al, 2012) and therefore, do not experience this stressor.

The mechanisms for AB and behaviour are polygenic systems and involve a variety of pathways in the brain and nervous system (National Research Council, 1989) and any member of these pathways may represent a candidate gene for study. Candidate genes are used, as they are cheaper than full genome sequencing, which in humans can cost up to around £7,000 per individual

depending on number of samples (Schwarze et al, 2020). However, selecting appropriate candidate genes is challenging as there is currently little physiological, biochemical, or functional knowledge relating to AB (Zhu & Zhao, 2007). It is convenient to select a small number of polymorphisms in a small number of genes; however, there are many more candidate genes, SNPs and variants that could be relevant and have an underlying effect on AB (Trask et al, 2011). Therefore, finding significant effects of individual alleles and genotypes with small sample sizes is challenging.

6.6 Conclusion

The genotype for 5-HTTLPR, *DRD4* and *OPRM1* was found to have a significant relationship or non-significant trend that approached significance with AB measures when pedigree information was included. However, although overall sample size was an improvement on previous studies and was within the suggested acceptable range for NHP genetics, the number of individuals with specific genotypes of interest was low and the results may not be as meaningful due to small sample size and low statistical power. When pedigree was not included *AVPR1a*, *DRD4*, HTR2A, OXTR, STin and *TPH2* had a significant association with looking time. This research demonstrates the potential for type I error if relatedness or family information is not included in genetic studies.

Chapter 7 – Thesis conclusion

7.1 The utility of AB for understanding primate welfare

Each year around 100,000 NHPs are used in biomedical research worldwide (Chatfield & Morton, 2018). Due to their evolutionary proximity to humans, high sentience and cognitive sophistication, resulting in similarities in their behavioural and physiological needs and ability to experience pain, distress and anxiety, there are numerous legal and ethical considerations (Zoo Licencing Act, 1981; Animals (Scientific Procedures) Act, 1986; Festing & Wilkinson, 2007; Sughrue et al, 2009; APC, 2013; DeGrazia & Sebo, 2015; Schönfelder, 2015; Friedman et al, 2017; Kagan et al, 2018; Walker, 2018; Siani, 2019). The housing and husbandry of captive NHPs must meet the required standards to minimise animal suffering and promote good welfare (Zoo Licencing Act, 1981; Animals (Scientific Procedures) Act, 1986; FAWC, 1979). Further, public support for the use of any animal in research is dependent on avoiding unnecessary suffering (Ipsos MORI, 2016). To meet these standards and avoid suffering, we must consider an animal's environment, behaviour, physiology, health, and psychological well-being. To do this we must develop tools to quantify the impact of stressors including husbandry, housing, and veterinary interventions, on psychological wellbeing.

At present, minimising suffering for captive NHPs is challenging; welfare assessment is notoriously difficult as there is no single measure of well-being (Wolfensohn & Honess, 2008). On their own, traditional welfare assessment methods such as behavioural, physiological, and physical health indicators, do not capture the psychological component of wellbeing and interpretation can be challenging. Triangulation of these methods is the current best method (Webster, 2008; Jennings & Prescott, 2009; Tasker, 2012); however, the animal's emotional or psychological response is still not considered. Therefore, there is currently a need for the development of new methods of welfare assessment for captive NHPs that access the psychological aspect of wellbeing (Mendl et al, 2009; Bethell et al, 2012ab, Crump et al, 2018). I propose methods from human psychology that utilise cognitive or attentional biases. These have shown promise in several species (Crump et al, 2018) and here, I have conducted the first studies to look at the factors that underlie attention bias in rhesus macaques including genetic predisposition, endocrine response, life-history and husbandry factors and factors associated with the AB test itself.

7.2 Key findings

In this thesis, I aimed to utilise the innate survival mechanism of automatic allocation of attention and rapid processing in response to threat-relevant cues (LeDoux, 1996; Öhman & Mineka, 2001; Anderson & Adolphs, 2014; Crump et al, 2020). This innate mechanism has been utilised to develop AB tasks to assess emotion in humans using paradigms such as the looking time task (Fantz, 1958), the dot-probe task (MacLeod et al, 1986), the visual search task (Green & Anderson, 1956) and emotional Stroop task (Stroop, 1935). In humans, cognitive studies have revealed shifts in AB with both trait anxiety (individual differences; MacLeod et al, 1986; Mogg and Bradley, 1998; Bar-Haim et al, 2007; Richards et al, 2013; Veerapa et al, 2020) and state anxiety (induced by temporary stressors; Quigley et al, 2012). AB tasks have previously been shown to detect shifts in emotional state in humans, with some recent data suggesting they can be adapted for use with animals (Crump et al, 2018), including NHPs (Bethell et al, 2012b; Marzouki et al, 2014; Allritz et al, 2016; Boggiani et al, 2018; Morin et al, 2019). Here, I used a looking time task with 61 socially housed captive rhesus macaques to identify the biological and environmental factors (life history, hormonal, genetic and potentially stress-inducing husbandry procedures) that influence an individual's AB profile and the extent to which this can be used to identify state (e.g., response to veterinary intervention) and trait (e.g., individual differences in personality) affect.

Previous studies have focused on shifts in AB following environmental or pharmacological manipulation (Crump et al, 2018) and, to date, only one study has examined trait differences in AB (birds; Cussen & Mench, 2014). The interaction between trait and state anxiety has not been previously discussed; however, Bethell et al (2012b) did note a uniform pattern of AB following a stressor indicating no apparent influence of trait affect on shifts in AB, and a large degree of variation between individual macaques in baseline AB. In this thesis, I presented evidence for both state and trait influence on AB. These factors should be used as possible indicators for individuals that may be more vulnerable following stressful life events and should, therefore, be included in the design and analysis of future AB studies.

State affect influences social attention: influence of vet check, time of day and stimulus ID

AB to threat was not associated with condition. In Chapter 3, I found no significant change in AB to threat face stimuli following the macaques' annual veterinary health check (stressor). This differs from previous macaque literature (Bethell et al, 2012b). However, as discussed in Chapter 3 there were some methodological differences between that study and this thesis including the use of an enriched baseline rather than non-stressed baseline and, most notably, singly housed macaques rather than socially housed macaques. The macaques at MRC-CFM live in stable social groups with species-typical matrilineal hierarchies (Dr Claire Witham, Scientific Project Co-ordinator at MRC-CFM, personal communication, June 2017). Familiar conspecifics are a source of comfort (Suomi et al, 1973) and there is a selective advantage of living in a stable group (Markham & Gesquiere, 2017). In NHPs, the presence of conspecifics can have a stress attenuation effect (Meyer & Hamel, 2014) with positive social interaction blunting the activation of the physiological stress response including reducing changes in cortisol concentration (DeVries et al, 2003; Hennessy et al, 2009). Social stability has been shown to influence the SNS which in turn mediates many of the endocrine, immune and health responses to stress (Capitanio & Cole, 2015). Singly housed macaques are known to have exaggerated fear responses compared to group housed animals (Clay et al, 2009b). These differences in housing and social conditions may have exaggerated the negative impact of veterinary intervention on macaque welfare and resulted in a larger negative shift in AB measures in Bethell et al (2012b) compared to this thesis. For social species, future AB studies must account for the differences in stress response between singly and group housed animals.

Social attention towards faces was associated with time of day. Social attention for digitally presented face pairs (duration of TL) increased throughout the day. Attention in humans shows a circadian rhythm (Kraemer et al, 2000; Valdez et al, 2005; Knight & Mather, 2013). It is therefore likely the effect of time of day reflects circadian changes in alertness and arousal that have been documented across animal species (e.g., cows: Niu et al, 2014; birds: Ramli & Norazlimi, 2016; primates: Kappeler & Erkert, 2003; Plant, 1981; Novak et al, 2013). Daily husbandry schedules, such

as cleaning and feeding, may also influence engagement with tasks at certain times. In future AB studies, time should be controlled for where testing time varies.

Stimulus ID was associated with differences in physiological stress response. Macaques that had been shown stimulus ID 2 had significantly higher salivary cortisol concentrations than macaques shown other stimuli. Sustained eye contact is a threatening display in macaques (van Hooff, 1967; McFarland et al, 2013) and stimulus ID 2 had more prominent front facing eyes than the other stimuli used in the present study. The prominent eyes may have been perceived as more threatening which may explain the elevated cortisol associated with stimulus ID 2. Howarth et al (2021) reported an association between stimulus ID and ABDiff when only baseline data were considered. In this study, stimulus ID 3 (Figure 3.2 in this thesis) was found to have a significantly larger mean ABDiff score compared to stimuli 1, 5 and 6. The images used as stimuli were opportunistically taken photographs of real animals (Bethell, 2009; Witham & Bethell, 2019). It is therefore likely that the images varied from each other in brightness, colour, contrast energy and luminance, as well as age, attractiveness, degree of head tilt, dominance, emotional intensity, and orientation, all of which can influence attention to faces (Waitt & Buchanan-Smith, 2006; Hess et al, 2007; Palumbo et al, 2017). Human studies have shown variation in AB for pain, disgust, and angry facial expressions (Schofield et al, 2013; Hommer et al, 2014; Heathcote et al, 2015). Researchers developing AB protocols for animals should work collaboratively to develop validated picture libraries to reduce the influence of individual stimuli on study results. Stimulus set libraries are becoming freely available (Murphy & Leopold, 2019; Witham & Bethell, 2019; Wilson et al. 2020) and future AB studies should use these, or pilot potential stimulus sets to ensure uniformity in attention across the images; this will allow AB tests to measure changes in affect caused by specific interventions rather than noise introduced by an emotive or distracting stimulus.

Trait affect influences social attention to threat: influence of sex, age, and genotype

There is evidence for consistent individual differences in social attention. Repeatability of the AB signal (TL) was found to be 0.093 ± 0.243, which is within the range of the animal social behaviour

and human AB literature (e.g., Bell et al, 2009, reported on a range of behaviours across species, mean R = 0.37, range = 0-1). This suggests that an individual's social attention to faces is relatively consistent across trials and AB profile can be considered a trait. The other AB measures (THR, ABDiff, ABDiff/TL) did not have significant repeatabilities; however, with a larger sample size (n = 110), Howarth et al (2021) reported a significant repeatability for THR (R = 0.12, CI = 0.04 - 0.23, p<0.001) and a similar repeatability for TL to that reported in this thesis (R = 0.24, CI = 0.11 - 0.36, p<0.001). A lack of repeatability data has been previously identified as a threat to understanding the theory underlying AB (Rodebaugh et al, 2016).

Sex was significantly associated with social attention at baseline. Males have a greater duration of both THR and TL than females. Sex differences in AB are evident in humans, for example, females have a higher variability of AB compared to males (Carlson et al, 2019) and a greater AB for disgust (Kraines et al, 2017) and threat (Montagner et al, 2016) stimuli compared to males. Human studies have reported conflicting results with anxious males being more attentive (Zhang et al, 2017), less attentive (Tan et al, 2011) or having no difference in their attention (Kinney et al, 2017) to threat compared to anxious females. Human females exhibit an own-gender bias in attention to faces, which is not present in men (Lovén et al, 2011; Herlitz & Lovén, 2013). This gender bias in human is mirrored in primates with female capuchin monkeys showing an AB towards images of female conspecifics over male conspecifics while male capuchin monkeys showed no preference (Schino et al, 2020).

The difference in response between males and females may be due to the "tend and befriend" alternative stress response pathway, which has been studied in female humans (Taylor et al, 2000). Taylor et al (2000) suggested that a response geared towards aggression might not be adaptive for female animals as it could leave offspring unprotected. Instead, female behaviour is directed at retrieving and protecting offspring while anticipating and avoiding threats to increase the likelihood of offspring survival. MRC-CFM is a breeding colony and 60% of the females included in this study had offspring < 12 months old and 92% had offspring < two years old. Attentiveness and a drive to

ensure offspring survival may have contributed to the female macaques' avoidance of the threatening unfamiliar male face. Future AB studies must include sex as a factor due to biological and developmental differences that influence attention to social threat.

This thesis provided the first evidence for the relationship between age and AB in rhesus macaques. Here, young macaques (≤4 years old) were more attentive to the threat face stimulus that older macaques (≥7 years old). This may suggest that younger macaques may have better emotional well-being than older macaques. Evolved training practices in NHP laboratories means younger NHPs are better habituated to humans and experience only PRT methods (e.g., Perlman et al, 2012; Whittaker & Laule, 2012; Nightingale et al, 2015; Westlund, 2015). This shift to PRT promotes improved animal welfare during training (Laule et al, 2003, 2007; Prescott & Buchanan-Smith, 2003; NC3Rs, 2019) and time spent training and rewarding promotes a closer relationship between trainers and the NHPs involved (Buchanan-Smith, 2003; Prescott & Buchanan-Smith, 2003). Older macaques may not have experienced this positive interaction to the same degree or during critical periods of development (five of the older macaques were weaned at less than 12 months old) which may influence their lifelong relationship with keepers and their overall trainability.

The effect of age is an important consideration for development of this method for welfare assessment and for macaque welfare generally. At MRC-CFM, macaques retained for breeding are weaned between 12 and 30-months-old and moved into one of the single sex weaner groups where they remain until they are moved on to one of the universities at between four or five-year-old (Dr Claire Witham, Scientific Project Co-ordinator at MRC-CFM, personal communication, June 2017). Once at the university, most macaques spend the first-year training for the experimental protocol (Stuart Mason, Research Assistant at the Experimental Psychology department, University of Oxford, personal communication, August 2017) and do not experience their first procedure (e.g., implantation) until they are around six years old. This means that macaques begin highly stressful, often invasive protocols at an age where they may be more susceptible to stress and anxiety. It may

be of benefit to repeat this study with animals that started research protocols at a range of ages to establish if there is a definite relationship between age, anxiety, and attention.

Pedigree information must be included in genetic studies involving closely related individuals. When pedigree was not included, there was a significant association between social attention and genotype for *TPH2*, *AVPR1a*, STin, *HTR2A* and haplotypes for *OXTR* and *DRD4*. When pedigree was included, there was only one significant association found (*OPRM1*) and two relationships that approached significance (5-HTTLPR and *DRD4*).

OPRM1 is associated with reward and aversion behaviour and the C77G polymorphism is linked to aggressive-threat behaviours in rhesus macaques (Miller et al, 2004). Here, G-allele homozygotes were revealed to have greater attention to social stimuli than C-allele homozygotes, which is likely linked to the increased attachment and prosocial behaviour seen in G-allele homozygotes (Barr et al, 2008). The 5-HTTLPR low expressing s-allele has been previously shown to be associated with stress sensitivity, depression and heightened vigilance in humans and rhesus macaques (Bennett et al, 2002; Caspi et al, 2003; Barr et al, 2004; Bethea et al, 2004; Mrazek et al, 2013), which fits with the findings of this study. Individuals carrying the 5-HTTLPR s-allele had a longer duration of total looking time than those homozygous for the l-allele. Polymorphisms in *DRD4* can result in a lower affinity for dopamine (Ptacek et al, 2011) and are associated with psychiatric disorders including ADHD, bulimia, and alcoholism (Kaplan et al, 2007; Chen et al, 2011), behavioural problems including anger, aggression, and delinquency in humans (Hohmann et al, 2009; Dmitrieva et al, 2011) and avoidance of mothers and conspecifics in juvenile rhesus macaques (Coyne et al, 2015). Here, *DRD4* 3-4 haplotype individuals were more avoidant of the threat face and had a shorter duration of total looking time than 2-3 haplotype and 2-4 haplotype individuals. It is evident that genotype is associated with social attention; however, further research on this association is needed. Below (6.3 Limitations), I discuss some of the issues with the genetic analysis, and other areas of the thesis and suggest directions for future study. Of key importance for the genetic

component of this thesis is a collation and publication of a larger data set, which I plan to do this year.

7.3 Limitations

Training

The quality of the macaques training impacted the success of the whole thesis. It may have been better to focus on fewer training goals rather than attempting to complete such a range of tasks. In addition to station training, AB apparatus desensitisation, and saliva sample collection, the macaques were also trained for urine collection. Over 100 urine samples were collected; however, they were not used in the thesis. If urine collection training was not conducted it would have allowed more time to focus on the key study questions. This may have increased the number of saliva samples successfully collected and analysed (if greater volumes were collected), which would have then provided more data and may have improved the precision of the data.

Calculated measures introduced noise into the data

Four measures of AB were used in this thesis: duration looking at the threat face stimulus (THR), total duration looking at the threat and neutral face stimuli (TL), AB difference score (ABDiff) and ABDiff/TL. Both ABDiff and ABDiff/TL had a repeatability of 0 and did not shift with condition (baseline/post-stress). Calculated measures of AB (ABDiff, ABDiff/TL) add extra noise to the data and make the statistical models unstable compared with the raw data (TL, THR). The additional variable added extra noise and the models were unstable, so I did not include it in the thesis. Calculated measures of AB are less reliable than raw data. This noise may have been reduced with the inclusion of a larger sample size. When a sample of 110 monkeys were used (including those discussed in this thesis) the model stability and clarity of the signal for ABDiff were improved sufficiently for inclusion in the publication (Howarth et al, 2021).

Sample size

Sample size may have been an issue in Chapter 5 and 6. In Chapter 5, 77 samples from 17 monkeys were used for cortisol analysis while in Chapter 6, 61 monkeys were used for genetic analysis. Genotype studies involving humans typically include many more individuals than included here as larger sample sizes have higher statistical power (Hong & Park, 2012; Button et al, 2013). Although the sample size in Chapter 6 (n = 61) is small for genetic research, it is a significant improvement on other published rhesus macaque studies (e.g., n = 20 - Ferguson et al, 2007; n = 20 – McCormack et al, 2009; n = 9 - Watson et al, 2009; n = 7 – Ebitz et al, 2013; n = 29 – Qin et al, 2015) and is within the suggested range of individuals required for analysis (60 – 100; Brent & Melin, 2014; von Borell et al, 2019). I suggest that the required next step in NHP genotype analysis is to collate any unpublished data (e.g., Howarth et al (2021) published AB data on 110 rhesus macaques, for which genetic analysis has also been conducted and a paper is being prepped for publication) or conduct a meta-analysis on the published data to determine if the chosen polymorphisms are appropriate for further study.

The *OPRM1* genotype that showed a significant difference in social attention had a sample size of 3 and one of those individuals had a rhesus macaque form of undiagnosed Down or Williams syndrome. These syndromes in humans are associated with heightened social interest (Cicchetti & Beeghly, 1990; Javinen et al, 2013) and increased AB to positive emotional faces (Goldman et al, 2017). Therefore, this result is likely due to the adverse effect of this one individual in a small sample size and might be lost if a large sample size could be studied.

To increase sample size in Chapter 5, a higher than standard %CV was used (intra- and inter-assay %CV should be <10% and <15%, respectively; Thomsson et al, 2014). This strict cut off point would have resulted in very few samples from the same monkey collected before and after the stressor (55 samples from 14 monkeys). Therefore, a higher %CV (intra- and inter-assay %CV of 15% and 20%, respectively) was used. This higher %CV has been used in published literature (e.g., Reed et al, 2002) and allowed the inclusion of 77 samples from 17 monkeys. However, the precision of EIA

data is determined using %CV and reflects the researcher's ability to perform the EIA. A poor %CV can reflect inconsistent pipetting technique or mishandling of the samples; the high viscosity of saliva should be accounted for in pipetting technique and speed (Schultheiss & Stanton, 2009). In addition to focusing macaque training, a longer pipetting training period, prewetting pipette tips and regularly calibrating the pipettes may all have reduced the %CV (Mannonen et al, 2006; Hemmings, 2009).

Apparatus

The device used to collect AB trial footage was a relatively inexpensive automated apparatus which allowed for digital images to be presented on computer screens for a fixed amount of time. The device was an improvement on previous macaque AB studies that involved manually operated stimuli presented on card. Digital images do not discolour or become damaged or dirty. The presentation and removal of stimuli was not reliant on the timing of the researcher, which ensured the three second presentation was consistent for every trial. However, the device was large, heavy, and included a black sheet designed to block the monkeys' view of the researcher. The use of a smaller, hand-held AB device may have been preferable as it would have been practically easier allowing for quicker set-up and more opportunistic data collection. Further the use of a less intrusive device may also have decreased training and desensitisation time as the monkeys would have been less aware of its presence throughout training and testing. For AB to be adopted as a method of welfare assessment quick analysis of the AB trial footage is required. This will ultimately mean that eye-tracking technology should be implemented, and this study was the first step towards automation of the trial and analysis by using a digital tool. Future AB studies should look at methods to reduce the device size while ensuring the stability of the footage and that a consistent distance is maintained between the apparatus and the monkey.

In addition to the device size, the AB apparatus build quality resulted in sensitive cables and the risk of the computer disconnecting. This was overcome by turning the camera on at the start of the data collection session and allowing it to record for the whole 20 to 30 minutes while AB trials were

conducted for the group. This set up did not impact the quality of the footage collected; however, it resulted in hours of irrelevant footage that had to be watched to snip out the three second clip of interest. This was time consuming and detracted from time that could have been used improving, for example, salivary cortisol analysis skills.

Veterinary stressor

This project piggybacked onto the macaques' routine annual veterinary health check to ensure further stress was not caused as a result of this research. Thesis results (Chapter 3, 4 and 5) suggest that the veterinary intervention was not sufficiently stressful to result in the expected changes in AB, behaviour, and cortisol. The procedures involved in the health check have previously been shown to be acutely stressful and compromise welfare (Ruys et al, 2004; Heistermann et al, 2006; Bethell et al, 2012a). However, recent refinements in veterinary and scientific practice and techniques (NC3Rs, 2020) may have sufficiently improved the veterinary and husbandry practices involved in the annual health check so that they no longer significantly compromise macaque welfare. As the expected shift in AB, behaviour and cortisol was not seen, I recommend that future AB studies should involve macaques involved in neurological or toxicology studies. The severity of these procedures should be included as a factor within the analysis as more severe procedures may be associated with larger shifts in AB than mild or moderate procedures.

Social stressor

A social stressor may be required for large shifts in AB to be seen. Social behaviour was unchanged following the veterinary stressor (health check). Although the stressor was associated with a greater duration of anxiety behaviour and inactive behaviour, the durations of prosocial approach behaviour and antagonistic behaviour were constant from baseline to post-stressor. Primate social behaviour is known to change in response to stress and, in this thesis, it was expected that the duration of prosocial approach behaviour would decrease, and the duration antagonistic behaviour would increase in line with previous primate stress response literature (Maestripieri & Wallen,

1997; Mallapur et al, 2005; Arnold et al, 2011; Camus et al, 2013). It may be that the type and intensity of the stressor were not appropriate for shifts in social behaviour and social attention.

Visual social recognition and social behaviour are controlled by the same areas of the brain (Adolphs, 2009). The amygdala and prefrontal cortex are responsible for social perception, communication, social emotion, and social behaviour (Adolphs, 2009). Social behaviour involves active detection and response to cues from conspecifics via multiple sensory systems (Chen & Hong, 2018). Depending on the type of the information, these signals are then processed by specific neuronal circuits and brain regions before the animal responds. For example, olfactory cues are processed by the olfactory bulb which signals to the posterolateral and posteromedial cortical amygdala. If these cues are interpreted as a threatening encounter, signals are sent to the posterodorsal and posteroventral medial amygdala, principal nucleus of the bed nucleus of the stria terminalis and anterolateral and dorsomedial subregions of the ventromedial hypothalamic nucleus, which then results in aggression as the behavioural outcome including orientation towards threat (Chen & Hong, 2018).

In humans, abnormal social behaviour is linked to extreme AB scores. AB to disgust and emotional reactivity have been linked to post-event processing of social stressors (Cek et al, 2016). Social withdrawal is associated with more extreme AB scores compared to those with normally developed social skills (Thai et al, 2016). Individuals with social anxiety disorder have significantly greater difficulty disengaging with threatening face stimuli and a more negative AB to ambiguous social scenarios than non-anxious controls (Pergamin-Hight et al, 2016).

This suggests that social behaviour and social attention to threat are linked and that a social stressor may be the most appropriate stressor for inducing shifts in AB. It may be that AB to social threat and social behaviour are sufficiently linked for one to provide a predictor of the other. Perhaps AB to social stimuli would be more extreme following social stress rather than veterinary intervention. Future studies should look more specifically at the interaction between social behaviour and social attention to unpick the potentially important relationship.

7.4 Take homes and future directions

AB is a promising tool for welfare assessment showing within individual repeatability. At present the methodology is not suitable for real-time welfare assessment as the apparatus is too large and inconvenient and the editing, coding and analysis take too long for data to be useful for care staff. AB research needs to move in the direction of developing an automated eye-tracker that will work in low light levels, with bars and fast-moving animals. This automated approach would remove the major limitation of the research in that a much larger sample size could be collected.

This study highlights that affective state, sex, age, time of the AB trial, 5-HTTLP genotype, *DRD4* haplotype and stimulus ID should be included in the analysis in future AB studies. Future studies should also include more severe stressors, or investigate the effectiveness of social stressors, for shifts in AB to be adequately detected at the early stages of development of this method. Once the underlying factors are established and methods are developed, the approach could be refined to be more sensitive for milder procedures.

Agreement between new measures (AB) and established methods of welfare assessment is essential to determine if the new method is appropriate. The ultimate aim of AB studies should be to triangulate the cognitive data with other factors, such as behavioural observations, physiological measures, and key genetic polymorphisms to properly validate this method for detecting shifts in affective state and, therefore, welfare. Triangulation proved difficult in this thesis due to a milder than expected stressor; however, with a more intense stressor of known severity rating this important next step can occur.

To reduce noise, studies should primarily use raw data response variables (THR, TL) with calculated measures (ABDiff) included if sample size is sufficient (110 macaques was sufficient for calculated variables to be used; Howarth et al, 2021). To further reduce noise, including a measure of fearful temperament in the analysis may be beneficial (Bethell et al, 2019). Rhesus macaques show individual differences in stress responsivity and temperament (Kalin et al, 1998; Gottlieb & Capitanio, 2013; Capitanio et al, 2017; Bethell et al, 2019) and the inclusion of temperament as a

factor would aid standardisation of AB methods and improve scientific outcomes. Personality was not included as a factor within this thesis as the response slowing methodology proposed by Bethell et al (2019) was developed after data collection for this thesis.

AB studies involving genetic analysis should include pedigree (relatedness) to avoid type 1 errors. Although further study is needed to determine the effectiveness of this method for detecting shifts in emotional state in captive NHPs, the PhD research highlights some important influencing variables and should act as a guide for future research.

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Appendices

Appendix 2a

Flash cards

During the pilot trials in May and July 2017 stimuli flash cards were trialled. During the pilot the threat and neutral stimuli both had eyes open face images as the final stimulus set with eye-closed neutral images was not yet complete. Stimulus pairs were matched for brightness and luminosity and printed using a Xerox Coloured Printer at the Liverpool John Moores University Library. Each image was A5 size and laminated using a Texet (LMA4-V) laminator and laminator pouches. The images were labelled on the reverse with the stimulus ID and aggressive or neutral. Velcro patches on the back of each image attached the stimuli to hand-held presentation boards. The boards consisted of B&Q grey oak effect laminate floor tile samples and white straight interior door pull cabinet handles (Figure 2.8). Responses were filmed with a Sony HD video camera. Although initially promising, as the macaques were engaged with the stimuli and responding during the pilot trials, the flash cards soon became dirty and discoloured. Previous studies had reprinted stimulus sets weekly to prevent fading and loss of colour over time (El-Molla et al, 2012; Thatcher, 2015); however, due to relatively large stimulus set and the preparation time and cost involved, it was decided re-printing was not an option. In addition, we realised that the laminated covering reflected the ceiling lights and resulted in a glare that blocked the stimuli from view of the macaque unless held exactly at a 90° angle. Changing the cards between threat-neutral and filler trials and between AB sessions with different individuals was time consuming and frequently macaques would walk

Figure 0.1. Fruit stimuli flash cards on hand-held boards for pilot attention bias trial with rhesus macaques. Photograph: E. Howarth.

away before the trial could be completed. As a result, we decided to design and build a more automated computer-based apparatus.

Training diary **02/08/17 – 11/08/17, University of Oxford.**

Date: 02/08/17, Time: AM

Training type: Urine collection

Trainers: Emmeline Howarth & Rhyanne Dale

Monkey ID	**Training record**
Vadoka	Stationing well in correct location and position. Tolerated pot, held in trainer's hand, touching the bars of the enclosure. Training session finished early as monkey stole juice syringe.
Victory	Stationing okay; however, need to move closer to bars. Tolerated pot, held in trainer's hand, touching and being placed through the bars of the enclosure. Trainer did t touch monkey with pot. Monkey touched pot with hands and mouth.
Ward	Not stationing well at the start of the training session. Monkey willing to come forward but t sitting with all four feet on perch. Good improvement within session seen after trainer started only rewarding monkey when all four feet on perch. Pot t introduced during this session.
Wayne	Stationing well. Monkey is very confident and immediately sat on perch for rewards. Tolerated pot, held in trainer's hand touching and being placed through the bars of the enclosure. Trainer did t touch monkey with pot. Monkey touched pot with hands and mouth.
Wombat	Not stationing well at the start of the training session. Monkey would approach for a reward but then move away. After 10-15 minutes approached and sat on the perch for rewards and mash. Pot t introduced during this session.
Wretch	Stationing well in correct location. Monkey is very confident. Initially wary of pot, held in trainer's hand touching the bars of the enclosure; however, by the end of the session, monkey was taking rewards from next to pot.

Date: 02/08/17, Time: PM
Training type: Urine collection

Trainers: Emmeline Howarth & Rhyanne Dale

Monkey ID	**Training record**
Umbongo	Stationing well in correct position with foot up on the bars but would t stay in position for very long. Monkey was distracted by neighbour and drank a lot when unsure what trainer wanted from him. Tolerated pot held next to foot for a short time.
Ward	Stationing well in correct location with trainer close to enclosure. Fruit given as a reward. Tolerated pot held in view but outside of enclosure.
Wayne	Stationing well in correct location and position. Tolerated pot through bars and within a few centimetres of feet. Training session finished early as monkey stole urine collection pot.

Wombat	Not stationing well at the start of the session and took a long time to move to perch. Once on perch worked well with good position. Tried to work even when cage mate's (Wretch) turn.
Wretch	Stationing well in correct position and location. Tolerated pot through bars for a second before moving away.

Date: 03/08/17, Time: AM

Training type: Urine collection

Trainers: Emmeline Howarth & Rhyanne Dale

Monkey ID	**Training record**
Pat	Stationing very well in correct location. Tolerated hosepipe through bars and touching stomach and feet. Monkey tried to bite hosepipe but stopped after told "" by trainer.
Vadoka	Stationing well in correct position but need to ensure trainer does t over treat for moving back and then forwards again on the perch. Monkey initially confident with pot touching bar but became very nervous when pot moved through bars.
Victory	Stationing very well in correct position and location, closer to bars. Getting correct location required trainer to be very patient. Tolerate pot through bars.
Ward	Stationing very well in correct position and location. Tolerated pot held just through bars. Trainer stepping away from monkey if he moved from perch or tried to grab pot.
Wayne	Stationing very well in correct position and location. Remaining stationed for good length of time. Monkey is habituated to the pot; he tolerated it being held in front of genitals for several seconds. Trainer stepping away from monkey if he moved position or tried to grab pot
Wombat	Stationing well. Monkey approaches perch much faster and, occasionally, without prompting. Tolerated pot through bars.
Wretch	Stationing ok. Monkey needs encouragement to stay on perch. Tolerated pot in view outside of enclosure. Moved away if pot moved closer to enclosure.

Date: 03/08/17, Time: PM
Training type: Urine collection

Trainers: Emmeline Howarth & Rhyanne Dale

Monkey ID	**Training record**
Umbongo	Stationing ok with leg on bar. Trained with RD as hesitant with EH. Monkey made very little / no progress from day before.
Ward	Stationing ok in correct position but taking a long time to approach perch. Training session finished early as monkey appeared stressed. Pot held in trainer's hand outside of enclosure.
Wayne	Stationing very well in correct location. Monkey needs to twist body slightly to be in correct position. Allowed pot through bars and to touch foot.
Wombat	Stationing well in correct position and location but t for very long. Tolerated pot being held in front of genitals while receiving rewards.

Wretch	Stationing ok but seemed wary to approach perch. Tolerated pot held in front of bars while receiving fruit.

Date: 04/08/17, Time: AM

Training type: Urine collection

Trainers: Emmeline Howarth & Rhyanne Dale

Monkey ID	**Training record**
Vadoka	Stationing well in correct position for around 10 seconds. Large improvement from previous session. Tolerated pot through bars. Training session finished early as monkey stole pot.
Victory	Stationing well but still sitting slightly too far back on perch. Allowed pot to touch foot.
Ward	Not stationing well. Monkey repeatedly attempted to run away with reward. Need to work on stationing for longer periods. Pot may have touched foot.
Wayne	Stationing very well. Allowed pot to touch genitals in correct position. Try juice before PM session on Monday. Urinated twice after training session had finished.
Wombat	Stationing very well. Urinated on perch. Allowed pot to touch foot very briefly.
Wretch	Stationing well. Large improvement from previous session. Monkey still wary of pot; however, allowed pot through bars if t too close to him. Needs constantly rewarded to keep interest. Urinated on perch.

Date: 04/08/17, Time: PM
Training type: Juice & syringe habituation

Trainers: Emmeline Howarth & Rhyanne Dale

Monkey ID	**Training record**
Ward	Seemed very wary of syringe. Took banana by hand from near syringe 3 times but would not take any food with mouth.
Wayne	Taking food with mouth next to syringe. Allowing juice to be squirted into mouth. Tries to push syringe away.
Wombat	Seemed very wary of syringe. Took treat from near the end of the syringe using hand a couple of times.
Wretch	Took treat with mouth next to end of syringe. Seems to like juice.

Training type: Urine collection

Pat	Allowed hosepipe to touch rump, legs and stomach. Seems to really like juice. Try getting monkey to stand over hosepipe.

Date: 07/08/17, Time: AM
Training type: Juice & syringe habituation

Trainers: Emmeline Howarth & Stuart Mason

Ward	Still very wary of syringe. Took food with hand from next to end of syringe. Work on taking treat with mouth. Reluctant to move across form home cage to crush back area.
Wayne	Took food with mouth from next to end of syringe. Drank some juice from end without having syringe in mouth. Work on having syringe in mouth. Reluctant to move across from home cage to crush back area
Wombat	Seemed quite wary of syringe. Took treat next to syringe with hand. Took with mouth couple of times. Finished session there. Next time work on taking with mouth more consistently.
Wretch	Not stationing well when alone in crush back area. Once in the home cage, took treat next to syringe with mouth. Seems to like juice a lot but still wary of syringe.

Date: 07/08/17, Time: PM

Training type: Urine collection

Trainers: Emmeline Howarth & Rhyanne Dale

Pat	Stationing well in correct location. Allowed hosepipe to touch rump. Working on her turning monkey turning with backend towards trainer using "round" and hosepipe as cue. Need to work on position and standing.
Umbongo	Stationing very well. Allowed pot to touch genitals for a few seconds before getting distracted by other monkey (Suggs) in next cage. Very eager to participate. Need to work on reinforcing urinating.
Ward	Not stationing well at start of session. Took time to approach perch. Managed to build up again to stationing in correct position and location with pot held in good position. Allowed pot to touch foot. Urinated on perch.
Wayne	Stationing very well. Allows pot to be held in perfect position next to genitals. Urinated on perch in good position but while pot was away. Need to capture urination at correct time.
Wombat	Stationing very well. Urinated on perch as soon as pot was put through bars. Training session finished early as monkey performed correct behaviour and was given lots of rewards. Need to work on monkey allowing pot to stay near genitals for longer periods.
Wretch	Stationing well. Still seems wary of pot. Tolerated pot all the way through bars with him on perch. Urinated on perch.

Date: 08/08/17, Time: AM

Training type: Juice & syringe habituation

Trainers: Emmeline Howarth & Stuart Mason

Ward	Had small amount (~20ml) of fluid. Syringe wasn't very good – only dribbled then lots at once. Cut end of that monkey had chewed and worked much better.
Wayne	Stationing well. Slightly distracted by trainer working with cage mate (Ward). Allowed syringe into mouth before treat was given. Still trying to push syringe away but t as often.

Wombat	Not stationing well. Preferred top perch again. Finally came down to lower perch. Only took treats with hand. Would not train in crush back area when monkeys swapped.
Wretch	Seemed very nervous and paced in crush back area. Would not station for SM so worked with EH. Trained much better in the home cage. Took treats with hand next to end of syringe.

Date: 08/08/17, Time: PM

Training type: Urine collection

Trainers: Emmeline Howarth & Rhyanne Dale

Pat	Stationing well in correct location. Understands "round" command and showing hosepipe. Positioning backend in air and allowing tube to touch rump for ~5 seconds. May be unnatural position for urinating. Need to build association between natural urination and the hosepipe touching anywhere on body.
Ward	Not stationing reliably. Not tolerating and would move way when pot through bars. Monkey approached perch for rewards but would t station.
Wayne	Stationing very well in correct position and location and for a good length of time. Urinated on perch and managed to collect very small amount of urine in pot. Need to work on urinating only on perch in pot and t elsewhere in cage.
Wombat	Stationing well in correct position and location for good length of time. Trainer waiting 5-10 seconds between rewards. Pot held in correct position next to genitals. Appeared to be attempting to urinate when working with trainer but would move away and urinate on wrong perch. Only reward when urinates on correct perch.
Wretch	Stationing very well. Large improvement from previous session. Seemed a bit timid of pot at first but got a lot better and managed to get pot held in a good position by the end of the session. Urinated on perch. Trainer tried to collect in pot but missed. Stayed on perch during and after urination.

Date: 09/08/17, Time: AM

Training type: Juice & syringe habituation

Trainers: Emmeline Howarth & Stuart Mason

Ward	Took treat with mouth with syringe ~3 rungs down while standing on perch. Continue getting to take treat with mouth and slowly move syringe closer.
Wayne	Took treat with mouth next to end of syringe. Squirted some juice into mouth with syringe in mouth. Need to work on increasing the volume of juice squirted before reward given.
Wombat	Took treat with mouth next to end of syringe. Prefers to work on top level. Work on getting more juice into mouth.
Wretch	Drank from syringe. Does not drink every time but consistently taking treat with moth from next to end of syringe. Likes to work on top level. Work on increasing volume of juice and reducing number of rewards.

Date: 09/08/17, Time: PM

Training type: Urine collection

Trainers: Emmeline Howarth & Rhyanne Dale

Pat	Stationing very well in correct location. Understand "round" command and hosepipe cue. Will stand with backend in air for~10 seconds. Unable to capture urination. Monkey tries to grab tube sometimes but stops when told "" by trainer.
Ward	Stationing ok but was t making progress from previous sessions. Took step back in training and built from stationing without pot for ~20 seconds to pot being through bars and around 5 cm from monkey's genitals. Monkey given whole nuts a distraction but moved away once finished and realised pot was close.
Wayne	Stationing very well in correct position and location, for extended time and with long gap between rewards. Urinated elsewhere in enclosure. Only reward when urinates on correct perch.
Wombat	Stationing very well in correct position and location with pot in place for long periods. Managed to get monkey sitting in right position for 15 minutes (sometimes moved away for 10-30 seconds) until urinated in the pot. Lots of treats given and training session finished.
Wretch	Stationing well; however, need to work on position and confidence with pot. Urinated on perch at the start of the session. Moved to top perch and continued to urinate. Allowed pot to touch foot but would still prefer to sit slightly out of reach of pot; however, by end of session was sitting very well with pot close to genitals. Only reward when urinates on correct perch.

Date: 10/08/17, Time: AM

Training type: Juice & syringe habituation

Trainers: Emmeline Howarth & Stuart Mason

Notes: Wet clean

Ward	Stationing well and large improvement from previous session. Took treats from next to syringe using mouth and juice dribbled into mouth. Licked end of syringe too. Work on putting syringe into mouth. Seems to only like raisins as reward (not nuts).
Wayne	Not stationing well. Seemed very distracted at start of session. Took treats and small amount of juice with mouth. Continues to push syringe away. Not progress from yesterday but lots of distractions (wet clean).
Wombat	Not stationing well. Seemed very distracted at start of session. Would only work in home cage. Took treats with mouth next to syringe and small amount of juice squirted into mouth. Work on putting syringe into mouth.
Wretch	Not stationing well. Seemed very distracted at start of session. Drank juice from syringe with treats held visible but away from cage and out of reach. Work on no treats visible.

Date: 10/08/17, Time: PM

Training type: Urine collection

Trainers: Emmeline Howarth & Rhyanne Dale

Pat	Stationing for ~20 seconds with backend in air and hosepipe in position. Given juice. Need to capture urination. Waited with monkey for 15 mins but did not urinate.
Ward	Stationing ok. Took a little while to come approach perch but managed to get sitting in right location and position with pot in good position but only for short periods. (Very annoying during Wards training, grabbed at over shoes, coat etc.)
Wayne	Not stationing well at start of session and did t seem to want to train to begin with. Left alone for 10 minutes and swapped trainer. Started stationing ok in good position but t for very long. Urinated on perch into pot.
Wombat	Stationing perfectly. Urinated twice but only on top perch. Only reward when urinates in correct location.
Wretch	Not stationing well at start of session. Sitting at other end of perch with body twisted away from pot and trainer. Started stationing in correct position. Urinated on perch. Managed to collect urine in pot. Did not move away from pot but tried to twist body away from pot.

Date: 11/08/17, Time: AM

Training type: Juice & syringe habituation

Trainers: Emmeline Howarth & Stuart Mason

Notes: cage maintenance workmen in until 5 minutes before training session

Ward	Stationing well. Taking rewards with mouth next to syringe and small amount of juice with reward straight after. Work on putting syringe into mouth and monkey drinking before rewarding.
Wayne	Would not move down from top level. Took ~40 ml of juice over 3-4 drinks with date held away from enclosure. Got quite aggressive at points. Managed to get syringe a few centimetres into mouth.

Training type: Urine collection

Trainers: Emmeline Howarth & Rhyanne Dale

Pat	Stationing very well in correct location and in good position. Will hold position for ~30 seconds. Given lots of juice as reward. Urinated and hosepipe placed on rump.
Victory	Stationing well in correct position and location. Allowed pot to rest on foot. Need to capture urination.

Appendix 2b

Table 2b.1. Life history data of rhesus macaques involved in attention bias trials for this study.

Group (group size adults)	Sex	Animal ID	Mother DOB	Mother age at birth (months)	Mother number previous offspring	Maternal rank	Retained in natal group	Group composition	Matriline	Mother ID	Father ID	DOB	Rank	Wean early	Date wean	Age wean (months)	Reproductive status	Youngest offspring DOB	Has dependent offspring	Total number offspring	Male present	Weight	Alopecia score
Abbott (3)	M	ABBOTT	UNKNOWN	UNK	UNK	UNK	No	Mixed Sex	39MPorton	39M	20_GRP	19/04/2002	High	No	19/08/2003	16	Male Breeding	NA	No	25	Yes	14.07	5
	F	ORINOCO	15/06/1996	108	2	MID	No	Mixed Sex	Perky StAndrews	DIP	BART	13/07/2005	High	No	12/10/2007	26	Implanted	19/08/2017	No	5	Yes	8.73	4
Linz (7)	F	LINZ	UNKNOWN	UNK	UNK	UNK	No	Mixed Sex	LinzPorton	67T	18 GRP	01/04/2002	High	No	09/09/2003	17	Implanted	17/09/2015	No	6	No	8.92	5
		MAJ	UNKNOWN	UNK	UNK	UNK	No	Mixed Sex	X33Porton	X33	20_GRP	15/06/2002	Mid	Yes	20/03/2003	9	Implanted	20/09/2015	No	7	No	10.94	5

		VENUS	01/04/2002	112	1	MID	Yes	Same Sex	LinzPorton	LINZ	LUCAS	06/08/2011	High	No	Remained Natal Group	60	Cycling	03/04/2017	No	2	No	6.22	5
		VERITY	15/06/2002	109	2	HIGH	Yes	Same Sex	X33Porton	MAJ	LUCAS	13/08/2011	Mid	No	Remained Natal Group	60	Cycling	12/05/2017	No	3	No	6.97	5
		WINE	01/04/2002	124	2	MID	Yes	Same Sex	LinzPorton	LINZ	LUCAS	23/08/2012	High	No	23/08/2013	12	Cycling	NA	No	0	No	6.41	5
Nodon	M			UNK	UNK	UNK	No	Mixed Sex					High	Yes		10			No	16	Yes	12.6	4
(7)		NODON	UNKNOWN						D25Porton	D25	G24	15/05/2004			12/04/2005		Male Breeding	NA					
	F			177	5	LOW	No	Mixed Sex					Mid	No		21			No	6	Yes	12.38	5
		RAZZ	01/09/1992						Alice Cambridge	ALICE	BLUES	22/06/2007			26/03/2009		Pregnant	25/06/2016					
				176	5	MID	No	Mixed Sex					High	No		20			Yes	6	Yes	8.97	5
		RENE	03/11/1992						258Park Farm	ASTRID	BLUES	22/07/2007			26/03/2009		Nurse	09/07/2018					
				191	6	MID	No	Mixed Sex					High	Yes		5			No	4	Yes	8.77	5
		SHALLOT	03/11/1992						258Park Farm	ASTRID	BLUES	14/10/2008			26/03/2009		Pregnant	21/07/2016					

		YOANA	22/07/2007	76	2	HIGH	Yes	Mixed Sex	258Park Farm	RENE	PLUM	26/11/2013	High	No	Remained Natal Group	60	Pregnant	NA	No	1	Yes	9.29	5
Plum (7)	M	PLUM	06/06/1994	145	3	MID	No	Mixed Sex	258Park Farm	BIANCA	JOE	08/07/2006	High	No	26/03/2009	32	Male Breeding	NA	No	20	Yes	14.43	5
	F	RACH	15/02/2000	86	1	LOW	Yes	Mixed Sex	149Park Farm	JANE	JOSEPH	03/05/2007	High	No	26/03/2009	22	Nurse	12/08/2018	Yes	4	Yes	10.49	5
		ROZANNE	11/03/2003	49	0	HIGH	Yes	Mixed Sex	Irene Cambridge	MADDY	JOSEPH	23/04/2007	Low	No	26/03/2009	23	Pregnant	06/07/2017	Yes	5	Yes	7.23	5
Sequel (9)	M	SEQUEL	04/11/1992	190	4	MID	No	Mixed Sex	F265Park Farm	ARIF	BLUES	02/10/2008	High	Yes	24/08/2009	10	Male Breeding	NA	No	18	Yes	11	5
	F	ORLANDA	21/03/1990	188	8	UNK	Yes	Mixed Sex	Eileen Cambridge	156	DOYLE	08/12/2005	Mid	No	17/10/2008	34	Implanted	22/09/2017	Yes	7	Yes	7.66	5
		PANSY	17/04/1992	164	7	HIGH	Yes	Mixed Sex	45Park Farm	NOVA	DOYLE	06/01/2006	Mid	No	17/10/2008	33	Pregnant	21/07/2017	No	8	Yes	9.66	5

		TULIP	06/01/2006	41	0	HIGH	Yes	Mixed Sex	45Park Farm	PANSY	BARNEY	04/07/2009	Low	No	04/07/2010	12	Pregnant	18/07/2017	Yes	3	Yes	8.94	5
		YEVA	08/12/2005	94	3	LOW	Yes	Mixed Sex	Eileen Cambridge	ORLANDA	BARNEY	26/10/2013	Mid	No	Remained Natal Group	60	Pregnant	30/06/2017	Yes	1	Yes	6.76	5
		YIBBI	06/01/2006	92	4	HIGH	Yes	Mixed Sex	45Park Farm	PANSY	BARNEY	11/09/2013	High	No	Remained Natal Group	60	Cycling	16/06/2017	No	1	Yes	8.26	5
Sol (8)	M	SOL	UNKNOWN	UNK	UNK	UNK	No	Mixed Sex	B8Porton	B8	Z18(1)	06/03/2002	High	No	18/08/2003	17	Male Breeding	NA	No	22	Yes	15	5
	F	TALLULAH	22/07/2001	94	2	HIGH	Yes	Mixed Sex	Yvette Cambridge	KELLY	DEAN	05/06/2009	High	No	05/06/2010	12	Nurse	05/07/2018	Yes	2	Yes	8.2	5
		TILLY	27/08/2001	94	2	LOW	Yes	Mixed Sex	7EPorton	KAY	DEAN	07/07/2009	Low	No	07/07/2010	12	Pregnant	08/06/2017	Yes	2	Yes	7.75	5
		V	22/07/2001	122	4	HIGH	Yes	Mixed Sex	Yvette Cambridge	KELLY	DEAN	12/10/2011	Low	No	12/10/2012	12	Cycling	18/06/2017	No	1	Yes	7.04	5

		ZENA	22/07/2001	156	6	HIGH	Yes	Mixed Sex		Yvette Cambridge	KELLY	SIMO	19/08/2014	Mid	No	Remained Natal Group	60	Pregnant	NA	No	0	Yes	6.95	5
		ZUMBA	02/09/2001	156	5	MID	Yes	Mixed Sex		Eileen Cambridge	KAS	SIMO	21/09/2014	Low	No	Remained Natal Group	60	Pregnant	NA	No	0	Yes	6.7	5
Star (4)	M	STAR	20/01/1992	198	9	HIGH	No	Mixed Sex		258ParkFarm	CHARLOTTE	DOYLE	26/07/2008	High	No	24/08/2009	12	Male Breeding	NA	No	17	Yes	16.48	5
	F	VALENTINE	27/03/2003	94	3	LOW	Yes	Mixed Sex		T63Porton	MAUREEN	ALI	14/02/2011	High	No	14/02/2012	12	Pregnant	26/06/2016	No	3	Yes	7	5
		ZARITA	19/03/2009	67	1	LOW	Yes	Mixed Sex		T63Porton	TOOTS	THORN	22/10/2014	Low	No	Remained Natal Group	60	Cycling	NA	No	0	Yes	4.09	4.5
		ZSA-ZSA	14/02/2011	45	0	MID	Yes	Mixed Sex		T63Porton	VALENTINE	THORN	21/11/2014	High	No	Remained Natal Group	60	Pregnant	NA	No	0	Yes	7.28	3
Thorn (7)	M	THORN	19/09/2001	94	3	HIGH	No	Mixed Sex		7ParkFarm	KRACKER	DEAN	09/08/2009	High	No	18/08/2011	24	Male Breeding	NA	No	19	Yes	15.02	4

	F	SENGA	12/06/1996	141	2	HIGH	Yes	Mixed Sex	Irene Cambridge	DIME	BART	08/04/2008	High	Yes	26/03/2009	11	Pregnant	02/09/2017	Yes	2	Yes	9.92	4.5
		SIENNA	08/05/1996	143	4	MID	Yes	Mixed Sex	3FPark Farm	DOREEN	BART	24/04/2008	Mid	Yes	26/03/2009	11	Pregnant	05/08/2017	Yes	3	Yes	8.31	5
		UNO	15/06/1996	169	5	MID	Yes	Mixed Sex	Perky StAndrews	DIP	BART	21/07/2010	Low	No	21/07/2011	12	Pregnant	11/09/2017	Yes	2	Yes	6.44	4.5
		VENICE	12/06/1996	186	3	HIGH	Yes	Mixed Sex	Irene Cambridge	DIME	BART	13/12/2011	High	No	Remained Natal Group	60	Pregnant	26/08/2017	Yes	2	Yes	9.8	3
Utah (8)	M	UTAH	UNKNOWN	UNK	UNK	UNK	No	Mixed Sex	3F1Porton	C83	23_GRP	18/05/2010	Mid	No	16/06/2011	12	Male Breeding	NA	No	10	Yes	6.8	5
	F	RUPEE	14/03/2002	59	1	HIGH	No	Mixed Sex	258Park Farm	LEAH	JUDD	15/02/2007	Low	No	20/11/2008	21	Nurse	18/06/2018	Yes	5	Yes	7.05	3.5
		SAPHY	19/04/2003	58	1	MID	No	Mixed Sex	Phylis Cambridge	MEESHA	JUDD	08/03/2008	High	No	19/01/2010	22	Pregnant	11/06/2017	Yes	5	Yes	10.06	5

		SPICE	05/04/2002	74	2	HIGH	No	Mixed Sex	F265Park Farm	LYDIA	JUDD	16/06/2008	High	No	19/01/2010	19	Pregnant	29/05/2017	No	3	Yes	8.64	5
		SUGAR	15/05/2003	59	1	LOW	No	Mixed Sex	AnnPark Farm	MELODY	JUDD	09/05/2008	Mid	No	08/07/2010	25	Nurse	25/05/2018	Yes	4	Yes	7.29	2
		TEA	15/05/2003	71	2	LOW	No	Mixed Sex	AnnPark Farm	MELODY	JUDD	19/04/2009	Mid	No	08/07/2010	14	Nurse	07/06/2018	Yes	4	Yes	7.67	5
		YAZZOO	08/03/2008	64	1	HIGH	Yes	Mixed Sex	Phylis Cambridge	SAPHY	RUGER	25/07/2013	High	No	Remained Natal Group	60	Nurse	04/06/2018	Yes	2	Yes	8.1	5
		YLANG-YLANG	15/02/2007	76	0	LOW	Yes	Mixed Sex	258Park Farm	RUPEE	RUGER	18/06/2013	Low	No	Remained Natal Group	60	Cycling	14/05/2017	No	1	Yes	6.93	4.5
Viktor (8)	M	VIKTOR	18/02/2003	100	1	LOW	No	Mixed Sex	B57Porton	MINDY	LUCAS	21/06/2011	High	No	14/01/2014	30	Male Breeding	NA	No	9	Yes	10.13	5
	F	SERENA	17/03/2002	72	2	MID	No	Mixed Sex	152Park Farm	LALA	JUDD	19/03/2008	High	No	08/07/2010	27	Nurse	07/06/2018	Yes	6	Yes	9.38	5

		THYME	17/03/2002	86	3	MID	No	Mixed Sex		152Park Farm	LALA	JUDD	19/05/2009	High	No	08/07/2010	13	Pregnant	31/07/2017	Yes	3	Yes	8.78	5
		YOYO	19/03/2008	61	1	HIGH	Yes	Mixed Sex		152Park Farm	SERENA	RUGER	09/05/2013	High	No	Remained Natal	60	Pregnant	21/06/2017	Yes	1	Yes	6.99	5
		ZELDA	19/05/2009	62	1	HIGH	Yes	Mixed Sex		152Park Farm	THYME	DAN	15/08/2014	Mid	No	Remained Natal Group	60	Nurse	07/07/2018	Yes	1	Yes	5.69	5
Vincent (6)	M	VINCENT	27/08/2002	106	0	MID	No	Mixed Sex		X69Porton	LARCH	SOL	22/07/2011	High	No	07/08/2012	12	Male Breeding	NA	No	5	Yes	10.25	5
	F	TANYA	12/09/1999	114	3	HIGH	Yes	Mixed Sex		A76Park Farm	HELGA	DIESEL	23/03/2009	Mid	No	Remained Natal Group	60	Pregnant	22/05/2017	No	4	Yes	7.57	5
		VERSA	22/06/2007	47	0	HIGH	No	Mixed Sex		Alice Cambridge	RAZZ	PLUM	06/06/2011	High	Yes	08/06/2011	0	Pregnant	02/06/2017	Yes	2	Yes	7.61	5
William (6)	M	WILL.I.AM	03/05/2003	107	4	HIGH	No	Mixed Sex		M20Porton	LAKE	ALI	02/05/2012	High	No	10/04/2014	23	Male Breeding	NA	No	4	Yes	10.89	4

	F	OCELOT	26/06/1999	73	0	HIGH	Yes	Mixed Sex	F265Park Farm	HELEN	DAN	20/08/2005	High	No	22/08/2007	24	Pregnant	11/08/2017	Yes	5	Yes	8.56	3
		SIZZLE	25/03/1999	111	2	HIGH	Yes	Mixed Sex	F265ParkFarm	HONEY	DAN	11/07/2008	Low	Yes	26/03/2009	8	Nurse	22/06/2018	Yes	3	Yes	7.13	4.5
		TES	25/03/1999	124	3	HIGH	Yes	Mixed Sex	F265ParkFarm	HONEY	DIESEL	20/08/2009	Mid	No	20/08/2010	12	Nurse	23/09/2016	No	4	Yes	10.68	5
Zorro (8)	M	ZACHARIAH	21/07/2010	46	0	LOW	No	Same Sex	Monika Cambridge	Uno	JEREMY	05/06/2014	Mid	No	04/07/2016	24	Weaner Group	NA	No	0	Yes	7.82	4
		ZAVIER	17/06/2009	62	1	LOW	No	Same Sex	258Park Farm	TEAL	DAN	08/09/2014	High	No	04/07/2016	21	Weaner Group	NA	No	0	Yes	6.98	4
		ZEBEDEE	12/06/1996	218	4	HIGH	No	Same Sex	Irene Cambridge	DIME	JEREMY	16/08/2014	Mid	No	04/07/2016	22	Weaner Group	NA	No	0	Yes	6.02	2
		ZOIDBERG	20/05/2007	85	2	LOW	No	Same Sex	Yvette Cambridge	RIANNE	ABBOTT	12/07/2014	Low	No	04/07/2016	23	Weaner Group	NA	No	0	Yes	8.47	5

ZULU	11/07/2008	70	0	LOW	No	Same Sex	F265Park Farm	SIZZLE	JUDD	26/05/2014	Low	No	04/07/2016	25	Weaner Group	NA	No	0	Yes	6.95	2.5

Appendix 2c

Example training diary for groups of rhesus macaques at MRC-CFM. Each monkey's station is shown in brackets.

Date: 07/09/17, Time: AM

Training type: Desensitisation to trainer. Did not station monkeys in this session.

G13 (1)	**Plum** (red tube) – sat on middle level right in front of trainer. Took food by hand from trainer. Did t bother or chase females. **Rach** (green shoehorn) – came forward on bottom level underneath Plum. Took food confidently by hand from trainer. **Rozanne** (blue tube) – approached with baby on top level and took food. **May** (white loop) – approached on middle level and took food. **Zola** (yellow cup) – did not come into cage room. **Yardley** (red cup) – did not come into cage room. **Reya** (green cup) – did not come into cage room. **Thorn** (green loop) – displaces females. Very food possessive. Will not approach for food. Have to leave nut on the bars of the enclosure. **Senga** (blue shoehorn) - approaching and will take food by hand from trainer. **Sienna** (blue tube) – approached on lower level and took food by hand from trainer.
G15 (1)	**Tia** (red cup) – sat in corner of cage room and did t approach. **U** (white loop) – approached on top level for food. Want to interact with trainer but very wary of other monkeys. **Venice** (blank) – approach for food on middle level. **Vixon** (green cup) – did not come into cage room. Appeared at doorway between cage room and free roaming area. **Zorilla** (orange cup) – did not come into cage room. **Sequel** (green loop) – very confident and food motivated. **Tulip** (rope) – did not appear in cage room. **Wench** (green cup) – did not appear in cage room
G16 (1)	**Yeva** (yellow hose) – approached for food from trainer's hand. **Yibbi** (red tube) – approached front of enclosure on middle level. Took food from trainer. **Ruby** (red cup) – did not come into cage room. **Orlanda** (purple loop) and **Pansy** (blue tube) are confident and may be good for repeatability study in July/August 2018. **Yoanna** (yellow hose) – very good. Will approach confidently.
G18 (1)	**Nodon** (green shoehorn) – approached but seems wary of new trainer. Does lots of grimacing. **Robyn** (red tube) – did not come into cage room

Rhumba (white loop) – did not come into cage room

Shallot (purple square), **Razz** (pink loop) and **Rene** (blank) would all be good for repeatability study in July/August 2018.

Varsalla (green loop) – approached front of enclosure for food on top level

Versa (red tube) – approached front of enclosure on bottom level for food.

Vincent (yellow loop) – chases females and very food possessive.

G55 (1)

Umber (white loop) – did not come into cage room

Spangle (blank) – did not approach bars of enclosure for food.

Tanya (orange disk) – confident. Will be good for repeatability study in July/August 2018.

Sol (green loop) – very confident and took food by hand from trainer. Seems very relaxed.

Tilly (purple cup) – did not come into cage room.

Vanquish (red cup) – did not come into cage room.

G57 (1)

Wanganui (green cup) – did not approach front of enclosure. Sat on bottom level in corner.

Tallulah (blue tube) and **V** (white loop) - confident. Will be good for repeatability study in July/August 2018.

Zena (blue cup) – confident to approach on top level. Ran away after getting food from trainer.

Zumba (yellow cup) – confident to approach. Took food from trainer.

Viktor (purple loop) – approaches well but wary of females, especially Serena.

Sonja (white loop) – did not come into cage room.

Tamara (orange cup) – approached on top shelf. Ran away once taken food from trainer.

G60 (1)

Yoyo (purple cup) – confident and approached near to Serena. Boards will be important to separate pair.

Zelda (blue cup) – very keen but wary of Serena and Yoyo. Prefers the bottom level.

Teal (red cup) – did not approach. Sat at the back on the top level.

Serena (green cup) and **Thyme** (yellow cup) very confident – will be good for repeatability study in July/August 2018

Date: 07/09/17, Time: PM

Training type: Desensitisation to visual barriers (boards) and trainer

Boards added and group generally okay.

G01 (1)

Sizzle (yellow loop) approached for food but mainly on top level of cage room. Wants to come forward but prefers to be on a different level to the other monkeys, except Aqua. Did not try to station but whole group will need stationed when station training starts. Sizzle is low ranking.

Shirley and **Tass** very wary and did t approach.

Ocelot and **Tes** (involved in previous AB studies) approached confidently and may be included in repeatability study in July/August 2018.

Boards added. Group already good at stationing as previously trained by Claire Witham. Need to work on moving them around so that different monkeys are in correct location for filming.

Utah (red tube) – very wary of new trainer would t approach.

Rupee (orange cup) – initially wary but quickly came forward and took food from trainer by hand. Stations very well but does not stay at station for long.

Saphy (yellow cup) – confident and stations well. Does not hold the cup just touches. Stays at the station for a good length of time.

G03 (1)

Spice (purple cup) – Unlikely to station with boards as very slow to approach. Desensitisation of boards will be important, as likes to sit next to Saphy so need boards to separate pair.

Sugar (red cup) – did not come close enough to bars to give food.

Tea (orange disk) – seems very nervous/wary of other monkeys. Sits next to station but does t hold.

Sugar (red cup) – did not come close enough to bars to give food.

Yazzoo (green cup) – keen to take part. Will station but needs prompted/reminded which one is hers.

Ylang-Ylang (blue cup) – very keen to take part. Stations well but will t hold. Seems very greedy and will follow trainer for food.

Boards not added immediately. Group good with boards and new trainer.

Umbrella (blue cup) – did not appear in cage room with or without boards added. Will need to station all other monkeys as seems to be very wary of them, especially the male, **Star** (yellow cup).

G04 (1)

Wine (purple cup) – stationed okay and took food until she stole her sister's baby and ran away.

Mindy (purple loop) – did not come into cage room.

Linz (green loop), **Maj** (white loop), **Verity** (red cup), **Venus** (yellow loop) all good and will be included in repeatability study in July/August 2018.

Boards not t added immediately. Whole group left cage room when boards added. Need to station others in-group – Maureen (yellow cup) and Toots (orange cup) are both leaving MRC-CFM in October 2017.

G06 (1)

Valentine (purple cup) – did t station very well. Would approach for food but would not stay sitting next to station.

Zarita (red cup) – Stations okay but needs to work on knowing own station. Will need to keep Apple (blue cup) out of the way.

Zsa-Zsa (green shoehorn) – very grabby and flightily. Need to work on her staying near station.

Date: 08/09/17, Time: AM

Training type: Desensitisation to trainer. Did not station monkeys in this session.

G13 (2)

Plum (red tube) – will need to be stationed as started to displace females.

Rach (green shoehorn) – came forward on top level. Took food.

	Rozanne (blue tube) – approached on bottom level. Took food.
	May (white loop) and **Reya** (green cup) did t approach. Sat in corner.
	Zola (yellow cup) – very wary of other monkeys. Want to engage but did not come forward.
	Yardley (red cup) – did not come into cage room.
	Senga (blue shoehorn), **Sienna** (blue tube) and **U** (white loop) approached for food.
	Vixon (green cup) – did t approach. Sat in corner of cage room.
G15 (2)	**Venice** (blank) and **Zorilla** (orange cup) – did not come into cage room.
	Tia (red cup) – very wary of other monkeys. Ran up, grabbed food and ran away.
	Thorn (green loop) – initially wary but approached front of enclosure for nut.
	One of the "A" age monkeys (tattoo AO) will need to be stationed.
	Sequel (green loop) – very confident and food motivated will need to be stationed to stop him displacing females.
	Tulip (rope) – approached on top level and took food.
	Wench (green cup) – did not approach the front of the enclosure.
G16 (2)	**Yeva** (yellow hose) – very nervous. Ran up, grabbed food, screamed and unsettled group.
	Yibbi (red tube) – very confident. Approached near Sequel and sat eating next to him.
	Ruby (red cup) – did not come into cage room.
	Orlanda (purple loop) and **Pansy** (blue tube) approached and sat on bottom level.
	Yoanna (yellow hose) – approached confidently and took food on top level.
	Nodon (green shoehorn) – will need to be stationed as displacing females.
G18 (2)	**Robyn** (red tube) – did not come into cage room
	Rhumba (white loop), **Shallot** (purple square), **Razz** (pink loop) and **Rene** (blank) will all need to be stationed. All approaching front of enclosure well.
	Varsalla (green loop) – did not come into cage room
G55 (2)	**Versa** (red tube), **Umber** (white loop), **Spangle** (blank) and **Tanya** (orange disk) all good and approached front of enclosure for food.
	Vincent (yellow loop) – will need to be stationed.
	Sol (green loop) – will need to be stationed as will displace females if they get food.
G57 (2)	**Tilly** (purple cup), **Zena** (blue cup), **Zumba** (yellow cup) and **Tallulah** (blue tube) all approached confidently for food.
	Vanquish (red cup) – did not come into cage room.
	Wanganui (green cup) and **V** (white loop) – approached a few times on bottom level.
	Viktor (purple loop) – did t approach. Sat in cage room watching females.
G60 (2)	**Sonja** (white loop), **Tamara** (orange cup) and **Teal** (red cup) – did not come into cage room.
	Yoyo (purple cup) – approached for food but very wary and took food away to eat.

Zelda (blue cup) – approached lots of times for food on bottom level.

– did t approach. Sat at the back on the top level.

Serena (green cup) and **Thyme** (yellow cup) will need to be stationed.

Date: 11/09/17, Time: AM

Training type: Station training

Yoanna (yellow hose), **Nodon** (green shoehorn) and **Rene** (blank) – stationed well. Rene grabbed at Yoanna's station sometimes. Need to work on separating them more. Responded well to juice but don stole syringe so had to stop session.

G18 (3)

Robyn (red tube) – did not come into cage room

Rhumba (white loop), **Shallot** (purple square), **Razz** (pink loop) – stationed well on bottom level.

Varsalla (green loop) – did not come into cage room

G55 (3) **Spangle** (blank) and **Tanya** (orange disk) got in the way of

Vincent (yellow loop) – will need to be stationed.

Sol (green loop), **Tilly** (purple cup), **Zena** (blue cup), **Zumba** (yellow cup) and **Tallulah** (blue tube), **Wanganui** (green cup) and **V** (white loop) – all approach near their station for food.

G57 (3)

Vanquish (red cup) – did not come into cage room.

Date: 11/09/17, Time: PM

Training type: Board desensitisation and station training

G01(2) Boards added and whole group confident to approach for food. **Sizzle** (yellow loop) approached well on top level.

Boards added. All seemed relaxed and confident to take food with boards in place. **Saphy** (yellow cup) – stationed near to crush back area. Confident to station on edge of crush back area.

Tea (orange disk) – stationing well (will not hold) on top level.

Utah (red tube) – approached a couple of time but still displaying fear/aggression towards trainer.

G03 (2)

Rupee (orange cup) – stationing well on bottom level. Will hold station when reminded.

Spice (purple cup) and **Sugar** (red cup) – both still a bit wary. Approached for food but t near stations. Make sure this does not continue for longer than next training session.

Yazzoo (green cup) – stationing really well on middle level near to Saphy.

Ylang-Ylang (blue cup) – need to work on her staying near her station. This might improve if she has to hold station before reward.

Group fine with boards in place.

G04 (2) **Wine** (purple cup), **Linz** (green loop), **Verity** (red cup) and **Star** (yellow cup) all stationed well. Star moved away to the top level after a while which disturbed the females.

Umbrella (blue cup), **Mindy** (purple loop), **Maj** (white loop) and **Venus** (yellow loop) – did not appear in cage room.

G06 (2) Group a lot better with boards this time.

Maureen (yellow cup) and Toots (orange cup) stationed really well on middle level. **Zsa-Zsa** (green shoehorn) – grabbed food from next to station and ran away.

Valentine (purple cup), **Zarita** (red cup) and Apple (blue cup) all good but did not stay near station for long and were displaced when Maureen moved to top level.

Date: 12/09/17, Time: AM

Training type: Board desensitisation and station training

Boards added but t stationed.

Plum (red tube) – approached on middle level for food.

Rach (green shoehorn) – approached on top level

G13 (3)

Rozanne (blue tube) and **May** (white loop) – approached on bottom level

Zola (yellow cup) and **Reya** (green cup) – in cage room but did t approach

Yardley (red cup) – did not come into cage room.

Boards added and group seem fine.

Sequel (green loop), **Yibbi** (red tube) and **Orlanda** (purple loop) – stationing well.

Tulip (rope) – in cage room but did t approach

G16 (3)

Wench (green cup), **Ruby** (red cup) and **Pansy** (blue tube) - did not come into cage room.

Yeva (yellow hose) – approached near to station. Rewarded when she sat down in same section as seems to be very nervous still.

Date: 14/09/17, Time: PM

Training type: "Toilet board" (TB) desensitisation in crush back

Rach (green shoehorn) and **May** (white loop) sat on TB when t displaced by Plum. **Rozanne** (blue tube) on top right above board. **Zola** (yellow cup) sat and explored on TB but retreated when trainer approached.

G13 (4)

Reya (green cup) and **Yardley** (red cup) did not come into cage room.

Plum (red tube) would go onto TB when female was there receiving food and would displace female, but would t stay on TB or take food while on TB.

Yibbi (red tube), **Yeva** (yellow hose) and **Orlanda** (purple loop) approached on TB. **Tulip** (rope) – in cage room but did t approach

G16 (4)

Wench (green cup), **Ruby** (red cup) and **Pansy** (blue tube) - did not come into cage room. **Sequel** (green loop) would t go onto TB and grabbed at females including Yeva's baby which unsettled group.

Senga (blue shoehorn), **Sienna** (blue tube) and **U** (white loop) approached for food on TB. U particularly keen but wary when other females and Thorn approached in cage room.

G15 (3)

Vixon (green cup), **Zorilla** (orange cup) and **Tia** (red cup) – did not come into cage room.

Venice (blank) – approached in cage room but not on TB.

Thorn (green loop) – did t approach front of enclosure while TB was in place.

G03 (3)

TB and half boards added. All good.

	Spice (purple cup)**, Saphy** (yellow cup), **Utah** (red tube), **Rupee** (orange cup), **Yazzoo** (green cup) and **Ylang-Ylang** (blue cup) all sat on TB. Rupee sat on TB but was displaced by others.
	Sugar (red cup) and **Tea** (orange disk) – did not sit on TB but approached near TB on middle level (Sugar) and top level (Tea).
G01(3)	**Sizzle** (yellow loop) approached well on top level above TB. Still very wary of others. Ate raisins off TB when trainer walked away. Ocelot, Arya, Aqua and Tass all walked onto TB and took food.
	Group fine with boards in place.
G04 (3)	**Wine** (purple cup) initially on top level to right of TB when **Star** (yellow cup) walked on TB. Wine came onto TB when Star left cage room. Took raisins from trainer.
	Linz (green loop), **Verity** (red cup) interested but did not go on TB.
	Umbrella (blue cup), **Mindy** (purple loop), **Maj** (white loop) and **Venus** (yellow loop) – did not appear in cage room.
	Yoanna (yellow hose) – sat on the top level and hung down over TB for food.
G18 (3)	**Nodon** (green shoehorn) – very unsettled and displacing any females wanting to approach TB.
	Robyn (red tube) – did not come into cage room
	Rhumba (white loop), **Shallot** (purple square), **Razz** (pink loop) and **Rene** (blank) did t approach near the TB. Group seem very wary of TB. Left TB in with group while working with G16 (opposite group) – juveniles all walking on TB.
G55 (4)	**Spangle** (blank) and **Tanya** (orange disk) approached on TB but left once given food. **Varsalla** (green loop) – sat on top level to right of TB. **Versa** (red tube) – sat on top level to left of TB.
	Vincent (yellow loop) – stationed well on top level at other end of CR to allow females to explore TB. Before stationing walked on TB and took food confidently.
	Sol (green loop) explored TB and took food. Stationed at other end of cage room away from TB to allow females to explore.
G57 (4)	**Tilly** (purple cup), **Zena** (blue cup), **Zumba** (yellow cup) approached on top and bottom levels but not on TB.
	Tallulah (blue tube), **Wanganui** (green cup) and **V** (white loop) – explored TB and took food while on TB.
	Vanquish (red cup) – did not come into cage room.
	TB put into enclosure and left while trainer gave food to opposite group (Lucas et al – not included in study).
	Viktor (purple loop) – very wary and would t go near TB. Females were much better.
G60 (3)	**Tamara** (orange cup) – sat on top level to right of TB.
	Yoyo (purple cup), **Serena** (green cup) and **Thyme** (yellow cup) – all sat on TB and took food by hand from trainer.
	Zelda (blue cup), **Teal** (red cup) and **Sonja** (white loop) – did not come into cage room.
G06 (3)	Maureen (yellow cup), Toots (orange cup) and Apple (blue cup) ate food off the TB when trainer walked away. **Valentine** (purple cup) and **Zarita** (red cup) sat on top level either side of TB.

Zsa-Zsa (green shoehorn) – approached and took food from trainer on TB but then moved away.

Date: 18/09/17, Time: AM

Training type: Visual barrier desensitisation

G01(4)

Sizzle (yellow loop) – given food when initially approached on top level. After this food only given when approached on middle level. Had to station / distract other females (**Shirley, Tass, Ocelot** and **Tes**) on other side of board but this allowed Sizzle to approach multiple time on middle level.

G03 (4)

Spice (purple cup), **Saphy** (yellow cup), **Sugar** (red cup), **Utah** (red tube), **Yazzoo** (green cup) and **Ylang-Ylang** (blue cup) **–** all approached on middle level either side of boards. **Rupee** (orange cup) also approached on middle level but then displaced to bottom level. **Tea** (orange disk) – only on top level. May only be able to collect AB trial and no samples from Tea.

G04 (4)

Star (yellow cup) sat on middle level and did not move much. **Wine** (purple cup) approached on other side of board to Star 3 times. Session finished early because Wine stole a baby again which disrupted the group. Group generally good with boards. Juveniles try to break them. Need to station Star next time.

G06 (4)

Valentine (purple cup) and **Zsa-Zsa** (green shoehorn) only approach on top level (AB only?). **Zarita** (red cup) sat on middle level and took food from trainer. Apple (blue cup) got in the way again. Need to station Apple next time.

Rach (green shoehorn) climbed up bars but t properly on middle level.

G13 (4)

Rozanne (blue tube) – on bottom level below **Plum** (red tube). Plum approach on middle level for nuts. Without nuts would only sit on top level.

Zola (yellow cup) approached on middle level but would not approach if Plum on middle level. Juveniles very confident and tried to break boards.

Senga (blue shoehorn), **Sienna** (blue tube), **Venice** (blank) and **U** (white loop) – approached on middle level for food.

G15 (4)

Vixon (green cup), and **Tia** (red cup) – did not come into cage room.

– approached in cage room but not on TB.

Thorn (green loop) and **Zorilla** (orange cup) – in cage room but did t approach

Sequel (green loop) sat well on middle level and took food by mouth.

G16 (5)

Yibbi (red tube), **Yeva** (yellow hose), **Tulip** (rope) and **Orlanda** (purple loop) all approached on top level. Yeva also went onto middle level but would not stay there for long. Yibbi needs to be stationed as follows food and gets in the way.

Wench (green cup), **Ruby** (red cup) and **Pansy** (blue tube) - did not come into cage room.

Yoanna (yellow hose) and **don** (green shoehorn) sitting perfectly either side of a board. Yoana prefers to be on the right and don on the left.

G18 (4)

Rhumba (white loop), **Shallot** (purple square), **Razz** (pink loop) and **Rene** (blank) – approached on top and bottom levels.

Robyn (red tube) – did not come into cage room

G55 (5)

Spangle (blank) and **Tanya** (orange disk) and **Umber** (white loop) did not come into cage room. **Varsalla** (green loop) and **Versa** (red tube) sat in doorway to the free roaming area and would not approach.

	Vincent (yellow loop) very unsettled (aggressive/nervous). Need to station Vincent to get females to approach. Vincent bent one of the boards.
	Tilly (purple cup) only approach on top level.
	Sol (green loop) – sat next to board well and took food.
G57 (5)	**Zena** (blue cup), **Zumba** (yellow cup) – good with board and would approach for food on middle level but t when Sol was in cage room.
	Tallulah (blue tube) and **V** (white loop) – approached on middle level. Will have to station Tallulah to keep her way from Tilly.
	Vanquish (red cup) and **Wanganui** (green cup) – did not come into cage room.
	Viktor (purple loop) – started well. Approached on middle level next to boards. Had to finish session early as tried to break boards.
	Tamara (orange cup) – approached on top level.
G60 (4)	**Yoyo** (purple cup) very confident and approached multiple times on middle level next to board
	Serena (green cup) and **Thyme** (yellow cup) – approach on middle level.
	Zelda (blue cup) – approached on bottom level
	Teal (red cup) and **Sonja** (white loop) – did not come into cage room. Remove from training as t coming into cage room.

Date: 19/09/17, Time: PM

Training type: Visual barrier and "toilet board" desensitisation

G01(5)	Only TB added. **Sizzle** (yellow loop) – approached TB and stretched over to get food. Aqua very confident and sat on board to get food. Sizzle is very wary of other females and would t station in training session with another trainer (Elise) earlier in the day.
G03 (5)	All okay with both boards. **Spice** (purple cup), **Saphy** (yellow cup), **Sugar** (red cup), **Utah** (red tube), **Yazzoo** (green cup) and **Ylang-Ylang** (blue cup) **–** all approached on TB with board in down the side.
	Rupee (orange cup) walked on TB but mainly approaching below TB.
	Tea (orange disk) – still only on top level.
G04 (5)	**Star** (yellow cup) sat on TB and took food. **Wine** (purple cup) approached on top level to right of TB.
G06 (5)	Only visual barrier added. **Valentine** (purple cup) and **Zsa-Zsa** (green shoehorn) stationing well on middle level with boards. **Zarita** (red cup) would t come to middle level. Stationing well on top level.
	Only TB added. **Plum** (red tube) stationed but does t kw what to do. Will take a lot more training to be able to use station with Plum. Attempted to desensitise Plum to pee pot – very wary and aggressive when pot near bars.
G13 (5)	**Rach** (green shoehorn) sat on TB over holes. Became a bit aggressive and tried to bite trainer's fingers when giving food.
	Rozanne (blue tube) stationed on top level to right of boards.
	Zola (yellow cup) – did not appear in cage room.
G15 (5)	Visual barriers only.

Senga (blue shoehorn), **Sienna** (blue tube), **Venice** (blank) – approached on middle level for food.

Vixon (green cup), and **Tia** (red cup) – did not come into cage room. Remove from study. **Thorn** (green loop), **U** (white loop) and **Zorilla** (orange cup) – in cage room but did t approach

Spangle (blank) and **Tanya** (orange disk) and **Umber** (white loop) did not come into cage room.

Varsalla (green loop) moving between middle level and top level. **Versa** (red tube) sat on TB.

G55 (6)

Vincent (yellow loop) stationed at other end of cage room away from TB. Stayed for around 30 seconds with lots of verbal praise and food rewards. Vincent did t like the pee pot being near to the bars. Held pot close to me and was fine. Moved it even a few cm closer and he ran off.

Sol (green loop) – stationed at other end to TB and started pee pot desensitisation. Allowed the pot to touch foot. Will station for very long period without wandering off.

Tilly (purple cup) stationing on top level. Good but very wary of Tallulah.

Zena (blue cup), **Zumba** (yellow cup) – would t come up to middle or top levels. Only stationing on bottom. Might come up if Tallulah t there.

G57 (6)

Tallulah (blue tube) and **V** (white loop) – stationing well on middle level but would wander off to see what other females were getting.

Vanquish (red cup) and **Wanganui** (green cup) – did not come into cage room. Remove from study.

Appendix 3a

```
##############################Full AB model##################################
#############################################################################
#Step 1. Clear workspace, set working directory and load packages
#############################################################################
#Clear workspace
ls() #this looks at what is loaded
rm(list=ls()) #this clears everything

#Set the working directory
#setwd('M:/Emily/Writing/1Papers in prep/2019 AB Heritability/AB Heritability')
#setwd("C:/Users/emmel/Desktop/R Studio and R")
setwd("D:/R Studio and R/")
#setwd("E:/R Studio and R/")

#Load Package
#install.packages("lme4")
#install.packages("tidyverse")
#install.packages("car")
#install.packages("CarData")
#install.packages("rcompanion")

library(tidyverse)
library(rcompanion)
library(lme4)
library(car)#or CarData in earlier forms of R
#library(carData)
#install.packages("MuMIn")
library(MuMIn)
#citation("MuMIn")

#load Roger Functions #source files need to be in the working directory
#source("C:/Users/emmel/Desktop/R Studio and R/Functions/diagnostic_fcns.r")
#source("C:/Users/emmel/Desktop/R Studio and R/Functions/glmm_stability.r")
source("D:/R Studio and R/Functions/diagnostic_fcns.r")
source("D:/R Studio and R/Functions/glmm_stability.r")
#source("M:/Emily/Doing/R Users Group/R Training/Mundry/Functions/Functions/diagnostic_fcns.r")
#source("M:/Emily/Doing/R Users Group/R Training/Mundry/Functions/Functions/glmm_stability.r")

#############################################################################
#Step 2. Import and check data:
#############################################################################
#Load data
AB1KempThatcherHowarth_20200707<-read.csv(file.choose(), header=T) #select file from pop up window
d <- AB1KempThatcherHowarth_20200707

nrow(d) #1188 (if higher do the following go back to CSV and delete ghost cells from bottom)
ncol(d) #246

str(d)
View(d)

e.data <- subset(d, StudyNo_EH_1HC_2Rep == "1")
nrow(e.data)#332

#############################################################################
#Step 3. Ensure variables accurately labelled as factors and correct levels of each factor are being read.
#############################################################################
MData<-e.data

#factors
MData$animalID <- as.factor(MData$animalID)
str(MData$animalID) #Factor w/ 110 levels
MData$Sex <- as.factor(MData$Sex)
```

```
str(MData$Sex) #Factor w/ 2 levels
MData$WeanEarlyR <- as.factor(MData$WeanEarlyR) #save for another analysis
str(MData$WeanEarlyR) #Factor w/ 2 levels
MData$ReproStat <- as.factor(MData$ReproStatR)
str(MData$ReproStatR) #Factor w/ 6 levels
MData$MalePres <- as.factor(MData$MalePres)
str(MData$MalePres) #Factor w/ 2 levels
MData$Treatment <- as.factor(MData$Treatment)
str(MData$Treatment) #Factor w/ 2 level
MData$AggLoc <- as.factor(MData$AggLoc)
str(MData$AggLoc) #Factor w/ 2 levels
MData$StimulusID<- as.factor(MData$StimulusID)
str(MData$StimulusID) #Factor w/ 7 levels
MData$HasDependentOffspring<-as.factor(MData$HasDependentOffspring)
str(MData$HasDependentOffspring) #Factor w/ 2 levels
MData$DisruptionInGrpOtherYN<-as.factor(MData$DisruptionInGrpOtherYN)
str(MData$DisruptionInGrpOtherYN) #Factor w/ 2 levels
MData$AnyOtherTreatment<-as.factor(MData$AnyOtherTreatment)
str(MData$AnyOtherTreatment) #Factor w/ 2 levels
MData$DrugKHClLast24HoursYN<-as.factor(MData$DrugKHClLast24HoursYN)
str(MData$DrugKHClLast24HoursYN) #Factor w/ 2 levels
MData$InjuryLast48HrsYN<-as.factor(MData$InjuryLast48HrsYN)
str(MData$InjuryLast48HrsYN) #Factor w/ 2 levels
MData$CleaningLast24HrsYN<-as.factor(MData$CleaningLast24HrsYN)
str(MData$CleaningLast24HrsYN) #Factor w/ 2 levels
MData$GrpChangeLast7DaysYN<-as.factor(MData$GrpChangeLast7DaysYN)
str(MData$GrpChangeLast7DaysYN) #Factor w/ 2 levels
MData$BabyBornLast24HrsGrp<-as.factor(MData$BabyBornLast24HrsGrp)
str(MData$BabyBornLast24HrsGrp) #Factor w/ 2 levels

#numeric
MData$AlopeciaScoreHC<-as.numeric(MData$AlopeciaScoreHC)
str(MData$AlopeciaScoreHC) #num
MData$AgeMos<-as.numeric(MData$AgeMos)
str(MData$AgeMos) #num
MData$Weight<-as.numeric(MData$WeightHC)
str(MData$Weight) #num
MData$TimeR<-as.numeric(MData$TimeR)
str(MData$TimeR) #num
MData$TimeF<-as.factor(MData$TimeR)
str(MData$TimeF) #factor w/9 levels
MData$GroupSizeAdults<-as.numeric(MData$GroupSizeAdults)
str(MData$GroupSizeAdults) #num
MData$DaysSinceLastHC<-as.numeric(MData$DaysSinceLastHC)
str(MData$DaysSinceLastHC) #num
MData$TrialStudentFile<-as.numeric(MData$TrialStudentFile)
str(MData$TrialStudentFile) #num
MData$TrialChronological<-as.numeric(MData$TrialChronological)
str(MData$TrialChronological) #num
MData$TotalNoffspring<-as.numeric(MData$TotalNoffspring)
str(MData$TotalNoffspring) #num
MData$YoungestOffspringAgeatTestMos<-as.numeric(MData$YoungestOffspringAgeatTestMos)
str(MData$YoungestOffspringAgeatTestMos) #num
MData$Trial14<-as.numeric(MData$Trial14or5InWeekorBlock)
str(MData$Trial14) #num

MData$Rank <- as.integer(MData$Rank)
str(MData$Rank) #int

nrow(MData)#332

#############################################################################
#Step 4. Select variables by checking correlations
#############################################################################
#check correlations between predictors INCLUDING those that are not numeric.
```

```
corr.tab=data.frame(cbind(Sex=as.numeric(MData$Sex), AgeMos=as.numeric(MData$AgeMos),
Rank=as.numeric(MData$RankR),
                ReproStat=as.numeric(MData$ReproStatR), Weight=as.numeric(MData$WeightHC),
AgeWean=as.numeric(MData$AgeWean),
                Alopecia=as.numeric(MData$AlopeciaScoreHC), TotalNoffspring=as.numeric(MData$TotalNoffspring),
DependOffspring=as.numeric(MData$HasDependentOffspring),
                OffspringAge=as.numeric(MData$YoungestOffspringAgeatTestMos), Time=as.numeric(MData$TimeR),
                GroupSize=as.numeric(MData$GroupSizeAdults), Days = as.numeric (MData$DaysSinceLastHC),
                Trial14=as.numeric(MData$Trial14or5InWeekorBlock),
TrialChron=as.numeric(MData$TrialChronological), AggLoc=as.numeric(MData$AggLoc),
                StimulusID=as.numeric(MData$StimulusID), MalePres=as.numeric(MData$MalePres),
TrialStudent=as.numeric(MData$TrialStudentFile),
                Disruption=as.numeric(MData$DisruptionInGrpOtherYN),
OtherTreatment=as.numeric(MData$AnyOtherTreatment),
                Drug=as.numeric(MData$DrugKHClLast24HoursYN), Inury=as.numeric(MData$InjuryLast48HrsYN),
                Cleaning=as.numeric(MData$CleaningLast24HrsYN),
GroupChange=as.numeric(MData$GrpChangeLast7DaysYN), BabyGrp=as.numeric(MData$BabyBornLast24HrsGrp),
                Treatment=as.numeric(MData$Treatment)))

str(corr.tab)
spear=cor(corr.tab[,1:27],method ="spearman")
spear

#                  Sex     AgeMos       Rank   ReproStat      Weight     AgeWean     Alopecia TotalNoffspring
# Sex              1.000000000  0.28678615 -0.345408090 -0.599796304  0.5410536655 -0.304326545  0.0778327037
0.733899630
# AgeMos           0.286786150  1.00000000 -0.098689732 -0.093546417  0.5564091269 -0.531094725 -0.1034942047
0.734496303
# Rank            -0.345408090 -0.09868973  1.000000000  0.197140418 -0.6146196785  0.094760701 -0.2067353270 -
0.279399393
# ReproStat       -0.599796304 -0.09354642  0.197140418  1.000000000 -0.1699450024  0.092288209  0.1663921731 -
0.392817360
# Weight           0.541053665  0.55640913 -0.614619679 -0.169945002  1.0000000000 -0.273475528  0.0771977319
0.672201520
# AgeWean         -0.304326545 -0.53109473  0.094760701  0.092288209 -0.2734755276  1.000000000 -0.1170759666 -
0.432563803
# Alopecia         0.077832704 -0.10349420 -0.206735327  0.166392173  0.0771977319 -0.117075967  1.0000000000 -
0.033234993
# TotalNoffspring  0.733899630  0.73449630 -0.279399393 -0.392817360  0.6722015199 -0.432563803 -0.0332349930
1.000000000
# DependOffspring -0.551214371  0.14230172  0.140710410  0.441471254 -0.1032389266 -0.072190465 -0.1060994263
-0.084317775
# OffspringAge            NA         NA         NA         NA          NA          NA          NA           NA
# Time             0.026364956  0.12732399 -0.040207500  0.006218328  0.0877201135 -0.089163381 -0.1137794652
0.068654140
# GroupSize       -0.109153155 -0.05312871  0.252676091  0.079516045 -0.1185917080  0.250459770  0.2128640763 -
0.068505700
# Days            -0.035310383 -0.08118105  0.019042950  0.031097659 -0.0059703144 -0.003780543  0.0240565492 -
0.071120554
# Trial14          0.002325430  0.01434436  0.012922624 -0.045584223 -0.0248546910 -0.017677603  0.0082178231
0.006436554
# TrialChron      -0.089320842  0.06243571  0.039971931 -0.106283347 -0.0581204742  0.060143489 -0.1449766303
0.049500456
# AggLoc          -0.001187191  0.03303598  0.027772297  0.009190506  0.0076045740 -0.004119649 -0.0008069027
0.034543534
# StimulusID      -0.030520552 -0.01178759 -0.003302855  0.037491604 -0.0106454966 -0.032186398 -0.0265708121 -
0.013324324
# MalePres         0.100110058  0.12324673  0.149802055  0.294200751  0.2685782331  0.164179310 -0.1058434071
0.266538816
# TrialStudent    -0.077837562  0.07085784  0.077446690 -0.132008599 -0.0892647983  0.032727141 -0.0839420200
0.055253101
# Disruption       0.072695089 -0.06466228 -0.167649456 -0.012993854  0.0503090963 -0.153856186  0.0349130660 -
0.029317892
```

```
# OtherTreatment   0.033715786  0.03618372 -0.005760035 -0.063404626  0.0326755712 -0.024728915 -0.0390967553
0.055121110
# Drug              0.010524460 -0.01822369 -0.003255033 -0.015723547 -0.0001023844  0.021094898  0.0078544944
0.004481289
# Inury           -0.054246076 -0.02342389  0.068940942  0.099239667 -0.0981849224 -0.114041994 -0.0607514565 -
0.050148574
# Cleaning        -0.033000651  0.12796743  0.023311515 -0.038991505  0.0370099918  0.012848780 -0.1493290490
0.086416294
# GroupChange     -0.004696096 -0.08251866 -0.009388476  0.073199617 -0.0849469755  0.140260572  0.0441382247
-0.056120680
# BabyGrp          0.028226446 -0.11824474 -0.047129350  0.046246841 -0.0656154729  0.136501349  0.0814814169 -
0.066971587
# Treatment       -0.004273396 -0.01234286 -0.007025124 -0.043254958 -0.0196800892  0.027717258 -0.0325708362
0.003485136
#              DependOffspring OffspringAge       Time  GroupSize       Days     Trial14  TrialChron      AggLoc  StimulusID
# Sex           -0.55121437         NA  0.026364956 -0.10915315 -0.035310383  0.002325430 -0.089320842 -0.0011871911
-0.030520552
# AgeMos         0.14230172         NA  0.127323986 -0.05312871 -0.081181049  0.014344363  0.062435706  0.0330359788
-0.011787593
# Rank           0.14071041         NA -0.040207500  0.25267609  0.019042950  0.012922624  0.039971931  0.0277722974
-0.003302855
# ReproStat      0.44147125         NA  0.006218328  0.07951605  0.031097659 -0.045584223 -0.106283347
0.0091905059  0.037491604
# Weight        -0.10323893         NA  0.087720113 -0.11859171 -0.005970314 -0.024854691 -0.058120474  0.0076045740
-0.010645497
# AgeWean       -0.07219046         NA -0.089163381  0.25045977 -0.003780543 -0.017677603  0.060143489 -
0.0041196492 -0.032186398
# Alopecia      -0.10609943         NA -0.113779465  0.21286408  0.024056549  0.008217823 -0.144976630 -0.0008069027
-0.026570812
# TotalNoffspring -0.08431778       NA  0.068654140 -0.06850570 -0.071120554  0.006436554  0.049500456
0.0345435336 -0.013324324
# DependOffspring  1.00000000       NA  0.029511555  0.14585148 -0.016101466  0.015116551  0.232546055
0.0760784147  0.076775729
# OffspringAge           NA        1         NA         NA         NA         NA         NA         NA         NA
# Time           0.02951156         NA 1.000000000 -0.29572223 -0.146460849  0.088755467  0.247405555 -0.0543002343
0.066873880
# GroupSize      0.14585148         NA -0.295722230  1.00000000  0.059967157 -0.020471479  0.031566084
0.0243391021 -0.021074141
# Days          -0.01610147         NA -0.146460849  0.05996716  1.000000000  0.196527177 -0.513950786 -0.0808217399
0.030281629
# Trial14        0.01511655         NA  0.088755467 -0.02047148  0.196527177  1.000000000  0.427553932 -0.0817676749
-0.023155015
# TrialChron     0.23254606         NA  0.247405555  0.03156608 -0.513950786  0.427553932  1.000000000  0.0318947089
-0.019987852
# AggLoc         0.07607841         NA -0.054300234  0.02433910 -0.080821740 -0.081767675  0.031894709  1.0000000000
0.047995029
# StimulusID     0.07677573         NA  0.066873880 -0.02107414  0.030281629 -0.023155015 -0.019987852
0.0479950292  1.000000000
# MalePres       0.17099639         NA  0.019855841  0.14665044  0.088542334 -0.039206336  0.008216924  0.0204557424
0.012549420
# TrialStudent   0.19785759         NA  0.240346292  0.06174827 -0.519178977  0.454091260  0.969809080
0.0321868368 -0.024847727
# Disruption     0.01328108         NA  0.077969513 -0.24193890  0.033240970 -0.146528779 -0.057211595
0.0700665962  0.111979592
# OtherTreatment 0.02990517         NA  0.069708491 -0.10725487 -0.640765047 -0.453977284  0.232586190
0.0560719259  0.058260245
# Drug           0.02015724         NA  0.054050686 -0.02327286 -0.500384768 -0.456244900  0.103643214  0.0782714966
-0.017125117
# Inury          0.09841194         NA  0.073087589 -0.09915184 -0.116824532 -0.050217409  0.045191622 -0.0238619023
0.088485039
# Cleaning       0.09522534         NA  0.504082767 -0.10930223 -0.272814790  0.203719462  0.423589922  0.0027146702
0.029607582
# GroupChange   -0.08870028         NA -0.026780587  0.13846010 -0.072017213 -0.025797988 -0.059636546
0.0333767143  0.035825681
```

```
# BabyGrp              -0.04115395            NA -0.102507826  0.08085786 -0.098590970  0.092142801  0.102228877
0.1022490134  0.025285366
# Treatment             0.10700656            NA  0.287523358 -0.04782994 -0.824471471  0.063560637  0.773224910
0.0741134118 -0.012665783
#                 MalePres TrialStudent   Disruption OtherTreatment        Drug      Inury   Cleaning GroupChange    BabyGrp
# Sex             0.100110058 -0.077837562  0.072695089     0.033715786  0.0105244598 -0.05424608 -0.03300065 -
0.004696096  0.02822645
# AgeMos          0.123246730  0.070857837 -0.064662283     0.036183724 -0.0182236890 -0.02342389  0.12796743 -
0.082518660 -0.11824474
# Rank            0.149802055  0.077446690 -0.167649456    -0.005760035 -0.0032550335  0.06894094  0.02331152 -
0.009388476 -0.04712935
# ReproStat       0.294200751 -0.132008599 -0.012993854    -0.063404626 -0.0157235469  0.09923967 -0.03899151
0.073199617  0.04624684
# Weight          0.268578233 -0.089264798  0.050309096     0.032675571 -0.0001023844 -0.09818492  0.03700999 -
0.084946976 -0.06561547
# AgeWean         0.164179310  0.032727141 -0.153856186    -0.024728915  0.0210948981 -0.11404199  0.01284878
0.140260572  0.13650135
# Alopecia       -0.105843407 -0.083942020  0.034913066    -0.039096755  0.0078544944 -0.06075146 -0.14932905
0.044138225  0.08148142
# TotalNoffspring 0.266538816  0.055253101 -0.029317892     0.055121110  0.0044812887 -0.05014857  0.08641629 -
0.056120680 -0.06697159
# DependOffspring 0.170996392  0.197857592  0.013281083     0.029905172  0.0201572434  0.09841194  0.09522534 -
0.088700276 -0.04115395
# OffspringAge           NA         NA         NA          NA         NA         NA         NA         NA         NA
# Time            0.019855841  0.240346292  0.077969513     0.069708491  0.0540506858  0.07308759  0.50408277 -
0.026780587 -0.10250783
# GroupSize       0.146650435  0.061748274 -0.241938899    -0.107254866 -0.0232728613 -0.09915184 -0.10930223
0.138460095  0.08085786
# Days            0.088542334 -0.519178977  0.033240970    -0.640765047 -0.5003847682 -0.11682453 -0.27281479 -
0.072017213 -0.09859097
# Trial14        -0.039206336  0.454091260 -0.146528779    -0.453977284 -0.4562448995 -0.05021741  0.20371946 -
0.025797988  0.09214280
# TrialChron      0.008216924  0.969809080 -0.057211595     0.232586190  0.1036432137  0.04519162  0.42358992 -
0.059636546  0.10222888
# AggLoc          0.020455742  0.032186837  0.070066596     0.056071926  0.0782714966 -0.02386190  0.00271467
0.033376714  0.10224901
# StimulusID      0.012549420 -0.024847727  0.111979592     0.058260245 -0.0171251172  0.08848504  0.02960758
0.035825681  0.02528537
# MalePres        1.000000000  0.004247883 -0.113338903    -0.005993038  0.0031112315  0.01682809  0.06429402
0.040939379  0.02390776
# TrialStudent    0.004247883  1.000000000 -0.080019742     0.228789708  0.0990403359  0.04770348  0.42250651 -
0.054207425  0.10699068
# Disruption     -0.113338903 -0.080019742  1.000000000     0.082006136 -0.0715658764  0.04416427 -0.05940106
0.008699526  0.12401253
# OtherTreatment -0.005993038  0.228789708  0.082006136     1.000000000  0.6660414004  0.11759574 -0.03080107 -
0.111322550 -0.06501009
# Drug            0.003111231  0.099040336 -0.071565876     0.666041400  1.0000000000  0.07086879 -0.12524324 -
0.079748951 -0.04657176
# Inury           0.016828087  0.047703483  0.044164271     0.117595741  0.0708687927  1.00000000  0.06401972 -
0.022183592 -0.01295476
# Cleaning        0.064294017  0.422506509 -0.059401061    -0.030801067 -0.1252432391  0.06401972  1.00000000 -
0.084755458 -0.04949545
# GroupChange     0.040939379 -0.054207425  0.008699526    -0.111322550 -0.0797489514 -0.02218359 -0.08475546
1.000000000  0.58397955
# BabyGrp         0.023907760  0.106990683  0.124012531    -0.065010093 -0.0465717570 -0.01295476 -0.04949545
0.583979553  1.00000000
# Treatment      -0.009568541  0.786925427 -0.008764595     0.505921529  0.3624307149  0.10081656  0.38518351
0.026299173  0.14323067
# Treatment
# Sex          -0.004273396
# AgeMos       -0.012342859
# Rank         -0.007025124
# ReproStat    -0.043254958
# Weight       -0.019680089
# AgeWean       0.027717258
```

```
# Alopecia       -0.032570836
# TotalNoffspring  0.003485136
# DependOffspring  0.107006564
# OffspringAge            NA
# Time           0.287523358
# GroupSize      -0.047829940
# Days           -0.824471471
# Trial14         0.063560637
# TrialChron      0.773224910
# AggLoc          0.074113412
# StimulusID      -0.012665783
# MalePres       -0.009568541
# TrialStudent    0.786925427
# Disruption     -0.008764595
# OtherTreatment   0.505921529
# Drug           0.362430715
# Inury          0.100816560
# Cleaning        0.385183515
# GroupChange     0.026299173
# BabyGrp         0.143230670
# Treatment       1.000000000

#Remove Correlations >0.4
################################################################################
#Step 5. Transform variables
################################################################################
HData<-MData

#response variable AGG
hist(HData$AGG)
hist(transformTukey(HData$AGG))
#      lambda      W Shapiro.p.value
# 421    0.5 0.9698      1.685e-06
hist(sqrt(HData$AGG))
HData$sqrt.AGG<-sqrt(HData$AGG)
#ABDiff
hist(HData$ABDiff)# normal distribution nice!
hist(transformTukey(HData$ABDiff))
#     lambda      W Shapiro.p.value
# 441      1 0.9588      4.727e-08
#Total Look
hist(transformTukey(HData$TotalLook))
#     lambda      W Shapiro.p.value
# 421    0.5 0.9943         0.2461
HData$sqrt.TotalLook<-sqrt(HData$TotalLook)
#ABDiff/TL
hist(transformTukey(HData$ABDiff.TL)) # normal distribution
# lambda      W Shapiro.p.value
# 441      1 0.9492      2.786e-09

#Rank
table(HData$RankR)
# 1   2   3
# 186  73  73
#Sex
table(HData$Sex)
# F   M
# 251  81
#Treatment
table(HData$Treatment)
# BL Stress
# 175    157
#Aggloc
table(HData$AggLoc)
# Lmonkeyview Rmonkeyview
```

```
# 180       152
#StimulusID
table(HData$StimulusID)
# 1  2  3  4  5  6  7
# 53 58 40 53 42 49 37
#Trial14
table(HData$Trial14)
# 1  2  3  4  5
# 79 78 72 74 29
#Age
hist(HData$AgeMos)
hist(transformTukey(HData$AgeMos))
#     lambda      W Shapiro.p.value
# 427   0.65 0.9499      3.348e-09
HData$Tukey.AgeMos<-transformTukey(HData$AgeMos)
#Time
hist(HData$TimeR)
hist(transformTukey(HData$TimeR))
#     lambda      W Shapiro.p.value
# 357   -1.1 0.9351      7.305e-11
HData$Tukey.TimeR<-transformTukey(HData$TimeR)
table(HData$TimeF)
# 9 10 11 12 13 14 15
# 42 78 99 36 45 16 16
#Trial14
hist(HData$Trial14)
hist(transformTukey(HData$Trial14))
#     lambda     W Shapiro.p.value
# 437    0.9 0.895      2.293e-14
HData$Tukey.Trial14<-transformTukey(HData$Trial14)

#now z-transform all the covariates
HData$z.Tukey.AgeMos <- as.vector(scale(HData$Tukey.AgeMos))
HData$z.RankR <- as.vector(scale(HData$RankR))
HData$z.Tukey.Trial14 <- as.vector(scale(HData$Tukey.Trial14))
#HData$z.Tukey.TimeR<- as.vector(scale(HData$Tukey.TimeR))

###############################################################################
#Step 6. Save and / or load HData
###############################################################################
EData<-HData
write.csv(EData,
      file='EData.txt', row.names=T)
EData<-read.csv(file.choose(), row.names = 1, header=T) #select file from pop up window
View(EData)
nrow(EData)#332
ncol(EData)#258
str(EData$animalID)#Factor w/ 36 levels
##########################END of TRANSFORMATIONS#########################
###############################################################################
#Step 7. Start to build model
###############################################################################
a<-HData

#Set the working directory
#setwd('M:/Emily/Writing/1Papers in prep/2019 AB Heritability/AB Heritability')
#setwd("C:/Users/emmel/Desktop/R Studio and R")
setwd("D:/R Studio and R/Functions")
#setwd("E:/R Studio and R/Functions")

library(tidyverse)
library(rcompanion)
library(lme4)
library(car)
library(MuMIn)
```

```
#load Roger Functions #source files need to be in the working directory
source("C:/Users/emmel/Desktop/R Studio and R/Functions/diagnostic_fcns.r")
source("C:/Users/emmel/Desktop/R Studio and R/Functions/glmm_stability.r")
#source("M:/Emily/Doing/R Users Group/R Training/Mundry/Functions/Functions/diagnostic_fcns.r")
#source("M:/Emily/Doing/R Users Group/R Training/Mundry/Functions/Functions/glmm_stability.r")

##############################################################################
#Step 8. Full model for Agg with M&Fs
##############################################################################
AGGFull1<-lmer(sqrt.AGG ~
            Treatment*Sex +
            z.Tukey.Trial14 +  StimulusID + TimeF + AggLoc +
            z.RankR + z.Tukey.AgeMos + #Sex +
           (1|animalID), data=a, REML = "F")

diagnostics.plot(AGGFull1)# ok
ranef.diagn.plot(AGGFull1)# ok
plot(AGGFull1)
plot(residuals(AGGFull1)) #ok
as.data.frame(drop1(AGGFull1, test = "Chisq"))
#               Df     AIC         LRT    Pr(Chi)
# <none>         NA 2534.625          NA        NA
# z.Tukey.Trial14  1 2533.565 9.401512e-01 0.33223897 #remove
# StimulusID       6 2527.293 4.668258e+00 0.58700918 #remove
# TimeF            6 2533.763 1.113787e+01 0.08420786
# AggLoc           1 2532.625 1.970813e-05 0.99645790 #remove
# z.RankR          1 2532.625 5.516582e-04 0.98126148 #remove
# z.Tukey.AgeMos   1 2532.994 3.691151e-01 0.54348699 #remove
# Treatment:Sex    1 2533.442 8.170987e-01 0.36602980 #remove interaction

summary(AGGFull1)
# Linear mixed model fit by maximum likelihood  ['lmerMod']
# Formula: sqrt.AGG ~ Treatment * Sex + z.Tukey.Trial14 + StimulusID + TimeF +
#   AggLoc + z.RankR + z.Tukey.AgeMos + (1 | animalID)
# Data: a
#
# AIC      BIC   logLik deviance df.resid
# 2534.6   2618.3  -1245.3   2490.6     310
#
# Scaled residuals:
#   Min      1Q  Median      3Q     Max
# -2.16924 -0.68167  0.06877  0.58046  2.73249
#
# Random effects:
#   Groups   Name        Variance Std.Dev.
# animalID (Intercept) 18.74    4.329
# Residual             94.87    9.740
# Number of obs: 332, groups:  animalID, 36
#
# Fixed effects:
#   Estimate Std. Error t value
# (Intercept)         17.983892   3.182676   5.651
# TreatmentStress     -1.235838   1.359565  -0.909
# SexM               -0.833862   2.722868  -0.306
# z.Tukey.Trial14      0.537273   0.553640   0.970
# StimulusID2          0.567243   1.912922   0.297
# StimulusID3         -2.226476   2.087651  -1.066
# StimulusID4          1.417928   1.946649   0.728
# StimulusID5         -1.837450   2.065529  -0.890
# StimulusID6         -0.762716   1.994633  -0.382
# StimulusID7          0.164819   2.154218   0.077
# TimeF9               2.167537   3.054666   0.710
# TimeF10              4.259624   2.855397   1.492
# TimeF11              2.356285   2.790763   0.844
```

```
# TimeF12             6.479907   3.099259   2.091
# TimeF13             3.691337   2.978502   1.239
# TimeF15             8.584514   3.590817   2.391
# AggLocRmonkeyview    0.005025   1.131618   0.004
# z.RankR             0.022743   0.968280   0.023
# z.Tukey.AgeMos       0.595725   0.978016   0.609
# TreatmentStress:SexM  2.288860   2.530234   0.905

AGGFull2<-lmer(sqrt.AGG ~
            Treatment + Sex +
            TimeF +
           (1|animalID), data=a, REML = "F")

diagnostics.plot(AGGFull2)# ok
ranef.diagn.plot(AGGFull2)# ok
plot(AGGFull2)
plot(residuals(AGGFull2)) #ok
as.data.frame(drop1(AGGFull2, test = "Chisq"))
#          Df     AIC      LRT   Pr(Chi)
# <none>    NA 2519.435       NA       NA
# Treatment  1 2517.786 0.3509255 0.5535897 #keep in model
# Sex        1 2517.613 0.1778040 0.6732673 #remove
# TimeF      6 2517.340 9.9043748 0.1287372

summary(AGGFull2)
# Linear mixed model fit by maximum likelihood  ['lmerMod']
# Formula: sqrt.AGG ~ Treatment + Sex + TimeF + (1 | animalID)
# Data: a
#
# AIC     BIC   logLik deviance df.resid
# 2519.4   2561.3  -1248.7   2497.4     321
#
# Scaled residuals:
#   Min      1Q   Median      3Q     Max
# -2.14468 -0.67772  0.06888  0.61759  2.76969
#
# Random effects:
#   Groups   Name        Variance Std.Dev.
# animalID (Intercept) 18.63    4.316
# Residual             97.03    9.851
# Number of obs: 332, groups:  animalID, 36
#
# Fixed effects:
#   Estimate Std. Error t value
# (Intercept)     17.7174     2.8375   6.244
# TreatmentStress  -0.7068     1.1919  -0.593
# SexM             0.8840     2.0935   0.422
# TimeF9           1.6806     3.0527   0.551
# TimeF10          3.4894     2.8440   1.227
# TimeF11          2.0595     2.7831   0.740
# TimeF12          5.7800     3.0510   1.894
# TimeF13          3.6413     2.9624   1.229
# TimeF15          8.1450     3.5842   2.272
#
# Correlation of Fixed Effects:
#   (Intr) TrtmnS SexM   TimeF9 TimF10 TimF11 TimF12 TimF13
# TrtmntStrss -0.303
# SexM        -0.204  0.006
# TimeF9      -0.807  0.134  0.027
# TimeF10     -0.856  0.140  0.020  0.793
# TimeF11     -0.875  0.161  0.024  0.801  0.850
# TimeF12     -0.776  0.110  0.003  0.714  0.760  0.774
# TimeF13     -0.767 -0.034  0.018  0.728  0.778  0.786  0.710
# TimeF15     -0.605 -0.105  0.026  0.582  0.622  0.637  0.567  0.616
```

```
AGGFull3<-lmer(sqrt.AGG ~
            Treatment +
            TimeF +
            (1|animalID), data=a, REML = "F")

diagnostics.plot(AGGFull3)# ok
ranef.diagn.plot(AGGFull3)# ok
plot(AGGFull3)
plot(residuals(AGGFull3)) #ok
as.data.frame(drop1(AGGFull3, test = "Chisq"))
#          Df     AIC      LRT   Pr(Chi)
# <none>    NA 2517.613       NA       NA
# Treatment  1 2515.966 0.3527973 0.5525339
# TimeF      6 2515.525 9.9121928 0.1283988

summary(AGGFull3)
# Linear mixed model fit by maximum likelihood  ['lmerMod']
# Formula: sqrt.AGG ~ Treatment + TimeF + (1 | animalID)
# Data: a
#
# AIC      BIC   logLik deviance df.resid
# 2517.6   2555.7  -1248.8   2497.6      322
#
# Scaled residuals:
#   Min      1Q  Median      3Q     Max
# -2.13598 -0.68397  0.08344  0.62635  2.76081
#
# Random effects:
#   Groups   Name        Variance Std.Dev.
# animalID (Intercept) 18.78    4.334
# Residual             97.03    9.850
# Number of obs: 332, groups:  animalID, 36
#
# Fixed effects:
#               Estimate Std. Error t value
# (Intercept)     17.9625     2.7786   6.465
# TreatmentStress  -0.7087     1.1919  -0.595
# TimeF9           1.6465     3.0521   0.539
# TimeF10          3.4645     2.8438   1.218
# TimeF11          2.0308     2.7826   0.730
# TimeF12          5.7746     3.0512   1.893
# TimeF13          3.6165     2.9622   1.221
# TimeF15          8.1060     3.5832   2.262
#
# Correlation of Fixed Effects:
#   (Intr) TrtmnS TimeF9 TimF10 TimF11 TimF12 TimF13
# TrtmntStrss -0.308
# TimeF9      -0.819  0.134
# TimeF10     -0.870  0.140  0.793
# TimeF11     -0.889  0.161  0.801  0.850
# TimeF12     -0.792  0.110  0.714  0.760  0.774
# TimeF13     -0.779 -0.034  0.728  0.777  0.786  0.710
# TimeF15     -0.613 -0.106  0.581  0.622  0.636  0.568  0.616

AGGFull4<-lmer(sqrt.AGG ~
             Treatment +
            (1|animalID), data=a, REML = "F")

diagnostics.plot(AGGFull4)# ok
ranef.diagn.plot(AGGFull4)# ok
plot(AGGFull4)
plot(residuals(AGGFull4)) #ok
as.data.frame(drop1(AGGFull4, test = "Chisq"))
#          Df     AIC        LRT   Pr(Chi)
# <none>    NA 2515.525         NA       NA
```

```
# Treatment  1 2513.529 0.003461228 0.9530858

anova(AGGFull1, AGGFull2) #0.8768
anova(AGGFull2, AGGFull3) #0.6733
anova(AGGFull1, AGGFull3) #0.8584
anova(AGGFull3, AGGFull4) #0.1284

AGGnull<-lmer(sqrt.AGG ~
            1 +
            (1|animalID), data=a, REML = "F")

anova(AGGnull, AGGFull1) #0.5964
anova(AGGnull, AGGFull2) #0.2585
anova(AGGnull, AGGFull3) #0.1934
anova(AGGnull, AGGFull4) #0.9531

#full3 is best model
# Data: a
# Models:
#   AGGnull: sqrt.AGG ~ 1 + (1 | animalID)
# AGGFull3: sqrt.AGG ~ Treatment + TimeF + (1 | animalID)
#          Df    AIC    BIC  logLik deviance  Chisq Chi Df Pr(>Chisq)
# AGGnull   3 2513.5 2524.9 -1253.8   2507.5
# AGGFull3 10 2517.6 2555.7 -1248.8   2497.6 9.9157      7     0.1934

FinalAgg<-lmer(sqrt.AGG ~
             Treatment +
             TimeF +
             (1|animalID), data=a, REML = "F")

mstab=glmm.model.stab(model.res=FinalAgg, contr=lmerControl(optimizer = "bobyqa",optCtrl = list(maxfun=2e5)))
mstab$detailed$warnings
# [1] none none none none none none none none none none none none none none none none none none none none none none none none none none none
# [21] none none
# Levels: none
mstab$summary[,-1]
#                         orig      min       max
# (Intercept)           17.962473 16.284225 19.0830050
# TreatmentStress       -0.708653 -1.081907 -0.1511949
# TimeF9                 1.646468  0.456106  3.2598605
# TimeF10                3.464504  2.627648  4.7740888
# TimeF11                2.030764  1.056985  3.8869265
# TimeF12                5.774552  4.465914  7.2534051
# TimeF13                3.616450  3.029316  5.5810704
# TimeF15                8.106038  6.785144  9.6131810
# animalID@(Intercept)@NA  4.333707  3.698262  4.4718218
# Residual               9.850347  9.549925 10.0123671

r.squaredGLMM(FinalAgg)
#        R2m       R2c
# [1,] 0.02550976 0.1835433

confint.merMod(object=FinalAgg)
#                    2.5 %    97.5 %
# .sig01          2.8445258  6.195085
# .sigma          9.1059749 10.702141
# (Intercept)     12.5005419 23.424699
# TreatmentStress -3.0568410  1.634787
# TimeF9          -4.3534997  7.646045
# TimeF10         -2.1254770  9.057704
# TimeF11         -3.4389749  7.502317
# TimeF12         -0.2238091 11.780529
# TimeF13         -2.2071430  9.447279
# TimeF15          1.0613979 15.150840
```

```
##############################################################################
#Step 9. Full  model for TL
##############################################################################
TLFull1<-lmer(sqrt.TotalLook ~
            Treatment*Sex +
            z.Tukey.Trial14 +  StimulusID + TimeF + AggLoc +
            z.RankR + z.Tukey.AgeMos + #Sex +
            (1|animalID), data=a, REML = "F")

diagnostics.plot(TLFull1)# ok
ranef.diagn.plot(TLFull1)# ok
plot(TLFull1)
plot(residuals(TLFull1)) #ok
as.data.frame(drop1(TLFull1, test = "Chisq"))
#               Df     AIC        LRT    Pr(Chi)
# <none>         NA 2478.993         NA        NA
# z.Tukey.Trial14  1 2477.011  0.01864884 0.89137798 #remove
# StimulusID       6 2471.948  4.95486205 0.54961534 #remove
# TimeF            6 2481.188 14.19523555 0.02752970
# AggLoc           1 2478.856  1.86311547 0.17226566
# z.RankR          1 2477.436  0.44327680 0.50554511 #remove
# z.Tukey.AgeMos   1 2477.202  0.20955946 0.64711292 #remove
# Treatment:Sex    1 2480.224  3.23172484 0.07222458

summary(TLFull1)
# Linear mixed model fit by maximum likelihood  ['lmerMod']
# Formula: sqrt.TotalLook ~ Treatment * Sex + z.Tukey.Trial14 + StimulusID +
#   TimeF + AggLoc + z.RankR + z.Tukey.AgeMos + (1 | animalID)
# Data: a
#
# AIC     BIC   logLik deviance df.resid
# 2479.0   2562.7  -1217.5   2435.0     310
#
# Scaled residuals:
#   Min      1Q  Median      3Q     Max
# -2.89054 -0.72028  0.02776  0.73379  2.40219
#
# Random effects:
#   Groups   Name        Variance Std.Dev.
# animalID (Intercept) 31.85    5.643
# Residual             75.65    8.698
# Number of obs: 332, groups:  animalID, 36
#
# Fixed effects:
#   Estimate Std. Error t value
# (Intercept)         28.00271    2.98260   9.389
# TreatmentStress     -1.05683    1.21882  -0.867
# SexM                -0.43869    3.07165  -0.143
# z.Tukey.Trial14     -0.06761    0.49487  -0.137
# StimulusID2         -0.66448    1.71541  -0.387
# StimulusID3         -3.34250    1.86877  -1.789
# StimulusID4         -0.22500    1.74285  -0.129
# StimulusID5          0.16193    1.84775   0.088
# StimulusID6         -1.09502    1.78730  -0.613
# StimulusID7          0.35473    1.92940   0.184
# TimeF9               1.77570    2.75941   0.644
# TimeF10              2.57283    2.57652   0.999
# TimeF11              3.87743    2.51524   1.542
# TimeF12              7.18886    2.78725   2.579
# TimeF13              5.27031    2.68051   1.966
# TimeF15              7.63312    3.22331   2.368
# AggLocRmonkeyview   -1.38366    1.01197  -1.367
# z.RankR              0.75752    1.13474   0.668
# z.Tukey.AgeMos       0.52349    1.14237   0.458
# TreatmentStress:SexM  4.08032    2.26299   1.803
```

```
TLFull2<-lmer(sqrt.TotalLook ~
            Treatment*Sex +
            TimeF + AggLoc +
            (1|animalID), data=a, REML = "F")

diagnostics.plot(TLFull2)# ok
ranef.diagn.plot(TLFull2)# ok
plot(TLFull2)
plot(residuals(TLFull2)) #ok
as.data.frame(drop1(TLFull2, test = "Chisq"))
#             Df     AIC      LRT    Pr(Chi)
# <none>       NA 2466.542       NA        NA
# TimeF         6 2469.335 14.792846 0.02193057
# AggLoc        1 2466.606  2.063949 0.15081891 #remove
# Treatment:Sex  1 2467.795  3.252521 0.07131369

summary(TLFull2)
# Linear mixed model fit by maximum likelihood  ['lmerMod']
# Formula: sqrt.TotalLook ~ Treatment * Sex + TimeF + AggLoc + (1 | animalID)
# Data: a
#
# AIC     BIC   logLik deviance df.resid
# 2466.5   2516.0  -1220.3   2440.5     319
#
# Scaled residuals:
#   Min      1Q   Median      3Q     Max
# -2.94516 -0.68757  0.01849  0.66808  2.33223
#
# Random effects:
#   Groups   Name        Variance Std.Dev.
# animalID (Intercept) 32.14    5.670
# Residual             76.97    8.774
# Number of obs: 332, groups:  animalID, 36
#
# Fixed effects:
#   Estimate Std. Error t value
# (Intercept)          27.8148     2.7646  10.061
# TreatmentStress      -1.1236     1.2176  -0.923
# SexM                 -0.6062     2.6872  -0.226
# TimeF9                1.3373     2.7584   0.485
# TimeF10               2.0356     2.5717   0.792
# TimeF11               3.4131     2.5108   1.359
# TimeF12               6.7894     2.7788   2.443
# TimeF13               4.9195     2.6889   1.830
# TimeF15               7.6344     3.2133   2.376
# AggLocRmonkeyview    -1.4465     1.0050  -1.439
# TreatmentStress:SexM   4.1041     2.2685   1.809
#
# Correlation of Fixed Effects:
#   (Intr) TrtmnS SexM   TimeF9 TimF10 TimF11 TimF12 TimF13 TimF15 AggLcR
# TrtmntStrss -0.280
# SexM        -0.258  0.196
# TimeF9      -0.759  0.128  0.028
# TimeF10     -0.808  0.122  0.017  0.797
# TimeF11     -0.825  0.145  0.022  0.804  0.851
# TimeF12     -0.747  0.090  0.005  0.713  0.761  0.773
# TimeF13     -0.734 -0.042  0.009  0.729  0.781  0.788  0.718
# TimeF15     -0.563 -0.104  0.009  0.582  0.622  0.638  0.564  0.616
# AggLcRmnkyv -0.228 -0.089 -0.015  0.045  0.083  0.068  0.168  0.142  0.033
# TrtmntSt:SM  0.104 -0.477 -0.402 -0.023 -0.008 -0.013 -0.009  0.007  0.021  0.030

TLFull3<-lmer(sqrt.TotalLook ~
            Treatment*Sex +
            TimeF +
```

```
          (1|animalID), data=a, REML = "F")

diagnostics.plot(TLFull3)# ok
ranef.diagn.plot(TLFull3)# ok
plot(TLFull3)
plot(residuals(TLFull3)) #ok
as.data.frame(drop1(TLFull3, test = "Chisq"))
#             Df     AIC      LRT     Pr(Chi)
# <none>       NA 2466.606       NA         NA
# TimeF         6 2471.595 16.989078 0.009323469
# Treatment:Sex  1 2467.991  3.384866 0.065797529

summary(TLFull3)
# Linear mixed model fit by maximum likelihood  ['lmerMod']
# Formula: sqrt.TotalLook ~ Treatment * Sex + TimeF + (1 | animalID)
# Data: a
#
# AIC      BIC   logLik deviance df.resid
# 2466.6   2512.3  -1221.3   2442.6      320
#
# Scaled residuals:
#   Min      1Q   Median      3Q     Max
# -2.99474 -0.72601  0.00964  0.71479  2.21698
#
# Random effects:
#   Groups   Name       Variance Std.Dev.
# animalID (Intercept) 32.04    5.660
# Residual             77.52    8.805
# Number of obs: 332, groups:  animalID, 36
#
# Fixed effects:
#   Estimate Std. Error t value
# (Intercept)          26.9101     2.6989   9.971
# TreatmentStress      -1.2801     1.2171  -1.052
# SexM                 -0.6654     2.6871  -0.248
# TimeF9                1.5123     2.7651   0.547
# TimeF10               2.3446     2.5716   0.912
# TimeF11               3.6571     2.5137   1.455
# TimeF12               7.4601     2.7488   2.714
# TimeF13               5.4701     2.6708   2.048
# TimeF15               7.7825     3.2229   2.415
# TreatmentStress:SexM   4.2003     2.2756   1.846
#
# Correlation of Fixed Effects:
#   (Intr) TrtmnS SexM   TimeF9 TimF10 TimF11 TimF12 TimF13 TimF15
# TrtmntStrss -0.310
# SexM        -0.268  0.196
# TimeF9      -0.771  0.133  0.028
# TimeF10     -0.814  0.130  0.018  0.796
# TimeF11     -0.833  0.152  0.023  0.803  0.850
# TimeF12     -0.739  0.107  0.007  0.716  0.760  0.774
# TimeF13     -0.728 -0.029  0.011  0.730  0.780  0.788  0.712
# TimeF15     -0.572 -0.101  0.009  0.581  0.622  0.638  0.567  0.618
# TrtmntSt:SM  0.114 -0.477 -0.403 -0.024 -0.011 -0.015 -0.014  0.003  0.020

anova(TLFull1, TLFull2) #0.784
anova(TLFull2, TLFull3) #0.1508
anova(TLFull1, TLFull3) #0.6665

TLnull<-lmer(sqrt.TotalLook ~
          1 +
          (1|animalID), data=a, REML = "F")

anova(TLnull, TLFull1) #0.08345
anova(TLnull, TLFull2) #0.01297
```

```
anova(TLnull, TLFull3) #0.01568

#full3 is best model
# Data: a
# Models:
#   TLnull: sqrt.TotalLook ~ 1 + (1 | animalID)
# TLFull3: sqrt.TotalLook ~ Treatment * Sex + TimeF + (1 | animalID)
# Df    AIC    BIC  logLik deviance  Chisq Chi Df Pr(>Chisq)
# TLnull   3 2469.0 2480.4 -1231.5   2463.0
# TLFull3 12 2466.6 2512.3 -1221.3   2442.6 20.385      9    0.01568
  ---
a$TimeF<- as.factor(a$TimeF)
a$TimeF<-relevel(a$TimeF, ref="14")
table(a$TimeF)
# 14  9 10 11 12 13 15
# 16 42 78 99 36 45 16

FinalTL<-lmer(sqrt.TotalLook ~
              Treatment*Sex +
              TimeF +
              (1|animalID), data=a, REML = "F")

diagnostics.plot(FinalTL)# ok
ranef.diagn.plot(FinalTL)# ok
plot(FinalTL)
plot(residuals(FinalTL)) #ok
as.data.frame(drop1(FinalTL, test = "Chisq"))
#              Df     AIC      LRT     Pr(Chi)
# <none>       NA 2466.606       NA         NA
# TimeF         6 2471.595 16.989078 0.009323469
# Treatment:Sex  1 2467.991  3.384866 0.065797529

summary(FinalTL)
# Linear mixed model fit by maximum likelihood  ['lmerMod']
# Formula: sqrt.TotalLook ~ Treatment * Sex + TimeF + (1 | animalID)
# Data: a
#
# AIC      BIC   logLik deviance df.resid
# 2466.6   2512.3  -1221.3   2442.6     320
#
# Scaled residuals:
#   Min      1Q   Median      3Q     Max
# -2.99474 -0.72601  0.00964  0.71479  2.21698
#
# Random effects:
#   Groups   Name        Variance Std.Dev.
# animalID (Intercept) 32.04    5.660
# Residual             77.52    8.805
# Number of obs: 332, groups:  animalID, 36
#
# Fixed effects:
#                 Estimate Std. Error t value
# (Intercept)         26.9101     2.6989   9.971
# TreatmentStress     -1.2801     1.2171  -1.052
# SexM                -0.6654     2.6871  -0.248
# TimeF9               1.5123     2.7651   0.547
# TimeF10              2.3446     2.5716   0.912
# TimeF11              3.6571     2.5137   1.455
# TimeF12              7.4601     2.7488   2.714
# TimeF13              5.4701     2.6708   2.048
# TimeF15              7.7825     3.2229   2.415
# TreatmentStress:SexM   4.2003     2.2756   1.846
#
# Correlation of Fixed Effects:
#   (Intr) TrtmnS SexM   TimeF9 TimF10 TimF11 TimF12 TimF13 TimF15
```

```
# TrtmntStrss -0.310
# SexM       -0.268  0.196
# TimeF9     -0.771  0.133  0.028
# TimeF10    -0.814  0.130  0.018  0.796
# TimeF11    -0.833  0.152  0.023  0.803  0.850
# TimeF12    -0.739  0.107  0.007  0.716  0.760  0.774
# TimeF13    -0.728 -0.029  0.011  0.730  0.780  0.788  0.712
# TimeF15    -0.572 -0.101  0.009  0.581  0.622  0.638  0.567  0.618
# TrtmntSt:SM  0.114 -0.477 -0.403 -0.024 -0.011 -0.015 -0.014  0.003  0.020
mstab=glmm.model.stab(model.res=FinalTL, contr=lmerControl(optimizer = "bobyqa",optCtrl = list(maxfun=2e5)))
mstab$detailed$warnings
# [1] none none none none none none none none none none none none none none none none none none none none
# [21] none none
# Levels: none
mstab$summary[,-1]
#                        orig        min        max
# (Intercept)           26.9101005 25.0094070 28.0251026
# TreatmentStress        -1.2801058 -1.7979271 -0.5715827
# SexM                  -0.6654064 -2.7376884  2.1466493
# TimeF9                 1.5122517  0.3378105  3.4530354
# TimeF10                2.3446497  0.9289907  4.1283658
# TimeF11                3.6570810  2.4709763  5.7578415
# TimeF12                7.4600696  6.1825647  9.3376776
# TimeF13                5.4701024  4.0709071  7.4488499
# TimeF15                7.7824919  5.8428605  9.5887039
# TreatmentStress:SexM    4.2003239  3.3245123  5.8121058
# animalID@(Intercept)@NA  5.6600160  4.3011720  5.7727901
# Residual               8.8047808  8.6656074  8.9064391
r.squaredGLMM(FinalTL)
#        R2m       R2c
# [1,] 0.04748649 0.3260054

confint.merMod(object=FinalTL)
#                      2.5 %    97.5 %
# .sig01              4.1832890  7.677670
# .sigma              8.1393677  9.566222
# (Intercept)         21.5978939 32.215355
# TreatmentStress     -3.6764443  1.112355
# SexM                -6.0450247  4.699469
# TimeF9              -3.9274941  6.947461
# TimeF10             -2.7106456  7.400852
# TimeF11             -1.2882132  8.598046
# TimeF12              2.0541331 12.863571
# TimeF13              0.2195835 10.721537
# TimeF15              1.4410551 14.117533
# TreatmentStress:SexM -0.2761576  8.673587

install.packages("emmeans")
library(emmeans)

emmeans(FinalTL, list(pairwise ~ TimeF), adjust = "tukey")
# $`emmeans of TimeF`
# TimeF emmean   SE    df lower.CL upper.CL
# 14      27.0 2.61 285.2     19.9     34.0
# 9       28.5 1.85 148.2     23.5     33.5
# 10      29.3 1.58  87.7     25.0     33.7
# 11      30.6 1.50  73.6     26.5     34.8
# 12      34.4 1.91 164.5     29.2     39.6
# 13      32.5 1.82 143.1     27.5     37.4
# 15      34.8 2.65 295.8     27.6     41.9
#
# Results are averaged over the levels of: Treatment, Sex
# Degrees-of-freedom method: kenward-roger
# Confidence level used: 0.95
# Conf-level adjustment: sidak method for 7 estimates
```

```
#
# $`pairwise differences of TimeF`
# contrast estimate   SE  df t.ratio p.value
# 14 - 9     -1.512 2.81 324 -0.538  0.9983
# 14 - 10    -2.345 2.61 322 -0.897  0.9728
# 14 - 11    -3.657 2.55 320 -1.432  0.7840
# 14 - 12    -7.460 2.79 317 -2.673  0.1086
# 14 - 13    -5.470 2.71 318 -2.017  0.4059
# 14 - 15    -7.782 3.27 313 -2.380  0.2108
# 9 - 10     -0.832 1.74 310 -0.479  0.9991
# 9 - 11     -2.145 1.70 315 -1.264  0.8680
# 9 - 12     -5.948 2.11 315 -2.820  0.0747
# 9 - 13     -3.958 2.03 315 -1.952  0.4479
# 9 - 15     -6.270 2.81 317 -2.229  0.2831
# 10 - 11    -1.312 1.41 319 -0.928  0.9679
# 10 - 12    -5.115 1.88 316 -2.724  0.0956
# 10 - 13    -3.125 1.77 312 -1.767  0.5712
# 10 - 15    -5.438 2.62 315 -2.072  0.3720
# 11 - 12    -3.803 1.81 315 -2.104  0.3531
# 11 - 13    -1.813 1.72 314 -1.053  0.9408
# 11 - 15    -4.125 2.56 311 -1.611  0.6755
# 12 - 13     1.990 2.09 313  0.952  0.9635
# 12 - 15    -0.322 2.85 315 -0.113  1.0000
# 13 - 15    -2.312 2.66 311 -0.869  0.9769

emmeans(FinalTL, list(pairwise ~ Treatment*Sex), adjust = "tukey")
# $`emmeans of Treatment, Sex`
# Treatment Sex emmean   SE   df lower.CL upper.CL
# BL        F     30.9 1.50 72.3     27.1     34.8
# Stress    F     29.7 1.42 60.4     26.0     33.3
# BL        M     30.3 2.45 58.9     24.0     36.6
# Stress    M     33.2 2.45 59.5     26.9     39.5
#
# Results are averaged over the levels of: TimeF
# Degrees-of-freedom method: kenward-roger
# Confidence level used: 0.95
# Conf-level adjustment: sidak method for 4 estimates
#
# $`pairwise differences of Treatment, Sex`
# contrast           estimate   SE    df t.ratio p.value
# BL F - Stress F       1.280 1.23 311.2  1.037  0.7280
# BL F - BL M           0.665 2.76  54.2  0.241  0.9950
# BL F - Stress M      -2.255 2.83  59.7 -0.796  0.8561
# Stress F - BL M      -0.615 2.80  57.1 -0.219  0.9962
# Stress F - Stress M  -3.535 2.81  58.1 -1.258  0.5931
# BL M - Stress M      -2.920 2.03 306.7 -1.437  0.4775
#
# Results are averaged over the levels of: TimeF
# Degrees-of-freedom method: kenward-roger
# P value adjustment: tukey method for comparing a family of 4 estimates

#############################################################################
#Step 10. Full for AB
#############################################################################
ABFull1<-lmer(ABDiff ~
              Treatment*Sex +
              z.Tukey.Trial14 +  StimulusID + TimeF + AggLoc +
              z.RankR + z.Tukey.AgeMos + #Sex +
              (1|animalID), data=a, REML = "F")

diagnostics.plot(ABFull1)# ok
ranef.diagn.plot(ABFull1)# no data
plot(ABFull1)
plot(residuals(ABFull1)) #ok
as.data.frame(drop1(ABFull1, test = "Chisq"))
```

```
#               Df     AIC      LRT   Pr(Chi)
# <none>         NA 5285.734       NA       NA
# z.Tukey.Trial14  1 5286.148 2.4132163 0.1203149
# StimulusID       6 5278.646 4.9116396 0.5551950 #remove
# TimeF            6 5282.022 8.2871676 0.2178105 #remove
# AggLoc           1 5285.050 1.3159132 0.2513268 #remove
# z.RankR          1 5286.278 2.5432252 0.1107683
# z.Tukey.AgeMos   1 5285.225 1.4903271 0.2221657 #remove
# Treatment:Sex    1 5283.955 0.2205712 0.6386051 #remove interaction

summary(ABFull1)
# Linear mixed model fit by maximum likelihood  ['lmerMod']
# Formula: ABDiff ~ Treatment * Sex + z.Tukey.Trial14 + StimulusID + TimeF +
#   AggLoc + z.RankR + z.Tukey.AgeMos + (1 | animalID)
# Data: a
#
# AIC      BIC   logLik deviance df.resid
# 5285.7   5369.4  -2620.9   5241.7      310
#
# Scaled residuals:
#   Min      1Q  Median      3Q     Max
# -3.9766 -0.6410 -0.0615  0.5121  4.2709
#
# Random effects:
#   Groups   Name       Variance Std.Dev.
# animalID (Intercept)     0    0.0
# Residual            421039   648.9
# Number of obs: 332, groups:  animalID, 36
#
# Fixed effects:
#                   Estimate Std. Error t value
# (Intercept)         -140.47     198.32  -0.708
# TreatmentStress      -59.23      89.29  -0.663
# SexM                 -40.02     123.17  -0.325
# z.Tukey.Trial14       57.18      36.74   1.556
# StimulusID2           93.35     125.25   0.745
# StimulusID3           19.03     137.70   0.138
# StimulusID4          102.62     128.34   0.800
# StimulusID5         -157.85     136.48  -1.157
# StimulusID6           68.86     131.12   0.525
# StimulusID7           50.70     141.84   0.357
# TimeF9               175.82     195.12   0.901
# TimeF10              278.68     182.97   1.523
# TimeF11               95.71     179.81   0.532
# TimeF12              252.22     201.48   1.252
# TimeF13               96.33     192.51   0.500
# TimeF15              451.00     234.25   1.925
# AggLocRmonkeyview     86.03      74.92   1.148
# z.RankR              -61.13      38.26  -1.598
# z.Tukey.AgeMos        47.95      39.23   1.222
# TreatmentStress:SexM  -78.65     167.44  -0.470
#
# Correlation matrix not shown by default, as p = 20 > 12.
# Use print(x, correlation=TRUE)  or
# vcov(x)       if you need it
#
# convergence code: 0
# boundary (singular) fit: see ?isSingular

ABFull2<-lmer(ABDiff ~
            Treatment +
            z.Tukey.Trial14 +
            z.RankR + Sex +
            (1|animalID), data=a, REML = "F")
```

```
diagnostics.plot(ABFull2)# ok but vertical lines
ranef.diagn.plot(ABFull2)# no data
plot(ABFull2)
plot(residuals(ABFull2)) #ok
as.data.frame(drop1(ABFull2, test = "Chisq"))
#               Df     AIC      LRT   Pr(Chi)
# <none>         NA 5270.643       NA       NA
# Treatment       1 5269.380 0.7370497 0.3906076 #keep in model
# z.Tukey.Trial14  1 5270.063 1.4194091 0.2335007 #remove
# z.RankR         1 5270.961 2.3182546 0.1278634
# Sex             1 5268.752 0.1090171 0.7412659 #remove

summary(ABFull2)
# Linear mixed model fit by maximum likelihood  ['lmerMod']
# Formula: ABDiff ~ Treatment + z.Tukey.Trial14 + z.RankR + Sex + (1 | animalID)
# Data: a
#
# AIC      BIC   logLik deviance df.resid
# 5270.6   5297.3  -2628.3   5256.6     325
#
# Scaled residuals:
#   Min      1Q  Median      3Q     Max
# -3.7752 -0.5691 -0.0869  0.5341  4.3339
#
# Random effects:
#   Groups   Name        Variance Std.Dev.
# animalID (Intercept)     0     0.0
# Residual             440377   663.6
# Number of obs: 332, groups:  animalID, 36
#
# Fixed effects:
#               Estimate Std. Error t value
# (Intercept)       98.36      54.95   1.790
# TreatmentStress   -62.81      73.13  -0.859
# z.Tukey.Trial14    43.61      36.57   1.193
# z.RankR          -59.35      38.91  -1.525
# SexM             -29.87      90.46  -0.330
#
# Correlation of Fixed Effects:
#   (Intr) TrtmnS z.T.T1 z.RnkR
# TrtmntStrss -0.632
# z.Tky.Trl14  0.046 -0.069
# z.RankR     -0.144  0.007 -0.013
# SexM        -0.406  0.007 -0.007  0.348
# convergence code: 0
# boundary (singular) fit: see ?isSingular

ABFull3<-lmer(ABDiff ~
              Treatment +
              z.RankR +
              (1|animalID), data=a, REML = "F")

diagnostics.plot(ABFull3)# ok but vertical lines
ranef.diagn.plot(ABFull3)# no data
plot(ABFull3)
plot(residuals(ABFull3)) #ok
as.data.frame(drop1(ABFull3, test = "Chisq"))
#          Df     AIC      LRT   Pr(Chi)
# <none>    NA 5268.166       NA       NA
# Treatment  1 5266.765 0.5994039 0.4388056
# z.RankR    1 5268.373 2.2068094 0.1374026

summary(ABFull3)
# Linear mixed model fit by maximum likelihood  ['lmerMod']
# Formula: ABDiff ~ Treatment + z.RankR + (1 | animalID)
```

```
# Data: a
#
# AIC      BIC   logLik deviance df.resid
# 5268.2   5287.2  -2629.1   5258.2      327
#
# Scaled residuals:
#    Min      1Q  Median      3Q     Max
# -3.8325 -0.5201 -0.0811  0.4923  4.3118
#
# Random effects:
#  Groups   Name        Variance Std.Dev.
# animalID (Intercept)     0     0.0
# Residual             442401   665.1
# Number of obs: 332, groups:  animalID, 36
#
# Fixed effects:
#                 Estimate Std. Error t value
# (Intercept)       88.15      50.28   1.753
# TreatmentStress   -56.63      73.12  -0.775
# z.RankR           -54.40      36.56  -1.488
#
# Correlation of Fixed Effects:
#   (Intr) TrtmnS
# TrtmntStrss -0.688
# z.RankR     -0.003  0.004
# convergence code: 0
# boundary (singular) fit: see ?isSingular

ABNull<-lmer(sqrt.TotalLook ~
           1 +
           (1|animalID), data=a, REML = "F")

anova(ABNull, ABFull1) #1
anova(ABNull, ABFull2) #1
anova(ABNull, ABFull3) #1
# Data: a
# Models:
#   ABNull: sqrt.TotalLook ~ 1 + (1 | animalID)
# ABFull3: ABDiff ~ Treatment + z.RankR + (1 | animalID)
#         Df    AIC    BIC  logLik deviance Chisq Chi Df Pr(>Chisq)
# ABNull   3 2469.0 2480.4 -1231.5   2463.0
# ABFull3  5 5268.2 5287.2 -2629.1   5258.2     0      2          1

FinalAB<-lmer(ABDiff ~
            Treatment +
            z.RankR +
            (1|animalID), data=a, REML = "F")

r.squaredGLMM(FinalAB)
#        R2m       R2c
# [1,] 0.008401753 0.008401753

confint.merMod(object=FinalAB)
#                  2.5 %    97.5 %
# .sig01            0.00000 156.80048
# .sigma          617.52556 719.11837
# (Intercept)     -10.68053 186.98332
# TreatmentStress -200.35294  87.08737
# z.RankR         -126.26238  17.46115

##############################################################################
#Step 11. Full model for ADDiff/TL with M&Fs
##############################################################################
ABDiff.TLFull1<-lmer(ABDiff.TL ~
                  Treatment*Sex +
```

```
                z.Tukey.Trial14 +  StimulusID + TimeF + AggLoc +
                z.RankR + z.Tukey.AgeMos + #Sex +
                (1|animalID), data=a, REML = "F")

diagnostics.plot(ABDiff.TLFull1)# ok
ranef.diagn.plot(ABDiff.TLFull1)# no data on graph
plot(ABDiff.TLFull1)
plot(residuals(ABDiff.TLFull1)) #ok
as.data.frame(drop1(ABDiff.TLFull1, test = "Chisq"))
#                Df     AIC         LRT   Pr(Chi)
# <none>         NA 634.6015          NA       NA
# z.Tukey.Trial14  1 633.0486 4.470909e-01 0.5037197 #remove
# StimulusID       6 632.4835 9.882006e+00 0.1297098
# TimeF            6 633.1857 1.058421e+01 0.1021089
# AggLoc           1 635.1967 2.595175e+00 0.1071896
# z.RankR          1 633.1104 5.089278e-01 0.4756034 #remove
# z.Tukey.AgeMos   1 633.0788 4.772449e-01 0.4896729 #remove
# Treatment:Sex    1 632.6016 1.061538e-04 0.9917795 #remove interaction

summary(ABDiff.TLFull1)
# Linear mixed model fit by maximum likelihood  ['lmerMod']
# Formula: ABDiff.TL ~ Treatment * Sex + z.Tukey.Trial14 + StimulusID +
#   TimeF + AggLoc + z.RankR + z.Tukey.AgeMos + (1 | animalID)
# Data: a
#
# AIC      BIC   logLik deviance df.resid
# 634.6    718.3   -295.3    590.6      310
#
# Scaled residuals:
#   Min      1Q   Median      3Q     Max
# -2.16693 -0.75784 -0.01838  0.84921  1.94458
#
# Random effects:
#   Groups   Name        Variance Std.Dev.
# animalID (Intercept) 0.0000   0.0000
# Residual             0.3468   0.5889
# Number of obs: 332, groups:  animalID, 36
#
# Fixed effects:
#   Estimate Std. Error t value
# (Intercept)         -0.147521   0.179994  -0.820
# TreatmentStress     -0.022135   0.081036  -0.273
# SexM                -0.079100   0.111789  -0.708
# z.Tukey.Trial14      0.022303   0.033344   0.669
# StimulusID2          0.074012   0.113680   0.651
# StimulusID3          0.003323   0.124976   0.027
# StimulusID4          0.115942   0.116481   0.995
# StimulusID5         -0.219704   0.123872  -1.774
# StimulusID6         -0.085233   0.119000  -0.716
# StimulusID7         -0.091446   0.128737  -0.710
# TimeF9               0.204812   0.177091   1.157
# TimeF10              0.297655   0.166059   1.792
# TimeF11              0.063281   0.163193   0.388
# TimeF12              0.213545   0.182862   1.168
# TimeF13              0.132185   0.174722   0.757
# TimeF15              0.396431   0.212600   1.865
# AggLocRmonkeyview    0.109754   0.067996   1.614
# z.RankR             -0.024781   0.034723  -0.714
# z.Tukey.AgeMos       0.024607   0.035607   0.691
# TreatmentStress:SexM -0.001566   0.151965  -0.010

ABDiff.TLFull2<-lmer(ABDiff.TL ~
                Treatment + Sex +
                StimulusID + AggLoc +
                TimeF +
```

```
                (1|animalID), data=a, REML = "F")

diagnostics.plot(ABDiff.TLFull2)# ok
ranef.diagn.plot(ABDiff.TLFull2)# no data on graph
plot(ABDiff.TLFull2)
plot(residuals(ABDiff.TLFull2)) #ok
as.data.frame(drop1(ABDiff.TLFull2, test = "Chisq"))
#           Df     AIC       LRT   Pr(Chi)
# <none>    NA 628.0201        NA       NA
# Treatment   1 626.1204  0.1002592 0.7515188 #keep in model
# Sex         1 626.2777  0.2575516 0.6118072 #remove
# StimulusID  6 626.0071  9.9870151 0.1251999 #remove
# AggLoc      1 628.4393  2.4191994 0.1198562
# TimeF       6 626.0952 10.0750568 0.1215266

summary(ABDiff.TLFull2)
# Linear mixed model fit by maximum likelihood  ['lmerMod']
# Formula: ABDiff.TL ~ Treatment + Sex + StimulusID + AggLoc + TimeF + (1 |      animalID)
# Data: a
#
# AIC      BIC   logLik deviance df.resid
# 628.0    696.5   -296.0    592.0      314
#
# Scaled residuals:
#   Min      1Q   Median      3Q     Max
# -2.10124 -0.76526 -0.04863  0.85407  1.88009
#
# Random effects:
#   Groups   Name        Variance Std.Dev.
# animalID (Intercept) 0.0000   0.0000
# Residual             0.3483   0.5902
# Number of obs: 332, groups:  animalID, 36
#
# Fixed effects:
#               Estimate Std. Error t value
# (Intercept)      -0.1453941  0.1783393  -0.815
# TreatmentStress   -0.0224233  0.0708115  -0.317
# SexM             -0.0384477  0.0757449  -0.508
# StimulusID2       0.0682342  0.1134234   0.602
# StimulusID3      -0.0006548  0.1245858  -0.005
# StimulusID4       0.1132434  0.1155778   0.980
# StimulusID5      -0.2261516  0.1235076  -1.831
# StimulusID6      -0.0917569  0.1183871  -0.775
# StimulusID7      -0.0908503  0.1287320  -0.706
# AggLocRmonkeyview  0.1057335  0.0678555   1.558
# TimeF9            0.1872137  0.1760952   1.063
# TimeF10           0.2855909  0.1654774   1.726
# TimeF11           0.0621144  0.1623734   0.383
# TimeF12           0.2054642  0.1827184   1.124
# TimeF13           0.1282148  0.1740249   0.737
# TimeF15           0.4007254  0.2125953   1.885

ABDiff.TLFull3<-lmer(ABDiff.TL ~
                Treatment +
                AggLoc + TimeF +
                (1|animalID), data=a, REML = "F")

diagnostics.plot(ABDiff.TLFull3)# ok
ranef.diagn.plot(ABDiff.TLFull3)# no data on graph
plot(ABDiff.TLFull3)
plot(residuals(ABDiff.TLFull3)) #ok
as.data.frame(drop1(ABDiff.TLFull3, test = "Chisq"))
#           Df     AIC       LRT   Pr(Chi)
# <none>    NA 624.1527        NA       NA
# Treatment  1 622.2874 0.1346435 0.7136653
```

```
# AggLoc     1 624.3195 2.1668158 0.1410180
# TimeF      6 620.2728 8.1201311 0.2294337 #remove

summary(ABDiff.TLFull3)
# Linear mixed model fit by maximum likelihood  ['lmerMod']
# Formula: ABDiff.TL ~ Treatment + AggLoc + TimeF + (1 | animalID)
# Data: a
#
# AIC      BIC   logLik deviance df.resid
# 624.2    666.0   -301.1    602.2      321
#
# Scaled residuals:
#   Min      1Q   Median      3Q     Max
# -2.06477 -0.75588  0.00888  0.84328  1.84942
#
# Random effects:
#   Groups   Name        Variance Std.Dev.
# animalID (Intercept) 0.0000   0.0000
# Residual             0.3591   0.5992
# Number of obs: 332, groups:  animalID, 36
#
# Fixed effects:
#                 Estimate Std. Error t value
# (Intercept)      -0.14696    0.16331  -0.900
# TreatmentStress   -0.02627    0.07160  -0.367
# AggLocRmonkeyview  0.10043    0.06812   1.474
# TimeF9            0.17116    0.17757   0.964
# TimeF10           0.25339    0.16654   1.521
# TimeF11           0.04155    0.16369   0.254
# TimeF12           0.14128    0.18339   0.770
# TimeF13           0.12549    0.17628   0.712
# TimeF15           0.34112    0.21312   1.601
#
# Correlation of Fixed Effects:
#   (Intr) TrtmnS AggLcR TimeF9 TimF10 TimF11 TimF12 TimF13
# TrtmntStrss -0.277
# AggLcRmnkyv -0.261 -0.084
# TimeF9      -0.822  0.119  0.043
# TimeF10     -0.887  0.128  0.081  0.779
# TimeF11     -0.902  0.144  0.067  0.794  0.849
# TimeF12     -0.821  0.080  0.165  0.708  0.761  0.773
# TimeF13     -0.805 -0.048  0.140  0.719  0.771  0.782  0.714
# TimeF15     -0.621 -0.107  0.029  0.581  0.620  0.630  0.569  0.605
# convergence code: 0
# boundary (singular) fit: see ?isSingular

ABDiff.TLFull4<-lmer(ABDiff.TL ~
                     Treatment +
                     AggLoc +
                     (1|animalID), data=a, REML = "F")

diagnostics.plot(ABDiff.TLFull4)# ok
ranef.diagn.plot(ABDiff.TLFull4)# no data on graph
plot(ABDiff.TLFull4)
plot(residuals(ABDiff.TLFull4)) #ok
as.data.frame(drop1(ABDiff.TLFull4, test = "Chisq"))
#           Df     AIC       LRT   Pr(Chi)
# <none>    NA 620.2728        NA       NA
# Treatment  1 618.2991 0.02624007 0.8713154
# AggLoc     1 620.3988 2.12594536 0.1448234

summary(ABDiff.TLFull4)
# Linear mixed model fit by maximum likelihood  ['lmerMod']
# Formula: ABDiff.TL ~ Treatment + AggLoc + (1 | animalID)
# Data: a
```

```
#
# AIC      BIC   logLik deviance df.resid
# 620.3    639.3   -305.1    610.3      327
#
# Scaled residuals:
#   Min      1Q  Median      3Q     Max
# -1.7569 -0.7561 -0.0278  0.8282  1.6654
#
# Random effects:
#   Groups   Name        Variance Std.Dev.
# animalID (Intercept) 0.000    0.0000
# Residual             0.368    0.6066
# Number of obs: 332, groups:  animalID, 36
#
# Fixed effects:
#                 Estimate Std. Error t value
# (Intercept)       -0.01074    0.05390  -0.199
# TreatmentStress   -0.01083    0.06687  -0.162
# AggLocRmonkeyview  0.09786    0.06701   1.460
#
# Correlation of Fixed Effects:
#   (Intr) TrtmnS
# TrtmntStrss -0.544
# AggLcRmnkyv -0.526 -0.074
# convergence code: 0
# boundary (singular) fit: see ?isSingular

anova(ABDiff.TLFull1, ABDiff.TLFull2) #0.841
anova(ABDiff.TLFull1, ABDiff.TLFull3) #0.3983
anova(ABDiff.TLFull1, ABDiff.TLFull4) #0.2914

ABDiff.TLnull<-lmer(ABDiff.TL ~
                1 +
                (1|animalID), data=a, REML = "F")

anova(ABDiff.TLnull, ABDiff.TLFull1) #0.2943
anova(ABDiff.TLnull, ABDiff.TLFull2) #0.1578
anova(ABDiff.TLnull, ABDiff.TLFull3) #0.248
anova(ABDiff.TLnull, ABDiff.TLFull4) #0.3449

#full2 is the best model
# Data: a
# Models:
#   ABDiff.TLnull: ABDiff.TL ~ 1 + (1 | animalID)
# ABDiff.TLFull2: ABDiff.TL ~ Treatment + Sex + StimulusID + AggLoc + TimeF + (1 |
#                                                       ABDiff.TLFull2:     animalID)
#               Df    AIC    BIC  logLik deviance  Chisq Chi Df Pr(>Chisq)
# ABDiff.TLnull   3 618.40 629.82 -306.20   612.40
# ABDiff.TLFull2 18 628.02 696.51 -296.01   592.02 20.381     15     0.1578

FinalABDiff.TLFull<-lmer(ABDiff.TL ~
                 Treatment + Sex +
                 AggLoc + StimulusID + TimeF +
                 (1|animalID), data=a, REML = "F")

r.squaredGLMM(FinalABDiff.TLFull)

#       R2m       R2c
# [1,] 0.05971293 0.05971293
confint.merMod(object=FinalABDiff.TLFull)
#                   2.5 %    97.5 %
# .sig01            0.00000000 0.1189843
# .sigma            0.54797648 0.6380732
# (Intercept)       -0.49594620 0.2051580
# TreatmentStress   -0.16161368 0.1167672
```

SexM -0.18735895 0.1104454
AggLocRmonkeyview -0.02764652 0.2391136
StimulusID2 -0.15471618 0.2911846
StimulusID3 -0.24554650 0.2442370
StimulusID4 -0.11394186 0.3404287
StimulusID5 -0.46892408 0.0166208
StimulusID6 -0.32446412 0.1409504
StimulusID7 -0.34389208 0.1621915
TimeF9 -0.15892738 0.5333548
TimeF10 -0.03967926 0.6108610
TimeF11 -0.25705448 0.3812832
TimeF12 -0.15369578 0.5646242
TimeF13 -0.21385676 0.4702863
TimeF15 -0.01716198 0.8186128

Appendix 3b

```
################################################################################
################################################################################
###################Repeatability & Heritability ##########################

#Sheet 1 - building the model with sex

################################################################################
#Step 1. Clear workspace, set working directory and load packages
################################################################################
#Clear workspace
ls() #this looks at what is loaded
rm(list=ls()) #this clears everything

#Set the working directory
#setwd('M:/Emily/Writing/1Papers in prep/2019 AB Heritability/AB Heritability')
#setwd("C:/Users/emmel/Desktop/R Studio and R")
setwd("D:/R Studio and R/")
#setwd("E:/R Studio and R/")

#Load Package
# install.packages("lme4")
# install.packages("tidyverse")
# install.packages("car")
# install.packages("CarData")
# install.packages("rcompanion")
install.packages("rptR")

library(tidyverse)
library(rcompanion)
library(lme4)
library(car)#or CarData in earlier forms of R
#library(carData)
library(rptR)

#load Roger Functions #source files need to be in the working directory

#source("C:/Users/emmel/Desktop/R Studio and R/Functions/diagnostic_fcns.r")
#source("C:/Users/emmel/Desktop/R Studio and R/Functions/glmm_stability.r")
source("D:/R Studio and R/Functions/diagnostic_fcns.r")
source("D:/R Studio and R/Functions/glmm_stability.r")
# #source("M:/Emily/Doing/R Users Group/R Training/Mundry/Functions/Functions/diagnostic_fcns.r")
# #source("M:/Emily/Doing/R Users Group/R Training/Mundry/Functions/Functions/glmm_stability.r")
# source("E:\R Studio and R\Functions/diagnostic_fcns.r")
# source("E:\R Studio and R\Functions/glmm_stability.r")

################################################################################
#Step 2. Import and check data:
################################################################################
#Load data
AB1KempThatcherHowarth_20200707<-read.csv(file.choose(), header=T) #select file from pop up window
d <- AB1KempThatcherHowarth_20200707

nrow(d)  #1188 (if higher do the following go back to CSV and delete ghost cells from bottom)
ncol(d) #246

str(d)
View(d)

################################################################################
#Step 3. Select data for analysis:
################################################################################
m.data<-d
#subsetting the data to remove conditions we are not interested in:
```

```
m.data<-subset(m.data, StudyNo_EH_1HC_2Rep == "2")
nrow(m.data)#147
# #Treatment
# m.data <- subset(m.data, Treatment == "BL")#| Treatment== "Stress")
# nrow(m.data)#280
# # #Baby born last 24 hours to that monkey
# m.data <- subset(m.data, BabyBornLast24HrsMum != 1)
# nrow(m.data)#277
# #Chronic illness
# m.data <- subset(m.data, IllnessChronicYN == "No")
# nrow(m.data)#273
# #Injury last 48 hours
# m.data <- subset(m.data, InjuryLast48HrsYN == "No")
# nrow(m.data)#270
# #Cleaning last 24 hours
# m.data <- subset(m.data, CleaningLast24HrsYN == "No")
# nrow(m.data)#194
# #Drug
# m.data<-subset(m.data, DrugKHClLast24HoursYN == "No")
# nrow(m.data) #193
# #Anyothertreatment
# m.data<-subset(m.data, AnyOtherTreatment == "No")
# nrow(m.data)#190
# #DisruptionInGrpOtherYN
# m.data<-subset(m.data, DisruptionInGrpOtherYN =="No")
# nrow(m.data)#171
# #OrderTreatment
# m.data<-subset(m.data, OrderTreatR =="PreFirst")
# nrow(m.data)#157
View(m.data)
summary(m.data)
str(m.data)#look for NAs in any potentialpredictor variables
MData<-m.data
nrow(MData) #147

#############################################################################
#Step 5. Ensure variables accurately labelled as factors and correct levels of each factor are being read.
#############################################################################
#Ensure random factors are coded as factors
MData$animalID <- as.factor(MData$animalID)
MData$ID <- as.factor(MData$animalID)
MData$Rank <- as.integer(MData$Rank)
MData$Sex <- as.factor(MData$Sex)
MData$MalePres <- as.factor(MData$MalePres)
MData$AggLoc <- as.factor(MData$AggLoc)
MData$StimulusID<- as.factor(MData$StimulusID)
nrow(MData)#147

#############################################################################
#Step 6. Select variables by checking correlations
#############################################################################
#check correlations between predictors INCLUDING those that are not numeric.

corr.tab=data.frame(cbind(Sex = as.numeric(MData$Sex), AgeMos=as.numeric(MData$AgeMos),
Rank=as.numeric(MData$RankR),
                ReproStat=as.numeric(MData$ReproStatR), Weight=as.numeric(MData$WeightHC),
                Alopecia=as.numeric(MData$AlopeciaScoreHC), TotalNoffspring=as.numeric(MData$TotalNoffspring),
DependOffspring=as.numeric(MData$HasDependentOffspring),
                Time=as.numeric(MData$TimeR),
                GroupSize=as.numeric(MData$GroupSizeAdults), Days = as.numeric (MData$DaysSinceLastHC),
                TrialChron=as.numeric(MData$TrialNoForStudy2), AggLoc=as.numeric(MData$AggLoc),
                StimulusID = as.numeric(MData$StimulusID), MalePres=as.numeric(MData$MalePres)))

str(corr.tab)
spear=cor(corr.tab[,1:15],method ="spearman")
```

```
spear

#                  Sex      AgeMos        Rank    ReproStat      Weight      Alopecia
# Sex           1.00000000 -0.358055101 -0.063889553  0.229671838  0.185051278 -0.4682198673
# AgeMos          -0.35805510  1.000000000 -0.197073590 -0.364753040  0.631406292  0.2701716261
# Rank          -0.06388955 -0.197073590  1.000000000  0.317039225 -0.363125565 -0.1576713918
# ReproStat       0.22967184 -0.364753040  0.317039225  1.000000000 -0.257691496 -0.4499814626
# Weight          0.18505128  0.631406292 -0.363125565 -0.257691496  1.000000000  0.2504614253
# Alopecia       -0.46821987  0.270171626 -0.157671392 -0.449981463  0.250461425  1.0000000000
# TotalNoffspring -0.08132371  0.820729627 -0.348275949 -0.495097292  0.759942565  0.4383247906
# DependOffspring -0.60200468  0.273547433 -0.072729493  0.006667801 -0.118416111  0.1722250113
# Time           0.12391750 -0.105801612  0.054703781  0.192955565 -0.077199546 -0.2570355907
# GroupSize       0.07017672 -0.106067709  0.044738673 -0.015593727 -0.074407657  0.2595926395
# Days          -0.14789715 -0.009658321 -0.170547805 -0.195018463  0.038990203  0.3604818553
# TrialChron      0.03310212 -0.026141679 -0.058772107  0.016244240 -0.027090938 -0.0210890839
# AggLoc          0.11612081  0.028558881 -0.052445145  0.078862978  0.150178484  0.0512882443
# StimulusID     -0.10596460  0.111619241 -0.004536289 -0.034120702 -0.001323898 -0.0003480307
# MalePres        0.19497360 -0.175579883  0.035367315  0.415257353  0.079256757 -0.1544217659
#                TotalNoffspring DependOffspring       Time   GroupSize        Days
# Sex             -0.08132371    -0.602004683  0.12391750  0.07017672 -0.147897147
# AgeMos            0.82072963     0.273547433 -0.10580161 -0.10606771 -0.009658321
# Rank             -0.34827595    -0.072729493  0.05470378  0.04473867 -0.170547805
# ReproStat         -0.49509729     0.006667801  0.19295556 -0.01559373 -0.195018463
# Weight            0.75994256    -0.118416111 -0.07719955 -0.07440766  0.038990203
# Alopecia          0.43832479     0.172225011 -0.25703559  0.25959264  0.360481855
# TotalNoffspring      1.00000000     0.116696652 -0.19170701 -0.01800253  0.102541745
# DependOffspring      0.11669665     1.000000000 -0.04745100 -0.22405148  0.107050324
# Time             -0.19170701    -0.047450996  1.00000000 -0.04110963 -0.035733043
# GroupSize         -0.01800253    -0.224051476 -0.04110963  1.00000000  0.158070814
# Days              0.10254175     0.107050324 -0.03573304  0.15807081  1.000000000
# TrialChron         0.01685362     0.106096177  0.34064504  0.20034361  0.440851165
# AggLoc            0.05214035    -0.036969134 -0.14250016  0.06928086 -0.034213561
# StimulusID         0.05211016     0.068791209  0.20108518  0.05625011  0.082267449
# MalePres          -0.06961909     0.249734090 -0.09346477  0.12537454  0.260009585
#               TrialChron     AggLoc    StimulusID    MalePres
# Sex            0.03310212  0.11612081 -0.1059645959  0.19497360
# AgeMos          -0.02614168  0.02855888  0.1116192406 -0.17557988
# Rank           -0.05877211 -0.05244514 -0.0045362893  0.03536731
# ReproStat       0.01624424  0.07886298 -0.0341207015  0.41525735
# Weight         -0.02709094  0.15017848 -0.0013238980  0.07925676
# Alopecia        -0.02108908  0.05128824 -0.0003480307 -0.15442177
# TotalNoffspring  0.01685362  0.05214035  0.0521101558 -0.06961909
# DependOffspring  0.10609618 -0.03696913  0.0687912091  0.24973409
# Time           0.34064504 -0.14250016  0.2010851804 -0.09346477
# GroupSize       0.20034361  0.06928086  0.0562501143  0.12537454
# Days           0.44085116 -0.03421356  0.0822674487  0.26000959
# TrialChron      1.00000000 -0.20473736  0.3721709469  0.18533244
# AggLoc         -0.20473736  1.00000000 -0.0538048078  0.12349360
# StimulusID      0.37217095 -0.05380481  1.0000000000 -0.06219643
# MalePres        0.18533244  0.12349360 -0.0621964294  1.00000000

#Remove variables that corrolarte >0.4

################################################################################
#Step 7. Transform variables
################################################################################
HData<-MData

#response variable AGG
hist(HData$AGG)
hist(transformTukey(HData$AGG))
#    lambda      W Shapiro.p.value
# 423   0.55 0.9765         0.0127
HData$Tukey.AGG<-transformTukey(HData$AGG)
```

```
hist(HData$ABDiff)# normal distribution nice!
hist(transformTukey(HData$ABDiff))
#     lambda      W Shapiro.p.value
# 441      1 0.9879         0.2297

#Total Look
hist(transformTukey(HData$TotalLook))
#     lambda      W Shapiro.p.value
# 423   0.55 0.9925         0.6323
HData$Tukey.TotalLook<-transformTukey(HData$TotalLook)

#ABDiff/TL
hist(transformTukey(HData$ABDiff.TL))
#       lambda      W Shapiro.p.value
# 441      1 0.9648       0.0008075

#AgeMos
hist(HData$AgeMos) # not great but transformations do not improve it.
hist(transformTukey(HData$AgeMos))
#     lambda      W Shapiro.p.value
# 414  0.325 0.9248       5.492e-07
HData$Tukey.AgeMos<-(transformTukey(HData$AgeMos))

#Transformation needed for Time of day
hist(HData$TimeR)
hist(transformTukey(HData$TimeR))
#      lambda      W Shapiro.p.value
# 334 -1.675 0.9284       9.533e-07
HData$Tukey.TimeR<-(transformTukey(HData$TimeR))

#Transformation needed for TrialChronological
hist(HData$TrialNoForStudy2)
hist(transformTukey(HData$TrialNoForStudy2))
#       lambda      W Shapiro.p.value
#421    0.5 0.9216       3.382e-07
HData$Tukey.TrialChronological<-(transformTukey(HData$TrialNoForStudy2))

#Transformation needed for Groupsize
hist(HData$GroupSizeAdults)
hist(transformTukey(HData$GroupSizeAdults))
# lambda      W Shapiro.p.value
#535   3.35 0.8414       2.626e-11
HData$Tukey.GroupSizeAdults<-(transformTukey(HData$GroupSizeAdults))

#Sex
summary(HData$Sex)
# F   M
# 100  47

#AggLoc
summary(HData$AggLoc)
# Lmonkeyview Rmonkeyview
# 75         72

#StimulusID
table(HData$StimulusID)
# 1  2  3  4  5  6  7
# 18 21 19 24 24 19 22

#Rank
table(HData$RankR)
# 1   2   3
# 78 53 16

#TrialChrono
```

```
table(HData$TrialNoForStudy2)
# 1  2  3  4  5  6  7
# 33 33 32 21 14 10  4

#now z-transform all the covariates
HData$z.RankR <- as.vector(scale(HData$RankR))
HData$z.Tukey.TrialChronological <- as.vector(scale(HData$Tukey.TrialChronological))
#HData$z.Tukey.AlopeciaScoreHC <- as.vector(scale(HData$Tukey.AlopeciaScoreHC))
HData$z.Tukey.TimeR <- as.vector(scale(HData$Tukey.TimeR))#transformation not needed for the VIG data set
HData$z.Tukey.AgeMos <- as.vector(scale(HData$Tukey.AgeMos))
HData$z.Tukey.GroupSize <- as.vector(scale(HData$Tukey.GroupSizeAdults))

HData$gr
par(family = "sans",mfrow = c(1,3))

plot(x = HData$animalID, y = HData$TimeR, main="Time")# yes keep in
plot(x = HData$animalID, y = HData$TrialChronological, main="Trial")# yes keep in
plot(x = HData$StimulusID, y = HData$AGG, main="StimulusID")# yes keep in

##########################END of TRANSFORMATIONS########################

#############################################################################
#Step 8. Save and / or load HData
#############################################################################
RData<-HData

write.csv(RData,
      file='RData.txt', row.names=T)

RData<-read.csv(file.choose(), row.names = 1, header=T) #select file from pop up window
View(RData)
nrow(RData)#147
ncol(RData)#258
str(RData$animalID)#Factor w/ 35 levels
table(RData$animalID)

#############################################################################
#Step 9. Start to build model
#############################################################################
a<-RData

#Set the working directory
#setwd('M:/Emily/Writing/1Papers in prep/2019 AB Heritability/AB Heritability')
#setwd("C:/Users/emmel/Desktop/R Studio and R")
#setwd("D:/R Studio and R/Functions")

library(tidyverse)
library(rcompanion)
library(lme4)
library(car)

#load Roger Functions #source files need to be in the working directory

source("D:/R Studio and R/Functions/diagnostic_fcns.r")
source("D:/R Studio and R/Functions/glmm_stability.r")
#source("C:/Users/emmel/Desktop/R Studio and R/Functions/diagnostic_fcns.r")
#source("C:/Users/emmel/Desktop/R Studio and R/Functions/glmm_stability.r")

#source("M:/Emily/Doing/R Users Group/R Training/Mundry/Functions/Functions/diagnostic_fcns.r")
#source("M:/Emily/Doing/R Users Group/R Training/Mundry/Functions/Functions/glmm_stability.r")

#############################################################################
#Step 10. AGG model
#############################################################################
AGGFull1<-lmer(Tukey.AGG~
```

```
            z.Tukey.AgeMos + z.Tukey.GroupSize +  z.RankR + Sex +
            AggLoc + StimulusID + z.Tukey.TimeR + z.Tukey.TrialChronological +
            (1|animalID), data=a, REML = "F")

diagnostics.plot(AGGFull1)# ok - clustered to left
ranef.diagn.plot(AGGFull1)# no data
plot(AGGFull1)
plot(residuals(AGGFull1))
#now look at any interaction terms
as.data.frame(drop1(AGGFull1, test = "Chisq"))

#                       Df     AIC       LRT     Pr(Chi)
# <none>                 NA 1220.359        NA         NA
# z.Tukey.AgeMos          1 1228.157 9.798922802 0.001746141
# z.Tukey.GroupSize       1 1218.615 0.256717395 0.612384371 #remove
# z.RankR                 1 1223.563 5.204936652 0.022522836
# Sex                     1 1221.738 3.379138195 0.066026571
# AggLoc                  1 1218.897 0.537988315 0.463267632 #remove
# StimulusID              1 1218.543 0.184434796 0.667589426 #remove
# z.Tukey.TimeR           1 1218.361 0.002740757 0.958248027 #remove
# z.Tukey.TrialChronological  1 1218.436 0.077417969 0.780827480 #remove

# a$StimulusID<- as.factor(a$StimulusID)
# a$StimulusID<-relevel(a$StimulusID, ref="6")
# table(a$StimulusID)
#
# # 6  1  2  3  4  5  7
# #36 41 39 38 44 41 36

AGGFull2<-lmer(Tukey.AGG~
            z.Tukey.AgeMos + z.RankR + Sex +
            (1|animalID), data=a, REML = "F")

#look at plots first - lookong for no pattern in residuals
diagnostics.plot(AGGFull2)# ok
ranef.diagn.plot(AGGFull2)# no data
plot(AGGFull2)
plot(residuals(AGGFull2))

#now look at any interaction terms
as.data.frame(drop1(AGGFull2, test = "Chisq"))

#              Df     AIC     LRT     Pr(Chi)
# <none>        NA 1211.804      NA         NA
# z.Tukey.AgeMos  1 1219.564 9.759825 0.001783668
# z.RankR         1 1215.068 5.264024 0.021770621
# Sex             1 1213.835 4.030875 0.044674772

summary(AGGFull2)
# Linear mixed model fit by maximum likelihood  ['lmerMod']
# Formula: Tukey.AGG ~ z.Tukey.AgeMos + z.RankR + Sex + (1 | animalID)
# Data: a
#
# AIC     BIC   logLik deviance df.resid
# 1211.8   1229.7   -599.9   1199.8      141
#
# Scaled residuals:
#   Min      1Q   Median      3Q     Max
# -2.25237 -0.63529  0.07209  0.57421  2.88247
#
# Random effects:
#   Groups   Name        Variance Std.Dev.
# animalID (Intercept)   0.0     0.00
# Residual             205.2    14.33
# Number of obs: 147, groups:  animalID, 33
```

```
#
# Fixed effects:
#             Estimate Std. Error t value
# (Intercept)     29.674      1.467  20.231
# z.Tukey.AgeMos   -4.272      1.298  -3.293
# z.RankR         -2.818      1.216  -2.317
# SexM             5.679      2.718   2.089
#
# Correlation of Fixed Effects:
#   (Intr) z.T.AM z.RnkR
# z.Tuky.AgMs -0.214
# z.RankR     -0.061  0.222
# SexM        -0.593  0.361  0.103
# convergence code: 0
# boundary (singular) fit: see ?isSingular

nullAGG<-lmer(Tukey.AGG~ 1+
            (1|animalID), data=a, REML = "F")

anova(nullAGG, AGGFull2)
# Data: a
# Models:
#   nullAGG: Tukey.AGG ~ 1 + (1 | animalID)
# AGGFull2: Tukey.AGG ~ z.Tukey.AgeMos + z.RankR + Sex + (1 | animalID)
# Df    AIC    BIC  logLik deviance  Chisq Chi Df Pr(>Chisq)
# nullAGG   3 1225.7 1234.7 -609.86   1219.7
# AGGFull2  6 1211.8 1229.8 -599.90   1199.8 19.911      3  0.0001771

mstab=glmm.model.stab(model.res=AGGFull2, contr=lmerControl(optimizer = "bobyqa",optCtrl = list(maxfun=2e5)))
# Error in data.frame(what = colnames(all.coeff.mat), orig = orig[colnames(all.coeff.mat)],  :
#                   arguments imply differing number of rows: 0, 1
mstab$detailed$warnings
# [1] none none none none none none none none none none none none none none none none none none none none none none none none none
# [21] none none
# Levels: none
mstab$summary[,-1]
#                       orig       min       max
# (Intercept)           29.673721 29.078780 30.492275
# z.Tukey.AgeMos        -4.272371 -4.914384 -3.751804
# z.RankR               -2.818103 -3.341223 -2.224156
# SexM                  5.678746  3.992013  6.553746
# animalID@(Intercept)@NA  0.000000  0.000000  2.276734
# Residual              14.325316 13.569939 14.566255

#remove stimulus ID to help model stability

AGGFull3<-lmer(Tukey.AGG~
            z.Tukey.AgeMos + Sex +
            (1|animalID), data=a, REML = "F")

#look at plots first - lookong for no pattern in residuals
diagnostics.plot(AGGFull3)# ok
ranef.diagn.plot(AGGFull3)# look fine
plot(AGGFull3)
plot(residuals(AGGFull3))

#now look at any interaction terms
as.data.frame(drop1(AGGFull3, test = "Chisq"))

#              Df     AIC      LRT     Pr(Chi)
# <none>        NA 1215.068       NA         NA
# z.Tukey.AgeMos  1 1219.836 6.767319 0.009284218
# Sex           1 1217.634 4.565487 0.032622404

summary(AGGFull3)
```

```
# Linear mixed model fit by maximum likelihood  ['lmerMod']
# Formula: Tukey.AGG ~ z.Tukey.AgeMos + Sex + (1 | animalID)
# Data: a
#
# AIC      BIC   logLik deviance df.resid
# 1215.1   1230.0   -602.5   1205.1      142
#
# Scaled residuals:
#   Min       1Q   Median       3Q      Max
# -2.50712 -0.65906  0.06024  0.57574  2.79077
#
# Random effects:
#   Groups   Name        Variance Std.Dev.
# animalID (Intercept)   1.245   1.116
# Residual             211.466  14.542
# Number of obs: 147, groups:  animalID, 33
#
# Fixed effects:
#               Estimate Std. Error t value
# (Intercept)      29.448      1.508  19.530
# z.Tukey.AgeMos   -3.604      1.302  -2.767
# SexM             6.316      2.785   2.268
#
# Correlation of Fixed Effects:
#   (Intr) z.T.AM
# z.Tuky.AgMs -0.208
# SexM       -0.591  0.350

mstab2=glmm.model.stab(model.res=AGGFull3, contr=lmerControl(optimizer = "bobyqa",optCtrl = list(maxfun=2e5)))

mstab2$detailed$warnings
# [1] none none none none none none none none none none none none none none none none none none none none
# [21] none none
# Levels: none
mstab2$summary[,-1]
#                         orig       min       max
# (Intercept)             29.448245 28.926110 30.191557
# z.Tukey.AgeMos          -3.604296 -4.291787 -3.071773
# SexM                    6.316043  4.906147  7.390715
# animalID@(Intercept)@NA  1.115805  0.000000  3.057214
# Residual                14.541859 13.715463 14.799304

anova(nullAGG, AGGFull2)#0.0001771
anova(nullAGG, AGGFull3)
# Data: a
# Models:
#   nullAGG: Tukey.AGG ~ 1 + (1 | animalID)
# AGGFull3: Tukey.AGG ~ z.Tukey.AgeMos + Sex + (1 | animalID)
# Df    AIC    BIC  logLik deviance  Chisq Chi Df Pr(>Chisq)
# nullAGG   3 1225.7 1234.7 -609.86   1219.7
# AGGFull3  5 1215.1 1230.0 -602.53   1205.1 14.646      2    0.00066

#model stability improved without rank

confint.merMod(object=AGGFull3)
#                 2.5 %     97.5 %
# .sig01          0.000000  6.2867649
# .sigma         12.822385 16.4227350
# (Intercept)    26.269961 32.4488504
# z.Tukey.AgeMos -6.253782 -0.9592875
# SexM           0.567838 11.9353376#


repAGG<-rpt(Tukey.AGG~ z.Tukey.AgeMos + Sex   + (1  |  animalID),  grname  =  c("animalID"),  data  =  a,  datatype  =
"Gaussian",
```

```
        nboot = 1000, npermut = 0)

print(repAGG)
# Repeatability estimation using the lmm method
#
# Repeatability for animalID
# R  = 0.033
# SE = 0.056
# CI = [0, 0.188]
# P  = 0.465 [LRT]
# NA [Permutation]

summary(repAGG)
# Repeatability estimation using the lmm method
#
# Call = rpt(formula = Tukey.AGG ~ z.Tukey.AgeMos + Sex + (1 | animalID), grname = c("animalID"), data = a, datatype =
"Gaussian", nboot = 1000, npermut = 0)
#
# Data: 147 observations
# ----------------------------------------
#
#   animalID (33 groups)
#
# Repeatability estimation overview:
#   R    SE   2.5%  97.5% P_permut  LRT_P
# 0.0334 0.0564     0  0.188      NA  0.465
#
# Bootstrapping and Permutation test:
#   N   Mean Median   2.5%  97.5%
#   boot    1000  0.051 0.0337     0  0.188
# permut     1    NA    NA    NA    NA
#
# Likelihood ratio test:
#   logLik full model = -602.5341
# logLik red. model = -602.5381
# D  = 0.00789, df = 1, P = 0.465

#############################################################################
#Step 11. Total Look model
#############################################################################

a$StimulusID<- as.factor(a$StimulusID)
a$StimulusID<-relevel(a$StimulusID, ref="6")
table(a$StimulusID)

# 6  1  2  3  4  5  7
# 19 18 21 19 24 24 22

TLFull1<-lmer(Tukey.TotalLook~
            z.Tukey.AgeMos + z.Tukey.GroupSize +  z.RankR + Sex +
            AggLoc + StimulusID + z.Tukey.TimeR + z.Tukey.TrialChronological +
            (1|animalID), data=a, REML = "F")

#look at plots first - lookong for no pattern in residuals
diagnostics.plot(TLFull1)# ok
ranef.diagn.plot(TLFull1)# look fine
plot(TLFull1)
plot(residuals(TLFull1))
as.data.frame(drop1(TLFull1, test = "Chisq"))
#                      Df     AIC        LRT     Pr(Chi)
# <none>                NA 1205.844         NA         NA
# z.Tukey.AgeMos         1 1213.656 9.8118558245 0.001733905
# z.Tukey.GroupSize      1 1204.196 0.3514626411 0.553286330 #remove
# z.RankR                1 1203.918 0.0740847228 0.785479706 #remove
# Sex                    1 1207.111 3.2665043232 0.070708143
```

```
# AggLoc                  1 1204.735 0.8912489298 0.345139084 #remove
# StimulusID              6 1201.210 7.3658589731 0.288333338
# z.Tukey.TimeR           1 1205.903 2.0587459216 0.151334673
# z.Tukey.TrialChronological  1 1203.845 0.0009065657 0.975979940 #remove

TLFull2<-lmer(Tukey.TotalLook~
            z.Tukey.AgeMos +  Sex +
            z.Tukey.TimeR + StimulusID +
            (1|animalID), data=a, REML = "F")

#look at plots first - lookong for no pattern in residuals
diagnostics.plot(TLFull2)# ok
ranef.diagn.plot(TLFull2)# look fine
plot(TLFull2)
plot(residuals(TLFull2))
as.data.frame(drop1(TLFull2, test = "Chisq"))
#             Df     AIC      LRT      Pr(Chi)
# <none>        NA 1199.090       NA          NA
# z.Tukey.AgeMos  1 1208.220 11.129759 0.0008495361
# Sex           1 1199.873  2.782852 0.0952783636
# z.Tukey.TimeR   1 1199.372  2.282374 0.1308514162
# StimulusID     6 1195.036  7.945942 0.2420905459 #remove

summary(TLFull2)

TLFull3<-lmer(Tukey.TotalLook~
            Sex +
            z.Tukey.AgeMos + z.Tukey.TimeR +
            (1|animalID), data=a, REML = "F")

#look at plots first - lookong for no pattern in residuals
diagnostics.plot(TLFull3)# ok
ranef.diagn.plot(TLFull3)# look fine
plot(TLFull3)
plot(residuals(TLFull3))
as.data.frame(drop1(TLFull3, test = "Chisq"))
#              Df     AIC      LRT      Pr(Chi)
# <none>        NA 1195.036       NA          NA
# Sex           1 1196.743  3.707662 0.0541631976
# z.Tukey.AgeMos  1 1203.876 10.840515 0.0009930309
# z.Tukey.TimeR   1 1194.308  1.272011 0.2593897323

TLFull4<-lmer(Tukey.TotalLook~
            Sex +
            #z.Tukey.AgeMos +
            (1|animalID), data=a, REML = "F")

#look at plots first - lookong for no pattern in residuals
diagnostics.plot(TLFull4)# ok
ranef.diagn.plot(TLFull4)# look fine
plot(TLFull4)
plot(residuals(TLFull4))
as.data.frame(drop1(TLFull4, test = "Chisq"))
#        Df     AIC     LRT     Pr(Chi)
# <none> NA 1203.279       NA          NA
# Sex     1 1209.043 7.763613 0.005330923

summary(TLFull4)
# Linear mixed model fit by maximum likelihood  ['lmerMod']
# Formula: Tukey.TotalLook ~ Sex + (1 | animalID)
# Data: a
#
# AIC      BIC   logLik deviance df.resid
# 1203.3   1215.2   -597.6   1195.3      143
#
```

```
# Scaled residuals:
#   Min      1Q  Median      3Q     Max
# -2.96870 -0.60666 -0.07345  0.66347  2.70960
#
# Random effects:
#  Groups   Name        Variance Std.Dev.
# animalID (Intercept)  18.73    4.328
# Residual             183.13   13.532
# Number of obs: 147, groups:  animalID, 33
#
# Fixed effects:
#             Estimate Std. Error t value
# (Intercept)   44.446      1.648  26.976
# SexM           8.747      2.931   2.985
#
# Correlation of Fixed Effects:
#   (Intr)
# SexM -0.562

nullTL<-lmer(Tukey.TotalLook~ 1+
          (1|animalID), data=a, REML = "F")

anova(nullTL, TLFull1) #0.006134
anova(nullTL, TLFull2) #0.0009712
anova(nullTL, TLFull3) #0.0001692
anova(nullTL, TLFull4) #0.005331
# Data: a
# Models:
#   nullTL: Tukey.TotalLook ~ 1 + (1 | animalID)
# TLFull4: Tukey.TotalLook ~ Sex + (1 | animalID)
# Df    AIC    BIC  logLik deviance  Chisq Chi Df Pr(>Chisq)
# nullTL   3 1209.0 1218.0 -601.52   1203.0
# TLFull4  4 1203.3 1215.2 -597.64   1195.3 7.7636      1   0.005331

mstab=glmm.model.stab(model.res=TLFull4, contr=lmerControl(optimizer = "bobyqa",optCtrl = list(maxfun=2e5)))

mstab$detailed$warnings
# [1] none none none none none none none none none none none none none none none none none none none none none
none none none none none none none none none none none none none none none none none none none none none none none
# [35] none
# Levels: none
mstab$summary[,-1]
#                      orig      min      max
# (Intercept)          44.446182 43.913869 45.084043
# SexM                 8.746761  6.311536 10.315481
# animalID@(Intercept)@NA  4.328311  1.802593  5.043591
# Residual             13.532397 12.702848 13.823016

mstab=glmm.model.stab(model.res=TLFull3, contr=lmerControl(optimizer = "bobyqa",optCtrl = list(maxfun=2e5)))
# boundary (singular) fit: see ?isSingular
# boundary (singular) fit: see ?isSingular
#boundary (singular) fit: see ?isSingular
mstab$detailed$warnings
# [1] none none none none none none none none none none none none none none none none none none none none none
none none none none none none none none none none none none none none none none none none none none none none none
# [35] none
# Levels: none
mstab$summary[,-1]
#                      orig      min      max
# (Intercept)          45.815085 45.361988 46.354273
# SexM                 5.245389  3.679512  6.555433
# z.Tukey.AgeMos       -4.327716 -4.763758 -3.826694
# z.Tukey.TimeR        1.287554  0.798343  2.176980
# animalID@(Intercept)@NA  1.294752  0.000000  2.361149
# Residual             13.470135 12.561430 13.684783
```

```
#model more stable without age

confint.merMod(object=TLFull4)
#                2.5 %    97.5 %
# .sig01       0.000000  7.796241
# .sigma      11.963075 15.470313
# (Intercept) 41.054156 47.723655
# SexM         2.794781 14.644192

repTL<-rpt(Tukey.TotalLook~ Sex +
        (1 | animalID), grname = c("animalID"), data = a, datatype = "Gaussian",
        nboot = 1000, npermut = 0)

print(repTL)
# Repeatability estimation using the lmm method
#
# Repeatability for animalID
# R  = 0.11
# SE = 0.076
# CI = [0, 0.275]
# P  = 0.0706 [LRT]
# NA [Permutation]

summary(repTL)
# Repeatability estimation using the lmm method
#
# Call = rpt(formula = Tukey.TotalLook ~ Sex + (1 | animalID), grname = c("animalID"), data = a, datatype = "Gaussian",
nboot = 1000, npermut = 0)
#
# Data: 147 observations
# ----------------------------------------
#
#   animalID (33 groups)
#
# Repeatability estimation overview:
#   R    SE   2.5%  97.5% P_permut  LRT_P
# 0.11 0.0759     0  0.275       NA  0.071
#
# Bootstrapping and Permutation test:
#   N   Mean Median   2.5%  97.5%
#   boot    1000  0.112  0.106     0  0.275
# permut     1    NA    NA    NA    NA
#
# Likelihood ratio test:
#   logLik full model = -597.6397
# logLik red. model = -598.7221
# D  = 2.16, df = 1, P = 0.0706

##############################################################################
#Step 12c. ABDiff model
##############################################################################
a$StimulusID<- as.factor(a$StimulusID)
a$StimulusID<-relevel(a$StimulusID, ref="6")
table(a$StimulusID)
# 6  1  2  3  4  5  7
# 19 18 21 19 24 24 22

ABFull1<-lmer(ABDiff~
            z.Tukey.AgeMos + z.Tukey.GroupSize +  z.RankR + Sex +
            AggLoc + StimulusID + z.Tukey.TimeR + z.Tukey.TrialChronological +
            (1|animalID), data=a, REML = "F")

#look at plots first - lookong for no pattern in residuals
diagnostics.plot(ABFull1)# ok clustered
```

```
ranef.diagn.plot(ABFull1)#
plot(ABFull1)
plot(residuals(ABFull1))
as.data.frame(drop1(ABFull1, test = "Chisq"))
#                       Df     AIC        LRT     Pr(Chi)
# <none>                 NA 2341.202         NA         NA
# z.Tukey.AgeMos          1 2344.852 5.6498436194 0.017456929
# z.Tukey.GroupSize       1 2339.204 0.0014421822 0.969706758 #remove
# z.RankR                 1 2347.595 8.3924686462 0.003767788
# Sex                     1 2339.379 0.1771644359 0.673821490 #remove
# AggLoc                  1 2342.334 3.1322652616 0.076757017
# StimulusID              6 2336.638 7.4355598921 0.282436448 #remove
# z.Tukey.TimeR           1 2339.756 0.5540035492 0.456686445 #remove
# z.Tukey.TrialChronological  1 2339.203 0.0008335541 0.976967206 #remove

ABFull2<-lmer(ABDiff~
            z.Tukey.AgeMos + z.RankR +
            AggLoc +
            (1|animalID), data=a, REML = "F")

#look at plots first - lookong for no pattern in residuals
diagnostics.plot(ABFull2)# ok clustered
ranef.diagn.plot(ABFull2)#
plot(ABFull2)
plot(residuals(ABFull2))
as.data.frame(drop1(ABFull2, test = "Chisq"))

#              Df     AIC     LRT     Pr(Chi)
# <none>        NA 2329.107      NA         NA
# z.Tukey.AgeMos  1 2332.071 4.963484 0.025887998
# z.RankR         1 2336.196 9.088612 0.002572059
# AggLoc          1 2329.589 2.482135 0.115145851 #remove

ABFull3<-lmer(ABDiff~
             z.RankR + z.Tukey.AgeMos +
            (1|animalID), data=a, REML = "F")

#look at plots first - lookong for no pattern in residuals
diagnostics.plot(ABFull3)# ok clustered
ranef.diagn.plot(ABFull3)#
plot(ABFull3)
plot(residuals(ABFull3))
as.data.frame(drop1(ABFull3, test = "Chisq"))
#              Df     AIC     LRT     Pr(Chi)
# <none>        NA 2329.589      NA         NA
# z.Tukey.AgeMos  1 2332.230 4.640633 0.031223483
# z.RankR         1 2336.950 9.360376 0.002217267

summary(ABFull3)
# Linear mixed model fit by maximum likelihood  ['lmerMod']
# Formula: ABDiff ~ z.RankR + z.Tukey.AgeMos + (1 | animalID)
# Data: a
#
# AIC     BIC   logLik deviance df.resid
# 2329.6   2344.5  -1159.8   2319.6     142
#
# Scaled residuals:
#   Min      1Q  Median      3Q     Max
# -2.89608 -0.68180  0.00564  0.58121  3.06740
#
# Random effects:
#   Groups   Name       Variance Std.Dev.
# animalID (Intercept)     0    0
# Residual           417334   646
# Number of obs: 147, groups:  animalID, 33
```

```
#
# Fixed effects:
#             Estimate Std. Error t value
# (Intercept)      53.26      53.28   1.000
# z.RankR        -169.62      54.56  -3.109
# z.Tukey.AgeMos  -118.47      54.56  -2.171
#
# Correlation of Fixed Effects:
#   (Intr) z.RnkR
# z.RankR     0.000
# z.Tuky.AgMs 0.000  0.199
# convergence code: 0
# boundary (singular) fit: see ?isSingular

nullAB<-lmer(ABDiff~ 1+
            (1|animalID), data=a, REML = "F")

anova(nullAB, ABFull1) #0.05588
anova(nullAB, ABFull2) #0.002822
anova(nullAB, ABFull3) #0.003058
# Data: a
# Models:
#   nullAB: ABDiff ~ 1 + (1 | animalID)
# ABFull3: ABDiff ~ z.RankR + z.Tukey.AgeMos + (1 | animalID)
# Df    AIC    BIC  logLik deviance Chisq Chi Df Pr(>Chisq)
# nullAB   3 2337.2 2346.1 -1165.6   2331.2
# ABFull3  5 2329.6 2344.5 -1159.8   2319.6 11.58      2   0.003058 **

mstab=glmm.model.stab(model.res=ABFull3, contr=lmerControl(optimizer = "bobyqa",optCtrl = list(maxfun=2e5)))
# Error in data.frame(what = colnames(all.coeff.mat), orig = orig[colnames(all.coeff.mat)],  :
#                   arguments imply differing number of rows: 0, 1
mstab$detailed$warnings
# [1] none none none none none none none none none none none none none none none none none none none none
none none none none none none none none none none none none none none
# [35] none
# Levels: none
mstab$summary[,-1]
#                        orig       min          max
# (Intercept)             53.2585   33.32686  7.874903e+01
# z.RankR               -169.6179 -196.36619 -1.360905e+02
# z.Tukey.AgeMos         -118.4677 -143.43826 -9.356806e+01
# animalID@(Intercept)@NA    0.0000    0.00000  3.655614e-05
# Residual               646.0139  617.90413  6.592031e+02

confint.merMod(object=ABFull3)
#                2.5 %    97.5 %
# .sig01           0.0000 202.18150
# .sigma         578.6561 727.52976
# (Intercept)    -51.8552 158.37693
# z.RankR       -277.2537 -61.97971
# z.Tukey.AgeMos -226.1050 -10.82992

repABD<-rpt(ABDiff~ z.RankR + z.Tukey.AgeMos +
          (1 | animalID), grname = c("animalID"), data = a, datatype = "Gaussian",
        nboot = 1000, npermut = 0)

print(repABD)
# Repeatability for animalID
# R  = 0
# SE = 0.041
# CI = [0, 0.141]
# P  = 1 [LRT]
# NA [Permutation]

##########################################################################
```

```
#Step 11. Full model for ADDiff/TL with M&Fs
################################################################################

ABDiffTLFull1<-lmer(ABDiff.TL~
          z.Tukey.AgeMos + z.Tukey.GroupSize +  z.RankR + Sex +
          AggLoc + StimulusID + z.Tukey.TimeR + z.Tukey.TrialChronological +
          (1|animalID), data=a, REML = "F")

#look at plots first - lookong for no pattern in residuals
diagnostics.plot(ABDiffTLFull1)# ok clustered
ranef.diagn.plot(ABDiffTLFull1)#
plot(ABDiffTLFull1)
plot(residuals(ABDiffTLFull1))
as.data.frame(drop1(ABDiffTLFull1, test = "Chisq"))
#                      Df     AIC      LRT     Pr(Chi)
# <none>                NA 248.5458       NA         NA
# z.Tukey.AgeMos         1 248.2148 1.6689940 0.196393340
# z.Tukey.GroupSize      1 247.3270 0.7811594 0.376786798 #remove
# z.RankR                1 255.1559 8.6100545 0.003343123
# Sex                    1 247.3310 0.7851351 0.375575227 #remove
# AggLoc                 1 251.8797 5.3338293 0.020915383
# StimulusID             6 242.6691 6.1232998 0.409520772 #remove
# z.Tukey.TimeR          1 247.7332 1.1873452 0.275865717 #remove
# z.Tukey.TrialChronological  1 246.8021 0.2562591 0.612701907 #remove

ABDiffTLFull2<-lmer(ABDiff.TL~
               z.Tukey.AgeMos + z.RankR +
               AggLoc +
               (1|animalID), data=a, REML = "F")

#look at plots first - lookong for no pattern in residuals
diagnostics.plot(ABDiffTLFull2)# ok clustered
ranef.diagn.plot(ABDiffTLFull2)#
plot(ABDiffTLFull2)
plot(residuals(ABDiffTLFull2))
as.data.frame(drop1(ABDiffTLFull2, test = "Chisq"))
#            Df     AIC     LRT     Pr(Chi)
# <none>       NA 236.8440      NA         NA
# z.Tukey.AgeMos  1 236.0152 1.171189 0.279156986 #remove
# z.RankR        1 244.4047 9.560698 0.001987875
# AggLoc         1 239.9507 5.106734 0.023833150

ABDiffTLFull3<-lmer(ABDiff.TL~
               z.RankR +
               AggLoc +
               (1|animalID), data=a, REML = "F")

#look at plots first - lookong for no pattern in residuals
diagnostics.plot(ABDiffTLFull3)# ok clustered
ranef.diagn.plot(ABDiffTLFull3)#
plot(ABDiffTLFull3)
plot(residuals(ABDiffTLFull3))
as.data.frame(drop1(ABDiffTLFull3, test = "Chisq"))
#        Df     AIC     LRT     Pr(Chi)
# <none>  NA 236.0152      NA         NA
# z.RankR  1 242.5916 8.576413 0.003405456
# AggLoc   1 238.9146 4.899469 0.026864953

summary(ABDiffTLFull3)
# Linear mixed model fit by maximum likelihood  ['lmerMod']
# Formula: ABDiff.TL ~ z.RankR + AggLoc + (1 | animalID)
# Data: a
#
# AIC     BIC   logLik deviance df.resid
# 236     251    -113     226     142
```

```
#
# Scaled residuals:
#   Min      1Q   Median      3Q      Max
# -1.95514 -0.70484 -0.00254  0.81549  2.53164
#
# Random effects:
#   Groups   Name        Variance Std.Dev.
# animalID (Intercept) 0.0000   0.000
# Residual             0.2724   0.522
# Number of obs: 147, groups:  animalID, 33
#
# Fixed effects:
#                  Estimate Std. Error t value
# (Intercept)       -0.06711    0.06031  -1.113
# z.RankR           -0.13095    0.04326  -3.027
# AggLocRmonkeyview  0.19249    0.08624   2.232
#
# Correlation of Fixed Effects:
#   (Intr) z.RnkR
# z.RankR     -0.037
# AggLcRmnkyv -0.700  0.053
# convergence code: 0
# boundary (singular) fit: see ?isSingular

nullABDiff.TL<-lmer(ABDiff.TL~ 1+
           (1|animalID), data=a, REML = "F")

anova(nullABDiff.TL, ABDiffTLFull1) #0.03904
anova(nullABDiff.TL, ABDiffTLFull2) #0.001879
anova(nullABDiff.TL, ABDiffTLFull3) #0.00103
# Data: a
# Models:
#   nullABDiff.TL: ABDiff.TL ~ 1 + (1 | animalID)
# ABDiffTLFull3: ABDiff.TL ~ z.RankR + AggLoc + (1 | animalID)
# Df    AIC    BIC  logLik deviance  Chisq Chi Df Pr(>Chisq)
# nullABDiff.TL  3 245.77 254.74 -119.89   239.77
# ABDiffTLFull3  5 236.01 250.97 -113.01   226.01 13.757      2    0.00103 **

mstab=glmm.model.stab(model.res=ABDiffTLFull3, contr=lmerControl(optimizer = "bobyqa",optCtrl = list(maxfun=2e5)))
# Error in data.frame(what = colnames(all.coeff.mat), orig = orig[colnames(all.coeff.mat)],  :
#                    arguments imply differing number of rows: 0, 1
mstab$detailed$warnings
# [1] none none none none none none none none none none none none none none none none none none none none
none none none none none none none none none none none none none none
# [35] none
# Levels: none
mstab$summary[,-1]
#                          orig        min        max
# (Intercept)            -0.0671114 -0.09293115 -0.04152639
# z.RankR                -0.1309543 -0.14827637 -0.10411293
# AggLocRmonkeyview       0.1924865  0.15457821  0.22847696
# animalID@(Intercept)@NA  0.0000000  0.00000000  0.04682630
# Residual                0.5219521  0.51076634  0.5278986

confint.merMod(object=ABDiffTLFull3)
#                   2.5 %      97.5 %
# .sig01            0.0000000  0.20598277
# .sigma            0.4627671  0.58781346
# (Intercept)       -0.1863702  0.05189221
# z.RankR           -0.2188916 -0.04549527
# AggLocRmonkeyview  0.0223535  0.36261959

repABDTL<-rpt(ABDiff.TL~ z.RankR + AggLoc +
          (1 | animalID), grname = c("animalID"), data = a, datatype = "Gaussian",
        nboot = 1000, npermut = 0)
```

```
print(repABDTL)
# Repeatability for animalID
# R  = 0.006
# SE = 0.043
# CI = [0, 0.145]
# P  = 1 [LRT]
# NA [Permutation]
```

Appendix 3c

```
################################################################################
###################Repeatability & Heritability ##########################
#Sheet 2 - running the repeatability with the model
################################################################################
#Step 1. Clear workspace, set working directory and load packages
################################################################################

#Clear workspace
ls() #this looks at what is loaded
rm(list=ls()) #this clears everything

#Set the working directory
#setwd('M:/Emily/Writing/1Papers in prep/2019 AB Heritability/AB Heritability')
#setwd("C:/Users/emmel/Desktop/R Studio and R")
setwd("D:/R Studio and R/")

#Load Package
#install.packages("lme4")
#install.packages("tidyverse")
#install.packages("car")
#install.packages("CarData")
#install.packages("rcompanion")
#install.packages("MCMCglmm")
#install.packages("pedantics")

library(tidyverse)
library(rcompanion)
library(lme4)
library(car)#or CarData in earlier forms of R
library(MCMCglmm)
library(pedantics)
#library(carData)

#load Roger Functions #source files need to be in the working directory
#source("C:/Users/emmel/Desktop/R Studio and R/Functions/diagnostic_fcns.r")
#source("C:/Users/emmel/Desktop/R Studio and R/Functions/glmm_stability.r")
source("D:/R Studio and R/Functions/diagnostic_fcns.r")
source("D:/R Studio and R/Functions/glmm_stability.r")
#source("M:/Emily/Doing/R Users Group/R Training/Mundry/Functions/Functions/diagnostic_fcns.r")
#source("M:/Emily/Doing/R Users Group/R Training/Mundry/Functions/Functions/glmm_stability.r")

################################################################################
#Step 2. Import and check data:
################################################################################
#Data were previously transformed and scaled - models were built using lmer function

RData<-read.csv(file.choose(), row.names = 1, header=T) #select file from pop up window
View(RData)
nrow(RData)#157
ncol(RData)#256
str(RData$animalID)#Factor w/ 35 levels

NData<-RData
################################################################################
#Step 3. Set the prior for Agg
################################################################################

#first we set the prior. I tried several and compared them and they did not alter the results materially (with some giving
the same exactly) #Therefore use prior1

p.var<-var(NData$Tukey.AGG ,na.rm=TRUE)
prior1<-list(R=list(V=matrix(p.var/2),nu=1), # this is for the residual variance
        G=list(G1=list(V=matrix(p.var/2),nu=1))) # this is for monkey  (/2 because we have R + G1 )
```

```
# prior2<-list(R=list(V=matrix(p.var/2),nu=0.001), # this is for the residual variance
#            G=list(G1=list(V=matrix(p.var/2),nu=0.002))) # this is for monkey  (/2 because we have R + G1 )
#
# priorflat <- list(R = list(V=1, nu=0.002), G = list(G1 = list(V=1, nu=0.002)))
# prior1#nu=1
# prior2#nu=0.001
# priorflat #V=1, nu=0.002
##############################################################################
#Step 4. Agg model
##############################################################################
NData$StimulusID<- as.factor(NData$StimulusID)
NData$StimulusID<-relevel(NData$StimulusID, ref="6")
table(NData$StimulusID)
# 6  1  2  3  4  5  7
# 21 19 21 21 27 25 23

AGGFull<-(MCMCglmm(Tukey.AGG~
            AggLoc + as.factor(StimulusID) + z.Tukey.TimeR + z.Tukey.TrialChronological +
            z.RankR + z.Tukey.GroupSizeAdults + HasDependentOffspring + ReproStat,
             random=~animalID, data=NData, nitt=501000,burnin=1000,thin=500, verbose = FALSE, prior=prior1))#>1hr to
run

autocorr(AGGFull$VCV) # good - all <0.1 @10000 lag
# , , animalID
#
# animalID       units
# Lag 0     1.000000000 -0.090957498
# Lag 500   -0.020114276  0.032925697
# Lag 2500  -0.026629100  0.006085755
# Lag 5000  -0.015333293  0.014048167
# Lag 25000 -0.002071926  0.057586604
#
# , , units
#
# animalID      units
# Lag 0    -0.090957498  1.00000000
# Lag 500   0.015718044  0.01194446
# Lag 2500  -0.008891017  0.07885415
# Lag 5000  -0.034999192 -0.00875961
# Lag 25000 -0.058954107 -0.03281430
save(AGGFull, file = "AGGFull.rda")
load("AGGFull.rda")
plot(AGGFull)#plots look ok

summary(AGGFull)
# post.mean l-95% CI u-95% CI eff.samp pMCMC
# (Intercept)               18.6399   8.7046  29.1340   1000.0 0.002 **
# AggLocRmonkeyview          2.2265  -2.0785   5.9412   1000.0 0.300
# as.factor(StimulusID)1     6.2254  -2.1179  14.6153    946.9 0.154
# as.factor(StimulusID)2     9.3113   1.6655  17.0296   1000.0 0.018 *
# as.factor(StimulusID)3     8.4267   0.6557  15.0085   1000.0 0.018 *
# as.factor(StimulusID)4     6.9263   0.4750  13.8159   1000.0 0.042 *
# as.factor(StimulusID)5     8.7000   0.9179  15.4324   1000.0 0.016 *
# as.factor(StimulusID)7     8.4019   0.9553  15.4633   1000.0 0.018 *
# z.Tukey.TimeR             -0.5098  -2.7128   1.4917   1000.0 0.658
# z.Tukey.TrialChronological  -0.6070  -3.7862   2.6637   1000.0 0.740
# z.RankR                   -3.7019  -6.8815  -0.3874   1000.0 0.030 *
# z.Tukey.GroupSizeAdults    0.6625  -2.6372   3.4321   1000.0 0.682
# HasDependentOffspringYes   -3.9014 -13.6717   5.3488   1000.0 0.408
# ReproStatImplanted         -6.6205 -20.9281   7.0269   1000.0 0.354
# ReproStatMaleBreeding      -2.0992 -13.2030   8.2008   1000.0 0.706
# ReproStatNurse             1.6240 -12.3606  14.4437   1000.0 0.796
# ReproStatPregnant          0.2095  -9.9041  11.9304   1000.0 0.954
# ReproStatWeanerGroup       8.1360  -3.5322  21.0662   1000.0 0.204
```

```
RAGGFull<-AGGFull$VCV[,"animalID"]/(AGGFull$VCV[,"animalID"]+AGGFull$VCV[,"units"])
HPDinterval(RAGGFull)
# lower     upper
# var1 0.07463268 0.3569047
# attr(,"Probability")
# [1] 0.95
posterior.mode(RAGGFull)
# var1
# 0.07463268 0.3569047

FinalAgg<-(MCMCglmm(Tukey.AGG~
             AggLoc + as.factor(StimulusID) + z.RankR + ReproStat,
           random=~animalID, data=NData, nitt=501000,burnin=1000,thin=500, verbose = FALSE, prior=prior1))#>1hr to
run

autocorr(FinalAgg$VCV) # good - all <0.1 @10000 lag
# , , animalID
#
# animalID       units
# Lag 0      1.000000000 -0.007677455
# Lag 500    0.059381648 -0.030290275
# Lag 2500  -0.003149543 -0.027431039
# Lag 5000   0.049039421 -0.005754231
# Lag 25000 -0.024414953 -0.002980791
#
# , , units
#
# animalID       units
# Lag 0     -0.007677455  1.000000000
# Lag 500   -0.003622680  0.018661566
# Lag 2500   0.027819632 -0.027447896
# Lag 5000  -0.037953570 -0.009471895
# Lag 25000  0.023536333  0.007922029

save(FinalAgg, file = "FinalAgg.rda")
load("FinalAgg.rda")
plot(FinalAgg)#plots look ok
###trace for ID suggests between between-individual variance

summary(FinalAgg)
# post.mean l-95% CI u-95% CI eff.samp  pMCMC
# (Intercept)            18.3015   8.5494  28.0314     1000 <0.001 ***
# AggLocRmonkeyview       2.2961  -1.6860   6.5750     1000  0.244
# as.factor(StimulusID)1    6.5889  -0.8643  13.9788     1142  0.090 .
# as.factor(StimulusID)2    9.8697   2.3536  17.1811     1000  0.012 *
# as.factor(StimulusID)3    8.2174   0.8924  15.2562     1330  0.028 *
# as.factor(StimulusID)4    7.0427   0.8320  14.3755     1112  0.046 *
# as.factor(StimulusID)5    8.4206   1.2033  14.8955     1348  0.024 *
# as.factor(StimulusID)7    8.5636   1.0549  15.3675     1182  0.012 *
# z.RankR                -3.4210  -6.4819  -0.4431     1096  0.038 *
# ReproStatImplanted      -7.0435 -18.3168   6.8166     1000  0.280
# ReproStatMaleBreeding   -1.7422 -11.5236   9.2516     1000  0.744
# ReproStatNurse          -2.3935 -11.0234   7.2488     1000  0.600
# ReproStatPregnant       -0.4131 -10.3784   9.4806     1000  0.940
# ReproStatWeanerGroup     8.8653  -3.0197  18.9805     1000  0.120

#Now we look at repeatability by comparing within animal v between animal variance
RAGGFinal<-FinalAgg$VCV[,"animalID"]/(FinalAgg$VCV[,"animalID"]+FinalAgg$VCV[,"units"])

HPDinterval(RAGGFinal)
# lower     upper
# var1 0.07650525 0.314328
# attr(,"Probability")
# [1] 0.95
```

```
posterior.mode(RAGGFinal)
# var1
#0.1560441

#Rep of Agg(full) = 0.17 (0.07-0.36)
#Rep of Agg(final) = 0.16 (0.08-0.32)

##############################################################################
#Step 5. Set the prior for ABDiff
##############################################################################
#first we set the prior. I tried several and compared them and they did not alter the results materially (with some giving the same exactly) #Therefore use prior1

p.var<-var(NData$ABDiff ,na.rm=TRUE)
prior1<-list(R=list(V=matrix(p.var/2),nu=1), # this is for the residual variance
         G=list(G1=list(V=matrix(p.var/2),nu=1))) # this is for monkey  (/2 because we have R + G1 )

# prior2<-list(R=list(V=matrix(p.var/2),nu=0.001), # this is for the residual variance
#            G=list(G1=list(V=matrix(p.var/2),nu=0.002))) # this is for monkey  (/2 because we have R + G1 )
#
# priorflat <- list(R = list(V=1, nu=0.002), G = list(G1 = list(V=1, nu=0.002)))
# prior1#nu=1
# prior2#nu=0.001
# priorflat #V=1, nu=0.002

##############################################################################
#Step 6. ABDiff Model
##############################################################################
NData$StimulusID<- as.factor(NData$StimulusID)
NData$StimulusID<-relevel(NData$StimulusID, ref="1")
table(NData$StimulusID)
# 1  6  2  3  4  5  7
# 19 21 21 21 27 25 23

FullAB<-(MCMCglmm(ABDiff~
               AggLoc + as.factor(StimulusID) + z.Tukey.TimeR + z.Tukey.TrialChronological +
               z.RankR + z.Tukey.GroupSizeAdults + HasDependentOffspring + ReproStat,
               random=~animalID, data=NData, nitt=501000,burnin=1000,thin=500, verbose = FALSE, prior=prior1))#>1hr to run

autocorr(FullAB$VCV)# good - all <0.1 @10000 lag
# , , animalID
#
# animalID      units
# Lag 0     1.000000000 -0.04352061
# Lag 500    0.003665766  0.01144281
# Lag 2500   0.013214922 -0.01873841
# Lag 5000  -0.028412439 -0.05834985
# Lag 25000  0.001299733 -0.01400843
#
# , , units
#
# animalID       units
# Lag 0     -0.043520609  1.000000000
# Lag 500    0.082612246  0.001038968
# Lag 2500  -0.029299305  0.044685131
# Lag 5000  -0.003256222 -0.032304328
# Lag 25000 -0.008991266 -0.057319917

save(FullAB, file = "FullAB.rda")
load("FullAB.rda")
plot(FullAB)#plots look ok

summary(FullAB)
# post.mean l-95% CI u-95% CI eff.samp pMCMC
```

```
# (Intercept)               -233.44  -836.74   286.30   1223.7 0.422
# AggLocRmonkeyview          162.94   -84.80   385.87    824.4 0.144
# as.factor(StimulusID)6      41.25  -402.12   484.35   1122.5 0.864
# as.factor(StimulusID)2     331.85  -113.82   796.62    507.3 0.152
# as.factor(StimulusID)3     379.30   -59.64   817.87    835.1 0.088 .
# as.factor(StimulusID)4     134.86  -299.50   574.19   1000.0 0.568
# as.factor(StimulusID)5     364.86   -39.58   795.07   1118.5 0.072 .
# as.factor(StimulusID)7     267.76  -143.66   704.37   1000.0 0.230
# z.Tukey.TimeR             -55.89  -179.59    68.15   1108.7 0.378
# z.Tukey.TrialChronological  -38.40  -219.10   137.59   1229.8 0.678
# z.RankR                   -190.69  -365.80   -18.83   1000.0 0.032 *
# z.Tukey.GroupSizeAdults     28.14  -150.58   187.79    850.3 0.740
# HasDependentOffspringYes   -175.15  -726.18   339.50    898.0 0.522
# ReproStatImplanted         -331.20  -985.34   379.20   1115.4 0.362
# ReproStatMaleBreeding      -108.24  -670.78   466.84   1000.0 0.690
# ReproStatNurse              123.30  -597.88   845.29    819.9 0.722
# ReproStatPregnant            94.95  -427.88   676.96   1000.0 0.702
# ReproStatWeanerGroup        112.50  -552.49   700.88   1000.0 0.738

#Now we look at repeatability by comparing within animal v between animal variance
RFullAB<-FullAB$VCV[,"animalID"]/(FullAB$VCV[,"animalID"]+FullAB$VCV[,"units"])
HPDinterval(RFullAB)
# lower     upper
# var1 0.04660585 0.2694086
# attr(,"Probability")
# [1] 0.95
posterior.mode(RFullAB)
# var1
# 0.1153922

FinalAB<-(MCMCglmm(ABDiff~
            AggLoc + z.RankR,
            random=~animalID, data=NData, nitt=501000,burnin=1000,thin=500, verbose = FALSE, prior=prior1))#>1hr to
run

autocorr(FinalAB$VCV)# good - all <0.1 @10000 lag
# , , animalID
#
# animalID       units
# Lag 0     1.000000000 -0.03947822
# Lag 500   -0.009362651  0.03260650
# Lag 2500  -0.023798359  0.01263998
# Lag 5000  -0.017928942  0.03013636
# Lag 25000  0.028933225  0.03664499
#
# , , units
#
# animalID       units
# Lag 0    -0.039478224  1.000000000
# Lag 500    0.001755346  0.051721477
# Lag 2500   0.016995956  0.002563551
# Lag 5000  -0.001714756  0.015015046
# Lag 25000  0.041351556 -0.072542482

save(FinalAB, file = "FinalAB.rda")
load("FinalAB.rda")
plot(FinalAB)#plots look ok

summary(FinalAB)
# post.mean l-95% CI u-95% CI eff.samp pMCMC
# (Intercept)         -47.94  -212.58   114.27    690.5 0.540
# AggLocRmonkeyview    168.06   -69.74   350.28    902.3 0.120
# z.RankR             -150.88  -277.86   -21.23   1000.0 0.028 *

#Now we look at repeatability by comparing within animal v between animal variance
```

```
RAB<-FinalAB$VCV[,"animalID"]/(FinalAB$VCV[,"animalID"]+FinalAB$VCV[,"units"])
HPDinterval(RAB)
# lower     upper
# var1 0.0347673 0.2132721
# attr(,"Probability")
# [1] 0.95

posterior.mode(RAB)
# var1
# 0.108593

#Rep for AB(full) = 0.12 (0.05-0.27)
#Rep fo AB(final) = 0.11 (0.03-0.21)

##############################################################################
#Step 7. Set the prior for Total Look
##############################################################################

#first we set the prior. I tried several and compared them and they did not alter the results materially (with some giving the same exactly) #Therefore use prior1

p.var<-var(NData$Tukey.TotalLook ,na.rm=TRUE)
prior1<-list(R=list(V=matrix(p.var/2),nu=1), # this is for the residual variance
         G=list(G1=list(V=matrix(p.var/2),nu=1))) # this is for monkey  (/2 because we have R + G1 )

# prior2<-list(R=list(V=matrix(p.var/2),nu=0.001), # this is for the residual variance
#            G=list(G1=list(V=matrix(p.var/2),nu=0.002))) # this is for monkey  (/2 because we have R + G1 )
#
# priorflat <- list(R = list(V=1, nu=0.002), G = list(G1 = list(V=1, nu=0.002)))
# prior1#nu=1
# prior2#nu=0.001
# priorflat #V=1, nu=0.002

##############################################################################
#Step 8. TL Model
##############################################################################
FullTL<-(MCMCglmm(Tukey.TotalLook ~
               AggLoc + as.factor(StimulusID) + z.Tukey.TimeR + z.Tukey.TrialChronological +
               z.RankR + z.Tukey.GroupSizeAdults + HasDependentOffspring + ReproStat,
             random=~animalID, data=NData, nitt=501000,burnin=1000,thin=500, verbose = FALSE, prior=prior1))#>1hr to run

autocorr(FullTL$VCV)# good - all <0.1 @10000 lag
# , , animalID
#
# animalID      units
# Lag 0     1.00000000 -0.10655117
# Lag 500   -0.03824092 -0.05835682
# Lag 2500   0.03448704 -0.06831792
# Lag 5000  -0.05535022 -0.01418915
# Lag 25000 -0.03419272  0.03143798
#
# , , units
#
# animalID       units
# Lag 0     -0.106551172  1.000000000
# Lag 500    0.007868086 -0.029287916
# Lag 2500   0.039211734  0.010850037
# Lag 5000  -0.013913249  0.002068844
# Lag 25000 -0.033647035  0.046425628

save(FullTL, file = "FullTL.rda")
load("FullTL.rda")
plot(FullTL)#plots look ok
###trace for ID suggests between between-individual variance
```

```
summary(FullTL)
# post.mean  l-95% CI  u-95% CI eff.samp  pMCMC
# (Intercept)              50.76328  39.81404  61.75312   1000.0 <0.001 ***
# AggLocRmonkeyview         -1.50969  -5.84206   3.30162   1000.0  0.522
# as.factor(StimulusID)6    -9.75202 -19.21852  -0.06196   1000.0  0.040 *
# as.factor(StimulusID)2    -0.53791  -9.34248   8.50481   1113.6  0.908
# as.factor(StimulusID)3    -1.39877 -11.23229   7.71753   1000.0  0.758
# as.factor(StimulusID)4    -1.01146  -9.86814   7.29756   1000.0  0.826
# as.factor(StimulusID)5    -3.48593 -11.90249   5.75255   1000.0  0.410
# as.factor(StimulusID)7    -1.89040 -10.42446   7.70347   1000.0  0.672
# z.Tukey.TimeR             1.24758  -1.45934   3.68978   1000.0  0.342
# z.Tukey.TrialChronological  -0.32120  -3.84604   3.54228   1237.3  0.850
# z.RankR                  -0.93332  -4.32172   2.38692   1000.0  0.592
# z.Tukey.GroupSizeAdults    0.98114  -2.57191   4.19916   1000.0  0.568
# HasDependentOffspringYes   -2.88586 -13.26170   7.98537    647.9  0.612
# ReproStatImplanted        -3.95323 -20.31441  10.60084   1000.0  0.620
# ReproStatMaleBreeding     -1.53220 -12.96253  12.20652   1096.6  0.838
# ReproStatNurse             0.13415 -16.95243  14.53675    897.0  0.982
# ReproStatPregnant         -2.40784 -15.30787   9.27638   1000.0  0.732
# ReproStatWeanerGroup       9.49068  -3.95561  23.82781   1000.0  0.170

#Now we look at repeatability by comparing within animal v between animal variance
RFullTL<-FullTL$VCV[,"animalID"]/(FullTL$VCV[,"animalID"]+FullTL$VCV[,"units"])
HPDinterval(RFullTL)
# lower     upper
# var1 0.04489078 0.3094399
# attr(,"Probability")
# [1] 0.95
posterior.mode(RFullTL)
# var1
# 0.1530909

FinalTL<-(MCMCglmm(Tukey.TotalLook ~
            ReproStat,
            random=~animalID, data=NData, nitt=501000,burnin=1000,thin=500, verbose = FALSE, prior=prior1))#>1hr to
run

autocorr(FinalTL$VCV)# good - all <0.1 @10000 lag
# , , animalID
#
# animalID       units
# Lag 0     1.000000000 -0.104382730
# Lag 500   -0.017845920  0.005410027
# Lag 2500  -0.018187134  0.008888926
# Lag 5000  -0.009480837 -0.020958932
# Lag 25000 -0.011755793 -0.001716847
#
# , , units
#
# animalID      units
# Lag 0     -0.1043827299 1.000000000
# Lag 500    0.0421323976 0.015969437
# Lag 2500   0.0425262673 0.047376271
# Lag 5000   0.0006728327 0.065644386
# Lag 25000 -0.0357828151 0.001855337

save(FinalTL, file = "FinalTL.rda")
load("FinalTL.rda")
plot(FinalTL)#plots look ok
###trace for ID suggests between between-individual variance

summary(FinalTL)
# post.mean l-95% CI u-95% CI eff.samp  pMCMC
# (Intercept)            47.802   38.666   56.299     1136 <0.001 ***
```

```
#   ReproStatImplanted      -5.360  -20.005    8.380     1000  0.452
# ReproStatMaleBreeding    -1.399  -12.643   10.039     1000  0.826
# ReproStatNurse          -3.990  -13.332    7.251     1066  0.474
# ReproStatPregnant       -3.601  -15.119    7.372     1000  0.558
# ReproStatWeanerGroup     9.678   -1.534   21.581     1000  0.096 .

#Now we look at repeatability by comparing within animal v between animal variance
RTOTAL<-FinalTL$VCV[,"animalID"]/(FinalTL$VCV[,"animalID"]+FinalTL$VCV[,"units"])
HPDinterval(RTOTAL)
# lower     upper
# var1 0.04856367 0.2833076
# attr(,"Probability")
# [1] 0.95
posterior.mode(RTOTAL)
# var1
# 0.1277456

#Rep for TL(full) = 0.14 (0.05-0.31)
#Rep for TL(final) = 0.12 (0.05-0.28)
```

Appendix 3d

```
#Clear workspace
#Clear workspace
ls() #this looks at what is loaded
rm(list=ls()) #this clears everything
setwd("D:/R Studio and R/")
#par(mfrow = c(2,1))

#Time
d<-read.csv(file.choose(), header=T)
View(d)
nrow(d)  #7
ncol(d) #5

plot(d$Time, d$Mean_TL,
    ylim=range(c(d$Mean_TL-d$SE_TL, d$Mean_TL+d$SE_TL)),
    xlab="Time of AB trial", ylab="mean TL (ms)", col="black", pch=16, cex=(d$Count)/20,
    arrows(d$Time, d$Mean_TL-d$SE_TL, d$Time, d$Mean_TL+d$SE_TL, code =3, angle = 90, length=0.1))

tmp <- lm(d$Mean_TL ~ d$Time, na.action = na.omit)
abline(tmp)
#title("a)", adj=0, line=0.5)

#AggLoc
s<-read.csv(file.choose(), header=T)
View(s)
nrow(s)  #2
ncol(s) #5

y<-s[1:2, "Mean"]
y.SE <- s[1:2, "SE"]

y
# 1109.4611  973.3947
y.SE
#[1] 49.60222 52.56709

mid<-barplot(y)

barplot(y, names.arg=c("Left", "Right"), beside=TRUE, ylim= c(800, 1200),
      xlab="Side of threat face presentation", ylab="mean TL (ms)", xpd=FALSE)
box(bty="l")
arrows(mid, y-y.SE, mid, y+y.SE, code =3, angle = 90, length=0.1)
#title("c)", adj=0, line=0.5)

#barplot

s<-read.csv(file.choose(), header=T)
View(s)
nrow(s)  #4
ncol(s) #5

test<- structure(c(s$TL_mean), .Dim=c(2L,2L), .Dimnames=list(c("Female", "Male"), c("Baseline", "Post-stressor")))
mid<-barplot(test, beside=T)

barplot(test, beside=T, xlab="Treatment", ylab="mean TL (ms)", col=c("grey80", "grey40"), ylim=range(0, 1700))

legend("topright", inset=.02, title="Sex",
     c("Female","Male"), fill=c("grey80", "grey40"), horiz=T, cex=1, box.lty = 0)

arrows(mid, s$TL_mean-s$TL_SE, mid, s$TL_mean+s$TL_SE, code =3, angle = 90, length=0.1)

title("d)", adj=0, line=0.5)
```

Appendix 3e

```
#Clear workspace
ls() #this looks at what is loaded
rm(list=ls()) #this clears everything
setwd("D:/R Studio and R/")

#par(mfrow = c(2,1))

#Sex&Agg
d<-read.csv(file.choose(), header=T)
View(d)
nrow(d)  #2
ncol(d) #5

y<-d[1:2, "mean_AGG"]
y.SE <- d[1:2, "SE_Agg"]

y
# 599.5657 837.1398
y.SE
#[1] 36.92714 59.35883

mid<-barplot(y)

barplot(y, names.arg=c("Female", "Male"), beside=TRUE, ylim= c(400, 1000),
      xlab="Sex", ylab="mean THR (ms)", xpd=FALSE)
box(bty="l")
arrows(mid, y-y.SE, mid, y+y.SE, code =3, angle = 90, length=0.1)
#title("c)", adj=0, line=0.5)

#Age & Agg
s<-read.csv(file.choose(), header=T)
View(s)
nrow(s)  #13
ncol(s) #11

plot(s$Age, s$Mean_agg,
    ylim=range(c(s$Mean_agg-s$SE_agg, s$Mean_agg+s$SE_agg)),
    xlab="Age of macaque (years)", ylab="mean THR (ms)", col="black", pch=16, cex=(s$Count)/10,
    arrows(s$Age, s$Mean_agg-s$SE_agg, s$Age, s$Mean_agg+s$SE_agg, code =3, angle = 90, length=0.1))

tmp <- lm(s$Mean_agg ~ s$Age, na.action = na.omit)
abline(tmp)
#title("a)", adj=0, line=0.5)

plot(s$Age, s$Mean_TL,
    ylim=range(c(s$Mean_TL-s$SE_TL, s$Mean_TL+s$SE_TL)),
    xlab="Age of macaque (years)", ylab="mean TL (ms)", col="black", pch=16, cex=(s$Count)/10,
    arrows(s$Age, s$Mean_TL-s$SE_TL, s$Age, s$Mean_TL+s$SE_TL, code =3, angle = 90, length=0.1))

tmp <- lm(s$Mean_TL ~ s$Age, na.action = na.omit)
abline(tmp)
#title("a)", adj=0, line=0.5)

plot(s$Age, s$mean_ABD,
    ylim=range(c(s$mean_ABD-(s$SE_ABD+100), s$mean_ABD+(s$SE_ABD+100))),
    xlab="Age of macaque (years)", ylab="mean ABDiff (ms)", col="black", pch=16, cex=(s$Count)/10,
    arrows(s$Age, s$mean_ABD-s$SE_ABD, s$Age, s$mean_ABD+s$SE_ABD, code =3, angle = 90, length=0.1))

tmp <- lm(s$mean_ABD ~ s$Age, na.action = na.omit)
abline(tmp)
#title("a)", adj=0, line=0.5)

#Sex&Agg
```

```
d<-read.csv(file.choose(), header=T)
View(d)
nrow(d)  #2
ncol(d) #10

y<-d[1:2, "mean_TL"]
y.SE <- d[1:2, "SE_TL"]

y
# 112.424 1601.065
y.SE
#[1] 40.57520 72.93564

mid<-barplot(y)

barplot(y, names.arg=c("Female", "Male"), beside=TRUE, ylim= c(800, 1800),
      xlab="Sex", ylab="mean TL (ms)", xpd=FALSE)
box(bty="l")
arrows(mid, y-y.SE, mid, y+y.SE, code =3, angle = 90, length=0.1)
#title("c)", adj=0, line=0.5)

#ABD_Rank
d<-read.csv(file.choose(), header=T)
View(d)
nrow(d)  #3
ncol(d) #8

y<-d[1:3, "ABDIff_mean"]
y.SE <- d[1:3, "ABD_SE"]

y
# 121.38690 -21.41379  44.73404
y.SE
#[1] 55.05098 156.63501  86.52487

mid<-barplot(y)

barplot(y, names.arg=c("High", "Middle", "Low"), beside=TRUE, ylim= c(-200, 250),
      xlab="Macaque rank", ylab="mean ABDiff (ms)", xpd=FALSE)
#box(bty="0")
arrows(mid, y-y.SE, mid, y+y.SE, code =3, angle = 90, length=0.1)
#title("c)", adj=0, line=0.5)

y<-d[1:3, "ABDIff.TL_mean"]
y.SE <- d[1:3, "ABDTL_SE"]

y
# 0.09140556 -0.03096812 -0.09818820
y.SE
#[1] 0.04010396 0.06193093 0.11628346

mid<-barplot(y)

barplot(y, names.arg=c("High", "Middle", "Low"), beside=TRUE, ylim= c(-0.25, 0.2),
      xlab="Macaque rank", ylab="mean ABDiff/TL", xpd=FALSE)
#box(bty="0")
arrows(mid, y-y.SE, mid, y+y.SE, code =3, angle = 90, length=0.1)
#title("c)", adj=0, line=0.5)

#Aggloc
d<-read.csv(file.choose(), header=T)
View(d)
nrow(d)  #2
ncol(d) #5
```

```
y<-d[1:2, "mean"]
y.SE <- d[1:2, "SE"]

y
# -0.0625406  0.125276
y.SE
#[1]0.04997658 0.04168618

mid<-barplot(y)

barplot(y, names.arg=c("Left", "Right"), beside=TRUE, ylim= c(-0.2, 0.2),
      xlab="Side of threat face presentation", ylab="mean ABDiff/TL", xpd=FALSE)
#box(bty="l")
arrows(mid, y-y.SE, mid, y+y.SE, code =3, angle = 90, length=0.1)
#title("c)", adj=0, line=0.5)
```

Appendix 4a

```
##############################################################################
############################Behvaiour#########################################

##############################################################################
#Step 1. Clear workspace, set working directory and load packages
##############################################################################
#Clear workspace
ls() #this looks at what is loaded
rm(list=ls()) #this clears everything

#Set the working directory
#setwd('M:/Emily/Writing/1Papers in prep/2019 AB Heritability/AB Heritability')
#setwd("C:/Users/emmel/Desktop/R Studio and R")
setwd("D:/R Studio and R")
#setwd("M:/Emily/Doing/Postgraduates/Emmeline Howarth/Data/Chapt6 Genetics")

#Load Package
# install.packages("lme4")
# install.packages("tidyverse")
# install.packages("car")
# install.packages("CarData")
# install.packages("rcompanion")

library(tidyverse)
library(rcompanion)
library(lme4)
library(car)#or CarData in earlier forms of R
#library(carData)

#install.packages("MuMIn")
library(MuMIn)
#citation("MuMIn")

#load Roger Functions #source files need to be in the working directory

#source("C:/Users/emmel/Desktop/R Studio and R/Functions/diagnostic_fcns.r")
#source("C:/Users/emmel/Desktop/R Studio and R/Functions/glmm_stability.r")
source("D:/R Studio and R/Functions/diagnostic_fcns.r")
source("D:/R Studio and R/Functions/glmm_stability.r")
#source("M:/Emily/Doing/R Users Group/R Training/Mundry/Functions/Functions/diagnostic_fcns.r")
#source("M:/Emily/Doing/R Users Group/R Training/Mundry/Functions/Functions/glmm_stability.r")

##############################################################################
#Step 2. Import and check data:
##############################################################################

#Load data
GroupedBehaviourandABDifAverages_20200728<-read.csv(file.choose(), header=T) #select file from pop up window
d <- GroupedBehaviourandABDifAverages_20200728

nrow(d)  #72 (if higher do the following go back to CSV and delete ghost cells from bottom)
ncol(d) #36

str(d)
View(d)

MData<-d
##############################################################################
#Step 4. Check data reading correctly
##############################################################################

MData$Sex <- as.factor(MData$Sex)
str(MData$Sex) #Factor w/ 2 levels
```

```
MData$Treatment <- as.factor(MData$Treatment)
str(MData$Treatment) #Factor w/ 2 level
MData$Age <- as.numeric(MData$Age)
str(MData$Age) #num

MData$AggBehav<-as.numeric(MData$AGGRESSIVEAPPROACHSUM_mean)
str(MData$AggBehav)
MData$FearBehav<-as.numeric(MData$FEARAFFILIATIVEAPPROCHSUM_mean)
str(MData$FearBehav)
MData$DistractBehav<-as.numeric(MData$DISTRACTIONSUM_mean)
str(MData$DistractBehav)
MData$AnxietyBehav<-as.numeric(MData$ANXIETYSUM_mean)
str(MData$AnxietyBehav)
MData$ForageBehav<-as.numeric(MData$FORAGINGSUM_mean)
str(MData$ForageBehav)
MData$InactiveBehav<-as.numeric(MData$INACTIVITYSUM_mean)
str(MData$InactiveBehav)

MData$Inactive_mean<-as.numeric(MData$Inactive_mean)
str(MData$Inactive_mean)
MData$Positive_social_mean<-as.numeric(MData$Positive_social_mean)
str(MData$Positive_social_mean)
MData$Negative_social_mean<-as.numeric(MData$Negative_social_mean)
str(MData$Negative_social_mean)
MData$exploratory_mean<-as.numeric(MData$exploratory_mean)
str(MData$exploratory_mean)
MData$Stress_mean<-as.numeric(MData$Stress_mean)
str(MData$Stress_mean)
MData$foraging_mean<-as.numeric(MData$foraging_mean)
str(MData$foraging_mean)
MData$locomotion_mean<-as.numeric(MData$locomotion_mean)
str(MData$locomotion_mean)
MData$manipulate_object_mean<-as.numeric(MData$manipulate_object_mean)
str(MData$manipulate_object_mean)

#############################################################################
#Step 5. TRANSFORM DATA - BEHAVIOUR
#############################################################################

hist(MData$AggBehav)
hist(transformTukey(MData$AggBehav))
  # lambda    W Shapiro.p.value
# 413    0.3 0.61      1.559e-12
MData$Tukey.AggBehav<-transformTukey(MData$AggBehav)

hist(MData$FearBehav)
hist(transformTukey(MData$FearBehav))
#    lambda      W Shapiro.p.value
# 436  0.875 0.9607        0.02422
MData$Tukey.FearBehav<-transformTukey(MData$FearBehav)

hist(MData$DistractBehav)
hist(transformTukey(MData$DistractBehav))
#     lambda      W Shapiro.p.value
# 415   0.35 0.9823         0.4061
MData$Tukey.DistractBehav<-transformTukey(MData$DistractBehav)

hist(MData$AnxietyBehav)
hist(transformTukey(MData$AnxietyBehav))
#     lambda      W Shapiro.p.value
# 411   0.25 0.9829          0.438
MData$Tukey.AnxietyBehav<-transformTukey(MData$AnxietyBehav)

hist(MData$ForageBehav)
hist(transformTukey(MData$ForageBehav))
```

```
#    lambda     W Shapiro.p.value
# 431   0.75 0.9607         0.0242
MData$Tukey.ForageBehav<-transformTukey(MData$ForageBehav)

hist(MData$InactiveBehav)
hist(transformTukey(MData$InactiveBehav))
#    lambda     W Shapiro.p.value
# 416  0.375 0.985         0.5485
MData$Tukey.InactiveBehav<-transformTukey(MData$InactiveBehav)

hist(MData$Inactive_mean)
hist(transformTukey(MData$Inactive_mean))
#    lambda     W Shapiro.p.value
# 416  0.375 0.985         0.5485
MData$Tukey.Inactive<-transformTukey(MData$Inactive_mean)

hist(MData$Positive_social_mean)
hist(transformTukey(MData$Positive_social_mean))
#    lambda     W Shapiro.p.value
# 436  0.875 0.9519       0.007898
MData$Tukey.Positive<-transformTukey(MData$Positive_social_mean)

hist(MData$Negative_social_mean)
hist(transformTukey(MData$Negative_social_mean))
#    lambda     W Shapiro.p.value
# 417    0.4 0.7736      3.554e-09
MData$Tukey.Negative<-transformTukey(MData$Negative_social_mean)

hist(MData$Stress_mean)
hist(transformTukey(MData$Stress_mean))
#      lambda     W Shapiro.p.value
# 411   0.25 0.9829          0.438
MData$Tukey.Stress<-transformTukey(MData$Stress_mean)

hist(MData$exploratory_mean)
hist(transformTukey(MData$exploratory_mean))
#     lambda     W Shapiro.p.value
# 430  0.725 0.9805         0.3282
MData$Tukey.Explore<-transformTukey(MData$exploratory_mean)

#############################################################################
#Step 6. TRANSFORM DATA - THE REST
#############################################################################

HData<-MData

#AGG
hist(HData$AGG_mean)
hist(transformTukey(HData$AGG_mean))
#    lambda     W Shapiro.p.value
# 418  0.425 0.9732         0.1265
HData$Tukey.AGG<-transformTukey(HData$AGG_mean)

#ABDiff
hist(HData$ABD_mean)# normal distribution nice!
hist(transformTukey(HData$ABD_mean))
#    lambda     W Shapiro.p.value
# 441      1 0.9621        0.02888

#Total Look
hist(transformTukey(HData$TL_mean))
#    lambda     W Shapiro.p.value
# 426  0.625 0.989         0.7886
HData$Tukey.TotalLook<-transformTukey(HData$TL_mean)
```

```
#ABDiff/TL
hist(HData$ABDTL_mean)# normal distribution nice!
hist(transformTukey(HData$ABDTL_mean))
#    lambda     W Shapiro.p.value
# 441      1 0.9866         0.6463

#Treatment
table(HData$Treatment)
# BL Stress
# 36    36

#Sex
table(HData$Sex)
# F  M
# 54 18

#Age
hist(HData$Age)
hist(transformTukey(HData$Age))
#    lambda     W Shapiro.p.value
# 424  0.575 0.9505         0.0066
HData$Tukey.Agek<-transformTukey(HData$Age)
HData$z.Tukey.Age <- as.vector(scale(HData$Tukey.Age))

##########################END of TRANSFORMATIONS#########################

#############################################################################
#Step 7. Save and / or load HData
#############################################################################

write.csv(HData,
       file='BData.txt', row.names=T)

BData<-read.csv(file.choose(), row.names = 1, header=T) #select file from pop up window
View(BData)
nrow(BData)#72
ncol(BData)#52

#############################################################################
#Step 7. Start to build model - AggBehav
#############################################################################

a<-BData

AggBehavFull1<-lmer(Tukey.AggBehav~
                Treatment*Sex + z.Tukey.Age +
                (1|animalID), data=a, REML = "F")

#look at plots first - lookong for no pattern in residuals
diagnostics.plot(AggBehavFull1)# not good. All in line
ranef.diagn.plot(AggBehavFull1)# look fine
plot(AggBehavFull1) #not good
plot(residuals(AggBehavFull1)) #clustered

#now look at any interaction terms
as.data.frame(drop1(AggBehavFull1, test = "Chisq"))
#             Df     AIC      LRT   Pr(Chi)
# <none>       NA 410.0555       NA       NA
# z.Tukey.Age    1 408.5294 0.4738720 0.4912112
# Treatment:Sex  1 408.1620 0.1064787 0.7441892

summary(AggBehavFull1)
# Linear mixed model fit by maximum likelihood  ['lmerMod']
# Formula: Tukey.AggBehav ~ Treatment * Sex + z.Tukey.Age + (1 | animalID)
# Data: a
```

```
#
# AIC     BIC   logLik deviance df.resid
# 410.1    426.0   -198.0    396.1      65
#
# Scaled residuals:
#   Min     1Q  Median     3Q    Max
# -1.7613 -0.5104 -0.2171  0.2037  3.5216
#
# Random effects:
#   Groups   Name        Variance Std.Dev.
# animalID (Intercept)  0.2734  0.5228
# Residual             14.0670  3.7506
# Number of obs: 72, groups:  animalID, 36
#
# Fixed effects:
#                   Estimate Std. Error t value
# (Intercept)          0.4726     0.7407   0.638
# TreatmentStress      1.5066     1.0210   1.476
# SexM                 5.2760     1.5389   3.429
# z.Tukey.Age         -0.3465     0.5017  -0.691
# TreatmentStress:SexM  -0.6667     2.0416  -0.327
#
# Correlation of Fixed Effects:
#   (Intr) TrtmnS SexM   z.Tk.A
# TrtmntStrss -0.692
# SexM        -0.523  0.338
# z.Tukey.Age  0.179 -0.019 -0.321
# TrtmntSt:SM  0.345 -0.500 -0.664  0.003

#TOO MANY ZEROS - SUBSET FOR JUST ANIMALS WITH AGG BEHAV DATA

agg.data <- subset(a, AggresiveYN == "Y")
nrow(agg.data)#32

table(agg.data$Sex)
# F  M
# 16 16

AggBehavFull2<-lmer(Tukey.AggBehav~
                Treatment*Sex + z.Tukey.Age +
                (1|animalID), data=agg.data, REML = "F")

#look at plots first - lookong for no pattern in residuals
diagnostics.plot(AggBehavFull2)# not good. All in line
ranef.diagn.plot(AggBehavFull2)# look fine
plot(AggBehavFull2) #not good
plot(residuals(AggBehavFull2)) #clustered

#now look at any interaction terms
as.data.frame(drop1(AggBehavFull2, test = "Chisq"))
#             Df     AIC      LRT   Pr(Chi)
# <none>       NA 201.7446       NA       NA
# z.Tukey.Age    1 200.0733 0.3286384 0.5664619 #remove
# Treatment:Sex  1 201.3369 1.5922487 0.2070052 #remove interaction

summary(AggBehavFull2)
# Linear mixed model fit by maximum likelihood  ['lmerMod']
# Formula: Tukey.AggBehav ~ Treatment * Sex + z.Tukey.Age + (1 | animalID)
# Data: agg.data
#
# AIC     BIC   logLik deviance df.resid
# 201.7    212.0    -93.9    187.7       25
#
# Scaled residuals:
#   Min     1Q  Median     3Q    Max
```

```
# -1.6827 -0.5434  0.1103  0.4975  1.8586
#
# Random effects:
#  Groups   Name       Variance Std.Dev.
# animalID (Intercept)  0.00    0.000
# Residual             20.68    4.547
# Number of obs: 32, groups:  animalID, 16
#
# Fixed effects:
#                  Estimate Std. Error t value
# (Intercept)          1.8417     1.6114   1.143
# TreatmentStress      5.0610     2.2740   2.226
# SexM                 4.6768     2.3747   1.969
# z.Tukey.Age         -0.6338     1.1028  -0.575
# TreatmentStress:SexM  -4.1085     3.2156  -1.278
#
# Correlation of Fixed Effects:
#  (Intr) TrtmnS SexM   z.Tk.A
# TrtmntStrss -0.707
# SexM        -0.695  0.483
# z.Tukey.Age  0.067 -0.016 -0.288
# TrtmntSt:SM  0.499 -0.707 -0.677  0.001
# convergence code: 0
# boundary (singular) fit: see ?isSingur

AggBehavFull3<-lmer(Tukey.AggBehav~
                    Treatment + Sex +
                    (1|animalID), data=agg.data, REML = "F")

#look at plots first - lookong for no pattern in residuals
diagnostics.plot(AggBehavFull3)# not good. All in line
ranef.diagn.plot(AggBehavFull3)# look fine
plot(AggBehavFull3) #not good
plot(residuals(AggBehavFull3)) #clustered

#now look at any interaction terms
as.data.frame(drop1(AggBehavFull3, test = "Chisq"))
#          Df     AIC     LRT    Pr(Chi)
# <none>    NA 199.6478      NA        NA
# Treatment  1 200.7452 3.097486 0.07841328
# Sex        1 199.4111 1.763341 0.18420833 #remove

summary(AggBehavFull3)
# Linear mixed model fit by maximum likelihood  ['lmerMod']
# Formula: Tukey.AggBehav ~ Treatment + Sex + (1 | animalID)
# Data: agg.data
#
# AIC      BIC   logLik deviance df.resid
# 199.6    207.0   -94.8    189.6      27
#
# Scaled residuals:
#  Min      1Q  Median      3Q     Max
# -1.73906 -0.62543  0.00144  0.61679  2.04937
#
# Random effects:
#  Groups   Name       Variance Std.Dev.
# animalID (Intercept)  0.00    0.000
# Residual             21.95    4.685
# Number of obs: 32, groups:  animalID, 16
#
# Fixed effects:
#                Estimate Std. Error t value
# (Intercept)       2.930      1.434   2.043
# TreatmentStress   2.987      1.656   1.803
# SexM              2.230      1.656   1.346
```

```
#
# Correlation of Fixed Effects:
#   (Intr) TrtmnS
# TrtmntStrss -0.577
# SexM        -0.577  0.000
# convergence code: 0
# boundary (singular) fit: see ?isSingular

AggBehavFull4<-lmer(Tukey.AggBehav~
                  Treatment +
                  (1|animalID), data=agg.data, REML = "F")

#look at plots first - lookong for no pattern in residuals
diagnostics.plot(AggBehavFull4)# not good. All in line
ranef.diagn.plot(AggBehavFull4)# no data
plot(AggBehavFull4) #not good
plot(residuals(AggBehavFull4)) #clustered

#now look at any interaction terms
as.data.frame(drop1(AggBehavFull4, test = "Chisq"))
#           Df     AIC     LRT   Pr(Chi)
# <none>    NA 199.4111      NA       NA
# Treatment  1 200.3499 2.93881 0.0864744

summary(AggBehavFull4)
# Linear mixed model fit by maximum likelihood  ['lmerMod']
# Formula: Tukey.AggBehav ~ Treatment + (1 | animalID)
# Data: agg.data
#
# AIC      BIC   logLik deviance df.resid
# 199.4    205.3    -95.7    191.4       28
#
# Scaled residuals:
#   Min      1Q  Median      3Q     Max
# -1.4603 -0.8400  0.1571  0.6814  2.1093
#
# Random effects:
#   Groups   Name        Variance Std.Dev.
# animalID (Intercept)  0.00    0.000
# Residual             23.19    4.816
# Number of obs: 32, groups:  animalID, 16
#
# Fixed effects:
#                 Estimate Std. Error t value
# (Intercept)       4.045      1.204   3.360
# TreatmentStress    2.987      1.703   1.754
#
# Correlation of Fixed Effects:
#   (Intr)
# TrtmntStrss -0.707
# convergence code: 0
# boundary (singular) fit: see ?isSingular

nullAgg.data<-lmer(Tukey.AggBehav~ 1+
                  (1|animalID), data=agg.data, REML = "F")

anova(nullAgg.data, AggBehavFull2)#0.1583
anova(nullAgg.data, AggBehavFull3)#0.09527
anova(nullAgg.data, AggBehavFull4)#0.08647
# Data: agg.data
# Models:
# nullAgg.data: Tukey.AggBehav ~ 1 + (1 | animalID)
# AggBehavFull4: Tukey.AggBehav ~ Treatment + (1 | animalID)
#              Df    AIC    BIC  logLik deviance  Chisq Chi Df Pr(>Chisq)
# nullAgg.data   3 200.35 204.75 -97.175   194.35
```

```
# AggBehavFull4  4 199.41 205.27 -95.706   191.41 2.9388      1    0.08647

#USing the 16 macaques (8 female) that did display agg beaviour there was non-significant trend for them to be more agg following the HC.
r.squaredGLMM(AggBehavFull4)
#           R2m       R2c
# [1,] 0.0903218 0.0903218

confint.merMod(object=AggBehavFull4)
#                  2.5 %   97.5 %
# .sig01          0.0000000 2.938927
# .sigma          3.8169597 6.287773
# (Intercept)     1.6127648 6.477208
# TreatmentStress -0.4526592 6.426702

mstab=glmm.model.stab(model.res=AggBehavFull4, contr=lmerControl(optimizer = "bobyqa",optCtrl =
list(maxfun=2e5)))
mstab$detailed$warnings
# [1] none none none none none none none none none none none none none none none none none none none none
# [21] none none
# Levels: none
mstab$summary[,-1]
#                    orig      min         max
# (Intercept)           4.044986 3.367827 4.314652e+00
# TreatmentStress        2.987022 2.151641 3.914310e+00
# animalID@(Intercept)@NA 0.000000 0.000000 1.796546e-07
# Residual              4.815597 4.324776 4.914692e+00

##############################################################################
#Step 8. Start to build model - Fear and affiliaive behav
##############################################################################

FearBehavFull1<-lmer(Tukey.FearBehav~
                  Treatment*Sex + z.Tukey.Age +
                  (1|animalID), data=a, REML = "F")

#look at plots first - lookong for no pattern in residuals
diagnostics.plot(FearBehavFull1)# okay
ranef.diagn.plot(FearBehavFull1)# look fine
plot(FearBehavFull1) #not good
plot(residuals(FearBehavFull1)) #okay

#now look at any interaction terms
as.data.frame(drop1(FearBehavFull1, test = "Chisq"))
#             Df     AIC         LRT   Pr(Chi)
# <none>       NA 1597.09          NA       NA
# z.Tukey.Age    1 1596.41 1.3199468269 0.2506016
# Treatment:Sex  1 1595.09 0.0001452148 0.9903853 #remove interaction

summary(FearBehavFull1)
# Linear mixed model fit by maximum likelihood  ['lmerMod']
# Formula: Tukey.FearBehav ~ Treatment * Sex + z.Tukey.Age + (1 | animalID)
# Data: a
#
# AIC      BIC   logLik deviance df.resid
# 1597.1   1613.0   -791.5   1583.1       65
#
# Scaled residuals:
#   Min       1Q   Median       3Q      Max
# -1.77640 -0.58099 -0.04698  0.42726  2.20416
#
# Random effects:
#   Groups   Name        Variance  Std.Dev.
# animalID (Intercept)  40655986  6376
# Residual             170552999 13060
```

```
# Number of obs: 72, groups:  animalID, 36
#
# Fixed effects:
#                  Estimate Std. Error t value
# (Intercept)         30166.22    2850.36  10.583
# TreatmentStress      -4070.83    3555.27  -1.145
# SexM               -15542.20    5957.26  -2.609
# z.Tukey.Age          2413.62    2082.93   1.159
# TreatmentStress:SexM    -85.66    7108.78  -0.012
#
# Correlation of Fixed Effects:
#   (Intr) TrtmnS SexM   z.Tk.A
# TrtmntStrss -0.628
# SexM       -0.527  0.306
# z.Tukey.Age  0.193 -0.022 -0.344
# TrtmntSt:SM  0.312 -0.500 -0.598  0.003

FearBehavFull2<-lmer(Tukey.FearBehav~
                  Treatment + Sex + z.Tukey.Age +
                  (1|animalID), data=a, REML = "F")

#look at plots first - lookong for no pattern in residuals
diagnostics.plot(FearBehavFull2)# okay
ranef.diagn.plot(FearBehavFull2)# look fine
plot(FearBehavFull2) #not good
plot(residuals(FearBehavFull2)) #okay

#now look at any interaction terms
as.data.frame(drop1(FearBehavFull2, test = "Chisq"))
#            Df      AIC      LRT     Pr(Chi)
# <none>      NA 1595.090       NA         NA
# Treatment    1 1594.814 1.724388 0.189129162
# Sex          1 1602.431 9.341102 0.002240708
# z.Tukey.Age  1 1594.410 1.320044 0.250584223 #remove

FearBehavFull3<-lmer(Tukey.FearBehav~
                  Treatment + Sex +
                  (1|animalID), data=a, REML = "F")

#look at plots first - lookong for no pattern in residuals
diagnostics.plot(FearBehavFull3)# okay
ranef.diagn.plot(FearBehavFull3)# look fine
plot(FearBehavFull3) #not good
plot(residuals(FearBehavFull3)) #okay

#now look at any interaction terms
as.data.frame(drop1(FearBehavFull3, test = "Chisq"))
#          Df      AIC      LRT     Pr(Chi)
# <none>    NA 1594.410       NA         NA
# Treatment  1 1594.063 1.652771 0.198582136
# Sex        1 1600.480 8.070478 0.004499225

summary(FearBehavFull3)
# Linear mixed model fit by maximum likelihood  ['lmerMod']
# Formula: Tukey.FearBehav ~ Treatment + Sex + (1 | animalID)
# Data: a
#
# AIC      BIC   logLik deviance df.resid
# 1594.4   1605.8   -792.2   1584.4       67
#
# Scaled residuals:
#   Min      1Q   Median      3Q     Max
# -1.74691 -0.53257 -0.01579  0.47891  2.05636
#
# Random effects:
```

```
#  Groups   Name       Variance  Std.Dev.
# animalID (Intercept)  45071914  6714
# Residual             170780663 13068
# Number of obs: 72, groups:  animalID, 36
#
# Fixed effects:
#               Estimate Std. Error t value
# (Intercept)       29543       2684  11.007
# TreatmentStress   -4006       3080  -1.300
# SexM             -13223       4396  -3.008
#
# Correlation of Fixed Effects:
#   (Intr) TrtmnS
# TrtmntStrss -0.574
# SexM        -0.409  0.000

mstabFearBehav1=glmm.model.stab(model.res=FearBehavFull3, contr=lmerControl(optimizer = "bobyqa",optCtrl =
list(maxfun=2e5)))
mstabFearBehav1$detailed$warnings
# [1] none none none none none none none none none none none none none none none none none none none none none
# [21] none none
# Levels: none
mstabFearBehav1$summary[,-1]
#                        orig       min       max
# (Intercept)           29543.254  28563.936  30583.650
# TreatmentStress        -4005.836  -5081.575  -2780.992
# SexM                  -13223.144 -15235.249 -11433.495
# animalID@(Intercept)@NA   6713.562   6007.655   7611.745
# Residual               13068.308  12192.445  13253.299

nullFearBehav<-lmer(Tukey.FearBehav~ 1+
                (1|animalID), data=a, REML = "F")

anova(nullFearBehav, FearBehavFull1) #0.02608
anova(nullFearBehav, FearBehavFull2) #0.01149
anova(nullFearBehav, FearBehavFull3) #0.007738
# Data: a
# Models:
#   nullFearBehav: Tukey.FearBehav ~ 1 + (1 | animalID)
# FearBehavFull3: Tukey.FearBehav ~ Treatment + Sex + (1 | animalID)
# Df    AIC    BIC  logLik deviance  Chisq Chi Df Pr(>Chisq)
# nullFearBehav   3 1600.1 1607.0 -797.07   1594.1
# FearBehavFull3  5 1594.4 1605.8 -792.20   1584.4 9.7232      2   0.007738 **

r.squaredGLMM(FearBehavFull3)
#          R2m       R2c
# [1,] 0.1473911 0.3254234

confint.merMod(object=FearBehavFull3)
#                 2.5 %    97.5 %
# .sig01             0.00 11634.354
# .sigma         10545.56 16733.790
# (Intercept)     24202.80 34883.705
# TreatmentStress -10207.67  2195.992
# SexM           -22074.86 -4371.428

#############################################################################
#Step 9. Start to build model - DistratBehav
#############################################################################

DistractBehavFull1<-lmer(Tukey.DistractBehav~
                    Treatment*Sex + z.Tukey.Age +
                    (1|animalID), data=a, REML = "F")

#look at plots first - lookong for no pattern in residuals
```

```
diagnostics.plot(DistractBehavFull1)# okay
ranef.diagn.plot(DistractBehavFull1)# look fine
plot(DistractBehavFull1) #ok
plot(residuals(DistractBehavFull1)) #okay

#now look at any interaction terms
as.data.frame(drop1(DistractBehavFull1, test = "Chisq"))
#              Df      AIC       LRT   Pr(Chi)
# <none>       NA 561.8238       NA       NA
# z.Tukey.Age    1 560.0557 0.2318241 0.6301746
# Treatment:Sex  1 560.6662 0.8423471 0.3587264 #remove interaction

DistractBehavFull2<-lmer(Tukey.DistractBehav~
                    Treatment + Sex + z.Tukey.Age +
                    (1|animalID), data=a, REML = "F")

#look at plots first - lookong for no pattern in residuals
diagnostics.plot(DistractBehavFull2)# okay
ranef.diagn.plot(DistractBehavFull2)# look fine
plot(DistractBehavFull2) #ok
plot(residuals(DistractBehavFull2)) #okay

#now look at any interaction terms
as.data.frame(drop1(DistractBehavFull2, test = "Chisq"))
#           Df      AIC       LRT     Pr(Chi)
# <none>     NA 560.6662        NA         NA
# Treatment    1 569.0127 10.3464791 0.001297219
# Sex          1 558.8964  0.2302225 0.631358860
# z.Tukey.Age  1 558.9006  0.2343750 0.628298660 #remove

DistractBehavFull3<-lmer(Tukey.DistractBehav~
                    Treatment + Sex +
                    (1|animalID), data=a, REML = "F")

#look at plots first - lookong for no pattern in residuals
diagnostics.plot(DistractBehavFull3)# okay
ranef.diagn.plot(DistractBehavFull3)# look fine
plot(DistractBehavFull3) #ok
plot(residuals(DistractBehavFull3)) #okay

#now look at any interaction terms
as.data.frame(drop1(DistractBehavFull3, test = "Chisq"))
#           Df      AIC       LRT     Pr(Chi)
# <none>    NA 558.9006        NA         NA
# Treatment  1 567.1882 10.2876530 0.001339233
# Sex        1 557.4704  0.5698042 0.450336716 #remove

DistractBehavFull4<-lmer(Tukey.DistractBehav~
                    Treatment +
                    (1|animalID), data=a, REML = "F")

#look at plots first - lookong for no pattern in residuals
diagnostics.plot(DistractBehavFull4)# okay
ranef.diagn.plot(DistractBehavFull4)# look fine
plot(DistractBehavFull4) #ok
plot(residuals(DistractBehavFull4)) #okay

#now look at any interaction terms
as.data.frame(drop1(DistractBehavFull4, test = "Chisq"))
#           Df      AIC      LRT     Pr(Chi)
# <none>    NA 557.4704       NA         NA
# Treatment  1 565.7580 10.28765 0.001339233

summary(DistractBehavFull4)
# Linear mixed model fit by maximum likelihood  ['lmerMod']
```

```
# Formula: Tukey.DistractBehav ~ Treatment + (1 | animalID)
# Data: a
#
# AIC      BIC   logLik deviance df.resid
# 557.5    566.6   -274.7    549.5       68
#
# Scaled residuals:
#   Min      1Q  Median      3Q     Max
# -2.14375 -0.59946  0.04828  0.64105  2.12761
#
# Random effects:
#   Groups   Name        Variance Std.Dev.
# animalID (Intercept) 25.12    5.012
# Residual             98.20    9.910
# Number of obs: 72, groups:  animalID, 36
#
# Fixed effects:
#                Estimate Std. Error t value
# (Intercept)      27.931      1.851  15.091
# TreatmentStress   8.060      2.336   3.451
#
# Correlation of Fixed Effects:
#   (Intr)
# TrtmntStrss -0.631

mstabDistractBehav1=glmm.model.stab(model.res=DistractBehavFull4, contr=lmerControl(optimizer =
"bobyqa",optCtrl = list(maxfun=2e5)))
mstabDistractBehav1$detailed$warnings
# [1] none none none none none none none none none none none none none none none none none none none none none none none none none none none
# [21] none none
# Levels: none
mstabDistractBehav1$summary[,-1]
#                       orig       min       max
# (Intercept)          27.931094 27.222700 28.461033
# TreatmentStress       8.060320  7.291355  8.875863
# animalID@(Intercept)@NA  5.012356  4.274225  5.599008
# Residual              9.909814  9.436000 10.050376

nullDistractBehav<-lmer(Tukey.DistractBehav~ 1+
                    (1|animalID), data=a, REML = "F")

anova(nullDistractBehav, DistractBehavFull1) #0.01785
anova(nullDistractBehav, DistractBehavFull2) #0.01124
anova(nullDistractBehav, DistractBehavFull3) #0.004389
anova(nullDistractBehav, DistractBehavFull4) #0.001339
# Data: a
# Models:
#   nullDistractBehav: Tukey.DistractBehav ~ 1 + (1 | animalID)
# DistractBehavFull4: Tukey.DistractBehav ~ Treatment + (1 | animalID)
#                  Df    AIC    BIC  logLik deviance  Chisq Chi Df Pr(>Chisq)
# nullDistractBehav   3 565.76 572.59 -279.88   559.76
# DistractBehavFull4  4 557.47 566.58 -274.74   549.47 10.288      1   0.001339

r.squaredGLMM(DistractBehavFull4)
#           R2m       R2c
# [1,] 0.1178187 0.2975317

confint.merMod(object=DistractBehavFull4)
#                 2.5 %    97.5 %
# .sig01          0.000000  8.749539
# .sigma          7.996772 12.678428
# (Intercept)    24.252283 31.609905
# TreatmentStress  3.357415 12.763224

##############################################################################
```

```
#Step 10. Start to build model - AnxietyBehav
################################################################################
AnxietyBehavFull1<-lmer(Tukey.AnxietyBehav~
                    Treatment*Sex + z.Tukey.Age +
                    (1|animalID), data=a, REML = "F")

diagnostics.plot(AnxietyBehavFull1)# okay but line
ranef.diagn.plot(AnxietyBehavFull1)# look fine
plot(AnxietyBehavFull1) #ok
plot(residuals(AnxietyBehavFull1)) #okayish

#now look at any interaction terms
as.data.frame(drop1(AnxietyBehavFull1, test = "Chisq"))
#             Df     AIC      LRT    Pr(Chi)
# <none>       NA 368.6703       NA        NA
# z.Tukey.Age    1 374.4062 7.7358823 0.00541341
# Treatment:Sex  1 367.0220 0.3516592 0.55317540 #remove interaction

AnxietyBehavFull2<-lmer(Tukey.AnxietyBehav~
                    Treatment + Sex + z.Tukey.Age +
                    (1|animalID), data=a, REML = "F")

diagnostics.plot(AnxietyBehavFull2)# okay
ranef.diagn.plot(AnxietyBehavFull2)# look fine
plot(AnxietyBehavFull2) #ok
plot(residuals(AnxietyBehavFull2)) #okay

#now look at any interaction terms
as.data.frame(drop1(AnxietyBehavFull2, test = "Chisq"))
#           Df     AIC     LRT     Pr(Chi)
# <none>     NA 367.0220      NA         NA
# Treatment    1 372.2541 7.232080 0.007161217
# Sex          1 371.3167 6.294667 0.012110179
# z.Tukey.Age  1 372.7663 7.744274 0.005388313

summary(AnxietyBehavFull2)
# Linear mixed model fit by maximum likelihood  ['lmerMod']
# Formula: Tukey.AnxietyBehav ~ Treatment + Sex + z.Tukey.Age + (1 | animalID)
# Data: a
#
# AIC     BIC   logLik deviance df.resid
# 367.0    380.7   -177.5    355.0      66
#
# Scaled residuals:
#   Min      1Q   Median      3Q     Max
# -2.04098 -0.68289  0.06515  0.54276  2.25576
#
# Random effects:
#   Groups   Name        Variance Std.Dev.
# animalID (Intercept) 0.188    0.4336
# Residual             7.923    2.8148
# Number of obs: 72, groups:  animalID, 36
#
# Fixed effects:
#               Estimate Std. Error t value
# (Intercept)      9.4620     0.5231  18.090
# TreatmentStress   1.8493     0.6636   2.787
# SexM             2.2731     0.8670   2.622
# z.Tukey.Age     -1.1105     0.3781  -2.937
#
# Correlation of Fixed Effects:
#   (Intr) TrtmnS SexM
# TrtmntStrss -0.638
# SexM        -0.420  0.009
# z.Tukey.Age  0.190 -0.020 -0.427
```

```
mstabAnxietyBehav2=glmm.model.stab(model.res=AnxietyBehavFull2, contr=lmerControl(optimizer = "bobyqa",optCtrl
= list(maxfun=2e5)))
mstabAnxietyBehav2$detailed$warnings
# [1] none none none none none none none none none none none none none none none none none none none none none
# [21] none none
# Levels: none
mstabAnxietyBehav2$summary[,-1]
#                       orig      min       max
# (Intercept)           9.4620427  9.271167  9.6042323
# TreatmentStress       1.8493230  1.573300  2.0597051
# SexM                  2.2730955  1.826778  2.5480537
# z.Tukey.Age           -1.1104756 -1.324559 -0.8416917
# animalID@(Intercept)@NA  0.4336345  0.000000  0.9194420
# Residual              2.8148210  2.604704  2.8547183

nullAnxietyBehav<-lmer(Tukey.AnxietyBehav~ 1+
                  (1|animalID), data=a, REML = "F")

anova(nullAnxietyBehav, AnxietyBehavFull1) #0.002259
anova(nullAnxietyBehav, AnxietyBehavFull2) #0.0009844
# Data: a
# Models:
#   nullAnxietyBehav: Tukey.AnxietyBehav ~ 1 + (1 | animalID)
# AnxietyBehavFull2: Tukey.AnxietyBehav ~ Treatment + Sex + z.Tukey.Age + (1 | animalID)
# Df    AIC    BIC  logLik deviance  Chisq Chi Df Pr(>Chisq)
# nullAnxietyBehav   3 377.32 384.15 -185.66   371.32
# AnxietyBehavFull2  6 367.02 380.68 -177.51   355.02 16.299      3  0.0009844

r.squaredGLMM(AnxietyBehavFull2)
#          R2m       R2c
# [1,] 0.2061241 0.2245281

confint.merMod(object=AnxietyBehavFull2)
#                   2.5 %     97.5 %
# .sig01          0.0000000  1.7981189
# .sigma          2.2714407  3.3834704
# (Intercept)     8.4224420 10.5015956
# TreatmentStress  0.5190570  3.1795758
# SexM            0.5279156  4.0191541
# z.Tukey.Age     -1.8722266 -0.3496223

#############################################################################
#Step 11. Start to build model - ForageBehav
#############################################################################
ForageBehavFull1<-lmer(Tukey.ForageBehav~
                  Treatment*Sex + z.Tukey.Age +
                  (1|animalID), data=a, REML = "F")

diagnostics.plot(ForageBehavFull1)# vertical lines
ranef.diagn.plot(ForageBehavFull1)# no data
plot(ForageBehavFull1)
plot(residuals(ForageBehavFull1)) #okay

#now look at any interaction terms
as.data.frame(drop1(ForageBehavFull1, test = "Chisq"))
#             Df     AIC      LRT   Pr(Chi)
# <none>       NA 1357.302       NA       NA
# z.Tukey.Age    1 1356.581 1.2787260 0.2581360
# Treatment:Sex  1 1355.571 0.2690628 0.6039611 #remove interaction

ForageBehavFull2<-lmer(Tukey.ForageBehav~
                  Treatment + Sex + z.Tukey.Age +
                  (1|animalID), data=a, REML = "F")
```

```
diagnostics.plot(ForageBehavFull2)# vertical lines
ranef.diagn.plot(ForageBehavFull2)# no data
plot(ForageBehavFull2)
plot(residuals(ForageBehavFull2)) #okay

#now look at any interaction terms
as.data.frame(drop1(ForageBehavFull2, test = "Chisq"))
#           Df     AIC       LRT   Pr(Chi)
# <none>     NA 1355.571        NA       NA
# Treatment    1 1354.560 0.98944556 0.3198779
# Sex          1 1353.620 0.04893251 0.8249313 #remove
# z.Tukey.Age  1 1354.842 1.27112484 0.2595558

ForageBehavFull3<-lmer(Tukey.ForageBehav~
                    Treatment + z.Tukey.Age +
                    (1|animalID), data=a, REML = "F")

diagnostics.plot(ForageBehavFull3)# vertical lines
ranef.diagn.plot(ForageBehavFull3)# no data
plot(ForageBehavFull3)
plot(residuals(ForageBehavFull3)) #okay

#now look at any interaction terms
as.data.frame(drop1(ForageBehavFull3, test = "Chisq"))
#           Df     AIC     LRT   Pr(Chi)
# <none>     NA 1353.620      NA       NA
# Treatment    1 1352.613 0.992556 0.3191185
# z.Tukey.Age  1 1352.925 1.304639 0.2533676

nullForageBehav<-lmer(Tukey.ForageBehav~ 1+
                    (1|animalID), data=a, REML = "F")

anova(nullForageBehav, ForageBehavFull1) #0.6201
anova(nullForageBehav, ForageBehavFull2) # 0.4994
# Data: a
# Models:
#   nullForageBehav: Tukey.ForageBehav ~ 1 + (1 | animalID)
# ForageBehavFull2: Tukey.ForageBehav ~ Treatment + Sex + z.Tukey.Age + (1 | animalID)
# Df    AIC    BIC  logLik deviance  Chisq Chi Df Pr(>Chisq)
# nullForageBehav   3 1351.9 1358.8 -672.97   1345.9
# ForageBehavFull2  6 1355.6 1369.2 -671.79   1343.6 2.3692      3     0.4994

r.squaredGLMM(ForageBehavFull2)
#           R2m       R2c
# [1,] 0.03281132 0.03281132

confint.merMod(object=ForageBehavFull2)
#                   2.5 %    97.5 %
# .sig01            0.000 1588.1335
# .sigma         2230.226 3242.6444
# (Intercept)    3634.187 5620.8162
# TreatmentStress -1919.601  635.6334
# SexM           -1454.388 1818.2265
# z.Tukey.Age    -1119.412  307.8588

mstabForageBehavFull2=glmm.model.stab(model.res=ForageBehavFull2, contr=lmerControl(optimizer =
"bobyqa",optCtrl = list(maxfun=2e5)))
mstabForageBehavFull2$detailed$warnings
# [1] none none none none none none none none none none none none none none none none none none none none none none none none none
# [21] none none
# Levels: none
mstabForageBehavFull2$summary[,-1]
#                        orig       min       max
# (Intercept)          4627.5014 4366.7320 4828.0718
# TreatmentStress      -641.9837 -879.7949 -410.1777
```

```
# SexM                181.6559 -133.5042  423.4612
# z.Tukey.Age         -405.5077 -524.2977 -266.2463
# animalID@(Intercept)@NA    0.0000    0.0000  544.8659
# Residual            2728.2506 2590.2711 2765.6454
#############################################################################
#Step 12. Start to build model - InactiveBehav
#############################################################################
InactiveBehavFull1<-lmer(Tukey.InactiveBehav~
                  Treatment*Sex + z.Tukey.Age +
                  (1|animalID), data=a, REML = "F")

diagnostics.plot(InactiveBehavFull1)# okay
ranef.diagn.plot(InactiveBehavFull1)# look fine
plot(InactiveBehavFull1) #ok
plot(residuals(InactiveBehavFull1)) #ok

#now look at any interaction terms
as.data.frame(drop1(InactiveBehavFull1, test = "Chisq"))
#            Df     AIC       LRT   Pr(Chi)
# <none>       NA 637.1039        NA       NA
# z.Tukey.Age    1 635.1485 0.04459316 0.8327539
# Treatment:Sex  1 635.1207 0.01677717 0.8969409 #remove interaction

summary(InactiveBehavFull1)
# Linear mixed model fit by maximum likelihood  ['lmerMod']
# Formula: Tukey.InactiveBehav ~ Treatment * Sex + z.Tukey.Age + (1 | animalID)
# Data: a
#
# AIC     BIC   logLik deviance df.resid
# 637.1    653.0   -311.6    623.1       65
#
# Scaled residuals:
#   Min      1Q  Median      3Q     Max
# -2.31133 -0.52544  0.02209  0.57554  1.52746
#
# Random effects:
#   Groups   Name        Variance Std.Dev.
# animalID (Intercept) 155.6    12.47
# Residual             214.4    14.64
# Number of obs: 72, groups:  animalID, 36
#
# Fixed effects:
#   Estimate Std. Error t value
# (Intercept)         46.6221     3.7863  12.313
# TreatmentStress      7.3397     3.9871   1.841
# SexM                24.3423     7.9741   3.053
# z.Tukey.Age          0.6356     3.0091   0.211
# TreatmentStress:SexM  -1.0326     7.9710  -0.130
#
# Correlation of Fixed Effects:
#   (Intr) TrtmnS SexM   z.Tk.A
# TrtmntStrss -0.532
# SexM        -0.532  0.261
# z.Tukey.Age  0.210 -0.029 -0.371
# TrtmntSt:SM  0.264 -0.500 -0.501  0.004

InactiveBehavFull2<-lmer(Tukey.InactiveBehav~
                  Treatment + Sex + z.Tukey.Age +
                  (1|animalID), data=a, REML = "F")

diagnostics.plot(InactiveBehavFull2)# okay
ranef.diagn.plot(InactiveBehavFull2)# look fine
plot(InactiveBehavFull2) #ok
plot(residuals(InactiveBehavFull2)) #ok
```

```
#now look at any interaction terms
as.data.frame(drop1(InactiveBehavFull2, test = "Chisq"))
#           Df     AIC        LRT     Pr(Chi)
# <none>     NA 635.1207         NA         NA
# Treatment    1 637.0966  3.97592008 0.046155231
# Sex          1 643.4217 10.30099915 0.001329582
# z.Tukey.Age  1 633.1655  0.04480882 0.832355968 #remove

InactiveBehavFull3<-lmer(Tukey.InactiveBehav~
                    Treatment + Sex +
                    (1|animalID), data=a, REML = "F")

diagnostics.plot(InactiveBehavFull3)# okay
ranef.diagn.plot(InactiveBehavFull3)# look fine
plot(InactiveBehavFull3) #ok
plot(residuals(InactiveBehavFull3)) #ok

#now look at any interaction terms
as.data.frame(drop1(InactiveBehavFull3, test = "Chisq"))
#          Df     AIC      LRT     Pr(Chi)
# <none>    NA 633.1655       NA         NA
# Treatment  1 635.1692  4.003715 0.0454000901
# Sex        1 643.9367 12.771202 0.0003519966

summary(InactiveBehavFull3)
# Linear mixed model fit by maximum likelihood  ['lmerMod']
# Formula: Tukey.InactiveBehav ~ Treatment + Sex + (1 | animalID)
# Data: a
#
# AIC      BIC   logLik deviance df.resid
# 633.2    644.5   -311.6    623.2      67
#
# Scaled residuals:
#    Min      1Q   Median      3Q     Max
# -2.28353 -0.52251  0.00653  0.55701  1.51017
#
# Random effects:
#   Groups   Name        Variance Std.Dev.
# animalID (Intercept) 155.9    12.49
# Residual             214.5    14.65
# Number of obs: 72, groups:  animalID, 36
#
# Fixed effects:
#   Estimate Std. Error t value
# (Intercept)      46.584      3.567  13.058
# TreatmentStress   7.104      3.452   2.058
# SexM             24.448      6.244   3.915
#
# Correlation of Fixed Effects:
#   (Intr) TrtmnS
# TrtmntStrss -0.484
# SexM        -0.438  0.000

mstabInactiveBehav1=glmm.model.stab(model.res=InactiveBehavFull3, contr=lmerControl(optimizer =
"bobyqa",optCtrl = list(maxfun=2e5)))
mstabInactiveBehav1$detailed$warnings
# [1] none none none none none none none none none none none none none none none none none none none none none none none none none
# [21] none none
# Levels: none
mstabInactiveBehav1$summary[,-1]
#                       orig       min       max
# (Intercept)          46.584318 45.052626 47.528877
# TreatmentStress       7.104277  5.843342  8.219719
# SexM                 24.448004 22.033232 27.924456
# animalID@(Intercept)@NA 12.486639 11.198395 13.141606
```

```
# Residual             14.646630 13.857625 14.854093

nullInactiveBehav<-lmer(Tukey.InactiveBehav~ 1+
                  (1|animalID), data=a, REML = "F")

anova(nullInactiveBehav, InactiveBehavFull1) #0.00208
anova(nullInactiveBehav, InactiveBehavFull2) #0.0007697
anova(nullInactiveBehav, InactiveBehavFull3) #0.0002277
# Data: a
# Models:
#   nullInactiveBehav: Tukey.InactiveBehav ~ 1 + (1 | animalID)
# InactiveBehavFull3: Tukey.InactiveBehav ~ Treatment + Sex + (1 | animalID)
# Df    AIC    BIC  logLik deviance  Chisq Chi Df Pr(>Chisq)
# nullInactiveBehav   3 645.94 652.77 -319.97   639.94
# InactiveBehavFull3  5 633.17 644.55 -311.58   623.17 16.775      2  0.0002277

r.squaredGLMM(InactiveBehavFull3)
#          R2m       R2c
# [1,] 0.2544732 0.5682614

confint.merMod(object=InactiveBehavFull3)
#                 2.5 %   97.5 %
# .sig01         6.4445226 18.20637
# .sigma         11.8191947 18.81090
# (Intercept)    39.4693992 53.69924
# TreatmentStress  0.1534197 14.05513
# SexM           11.8758454 37.02016

##############################################################################
##############################################################################

PositiveFull1<-lmer(Tukey.Positive~
                Treatment*Sex + z.Tukey.Age +
                (1|animalID), data=a, REML = "F")

#look at plots first - lookong for no pattern in residuals
diagnostics.plot(PositiveFull1)# good
ranef.diagn.plot(PositiveFull1)# good
plot(PositiveFull1) #good
plot(residuals(PositiveFull1)) #good

#now look at any interaction terms
as.data.frame(drop1(PositiveFull1, test = "Chisq"))
#             Df     AIC       LRT   Pr(Chi)
# <none>       NA 1375.609        NA       NA
# z.Tukey.Age    1 1375.909 2.30016864 0.1293600
# Treatment:Sex  1 1373.635 0.02590983 0.8721207 #remove interaction

PositiveFull2<-lmer(Tukey.Positive~
                Treatment + Sex + #z.Tukey.Age +
                (1|animalID), data=a, REML = "F")

#look at plots first - lookong for no pattern in residuals
diagnostics.plot(PositiveFull2)# good
ranef.diagn.plot(PositiveFull2)# good
plot(PositiveFull2)#good
plot(residuals(PositiveFull2)) #good

#now look at any interaction terms
as.data.frame(drop1(PositiveFull2, test = "Chisq"))
#          Df     AIC     LRT     Pr(Chi)
# <none>    NA 1373.936      NA         NA
# Treatment  1 1372.990 1.054079 0.304569626
# Sex        1 1380.642 8.705395 0.003172697
```

```
summary(PositiveFull2)
# Linear mixed model fit by maximum likelihood  ['lmerMod']
# Formula: Tukey.Positive ~ Treatment + Sex + (1 | animalID)
# Data: a
#
# AIC      BIC   logLik deviance df.resid
# 1373.9   1385.3   -682.0   1363.9       67
#
# Scaled residuals:
#   Min      1Q  Median      3Q     Max
# -1.6521 -0.5083 -0.0392  0.5792  2.0281
#
# Random effects:
#   Groups   Name        Variance Std.Dev.
# animalID (Intercept) 2860748  1691
# Residual             7421863  2724
# Number of obs: 72, groups:  animalID, 36
#
# Fixed effects:
#                 Estimate Std. Error t value
# (Intercept)       6411.7      588.6  10.893
# TreatmentStress   -664.1      642.1  -1.034
# SexM             -3096.5      986.7  -3.138
#
# Correlation of Fixed Effects:
#   (Intr) TrtmnS
# TrtmntStrss -0.545
# SexM        -0.419  0.000

mstabPositiveFull2=glmm.model.stab(model.res=PositiveFull2, contr=lmerControl(optimizer = "bobyqa",optCtrl =
list(maxfun=2e5)))
mstabPositiveFull2$detailed$warnings
# [1] none none none none none none none none none none none none none none none none none none none none
# [21] none none
# Levels: none
mstabPositiveFull2$summary[,-1]
#                          orig        min        max
# (Intercept)             6411.6754  6218.0108  6639.1324
# TreatmentStress         -664.1153  -883.5234  -400.0824
# SexM                   -3096.4514 -3516.2426 -2723.5555
# animalID@(Intercept)@NA  1691.3747  1544.7740  1876.5988
# Residual                2724.3097  2525.6832  2762.7662

nullpositive<-lmer(Tukey.Positive~ 1+
                   (1|animalID), data=a, REML = "F")

anova(nullpositive, PositiveFull1) #0.01672
anova(nullpositive, PositiveFull2) # 0.007599
#Data: a
# Models:
#   nullpositive: Tukey.Positive ~ 1 + (1 | animalID)
# PositiveFull2: Tukey.Positive ~ Treatment + Sex + (1 | animalID)
#               Df    AIC    BIC  logLik deviance  Chisq Chi Df Pr(>Chisq)
# nullpositive   3 1379.7 1386.5 -686.85   1373.7
# PositiveFull2  5 1373.9 1385.3 -681.97   1363.9 9.7595      2   0.007599

r.squaredGLMM(PositiveFull2)
#           R2m       R2c
# [1,] 0.1583702 0.3925219

confint.merMod(object=PositiveFull2)
#                  2.5 %     97.5 %
# .sig01            0.000  2709.5886
# .sigma         2198.400  3499.4529
```

```
# (Intercept)     5239.686  7583.6649
# TreatmentStress -1956.992   628.7615
# SexM           -5083.110 -1109.7928

##############################################################################
##############################################################################
NegativeFull1<-lmer(Tukey.Negative~
                Treatment*Sex + z.Tukey.Age +
                (1|animalID), data=a, REML = "F")

#look at plots first - lookong for no pattern in residuals
diagnostics.plot(NegativeFull1)# not good, line in residuals - lots of zeros.
ranef.diagn.plot(NegativeFull1)# no data
plot(NegativeFull1)
plot(residuals(NegativeFull1))

#now look at any interaction terms
as.data.frame(drop1(NegativeFull1, test = "Chisq"))
#             Df     AIC      LRT   Pr(Chi)
# <none>       NA 489.4811       NA       NA
# z.Tukey.Age    1 488.2384 0.7572860 0.3841792
# Treatment:Sex  1 487.7705 0.2894129 0.5905970 #remove interaction

NegativeFull2<-lmer(Tukey.Negative~
                Treatment + Sex + #z.Tukey.Age +
                (1|animalID), data=a, REML = "F")
#look at plots first - lookong for no pattern in residuals
diagnostics.plot(NegativeFull2)# not good, lots of zeros
ranef.diagn.plot(NegativeFull2)# no data
plot(NegativeFull2)
plot(residuals(NegativeFull2))

#now look at any interaction terms
as.data.frame(drop1(NegativeFull2, test = "Chisq"))
#           Df     AIC      LRT    Pr(Chi)
# <none>     NA 487.7705       NA        NA
# Treatment    1 486.7073 0.9368506 0.33308910
# Sex          1 491.6299 5.8593765 0.01549428
# z.Tukey.Age  1 486.5248 0.7543604 0.38509922

NegativeFull3<-lmer(Tukey.Negative~
                Treatment + Sex +
                (1|animalID), data=a, REML = "F")

#look at plots first - lookong for no pattern in residuals
diagnostics.plot(NegativeFull3)# not good, lots of zeros
ranef.diagn.plot(NegativeFull3)#
plot(NegativeFull3)
plot(residuals(NegativeFull3))

#now look at any interaction terms
as.data.frame(drop1(NegativeFull3, test = "Chisq"))
#          Df     AIC      LRT    Pr(Chi)
# <none>    NA 486.5248       NA        NA
# Treatment  1 485.4212 0.8963139 0.34377202
# Sex        1 489.6728 5.1479738 0.02327351

summary(NegativeFull3)
# Linear mixed model fit by maximum likelihood  ['lmerMod']
# Formula: Tukey.Negative ~ Treatment + Sex + (1 | animalID)
# Data: a
#
# AIC     BIC   logLik deviance df.resid
# 486.5    497.9   -238.3    476.5       67
#
```

```
# Scaled residuals:
#   Min     1Q  Median     3Q    Max
# -1.4387 -0.7873 -0.5629  0.7371  2.9675
#
# Random effects:
#   Groups   Name        Variance Std.Dev.
# animalID (Intercept)  0.2845  0.5334
# Residual            43.5554  6.5997
# Number of obs: 72, groups:  animalID, 36
#
# Fixed effects:
#             Estimate Std. Error t value
# (Intercept)       3.773      1.192   3.164
# TreatmentStress    1.481      1.556   0.952
# SexM             4.253      1.808   2.352
#
# Correlation of Fixed Effects:
#   (Intr) TrtmnS
# TrtmntStrss -0.652
# SexM        -0.379  0.000

mstabNegativeFull=glmm.model.stab(model.res=NegativeFull3, contr=lmerControl(optimizer = "bobyqa",optCtrl =
list(maxfun=2e5)))
mstabNegativeFull$detailed$warnings
# [1] none none none none none none none none none none none none none none none none none none none none
# [21] none none
# Levels: none
mstabNegativeFull$summary[,-1]
#                         orig       min      max
# (Intercept)            3.7733452 3.3277245 3.925797
# TreatmentStress        1.4808586 0.8105547 2.005275
# SexM                   4.2530263 2.4491235 4.808064
# animalID@(Intercept)@NA 0.5333928 0.0000000 1.567519
# Residual               6.5996516 6.0591303 6.682240

nullnegative<-lmer(Tukey.Negative~ 1+
                 (1|animalID), data=a, REML = "F")

anova(nullnegative, NegativeFull1) #0.1314
anova(nullnegative, NegativeFull2) #0.07864
anova(nullnegative, NegativeFull3) #0.04873
# Data: a
# Models:
#   nullnegative: Tukey.Negative ~ 1 + (1 | animalID)
# NegativeFull2: Tukey.Negative ~ Treatment + Sex + (1 | animalID)
# Df    AIC    BIC  logLik deviance Chisq Chi Df Pr(>Chisq)
# nullnegative   3 488.57 495.40 -241.28   482.57
# NegativeFull2  5 486.52 497.91 -238.26   476.52 6.043      2    0.04873 *

r.squaredGLMM(NegativeFull3)
#           R2m       R2c
# [1,] 0.08352157 0.08946924

confint.merMod(object=NegativeFull3)
#                 2.5 %   97.5 %
# .sig01          0.0000000 4.072813
# .sigma          5.3256693 7.869158
# (Intercept)     1.4038039 6.142886
# TreatmentStress -1.6185432 4.580260
# SexM            0.6129887 7.893064

##############################################################################
##############################################################################

StressFull1<-lmer(Tukey.Stress~
```

```
                Treatment*Sex + z.Tukey.Age +
                (1|animalID), data=a, REML = "F")

#look at plots first - lookong for no pattern in residuals
diagnostics.plot(StressFull1)# good
ranef.diagn.plot(StressFull1)# good
plot(StressFull1) #good
plot(residuals(StressFull1)) #good

#now look at any interaction terms
as.data.frame(drop1(StressFull1, test = "Chisq"))
#           Df     AIC      LRT    Pr(Chi)
# <none>       NA 304.1670       NA        NA
# z.Tukey.Age    1 309.9029 7.7358823 0.00541341
# Treatment:Sex  1 302.5187 0.3516592 0.55317540 #remove interaction

StressFull2<-lmer(Tukey.Stress~
                Treatment + Sex + z.Tukey.Age +
                (1|animalID), data=a, REML = "F")

#look at plots first - lookong for no pattern in residuals
diagnostics.plot(StressFull2)# good
ranef.diagn.plot(StressFull2)# good
plot(StressFull2) #good
plot(residuals(StressFull2) #good

#now look at any interaction terms
as.data.frame(drop1(StressFull2, test = "Chisq"))
#           Df     AIC     LRT     Pr(Chi)
# <none>     NA 302.5187      NA         NA
# Treatment    1 307.7507 7.232080 0.007161217
# Sex          1 306.8133 6.294667 0.012110179
# z.Tukey.Age  1 308.2629 7.744274 0.005388313

summary(StressFull2)
# Linear mixed model fit by maximum likelihood  ['lmerMod']
# Formula: Tukey.Stress ~ Treatment + Sex + z.Tukey.Age + (1 | animalID)
# Data: a
#
# AIC      BIC   logLik deviance df.resid
# 302.5    316.2   -145.3    290.5       66
#
# Scaled residuals:
#   Min      1Q   Median      3Q     Max
# -2.04098 -0.68289  0.06515  0.54276  2.25576
#
# Random effects:
#   Groups   Name        Variance Std.Dev.
# animalID (Intercept) 0.07677  0.2771
# Residual             3.23464  1.7985
# Number of obs: 72, groups:  animalID, 36
#
# Fixed effects:
#               Estimate Std. Error t value
# (Intercept)      6.0457     0.3342  18.090
# TreatmentStress   1.1816     0.4240   2.787
# SexM             1.4524     0.5540   2.622
# z.Tukey.Age     -0.7095     0.2416  -2.937
#
# Correlation of Fixed Effects:
#   (Intr) TrtmnS SexM
# TrtmntStrss -0.638
# SexM        -0.420  0.009
```

```
mstabStressFull=glmm.model.stab(model.res=StressFull2, contr=lmerControl(optimizer = "bobyqa",optCtrl =
list(maxfun=2e5)))
mstabStressFull$detailed$warnings
# [1] none none none none none none none none none none none none none none none none none none none none none
# [21] none none
# Levels: none
mstabStressFull$summary[,-1]
#                      orig       min       max
# (Intercept)           6.0457069  5.9237479  6.1365580
# TreatmentStress       1.1816122  1.0052489  1.3160344
# SexM                  1.4523787  1.1672075  1.6280614
# z.Tukey.Age          -0.7095307 -0.8463178 -0.5377931
# animalID@(Intercept)@NA  0.2770678  0.0000000  0.5874711
# Residual              1.7985105  1.6642579  1.8240026

nullstress<-lmer(Tukey.Stress~ 1+
                 (1|animalID), data=a, REML = "F")

anova(nullstress, StressFull1) #0.002259
anova(nullstress, StressFull2) #0.0009844
# Data: a
# Models:
#   nullstress: Tukey.Stress ~ 1 + (1 | animalID)
# StressFull2: Tukey.Stress ~ Treatment + Sex + z.Tukey.Age + (1 | animalID)
# Df    AIC    BIC  logLik deviance  Chisq Chi Df Pr(>Chisq)
# nullstress   3 312.82 319.65 -153.41   306.82
# StressFull2  6 302.52 316.18 -145.26   290.52 16.299      3  0.0009844

r.squaredGLMM(StressFull2)
#           R2m       R2c
# [1,] 0.2061241 0.2245281

confint.merMod(object=StressFull2)
#                  2.5 %     97.5 %
# .sig01          0.0000000  1.1488957
# .sigma          1.4513214  2.1618451
# (Intercept)     5.3814612  6.7099221
# TreatmentStress  0.3316479  2.0315681
# SexM            0.3373080  2.5680108
# z.Tukey.Age     -1.1962463 -0.2233888

##############################################################################
##############################################################################
ExploratoryFull1<-lmer(Tukey.Explore~
                 Treatment*Sex + z.Tukey.Age +
                 (1|animalID), data=a, REML = "F")

#look at plots first - lookong for no pattern in residuals
diagnostics.plot(ExploratoryFull1)# good
ranef.diagn.plot(ExploratoryFull1)# no data
plot(ExploratoryFull1) #good
plot(residuals(ExploratoryFull1)) #good

#now look at any interaction terms
as.data.frame(drop1(ExploratoryFull1, test = "Chisq"))
#boundary (singular) fit: see ?isSingular
#              Df     AIC      LRT    Pr(Chi)
# <none>       NA 1190.424       NA        NA
# z.Tukey.Age    1 1191.744 3.3201875 0.06843402
# Treatment:Sex  1 1188.535 0.1113464 0.73861645 #remove interaction

ExploratoryFull2<-lmer(Tukey.Explore~
                 Treatment + Sex + z.Tukey.Age +
                 (1|animalID), data=a, REML = "F")
```

```
#look at plots first - lookong for no pattern in residuals
diagnostics.plot(ExploratoryFull2)# good
ranef.diagn.plot(ExploratoryFull2)# no data
plot(ExploratoryFull2) #good
plot(residuals(ExploratoryFull2) #good

#now look at any interaction terms
as.data.frame(drop1(ExploratoryFull2, test = "Chisq"))
#           Df     AIC         LRT    Pr(Chi)
# <none>     NA 1188.535          NA        NA
# Treatment    1 1186.535 4.857047e-07 0.99944393
# Sex          1 1186.878 3.433452e-01 0.55790467 #remove to improve stablity
# z.Tukey.Age  1 1189.847 3.312264e+00 0.06876471

ExploratoryFull3<-lmer(Tukey.Explore~
                       z.Tukey.Age + Treatment +
                      (1|animalID), data=a, REML = "F")

#look at plots first - lookong for no pattern in residuals
diagnostics.plot(ExploratoryFull3)# good
ranef.diagn.plot(ExploratoryFull3)# no data
plot(ExploratoryFull3) #good
plot(residuals(ExploratoryFull3) #good

as.data.frame(drop1(ExploratoryFull3, test = "Chisq"))

summary(ExploratoryFull3)
# Linear mixed model fit by maximum likelihood  ['lmerMod']
# Formula: Tukey.Explore ~ z.Tukey.Age + Treatment + (1 | animalID)
# Data: a
#
# AIC      BIC   logLik deviance df.resid
# 1186.9   1198.3   -588.4   1176.9      67
#
# Scaled residuals:
#   Min      1Q  Median      3Q     Max
# -2.26962 -0.78041  0.01166  0.68869  2.66721
#
# Random effects:
#   Groups   Name        Variance Std.Dev.
# animalID (Intercept)     0     0.0
# Residual             735021   857.3
# Number of obs: 72, groups:  animalID, 36
#
# Fixed effects:
#   Estimate Std. Error t value
# (Intercept)    1800.285    142.901  12.598
# z.Tukey.Age    -178.571    101.763  -1.755
# TreatmentStress   -1.147    202.108  -0.006
#
# Correlation of Fixed Effects:
#   (Intr) z.Tk.A
# z.Tukey.Age  0.013
# TrtmntStrss -0.707 -0.018
# convergence code: 0
# boundary (singular) fit: see ?isSingular

mstabExploreFull=glmm.model.stab(model.res=ExploratoryFull3, contr=lmerControl(optimizer = "bobyqa",optCtrl =
list(maxfun=2e5)))
mstabExploreFull$detailed$warnings
# [1] none none none none none none none none none none none none none none none none none none none none none none none none none
# [21] none none
# Levels: none
mstabExploreFull$summary[,-1]
#                       orig       min          max
```

```
# (Intercept)           1800.284660 1735.0557  1.856448e+03
# z.Tukey.Age            -178.570931 -209.3584 -1.484483e+02
# TreatmentStress         -1.146775  -77.5292  8.673271e+01
# animalID@(Intercept)@NA    0.000000    0.0000  7.796533e-05
# Residual               857.333679  822.5045  8.688833e+02

nullExplore<-lmer(Tukey.Explore~ 1+
                  (1|animalID), data=a, REML = "F")

anova(nullExplore, ExploratoryFull1) #0.4823
anova(nullExplore, ExploratoryFull2) #0.3394
anova(nullExplore, ExploratoryFull3) #0.2213

r.squaredGLMM(ExploratoryFull3)
#          R2m       R2c
# [1,] 0.04158443 0.04158443

confint.merMod(object=ExploratoryFull3)
#                    2.5 %     97.5 %
# .sig01            0.0000  411.32463
# .sigma          727.8528 1018.97797
# (Intercept)    1516.4270 2084.14235
# z.Tukey.Age    -380.7132   23.57127
# TreatmentStress -402.6148  400.32124

###############################################################################
###############################################################################
InactiveFull1<-lmer(Tukey.Inactive~
                    Treatment*Sex + z.Tukey.Age +
                    (1|animalID), data=a, REML = "F")

#look at plots first - lookong for no pattern in residuals
diagnostics.plot(InactiveFull1)# good
ranef.diagn.plot(InactiveFull1)# good
plot(InactiveFull1) #good
plot(residuals(InactiveFull1)) #good

#now look at any interaction terms
as.data.frame(drop1(InactiveFull1, test = "Chisq"))
#boundary (singular) fit: see ?isSingular
#              Df     AIC       LRT   Pr(Chi)
# <none>       NA 599.6740        NA       NA
# z.Tukey.Age   1 597.7186 0.04459316 0.8327539
# Treatment:Sex  1 597.6907 0.01677717 0.8969409 #remove interaction

InactiveFull2<-lmer(Tukey.Inactive~
                    Treatment + Sex + z.Tukey.Age +
                    (1|animalID), data=a, REML = "F")

#look at plots first - lookong for no pattern in residuals
diagnostics.plot(InactiveFull2)# good
ranef.diagn.plot(InactiveFull2)# good
plot(InactiveFull2) #good
plot(residuals(InactiveFull2) #good

#now look at any interaction terms
as.data.frame(drop1(InactiveFull2, test = "Chisq"))
#           Df     AIC        LRT     Pr(Chi)
# <none>     NA 597.6907         NA         NA
# Treatment   1 599.6667  3.97592008 0.046155231
# Sex         1 605.9917 10.30099915 0.001329582
# z.Tukey.Age  1 595.7355  0.04480882 0.832355968 #remove

InactiveFull3<-lmer(Tukey.Inactive~
                      Sex + Treatment +
```

```
                (1|animalID), data=a, REML = "F")

#look at plots first - lookong for no pattern in residuals
diagnostics.plot(InactiveFull3)# good
ranef.diagn.plot(InactiveFull3)# good
plot(InactiveFull3) #good
plot(residuals(InactiveFull3) #good

as.data.frame(drop1(InactiveFull3, test = "Chisq"))
#          Df      AIC       LRT      Pr(Chi)
# <none>    NA 595.7355        NA           NA
# Sex        1 606.5067 12.771202 0.0003519966
# Treatment  1 597.7393  4.003715 0.0454000901

summary(InactiveFull3)
# Linear mixed model fit by maximum likelihood  ['lmerMod']
# Formula: Tukey.Inactive ~ Sex + Treatment + (1 | animalID)
# Data: a
#
# AIC      BIC   logLik deviance df.resid
# 595.7    607.1   -292.9    585.7       67
#
# Scaled residuals:
#   Min       1Q   Median       3Q      Max
# -2.28353 -0.52251  0.00653  0.55701  1.51017
#
# Random effects:
#   Groups   Name        Variance Std.Dev.
# animalID (Intercept)  92.71    9.629
# Residual             127.56   11.294
# Number of obs: 72, groups:  animalID, 36
#
# Fixed effects:
#                Estimate Std. Error t value
# (Intercept)      35.921      2.751  13.058
# SexM             18.852      4.815   3.915
# TreatmentStress    5.478      2.662   2.058
#
# Correlation of Fixed Effects:
#   (Intr) SexM
# SexM       -0.438
# TrtmntStrss -0.484  0.000

mstabInactiveFull=glmm.model.stab(model.res=InactiveFull3, contr=lmerControl(optimizer = "bobyqa",optCtrl =
list(maxfun=2e5)))
mstabInactiveFull$detailed$warnings
# [1] none none none none none none none none none none none none none none none none none none none none
# [21] none none
# Levels: none
mstabInactiveFull$summary[,-1]
#                          orig       min      max
# (Intercept)            35.921420 34.740324 36.64977
# SexM                   18.851988 16.989944 21.53270
# TreatmentStress         5.478146  4.505832  6.33827
# animalID@(Intercept)@NA  9.628516  8.635143 10.13356
# Residual               11.294095 10.685690 11.45407

nullInactive<-lmer(Tukey.Inactive~ 1+
                (1|animalID), data=a, REML = "F")

anova(nullInactive, InactiveFull1) #0.00208
anova(nullInactive, InactiveFull2) #0.0007697
anova(nullInactive, InactiveFull3) #0.0002277
# Data: a
# Models:
```

```
#   nullInactive: Tukey.Inactive ~ 1 + (1 | animalID)
# InactiveFull3: Tukey.Inactive ~ Sex + Treatment + (1 | animalID)
#              Df    AIC    BIC  logLik deviance  Chisq Chi Df Pr(>Chisq)
# nullInactive   3 608.51 615.34 -301.25   602.51
# InactiveFull3  5 595.74 607.12 -292.87   585.74 16.775      2  0.0002277

r.squaredGLMM(InactiveFull3)
#       R2m       R2c
# [1,] 0.2544732 0.5682615

confint.merMod(object=InactiveFull3)
#                   2.5 %   97.5 %
# .sig01          4.9694063 14.03903
# .sigma          9.1138450 14.50519
# (Intercept)     30.4350673 41.40777
# SexM            9.1575287 28.54645
# TreatmentStress  0.1183027 10.83799
```

Appendix 4b

```
################################################################################
##########################Behvaiour & AB#######################################
################################################################################
#Step 1. Clear worksparce, set working directory and load packages
################################################################################
#Clear workspace
ls() #this looks at what is loaded
rm(list=ls()) #this clears everything

#Set the working directory
#setwd('M:/Emily/Writing/1Papers in prep/2019 AB Heritability/AB Heritability')
#setwd("C:/Users/emmel/Desktop/R Studio and R")
setwd("D:/R Studio and R")
#setwd("M:/Emily/Doing/Postgraduates/Emmeline Howarth/Data/Chapt6 Genetics")

#Load Package
#install.packages("lme4")
#install.packages("tidyverse")
#install.packages("car")
#install.packages("CarData")
#install.packages("rcompanion")
#install.packages("ggpubr")

library(tidyverse)
library(rcompanion)
library(lme4)
library(car)
library(ggpubr)

#install.packages("MuMIn")
library(MuMIn)
#citation("MuMIn")

#load Roger Functions #source files need to be in the working directory

#source("C:/Users/emmel/Desktop/R Studio and R/Functions/diagnostic_fcns.r")
#source("C:/Users/emmel/Desktop/R Studio and R/Functions/glmm_stability.r")
source("D:/R Studio and R/Functions/diagnostic_fcns.r")
source("D:/R Studio and R/Functions/glmm_stability.r")
#source("M:/Emily/Doing/R Users Group/R Training/Mundry/Functions/Functions/diagnostic_fcns.r")
#source("M:/Emily/Doing/R Users Group/R Training/Mundry/Functions/Functions/glmm_stability.r")

################################################################################
#Step 2. Import and check data:
################################################################################
#Load data
GroupedBehav_AB_Chpt4_S2<-read.csv(file.choose(), header=T) #select file from pop up window
d <- GroupedBehav_AB_Chpt4_S2

nrow(d)  #72 (if higher do the following go back to CSV and delete ghost cells from bottom)
ncol(d) #12

str(d)
View(d)

MData<-d

################################################################################
#Step 3. Check data reading correctly
################################################################################
# MData$AggBehav<-as.numeric(MData$AGGRESSIVEAPPROACHSUM)
# str(MData$AggBehav)
# MData$FearBehav<-as.numeric(MData$FEARAFFILIATIVEAPPROCHSUM)
```

```
# str(MData$FearBehav)
# MData$DistractBehav<-as.numeric(MData$DISTRACTIONSUM)
# str(MData$DistractBehav)
# MData$AnxietyBehav<-as.numeric(MData$ANXIETYSUM)
# str(MData$AnxietyBehav)
# MData$ForageBehav<-as.numeric(MData$FORAGINGSUM)
# str(MData$ForageBehav)
# MData$InactiveBehav<-as.numeric(MData$INACTIVITYSUM)
# str(MData$InactiveBehav)

MData$Inactive<-as.numeric(MData$Inactive_behav_mean)
str(MData$Inactive)
MData$Positive_social<-as.numeric(MData$Positive_social_mean)
str(MData$Positive_social)
MData$Negative_social<-as.numeric(MData$Negative_social_mean)
str(MData$Negative_social)
MData$exploratory<-as.numeric(MData$Exploratory_behav_mean)
str(MData$exploratory)
MData$Stress<-as.numeric(MData$Stress_behav_mean)
str(MData$Stress)

# MData$ABDiff_catagory<-as.integer(MData$ABDiff_catagory)
# str(MData$ABDiff_catagory) #int
# MData$ABDiff_BIAS<-as.factor(MData$ABDiff_BIAS)
# str(MData$ABDiff_BIAS) #Factor w/ 2 levels

#############################################################################
#Step 5. TRANSFORM DATA - BEHAVIOUR
#############################################################################
hist(MData$Inactive)
hist(transformTukey(MData$Inactive))
#     lambda     W Shapiro.p.value
# 416  0.375 0.985         0.5485
MData$Tukey.Inactive<-transformTukey(MData$Inactive)

hist(MData$Stress)
hist(transformTukey(MData$Stress))
#     lambda      W Shapiro.p.value
# 411   0.25 0.9829          0.438
MData$Tukey.Stress<-transformTukey(MData$Stress)

hist(MData$exploratory)
hist(transformTukey(MData$exploratory))
#      lambda      W Shapiro.p.value
# 430  0.725 0.9805         0.3282
MData$Tukey.Explore<-transformTukey(MData$exploratory)

hist(MData$Positive_social_mean)
hist(transformTukey(MData$Positive_social_mean))
#    lambda      W Shapiro.p.value
#436  0.875 0.9519       0.007898
MData$Tukey.Positive<-transformTukey(MData$Positive_social_mean)

hist(MData$Negative_social_mean)
hist(transformTukey(MData$Negative_social_mean))
#      lambda      W Shapiro.p.value
# 417    0.4 0.7736      3.554e-09
MData$Tukey.Negative<-transformTukey(MData$Negative_social_mean)

#############################################################################
#Step 6. TRANSFORM DATA - THE REST
#############################################################################

HData<-MData
```

```
#AGG
hist(HData$AGG_mean)
hist(transformTukey(HData$AGG_mean))
#    lambda      W Shapiro.p.value
# 418  0.425 0.9732         0.1265
HData$Tukey.AGG<-transformTukey(HData$AGG_mean)

#Total Look
hist(transformTukey(HData$TL_mean))
#    lambda     W Shapiro.p.value
#426  0.625 0.989         0.7886
HData$Tukey.TotalLook<-transformTukey(HData$TL_mean)

#Age
hist(transformTukey(HData$Age))
#    lambda      W Shapiro.p.value
# 424  0.575 0.9505         0.0066
HData$z.Tukey.Age<-scale(transformTukey(HData$Age))

a<-HData
#####################################################################
#Step 5: THR
#####################################################################
a$z.Tukey.Stress<-scale(transformTukey(a$Stress_behav_mean))
a$z.Tukey.Explore<-scale(transformTukey(a$Exploratory_behav_mean))
a$z.Tukey.Inactive<-scale(transformTukey(a$Inactive_behav_mean))
a$z.Tukey.Positive<-scale(transformTukey(a$Positive_social_mean))
a$z.Tukey.Negative<-scale(transformTukey(a$Negative_social_mean))

contr=lmerControl(optimizer="bobyqa", optCtrl=list(maxfun=1000000)) # optimiser needed for model to converge

AGGFull1<-lmer(Tukey.AGG~
            z.Tukey.Stress + Treatment*Sex + z.Tukey.Age +
            (1|animalID), data=a, REML = "F", control=contr)

diagnostics.plot(AGGFull1)# okay - some high and low
ranef.diagn.plot(AGGFull1)# look fine
plot(AGGFull1) #ok
plot(residuals(AGGFull1)) #fine

#now look at any interaction terms
as.data.frame(drop1(AGGFull1, test = "Chisq"))
#               Df     AIC      LRT   Pr(Chi)
# <none>        NA 386.4814       NA       NA
# z.Tukey.Stress  1 384.6899 0.2085192 0.6479306
# z.Tukey.Age     1 384.9601 0.4786690 0.4890258
# Treatment:Sex   1 384.7043 0.2229455 0.6368047 #remove interaction

AGGFull2<-lmer(Tukey.AGG~
            z.Tukey.Stress + Treatment + Sex + z.Tukey.Age +
            (1|animalID), data=a, REML = "F", control=contr)

diagnostics.plot(AGGFull2)# okay - some high and low
ranef.diagn.plot(AGGFull2)# look fine
plot(AGGFull2) #ok
plot(residuals(AGGFull2)) #fine

#now look at any interaction terms
as.data.frame(drop1(AGGFull2, test = "Chisq"))
#               Df     AIC       LRT   Pr(Chi)
# <none>        NA 384.7043        NA       NA
# z.Tukey.Stress  1 382.9322 0.22787989 0.6331003
# Treatment       1 382.7448 0.04042303 0.8406557 #remove
# Sex             1 382.7333 0.02893646 0.8649258 #remove
# z.Tukey.Age     1 383.1880 0.48362124 0.4867866 #remove
```

```
AGGFull3<-lmer(Tukey.AGG~
            z.Tukey.Stress +
            (1|animalID), data=a, REML = "F", control=contr)

diagnostics.plot(AGGFull3)# okay - some high and low
ranef.diagn.plot(AGGFull3)# look fine
plot(AGGFull3) #ok
plot(residuals(AGGFull3)) #fine

#now look at any interaction terms
as.data.frame(drop1(AGGFull3, test = "Chisq"))
#             Df     AIC      LRT   Pr(Chi)
# <none>        NA 379.4809       NA       NA
# z.Tukey.Stress  1 377.6027 0.1217757 0.7271165

mstabAGGFull=glmm.mode.stab(model.res=AGGFull3, contr=lmerControl(optimizer = "bobyqa",optCtrl =
list(maxfun=2e5)))
mstabAGGFull$detailed$warnings
# [1] none none none none none none none none none none none none none none none none none none none none none
# [21] none none
# Levels: none
mstabAGGFull$summary[,-1]
#                       orig       min       max
# (Intercept)          20.74603950 20.420052 21.1314466
# z.Tukey.Stress       -0.01239736 -0.250049  0.1829012
# animalID@(Intercept)@NA  4.39395710  3.885270  4.5276094
# Residual             9.99937448  9.705860 10.1626693

AGGnull<-lmer(Tukey.AGG~
            1 +
            (1|animalID), data=a, REML = "F")

anova(AGGnull, AGGFull3) #0.2124
anova(AGGnull, AGGFull4) #0.9853
# Data: a
# Models:
#   AGGnull: Tukey.AGG ~ 1 + (1 | animalID)
# AGGFull3: Tukey.AGG ~ z.Tukey.Stress + (1 | animalID)
#         Df    AIC    BIC  logLik deviance  Chisq Chi Df Pr(>Chisq)
# AGGnull   3 377.60 384.43 -185.80   371.60
# AGGFull3  4 379.48 388.59 -185.74   371.48 0.1218      1     0.7271

#no relationship between stress behaviour and THR looking time

#####################################################################
#AGG and Explore
#####################################################################
contr=lmerControl(optimizer="bobyqa", optCtrl=list(maxfun=1000000)) # optimiser needed for model to converge

ExploreFull1<-lmer(Tukey.AGG ~
             z.Tukey.Explore + Treatment*Sex + z.Tukey.Age +
             (1|animalID), data=a, REML = "F", control=contr)

diagnostics.plot(ExploreFull1)# ok
ranef.diagn.plot(ExploreFull1)# look fine
plot(ExploreFull1) #ok
plot(residuals(ExploreFull1)) #fine

#now look at any interaction terms
as.data.frame(drop1(ExploreFull1, test = "Chisq"))
#              Df     AIC      LRT    Pr(Chi)
# <none>         NA 383.9642       NA        NA
# z.Tukey.Explore  1 384.6899 2.7257122 0.09874406
# z.Tukey.Age      1 382.0786 0.1143585 0.73523557
```

```
# Treatment:Sex    1 382.1760 0.2117841 0.64537259 #remove interaction

ExploreFull2<-lmer(Tukey.AGG ~
                z.Tukey.Explore + Treatment + Sex + z.Tukey.Age +
                (1|animalID), data=a, REML = "F", control=contr)

diagnostics.plot(ExploreFull2)# ok
ranef.diagn.plot(ExploreFull2)# look fine
plot(ExploreFull2) #ok
plot(residuals(ExploreFull2)) #fine

#now look at any interaction terms
as.data.frame(drop1(ExploreFull2, test = "Chisq"))
#               Df     AIC         LRT    Pr(Chi)
# <none>         NA 382.1760          NA        NA
# z.Tukey.Explore  1 382.9322 2.7562342923 0.09687603
# Treatment        1 380.1770 0.0009802957 0.97502258 #remove
# Sex              1 380.3079 0.1319264197 0.71644296 #remove
# z.Tukey.Age      1 380.2878 0.1118535640 0.73804367 #remove

ExploreFull3<-lmer(Tukey.AGG ~
                z.Tukey.Explore +
                (1|animalID), data=a, REML = "F", control=contr)

diagnostics.plot(ExploreFull3)# ok
ranef.diagn.plot(ExploreFull3)# look fine
plot(ExploreFull3) #ok
plot(residuals(ExploreFull3)) #fine

#now look at any interaction terms
as.data.frame(drop1(ExploreFull3, test = "Chisq"))
#               Df     AIC     LRT    Pr(Chi)
# <none>         NA 376.5846      NA        NA
# z.Tukey.Explore  1 377.6027 3.018055 0.08234217

ExploreNull<-lmer(Tukey.AGG~
              1 +
              (1|animalID), data=a, REML = "F")

anova(ExploreNull, ExploreFull3)
# Data: a
# Models:
#   ExploreNull: Tukey.AGG ~ 1 + (1 | animalID)
# ExploreFull3: Tukey.AGG ~ z.Tukey.Explore + (1 | animalID)
#              Df    AIC    BIC  logLik deviance  Chisq Chi Df Pr(>Chisq)
# ExploreNull   3 377.60 384.43 -185.80   371.60
# ExploreFull3  4 376.58 385.69 -184.29   368.58 3.0181      1    0.08234 .

summary(ExploreFull3)
# Linear mixed model fit by maximum likelihood  ['lmerMod']
# Formula: Tukey.AGG ~ z.Tukey.Explore + (1 | animalID)
# Data: a
# Control: contr
#
# AIC      BIC   logLik deviance df.resid
# 376.6    385.7   -184.3    368.6       68
#
# Scaled residuals:
#   Min      1Q  Median      3Q     Max
# -2.33818 -0.57330 -0.01618  0.50256  2.64725
#
# Random effects:
#   Groups   Name        Variance Std.Dev.
# animalID (Intercept) 4.522    2.126
# Residual             6.262    2.502
```

```
# Number of obs: 72, groups:  animalID, 36
#
# Fixed effects:
#               Estimate Std. Error t value
# (Intercept)     14.0232     0.4611  30.415
# z.Tukey.Explore  -0.6078     0.3460  -1.757
#
# Correlation of Fixed Effects:
#   (Intr)
# z.Tky.Explr 0.000

mstabExploreFull=glmm.model.stab(model.res=ExploreFull3, contr=lmerControl(optimizer = "bobyqa",optCtrl =
list(maxfun=2e5)))

mstabExploreFull$detailed$warnings
# [1] none none none none none none none none none none none none none none none
# [16] none none none none none none none none none none none none none none none
# [31] none none none none none none
# Levels: none

mstabExploreFull$summary[,-1]
#                       orig       min       max
# (Intercept)           14.0231764 13.8687582 14.2524474
# z.Tukey.Explore       -0.6078471 -0.7429839 -0.2555364
# animalID@(Intercept)@NA  2.1264485  1.7082463  2.1987538
# Residual              2.5023910  2.2411123  2.5391149

r.squaredGLMM(ExploreFull3)
#          R2m       R2c
# [1,] 0.03312747 0.4385514

confint.merMod(object=ExploreFull3)
#                  2.5 %     97.5 %
# .sig01          1.080094  3.10681955
# .sigma          2.018057  3.21680244
# (Intercept)     13.094703 14.95164949
# z.Tukey.Explore -1.308361  0.07967555

plot(a$Exploratory_behav_mean, a$AGG_mean,
   xlab="Mean duration of exploratory behaviour (ms)", ylab="mean THR (ms)", col="black", pch=16)

tmp <- lm(a$AGG_mean ~ a$Exploratory_behav_mean, na.action = na.omit)
abline(tmp)

#####################################################################
#AGG and Inactive
#####################################################################
contr=lmerControl(optimizer="bobyqa", optCtrl=list(maxfun=1000000)) # optimiser needed for model to converge

InactiveFull1<-lmer(Tukey.AGG ~
                z.Tukey.Inactive + Treatment*Sex + z.Tukey.Age +
                (1|animalID), data=a, REML = "F", control=contr)

diagnostics.plot(InactiveFull1)# ok
ranef.diagn.plot(InactiveFull1)# look fine
plot(InactiveFull1) #ok
plot(residuals(InactiveFull1)) #fine

#now look at any interaction terms
as.data.frame(drop1(InactiveFull1, test = "Chisq"))
#               Df     AIC      LRT    Pr(Chi)
# <none>         NA 383.6099       NA        NA
# z.Tukey.Inactive  1 384.6899 3.0799857 0.07926122
# z.Tukey.Age       1 381.8543 0.2443511 0.62108101
# Treatment:Sex     1 381.8410 0.2310529 0.63074425 #remove interaction
```

```
InactiveFull2<-lmer(Tukey.AGG ~
                z.Tukey.Inactive + Treatment + Sex + z.Tukey.Age +
                (1|animalID), data=a, REML = "F", control=contr)

diagnostics.plot(InactiveFull2)# ok
ranef.diagn.plot(InactiveFull2)# look fine
plot(InactiveFull2) #ok
plot(residuals(InactiveFull2)) #fine

#now look at any interaction terms
as.data.frame(drop1(InactiveFull2, test = "Chisq"))
#                Df     AIC       LRT    Pr(Chi)
# <none>          NA 381.8410        NA        NA
# z.Tukey.Inactive  1 382.9322 3.09123901 0.07871485
# Treatment         1 380.0247 0.18370468 0.66820864 #remove
# Sex               1 379.9125 0.07150644 0.78915590 #remove
# z.Tukey.Age       1 380.0832 0.24226243 0.62257682 #remove

InactiveFull3<-lmer(Tukey.AGG ~
                z.Tukey.Inactive +
                (1|animalID), data=a, REML = "F", control=contr)

diagnostics.plot(InactiveFull3)# ok
ranef.diagn.plot(InactiveFull3)# look fine
plot(InactiveFull3) #ok
plot(residuals(InactiveFull3)) #fine

#now look at any interaction terms
as.data.frame(drop1(InactiveFull3, test = "Chisq"))
#                Df     AIC     LRT    Pr(Chi)
# <none>          NA 376.2650      NA        NA
# z.Tukey.Inactive  1 377.6027 3.337685 0.06770981

summary(InactiveFull3)
# Linear mixed model fit by maximum likelihood  ['lmerMod']
# Formula: Tukey.AGG ~ z.Tukey.Inactive + (1 | animalID)
# Data: a
# Control: contr
#
# AIC      BIC   logLik deviance df.resid
# 376.3    385.4   -184.1    368.3       68
#
# Scaled residuals:
#   Min      1Q  Median      3Q     Max
# -2.0507 -0.5822 -0.1126  0.5438  2.8724
#
# Random effects:
#   Groups   Name        Variance Std.Dev.
# animalID (Intercept) 3.870    1.967
# Residual             6.617    2.572
# Number of obs: 72, groups:  animalID, 36
#
# Fixed effects:
#   Estimate Std. Error t value
# (Intercept)      14.0232     0.4465  31.404
# z.Tukey.Inactive   0.7537     0.3970   1.899
#
# Correlation of Fixed Effects:
#   (Intr)

Inactivenull<-lmer(Tukey.AGG~
                1 +
                (1|animalID), data=a, REML = "F")
```

```
anova(Inactivenull, InactiveFull3)
# Data: a
# Models:
#   Inactivenull: Tukey.AGG ~ 1 + (1 | animalID)
# InactiveFull3: Tukey.AGG ~ z.Tukey.Inactive + (1 | animalID)
#              Df    AIC    BIC  logLik deviance  Chisq Chi Df Pr(>Chisq)
# Inactivenull   3 377.60 384.43 -185.80   371.60
# InactiveFull3  4 376.26 385.37 -184.13   368.26 3.3377      1    0.06771

mstabInactiveFull=glmm.model.stab(model.res=InactiveFull3, contr=lmerControl(optimizer = "bobyqa",optCtrl =
list(maxfun=2e5)))

mstabInactiveFull$detailed$warnings
# [1] none none none none none none none none none none none none none none none none none none none none none
none none none none none none
# [27] none none none none none none none none none none none
# Levels: none

mstabInactiveFull$summary[,-1]
#                      orig       min       max
# (Intercept)          14.0231764 13.8850710 14.2526874
# z.Tukey.Inactive      0.7537114  0.5325736  0.9191247
# animalID@(Intercept)@NA  1.9672653  1.4715395  2.0820275
# Residual              2.5722918  2.2541967  2.6205303

r.squaredGLMM(InactiveFull3)
#          R2m       R2c
# [1,] 0.05138725 0.4014703

confint.merMod(object=InactiveFull3)
#                   2.5 %    97.5 %
# .sig01           0.7230717  2.964463
# .sigma           2.0707089  3.312147
# (Intercept)     13.1235535 14.922799
# z.Tukey.Inactive -0.0565604  1.553965

plot(a$Inactive_behav_mean, a$AGG_mean,
   xlab="Mean duration of inactive behaviour (ms)", ylab="mean THR (ms)", col="black", pch=16)

tmp <- lm(a$AGG_mean ~ a$Inactive_behav_mean, na.action = na.omit)
abline(tmp)

#####################################################################
#AGG and +ve social
#####################################################################
contr=lmerControl(optimizer="bobyqa", optCtrl=list(maxfun=1000000)) # optimiser needed for model to converge

PositiveFull1<-lmer(Tukey.AGG ~
              z.Tukey.Positive + Treatment*Sex + z.Tukey.Age +
              (1|animalID), data=a, REML = "F", control=contr)

diagnostics.plot(PositiveFull1)# ok
ranef.diagn.plot(PositiveFull1)# look fine
plot(PositiveFull1) #ok
plot(residuals(PositiveFull1)) #fine

#now look at any interaction terms
as.data.frame(drop1(PositiveFull1, test = "Chisq"))
#               Df     AIC       LRT   Pr(Chi)
# <none>         NA 386.6073        NA       NA
# z.Tukey.Positive  1 384.6899 0.08263298 0.7737605
# z.Tukey.Age       1 384.9147 0.30738755 0.5792877
# Treatment:Sex     1 384.8627 0.25547388 0.6132469 #remove interaction

PositiveFull2<-lmer(Tukey.AGG ~
```

```
             z.Tukey.Positive + Treatment + Sex + z.Tukey.Age +
             (1|animalID), data=a, REML = "F", control=contr)

diagnostics.plot(PositiveFull2)# ok
ranef.diagn.plot(PositiveFull2)# look fine
plot(PositiveFull2) #ok
plot(residuals(PositiveFull2)) #fine

#now look at any interaction terms
as.data.frame(drop1(PositiveFull2, test = "Chisq"))
#                 Df      AIC          LRT   Pr(Chi)
# <none>          NA 384.8627           NA        NA
# z.Tukey.Positive  1 382.9322 6.946531e-02 0.7921169
# Treatment        1 382.8628 2.516434e-05 0.9959975 #remove
# Sex              1 382.9798 1.170412e-01 0.7322659 #remove
# z.Tukey.Age      1 383.1713 3.085291e-01 0.5785842 #remove

PositiveFull3<-lmer(Tukey.AGG ~
             z.Tukey.Positive +
             (1|animalID), data=a, REML = "F", control=contr)

diagnostics.plot(PositiveFull3)# ok
ranef.diagn.plot(PositiveFull3)# look fine
plot(PositiveFull3) #ok
plot(residuals(PositiveFull3)) #fine

#now look at any interaction terms
as.data.frame(drop1(PositiveFull3, test = "Chisq"))
#                  Df      AIC        LRT   Pr(Chi)
# <none>          NA 379.5686         NA        NA
# z.Tukey.Positive  1 377.6027 0.03411749 0.8534571

Positivenull<-lmer(Tukey.AGG~
            1 +
            (1|animalID), data=a, REML = "F")

anova(Positivenull, PositiveFull3)
# Data: a
# Models:
#   Positivenull: Tukey.AGG ~ 1 + (1 | animalID)
# PositiveFull3: Tukey.AGG ~ z.Tukey.Positive + (1 | animalID)
# Df    AIC    BIC  logLik deviance  Chisq Chi Df Pr(>Chisq)
# Positivenull   3 377.60 384.43 -185.80   371.60
# PositiveFull3  4 379.57 388.68 -185.78   371.57 0.0341      1     0.8535

mstabPositiveFull=glmm.model.stab(model.res=PositiveFull3, contr=lmerControl(optimizer = "bobyqa",optCtrl =
list(maxfun=2e5)))

mstabPositiveFull$detailed$warnings
# [1] none none none none none none none none none none none none none none none none none none none none
none none none none none none
# [27] none none none none none none none none none none
# Levels: none

mstabPositiveFull$summary[,-1]

#################################################################
#AGG and -ve social
#################################################################
contr=lmerControl(optimizer="bobyqa", optCtrl=list(maxfun=1000000)) # optimiser needed for model to converge

NegativeFull1<-lmer(Tukey.AGG ~
            z.Tukey.Negative + Treatment*Sex + z.Tukey.Age +
            (1|animalID), data=a, REML = "F", control=contr)
```

```
diagnostics.plot(NegativeFull1)# ok
ranef.diagn.plot(NegativeFull1)# look fine
plot(NegativeFull1) #ok -some v.high values
plot(residuals(NegativeFull1)) #fine

#now look at any interaction terms
as.data.frame(drop1(NegativeFull1, test = "Chisq"))
#                Df     AIC       LRT   Pr(Chi)
# <none>          NA 386.6409        NA       NA
# z.Tukey.Negative  1 384.6899 0.04904191 0.8247389
# z.Tukey.Age       1 384.9911 0.35023979 0.5539774
# Treatment:Sex     1 384.8649 0.22405719 0.6359658 #remove interaction

NegativeFull2<-lmer(Tukey.AGG ~
                z.Tukey.Negative + Treatment + Sex + z.Tukey.Age +
                (1|animalID), data=a, REML = "F", control=contr)

diagnostics.plot(NegativeFull2)# ok
ranef.diagn.plot(NegativeFull2)# look fine
plot(NegativeFull2) #ok -some v.high values
plot(residuals(NegativeFull2)) #fine

#now look at any interaction terms
as.data.frame(drop1(NegativeFull2, test = "Chisq"))
#                Df     AIC         LRT   Pr(Chi)
# <none>          NA 384.8649          NA       NA
# z.Tukey.Negative  1 382.9322 6.729092e-02 0.7953228
# Treatment         1 382.8649 1.225144e-05 0.9972072 #remove
# Sex               1 382.9550 9.004298e-02 0.7641225 #remove
# z.Tukey.Age       1 383.2123 3.473670e-01 0.5556074 #remove

NegativeFull3<-lmer(Tukey.AGG ~
                z.Tukey.Negative +
                (1|animalID), data=a, REML = "F", control=contr)

diagnostics.plot(NegativeFull3)# ok
ranef.diagn.plot(NegativeFull3)# look fine
plot(NegativeFull3) #ok -some v.high values
plot(residuals(NegativeFull3)) #fine

#now look at any interaction terms
as.data.frame(drop1(NegativeFull3, test = "Chisq"))
#                Df     AIC       LRT   Pr(Chi)
# <none>          NA 379.5540        NA       NA
# z.Tukey.Negative  1 377.6027 0.04866576 0.8254014

Negativenull<-lmer(Tukey.AGG~
                1 +
                (1|animalID), data=a, REML = "F")

anova(Negativenull, NegativeFull3)
# Data: a
# Models:
#   Negativenull: Tukey.AGG ~ 1 + (1 | animalID)
# NegativeFull3: Tukey.AGG ~ z.Tukey.Negative + (1 | animalID)
# Df    AIC    BIC  logLik deviance  Chisq Chi Df Pr(>Chisq)
# Negativenull   3 377.60 384.43 -185.80   371.60
# NegativeFull3  4 379.55 388.66 -185.78   371.55 0.0487      1     0.8254


mstabNegativeFull=glmm.model.stab(model.res=NegativeFull3, contr=lmerControl(optimizer = "bobyqa",optCtrl =
list(maxfun=2e5)))

mstabNegativeFull$detailed$warnings
# [1] none none none none none none none none none none none none none none none none none none none none none
```

```
# [16] none none none none none none none none none none none none none none none
# [31] none none none none none none
# Levels: none

mstabNegativeFull$summary[,-1]

#####################################################################
#TL
#####################################################################
contr=lmerControl(optimizer="bobyqa", optCtrl=list(maxfun=1000000)) # optimiser needed for model to converge

StressFull1<-lmer(Tukey.TotalLook~
            z.Tukey.Stress + Treatment*Sex + z.Tukey.Age +
            (1|animalID), data=a, REML = "F", control=contr)

diagnostics.plot(StressFull1)# okay - some high and low
ranef.diagn.plot(StressFull1)# look fine
plot(StressFull1) #ok
plot(residuals(StressFull1)) #fine

#now look at any interaction terms
as.data.frame(drop1(StressFull1, test = "Chisq"))
#             Df     AIC      LRT   Pr(Chi)
# <none>       NA 650.6622       NA       NA
# z.Tukey.Stress  1 649.0139 0.3517106 0.5531464
# z.Tukey.Age     1 648.9067 0.2445075 0.6209693
# Treatment:Sex   1 649.7070 1.0448715 0.3066912 #remove interaction

StressFull2<-lmer(Tukey.TotalLook~
            z.Tukey.Stress + Treatment + Sex + z.Tukey.Age +
            (1|animalID), data=a, REML = "F", control=contr)

diagnostics.plot(StressFull2)# okay - some high and low
ranef.diagn.plot(StressFull2)# look fine
plot(StressFull2) #ok
plot(residuals(StressFull2)) #fine

#now look at any interaction terms
as.data.frame(drop1(StressFull2, test = "Chisq"))
#              Df     AIC       LRT   Pr(Chi)
# <none>       NA 649.7070        NA       NA
# z.Tukey.Stress  1 648.1323 0.42521027 0.5143486
# Treatment       1 647.7179 0.01087889 0.9169297 #remove
# Sex             1 647.8659 0.15889672 0.6901743 #remove
# z.Tukey.Age     1 647.9639 0.25687591 0.6122746 #remove

StressFull3<-lmer(Tukey.TotalLook~
              z.Tukey.Stress +
              (1|animalID), data=a, REML = "F", control=contr)

diagnostics.plot(StressFull3)# okay - some high and low
ranef.diagn.plot(StressFull3)# look fine
plot(StressFull3) #ok
plot(residuals(StressFull3)) #fine

#now look at any interaction terms
as.data.frame(drop1(StressFull3, test = "Chisq"))
#              Df     AIC      LRT   Pr(Chi)
# <none>       NA 644.4382       NA       NA
# z.Tukey.Stress  1 642.8412 0.4029676 0.5255606

Stressnull<-lmer(Tukey.TotalLook~
            1 +
            (1|animalID), data=a, REML = "F")
```

```
anova(Stressnull, StressFull3)
# Data: a
# Models:
#   Stressnull: Tukey.TotalLook ~ 1 + (1 | animalID)
# StressFull3: Tukey.TotalLook ~ z.Tukey.Stress + (1 | animalID)
#            Df    AIC    BIC  logLik deviance Chisq Chi Df Pr(>Chisq)
# Stressnull   3 642.84 649.67 -318.42   636.84
# StressFull3  4 644.44 653.54 -318.22   636.44 0.403      1     0.5256

#no relationship between stress behaviour and THR looking time

#####################################################################
#AGG and Explore
#####################################################################
contr=lmerControl(optimizer="bobyqa", optCtrl=list(maxfun=1000000)) # optimiser needed for model to converge

ExploreFull1<-lmer(Tukey.TotalLook ~
                z.Tukey.Explore + Treatment*Sex + z.Tukey.Age +
                (1|animalID), data=a, REML = "F", control=contr)

diagnostics.plot(ExploreFull1)# ok
ranef.diagn.plot(ExploreFull1)# look fine
plot(ExploreFull1) #ok
plot(residuals(ExploreFull1)) #fine

#now look at any interaction terms
as.data.frame(drop1(ExploreFull1, test = "Chisq"))
#                Df     AIC       LRT   Pr(Chi)
# <none>         NA 650.5202        NA       NA
# z.Tukey.Explore  1 649.0139 0.49365214 0.4823027
# z.Tukey.Age      1 648.5793 0.05910115 0.8079223
# Treatment:Sex    1 649.5915 1.07129929 0.3006522 #remove interaction

ExploreFull2<-lmer(Tukey.TotalLook ~
                z.Tukey.Explore + Treatment + Sex + z.Tukey.Age +
                (1|animalID), data=a, REML = "F", control=contr)

diagnostics.plot(ExploreFull2)# ok
ranef.diagn.plot(ExploreFull2)# look fine
plot(ExploreFull2) #ok
plot(residuals(ExploreFull2)) #fine

#now look at any interaction terms
as.data.frame(drop1(ExploreFull2, test = "Chisq"))
#              Df     AIC       LRT   Pr(Chi)
# <none>         NA 649.5915        NA       NA
# z.Tukey.Explore  1 648.1323 0.54072405 0.4621328
# Treatment        1 647.6131 0.02155726 0.8832710 #remove
# Sex              1 647.9363 0.34479213 0.5570761 #remove
# z.Tukey.Age      1 647.6456 0.05407420 0.8161197 #remove

ExploreFull3<-lmer(Tukey.TotalLook ~
                z.Tukey.Explore +
                (1|animalID), data=a, REML = "F", control=contr)

diagnostics.plot(ExploreFull3)# ok
ranef.diagn.plot(ExploreFull3)# look fine
plot(ExploreFull3) #ok
plot(residuals(ExploreFull3)) #fine

#now look at any interaction terms
as.data.frame(drop1(ExploreFull3, test = "Chisq"))
#                Df     AIC      LRT   Pr(Chi)
# <none>         NA 644.2233       NA       NA
# z.Tukey.Explore  1 642.8412 0.6178628 0.4318426
```

```
ExploreNull<-lmer(Tukey.TotalLook~
              1 +
              (1|animalID), data=a, REML = "F")

anova(ExploreNull, ExploreFull3)
# Data: a
# Models:
#   ExploreNull: Tukey.TotalLook ~ 1 + (1 | animalID)
# ExploreFull3: Tukey.TotalLook ~ z.Tukey.Explore + (1 | animalID)
# Df    AIC    BIC  logLik deviance  Chisq Chi Df Pr(>Chisq)
# ExploreNull   3 642.84 649.67 -318.42   636.84
# ExploreFull3  4 644.22 653.33 -318.11   636.22 0.6179      1     0.4318

#####################################################################
#AGG and Inactive
#####################################################################
contr=lmerControl(optimizer="bobyqa", optCtrl=list(maxfun=1000000)) # optimiser needed for model to converge

InactiveFull1<-lmer(Tukey.TotalLook ~
                z.Tukey.Inactive + Treatment*Sex + z.Tukey.Age +
                (1|animalID), data=a, REML = "F", control=contr)

diagnostics.plot(InactiveFull1)# ok
ranef.diagn.plot(InactiveFull1)# look fine
plot(InactiveFull1) #ok
plot(residuals(InactiveFull1)) #fine

#now look at any interaction terms
as.data.frame(drop1(InactiveFull1, test = "Chisq"))
#               Df     AIC      LRT   Pr(Chi)
# <none>          NA 650.8288       NA       NA
# z.Tukey.Inactive  1 649.0139 0.1850665 0.6670548
# z.Tukey.Age       1 648.9347 0.1059295 0.7448268
# Treatment:Sex     1 649.9211 1.0922541 0.2959723 #remove interaction

InactiveFull2<-lmer(Tukey.TotalLook ~
                z.Tukey.Inactive + Treatment + Sex + z.Tukey.Age +
                (1|animalID), data=a, REML = "F", control=contr)

diagnostics.plot(InactiveFull2)# ok
ranef.diagn.plot(InactiveFull2)# look fine
plot(InactiveFull2) #ok
plot(residuals(InactiveFull2)) #fine

#now look at any interaction terms
as.data.frame(drop1(InactiveFull2, test = "Chisq"))
#               Df     AIC        LRT   Pr(Chi)
# <none>          NA 649.9211         NA       NA
# z.Tukey.Inactive  1 648.1323 0.211183644 0.6458413
# Treatment         1 647.9221 0.001078304 0.9738041 #remove
# Sex               1 648.0724 0.151360602 0.6972385 #remove
# z.Tukey.Age       1 648.0229 0.101783449 0.7496998 #remove

InactiveFull3<-lmer(Tukey.TotalLook ~
                z.Tukey.Inactive +
                (1|animalID), data=a, REML = "F", control=contr)

diagnostics.plot(InactiveFull3)# ok
ranef.diagn.plot(InactiveFull3)# look fine
plot(InactiveFull3) #ok
plot(residuals(InactiveFull3)) #fine

#now look at any interaction terms
as.data.frame(drop1(InactiveFull3, test = "Chisq"))
```

```
#               Df     AIC      LRT   Pr(Chi)
# <none>         NA 644.3159       NA       NA
# z.Tukey.Inactive  1 642.8412 0.5252781 0.4685988

Inactivenull<-lmer(Tukey.TotalLook~
                1 +
                (1|animalID), data=a, REML = "F")

anova(Inactivenull, InactiveFull3)
# Data: a
# Models:
#   Inactivenull: Tukey.TotalLook ~ 1 + (1 | animalID)
# InactiveFull3: Tukey.TotalLook ~ z.Tukey.Inactive + (1 | animalID)
#              Df    AIC    BIC  logLik deviance  Chisq Chi Df Pr(>Chisq)
# Inactivenull   3 642.84 649.67 -318.42   636.84
# InactiveFull3  4 644.32 653.42 -318.16   636.32 0.5253      1     0.4686

#####################################################################
#AGG and +ve social
#####################################################################
contr=lmerControl(optimizer="bobyqa", optCtrl=list(maxfun=1000000)) # optimiser needed for model to converge

PositiveFull1<-lmer(Tukey.TotalLook ~
                 z.Tukey.Positive + Treatment*Sex + z.Tukey.Age +
                 (1|animalID), data=a, REML = "F", control=contr)

diagnostics.plot(PositiveFull1)# ok
ranef.diagn.plot(PositiveFull1)# look fine
plot(PositiveFull1) #ok
plot(residuals(PositiveFull1)) #fine

#now look at any interaction terms
as.data.frame(drop1(PositiveFull1, test = "Chisq"))
#               Df     AIC       LRT   Pr(Chi)
# <none>         NA 650.9615        NA       NA
# z.Tukey.Positive  1 649.0139 0.05242286 0.8188997
# z.Tukey.Age       1 649.1049 0.14339749 0.7049265
# Treatment:Sex     1 650.0555 1.09401647 0.2955830 #remove interaction

PositiveFull2<-lmer(Tukey.TotalLook ~
                 z.Tukey.Positive + Treatment + Sex + z.Tukey.Age +
                 (1|animalID), data=a, REML = "F", control=contr)

diagnostics.plot(PositiveFull2)# ok
ranef.diagn.plot(PositiveFull2)# look fine
plot(PositiveFull2) #ok
plot(residuals(PositiveFull2)) #fine

#now look at any interaction terms
as.data.frame(drop1(PositiveFull2, test = "Chisq"))
#               Df     AIC       LRT   Pr(Chi)
# <none>         NA 650.0555        NA       NA
# z.Tukey.Positive  1 648.1323 0.07677758 0.7817128
# Treatment         1 648.0674 0.01188311 0.9131948 #remove
# Sex               1 648.2591 0.20367054 0.6517743 #remove
# z.Tukey.Age       1 648.2001 0.14457624 0.7037733 #remove

PositiveFull3<-lmer(Tukey.TotalLook ~
                 z.Tukey.Positive +
                 (1|animalID), data=a, REML = "F", control=contr)

diagnostics.plot(PositiveFull3)# ok
ranef.diagn.plot(PositiveFull3)# look fine
plot(PositiveFull3) #ok
plot(residuals(PositiveFull3)) #fine
```

```
#now look at any interaction terms
as.data.frame(drop1(PositiveFull3, test = "Chisq"))
#               Df     AIC     LRT   Pr(Chi)
# <none>          NA 644.6592      NA       NA
# z.Tukey.Positive  1 642.8412 0.18193 0.6697199

Positivenull<-lmer(Tukey.AGG~
                1 +
                (1|animalID), data=a, REML = "F")

anova(Positivenull, PositiveFull3)
# Data: a
# Models:
#   Positivenull: Tukey.AGG ~ 1 + (1 | animalID)
# PositiveFull3: Tukey.TotalLook ~ z.Tukey.Positive + (1 | animalID)
#              Df    AIC    BIC  logLik deviance Chisq Chi Df Pr(>Chisq)
# Positivenull   3 377.60 384.43 -185.80   371.60
# PositiveFull3  4 644.66 653.77 -318.33   636.66     0     1        1

#####################################################################
#AGG and -ve social
#####################################################################
contr=lmerControl(optimizer="bobyqa", optCtrl=list(maxfun=1000000)) # optimiser needed for model to converge

NegativeFull1<-lmer(Tukey.TotalLook ~
                z.Tukey.Negative + Treatment*Sex + z.Tukey.Age +
                (1|animalID), data=a, REML = "F", control=contr)

diagnostics.plot(NegativeFull1)# ok
ranef.diagn.plot(NegativeFull1)# look fine
plot(NegativeFull1) #ok -some v.high values
plot(residuals(NegativeFull1)) #fine

#now look at any interaction terms
as.data.frame(drop1(NegativeFull1, test = "Chisq"))
#               Df     AIC         LRT   Pr(Chi)
# <none>          NA 651.0130          NA       NA
# z.Tukey.Negative  1 649.0139 0.0008829415 0.9762949
# z.Tukey.Age       1 649.1378 0.1248384607 0.7238449
# Treatment:Sex     1 650.1207 1.1076743719 0.2925880 #remove interaction

NegativeFull2<-lmer(Tukey.TotalLook ~
                z.Tukey.Negative + Treatment + Sex + z.Tukey.Age +
                (1|animalID), data=a, REML = "F", control=contr)

diagnostics.plot(NegativeFull2)# ok
ranef.diagn.plot(NegativeFull2)# look fine
plot(NegativeFull2) #ok -some v.high values
plot(residuals(NegativeFull2)) #fine

#now look at any interaction terms
as.data.frame(drop1(NegativeFull2, test = "Chisq"))
#               Df     AIC       LRT   Pr(Chi)
# <none>          NA 650.1207        NA       NA
# z.Tukey.Negative  1 648.1323 0.01157977 0.9143056
# Treatment         1 648.1465 0.02580126 0.8723866 #remove
# Sex               1 648.4276 0.30689493 0.5795919 #remove
# z.Tukey.Age       1 648.2415 0.12079507 0.7281738 #remove

NegativeFull3<-lmer(Tukey.TotalLook ~
                z.Tukey.Negative +
                (1|animalID), data=a, REML = "F", control=contr)

diagnostics.plot(NegativeFull3)# ok
```

```
ranef.diagn.plot(NegativeFull3)# look fine
plot(NegativeFull3) #ok -some v.high values
plot(residuals(NegativeFull3)) #fine

#now look at any interaction terms
as.data.frame(drop1(NegativeFull3, test = "Chisq"))
# Df      AIC        LRT   Pr(Chi)
# <none>          NA 644.8400         NA       NA
# z.Tukey.Negative  1 642.8412 0.001160802 0.9728209

Negativenull<-lmer(Tukey.AGG~
                1 +
                (1|animalID), data=a, REML = "F")

anova(Negativenull, NegativeFull3)
# Data: a
# Models:
#   Negativenull: Tukey.AGG ~ 1 + (1 | animalID)
# NegativeFull3: Tukey.TotalLook ~ z.Tukey.Negative + (1 | animalID)
# Df    AIC    BIC  logLik deviance Chisq Chi Df Pr(>Chisq)
# Negativenull   3 377.60 384.43 -185.80   371.60
# NegativeFull3  4 644.84 653.95 -318.42   636.84     0      1         1
```

Appendix 5a

Oxytocin rational and collection

Tend and befriend

The "fight or flight" response was first described by Canon in 1914. More recently, Taylor et al (2000) suggested "tend and befriend" as an alternative stress response pathway in females. The authors argued that a response geared toward aggression or retreat may not be adaptive for female animals as it could leave offspring fatally unprotected. Instead, female behaviour is directed at retrieving and protecting offspring while anticipating and avoiding threats to increase the likelihood of offspring survival (Taylor et al, 2000). "Tend and befriend" utilises neuromodulators and hormones with known anxiolytic functions and significant roles in affiliation and pair bonding (Froehlich, 1997; Young et al, 2011; Carrier et al, 2015). It is mediated by oxytocin, the endogenous opioid peptides and the female sex hormone oestrogen.

Oxytocin and stress response

Oxytocin is released by action of the HPA axis in response to a stressor. This posterior pituitary hormone is associated with increased parasympathetic functioning and plays a counterregulatory role in the stress and fear responses (Swanson & Sawchenko, 1980, Sawchenko & Swanson, 1982; Dreifuss et al, 1992). The endogenous application of oxytocin inhibits corticotrophin releasing factor (CRF) neurons, decreases corticosteroid release and reduces fearful behaviour in both humans and rodents (Dreifuss et al, 1992; McCarthy et al, 1996; Windle et al, 2004).

Oxytocin increases activity in the capsular and lateral portions of the central amygdala blocking the fear inducing effect of vasopressin (Campbell, 2013), reduces activity in the basolateral amygdala (Huber et al, 2005) and downgrades the connection between the amygdala and the brain stem areas responsible for SNS activation (Ferguson et al, 2002; Campbell, 2013). Reduced SNS activity reduces the production of stress hormones and mutes the hormone cascade that mediates the "fight or flight" response (Light et al, 2000; Heinrichs et al, 2001). In response to a stressor, females

with higher oxytocin levels are calmer and perform more pro-social, affiliative and maternal behaviour, which results in increase offspring survival (Kendrick et al, 1997; Taylor et al, 2000).

Oxytocin

At the time of sample collection urine was the only suitable, non-invasive, properly validated method for oxytocin analysis (Rault et al, 2017 - review of 32 papers on oxytocin and welfare in domesticated species published since 1993) (Saliva has since been validated as a non-invasive sampling method for oxytocin analysis (MacLean et al, 2018; Salimetrics, 2018)). Urinary oxytocin has a response time of around one hour (Reyes et al, 2014), is highly correlated with plasma oxytocin level (Amico et al, 1987; Romero et al, 2014) and, although oxytocin in the cerebrospinal fluid of rhesus macaques has a circadian rhythm, this rhythm is not present in the urine (Perlow et al, 1982).

Influence of oxytocin on attention bias and looking time

Oxytocin is a neuromodulatory hormone that modulates social behaviour, for example, maternal bonding, partner preference (Lim & Young, 2006), female sexual intercourse, parturition, lactation (Donaldson & Young, 2008) and encourages altruism (Dreu et al, 2010) and affiliation (Romero et al, 2014). Oxytocin is also known to have anxiolytic (mediation / inhibition of anxiety) functions (Donaldson & Young, 2008) and has been shown to reduce macaque response to aggressive, dominant or unfamiliar faces and supress vigilance towards potentially threatening social stimuli (Ebitz et al, 2013; Lui et al, 2015). Parr et al (2013) reported that oxytocin significantly reduced monkeys' attention to negative facial expressions, but not neutral social or non-social images. Intranasal oxytocin administered to infant rhesus macaques resulted in increased time spent viewing expression videos compared to the placebo, but selectively reduced attention to eyes in a neutral face in a dose dependent manner (3 times/week or 1 time/week;Parr et al, 2016).

We hypothesise that macaques with higher urinary oxytocin will have lower overall looking times and will be more avoidant of the aggressive stimuli than macaques with lower oxytocin.

Oxytocin procedures

The first step was to identify the most suitable substrate for oxytocin sample collection. Potential methods of urine collection were discussed with a veterinarian at the Comparative Biology Centre (CBC) at Newcastle University and the Scientific Project Co-ordinator at MRC-CFM; both agreed that

Figure 5a.1. Preliminary urine collection device for rhesus macaques trialled at MRC-CFM in May and July 2017.

collecting non-contaminated urine is complex and would require a large investment of time to train animals. At the CBC, urine had only been collected during sedation via cystocentesis (direct puncture of the bladder) guided by ultrasound (Gray et al, 2016).

I trialled four methods: a urine collection tube, pot, board, and tray. Prior to the training at the University of Oxford in August 2017, a preliminary urine collection device was trialled at MRC-CFM in May and July 2017. At the University of Oxford, the urine collection pot was placed through the bars; this method was not suitable for MRC-CFM as most of the bars in the cage room have square holes of 40 mm by 40 mm, which are too small for sample pots to fit through. There was one larger 70 mm by 40 mm hole at one end of the cage room; however, use of this alone would have reduced flexibility and made collecting samples more difficult as it required animals to be stationed above this location. A plastic urine collection bottle was trialled trough the larger hole. The material was too weak when manipulated by the macaques – the plastic human urine sample pot was broken within 30-seconds.

The robust device was made of copper endfeed 35 mm cap attached by a 5 mm stainless steel hexagon socket mushroom head screw to a 500 mm long flat steel bar 10 mm x 1 mm (Figure 2.12). The copper pot would have needed to be coated with a non-toxic substance to prevent stress corrosion cracking of the copper caused by ammonia in the urine (Tromans, 1997).

A pilot trial was run in July 2017 in which the device was tested for destructibility, volume, water tightness and group engagement. Crockford et al (2013) collected urine from chimpanzees for subsequent analysis of oxytocin; they collected 5 ml of urine from 33 free-ranging chimpanzees and used just 1 ml for analysis. The new urine collection device was tested for retention when water was sprayed at it using a syringe form a height of 10 cm. Each time at least 5 ml of water could be collected from the pot suggesting that the volume and shape were sufficient for urine collection.

Although the pilot trial was initially promising, following the first week of desensitisation training, many of the macaques were still very wary of the pot, therefore, an alternative method was discussed with the animal technicians. The urine collection board or "toilet board" was a standard baseboard for the crush-back area modified with 16 drilled holes to allow urine to pass through (Figure 2.13) and be collect in a 28 cm stainless steel bowl placed on the enclosure floor. The floor of the enclosure could be sectioned off meaning the metal bowl, containing the urine sample, could easily and safely have been retrieved.

The initial plan was to train the monkeys to station over the holes and urinate into the bowl. However, because the urine collection board was inserted into the crush-back area, which is typically used for veterinary checks and isolation form the group, the macaques refused to sit on the board and would not stay in the area long enough to learn to urinate here.

Urine collection training protocol

Table 5a.1. Engagement of socially housed rhesus macaques at MRC-CFM with the urine collection device during pilot trial in July 2017.

Group Number	Engagement
G60	Approach within 1 minute, group generally very wary. Adult female approach for nut when food added. Juveniles ate raisins from pot.
G57	Approach within 1 minute. Wanganui took nut in lower section. Tallulah interested but did not go within 1 m of pot.
G55	Approach within 1 minute, group generally confident. Lots of juveniles approached and ate. Varsalla ate from pot. Versa ate from lower section. Adult male ate in upper section
G18	Approached within 1 minute. Adult male ate from pot, 3 adult females ate from / touched pot.
G16	Approached within 1 minute. Tulip ate from pot.
G15	Approached within 1 minute, group generally wary. Thorn ate raisins from lower section. Venice ate from pot.
G13	Approached within 1 minute. Juveniles very confident. Rozanne approached and ate from pot.
G04	Approached within 1 minute. Prune and 3 other adult females ate from pot. Juveniles seem very confident.
G03	so interested.
G01	quickly. Tes

Figure 5a.2. The urine collection board trialled at MRC-CFM in November 2017. The board could be placed into the enclosure like a normal base board in the crush-back area. Photograph: E. Howarth.

The protocol for urine collection training is shown in Figure 5a.2. This method using the urine collection trays and the protocol of PRT allowed the non-invasive collection of 96 urine samples from 29 macaques for oxytocin analysis.

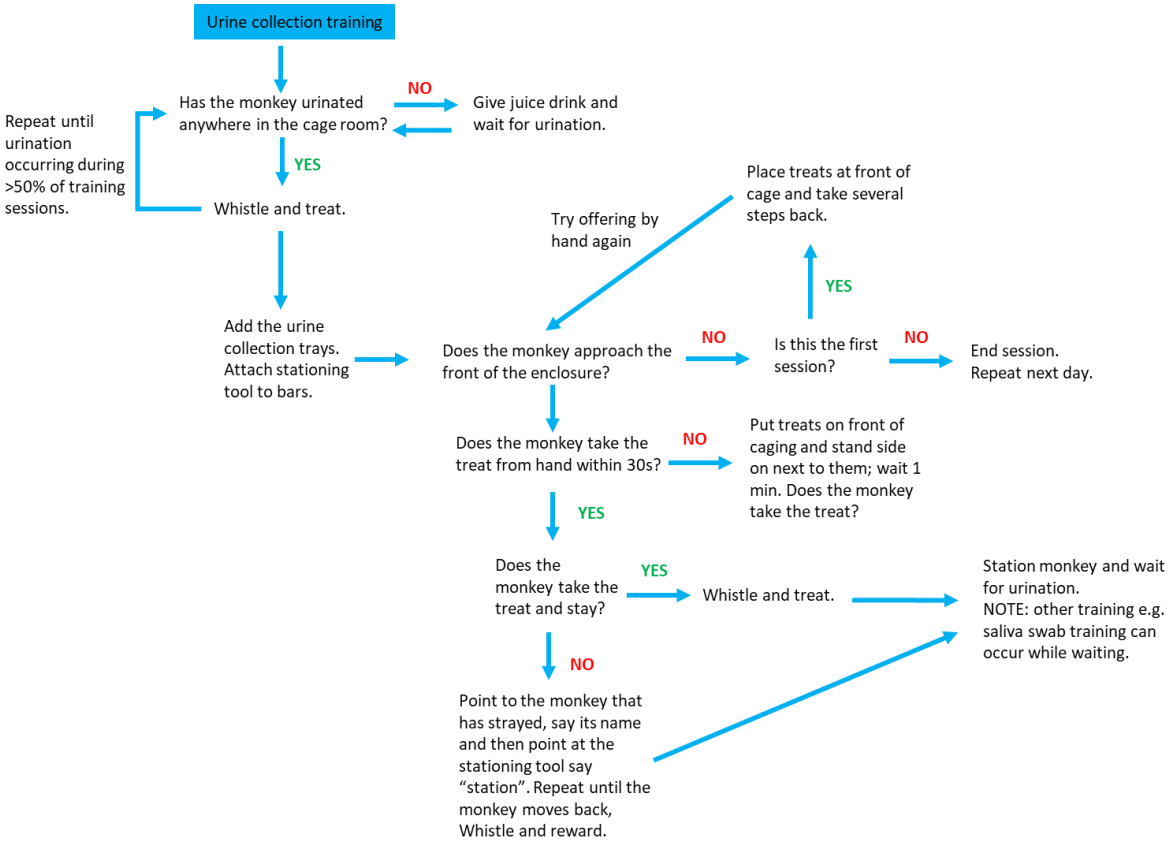


Figure 5.2a Urine collection training steps for rhesus macaques.

Urine collection

Crockford et al (2013) used plastic sheets or leaves on the forest floor to collect urine from chimpanzees. Urine was collected using 4 1m by 1m B&Q PVC corrugated roofing sheets (Figure 5a.1) placed on the floor of the middle two section in the cage room with standard side boards placed either side of this middle section to prevent macaques interfering with the plastic and reduce injury and contamination risk. See Appendix 2g for the urine training and collection protocol. Boards were washed with disinfectant and thoroughly rinsed with water between sample collections. Urine was collected into 5 ml plastic bottles using a syringe or by pouring from the plastic sheets. The urine was then frozen at -20°C in a storage freezer at MRC-CFM within one hour of collection (Stock et al, 1991). Ninety-six urine samples were collected from 29 monkeys (21 female, mean age = 7.81 ± 3.38 years, range = 3.5 - 16.2 years).

Figure 5a.3. B&Q PVC corrugated roofing sheets used as urine collection board for rhesus macaques at MRC-CFM. Photograph: https://www.diy.com/departments/pvc-corrugated-roofing-sheet-2m-x-950mm/1932744_BQ.prd

Sample analysis

Radioimmunoassay (RIA) is the most common method of oxytocin analysis and yet research published by Lefevre et al (2017) showed there to be no correlation with RIA and commercial or laboratory developed EIA. As a result, liquid chromatography mass spectrometry (LCMS) would be the preferred method (Zhang et al, 2011); however, due to funding constraints, this analysis for Part C of this study has not yet been completed.

Appendix 5b

For this assay, competitive binding occurred when a fixed amount of the enzyme-labelled antigen, horseradish peroxidase (COSMO FKA 403, cortisol-3-CMO-HPR 200 µg/ 20 µl), and the unknown amount of antigen in the saliva sample compete for binding sites within the wells of a protein A coated microtiter plate. A Pierce™ protein A coated clear 96-well microtiter plate (Corning™) was washed three times with 250 µl of phosphate buffered saline (PBS) on a Biochrom Asys Atlantis Microplate Washer.

Standards, samples, B0, NSB and quality controls (high and low cortisol) were pipetted onto the assay plate in duplicate with a volume of 50 µl per well. Standard concentrations ranged from 50ng/ml to 0.098ng/ml. The saliva samples were diluted with PBS before pipetting onto the plate if required. Subsequently, 50 µl of cortisol conjugated with horseradish peroxidase (cortisol-3-CMO-HPR 200 µg/ 20 µl, Cosmo Bio Co. Ltd.) and 50 µl of the anti-cortisol-3-antibody (Cosmo Bio Co. Ltd.) was added to each well except for the NSB, instead an addition 50 µl of assay buffer was added. The plate was then incubated overnight.

After incubation, the plate was washed a further four times with 250 µl PBS and 250 µl of substrate solution (tetramethylbenzidine in a mildly acidic buffer) was dispensed into each well using the multi-channel pipette. The plate was incubated in the dark with shaking on a Stuart Miniorbital Shaker SSM1. After 20 minutes, 50 µl of stop reagent (sulphuric acid) was added to each well using the multi-channel pipette. Absorbance at 450 nm was measured using a Clariostar reader.

Appendix 5c

```
################################################################################
#Part A - influence of cortisol on AB, Agg and TL
################################################################################

################################################################################
#Step 1. Clear workspace, set working directory and load in required files and packages
################################################################################

ls() #this looks at what is loaded
rm(list=ls()) #this clears everything

#set the working directory
#setwd("M:/Emily/Doing/Postgraduates/Emmeline Howarth/Data")
#setwd("C:/Users/emily/OneDrive/Desktop/R_Code")
setwd("C:/Users/emmel/Desktop/R Studio and R")
setwd("//jmu.ac.uk/PFS/HS03H/Store10/HS226988/My Documents")

#Load Package
# install.packages("lme4")
# install.packages("tidyverse")
# install.packages("car")
# install.packages("carData")
# install.packages("rcompanion")
install.packages("MuMIn")

library(tidyverse)
library(rcompanion)
library(lme4)
library(car)
library(MuMIn)

#load Roger Functions #source files need to be in the working directory

source("C:/Users/emmel/Desktop/R Studio and R/Functions/diagnostic_fcns.r")
source("C:/Users/emmel/Desktop/R Studio and R/Functions/glmm_stability.r")
# source("D:/R Studio and R/Functions/diagnostic_fcns.r")
# source("D:/R Studio and R/Functions/glmm_stability.r")
#source("M:/Emily/Doing/R Users Group/R Training/Mundry/Functions/Functions/diagnostic_fcns.r")
#source("M:/Emily/Doing/R Users Group/R Training/Mundry/Functions/Functions/glmm_stability.r")
source("//jmu.ac.uk/PFS/HS03H/Store10/HS226988/My Documents/Functions/diagnostic_fcns.r")
source("//jmu.ac.uk/PFS/HS03H/Store10/HS226988/My Documents/Functions/glmm_stability.r")

################################################################################
#Step 2. Import and check data:
################################################################################

#Load data
AB1KempThatcherHowarth_20191201<-read.csv(file.choose(), header=T) #select file from pop up window
d <- AB1KempThatcherHowarth_20191201

nrow(d)  #1188 (if higher do the following go back to CSV and delete ghost cells from bottom)
ncol(d) #245

str(d)
View(d)

#For analysis, select monkeys with CORT data
SData<-subset(d, CortSelect =="Yes")
nrow(SData)#77
################################################################################
#Step 3. Ensure variables accurately labelled as factors and correct levels of each factor are being read.
################################################################################
MData<-SData
```

```
#factors
MData$animalID <- as.factor(MData$animalID)
str(MData$animalID) #Factor w/ 110 levels
MData$Treatment <- as.factor(MData$Treatment)
str(MData$Treatment) #Factor w/ 2 level
MData$AggLoc <- as.factor(MData$AggLoc)
str(MData$AggLoc) #Factor w/ 2 levels
MData$StimulusID<- as.factor(MData$StimulusID)
str(MData$StimulusID) #Factor w/ 7 levels
MData$AnyOtherTreatment<-as.factor(MData$AnyOtherTreatment)
str(MData$AnyOtherTreatment) #Factor w/ 2 levels

#numeric
MData$TimeR<-as.numeric(MData$TimeR)
str(MData$TimeR) #num
MData$DaysSinceLastHC<-as.numeric(MData$DaysSinceLastHC)
str(MData$DaysSinceLastHC) #num
MData$TrialStudentFile<-as.numeric(MData$TrialStudentFile)
str(MData$TrialStudentFile) #num
MData$TrialChronological<-as.numeric(MData$TrialChronological)
str(MData$TrialChronological) #num
MData$Trial14<-as.numeric(MData$Trial14or5InWeekorBlock)
str(MData$Trial14) #num
MData$Concentration_.xDil.Factor..pg.mL.<-as.numeric(MData$Concentration_.xDil.Factor..pg.mL.)
str(MData$Concentration_.xDil.Factor..pg.mL.)
MData$TrialStudentFile<-as.numeric(MData$TrialStudentFile)
str(MData$TrialStudentFile)

MData$Rank <- as.integer(MData$Rank)
str(MData$Rank) #int

nrow(MData)#77

#############################################################################
#Step 4. look at the response variables & check distribution
#############################################################################
RData<-MData

#AGG
hist(RData$AGG)
hist(transformTukey(RData$AGG, plotit=FALSE))
# lambda    W Shapiro.p.value
# 420  0.475 0.95       0.004286
RData$Tukey.AGG<-transformTukey(RData$AGG)

#ABDiff
hist(RData$ABDiff)#looks fine as usual
hist(transformTukey(RData$ABDiff, plotit=FALSE))
# lambda      W Shapiro.p.value
# 441      1 0.9818         0.3391

#TotalLook
hist(RData$TotalLook)
hist(transformTukey(RData$TotalLook, plotit=FALSE))
# lambda      W Shapiro.p.value
# 421    0.5 0.9834         0.4143
hist(sqrt(RData$TotalLook))
RData$sqrt.TotalLook<-sqrt(RData$TotalLook)

#Cortisol
summary(RData$Concentration_.xDil.Factor..pg.mL.)
# Min.  1st Qu.   Median     Mean  3rd Qu.     Max.
# 141.5    438.1   1178.1  10671.9   4451.6 259962.0
hist(RData$Concentration_.xDil.Factor..pg.mL.)
```

```
hist(transformTukey(RData$Concentration_.xDil.Factor..pg.mL.))
# lambda      W Shapiro.p.value
# 391  -0.25 0.9805         0.2847
RData$Tukey.CORT<-transformTukey(RData$Concentration_.xDil.Factor..pg.mL.)

#TimeR
hist(RData$TimeR) #right skewed. Try transforming:
hist(transformTukey(RData$TimeR))
# lambda      W Shapiro.p.value
# 357   -1.1 0.9542       0.007392
RData$z.Tukey.TimeR<-scale(transformTukey(RData$TimeR))

#InjuryLast48HrsYN
summary(RData$InjuryLast48HrsYN)
#No Yes
#74   3    #do not include not enough data

#CleaningLast24HrsYN
summary(RData$CleaningLast24Hrs)
# No Yes
# 70   7   #do not include not enough data

#Trial14InBlock
hist(RData$Trial14or5InWeekorBlock)
hist(transformTukey(RData$Trial14or5InWeekorBlock, plotit=FALSE))
# lambda      W Shapiro.p.value
# 434  0.825 0.8971      1.299e-05
RData$z.Tukey.Trial14or5InWeekorBlock<-scale(transformTukey(RData$Trial14or5InWeekorBlock))

#TrialStudentFile
hist(RData$TrialStudentFile)
hist(transformTukey(RData$TrialStudentFile, plotit=FALSE))
# lambda     W Shapiro.p.value
# 435   0.85 0.948       0.003312
RData$z.Tukey.TrialStudent<-scale(transformTukey(RData$TrialStudentFile))

#AggLoc
summary(RData$AggLoc)
#Lmonkeyview Rmonkeyview
#   41          36

#StimulusID
str(RData$StimulusID)
RData$StimulusID<-as.factor(RData$StimulusID)
table(RData$StimulusID)
#1  2  3  4  5  6  7
#12 16  5 12 10 12 10      #may not be enough of each to include in model


#DrugKHCL
summary(RData$DrugKHClLast24HoursYN)
#No Yes
#67  10

#BabyBornGrp
RData$BabyBornLast24HrsGrp<-as.factor(RData$BabyBornLast24HrsGrp)
summary(RData$BabyBornLast24HrsGrp)
#0  1
#74  3     #do not include not enough data

#DiruptioninGrp
summary(RData$DisruptionInGrpOtherYN)
#No Yes
#62  15
```

```
#OtherTreatment
summary(RData$AnyOtherTreatment)
#No Yes
#60  17

#Treatment
table(RData$Treatment)
# BL Stress
# 38    39

#AnxietyBehaviour
str(RData$ANXIETYSUM)#int
Anxiety<-as.numeric(RData$ANXIETYSUM)
summary(Anxiety)
# Min. 1st Qu.  Median    Mean 3rd Qu.    Max.
# 0      0   8926   22495   24978  163106
str(Anxiety)#num
hist(Anxiety)
hist(transformTukey(Anxiety))
# lambda      W Shapiro.p.value
# 416  0.375 0.8992       1.58e-05
RData$Tukey.Anxiety<-scale(transformTukey(Anxiety))

#Vigilance
str(RData$vigilance)#int
hist(RData$vigilance)
hist(transformTukey(RData$vigilance))
# lambda     W Shapiro.p.value
# 411   0.25 0.677      9.948e-12
RData$Tukey.Vigilance<-scale(transformTukey(RData$vigilance))

#############################################################################
#Step 5. check all variables are reading correctly
#############################################################################
CData<-RData

str(CData$Tukey.AGG) #num
str(CData$ABDiff) #int
str(CData$sqrt.TotalLook) #num
str(CData$Tukey.CORT) #num
str(CData$z.Tukey.TimeR) #num
str(CData$z.Tukey.Trial14or5InWeekorBlock) #num
AggLoc<-as.factor(CData$AggLoc)
str(AggLoc) #factor
str(CData$z.Tukey.TrialStudent) #num
str(CData$DrugKHClLast24HoursYN) #factor w/ 2 levels
str(CData$DisruptionInGrpOtherYN) #factor w/ 2 levels
str(CData$AnyOtherTreatment) #factor w/ 2 levels
str(CData$Treatment)#factor w/ 2 levels
str(CData$Tukey.Anxiety) #num

#############################################################################
#Step 6. Check for correlation in variables
#############################################################################

corr.tab=data.frame(cbind(Cort=as.numeric(CData$Concentration_.xDil.Factor..pg.mL.),
Time=as.numeric(CData$TimeR),
                  Trial14=as.numeric(CData$Trial14or5InWeekorBlock),
                   AggLoc=as.numeric(CData$AggLoc),                    TrialStudent=as.numeric(CData$TrialStudent),
Drug=as.numeric(CData$DrugKHClLast24HoursYN),

Disruption=as.numeric(CData$DisruptionInGrpOtherYN),OtherTreat=as.numeric(CData$AnyOtherTreatment),
Treatment=as.numeric(CData$Treatment)))
str(corr.tab)
```

```
spear=cor(corr.tab[,1:9], method ="spearman")
spear # look for correlations between variables >0.4

#            Cort        Time        Trial14    AggLoc     Trialstudent    Drug    Disruption  OtherTreat
# Cort      1.000000000 -0.17004843 -0.07640331  0.1229653204  0.07144075 -0.003476395 -0.0309823493 -0.04367150
# Time     -0.170048429  1.00000000 -0.11757556 -0.0652451140  0.08465020  0.026653149  0.1191462253  0.17569298
# Trial14  -0.076403311 -0.11757556  1.00000000 -0.2462373024  0.43951115 -0.517015733 -0.1694804899 -0.47176559
# AggLoc    0.122965320 -0.06524511 -0.24623730  1.0000000000  0.01354972  0.257430135 -0.0008535232  0.06601487
# TrialStudent0.071440754  0.08465020  0.43951115  0.0135497195  1.00000000  0.087439617 -0.0994509579  0.18708953
# Drug     -0.003476395  0.02665315 -0.51701573  0.2574301355  0.08743962  1.000000000 -0.1900257186  0.63265100
# Disruption -0.030982349  0.11914623 -0.16948049 -0.0008535232 -0.09945096 -0.190025719  1.0000000000 -0.02464164
# OtherTreat -0.043671502  0.17569298 -0.47176559  0.0660148672  0.18708953  0.632651004 -0.0246416441  1.00000000
# Treatment   0.129728502  0.17443090 -0.05034639  0.1440165235  0.86542484  0.381348545 -0.0391825102  0.46279438

#            Treatment
# Cort       0.12972850
# Time       0.17443090
# Trial14   -0.05034639
# AggLoc     0.14401652
# TrialStudent0.86542484
# Drug       0.38134854
# Disruption -0.03918251
# OtherTreat  0.46279438
# Treatment   1.00000000

#check for correlations >0.4
################################################################################
#Step 7. Save and / or load HData
################################################################################
HData<-CData

write.csv(HData,
      file='HData.txt', row.names=T)

HData<-read.csv(file.choose(), row.names = 1, header=T) #select file from pop up window
View(HData)
nrow(HData)#77
ncol(HData)#255
str(HData$animalID)#Factor w/ 17 levels

################################################################################
#Step 8. Load packages
################################################################################
#Load Package
# install.packages("lme4")
# install.packages("tidyverse")
# install.packages("car")
# install.packages("carData")
# install.packages("rcompanion")
install.packages("MuMIn")

library(tidyverse)
library(rcompanion)
library(lme4)
library(car)
library(MuMIn)

#load Roger Functions #source files need to be in the working directory
```

```
# source("C:/Users/emmel/Desktop/R Studio and R/Functions/diagnostic_fcns.r")
# source("C:/Users/emmel/Desktop/R Studio and R/Functions/glmm_stability.r")
# source("D:/R Studio and R/Functions/diagnostic_fcns.r")
# source("D:/R Studio and R/Functions/glmm_stability.r")
#source("M:/Emily/Doing/R Users Group/R Training/Mundry/Functions/Functions/diagnostic_fcns.r")
#source("M:/Emily/Doing/R Users Group/R Training/Mundry/Functions/Functions/glmm_stability.r")
source("//jmu.ac.uk/PFS/HS03H/Store10/HS226988/My Documents/Functions/diagnostic_fcns.r")
source("//jmu.ac.uk/PFS/HS03H/Store10/HS226988/My Documents/Functions/glmm_stability.r")

C.Data<-HData

##############################################################################
#Step 9. Start to build the model - Tukey.AGG
##############################################################################
#include all variables to begin with
Aggfull1<-lmer(Tukey.AGG ~ Tukey.CORT +
            Treatment + AggLoc + z.Tukey.Trial14or5InWeekorBlock +
            z.Tukey.TimeR +
            (1|animalID),
            data=C.Data, REML=F)

diagnostics.plot(Aggfull1) #looks ok, lots of zeros in residual plot, qq plot wiggely
#visually check assumptions for random effect
ranef.diagn.plot(Aggfull1) #not great
#test sig of interaction
as.data.frame(drop1(Aggfull1, test = "Chisq"))

#                          Df     AIC       LRT   Pr(Chi)
# <none>                      NA 569.9862        NA       NA
# Tukey.CORT                   1 570.1185 2.132305006 0.1442237
# Treatment                    1 568.0320 0.045856165 0.8304375 #remove
# AggLoc                       1 568.7029 0.716743260 0.3972143 #remove
# z.Tukey.Trial14or5InWeekorBlock  1 567.9882 0.002030115 0.9640620 #remove
# z.Tukey.TimeR                1 568.6553 0.669177119 0.4133386 #remove

summary(Aggfull1)
# Linear mixed model fit by maximum likelihood  ['lmerMod']
# Formula: Tukey.AGG ~ Tukey.CORT + Treatment + AggLoc + z.Tukey.Trial14or5InWeekorBlock +      z.Tukey.TimeR + (1 |
animalID)
# Data: C.Data
#
# AIC      BIC   logLik deviance df.resid
# 570.0    588.7   -277.0    554.0       69
#
# Scaled residuals:
#    Min      1Q  Median      3Q     Max
# -1.9354 -0.8167  0.1356  0.6528  1.8986
#
# Random effects:
#  Groups   Name       Variance Std.Dev.
# animalID (Intercept) 24.30    4.930
# Residual             62.91    7.932
# Number of obs: 77, groups:  animalID, 17
#
# Fixed effects:
#                                 Estimate Std. Error t value
# (Intercept)                     22.59261    4.10551   5.503
# Tukey.CORT                      30.63865   19.87399   1.542
# TreatmentStress                 -0.42149    1.96155  -0.215
# AggLocRmonkeyview               -1.69713    1.99725  -0.850
# z.Tukey.Trial14or5InWeekorBlock -0.04444    0.97592  -0.046
# z.Tukey.TimeR                   -0.87518    1.02546  -0.853
#
# Correlation of Fixed Effects:
#  (Intr) T.CORT TrtmnS AggLcR z.T.T1
```

```
# Tukey.CORT   0.876
# TrtmntStrss -0.258 -0.064
# AggLcRmnkyv -0.256 -0.073 -0.161
# z.T.T145IWB  0.050  0.104 -0.039  0.243
# z.Tukey.TmR  0.159  0.169 -0.146  0.074  0.157

AggCort<-lmer(Tukey.AGG~ Tukey.CORT +
            (1|animalID),
          data=C.Data, REML=F)

diagnostics.plot(AggCort) #plots not too bad - residuals as slight line to bottom left side (lots of zeros)
#visually check assumptions for random effect
ranef.diagn.plot(AggCort) #not great
#test sig of interaction
as.data.frame(drop1(AggCort, test = "Chisq"))

#           Df     AIC     LRT   Pr(Chi)
# <none>     NA 563.5859      NA       NA
# Tukey.CORT  1 564.0536 2.467608 0.1162147  #no effect of cortisol on Agg

summary(AggCort)
# Linear mixed model fit by maximum likelihood  ['lmerMod']
# Formula: Tukey.AGG ~ Tukey.CORT + (1 | animalID)
# Data: C.Data
#
# AIC      BIC   logLik deviance df.resid
# 563.6    573.0   -277.8    555.6       73
#
# Scaled residuals:
#    Min      1Q  Median      3Q     Max
# -2.0253 -0.8057  0.1043  0.6393  1.8332
#
# Random effects:
#   Groups   Name        Variance Std.Dev.
# animalID (Intercept) 22.12    4.703
# Residual             65.35    8.084
# Number of obs: 77, groups:  animalID, 17
#
# Fixed effects:
#   Estimate Std. Error t value
# (Intercept)   21.812      3.711   5.878
# Tukey.CORT    32.090     19.534   1.643
#
# Correlation of Fixed Effects:
#   (Intr)
# Tukey.CORT 0.915

#check stability
mstabAggCort=glmm.model.stab(model.res=AggCort,       contr=lmerControl(optimizer       =       "bobyqa",optCtrl       =
list(maxfun=2e5)))
mstabAggCort$detailed$warnings
# [1] none none none none none none none none none none none none none none none none none none none none none none none
# [21] none none
# Levels: none
mstabAggCort$summary[,-1]
#                       orig       min       max
# (Intercept)           21.812456 18.335585 23.931234
# Tukey.CORT            32.089946 15.875274 42.478273
# animalID@(Intercept)@NA  4.703255  4.043304  5.098570
# Residual              8.084170  7.489335  8.318529

#stability okay

nullAG=lmer(Tukey.AGG~ 1 +
          (1|animalID),
```

```
        data=C.Data, REML=F)

anova(nullAG, Aggfull1)
# Df    AIC    BIC  logLik deviance  Chisq Chi Df Pr(>Chisq)
# nullAG    3 564.05 571.08 -279.03   558.05
# Aggfull1  8 569.99 588.74 -276.99   553.99 4.0674      5     0.5398

anova(nullAG, AggCort)
# Df    AIC    BIC  logLik deviance  Chisq Chi Df Pr(>Chisq)
# nullAG   3 564.05 571.08 -279.03   558.05
# AggCort  4 563.59 572.96 -277.79   555.59 2.4676      1     0.1162

anova(Aggfull1, AggCort) #0.7963

r.squaredGLMM(AggCort)
#        R2m       R2c
# [1,] 0.03720862 0.2806795

confint.merMod(object=AggCort)
#                2.5 %    97.5 %
#   .sig01       1.803624  8.190115
# .sigma         6.822597  9.786711
# (Intercept) 14.057509 29.316276
# Tukey.CORT  -8.123720 72.085045

#to get an effect size for the model (R2m = marginal and R2c = conditional effect size)
#R2m explains  the variance explained by the entirety of the fixed effects
# while R2c explains variance explained by fixed + random effects
#############################################################################
#Step 10. Model with ABDiff
#############################################################################

ABFull1<-lmer(ABDiff ~ Tukey.CORT +
            Treatment + AggLoc + z.Tukey.Trial14or5InWeekorBlock +
            z.Tukey.TimeR +
            (1|animalID),
          data=C.Data, REML=F)

diagnostics.plot(ABFull1) #looks okays
#visually check assumptions for random effect
ranef.diagn.plot(ABFull1) #fine
#test sig of interaction
as.data.frame(drop1(ABFull1, test = "Chisq"))

#                          Df     AIC       LRT   Pr(Chi)
# <none>                     NA 1219.987        NA       NA
# Tukey.CORT                  1 1219.713 1.72577253 0.1889517
# Treatment                   1 1218.408 0.42088629 0.5164951 #remove
# AggLoc                      1 1218.071 0.08367261 0.7723807 #remove
# z.Tukey.Trial14or5InWeekorBlock  1 1218.367 0.37980706 0.5377065 #remove
# z.Tukey.TimeR               1 1220.367 2.37951769 0.1229355

summary(ABFull1)
# Linear mixed model fit by maximum likelihood  ['lmerMod']
# Formula: ABDiff ~ Tukey.CORT + Treatment + AggLoc + z.Tukey.Trial14or5InWeekorBlock +       z.Tukey.TimeR + (1 |
animalID)
# Data: C.Data
#
# AIC      BIC   logLik deviance df.resid
# 1220.0   1238.7   -602.0   1204.0      69
#
# Scaled residuals:
#   Min      1Q  Median      3Q     Max
# -2.75830 -0.61074 -0.00879  0.64033  2.35952
#
```

```
# Random effects:
#  Groups   Name       Variance Std.Dev.
# animalID (Intercept)  28576   169.0
# Residual             337116   580.6
# Number of obs: 77, groups:  animalID, 17
#
# Fixed effects:
#  Estimate Std. Error t value
# (Intercept)                    288.94     262.17   1.102
# Tukey.CORT                    1765.88    1296.14   1.362
# TreatmentStress                -90.86     139.41  -0.652
# AggLocRmonkeyview               41.19     142.28   0.290
# z.Tukey.Trial14or5InWeekorBlock  -43.96      70.51  -0.624
# z.Tukey.TimeR                  -118.09      71.75  -1.646
#
# Correlation of Fixed Effects:
#  (Intr) T.CORT TrtmnS AggLcR z.T.T1
# Tukey.CORT   0.891
# TrtmntStrss -0.318 -0.104
# AggLcRmnkyv -0.265 -0.058 -0.152
# z.T.T145IWB  0.013  0.076 -0.034  0.257
# z.Tukey.TmR  0.176  0.184 -0.171  0.100  0.156

ABFull2<-lmer(ABDiff ~ Tukey.CORT +
              z.Tukey.TimeR +
              (1|animalID),
            data=C.Data, REML=F)

diagnostics.plot(ABFull2) #looks okay
#visually check assumptions for random effect
ranef.diagn.plot(ABFull2) #fine
#test sig of interaction
as.data.frame(drop1(ABFull2, test = "Chisq"))

#             Df     AIC     LRT   Pr(Chi)
# <none>       NA 1214.956      NA       NA
# Tukey.CORT    1 1214.820 1.864074 0.1721554
# z.Tukey.TimeR  1 1215.547 2.590985 0.1074735

summary(ABFull2)
# Linear mixed model fit by maximum likelihood  ['lmerMod']
# Formula: ABDiff ~ Tukey.CORT + z.Tukey.TimeR + (1 | animalID)
# Data: C.Data
#
# AIC     BIC   logLik deviance df.resid
# 1215.0   1226.7   -602.5   1205.0       72
#
# Scaled residuals:
#  Min     1Q  Median     3Q    Max
# -2.8081 -0.6019 -0.0065  0.6435  2.5088
#
# Random effects:
#  Groups   Name       Variance Std.Dev.
# animalID (Intercept)  26675   163.3
# Residual             343074   585.7
# Number of obs: 77, groups:  animalID, 17
#
# Fixed effects:
#  Estimate Std. Error t value
# (Intercept)    269.11     233.74   1.151
# Tukey.CORT    1796.24    1284.44   1.398
# z.Tukey.TimeR  -118.86      70.28  -1.691
#
# Correlation of Fixed Effects:
#  (Intr) T.CORT
```

```
# Tukey.CORT  0.941
# z.Tukey.TmR 0.155  0.166

mstabABFull2=glmm.model.stab(model.res=ABFull2, contr=lmerControl(optimizer = "bobyqa",optCtrl = list(maxfun=2e5)))
mstabABFull2$detailed$warnings
# [1] none none none none none none none none none none none none none none none none none none none none none none none none none none none
# [21] none none
# Levels: none
mstabABFull2$summary[,-1]
#                    orig       min       max
# (Intercept)          269.1119   75.76391  447.77425
# Tukey.CORT          1796.2361  817.49486 2606.04799
# z.Tukey.TimeR       -118.8633 -152.86973  -71.76511
# animalID@(Intercept)@NA  163.3257  108.56694  177.96629
# Residual             585.7251  542.11154  600.86998

#stability okay - animalID a bit high

ABCort<-lmer(ABDiff~ Tukey.CORT+
          (1|animalID),
        data=C.Data, REML=F)

diagnostics.plot(ABCort) #plots not too bad
#visually check assumptions for random effect
ranef.diagn.plot(ABCort) #ok
#test sig of interaction
as.data.frame(drop1(ABCort, test = "Chisq"))

#          Df     AIC     LRT    Pr(Chi)
# <none>     NA 1215.547      NA        NA
# Tukey.CORT   1  1216.479  2.931352  0.08687472   #Cortisol explaining some variance in ABDiff but fails to reach significance

mstabABCort=glmm.model.stab(model.res=ABCort, contr=lmerControl(optimizer = "bobyqa",optCtrl = list(maxfun=2e5)))
mstabABCort$detailed$warnings
# [1] none none none none none none none none none none none none none none none none none none none none none none none none none none none
# [21] none none
# Levels: none
mstabABCort$summary[,-1]
#                    orig       min       max
# (Intercept)          342.0635  148.02361  523.9624
# Tukey.CORT          2210.1105 1240.06450 3017.7084
# animalID@(Intercept)@NA  112.3665   30.76121  135.9075
# Residual             605.7576  572.95915  622.3871

#stability okay but animalID@(Intercept) towards max.

nullAB=lmer(ABDiff~ 1 +
          (1|animalID),
        data=C.Data, REML=F)

anova(nullAB, ABFull1) #0.2613
anova(nullAB, ABFull2) #0.06322 <- including time is best model
#        Df    AIC    BIC  logLik deviance  Chisq Chi Df Pr(>Chisq)
# nullAB   3 1216.5 1223.5 -605.24   1210.5
# ABFull2  5 1215.0 1226.7 -602.48   1205.0 5.5223      2    0.06322
anova(nullAB, ABCort) #0.08687

r.squaredGLMM(ABFull2)
#        R2m       R2c
# [1,] 0.07262097 0.1395261

confint.merMod(object=ABFull2)
```

```
#                2.5 %    97.5 %
# .sig01         0.0000  376.3568
# .sigma       496.4332  704.0730
# (Intercept)   -211.4548  733.2175
# Tukey.CORT    -809.6164 4349.6443
# z.Tukey.TimeR -262.7596   26.5696

##############################################################################
#Step 11. Model with sqrt.TotalLook
##############################################################################
TLFull1<-lmer(sqrt.TotalLook  ~ Tukey.CORT +
            Treatment + AggLoc + z.Tukey.Trial14or5InWeekorBlock +
            z.Tukey.TimeR +
            (1|animalID),
          data=C.Data, REML=F)

diagnostics.plot(TLFull1) #histogram of residual & qq-plot not great
#visually check assumptions for random effect
ranef.diagn.plot(TLFull1) # not good
#test sig of interaction
as.data.frame(drop1(TLFull1, test = "Chisq"))

#                          Df     AIC       LRT   Pr(Chi)
# <none>                      NA 595.6273        NA       NA
# Tukey.CORT                   1 596.9052 3.27788195 0.0702195
# Treatment                    1 593.6415 0.01414882 0.9053159 #remove
# AggLoc                       1 595.8039 2.17660274 0.1401235
# z.Tukey.Trial14or5InWeekorBlock  1 593.6533 0.02594903 0.8720249 #remove
# z.Tukey.TimeR                1 594.2782 0.65081957 0.4198198 #remove

summary(TLFull1)
# Linear mixed model fit by maximum likelihood  ['lmerMod']
# Formula: sqrt.TotalLook ~ Tukey.CORT + Treatment + AggLoc + z.Tukey.Trial14or5InWeekorBlock +      z.Tukey.TimeR +
(1 | animalID)
# Data: C.Data
#
# AIC      BIC   logLik deviance df.resid
# 595.6    614.4   -289.8    579.6      69
#
# Scaled residuals:
#   Min      1Q   Median      3Q     Max
# -2.76829 -0.65277  0.07834  0.58340  1.71911
#
# Random effects:
#   Groups   Name        Variance Std.Dev.
# animalID (Intercept) 37.23    6.102
# Residual             86.47    9.299
# Number of obs: 77, groups:  animalID, 17
#
# Fixed effects:
#   Estimate Std. Error t value
# (Intercept)                 39.4848     4.8725   8.104
# Tukey.CORT                  44.6441    23.5096   1.899
# TreatmentStress             -0.2757     2.3051  -0.120
# AggLocRmonkeyview           -3.4869     2.3468  -1.486
# z.Tukey.Trial14or5InWeekorBlock   0.1863     1.1453   0.163
# z.Tukey.TimeR                0.9830     1.2067   0.815
#
# Correlation of Fixed Effects:
#   (Intr) T.CORT TrtmnS AggLcR z.T.T1
# Tukey.CORT   0.873
# TrtmntStrss -0.253 -0.061
# AggLcRmnkyv -0.255 -0.074 -0.162
# z.T.T145IWB  0.053  0.106 -0.040  0.242
# z.Tukey.TmR  0.157  0.168 -0.144  0.072  0.157
```

```
TLFull2<-lmer(sqrt.TotalLook  ~ Tukey.CORT +
            AggLoc +
            (1|animalID),
          data=C.Data, REML=F)

diagnostics.plot(TLFull2) #histogram of residual & qq-plot not great
#visually check assumptions for random effect
ranef.diagn.plot(TLFull2) #not good
#test sig of interaction
as.data.frame(drop1(TLFull2, test = "Chisq"))

# Df     AIC     LRT    Pr(Chi)
# <none>    NA 590.2790      NA        NA
# Tukey.CORT  1 591.1467 2.867745 0.09037141
# AggLoc     1 590.8286 2.549620 0.11032079 #remove

summary(TLFull2)
# Linear mixed model fit by maximum likelihood  ['lmerMod']
# Formula: sqrt.TotalLook ~ Tukey.CORT + AggLoc + (1 | animalID)
# Data: C.Data
#
# AIC     BIC   logLik deviance df.resid
# 590.3    602.0   -290.1    580.3       72
#
# Scaled residuals:
#   Min      1Q   Median      3Q     Max
# -2.62623 -0.71832  0.04369  0.60649  1.62345
#
# Random effects:
#   Groups   Name       Variance Std.Dev.
# animalID (Intercept) 39.71    6.302
# Residual             86.41    9.296
# Number of obs: 77, groups:  animalID, 17
#
# Fixed effects:
#                 Estimate Std. Error t value
# (Intercept)        38.734      4.712   8.221
# Tukey.CORT         40.773     23.199   1.758
# AggLocRmonkeyview   -3.625      2.251  -1.611
#
# Correlation of Fixed Effects:
#   (Intr) T.CORT
# Tukey.CORT   0.885
# AggLcRmnkyv -0.334 -0.118

mstabTLFull2=glmm.model.stab(model.res=TLFull2,       contr=lmerControl(optimizer       =       "bobyqa",optCtrl       =
list(maxfun=2e5)))
mstabTLFull2$detailed$warnings
# [1] none none none none none none none none none none none none none none none none none none none none none none none none none
# [21] none none
# Levels: none
mstabTLFull2$summary[,-1]
#                       orig       min       max
# (Intercept)           38.733621 34.832063 40.420139
# Tukey.CORT            40.772526 20.321254 50.648086
# AggLocRmonkeyview     -3.625130 -4.676310 -2.295226
# animalID@(Intercept)@NA  6.301839  5.753412  6.725361
# Residual              9.295572  8.593605  9.595781

#stability okay but residuals a bit high

TLCort<-lmer(sqrt.TotalLook~ Tukey.CORT+
            (1|animalID),
          data=C.Data, REML=F)
```

```
diagnostics.plot(TLCort) #plots not good
#visually check assumptions for random effect
ranef.diagn.plot(TLCort) #not good
#test sig of interaction
as.data.frame(drop1(TLCort, test = "Chisq"))

#           Df      AIC      LRT   Pr(Chi)
# <none>     NA 590.8286       NA        NA
# Tukey.CORT  1 591.0461 2.217574 0.1364473 #no effect of cortisol on TotalLook

mstabTLCort=glmm.model.stab(model.res=TLCort, contr=lmerControl(optimizer = "bobyqa",optCtrl = list(maxfun=2e5)))
mstabTLCort$detailed$warnings
# [1] none none none none none none none none none none none none none none none none none none none none
# [21] none none
# Levels: none
mstabTLCort$summary[,-1]
#                         orig       min       max
# (Intercept)          36.149932 32.460447 37.887652
# Tukey.CORT            36.107462 19.356259 42.687187
# animalID@(Intercept)@NA  6.487325  5.876744  6.905493
# Residual              9.431021  8.650208  9.839132

#stability okay but not great.

nullTL=lmer(sqrt.TotalLook~ 1 +
            (1|animalID),
          data=C.Data, REML=F)

anova(nullTL, TLFull1)#0.3669
anova(nullTL, TLFull2)#0.09222
# Df    AIC    BIC  logLik deviance  Chisq Chi Df Pr(>Chisq)
# nullTL   3 591.05 598.08 -292.52   585.05
# TLFull2  5 590.28 602.00 -290.14   580.28 4.7672      2    0.09222
anova(nullTL, TLCort) #0.1364

r.squaredGLMM(TLFull2)
#        R2m       R2c
# [1,] 0.05933009 0.3555301

confint.merMod(object=TLFull2)
#                   2.5 %     97.5 %
# .sig01            2.954199 10.6440213
# .sigma            7.836580 11.2762195
# (Intercept)      28.993128 48.2487895
# Tukey.CORT       -6.548634 88.1462192
# AggLocRmonkeyview -8.139259  0.8422073
```

Appendix 5d

```
################################################################################
#Part B - Effect of veterinary, husbandry and life history stressors on salivary cortisol concentration
################################################################################

################################################################################
#Step 1. Clear workspace, set working directory and load in required files and packages
################################################################################

ls() #this looks at what is loaded
rm(list=ls()) #this clears everything

#set the working directory
#setwd("M:/Emily/Doing/Postgraduates/Emmeline Howarth/Data")
#setwd("C:/Users/emily/OneDrive/Desktop/R_Code")
setwd("C:/Users/emmel/Desktop/R Studio and R")
setwd("//jmu.ac.uk/PFS/HS03H/Store10/HS226988/My Documents")

#Load Package
# install.packages("lme4")
# install.packages("tidyverse")
# install.packages("car")
# install.packages("carData")
# install.packages("rcompanion")
# install.packages("MuMIn")

library(tidyverse)
library(rcompanion)
library(lme4)
library(car)
library(MuMIn)

#load Roger Functions #source files need to be in the working directory

source("C:/Users/emmel/Desktop/R Studio and R/Functions/diagnostic_fcns.r")
source("C:/Users/emmel/Desktop/R Studio and R/Functions/glmm_stability.r")
# source("D:/R Studio and R/Functions/diagnostic_fcns.r")
# source("D:/R Studio and R/Functions/glmm_stability.r")
#source("M:/Emily/Doing/R Users Group/R Training/Mundry/Functions/Functions/diagnostic_fcns.r")
#source("M:/Emily/Doing/R Users Group/R Training/Mundry/Functions/Functions/glmm_stability.r")
source("//jmu.ac.uk/PFS/HS03H/Store10/HS226988/My Documents/Functions/diagnostic_fcns.r")
source("//jmu.ac.uk/PFS/HS03H/Store10/HS226988/My Documents/Functions/glmm_stability.r")

################################################################################
#Step 2. Check data and subset required data for this analysis i.e. individuals with cortisol samples
################################################################################

#Load data
AB1KempThatcherHowarth_20191201<-read.csv(file.choose(), header=T) #select file from pop up window
d <- AB1KempThatcherHowarth_20191201

nrow(d)  #1188 (if higher do the following go back to CSV and delete ghost cells from bottom)
ncol(d) #245

str(d)
View(d)

#For analysis, select monkeys with CORT data
SData<-subset(d, CortSelect =="Yes")
nrow(SData)#77
################################################################################
#Step 3. Ensure variables accurately labelled as factors and correct levels of each factor are being read.
################################################################################
MData<-SData
```

```
#factors
MData$animalID <- as.factor(MData$animalID)
str(MData$animalID) #Factor w/ 110 levels
MData$Treatment <- as.factor(MData$Treatment)
str(MData$Treatment) #Factor w/ 2 level
MData$AggLoc <- as.factor(MData$AggLoc)
str(MData$AggLoc) #Factor w/ 2 levels
MData$StimulusID<- as.factor(MData$StimulusID)
str(MData$StimulusID) #Factor w/ 7 levels
MData$AnyOtherTreatment<-as.factor(MData$AnyOtherTreatment)
str(MData$AnyOtherTreatment) #Factor w/ 2 levels

#numeric
MData$TimeR<-as.numeric(MData$TimeR)
str(MData$TimeR) #num
MData$DaysSinceLastHC<-as.numeric(MData$DaysSinceLastHC)
str(MData$DaysSinceLastHC) #num
MData$TrialStudentFile<-as.numeric(MData$TrialStudentFile)
str(MData$TrialStudentFile) #num
MData$TrialChronological<-as.numeric(MData$TrialChronological)
str(MData$TrialChronological) #num
MData$Trial14<-as.numeric(MData$Trial14or5InWeekorBlock)
str(MData$Trial14) #num
MData$Concentration_.xDil.Factor..pg.mL.<-as.numeric(MData$Concentration_.xDil.Factor..pg.mL.)
str(MData$Concentration_.xDil.Factor..pg.mL.)
MData$TrialStudentFile<-as.numeric(MData$TrialStudentFile)
str(MData$TrialStudentFile)

MData$Rank <- as.integer(MData$Rank)
str(MData$Rank) #int

nrow(MData)#77

#############################################################################
#Step 4. look at the response variables & check distribution
#############################################################################
RData<-MData

#Cortisol
summary(RData$Concentration_.xDil.Factor..pg.mL.)
# Min.  1st Qu.   Median     Mean  3rd Qu.     Max.
# 141.5    438.1   1178.1  10671.9   4451.6 259962.0
hist(RData$Concentration_.xDil.Factor..pg.mL.)
hist(transformTukey(RData$Concentration_.xDil.Factor..pg.mL.))
# lambda      W Shapiro.p.value
# 391  -0.25 0.9805         0.2847
RData$Tukey.CORT<-transformTukey(RData$Concentration_.xDil.Factor..pg.mL.)

#TimeR
hist(RData$TimeR) #right skewed. Try transforming:
hist(transformTukey(RData$TimeR))
# lambda      W Shapiro.p.value
# 357   -1.1 0.9542       0.007392
RData$z.Tukey.TimeR<-scale(transformTukey(RData$TimeR))

#Trial14InBlock
hist(RData$Trial14or5InWeekorBlock)
hist(transformTukey(RData$Trial14or5InWeekorBlock, plotit=FALSE))
# lambda      W Shapiro.p.value
# 434  0.825 0.8971       1.299e-05
RData$z.Tukey.Trial14or5InWeekorBlock<-scale(transformTukey(RData$Trial14or5InWeekorBlock))

#TrialStudentFile
hist(RData$TrialStudentFile)
```

```
hist(transformTukey(RData$TrialStudentFile, plotit=FALSE))
# lambda    W Shapiro.p.value
# 435   0.85 0.948       0.003312
RData$z.Tukey.TrialStudent<-scale(transformTukey(RData$TrialStudentFile))

#AggLoc
summary(RData$AggLoc)
#Lmonkeyview Rmonkeyview
#   41          36

#StimulusID
str(RData$StimulusID)
RData$StimulusID<-as.factor(RData$StimulusID)
table(RData$StimulusID)
#1  2  3  4  5  6  7
#12 16  5 12 10 12 10     #small sample

#InjuryLast48HrsYN
summary(RData$InjuryLast48HrsYN)
#No Yes
#74   3    #small sample

#CleaningLast24HrsYN
summary(RData$CleaningLast24Hrs)
# No Yes
# 70   7   #small sample

#DrugKHCL
summary(RData$DrugKHClLast24HoursYN)
#No Yes
#67  10

#BabyBornGrp
RData$BabyBornLast24HrsGrp<-as.factor(RData$BabyBornLast24HrsGrp)
summary(RData$BabyBornLast24HrsGrp)
#0  1
#74  3     #small sample

#DiruptioninGrp
summary(RData$DisruptionInGrpOtherYN)
#No Yes
#62  15

#OtherTreatment
summary(RData$AnyOtherTreatment)
#No Yes
#60  17

#Treatment
table(RData$Treatment)
# BL Stress
# 38     39

#GroupChange
table(RData$GrpChangeLast7DaysYN)
# No Yes
# 72   5   #small sample

#Sex
table(RData$Sex)
# F  M
# 41 36

#Rank
table(RData$Rank)
```

```
# 1  2  3
# 58  2 17

#Chronic illness
table(RData$IllnessChronicYN)
# No Yes
# 77   0

#AlopeciaScore
table(RData$AlopeciaScoreHC)
# 3   4 4.5   5
# 5   4   2  66
hist(RData$AlopeciaScoreHC) #left skew - need to reverse
hist(6-(RData$AlopeciaScoreHC))
hist(transformTukey((6-(RData$AlopeciaScoreHC))))
#     lambda      W Shapiro.p.value
# 344 -1.425 0.4318      9.968e-16
RData$Tukey.AlopeciaScoreHC<-transformTukey((6-(RData$AlopeciaScoreHC)))

#Wean early
table(RData$WeanEarlyR)
# No Yes
# 66  11

#############################################################################
#Step 5. check all variables are reading correctly
#############################################################################
CData<-RData

str(CData$Tukey.CORT) #num
str(CData$z.Tukey.TimeR) #num
str(CData$z.Tukey.Trial14or5InWeekorBlock) #num
AggLoc<-as.factor(CData$AggLoc)
str(AggLoc) #factor
str(CData$z.Tukey.TrialStudent) #num
str(CData$DrugKHClLast24HoursYN) #factor w/ 2 levels
str(CData$DisruptionInGrpOtherYN) #factor w/ 2 levels
str(CData$AnyOtherTreatment) #factor w/ 2 levels
str(CData$Treatment)#factor w/ 2 levels
str(CData$StimulusID) #factor
str(CData$InjuryLast48HrsYN)

#############################################################################
#Step 6. Check for correlation in variables
#############################################################################

corr.tab=data.frame(cbind(Time=as.numeric(CData$TimeR), Trial14=as.numeric(CData$Trial14or5InWeekorBlock),
                  AggLoc=as.numeric(CData$AggLoc), TrialStudent=as.numeric(CData$TrialStudent),
                  StimulusID=as.numeric(CData$StimulusID),                Drug=as.numeric(CData$DrugKHClLast24HoursYN),
Disruption=as.numeric(CData$DisruptionInGrpOtherYN),
                  OtherTreat=as.numeric(CData$AnyOtherTreatment), Treatment=as.numeric(CData$Treatment),
                  Injury=as.numeric(CData$InjuryLast48HrsYN), Cleaning=as.numeric(CData$CleaningLast24Hrs),
                  Baby=as.numeric(CData$BabyBornLast24HrsGrp), Group=as.numeric(CData$GrpChangeLast7DaysYN),
                  rank=as.numeric(CData$Rank), Sex=as.numeric(CData$Sex),
                  Alopeciascore=as.numeric(CData$AlopeciaScoreHC), Wean=as.numeric(CData$WeanEarlyR)))

str(corr.tab)

spear=cor(corr.tab[,1:17], method ="spearman")
spear # look for correlations between variables >0.4

#              Time     Trial14       AggLoc TrialStudent  StimulusID       Drug    Disruption  OtherTreat
# Time        1.00000000 -0.11757556 -0.0652451140   0.08465020  0.11078269  0.02665315  0.1191462253  0.17569298
# Trial14    -0.11757556  1.00000000 -0.2462373024   0.43951115 -0.06600359 -0.51701573 -0.1694804899 -0.47176559
# AggLoc      -0.06524511 -0.24623730  1.0000000000   0.01354972  0.02253222  0.25743014 -0.0008535232  0.06601487
```

```
# TrialStudent     0.08465020   0.43951115   0.0135497195     1.00000000   0.05478036   0.08743962  -0.0994509579
0.18708953
# StimulusID       0.11078269  -0.06600359   0.0225322183     0.05478036   1.00000000   0.03344335   0.0530371779
0.14194383
# Drug         0.02665315 -0.51701573  0.2574301355   0.08743962  0.03344335  1.00000000 -0.1900257186  0.63265100
# Disruption       0.11914623  -0.16948049  -0.0008535232   -0.09945096   0.05303718  -0.19002572   1.0000000000  -
0.02464164
# OtherTreat       0.17569298  -0.47176559   0.0660148672    0.18708953   0.14194383   0.63265100  -0.0246416441
1.00000000
# Treatment        0.17443090  -0.05034639   0.1440165235    0.86542484   0.10888229   0.38134854  -0.0391825102
0.46279438
# Injury       0.26547072  0.01703450 -0.0541554324  0.08506623  0.20640529 -0.07778706 -0.0990363332  0.21645153
# Cleaning         0.34490915   0.10840479  -0.0246932399    0.32616570   0.04219387  -0.12216944  -0.0414780678
0.04950738
# Baby        -0.08488889  0.17653935  0.2148747802  0.26583197  0.13913245 -0.07778706  0.0704258369 -0.10717503
# Group        0.02060451  0.30888416  0.0699641798  0.37694803  0.11165938 -0.10180787  0.0034565056 -0.14027090
# rank        -0.05926998 -0.07160799  0.0679576542  0.06505479 -0.07360200  0.05449075 -0.0472347986 -0.02443068
# Sex          0.09696980 -0.01561505  0.0088075881  0.12548218  0.07826981  0.10256982  0.0648677657  0.12876974
# Alopeciascore  -0.23864719   0.02813082   0.0134788604   -0.16853223  -0.20043417   0.05144384  -0.0012129052  -
0.22584103
# Wean        -0.11009968 -0.01798308  0.0637576713  0.15539930 -0.04142775  0.17349208 -0.2008048322  0.14061025

#              Treatment     Injury   Cleaning       Baby      Group       rank        Sex Alopeciascore
# Time         0.17443090  0.26547072  0.34490915 -0.08488889  0.020604513 -0.05926998  0.096969802  -0.238647195
# Trial14      -0.05034639  0.01703450  0.10840479  0.17653935  0.308884157 -0.07160799 -0.015615048   0.028130819
# AggLoc       0.14401652 -0.05415543 -0.02469324  0.21487478  0.069964180  0.06795765  0.008807588   0.013478860
# TrialStudent     0.86542484   0.08506623   0.32616570   0.26583197   0.376948031   0.06505479   0.125482185  -
0.168532226
# StimulusID   0.10888229  0.20640529  0.04219387  0.13913245  0.111659385 -0.07360200  0.078269811 -0.200434170
# Drug         0.38134854 -0.07778706 -0.12216944 -0.07778706 -0.101807870  0.05449075  0.102569820   0.051443845
# Disruption       -0.03918251  -0.09903633  -0.04147807   0.07042584   0.003456506  -0.04723480   0.064867766  -
0.001212905
# OtherTreat       0.46279438   0.21645153   0.04950738  -0.10717503  -0.140270902  -0.02443068   0.128769741  -
0.225841029
# Treatment    1.00000000  0.06450615  0.31214724  0.19874868  0.260122697  0.07015857  0.196078835  -0.162379396
# Injury       0.06450615  1.00000000  0.16979054 -0.04054054 -0.053059545  0.12688970 -0.188670539  -0.461747737
# Cleaning     0.31214724  0.16979054  1.00000000 -0.06367145 -0.083333333 -0.09896625  0.156390519  -0.370956728
# Baby         0.19874868 -0.04054054 -0.06367145  1.00000000  0.764057448  0.20342634 -0.054155432   0.081922986
# Group        0.26012270 -0.05305954 -0.08333333  0.76405745  1.000000000  0.22459465 -0.035668013   0.107220976
# rank         0.07015857  0.12688970 -0.09896625  0.20342634  0.224594646  1.00000000 -0.534287764   0.114360844
# Sex          0.19607883 -0.18867054  0.15639052 -0.05415543 -0.035668013 -0.53428776  1.000000000   0.096277574
# Alopeciascore  -0.16237940  -0.46174774  -0.37095673   0.08192299   0.107220976   0.11436084   0.096277574
1.000000000
# Wean         0.18028068 -0.08219949  0.12909944 -0.08219949 -0.107582871 -0.05680207  0.286909521  -0.240235983

#              Wean
# Time         -0.11009968
# Trial14      -0.01798308
# AggLoc        0.06375767
# TrialStudent   0.15539930
# StimulusID    -0.04142775
# Drug          0.17349208
# Disruption    -0.20080483
# OtherTreat     0.14061025
# Treatment      0.18028068
# Injury       -0.08219949
# Cleaning      0.12909944
# Baby         -0.08219949
# Group        -0.10758287
# rank         -0.05680207
# Sex          0.28690952
# Alopeciascore -0.24023598
# Wean          1.00000000

# look for correlations between variables >0.4
```

```
##############################################################################
##########End of Data processing, start building model####################
##############################################################################

##############################################################################
#Step 7. Save and / or load HData
##############################################################################
BData<-CData

write.csv(BData,
      file='BData.txt', row.names=T)

BData<-read.csv(file.choose(), row.names = 1, header=T) #select file from pop up window
View(BData)
nrow(BData)#77
ncol(BData)#252
str(BData$animalID)#Factor w/ 17 levels

##############################################################################
#Step 8. Start to build model for effect of stressors on cort
##############################################################################
CData<-BData

StressFull1<-lmer(Tukey.CORT ~
              Treatment +
              DisruptionInGrpOtherYN + Sex +
              z.Tukey.TimeR +
            (1|animalID),
          data=CData, REML=F)

diagnostics.plot(StressFull1) #looks ok, qq plot wiggely
#visually check assumptions for random effect
ranef.diagn.plot(StressFull1) #ok
#test sig of interaction
as.data.frame(drop1(StressFull1, test = "Chisq"))

# Df      AIC       LRT   Pr(Chi)
# <none>              NA -226.3753        NA       NA
# Treatment            1 -228.1197 0.25554008 0.6132009
# DisruptionInGrpOtherYN  1 -228.3169 0.05839615 0.8090491 #remove
# Sex                  1 -227.5729 0.80233995 0.3703947
# z.Tukey.TimeR        1 -226.4842 1.89106765 0.1690816

summary(StressFull1)
# Linear mixed model fit by maximum likelihood  ['lmerMod']
# Formula: Tukey.CORT ~ Treatment + DisruptionInGrpOtherYN + Sex + z.Tukey.TimeR +      (1 | animalID)
# Data: CData
#
# AIC      BIC   logLik deviance df.resid
# -226.4   -210.0    120.2   -240.4       70
#
# Scaled residuals:
#    Min      1Q   Median      3Q     Max
# -1.77663 -0.69279 -0.07272  0.58063  2.31772
#
# Random effects:
#  Groups   Name        Variance Std.Dev.
# animalID (Intercept) 0.001020 0.03194
# Residual             0.002002 0.04474
# Number of obs: 77, groups:  animalID, 17
#
# Fixed effects:
#   Estimate Std. Error t value
```

```
# (Intercept)             -0.184072   0.013564 -13.571
# TreatmentStress          0.005606   0.011033   0.508
# DisruptionInGrpOtherYNYes -0.003469   0.014352  -0.242
# SexM                     0.017401   0.019279   0.903
# z.Tukey.TimeR           -0.007935   0.005717  -1.388
#
# Correlation of Fixed Effects:
#   (Intr) TrtmnS DIGOYN SexM
# TrtmntStrss -0.342
# DsrptIGOYNY -0.167  0.019
# SexM        -0.555 -0.120 -0.050
# z.Tukey.TmR  0.073 -0.120 -0.088 -0.020

StressFull2<-lmer(Tukey.CORT ~
                  Treatment + Sex +
                  z.Tukey.TimeR +
                  (1|animalID),
                data=CData, REML=F)

diagnostics.plot(StressFull2) #looks ok
#visually check assumptions for random effect
ranef.diagn.plot(StressFull2) #ok
#test sig of interaction
as.data.frame(drop1(StressFull2, test = "Chisq"))

# Df      AIC      LRT   Pr(Chi)
# <none>       NA -228.3169       NA       NA
# Treatment     1 -230.0567 0.2601181 0.6100390 #remove
# Sex           1 -229.5341 0.7827972 0.3762870 #remove
# z.Tukey.TimeR  1 -228.3548 1.9620629 0.1612929

StressFull3<-lmer(Tukey.CORT ~ z.Tukey.TimeR +
             (1|animalID),
           data=CData, REML=F)

diagnostics.plot(StressFull3) #looks ok, qq plot wiggely
#visually check assumptions for random effect
ranef.diagn.plot(StressFull3) #ok
#test sig of interaction
as.data.frame(drop1(StressFull3, test = "Chisq"))

#              Df     AIC      LRT   Pr(Chi)
# <none>       NA -231.170       NA       NA
# z.Tukey.TimeR  1 -231.484 1.685966 0.1941336

summary(StressFull3)
# Linear mixed model fit by maximum likelihood  ['lmerMod']
# Formula: Tukey.CORT ~ z.Tukey.TimeR + (1 | animalID)
# Data: CData
#
# AIC      BIC   logLik deviance df.resid
# -231.2   -221.8    119.6   -239.2       73
#
# Scaled residuals:
#   Min       1Q   Median       3Q      Max
# -1.77797 -0.70580 -0.04426  0.54432  2.24841
#
# Random effects:
#   Groups   Name        Variance Std.Dev.
# animalID (Intercept) 0.001119 0.03345
# Residual             0.002006 0.04479
# Number of obs: 77, groups:  animalID, 17
#
# Fixed effects:
#   Estimate Std. Error t value
```

```
# (Intercept)   -0.174473   0.009775 -17.849
# z.Tukey.TimeR -0.007419   0.005676  -1.307
#
# Correlation of Fixed Effects:
#   (Intr)
# z.Tukey.TmR -0.008

mstabStressCort=glmm.model.stab(model.res=StressFull3,     contr=lmerControl(optimizer     =     "bobyqa",optCtrl     =
list(maxfun=2e5)))
mstabStressCort$detailed$warnings
# [1] none none none none none none none none none none none none none none none none none none none none
# [21] none none
# Levels: none
mstabStressCort$summary[,-1]
#                          orig         min         max
# (Intercept)           -0.174473071 -0.178734789 -0.169625834
# z.Tukey.TimeR          -0.007419347 -0.008939106 -0.004022603
# animalID@(Intercept)@NA  0.033451463  0.029409366  0.035464049
# Residual               0.044785964  0.043013115  0.045753812

#stability okay - animal ID a bit high and time a bit low.

NullStC = lmer(Tukey.CORT ~ 1 +
             (1|animalID),
           data=CData, REML=F)


anova(NullStC, StressFull1) #0.5762
anova(NullStC, StressFull2) #0.3588
anova(NullStC, StressFull3) #0.1941

#no stressors have a significant effect on cort - model no better than the null
###########################################################################
#Step 9. Start to build model for effect of test on cort
###########################################################################
stimulusID <- as.factor(CData$StimulusID)

TestFull1<-lmer(Tukey.CORT ~
            AggLoc + z.Tukey.Trial14or5InWeekorBlock +
            stimulusID +
           (1|animalID),
          data=CData, REML=F)

diagnostics.plot(TestFull1) #looks ok
#visually check assumptions for random effect
ranef.diagn.plot(TestFull1) #ok
#test sig of interaction
as.data.frame(drop1(TestFull1, test = "Chisq"))

# Df      AIC       LRT    Pr(Chi)
# <none>                     NA -229.6437        NA        NA
# AggLoc                      1 -230.9108  0.7328476 0.39196173 #remove
# z.Tukey.Trial14or5InWeekorBlock  1 -230.9315  0.7121918 0.39871716 #remove
# stimulusID                  6 -229.1858 12.4578212 0.05250086

# summary(TestFull1)
# Linear mixed model fit by maximum likelihood  ['lmerMod']
# Formula: Tukey.CORT ~ AggLoc + z.Tukey.Trial14or5InWeekorBlock + stimulusID +     (1 | animalID)
# Data: CData
#
# AIC      BIC   logLik deviance df.resid
# -229.6   -203.9    125.8   -251.6      66
#
# Scaled residuals:
#   Min      1Q   Median      3Q      Max
```

```
# -2.08289 -0.48238 -0.05592  0.60215  2.65684
#
# Random effects:
#  Groups   Name       Variance Std.Dev.
# animalID (Intercept) 0.001056 0.03250
# Residual             0.001674 0.04091
# Number of obs: 77, groups:  animalID, 17
#
# Fixed effects:
#  Estimate Std. Error t value
# (Intercept)                  -0.186847   0.015575 -11.996
# AggLocRmonkeyview             0.009038   0.010522   0.859
# z.Tukey.Trial14or5InWeekorBlock -0.004417   0.005215  -0.847
# stimulusID2                   0.028971   0.017459   1.659
# stimulusID3                  -0.036613   0.024612  -1.488
# stimulusID4                   0.001358   0.017894   0.076
# stimulusID5                   0.006189   0.018676   0.331
# stimulusID6                   0.028079   0.017714   1.585
# stimulusID7                  -0.009403   0.018815  -0.500
#
# Correlation of Fixed Effects:
#  (Intr) AggLcR z.T.T1 stmID2 stmID3 stmID4 stmID5 stmID6
# AggLcRmnkyv -0.228
# z.T.T145IWB -0.192  0.213
# stimulusID2 -0.602 -0.131  0.177
# stimulusID3 -0.427 -0.186  0.178  0.456
# stimulusID4 -0.582 -0.029  0.208  0.543  0.418
# stimulusID5 -0.524 -0.142 -0.016  0.529  0.388  0.482
# stimulusID6 -0.568 -0.101  0.121  0.565  0.412  0.492  0.492
# stimulusID7 -0.567 -0.009  0.140  0.514  0.384  0.486  0.425  0.505

CData$StimulusID<- as.factor(CData$StimulusID)
CData$StimulusID<-relevel(CData$StimulusID, ref="2")
table(CData$StimulusID)
# 2  6  1  3  4  5  7
# 16 12 12  5 12 10 10

TestFull2<-lmer(Tukey.CORT ~
              StimulusID +
              (1|animalID),
            data=CData, REML=F)

diagnostics.plot(TestFull2) #looks ok
#visually check assumptions for random effect
ranef.diagn.plot(TestFull2) #ok
#test sig of interaction
as.data.frame(drop1(TestFull2, test = "Chisq"))
# Df      AIC     LRT    Pr(Chi)
# <none>     NA -231.8172      NA        NA
# stimulusID  6 -231.4840 12.3332 0.05493527

summary(TestFull2)
# Linear mixed model fit by maximum likelihood  ['lmerMod']
# Formula: Tukey.CORT ~ StimulusID + (1 | animalID)
# Data: CData
#
# AIC      BIC   logLik deviance df.resid
# -231.8   -210.7    124.9   -249.8      68
#
# Scaled residuals:
#  Min      1Q  Median      3Q     Max
# -1.95262 -0.54558 -0.00408  0.50705  2.32148
#
# Random effects:
#  Groups   Name       Variance Std.Dev.
```

```
# animalID (Intercept) 0.001081 0.03288
# Residual             0.001714 0.04140
# Number of obs: 77, groups:  animalID, 17
#
# Fixed effects:
#             Estimate Std. Error t value
# (Intercept) -0.151422   0.013723 -11.034
# StimulusID6 -0.002451   0.016555  -0.148
# StimulusID1 -0.034783   0.017121  -2.032
# StimulusID3 -0.061676   0.022845  -2.700
# StimulusID4 -0.028844   0.017015  -1.695
# StimulusID5 -0.026049   0.017439  -1.494
# StimulusID7 -0.041167   0.017987  -2.289
#
# Correlation of Fixed Effects:
#   (Intr) StmID6 StmID1 StmID3 StmID4 StmID5
# StimulusID6 -0.535
# StimulusID1 -0.535  0.452
# StimulusID3 -0.406  0.316  0.316
# StimulusID4 -0.543  0.421  0.465  0.334
# StimulusID5 -0.506  0.415  0.414  0.308  0.433
# StimulusID7 -0.517  0.439  0.425  0.310  0.424  0.361

mstabTestCort=glmm.model.stab(model.res=TestFull2,      contr=lmerControl(optimizer      =      "bobyqa",optCtrl      =
list(maxfun=2e5)))
mstabTestCort$detailed$warnings
# [1] none none none none none none none none none none none none none none none none none none none none none
# [21] none none
# Levels: none
mstabTestCort$summary[,-1]
#                        orig         min         max
# (Intercept)          -0.151422082 -0.158824059 -0.144253621
# StimulusID6           -0.002451025 -0.007507314  0.002756066
# StimulusID1           -0.034783429 -0.042016529 -0.025717726
# StimulusID3           -0.061675773 -0.076456896 -0.054591867
# StimulusID4           -0.028843554 -0.037243246 -0.020991666
# StimulusID5           -0.026049222 -0.032381835 -0.013234724
# StimulusID7           -0.041167292 -0.049557474 -0.030104994
# animalID@(Intercept)@NA  0.032881999  0.029068630  0.034661634
# Residual                0.041398095  0.038843028  0.042322373

#residual very high but all lie within stability range

NullTest<-lmer(Tukey.CORT ~
              (1|animalID),
             data=CData, REML=F)

anova(NullTest, TestFull1) #0.0777
anova(NullTest, TestFull2) #0.05494


#          Df     AIC     BIC logLik deviance  Chisq Chi Df Pr(>Chisq)
# NullTest   3 -231.48 -224.45 118.74  -237.48
# TestFull2  9 -231.82 -210.72 124.91  -249.82 12.333      6    0.05494

#Stimulus ID approaches significance.

r.squaredGLMM(TestFull2)
#        R2m       R2c
# [1,] 0.1108387 0.454801

confint.merMod(object=TestFull2)
#                2.5 %       97.5 %
# .sig01       0.01938913  0.0531740427
# .sigma       0.03495452  0.0500715711
```

```
# (Intercept) -0.17896272 -0.1240966515
# StimulusID6 -0.03532940  0.0309040125
# StimulusID1 -0.06885032 -0.0001360384
# StimulusID3 -0.10761264 -0.0163081074
# StimulusID4 -0.06295095  0.0049276318
# StimulusID5 -0.06088993  0.0085764481
# StimulusID7 -0.07686769 -0.0052682386
```

Appendix 6a

Life Sciences Building – COSHH Risk Assessment Form

Protocol/Procedure: EXTRACTION OF DNA FROM ANIMAL TISSUE USING QIAGEN DNEASY

Name of person carrying out procedure: Emmeline Howarth	
Job Title: student	Tel no. & e-mail: 07902494348 / e.r.howarth@2017.ljmu.ac.uk
Faculty: Science	School: NSP
Name of supervisor: Dr Craig Wilding Tel no. & e-mail: x2500 c.s.wilding@ljmu.ac.uk Signature of supervisor: Craig Wilding	
Location of procedure to be carried out: Room 2.13, Life Sciences Building or 356 James Parsons	
Has the person been trained in this protocol/procedure? YES Is training/supervision required? YES If yes, please give details: Supervisor to be present when undertaking procedure	
Date of assessment: 10/01/18 Signature of person carrying out procedure: Emmeline	

Description of Activity/Procedure/Process (include standard operating procedures (SOPs) as a Control): Extraction of DNA from animal tissue using Qiagen DNEasy blood and tissue kit. Involving lysis of tissue within proprietary buffer, addition of further proprietary buffers and use of centrifugation to remove contaminating proteins. Following addition of lysate to column and centrifugation, DNA is selectively bound to the DNeasy membrane as contaminants pass through. Pure DNA is subsequently eluted from column following dissolution in water, TE or proprietary AE buffer.
Who is at risk? (staff/students/others): staff and other lab users.
What is the duration of exposure? Whole process takes <3hr
What is the frequency of exposure? < 1x per week
What is the maximum number of people in the room/lab: 6
Are there any ethical issues Y/N? NO If yes, has consent been obtained?
Is health surveillance required? NO – although risk phrases R42/43 are relevant, this compound is only supplied in liquid form with this kit and therefore likelihood of inhalation of powder is reduced. Skin exposure is prevented by the user wearing laboratory coat and nitrile gloves. Health Surveillance is required if the procedure involves substances which are respiratory sensitisers or skin sensitisers (risk phrases R42, R43 or **R42/43**). If other substances with potential health effects are used and if any health effects are observed that is believed to have resulted from its use then Occupational Health should be contacted. Consideration should be made of the existing health status of the user of hazardous substances. Are special arrangements required? E.g., for types of PPE (ref:SCP9).
Will the individual be working outside of normal working hours, overnight or at weekends? NO If yes, has the appropriate form (SCP11 – Out of hours working) been completed and authorisation given? Will the experiment be left to run unattended out of hours? No If yes, has the appropriate form (SCP22 – Unattended experiments) been completed and authorisation given?

Starting substances:	Dissected invertebrate tissue (e.g., snails, mosquitoes, worms). Buffer AL, Buffer ATL, Buffer AW1, Buffer AW2, Buffer AE, Proteinase K.
End products:	Flow-through of buffer AL/ATL/Proteinase K mix. Flow-through from washes with AQ1 and AW2. Extracted DNA in TE (tris-EDTA) buffer
Hazard categories:	Buffer AL and Buffer AW1 (concentrate): Contains guanidine hydrochloride: **harmful**, **irritant**. H302 Harmful if swallowed. H315 Causes skin irritation. H319 Causes serious eye irritation Proteinase K:

	H315 Causes skin irritation. H319 Causes serious eye irritation. H334 May cause allergy or asthma symptoms or breathing difficulties if inhaled. H335 May cause respiratory irritation
What are the potential risks?	Buffer AL and Buffer AW1 contain guanidine hydrochloride, which is harmful and irritant and can form highly reactive compounds when combined with bleach. Proteinase K is a sensitiser and irritant.
How will these risks be controlled?	Proteinase K is provided as pre-prepared solution therefore preventing inhalational exposure. No dissolution of irritant powder is necessary. Do not use bleach to clean AL or AW1 spills. Wear appropriate PPE as detailed below when handling kit components.
What personal protective equipment (PPE) is required? (ref:SCP9)	Lab users should wear lab coat, laboratory gloves and protective eyewear at all times when using kit.

Chemical storage procedure:
All kit components can be stored at room temperature. No specialised storage required. Keep lids tightly shut when not in use. Following addition of ethanol to AW1 and AW2 store in white flammables cabinet.
Waste disposal procedure:
Contaminated plasticware (tips/tubes/columns) to be disposed of in yellow bio-bins. When full place inside large yellow bin in 1.29 for collection and disposal by a licenced contractor.
Surplus buffers to be retained in original bottles (including labels) and stored at room temperature before being sent to stores for disposal. Following addition of ethanol to AW1 and AW2 store in flammables cabinet prior to disposal and label containers appropriately.
Spillage/leaks procedure:
Buffer AL and Buffer AW1 contain guanidine hydrochloride, which can form highly reactive compounds when combined with bleach. If liquid containing this buffer is spilt, clean with suitable laboratory detergent and water. If the spilt liquid contains potentially infectious agents, clean the affected area first with laboratory detergent and water, and then with 1% (v/v) sodium hypochlorite
Emergency action procedures – action required if a substance is,
Ingested:
AL/ATL/AW1/Proteinase K: call for a doctor immediately.
Inhaled:
AL/ATL/AW1/Proteinase K: Supply fresh air; consult doctor in case of complaints.
Contact with skin:
AL/ATL/AW1/Proteinase K: Immediately wash with soap and water and rinse thoroughly.

Contact with eye:
AL/ATL/AW1/Proteinase K: Rinse open eye under running water for several minutes. Consult a doctor.
Injected:
N/A.

In case of an emergency please contact:

Please add names and contact details (telephone number required) for the following:

- your Supervisor(s) Craig Wilding
- Local First Aiders - a list can be found on the LJMU Health and Safety Unit webpage

Elaine Gascoigne	Reception	2509
Brian Birkett	G.01	8495
Hazel Clark	G.33c	2120
Tony Gerrard	Ground floor	2012
Jerry Crayden	Post/Print room	4174
Dave Wilson	Post/Print room	4174
John Hall	Post/Print room	4175
Anne-Marie Steen	1st floor 1.06	2315
Michelle Macdonnell	2nd floor 2.14	2643
Martin Lloyd	2nd floor 2.34	2322
Catherine Fay	4th floor 4.17	2239

Isabelle De-Groote	4th floor 4.36	2812
Joseph Furmedge	5th floor	2218
James Downing	6th floor 6.50	2256
Martin Hanneghan	6th floor 6.27	2577
Al-Jumeily Dhiya	6th floor 6.30	2578
Mark Wharton	7th floor 7.24	2108
David Lamb	7th Floor 7.44	2423
Stephen Tang	7th floor	2268
Andy Evans	9th floor 9.07	2145
Peter Elliott	9th floor 9.03	2097

Local Health and Safety Officers - a list can be found on the LJMU Health and Safety Unit webpage

Jerry Bird	206 (LSB)	2181
Ted Sayers	318 (TR)	6333

- In case of business interruption (e.g., power shutdown, flood), what are the Contingency Procedures for work and waste?

Leave area and return when advised it is safe to do so. No specialist procedures required .

In case of fire or explosion: Raise alarm, immediately evacuate area, call security on ext 2222 (0151 231 2222 from an external line) and inform them of the location and source of the fire, they will contact Merseyside Fire and Rescue Service. Inform fire warden on site of the location and source of the fire.

Based on the COSHH information below, the overall risk of the procedure/protocol is ~~high/medium~~/low (please delete as appropriate)

COSHH material safety data – This needs to be completed for each chemical used in the procedure that is classed as harmful, toxic, corrosive, an irritant or poses any other risk to human health.

Hazardous substance: Buffer AL	
What are the hazards and hazard codes? H302 Harmful if swallowed. H315 Causes skin irritation. H319 Causes serious eye irritation	
Is it solid, liquid or gas? Liquid	Quantity used: 12ml provided in 50 reaction kit. 200µl per extraction
What is the route of entry into the body? Ingestion, eye contact, skin contact, inhalation	What are the target organs? No information on acute toxicity. Irritating to eyes and skin
What is the working exposure limit (WEL)? No information	
What control measures, handling precautions and PPE are in place? Use nitrile gloves, safety glasses, lab coat. Small quantities are used.	
What is the disposal procedure (if different from page 2): See p2	
What is the spillage procedure (if different from page 2): See p2	
What are the emergency procedures (if different from page 2) if swallowed, inhaled, injected, contact with skin, contact with eyes? NA	

For further information contact Health and Safety Unit (ext. 8167), refer to supplier's data sheet, contact supervisor.

Hazardous substance: Buffer ATL	
What are the hazards and hazard codes? No specific hazard codes	
Is it solid, liquid or gas? Liquid	Quantity used: 10ml provided. 180µl used per reaction
What is the route of entry into the body? Ingestion, eye contact, skin contact, inhalation	What are the target organs? No information
What is the working exposure limit (WEL)? No information	
What control measures, handling precautions and PPE are in place? Use gloves, eyewear, lab coat. Small quantities are used	
What is the disposal procedure (if different from page 2): See p2	
What is the spillage procedure (if different from page 2): See p2	

What are the emergency procedures (if different from page 2) if swallowed, inhaled, injected, contact with skin, contact with eyes? NA

Hazardous substance: Buffer AW1	
What are the hazards and hazard codes? H302 Harmful if swallowed. H315 Causes skin irritation. H319 Causes serious eye irritation.	
Is it solid, liquid or gas? Liquid	Quantity used: 500µl per reaction of diluted AW1
What is the route of entry into the body? Ingestion, eye contact, skin contact, inhalation	What are the target organs? No information on acute toxicity. Irritating to eyes and skin
What is the working exposure limit (WEL)? No information	
What control measures, handling precautions and PPE are in place? Use gloves, eyewear, lab coat. Small quantities are used	
What is the disposal procedure (if different from page 2): See p2	
What is the spillage procedure (if different from page 2): See p2	
What are the emergency procedures (if different from page 2) if swallowed, inhaled, injected, contact with skin, contact with eyes? NA	

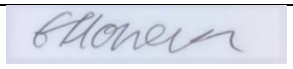
Hazardous substance: Proteinase K	
What are the hazards and hazard codes? H315 Causes skin irritation. H319 Causes serious eye irritation. H334 May cause allergy or asthma symptoms or breathing difficulties if inhaled. H335 May cause respiratory irritation	
Is it solid, liquid or gas? Liquid (as provided)	Quantity used: 20µl
What is the route of entry into the body? Ingestion, eye contact, skin contact, inhalation	What are the target organs? No information on acute toxicity. Irritating to eyes and skin
What is the working exposure limit (WEL)?	
What control measures, handling precautions and PPE are in place? Proteinase K is provided as liquid – no weighing of irritant dry powder is necessary. Small quantities provided and used. Use gloves, eyewear, lab coat.	
What is the disposal procedure (if different from page 2): See page 2	
What is the spillage procedure (if different from page 2): See page 2	
What are the emergency procedures (if different from page 2) if swallowed, inhaled, injected, contact with skin, contact with eyes? NA	

LIVERPOOL JOHN MOORES UNIVERSITY

Life Sciences Building – COSHH Risk Assessment Form

Protocol/Procedure: AGAROSE GEL ELECTROPHORESIS

Name of person carrying out procedure: Emmeline Howarth	
Job Title: student	Tel no. & e-mail: 07902494348 / e.r.howarth:2017.ljmu.ac.uk
Faculty: Science	School: NSP
Name of supervisor: Dr Craig Wilding Tel no. & e-mail: x2500 c.s.wilding@ljmu.ac.uk Signature of supervisor: Craig Wilding	
Location of procedure to be carried out: Room 2.13, Life Sciences Building or 356 James Parsons	
Has the person been trained in this protocol/procedure? YES Is training/supervision required? YES If yes, please give details: Supervisor to be present when undertaking procedure	
Date of assessment: 10/01/18	

Signature of person carrying out procedure: 
Description of Activity/Procedure/Process (include standard operating procedures (SOPs) as a Control): Preparation of agarose gels and separation of DNA/RNA fragments by running of samples through agarose gels. Gels stained with GelRed. Gels are viewed on UV transilluminators to visualise, and photograph, DNA/RNA.
Who is at risk? (staff/students/others): staff and other lab users.
What is the duration of exposure? Whole process takes <3hr
What is the frequency of exposure? < 3x per week
What is the maximum number of people in the room/lab: 6
Are there any ethical issues Y/N? NO If yes, has consent been obtained?
Is health surveillance required? NO Health Surveillance is required if the procedure involves substances which are respiratory sensitisers or skin sensitisers (risk phrases R42, R43 or **R42/43**). If other substances with potential health effects are used and if any health effects are observed that is believed to have resulted from its use then Occupational Health should be contacted. Consideration should be made of the existing health status of the user of hazardous substances. Are special arrangements required? E.g., for types of PPE (ref:SCP9).
Will the individual be working outside of normal working hours, overnight or at weekends? NO If yes, has the appropriate form (SCP11 – Out of hours working) been completed and authorisation given? Will the experiment be left to run unattended out of hours? No If yes, has the appropriate form (SCP22 – Unattended experiments) been completed and authorisation given?

Starting substances:	Agarose. Buffer (Tris-borate-EDTA or Tris-acetate-EDTA). GelRed nucleic acid stain. DNA samples (see relevant risk assessments for PCR and DNA extraction)
End products:	Agarose gel containing DNA and stain. Waste buffer.
Hazard categories:	Not hazardous
What are the potential risks?	Agarose gels are prepared by microwaving agarose and buffer together until boiling in order to dissolve agarose. **Burn risk**. Boiling solution can boil over if care is not taken. Process involves running at high voltages – **electrical risks**. Do not operate with wet gloves. Always turn off equipment before opening. Ultra Violet light may cause serious **burns** to the skin, may cause genetic damage leading to skin cancer and may cause serious eye damage. Ensure that the transilluminator is housed inside the safety cabinet and that the door of the cabinet is securely closed before the UV light is turned on.
How will these risks be controlled?	When microwaving agarose to dissolve, use short periods of operation (30s) and keep watch over flask to avoid boiling over. **ALWAYS** use heat resistant mitt to hold flask. Ensure flask is of sufficient volume to hold mixture with room for expansion whilst boiling. Cool flask under cold water before pouring into gel casting rig to avoid warping of material and leakage. Turn off electricity supply to gel tank before opening lid to remove agarose gel and do not operate electrical equipment with wet gloves. Ensure transilluminator has functional interlock to avoid exposure to UV. If using open transilluminator (e.g., to cut out DNA bands from gel) **ALWAYS WEAR UV OPAQUE FACEMASK AND GLOVES WITH NO BARE SKIN OPEN TO UV**. Use safe alternatives to ethidium bromide for staining of DNA in gel e.g., GelRed. All electrical equipment to undergo regular PAT testing.
What personal protective equipment (PPE) is required? (ref:SCP9)	Lab users should wear lab coat, laboratory gloves and protective eyewear at all times. Heat resistant mitts must be used to handle flasks whilst agarose is boiling. If cutting DNA bands from gel over transilluminator use UV opaque facemask, gloves and ensure no skin is open to UV exposure

Chemical storage procedure:

Agarose, buffer and stain can all be stored at room temperature.

Waste disposal procedure:

Waste agarose to be allowed to set then placed in yellow incineration bag for disposal. Contaminated plasticware (tips/tubes/columns) to be disposed of in yellow bio-bins. When full place inside large yellow bin in 1.29 for collection and disposal by a licenced contractor. Waste 1x (used) buffer can be poured down laboratory sinks.

Spillage/leaks procedure:

Spillage of agarose or buffer to be mopped up with laboratory roll, wiped with water plus laboratory detergent, and waste to be placed in yellow clinical waste bags for disposal.

Emergency action procedures – action required if a substance is,

Ingested:

Consult doctor in case of complaints

Inhaled:

Supply fresh air. Consult doctor in case of complaints

Contact with skin:

Wash off with water. There is no evidence of irritation of skin

Contact with eye:

Rinse open eye for several minutes under running water

Injected:

N/A.

In case of an emergency please contact:

Please add names and contact details (telephone number required) for the following:

- your Supervisor(s) Craig Wilding
- Local First Aiders - a list can be found on the LJMU Health and Safety Unit webpage

Elaine Gascoigne	Reception	2509
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Michelle Macdonnell	2nd floor 2.14	2643
Martin Lloyd	2nd floor 2.34	2322
Colin Armstrong	4th floor	2216
Catherine Fay	4th floor 4.17	2239
Isabelle De-Groote	4th floor 4.36	2812
Joseph Furmedge	5th floor	2218
James Downing	6th floor 6.50	2256
Martin Hanneghan	6th floor 6.27	2577
Al-Jumeily Dhiya	6th floor 6.30	2578
Mark Wharton	7th floor 7.24	2108
David Lamb	7th Floor 7.44	2423
Stephen Tang	7th floor	2268
Andy Evans	9th floor 9.07	2145
Peter Elliott	9th floor 9.03	2097

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Jerry Bird	206 (LSB)	2181
Ted Sayers	318 (TR)	6333

- In case of business interruption (e.g., power shutdown, flood), what are the Contingency Procedures for work and waste?

Leave area and return when advised it is safe to do so. No specialist procedures required.

In case of fire or explosion: Raise alarm, immediately evacuate area, call security on ext 2222 (0151 231 2222 from an external line) and inform them of the location and source of the fire, they

will contact Merseyside Fire and Rescue Service. Inform fire warden on site of the location and source of the fire.

Based on the COSHH information below, the overall risk of the procedure/protocol is ~~high/medium~~/low (please delete as appropriate)

COSHH material safety data – This needs to be completed for each chemical used in the procedure that is classed as harmful, toxic, corrosive, an irritant or poses any other risk to human health.

Hazardous substance: No hazardous substances	
What are the hazards and hazard codes?	
Is it solid, liquid or gas?	Quantity used:
What is the route of entry into the body?	What are the target organs?
What is the working exposure limit (WEL)?	
What control measures, handling precautions and PPE are in place?	
What is the disposal procedure (if different from page 2):	
What is the spillage procedure (if different from page 2):	
What are the emergency procedures (if different from page 2) if swallowed, inhaled, injected, contact with skin, contact with eyes?	

Appendix 6b

Table 6b. List of genotypes for all rhesus macaques (*Macaca mulatta*) involved in this study. Animals marked with a * were previously screened for 5-HTTLPR, *TPH2*, *OPRM1*, *MAOA* and *DRD4* by Szott (2015).

Animal ID	Sex	Dob	5-HTTLPR	*TPH2*	*OPRM1*	*MAOA*	*DRD4155*	*DRD4201*	*DRD4226*	*DRD4243*	*DRD4Hapl*	STin	*OXTR124*	*OXTR274*	*OXTR288*	*OXTR311*	*OXTR346*	*OXTR358*	*OXTR414*	*OXTRHapl*	*HTR2A1*	*HTR2A2*	*HTR2A3*	*HTR2A*	*AVPR1a*
ABBOTT	M	19/04/2002	SS	SS	CC	6-7	TT	GG	CG	AG	2-4	SL	GG	CT	CG	AG	CT	CT	AG	2-3	CG	AG	AA	1-2	BB
LINZ*	F	01/04/2002	SL	SS	CC	5-6	AT	CG	CG	AG	3-4	LL	TT	TT	GG	GG	CC	CC	GG	1-1	GG	AG	AC	2-4	BC
MAJ*	F	15/06/2002	SL	SS	CC	5-7	AA	GG	GG	GG	1-1	SL	GT	CT	CG	AG	CT	CT	AG	1-2	CG	AA	AC	1-4	BC
NODON	M	15/05/2004	LL	SS	CC	5-7	AT	CG	GG	GG	2-3	LL	GG	CT	CG	AG	CT	CT	AG	2-3	GG	AG	AC	2-4	BB
OCELOT*	F	20/08/2005	SL	SS	CC	6-6	AT	GG	GG	GG	1-2	SS	GG	CC	CC	AA	TT	TT	AA	2-2	GG	GG	CC	3-3	BB
ORINOCO*	F	13/07/2005	LL	SS	CG	7-7	AT	GG	GG	GG	1-2	SL	GT	CT	CG	AG	CT	CT	AG	1-2	CC	AA	AA	1-1	BB
ORLANDA*	F	08/12/2005	SS	SS	CC	7-7	AA	GG	GG	GG	1-1	SL	GG	CC	CC	AA	TT	TT	AA	2-2	CC	AA	AA	1-1	BB
PANSY*	F	06/01/2006	SL	SS	CG	5-7	AT	GG	GG	GG	1-2	LL	TT	TT	GG	GG	CC	CC	GG	1-1	CG	AG	AA	1-2	BB
PLUM	M	08/07/2006	LL	SL	CC	5-5	AA	GG	GG	GG	1-1	SS	GT	CT	CG	AG	CT	CT	AG	1-2	CG	AG	AA	1-2	BB

RACH	F	03/05/2007	SL	SS	CC	5-7	AT	GG	GG	GG	1-2	SL	GT	CT	CG	AG	CT	CT	AG	1-2	CG	AG	AA	1-2	BB
RAZZ*	F	22/06/2007	LL	SL	CG	5-7	AT	GG	GG	GG	1-2	SS	GT	CT	CG	AG	CT	CT	AG	1-2	GG	GG	AA	2-2	AB
RENE*	F	22/07/2007	SL	SS	CC	5-7	AA	GG	GG	GG	1-1	SL	GT	CT	CG	AG	CT	CT	AG	1-2	CG	AG	AA	1-2	AB
ROZANNE	F	23/04/2007	LL	SL	CC	6-7	AT	GG	GG	GG	1-2	LL	GG	CC	CC	AA	TT	TT	AA	2-2	CC	AA	AA	1-1	BB
RUPEE	F	15/02/2007	SL	SS	CC	6-7	AA	GG	GG	GG	1-1	SL	GG	CT	CG	AG	CT	CT	AG	2-3	GG	AG	AC	2-4	BC
SAPHY	F	08/03/2008	LL	SS	CC	5-7	AT	GG	GG	GG	1-2	SL	GG	CC	CC	AA	TT	TT	AA	2-2	CC	AA	AA	1-1	AB
SENGA	F	08/04/2008	SL	SS	CC	5-7	AT	GG	GG	GG	1-2	LL	GT	CT	CG	AG	CT	CT	AG	1-2	CC	AA	AA	1-1	BB
SEQUEL	M	02/10/2008	LL	SS	CC	7-7	TT	GG	GG	GG	2-2	LL	GG	CT	CG	AG	CT	CT	AG	2-3	CG	AG	AA	1-2	BB
SERENA*	F	19/03/2008	LL	SS	CC	6-7	AT	GG	GG	GG	1-2	LL	GG	CC	CC	AA	TT	TT	AA	2-2	CG	AG	AA	1-2	AC
SHALLOT*	F	14/10/2008	SL	SS	CC	5-7	AT	GG	GG	GG	1-2	SS	GT	CT	CG	AG	CT	CT	AG	1-2	CG	AG	AA	1-2	BB
SIENNA	F	24/04/2008	SL	SS	CC	5-7	AA	CG	GG	GG	1-3	LL	GT	CT	CG	AG	CT	CT	AG	1-2	CG	AG	AA	1-2	BC
SIZZLE	F	11/07/2008	SL	SS	CC	6-7	AT	GG	GG	GG	1-2	LL	GG	CC	CC	AA	TT	TT	AA	2-2	CG	AG	AA	1-2	BB

SOL	M	06/03/2002	LL	SS	CC	6-6	TT	GG	GG	GG	2-2	LL	GT	CT	CG	AG	CT	CT	AG	1-2	GG	GG	AA	2-2	BB
SPICE	F	16/06/2008	LL	SS	CC	5-6	TT	GG	GG	GG	2-2	LL	GG	CT	CG	AG	CT	CT	AG	2-3	CG	AA	AC	1-4	BB
STAR	M	26/07/2008	SL	SS	CG	5-5	AT	GG	GG	GG	1-2	SL	GT	CT	CG	AG	CT	CT	AG	1-2	CC	AA	AA	1-1	AB
SUGAR	F	09/05/2008	LL	SL	CC	5-6	AT	GG	GG	GG	1-2	LL	GG	CC	CC	AA	TT	TT	AA	2-2	CG	AA	AC	1-4	AB
TALLULAH*	F	05/06/2009	LL	SL	CG	6-6	AA	GG	GG	GG	1-1	LL	GT	CT	CG	AG	CT	CT	AG	1-2	CG	AG	AA	1-2	BB
TANYA*	F	23/03/2009	SL	SS	CC	6-6	AT	GG	GG	GG	1-2	LL	GT	CT	CG	AG	CT	CT	AG	1-2	CG	AG	AA	1-2	BB
TEA	F	19/04/2009	LL	SL	CC	6-6	TT	GG	GG	GG	2-2	LL	GG	CC	CC	AA	TT	TT	AA	2-2	GG	GG	AA	2-2	BB
TES*	F	20/08/2009	LL	SS	CC	5-6	AA	GG	GG	GG	1-1	SL	GT	CT	CG	AG	CT	CT	AG	1-2	GG	GG	AA	2-2	BB
THORN	M	09/08/2009	LL	SL	CC	6-6	AT	GG	GG	GG	1-2	LL	GT	TT	GG	GG	CC	CC	GG	1-3	CC	AA	AA	1-1	BB
THYME*	F	19/05/2009	LL	SS	CC	6-7	AA	GG	GG	GG	1-2	LL	GG	CC	CC	AA	TT	TT	AA	2-2	CG	AG	AA	1-2	AB
TILLY	F	07/07/2009	LL	SS	CC	6-7	TT	GG	GG	GG	2-2	SL	GT	CT	CG	AG	CT	CT	AG	1-2	CG	AA	AA	1-1	BB
TULIP	F	04/07/2009	SL	SS	CC	5-7	TT	GG	GG	GG	2-2	LL	GT	CT	CG	AG	CT	CT	AG	1-2	GG	GG	AA	2-2	BB

UNO	F	21/07/2010	LL	SS	CG	6-7	TT	GG	GG	GG	2-2	LL	TT	TT	GG	GG	CC	CC	GG	1-1	CG	AG	AA	1-2	BC
UTAH	M	18/05/2010	SS	SS	CC	5-5	AT	GG	GG	GG	1-2	SS	GT	CT	CG	AG	CT	CT	AG	1-2	GG	AG	AC	2-4	BB
V*	F	12/10/2011	LL	SL	CG	6-6	TT	GG	GG	GG	2-2	LL	GT	TT	GG	GG	CC	CC	GG	1-3	CG	AG	AA	1-2	BC
VALENTINE	F	14/02/2011	LL	SS	CC	6-7	AA	GG	GG	GG	1-1	SS	GG	CC	CC	AA	TT	TT	AA	2-2	CG	AG	AA	1-2	BC
VENICE*	F	13/12/2011	SL	SS	CG	5-7	AT	GG	GG	GG	1-2	LL	GT	CT	CG	AG	CT	CT	AG	1-2	CC	AA	AA	1-1	BC
VENUS*	F	06/08/2011	LL	SS	CC	6-6	AT	GG	CG	AG	1-4	LL	TT	TT	GG	GG	CC	CC	GG	1-1	CG	AG	AA	1-2	AB
VERITY*	F	13/08/2011	SL	SS	CC	6-7	AA	GG	GG	GG	1-1	SL	GT	CT	CG	AG	CT	CT	AG	1-2	CG	AA	AC	1-4	AB
VERSA	F	06/06/2011	SL	SL	CC	5-7	AT	GG	GG	GG	1-2	SS	GG	CC	CC	AA	TT	TT	AA	2-2	GG	GG	AA	2-2	BB
VIKTOR	M	21/06/2011	LL	SS	CC	6-6	AT	GG	GG	GG	1-2	SL	GG	CC	CC	AA	TT	TT	AA	2-2	CC	AA	AA	1-1	AB
VINCENT	M	22/07/2011	SL	SS	CC	6-6	AT	CG	GG	GG	2-3	SL	GT	CT	CG	AG	CT	CT	AG	1-2	CG	AG	AA	1-2	BC
WILL.I.AM	M	02/05/2012	SL	SS	GG	5-7	TT	GG	CG	AG	2-4	SL	GT	CT	CG	AG	CT	CT	AG	1-2	CG	AG	AA	1-2	BB
WINE	F	23/08/2012	LL	SS	CC	5-6	AA	CG	GG	GG	1-3	LL	GT	CT	CG	AG	CT	CT	AG	1-2	CG	AA	AC	1-4	AB

YAZZOO	F	25/07/2013	LL	SS	CC	6-7	TT	GG	GG	GG	2-2	LL	GT	CT	CG	AG	CT	CT	AG	1-2	CG	AG	AA	1-2	AB
YEVA	F	26/10/2013	SL	SS	CC	6-7	AA	GG	GG	GG	1-1	SS	GG	CC	CC	AA	TT	TT	AA	2-2	CG	AG	AA	1-2	BB
YIBBI	F	11/09/2013	SS	SL	CG	5-7	AT	GG	GG	GG	1-2	LL	TT	TT	GG	GG	CC	CC	GG	1-1	GG	GG	AA	2-2	BB
YLANG-YLANG	F	18/06/2013	SL	SS	CC	7-7	AT	GG	GG	GG	1-2	SL	GT	TT	GG	GG	CC	CC	GG	1-3	GG	AG	CC	3-4	BB
YOANA	F	26/11/2013	SL	SS	CC	5-7	AT	GG	GG	GG	1-2	SS	GT	CT	CG	AG	CT	CT	AG	1-2	GG	GG	AA	2-2	AB
YOYO	F	09/05/2013	LL	SS	CC	6-7	AT	GG	GG	GG	1-2	LL	GG	CC	CC	AA	TT	TT	AA	2-2	CC	AA	AA	1-1	AB
ZACHARIAH	M	05/06/2014	LL	SS	GG	6-6	TT	GG	GG	GG	2-2	LL	GT	CT	CG	AG	CT	CT	AG	1-2	GG	GG	AA	2-2	BB
ZARITA	F	22/10/2014	LL	SS	CC	5-7	AA	GG	GG	GG	1-1	LL	GT	TT	GG	GG	CC	CC	GG	1-3	CG	AG	AA	1-2	BC
ZAVIER	M	08/09/2014	LL	SL	CC	6-6	TT	GG	GG	GG	2-2	LL	GG	CC	CC	AA	TT	TT	AA	2-2	CG	AG	AA	1-2	BB
ZEBEDEE	M	16/08/2014	SL	SS	GG	7-7	TT	GG	GG	GG	2-2	LL	GT	TT	GG	GG	CC	CC	GG	1-3	CG	AG	AA	1-2	BB
ZELDA	F	15/08/2014	SL	SS	CC	5-5	AA	GG	GG	GG	1-1	LL	GG	CC	CC	AA	TT	TT	AA	2-2	CC	AA	AA	1-1	BB
ZENA	F	19/08/2014	SL	LL	CC	5-7	TT	GG	GG	GG	2-2	LL	GG	CT	CG	AG	CT	CT	AG	2-3	CG	AG	AA	1-2	BB

ZOIDBERG	M	12/07/2014	SL	SL	CC	7-7	AT	GG	GG	GG	1-2	LL	GT	CT	CG	AG	CT	CT	AG	1-2	CG	AG	AA	1-2	BB
ZSA-ZSA	F	21/11/2014	SL	SS	CC	5-6	AA	GG	GG	GG	1-1	SL	GT	CT	CG	AG	CT	CT	AG	1-2	CC	AA	AA	1-1	BB
ZULU	M	26/05/2014	LL	SS	CC	6-6	AT	GG	GG	GG	1-2	LL	GG	CC	CC	AA	TT	TT	AA	2-2	CG	AG	AA	1-2	AB
ZUMBA	F	21/09/2014	SS	SL	CC	5-6	TT	GG	GG	GG	2-2	SS	GG	CC	CC	AA	TT	TT	AA	2-2	CC	AA	AA	1-1	AB

Appendix 6c

Table 0c. Literature search of 5-HTTLPR primer sequences, locations within the gene, length and PCR conditions including temperature and timings.

Strand	**Name & length**	**Sequence in paper**	**Start in paper**	**Reference & name in paper**	**PCR**
R	**Name:** HTTLPR_stpr5 **Bp:** 20	GGCGTTGCCGCTCTGAATGC	16:24302938	Trefilov et al, 2000 HTTS-F	Denaturation: 17 min at 96°C 40 times: 30s at 60°C, 1 min at 72°C, 1 min at 96°C final extension: 3 min at 72°C 3% agarose gel
				Bennett et al, 2002 HTTLPR_stpr5	35 times: 30s at 95°C, 30s at 66°C, 1min at 72°C
			16:24302937	Barr et al, 2004 stpr5	Denaturation: 5 min at 96°C 30 times: 15 secs at 94°C, 15 secs at 60°C, 30 secs at 72°C Final extension: 3 min at 72°C 10% polyacrylamide gel with ethidium-bromide
				Kinnally et al, 2008 STR-F1	Denaturation: 5 min at 95°C 35 times: 30 secs at 52°C, 30 secs at 52°C, 30 secs at 74°C final extension: 5 mins at 74°C Following amplification, rh5-HTTLPR products were

				cleaved using restriction enzyme PstI for at least 1.5 h at 37°C. 3% agarose gel with ethidium bromide
			Spinelli et al, 2012 stpr5	Same as Barr et al, 2004
			Lesch et al, 1997,1996 stpr5	35 times: 30 secs at 61°C, 1 min at 72°C, 30 secs at 95°C
			McCormack et al, 2009 stpr5	Same as Lesch et al, 1996
	TATGGTACCGGCGTTGCCG CTCTGAATGC	16:24302928	Bennett et al, 2002 stprKPN5	35 times: 95°C for 30 s, 66°C for 30 s, 72°C for 1 min
Name: HTTLPR_stpr5 (+CT)	TCGACTGGCGTTGCCGCTCT GAATGC	16:24302936	Bethea et al, 2004 MutI	Denaturation: 5 mins at 95°C 30 times: 30 secs at 95°C, 30 secs at 60°C, 1 min at 72°C Final extension: 15 mins at 72°C 3.5% agarose gel cast with ethidium bromide at 23 V for

	Bp: 22				approximately 6 to 7 h, or until the bands had migrated for at least 4 cm
	Name: 5HTT-R **Bp:** 20	GCCGCTCTGAATACCAGCAC	16:24302921	Karere et al, 2012 5HTT-F	denaturation: 5 min at 95°C 40 times: 1 min at 94°C, 1 min at 55°C, 1 min at 72°C final extension: 10 min at 72°C
	Name: OLER-R **Bp:** 26	CAGCACCTAACCCCCTAATGTCCCTG	16:24302910	Rogers et al, 2006 OLER-F	Denaturation: 4 min at 95°C 37 times: 40 secs at 94°C, 30 secs at 61°C, 30 secs at 72°C Final extension: 7 min at 72°C
F	**Name:** HTTLPR_intl **Bp:** 20	CAGGGGAGATCCTGGGAGGG	16:24302537	Barr et al, 2004 intl Spinelli et al, 2012	Denaturation: 5 min at 96°C 30 times: 15 secs at 94°C, 15 secs at 60°C, 30 secs at 72°C Final extension: 3 min at 72°C 10% polyacrylamide gel with ethidium-bromide Same as Barr et al, 2004

			intl	
			McCormack et al, 2009 intl	Same as Lesch et al, 1996
Name: HTTLPR_intl (+A) **Bp:** 21	CAGGGGAGATCCTGGGAGGA	16:24302538	Lesch et al, 1997,1996 int1	36 times: 30 secs at 61°C, 1 min at 72°C, 30 secs at 95°C
			Bennett et al, 2002 HTTLPR_intl	36 times: 30s at 95°C, 30s at 66°C, 1 min at 72°C
			Trefilov et al, 2000 HTTSR	denaturation: 17 min at 96°C 40 times: 30s at 60°C, 1 min at 72°C, 1 min at 96°C final extension: 3 min at 72°C 3% agarose gel
		16:24302537	Bethea et al, 2004 Intl	Denaturation: 5 mins at 95°C 30 times: 30 secs at 95°C, 30 secs at 60°C, 1 min at 72°C Final extension: 15 mins at 72°C

				3.5% agarose gel cast with ethidium bromide at 23 V for approximately 6 to 7 h, or until the bands had migrated for at least 4 cm
Name: 5HTT-F **Bp:** 15	GGAGGGATGCAGGGGTTG	16:24302544	Karere et al, 2012 5HTT-R	denaturation: 5 min at 95°C 40 times: 1 min at 94°C, 1 min at 55°C, 1 min at 72°C final extension: 10 min at 72°C
Name: STR-F1 **Bp:** 20	GAGGGACTGAGCTGGACAACCAC	16:24302270	Kinnally et al, 2008 STR-R1	denaturation: 5 min at 95°C 35 times: 30 secs at 52°C, 30 secs at 52°C, 30 secs at 74°C final extension: 5 mins at 74°C Following amplification, rh5-HTTLPR products were cleaved using restriction enzyme PstI for at least 1.5 h at 37°C. 3% agarose gel with ethidium bromide
Name: OLER-F **Bp:** 25	GATTCTGGTGCCACCTAGACGCCAG	16:24302341	Rogers et al, 2006 OLER-R	Denaturation: 4 min at 95°C 37 times: 40 secs at 94°C, 30 secs at 61°C, 30 secs at 72°C Final extension: 7 min at 72°C

NA	**Unknown** Not found in Chromosome 16	GAGGATTGCTTGAGCCCAG GAATT	Unknown	Bennett et al, 2002 stprEC O3	Unknown

Appendix 6d

```
##############################################################################
################################Genetics######################################
##############################################################################
#Step 1. Clear workspace, set working directory and load packages
##############################################################################
#Clear workspace
ls() #this looks at what is loaded
rm(list=ls()) #this clears everything

#Set the working directory
#setwd('M:/Emily/Writing/1Papers in prep/2019 AB Heritability/AB Heritability')
setwd("G:/R Studio and R/")
#setwd("M:/Emily/Doing/Postgraduates/Emmeline Howarth/Data/Chapt6 Genetics")
#setwd("E:/R Studio and R")

#Load Package
#install.packages("lme4")
#install.packages("tidyverse")
#install.packages("car")
#install.packages("CarData")
#install.packages("rcompanion")

library(tidyverse)
library(rcompanion)
library(lme4)
library(car)#or CarData in earlier forms of R
#library(carData)

#install.packages("MuMIn")
library(MuMIn)
#citation("MuMIn")

#load Roger Functions #source files need to be in the working directory

source("G:/R Studio and R/Functions/diagnostic_fcns.r")
source("G:/R Studio and R/Functions/glmm_stability.r")
#source("E:/R Studio and R/Functions/diagnostic_fcns.r")
#source("E:/R Studio and R/Functions/glmm_stability.r")
#source("M:/Emily/Doing/R Users Group/R Training/Mundry/Functions/Functions/diagnostic_fcns.r")
#source("M:/Emily/Doing/R Users Group/R Training/Mundry/Functions/Functions/glmm_stability.r")

##############################################################################
#Step 2. Import and check data:
##############################################################################

#Load data
AB1KempThatcherHowarth_20200809<-read.csv(file.choose(), header=T) #select file from pop up window
d <- AB1KempThatcherHowarth_20200809

nrow(d)  #1188 (if higher do the following go back to CSV and delete ghost cells from bottom)
ncol(d) #245
str(d)
View(d)

e.data <- subset(d, Researcher == "Emmeline")
nrow(e.data)#634

#e.data <- subset(d, StudyNo_Genetics == "Yes")
#nrow(e.data)#319

table(e.data$WeanEarlyR)
# No Yes
# 428 206
```

```
#############################################################################
#Step 3. Ensure variables accurately labelled as factors and correct levels of each factor are being read.
#############################################################################
MData<-e.data

#Ensure random factors are coded as factors
MData$animalID <- as.factor(MData$animalID)
str(MData$animalID) #Factor w/ 110 levels
MData$HTTLPR <- as.factor(MData$HTTLPR)
str(MData$HTTLPR) #Factor w/ 3 levels "LL","SL","SS"
MData$TPH2 <- as.factor(MData$TPH2)
str(MData$TPH2) #Factor w/ 3 levels "LL","SL","SS"
MData$OPRM1 <- as.factor(MData$OPRM1)
str(MData$OPRM1) #Factor w/ 3 levels "CC","CG","GG"
MData$MAOA <- as.factor(MData$MAOA)
str(MData$MAOA) #Factor w/ 6 levels "55","56","57"
MData$AVPR <- as.factor(MData$AVPR)
str(MData$AVPR) #Factor w/ 5 levels "AB","AC","BB",.
MData$DRD4Haplotype <- as.factor(MData$DRD4Haplotype)
str(MData$DRD4Haplotype) #Factor w/ 8 levels "1.1","1.2","1.3",.
MData$STIN <- as.factor(MData$STIN)
str(MData$STIN) #Factor w/ 3 levels "LL","SL","SS"
MData$OXTRHaplotype <- as.factor(MData$OXTRHaplotype)
str(MData$OXTRHaplotype) #Factor w/ 5 levels "1.1","1.2","1.3",
MData$HTR2A <- as.factor(MData$HTR2A)
str(MData$HTR2A) #Factor w/ 7 levels "1.1","1.2","1.4",..
MData$AVPR <- as.factor(MData$AVPR)
str(MData$AVPR) #Factor w/ 5 levels "AB","AC","BB",.

MData$Sex <- as.factor(MData$Sex)
str(MData$Sex) #Factor w/ 2 levels
MData$WeanEarlyR <- as.factor(MData$WeanEarlyR)
str(MData$WeanEarlyR)  #Factor w/ 2 levels
MData$OrderTreatR <- as.factor(MData$OrderTreatR)
str(MData$OrderTreatR) #Factor w/ 2 levels
MData$AggLoc <- as.factor(MData$AggLoc)
str(MData$AggLoc) #Factor w/ 2 levels
MData$StimulusID<- as.factor(MData$StimulusID)
str(MData$StimulusID) #Factor w/ 7 levels
MData$HasDependentOffspring<-as.factor(MData$HasDependentOffspring)
str(MData$HasDependentOffspring) #Factor w/ 2 levels
MData$AlopeciaScoreHC<-as.numeric(MData$AlopeciaScoreHC)
str(MData$AlopeciaScoreHC) #num
MData$AgeMos<-as.numeric(MData$AgeMos)
str(MData$AgeMos) #num
MData$Weight<-as.numeric(MData$WeightHC)
str(MData$Weight) #num
MData$TimeR<-as.numeric(MData$TimeR)
str(MData$TimeR) #num
MData$GroupSizeAdults<-as.numeric(MData$GroupSizeAdults)
str(MData$GroupSizeAdults) #num
MData$DaysSinceLastHC<-as.numeric(MData$DaysSinceLastHC)
str(MData$DaysSinceLastHC) #num
MData$TrialStudentFile<-as.numeric(MData$TrialStudentFile)
str(MData$TrialStudentFile) #num
MData$TrialChronological<-as.numeric(MData$TrialChronological)
str(MData$TrialChronological) #num
MData$TotalNoffspring<-as.numeric(MData$TotalNoffspring)
str(MData$TotalNoffspring) #num
MData$YoungestOffspringAgeatTestMos<-as.numeric(MData$YoungestOffspringAgeatTestMos)
str(MData$YoungestOffspringAgeatTestMos) #num
MData$Trial14<-as.numeric(MData$Trial14or5InWeekorBlock)
str(MData$Trial14) #num
```

```
nrow(MData)#634

#############################################################################
#Step 4. Select variables by checking correlations
#############################################################################
#check correlations between predictors INCLUDING those that are not numeric.

corr.tab=data.frame(cbind(Wean=as.numeric(MData$WeanEarlyR), Sex = as.numeric(MData$Sex),
AgeMos=as.numeric(MData$AgeMos), Rank=as.numeric(MData$RankR),
                    Matreline=as.numeric(MData$Matriline), Time=as.numeric(MData$TimeR),
                    HTTLPR=as.numeric(MData$HTTLPR), TPH2=as.numeric(MData$TPH2),
OPRM1=as.numeric(MData$OPRM1), MAOA=as.numeric(MData$MAOA),
                    AVPR=as.numeric(MData$AVPR), DRD4=as.numeric(MData$DRD4Haplotype),
STIN=as.numeric(MData$STIN), OXTR=as.numeric(MData$OXTRHaplotype),
                    HTR2A=as.numeric(MData$HTR2A)))

str(corr.tab)
spear=cor(corr.tab[,1:15],method ="spearman")
spear

#             Wean        Sex       AgeMos       Rank   Matreline        Time      HTTLPR
# Wean 1.00000000 0.11140727 0.1392169530 -0.02534589 0.12859979 -0.011669930 -0.02356980
# Sex 0.11140727 1.00000000 0.0555391716 -0.24087300 0.02246407 0.062242108 -0.11527422
# AgeMos 0.13921695 0.05553917 1.0000000000 -0.20940595 -0.16907400 0.036729157 -0.12804856
# Rank -0.02534589 -0.24087300 -0.2094059476 1.00000000 0.07629818 -0.035464800 0.15789505
# Matreline 0.12859979 0.02246407 -0.1690739996 0.07629818 1.00000000 -0.010045778 -0.17506488
# Time -0.01166993 0.06224211 0.0367291574 -0.03546480 -0.01004578 1.000000000 -0.04198949
# HTTLPR -0.02356980 -0.11527422 -0.1280485570 0.15789505 -0.17506488 -0.041989490 1.00000000
# TPH2 0.12443085 0.08847509 0.1920566661 -0.13591324 -0.08694462 0.003939246 0.02355879
# OPRM1 -0.01555651 0.05964556 -0.1041652589 0.01776397 0.17030778 0.042676398 0.05462955
# MAOA -0.07556954 0.07159050 0.0037997006 0.13128921 0.12983087 0.004627953 -0.20327960
# AVPR 0.27587890 0.01443943 0.1061449149 0.22729481 0.23003284 0.051089393 0.24644668
# DRD4 0.15172892 0.38466996 0.0446097176 -0.15172522 0.22297866 -0.000669106 -0.15721003
# STIN 0.09188946 0.01199239 0.0580967920 -0.04920786 -0.14952082 -0.008443653 0.38215315
# OXTR -0.08205471 0.07258183 0.0000710535 0.06514131 -0.06964389 -0.028543655 -0.18832393
# HTR2A 0.13061084 0.02707165 0.2074705349 0.09261486 -0.06155017 -0.084828024 0.10220785
#              TPH2       OPRM1        MAOA        AVPR        DRD4        STIN
# Wean 0.124430850 -0.015556515 -0.075569542 0.27587890 0.151728917 0.091889456
# Sex 0.088475086 0.059645557 0.071590504 0.01443943 0.384669965 0.011992392
# AgeMos 0.192056666 -0.104165259 0.003799701 0.10614491 0.044609718 0.058096792
# Rank -0.135913236 0.017763974 0.131289209 0.22729481 -0.151725217 -0.049207860
# Matreline -0.086944621 0.170307778 0.129830867 0.23003284 0.222978665 -0.149520815
# Time 0.003939246 0.042676398 0.004627953 0.05108939 -0.000669106 -0.008443653
# HTTLPR 0.023558791 0.054629546 -0.203279604 0.24644668 -0.157210034 0.382153154
# TPH2 1.000000000 -0.118819259 0.154541164 0.02035991 -0.041530805 0.094616960
# OPRM1 -0.118819259 1.000000000 0.008331505 0.13386026 0.092801522 -0.170356098
# MAOA 0.154541164 0.008331505 1.000000000 0.04646637 0.070657347 -0.111589840
# AVPR 0.020359911 0.133860259 0.046466375 1.00000000 0.085650152 -0.035565872
# DRD4 -0.041530805 0.092801522 0.070657347 0.08565015 1.000000000 -0.341841279
# STIN 0.094616960 -0.170356098 -0.111589840 -0.03556587 -0.341841279 1.000000000
# OXTR -0.129969873 -0.418575013 0.188551645 -0.09776071 0.033129591 -0.020163092
# HTR2A -0.071423155 -0.043996522 -0.124347408 0.19329317 0.179636111 0.085279028
#              OXTR      HTR2A
# Wean      -0.0820547056  0.13061084
# Sex       0.0725818293  0.02707165
# AgeMos     0.0000710535  0.20747053
# Rank       0.0651413069  0.09261486
# Matreline -0.0696438860 -0.06155017
# Time      -0.0285436546 -0.08482802
# HTTLPR    -0.1883239282  0.10220785
# TPH2      -0.1299698729 -0.07142316
# OPRM1     -0.4185750132 -0.04399652
# MAOA       0.1885516452 -0.12434741
# AVPR      -0.0977607137  0.19329317
# DRD4       0.0331295908  0.17963611
```

```
# STIN      -0.0201630918  0.08527903
# OXTR       1.0000000000 -0.05920305
# HTR2A     -0.0592030547  1.00000000

##############################################################################
#Step 5. Transform variables
##############################################################################
NData<-MData

#response variable AGG
hist(NData$AGG)
hist(transformTukey(NData$AGG))
#     lambda      W Shapiro.p.value
# 421 0.5 0.9775 2.701e-08
hist(sqrt(NData$AGG))
NData$sqrt.AGG<-sqrt(NData$AGG)

#Total Look
hist(transformTukey(NData$TotalLook))
#     lambda      W Shapiro.p.value
# 423 0.55 0.9944 0.01959
NData$Tukey.TL<-transformTukey(NData$TotalLook)

NData$animalID <- as.factor(NData$animalID)
str(NData$animalID) #Factor w/ 110 levels

NData$HTTLPR <- as.factor(NData$HTTLPR)
str(NData$HTTLPR) #Factor w/ 3 levels "LL","SL","SS"

NData$TPH2 <- as.factor(NData$TPH2)
str(NData$TPH2) #Factor w/ 3 levels "LL","SL","SS"

NData$OPRM1 <- as.factor(NData$OPRM1)
str(NData$OPRM1) #Factor w/ 3 levels "CC","CG","GG"
NData$MAOA <- as.factor(NData$MAOA)
str(NData$MAOA) #Factor w/ 6 levels "55","56","57"

NData$AVPR <- as.factor(NData$AVPR)
str(NData$AVPR) #Factor w/ 5 levels "AB","AC","BB",.

NData$DRD4Haplotype <- as.factor(NData$DRD4Haplotype)
str(NData$DRD4Haplotype) #Factor w/ 8 levels "1.1","1.2","1.3",.

NData$STIN <- as.factor(NData$STIN)
str(NData$STIN) #Factor w/ 3 levels "LL","SL","SS"

NData$OXTRHaplotype <- as.factor(NData$OXTRHaplotype)
str(NData$OXTRHaplotype) #Factor w/ 5 levels "1.1","1.2","1.3",

NData$HTR2A <- as.factor(NData$HTR2A)
str(NData$HTR2A) #Factor w/ 7 levels "1.1","1.2","1.4",..

NData$AVPR <- as.factor(NData$AVPR)
str(NData$AVPR) #Factor w/ 5 levels "AB","AC","BB",.

NData$Sex <- as.factor(NData$Sex)
str(NData$Sex) #Factor w/ 2 levels
NData$WeanEarlyR <- as.factor(NData$WeanEarlyR)
str(NData$WeanEarlyR) #Factor w/ 3 levels
NData$Treatment <- as.factor(NData$Treatment)
str(NData$Treatment) #Factor w/ 2 level

#Treatment
table(NData$Treatment)
# BL Stress
```

```
# 460  174

HData<-NData

#############################################################################
#Step 5. TRANSFORM VARIABLES TO IMPROVE NORMAL DISTRIBUTION
#############################################################################

#Time
hist(HData$TimeR)
hist(transformTukey(HData$TimeR))
# lambda     W Shapiro.p.value
# 345 -1.4 0.9434 7.922e-15
HData$Tukey.Time<-transformTukey(HData$TimeR)

#Age
hist(HData$AgeMos)

#Time
hist(HData$TimeR)
hist(transformTukey(HData$AgeMos))
# lambda     W Shapiro.p.value
# 418 0.425 0.9546 4.585e-13
HData$Tukey.Age<-transformTukey(HData$AgeMos)

#Sex
table(HData$Sex)
# F   M
# 458 176

#HTTLPR
table(HData$HTTLPR)
# LL  SL  SS
# 306 275 53

#TPH2
table(HData$TPH2)
# LL  SL  SS
# 17 130 487

#OPRM1
table(HData$OPRM1)
# CC  CG  GG
# 528 82 24

#MAOA
table(HData$MAOA)
# 55 56 57 66 67 77
# 47 67 194 136 132 58

#AVPR
table(HData$AVPR)
# AB  AC  BB  BC  CC
# 153 8 376 97 0

#DRD4
table(HData$DRD4Haplotype)
# 1.1 1.2 1.3 1.4 2.2 2.3 2.4 3.4
# 124 270  19   8 155  34  16   8

#Stin
table(HData$STIN)
# LL  SL  SS
# 365 170 99
```

```
#OXTR
table(HData$OXTRHaplotype)
#1.1 1.2 1.3 2.2 2.3
#49 254 46 202 83

#HTP2A
table(HData$HTR2A)
# 1.1 1.2 1.4 2.2 2.4 3.3 3.4
# 148 263  52 101  50   8  12

#now z-transform all the covariates
HData$z.Tukey.Age <- as.vector(scale(HData$Tukey.Age))
HData$z.Tukey.Time <- as.vector(scale(HData$Tukey.Time))

##########################END of TRANSFORMATIONS#########################
#################################################################################
#Step 6. Save and / or load HData
#################################################################################
write.csv(HData,
      file='EData.txt', row.names=T)

EData<-read.csv(file.choose(), row.names = 1, header=T) #select file from pop up window
View(EData)
nrow(EData)#634
ncol(EData)#254
str(EData$animalID)#Factor w/ 61 levels
#################################################################################
#Step 7. Start to build model - AGG
#################################################################################
a<-EData

AGGFull1<-lmer(sqrt.AGG~
            Treatment*WeanEarlyR +
            z.Tukey.Age + Sex + z.Tukey.Time +
            HTTLPR + TPH2 + STIN + as.factor(HTR2A) + as.factor(MAOA) + OPRM1 + AVPR + as.factor(DRD4Haplotype)+
as.factor(OXTRHaplotype) +
            (1|animalID), data=a, REML = "F")

#look at plots first - lookong for no pattern in residuals
diagnostics.plot(AGGFull1)# ok
ranef.diagn.plot(AGGFull1)# ok
plot(AGGFull1) #fine
plot(residuals(AGGFull1)) #fine

#now look at any interaction terms
as.data.frame(drop1(AGGFull1, test = "Chisq"))
#                     Df     AIC        LRT    Pr(Chi)
# <none> NA 4862.056 NA NA
# z.Tukey.Age             1 4863.698  3.64262117 0.05631820
# Sex                   1 4860.325  0.26941032 0.60372761 #remove
# z.Tukey.Time            1 4861.486  1.43069514 0.23165103 #remove
# HTTLPR                2 4858.915  0.85932660 0.65072816 #remove
# TPH2                  2 4863.855  5.79953319 0.05503606
# STIN                  2 4868.281 10.22521657 0.00602036
# as.factor(HTR2A)         6 4862.103 12.04759769 0.06091534
# as.factor(MAOA)          5 4855.897  3.84157781 0.57244301 #remove
# OPRM1                 2 4859.664  1.60852585 0.44741759 #remove
# AVPR                  3 4866.593 10.53685228 0.01451298
# as.factor(DRD4Haplotype)  7 4862.872 14.81679627 0.03842084
# as.factor(OXTRHaplotype)  4 4857.572  3.51659212 0.47535997 #remove
# Treatment:WeanEarlyR      1 4860.145  0.08930762 0.76505923 #remove interaction

AGGFull2<-lmer(sqrt.AGG~
            Treatment + WeanEarlyR +
            z.Tukey.Age +
```

```
            TPH2 + STIN + as.factor(HTR2A) + AVPR + as.factor(DRD4Haplotype) +
            (1|animalID), data=a, REML = "F")

#look at plots first - lookong for no pattern in residuals
diagnostics.plot(AGGFull2)# ok
ranef.diagn.plot(AGGFull2)# fine
plot(AGGFull2) #fine
plot(residuals(AGGFull2)) #fine

#now look at any interaction terms
as.data.frame(drop1(AGGFull2, test = "Chisq"))
#                     Df     AIC        LRT     Pr(Chi)
# <none> NA 4840.327 NA NA
# Treatment               1 4838.457  0.13047405 0.717941030 #retain
# WeanEarlyR              1 4838.367  0.04061358 0.840285656 #remove
# z.Tukey.Age             1 4845.064  6.73748534 0.009440761
# TPH2                    2 4843.108  6.78144531 0.033684326
# STIN                    2 4844.493  8.16596801 0.016857089
# as.factor(HTR2A)        6 4836.597  8.27023217 0.218966389 #remove
# AVPR                    3 4843.423  9.09601892 0.028041131
# as.factor(DRD4Haplotype)  7 4842.358 16.03180813 0.024827341

AGGFull3<-lmer(sqrt.AGG~
            Treatment + z.Tukey.Age +
            TPH2 + STIN + AVPR + as.factor(DRD4Haplotype) +
            (1|animalID), data=a, REML = "F")

#look at plots first - lookong for no pattern in residuals
diagnostics.plot(AGGFull3)# ok
ranef.diagn.plot(AGGFull3)# look fine
plot(AGGFull3) #vertical lines
plot(residuals(AGGFull3)) #fine

#now look at any interaction terms
as.data.frame(drop1(AGGFull3, test = "Chisq"))
#                     Df     AIC       LRT    Pr(Chi)
# <none> NA 4832.966 NA NA
# Treatment               1 4832.966  0.3433866 0.55788089
# z.Tukey.Age             1 4836.609  3.9857081 0.04588781
# TPH2                    2 4835.149  4.5259066 0.10404276 #remove
# STIN                    2 4835.358  4.7351187 0.09370916
# AVPR                    3 4834.015  5.3920938 0.14523696 #remove
# as.factor(DRD4Haplotype)  7 4834.670 14.0470410 0.05035098

AGGFull4<-lmer(sqrt.AGG~
            Treatment + z.Tukey.Age + STIN + as.factor(DRD4Haplotype) +
            (1|animalID), data=a, REML = "F")

diagnostics.plot(AGGFull4)# ok
ranef.diagn.plot(AGGFull4)# look fine
plot(AGGFull4) #vertical lines
plot(residuals(AGGFull4)) #fine

#now look at any interaction terms
as.data.frame(drop1(AGGFull4, test = "Chisq"))
#                     Df     AIC       LRT   Pr(Chi)
# <none> NA 4831.590 NA NA
# Treatment               1 4831.590  0.4029706 0.5255591
# z.Tukey.Age             1 4832.926  1.7388204 0.1872884
# STIN                    2 4832.581  3.3938119 0.1832496
# as.factor(DRD4Haplotype)  7 4830.742 11.5549578 0.1161772

AGGFull5<-lmer(sqrt.AGG~
            Treatment + as.factor(DRD4Haplotype) +
            (1|animalID), data=a, REML = "F")
```

```
diagnostics.plot(AGGFull5)# ok
ranef.diagn.plot(AGGFull5)# look fine
plot(AGGFull5) #vertical lines
plot(residuals(AGGFull5)) #fine

#now look at any interaction terms
as.data.frame(drop1(AGGFull5, test = "Chisq"))
#                     Df     AIC     LRT   Pr(Chi)
# <none> NA 4829.957 NA NA
# Treatment              1 4829.957  0.2680257 0.6046592
# as.factor(DRD4Haplotype)  7 4828.627 10.9372793 0.1413784

nullAGG<-lmer(sqrt.AGG~ 1+
            (1|animalID), data=a, REML = "F")

anova(nullAGG, AGGFull1) #0.3067
anova(nullAGG, AGGFull2) #0.08731
anova(nullAGG, AGGFull3) #0.08201
anova(nullAGG, AGGFull4) #0.149
anova(nullAGG, AGGFull5) #0.1858

#full3 is best model
# Data: a
# Models:
#   nullAGG: sqrt.AGG ~ 1 + (1 | animalID)
# AGGFull3: sqrt.AGG ~ Treatment + z.Tukey.Age + TPH2 + STIN + AVPR + as.factor(DRD4Haplotype) +
#   AGGFull3:     (1 | animalID)
# Df    AIC    BIC  logLik deviance  Chisq Chi Df Pr(>Chisq)
# nullAGG   3 4827.0 4840.3 -2410.5   4821.0
# AGGFull3 19 4834.6 4919.2 -2398.3 4796.6 24.356 16 0.08201 .
# ---
#   Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

a$STIN<-relevel(a$STIN, ref="LL")
table(a$STIN)
#  LL  SS  SL
# 365  99 170


a$AVPR<-relevel(a$AVPR, ref="BC")
table(a$AVPR)
# BC  AB  AC  BB
# 97 153 8 376

FinalAGG<-lmer(sqrt.AGG~
             z.Tukey.Age +
             TPH2 + STIN + AVPR + as.factor(DRD4Haplotype) +
             (1|animalID), data=a, REML = "F")

as.data.frame(drop1(FinalAGG, test = "Chisq"))
#                     Df     AIC      LRT    Pr(Chi)
# <none> NA 4832.966 NA NA
# z.Tukey.Age 1 4834.815 3.848327 0.04979564
# TPH2 2 4833.402 4.435972 0.10882809
# STIN 2 4833.631 4.665106 0.09704768
# AVPR 3 4832.524 5.558107 0.13520419
# as.factor(DRD4Haplotype)  7 4832.986 14.019941 0.05082783

summary(FinalAGG)
# Linear mixed model fit by maximum likelihood  ['lmerMod']
# Formula: sqrt.AGG ~ z.Tukey.Age + TPH2 + STIN + AVPR + as.factor(DRD4Haplotype) +      (1 | animalID)
# Data: a
#
# AIC      BIC   logLik deviance df.resid
```

```
# 4833.0   4913.1  -2398.5   4797.0     616
#
# Scaled residuals:
#   Min      1Q  Median      3Q     Max
# -2.14751 -0.66293  0.03647  0.58632  3.10640
#
# Random effects:
#   Groups   Name        Variance Std.Dev.
# animalID (Intercept)   4.66    2.159
# Residual             109.23   10.452
# Number of obs: 634, groups:  animalID, 61
#
# Fixed effects:
#   Estimate Std. Error t value
# (Intercept)                 8.1279     4.1359   1.965
# z.Tukey.Age                -1.1293     0.5669  -1.992
# TPH2SL                      7.5003     3.7261   2.013
# TPH2SS                      7.9100     3.6405   2.173
# STINSL                      3.0689     1.4854   2.066
# STINSS                     -0.2446     1.5438  -0.158
# AVPRAB                      1.9282     1.8723   1.030
# AVPRAC                      3.9890     4.6767   0.853
# AVPRBB                      3.7379     1.6316   2.291
# as.factor(DRD4Haplotype)1.2   2.4792     1.5199   1.631
# as.factor(DRD4Haplotype)1.3   6.2228     3.3083   1.881
# as.factor(DRD4Haplotype)1.4   7.8162     4.7147   1.658
# as.factor(DRD4Haplotype)2.2   3.2944     1.7494   1.883
# as.factor(DRD4Haplotype)2.3   8.2247     2.6787   3.070
# as.factor(DRD4Haplotype)3.3   7.4091     3.3740   2.196
# as.factor(DRD4Haplotype)3.4   7.2834     4.8372   1.506

r.squaredGLMM(FinalAGG)
#           R2m       R2c
# [1,]  0.06279215 0.1011415

confint.merMod(object=FinalAGG)
#                              2.5 %      97.5 %
# .sig01 0.000000000 3.511178052
# .sigma 9.871408311 11.090986095
# (Intercept) -0.730000427 16.395862582
# z.Tukey.Age -2.266039865 -0.001043319
# TPH2SL -0.009569376 15.034358109
# TPH2SS 0.579378266 15.291644441
# STINLL -2.806228654 3.366216782
# STINSL -0.231212578 6.861730126
# AVPRAB -1.773325243 5.708906387
# AVPRAC -5.308947231 13.265547190
# AVPRBB 0.482269197 6.988398225
# as.factor(DRD4Haplotype)1.2 -0.554716136 5.516911479
# as.factor(DRD4Haplotype)1.3 -0.416799691 12.780718378
# as.factor(DRD4Haplotype)1.4 -1.606903311 17.143081755
# as.factor(DRD4Haplotype)2.2 -0.196034799 6.791167841
# as.factor(DRD4Haplotype)2.3 2.829061328 13.638411870
# as.factor(DRD4Haplotype)2.4 0.728687686 14.134394574
# as.factor(DRD4Haplotype)3.4 -2.330612731 16.888271045

mstabAgg=glmm.model.stab(model.res=FinalAGG, contr=lmerControl(optimizer = "bobyqa",optCtrl = list(maxfun=2e5)))
mstabAgg$detailed$warnings

mstaBagg$summary[,-1]

##############################################################################
#Step 8. Start to build model - TL
##############################################################################
TLFull1<-lmer(Tukey.TL~
```

```
        Treatment*WeanEarlyR +
        z.Tukey.Age + Sex + z.Tukey.Time +
        HTTLPR + TPH2 + STIN + as.factor(HTR2A) + as.factor(MAOA) + OPRM1 + AVPR + as.factor(DRD4Haplotype)+
as.factor(OXTRHaplotype) +
        (1|animalID), data=a, REML = "F")

#look at plots first - lookong for no pattern in residuals
diagnostics.plot(TLFull1)# ok - a bit clused in residuals plot
ranef.diagn.plot(TLFull1)# OK
plot(TLFull1) #a bit clustered
plot(residuals(TLFull1)) #fine

#now look at any interaction terms
as.data.frame(drop1(TLFull1, test = "Chisq"))

#                    Df     AIC        LRT      Pr(Chi)
# <none> NA 5251.899 NA NA
# z.Tukey.Age             1 5252.603  2.70345573 0.1001309982
# Sex                   1 5249.911  0.01117067 0.9158272067 #remove
# z.Tukey.Time            1 5261.624 11.72442248 0.0006168519
# HTTLPR                2 5252.810  4.91057123 0.0858386750
# TPH2                  2 5258.951 11.05101927 0.0039838379
# STIN                  2 5261.199 13.29977470 0.0012941679
# as.factor(HTR2A)        6 5259.646 19.74667233 0.0030719779
# as.factor(MAOA)         5 5244.496  2.59681060 0.7618497986 #remove
# OPRM1                 2 5250.953  3.05305886 0.2172884750 #remove
# AVPR                  3 5255.842  9.94287111 0.0190580466
# as.factor(DRD4Haplotype)  7 5256.732 18.83212405 0.0087298149
# as.factor(OXTRHaplotype)  4 5254.390 10.49060183 0.0329266938
# Treatment:WeanEarlyR      1 5250.558  0.65844470 0.4171095594 #remove interaction

TLFull2<-lmer(Tukey.TL~
        Treatment + WeanEarlyR +
        z.Tukey.Age + z.Tukey.Time +
        HTTLPR + TPH2 + STIN + as.factor(HTR2A) + AVPR + as.factor(DRD4Haplotype)+  as.factor(OXTRHaplotype) +
        (1|animalID), data=a, REML = "F")

#look at plots first - lookong for no pattern in residuals
diagnostics.plot(TLFull2)# ok - a bit clused in residuals plot
ranef.diagn.plot(TLFull2)# OK
plot(TLFull2) #a bit clustered
plot(residuals(TLFull2)) #fine

#now look at any interaction terms
as.data.frame(drop1(TLFull2, test = "Chisq"))

#                    Df     AIC        LRT      Pr(Chi)
# <none> NA 5241.005 NA NA
# Treatment 1 5240.605 1.599466e+00 0.2059788943 #retain
# WeanEarlyR 1 5239.006 1.671212e-04 0.9896856050 #remove
# z.Tukey.Age 1 5243.460 4.454169e+00 0.0348161554
# z.Tukey.Time 1 5251.112 1.210649e+01 0.0005024661
# HTTLPR 2 5240.546 3.540995e+00 0.1702482514 #remove
# TPH2 2 5248.805 1.179929e+01 0.0027404110
# STIN 2 5249.492 1.248636e+01 0.0019436623
# as.factor(HTR2A) 6 5250.320 2.131448e+01 0.0016105187
# AVPR 3 5246.834 1.182848e+01 0.0079944134
# as.factor(DRD4Haplotype) 7 5250.839 2.383370e+01 0.0012186155
# as.factor(OXTRHaplotype) 4 5243.667 1.066115e+01 0.0306481384

TLFull3<-lmer(Tukey.TL~
        Treatment +
        z.Tukey.Age + z.Tukey.Time +
        TPH2 + STIN + as.factor(HTR2A) + AVPR + as.factor(DRD4Haplotype)+  as.factor(OXTRHaplotype) +
        (1|animalID), data=a, REML = "F")
```

```
#look at plots first - looking for no pattern in residuals
diagnostics.plot(TLFull3)# ok - a bit clustered in residuals plot
ranef.diagn.plot(TLFull3)# OK
plot(TLFull3) #a bit clustered
plot(residuals(TLFull3)) #fine

#now look at any interaction terms
as.data.frame(drop1(TLFull3, test = "Chisq"))
#                     Df     AIC      LRT      Pr(Chi)
# <none> NA 5238.562 NA NA
# Treatment 1 5238.232 1.669964 0.1962632970
# z.Tukey.Age 1 5243.425 6.862654 0.0088015737
# z.Tukey.Time            1 5247.914 11.351926 0.0007536975
# TPH2                    2 5245.478 10.916050 0.0042619640
# STIN                    2 5248.584 14.021225 0.0009022560
# as.factor(HTR2A)        6 5247.388 20.825372 0.0019719469
# AVPR                    3 5246.822 14.259562 0.0025723128
# as.factor(DRD4Haplotype)  7 5249.155 24.592436 0.0008958931
# as.factor(OXTRHaplotype) 4 5239.829 9.267149 0.0547580875

summary(TLFull3)
# Linear mixed model fit by maximum likelihood  ['lmerMod']
# Formula: Tukey.TL ~ Treatment + z.Tukey.Age + z.Tukey.Time + TPH2 + STIN +
#   as.factor(HTR2A) + AVPR + as.factor(DRD4Haplotype) + as.factor(OXTRHaplotype) +
#   (1 | animalID)
# Data: a
#
# AIC      BIC   logLik deviance df.resid
# 5238.6 5372.1 -2589.3 5178.6 604
#
# Scaled residuals:
#   Min      1Q  Median      3Q     Max
# -3.1763 -0.7197 -0.0055 0.7161 2.6242
#
# Random effects:
#   Groups   Name       Variance Std.Dev.
# animalID (Intercept) 10.05 3.17
# Residual 198.37 14.08
# Number of obs: 634, groups:  animalID, 61
#
# Fixed effects:
#   Estimate Std. Error t value
# (Intercept) 16.4280 8.5067 1.931
# TreatmentStress -1.7491 1.3485 -1.297
# z.Tukey.Age -2.5099 0.9264 -2.709
# z.Tukey.Time 1.9789 0.5844 3.387
# TPH2SL 5.2788 6.4059 0.824
# TPH2SS 11.4256 6.0125 1.900
# STINLL 0.6659 2.4904 0.267
# STINSL 9.6797 2.9394 3.293
# as.factor(HTR2A)1.2 6.1433 2.2206 2.766
# as.factor(HTR2A)1.4 12.3795 3.3130 3.737
# as.factor(HTR2A)2.2 7.4462 2.7740 2.684
# as.factor(HTR2A)2.4 10.5785 4.3605 2.426
# as.factor(HTR2A)3.3 -3.2144 6.8456 -0.470
# as.factor(HTR2A)3.4 -16.6893 6.7707 -2.465
# AVPRAB 1.7549 2.9628 0.592
# AVPRAC 2.5983 6.7820 0.383
# AVPRBB 8.1614 2.4688 3.306
# as.factor(DRD4Haplotype)1.2 7.8241 2.3541 3.324
# as.factor(DRD4Haplotype)1.3 12.2062 4.8857 2.498
# as.factor(DRD4Haplotype)1.4 10.8677 7.5806 1.434
# as.factor(DRD4Haplotype)2.2 9.2154 2.5969 3.549
# as.factor(DRD4Haplotype)2.3 18.3677 4.0873 4.494
```

```
# as.factor(DRD4Haplotype)2.4 12.8623 5.0674 2.538
# as.factor(DRD4Haplotype)3.4 9.4070 8.3825 1.122
# as.factor(OXTRHaplotype)1.2 -2.7844 3.6674 -0.759
# as.factor(OXTRHaplotype)1.3 6.2274 4.4900 1.387
# as.factor(OXTRHaplotype)2.2 2.0213 3.7960 0.532
# as.factor(OXTRHaplotype)2.3 -5.6183 4.9100 -1.144

nullTL<-lmer(Tukey.TL~ 1+
          (1|animalID), data=a, REML = "F")

anova(nullTL, TLFull1) #0.00101
anova(nullTL, TLFull2) #0.0002251
anova(nullTL, TLFull3) #0.0001748

#Full3 is best model
# Data: a
# Models:
#   nullTL: Tukey.TL ~ 1 + (1 | animalID)
# TLFull3: Tukey.TL ~ Treatment + z.Tukey.Age + z.Tukey.Time + TPH2 + STIN +
#   TLFull3:     as.factor(HTR2A) + AVPR + as.factor(DRD4Haplotype) + as.factor(OXTRHaplotype) +
#   TLFull3:     (1 | animalID)
# Df    AIC    BIC  logLik deviance  Chisq Chi Df Pr(>Chisq)
# nullTL   3 5245.9 5259.3 -2620.0   5239.9
# TLFull3 30 5238.6 5372.1 -2589.3 5178.6 61.355 27 0.0001748 ***
#   ---
#   Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

a$HTR2A4<-as.factor(a$HTR2A)
a$HTR2A4<-relevel(a$HTR2A4, ref="1.1")
table(a$HTR2A4)
# 1.1 1.2 1.4 2.2 2.4 3.3 3.4
# 148 263  52 101  50   8  12

a$OXTR9<-as.factor(a$OXTRHaplotype)
a$OXTR9<-relevel(a$OXTR9, ref="1.2")
table(a$OXTR9)
# 1.2 1.1 1.3 2.2 2.3
# 254  49  46 202  83

a$TPH22<-as.factor(a$TPH2)
a$TPH22<-relevel(a$TPH22, ref="SS")
table(a$TPH22)
# SS  LL  SL
# 487  17 130

a$STIN2<-as.factor(a$STIN)
a$STIN2<-relevel(a$STIN2, ref="SL")
table(a$STIN2)
# LL  SL  SS
# 365 170  99

FinalTL<-lmer(Tukey.TL~
          Treatment +
          z.Tukey.Age + z.Tukey.Time +
          TPH22 + STIN2 + HTR2A4 + AVPR + as.factor(DRD4Haplotype)+  OXTR9 +
          (1|animalID), data=a, REML = "F")

as.data.frame(drop1(FinalTL, test = "Chisq"))
#                    npar     AIC      LRT     Pr(Chi)
# <none>                NA 5238.562       NA          NA
# Treatment              1 5238.232  1.669964 0.1962632970
# z.Tukey.Age            1 5243.425  6.862654 0.0088015737
# z.Tukey.Time           1 5247.914 11.351926 0.0007536975
# TPH22                  2 5245.478 10.916050 0.0042619640
# STIN2                  2 5248.584 14.021225 0.0009022560
```

```
# HTR2A4                    6 5247.388 20.825372 0.0019719469
# AVPR                      3 5246.822 14.259562 0.0025723128
# as.factor(DRD4Haplotype)    7 5249.155 24.592436 0.0008958931
# OXTR9                     4 5239.829  9.267149 0.0547580875

summary(FinalTL)
# Linear mixed model fit by maximum likelihood  ['lmerMod']
# Formula: Tukey.TL ~ Treatment + z.Tukey.Age + z.Tukey.Time + TPH22 + STIN2 +
#   HTR2A4 + AVPR + as.factor(DRD4Haplotype) + OXTR9 + (1 | animalID)
# Data: a
#
# AIC      BIC   logLik deviance df.resid
# 5238.6   5372.1  -2589.3   5178.6     604
#
# Scaled residuals:
#   Min      1Q  Median      3Q     Max
# -3.1763 -0.7197 -0.0055  0.7161  2.6242
#
# Random effects:
#   Groups   Name        Variance Std.Dev.
# animalID (Intercept)  10.05    3.17
# Residual             198.37   14.08
# Number of obs: 634, groups:  animalID, 61
#
# Fixed effects:
#   Estimate Std. Error t value
# (Intercept)               34.7488     3.0426  11.421
# TreatmentStress           -1.7491     1.3485  -1.297
# z.Tukey.Age               -2.5099     0.9264  -2.709
# z.Tukey.Time               1.9789     0.5844   3.387
# TPH22LL                  -11.4256     6.0125  -1.900
# TPH22SL                   -6.1468     2.1083  -2.916
# STIN2LL                   -9.0138     2.3531  -3.831
# STIN2SS                   -9.0137     2.9394  -3.293
# HTR2A41.2                  6.1433     2.2206   2.766
# HTR2A41.4                 12.3795     3.3130   3.737
# HTR2A42.2                  7.4462     2.7740   2.684
# HTR2A42.4                 10.5785     4.3605   2.426
# HTR2A43.3                 -3.2144     6.8456  -0.470
# HTR2A43.4                -16.6893     6.7707  -2.465
# AVPRAB                    1.7549     2.9628   0.592
# AVPRAC                    2.5983     6.7820   0.383
# AVPRBB                    8.1614     2.4688   3.306
# as.factor(DRD4Haplotype)1.2   7.8241     2.3541   3.324
# as.factor(DRD4Haplotype)1.3  12.2062     4.8857   2.498
# as.factor(DRD4Haplotype)1.4  10.8677     7.5806   1.434
# as.factor(DRD4Haplotype)2.2   9.2154     2.5969   3.549
# as.factor(DRD4Haplotype)2.3  18.3677     4.0873   4.494
# as.factor(DRD4Haplotype)3.3  12.8623     5.0674   2.538
# as.factor(DRD4Haplotype)3.4   9.4070     8.3825   1.122
# OXTR91.1                   2.7844     3.6674   0.759
# OXTR91.3                   9.0118     3.6883   2.443
# OXTR92.2                   4.8057     2.0245   2.374
# OXTR92.3                  -2.8338     3.4417  -0.823

r.squaredGLMM(FinalTL)
#           R2m       R2c
# [1,]  0.1750258 0.2148066

confint.merMod(object=FinalTL)
                          # 2.5 %     97.5 %
#   .sig01                0.8907982  4.9592943
# .sigma                 13.3034526 14.9447747
# (Intercept)            28.6100035 40.7452152
# TreatmentStress        -4.4073118  0.9054534
```

```
# z.Tukey.Age               -4.3534734 -0.6589567
# z.Tukey.Time               0.8308322  3.1282971
# TPH22LL                  -23.5883677  0.6393023
# TPH22SL                  -10.3201162 -1.9218355
# STIN2LL                  -13.7127115 -4.2929602
# STIN2SS                  -15.5211777 -3.7968401
# HTR2A41.2                  1.7416553 10.5921958
# HTR2A41.4                  5.7689081 18.9903586
# HTR2A42.2                  1.9627447 13.0285418
# HTR2A42.4                  1.8579189 19.3105560
# HTR2A43.3                 -16.8156257 10.3845430
# HTR2A43.4                 -30.1981230 -3.1420692
# AVPRAB                    -4.1109644  7.7079026
# AVPRAC                   -10.8897832 16.0511930
# AVPRBB                    3.2327164 13.0713285
# as.factor(DRD4Haplotype)1.2   3.1596219 12.5744771
# as.factor(DRD4Haplotype)1.3   2.4819697 21.9486159
# as.factor(DRD4Haplotype)1.4  -4.2314842 25.9256615
# as.factor(DRD4Haplotype)2.2   4.0588204 14.4176071
# as.factor(DRD4Haplotype)2.3  10.1601978 26.6198351
# as.factor(DRD4Haplotype)3.3   2.8106298 22.9661466
# as.factor(DRD4Haplotype)3.4  -7.2576852 26.1350354
# OXTR91.1                  -4.5136223 10.1141365
# OXTR91.3                   1.6908733 16.3593009
# OXTR92.2                   0.7905077  8.8613183
# OXTR92.3                  -9.6663253  4.1446140


mstabTL=glmm.model.stab(model.res=FinalTL, contr=lmerControl(optimizer = "bobyqa",optCtrl = list(maxfun=2e5)))
mstabTL$detailed$warnings

mstabTL$summary[,-1]

install.packages("emmeans")
library(emmeans)

emmeans(FinalTL, list(pairwise ~ STIN2), adjust = "tukey")
# $`pairwise differences of TPH22`
# contrast estimate   SE  df t.ratio p.value
# SL - LL     9.014 3.25 121 2.770   0.0177
# SL - SS     9.680 3.97 120 2.437   0.0427
# LL - SS     0.666 3.35 118 0.198   0.9785


emmeans(FinalTL, list(pairwise ~ HTR2A4), adjust = "tukey")
# $`emmeans of HTR2A4`
# HTR2A4 emmean   SE  df lower.CL upper.CL
# 1.1      37.8 5.13 118    23.76     51.8
# 1.2      43.9 4.16 122    32.54     55.3
# 1.4      50.1 5.80 117    34.31     66.0
# 2.2      45.2 5.22 119    30.97     59.4
# 2.4      48.3 6.32 116    31.07     65.6
# 3.3      34.5 9.62 120     8.30     60.8
# 3.4      21.1 9.96 115    -6.12     48.3
#
# Results are averaged over the levels of: Treatment, TPH22, STIN2, AVPR, DRD4Haplotype, OXTR9
# Degrees-of-freedom method: kenward-roger
# Confidence level used: 0.95
# Conf-level adjustment: sidak method for 7 estimates
#
# $`pairwise differences of HTR2A4`
# contrast  estimate    SE  df t.ratio p.value
# 1.1 - 1.2    -6.14  2.98 123 -2.061  0.3826
# 1.1 - 1.4   -12.38  4.50 117 -2.749  0.0956
# 1.1 - 2.2    -7.45  3.73 120 -1.997  0.4220
```

```
# 1.1 - 2.4   -10.58  6.04 115 -1.750  0.5841
# 1.1 - 3.3     3.21  9.02 120  0.356  0.9998
# 1.1 - 3.4    16.69  9.29 115  1.796  0.5536
# 1.2 - 1.4    -6.24  4.24 116 -1.471  0.7612
# 1.2 - 2.2    -1.30  3.27 118 -0.398  0.9997
# 1.2 - 2.4    -4.44  5.56 114 -0.798  0.9847
# 1.2 - 3.3     9.36  8.91 118  1.051  0.9408
# 1.2 - 3.4    22.83  9.53 116  2.395  0.2102
# 1.4 - 2.2     4.93  4.86 116  1.015  0.9497
# 1.4 - 2.4     1.80  6.41 114  0.281  1.0000
# 1.4 - 3.3    15.59  9.91 118  1.574  0.6990
# 1.4 - 3.4    29.07 10.45 115  2.783  0.0880
# 2.2 - 2.4    -3.13  6.23 115 -0.503  0.9988
# 2.2 - 3.3    10.66  9.14 119  1.166  0.9054
# 2.2 - 3.4    24.14  9.99 116  2.416  0.2013
# 2.4 - 3.3    13.79 10.02 117  1.376  0.8134
# 2.4 - 3.4    27.27 10.74 115  2.539  0.1552
# 3.3 - 3.4    13.47 12.45 118  1.082  0.9322
#
# Results are averaged over the levels of: Treatment, TPH22, STIN2, AVPR, DRD4Haplotype, OXTR9
# Degrees-of-freedom method: kenward-roger
# P value adjustment: tukey method for comparing a family of 7 estimates

emmeans(FinalTL, list(pairwise ~ DRD4Haplotype), adjust = "tukey")
$`pairwise differences of DRD4Haplotype`
contrast  estimate    SE  df t.ratio p.value
# 1.1 - 1.2   -7.824  3.22 117 -2.429  0.2376
# 1.1 - 1.3  -12.206  6.58 117 -1.856  0.5833
# 1.1 - 1.4  -10.868 10.08 118 -1.078  0.9602
# 1.1 - 2.2   -9.215  3.51 117 -2.623  0.1584
# 1.1 - 2.3  -18.368  5.86 114 -3.134  0.0440
# 1.1 - 3.3  -12.862  6.73 119 -1.912  0.5456
# 1.1 - 3.4   -9.407 11.26 118 -0.835  0.9907
# 1.2 - 1.3   -4.382  6.35 117 -0.691  0.9971
# 1.2 - 1.4   -3.044  9.36 118 -0.325  1.0000
# 1.2 - 2.2   -1.391  3.05 118 -0.457  0.9998
# 1.2 - 2.3  -10.544  6.06 114 -1.740  0.6613
# 1.2 - 3.3   -5.038  7.10 118 -0.710  0.9966
# 1.2 - 3.4   -1.583 10.81 117 -0.146  1.0000
# 1.3 - 1.4    1.338 11.18 118  0.120  1.0000
# 1.3 - 2.2    2.991  6.43 116  0.465  0.9998
# 1.3 - 2.3   -6.161  8.20 116 -0.752  0.9951
# 1.3 - 3.3   -0.656  9.49 118 -0.069  1.0000
# 1.3 - 3.4    2.799 12.23 118  0.229  1.0000
# 1.4 - 2.2    1.652  9.75 118  0.169  1.0000
# 1.4 - 2.3   -7.500 11.27 117 -0.665  0.9977
# 1.4 - 3.3   -1.995 11.80 118 -0.169  1.0000
# 1.4 - 3.4    1.461 12.97 118  0.113  1.0000
# 2.2 - 2.3   -9.152  6.06 114 -1.509  0.8011
# 2.2 - 3.3   -3.647  6.94 119 -0.526  0.9995
# 2.2 - 3.4   -0.192 11.44 118 -0.017  1.0000
# 2.3 - 3.3    5.505  8.21 117  0.670  0.9976
# 2.3 - 3.4    8.961 11.70 117  0.766  0.9945
# 3.3 - 3.4    3.455 13.74 118  0.251  1.0000

emmeans(FinalTL, list(pairwise ~ AVPR), adjust = "tukey")
# $`pairwise differences of AVPR`
# contrast estimate   SE  df t.ratio p.value
# BC - AB    -1.755 3.99 118 -0.439  0.9715
# BC - AC    -2.598 8.92 119 -0.291  0.9914
# BC - BB    -8.161 3.32 119 -2.456  0.0723
# AB - AC    -0.843 8.55 119 -0.099  0.9997
# AB - BB    -6.407 2.94 117 -2.176  0.1359
# AC - BB    -5.563 8.47 119 -0.657  0.9128
```

```
emmeans(FinalTL, list(pairwise ~ OXTR9), adjust = "tukey")
# $`pairwise differences of OXTR9`
# contrast  estimate   SE  df t.ratio p.value
# 1.2 - 1.1    -2.78 4.97 117 -0.561  0.9804
# 1.2 - 1.3    -9.01 4.89 118 -1.842  0.3550
# 1.2 - 2.2    -4.81 2.73 118 -1.763  0.4000
# 1.2 - 2.3     2.83 4.81 115  0.590  0.9764
# 1.1 - 1.3    -6.23 6.04 117 -1.030  0.8409
# 1.1 - 2.2    -2.02 5.17 116 -0.391  0.9950
# 1.1 - 2.3     5.62 6.71 116  0.837  0.9184
# 1.3 - 2.2     4.21 4.82 117  0.873  0.9061
# 1.3 - 2.3    11.85 6.57 116  1.802  0.3773
# 2.2 - 2.3     7.64 5.43 116  1.406  0.6251
```

Appendix 6e

```
##############################################################################################
########################################## MCMCGLMM ##########################################
##############################################################################################

##############################################################################################
###STEP 1 Set working directory to import files and check data
##############################################################################################

ls() #this looks at what is loaded
rm(list=ls()) #this clears everything

#setwd("M:/Emily/Doing/Postgraduates/Emmeline Howarth/Data/Chapt6 Genetics")
#setwd("M:/Emily/Writing/1Papers in prep/2018 JEvolBiol Julia/RWorkDir/3TEST MATERNAL EFFECTS")
#setwd("C:/Users/emmel/Desktop/R Studio and R")
#setwd("D:/R Studio and R/")
setwd("E:/R Studio and R/")

GData<-read.csv(file.choose(), row.names = 1, header=T) #select file from pop up window
View(GData)
nrow(GData)#634
ncol(GData)#261
str(GData$animalID)#Factor w/ 58 levels

library(MCMCglmm)
library(MasterBayes)
library(pedantics)

a<-GData
nrow(a) #634
a$animal<-a$animalID

##############################################################################################
###STEP 2 Read in the pedigree information
##############################################################################################

# first we read in the pedigree
ped<-read.csv(file.choose(), header=T)
nrow(ped)#261
head(ped)
View(ped)

pe<-insertPed(ped)
#then we order the pedigree so that parents come before their offspring
p<-orderPed(pe)

#p<-read.table("p.txt", header=T)
head(p)
nrow(p)#278

##############################################################################################
###STEP 3 Set working directory to save new ped files and all output
##############################################################################################
# now we do some stats and fancy graphs on the pedigree using pedantics package

colnames(p) <- c("id", "dam","sire")
par(family = "sans",mfrow = c(1,1))#this code specifies number of rows and columns to display the graphs in
drawPedigree(p)
View(p)
nrow(p)#278
#save that fixed pedigree to a file
write.table(p,
        file='fixedped.txt', sep='\t',
        row.names=F, col.names=T)
```

```
# I've added whether or not the individuals were typed or not for behaviour to see how well resolved your actual pedigree
is
pedigree<-read.table("fixedped.txt", header=T)
#pedigree<-read.table("pedTyped.txt", header=T)

nrow(pedigree)#278
View(pedigree)
names(pedigree)

# I'm computing stats for the pruned pedigree for AGG
# stats.pAGG<-pedigreeStats(p,dat=(pedigree$Typed==1)+0, graphicalReport='n')
# # and for the non-pruned one
# stats.m<-pedigreeStats(p,graphicalReport = "n")
# # and compare both
# pedStatSummary(stats.m, stats.pAGG)
# # now we print the pruned one over the other:
# drawPedigree(Ped=p,dat=(pedigree$Typed==1)+0)
# # and you can see that the remaining pedigree is not that deep at all.
# save(stats.m, file = "stats.m.rda")
# save(stats.pAGG, file = "stats.pAGG.rda")

#or just use this if already have .txt file called 'pedigree' in the folder :)
#p<-ped

##############################################################################################
###STEP 4 Final models previously developed
##############################################################################################
# FinalTL<-lmer(Tukey.TotalLook~
#               z.Tukey.Time +
#               z.Tukey.AlopeciaScoreHC + ReproStat +
#               (1|animalID), data=a, REML = "F")


# FinalAGG<-lmer(sqrt.AGG~
#                Treatment*WeanEarlyR +
#                z.Tukey.AlopeciaScoreHC + ReproStat +
#                z.Tukey.GroupSizeAdults +
#                (1|animalID), data=a, REML = "F")

##############################################################################################
#################################START AGG REPEATABILITY######################################
# first, set phenotypic variance of AGG
p.var<-var(a$sqrt.Agg,na.rm=TRUE)
prior2<-list(
  G=list(
    G1=list(V=matrix(p.var/3),n=1), # this is for animal
    G2=list(V=matrix(p.var/3),n=1)),# this is for ID
  R=list(V=matrix(p.var/3),n=1)) # this is for the residual variance
prior2
#uninformative prior
#priorA <- list(R = list(V=1, nu=0.002), G = list(G1 = list(V=1, nu=0.002), G2=list(V=1, nu=0.002)))
#priorA

#################################################################
#####################TL##########################################
#################################################################

# a$STIN<-relevel(a$STIN, ref="SS")
# table(a$STIN)
# # SS  LL  SL
# # 99 365 170

a$MAOA<-as.factor(a$MAOA)
a$MAOA<-relevel(a$MAOA, ref="57")
```

```
table(a$MAOA)
# 57  55  56  66  67  77
# 194  47  67 136 132  58

a$HTR2A<-as.factor(a$HTR2A)
a$HTR2A<-relevel(a$HTR2A, ref="3.4")
table(a$HTR2A)
# 3.4 1.1 1.2 1.4 2.2 2.4 3.3
# 12 148 263  52 101  50   8

a$DRD4Haplotype<-as.factor(a$DRD4Haplotype)
a$DRD4Haplotype<-relevel(a$DRD4Haplotype, ref="3.4")
table(a$DRD4Haplotype)
# 3.4 1.1 1.2 1.3 1.4 2.2 2.3 2.4
# 8 124 270  19   8 155  34  16

a$AVPR<-as.factor(a$AVPR)
a$AVPR<-relevel(a$AVPR, ref="BC")
table(a$AVPR)
# BC  AB  AC  BB
# 97 153   8 376
a$OXTRHaplotype<-as.factor(a$OXTRHaplotype)
a$OXTRHaplotype<-relevel(a$OXTRHaplotype, ref="1.2")
table(a$OXTRHaplotype)
# 1.2 1.1 1.3 2.2 2.3
# 254  49  46 202  83

TLAll<-(MCMCglmm(Tukey.TotalLook~
            z.Tukey.Time + z.Tukey.AlopeciaScoreHC + ReproStat +
              HTTLPR + TPH2 + STIN + as.factor(HTR2A) + as.factor(MAOA) + OPRM1 + AVPR + as.factor(DRD4Haplotype) +
as.factor(OXTRHaplotype),
            random=~animalID+animal, ped=p, data=a, nitt=501000,burnin=1000,thin=500, verbose = FALSE,
prior=prior2))#>1hr to run

autocorr(TLAll$VCV)

# , , animalID
#
# animalID     animal       units
# Lag 0     1.00000000 -0.18128122 -0.089289638
# Lag 500    0.02432315  0.02012181 -0.055331186
# Lag 2500   0.03920748 -0.05655126  0.026766562
# Lag 5000   0.02348969 -0.02332297 -0.025213236
# Lag 25000 -0.02120743  0.02196674  0.004779863
#
# , , animal
#
# animalID      animal       units
# Lag 0     -0.18128122  1.000000000  0.020805446
# Lag 500    0.03408022 -0.038916851 -0.018550549
# Lag 2500   0.02522908  0.002621885  0.008037134
# Lag 5000   0.00960303  0.025320924  0.063156938
# Lag 25000  0.04543528 -0.001386986  0.020972775
#
# , , units
#
# animalID      animal       units
# Lag 0     -0.089289638  0.020805446  1.0000000000
# Lag 500    0.033082194  0.026089735 -0.0530740146
# Lag 2500   0.006584343 -0.009801918  0.0007155441
# Lag 5000  -0.022434627  0.010074215  0.0046145824
# Lag 25000  0.058758309 -0.005160808 -0.0394444911

par(family = "sans",mfrow = c(1,1))#this code specifies number of rows and columns to display the graphs in
```

```
#CHECK PLOT OF RESIDUALS
plot(TLAll)

save(TLAll, file = "TLAll.rda")
load("TLAll.rda")
summary(TLAll)
#                          post.mean  l-95% CI  u-95% CI eff.samp pMCMC
# (Intercept)                2.7983 -55.2175  50.2784     1000 0.900
# z.Tukey.Time               1.6978   0.6880   2.8918     1000 0.006 **
# z.Tukey.AlopeciaScoreHC      -3.2153  -7.3677   0.5593     1000 0.114
# ReproStatImplanted          -8.9806 -27.8282  12.4546     1000 0.352
# ReproStatMaleBreeding       -10.0072 -26.5101   3.3676     1000 0.176
# ReproStatNurse              -6.2281 -15.9048   4.4913     1102 0.256
# ReproStatPregnant           -9.7118 -20.1814   1.1760     1000 0.078 .
# ReproStatWeanerGroup         5.8845 -11.5049  23.6588     1000 0.522
# HTTLPRSL                     4.9314  -2.4380  11.6592      864 0.144
# HTTLPRSS                     2.5777 -10.3522  14.6410     1000 0.654
# TPH2SL                      0.9802 -24.4021  26.0975     1071 0.962
# TPH2SS                      8.1656 -13.5604  30.2834     1000 0.470
# STINSL                     5.3506  -3.6943  13.7229     1000 0.216
# STINSS                     0.2217  -9.6615  10.4901     1000 0.980
# as.factor(HTR2A)1.1          17.8826  -7.4342  45.8003     1000 0.200
# as.factor(HTR2A)1.2          19.2514  -6.6355  45.5018     1000 0.166
# as.factor(HTR2A)1.4          24.6356  -8.2066  56.6247     1000 0.138
# as.factor(HTR2A)2.2          22.0669  -9.8255  48.6777     1000 0.136
# as.factor(HTR2A)2.4          26.9042  -4.9303  54.8028     1000 0.082 .
# as.factor(HTR2A)3.3          10.0086 -19.1120  42.4559     1000 0.528
# as.factor(MAOA)55             2.1720 -12.9570  18.9631     1000 0.820
# as.factor(MAOA)56             5.2359  -8.8121  17.8830     1000 0.444
# as.factor(MAOA)66             2.2952  -7.9827  13.5414     1000 0.674
# as.factor(MAOA)67             1.8669  -5.9168   9.6556     1000 0.632
# as.factor(MAOA)77             0.3720 -15.3827  15.2342     1000 0.940
# OPRM1CG                     3.7293  -8.6804  16.2398     1000 0.536
# OPRM1GG                     5.9456 -12.5028  22.9287     1000 0.508
# AVPRAB                     1.1107  -9.3841  12.2607     1115 0.834
# AVPRAC                     1.9420 -17.6699  23.8069     1000 0.864
# AVPRBB                     5.6670  -2.9462  15.8411     1202 0.240
# as.factor(DRD4Haplotype)1.1    3.3877 -47.3615  42.2998     1000 0.874
# as.factor(DRD4Haplotype)1.2   11.5294 -36.1106  55.4941     1000 0.590
# as.factor(DRD4Haplotype)1.3   11.3124 -32.9731  55.1732     1000 0.618
# as.factor(DRD4Haplotype)1.4   10.3818 -30.4539  51.5078     1000 0.622
# as.factor(DRD4Haplotype)2.2   12.1323 -35.1270  55.4103     1000 0.578
# as.factor(DRD4Haplotype)2.3   25.9030 -19.3736  68.8355     1000 0.280
# as.factor(DRD4Haplotype)2.4   21.0376 -36.6068  75.5771     1000 0.462
# as.factor(OXTRHaplotype)1.1    2.3483 -12.9371  20.1149     1000 0.768
# as.factor(OXTRHaplotype)1.3    9.3303  -3.5268  23.0315     1000 0.170
# as.factor(OXTRHaplotype)2.2    6.8667  -0.9593  15.1074     1000 0.098 .
# as.factor(OXTRHaplotype)2.3    0.5354 -14.3802  14.7208     1000 0.934

a$STIN<-relevel(a$STIN, ref="SS")
table(a$STIN)
# SS  LL  SL
# 99 365 170

a$MAOA<-as.factor(a$MAOA)
a$MAOA<-relevel(a$MAOA, ref="55")
table(a$MAOA)
# 55  57  56  66  67  77
# 47 194  67 136 132  58

#serotonin
TLm1<-(MCMCglmm(Tukey.TotalLook~
            z.Tukey.Time + z.Tukey.AlopeciaScoreHC + ReproStat +
            HTTLPR + TPH2 + STIN + as.factor(HTR2A) + as.factor(MAOA), #OPRM1 + AVPR + as.factor(DRD4Haplotype) +
as.factor(OXTRHaplotype),
```

```
          random=~animalID+animal, ped=p, data=a, nitt=501000,burnin=1000,thin=500, verbose = FALSE, prior=prior2))#>1hr to run

autocorr(TLm1$VCV)
# , , animalID
#
# animalID      animal       units
# Lag 0      1.000000000 -0.361101566 -2.903048e-02
# Lag 500   -0.009775929 -0.067397866 -7.298238e-05
# Lag 2500   0.014259615 -0.018106205  8.619452e-03
# Lag 5000   0.020613176  0.001502126  2.911088e-02
# Lag 25000 -0.043061816  0.070952655 -4.036684e-03
#
# , , animal
#
# animalID      animal      units
# Lag 0     -0.36110157  1.000000000 -0.04134928
# Lag 500    0.02226022  0.019542179 -0.03967511
# Lag 2500  -0.02559490  0.029664748 -0.01173834
# Lag 5000  -0.00228381 -0.030083082 -0.05372914
# Lag 25000  0.02481951 -0.004391453 -0.01405766
#
# , , units
#
# animalID      animal       units
# Lag 0     -0.02903048 -0.04134928 1.0000000000
# Lag 500   -0.01164750 -0.01741842 0.0418395933
# Lag 2500   0.01384612  0.04933635 0.0009551708
# Lag 5000   0.01430021 -0.04488214 0.0234707110
# Lag 25000 -0.01233587 -0.01284334 0.0311658026

par(family = "sans",mfrow = c(1,1))#this code specifies number of rows and columns to display the graphs in

#CHECK PLOT OF RESIDUALS
plot(TLm1)

save(TLm1, file = "TLm1.rda")
load("TLm1.rda")
summary(TLm1)
#post.mean  l-95% CI  u-95% CI eff.samp pMCMC
# (Intercept)             23.96332  -4.97895  51.72709    901.4 0.098 .
# z.Tukey.Time             1.65502   0.53931   2.85888   1000.0 0.008 **
# z.Tukey.AlopeciaScoreHC  -2.36707  -5.13143   0.75792   1000.0 0.124
# ReproStatImplanted      -12.92654 -25.83606   0.76333    905.9 0.082 .
# ReproStatMaleBreeding    -0.33762 -10.72469  10.17860   1000.0 0.916
# ReproStatNurse           -5.28569 -12.53434   4.25499    891.6 0.242
# ReproStatPregnant        -8.63232 -17.50965   0.05765    995.0 0.054 .
# ReproStatWeanerGroup     11.22867  -1.33004  25.00441   1213.1 0.094 .
# HTTLPRSL                  5.53835  -0.53177  11.27324    877.6 0.078 .
# HTTLPRSS                  8.05118  -0.44238  18.31882   1000.0 0.090 .
# TPH2SL                   4.26124 -13.81931  21.03933   1044.9 0.670
# TPH2SS                   9.28090  -6.49046  27.95383   1000.0 0.290
# STINLL                  2.66499  -5.16806  10.39972   1000.0 0.508
# STINSL                  3.40935  -5.39921  11.92837   1000.0 0.438
# as.factor(HTR2A)1.1      5.82734 -16.26433  27.50624    888.7 0.624
# as.factor(HTR2A)1.2      4.97151 -16.10532  27.51477    904.9 0.660
# as.factor(HTR2A)1.4      9.07173 -14.06896  31.80670    830.3 0.462
# as.factor(HTR2A)2.2      7.76117 -14.81202  31.97822    884.8 0.536
# as.factor(HTR2A)2.4      7.74396 -18.11583  31.96158    885.7 0.516
# as.factor(HTR2A)3.3      3.81485 -25.19411  34.25920   1000.0 0.802
# as.factor(MAOA)57        7.10222  -4.90216  19.07103   1000.0 0.256
# as.factor(MAOA)56        8.38572  -6.28256  21.03867   1000.0 0.230
# as.factor(MAOA)66        6.91582  -5.68134  18.28033   1000.0 0.282
# as.factor(MAOA)67        7.89850  -4.66393  19.87486   1000.0 0.192
# as.factor(MAOA)77        6.42740  -7.87810  19.47018    919.2 0.400
```

```
#Dopamine
a$MAOA<-as.factor(a$MAOA)
a$MAOA<-relevel(a$MAOA, ref="66")
table(a$MAOA)
# 66  55  57  56  67  77
# 136  47 194  67 132  58

TLm2<-(MCMCglmm(Tukey.TotalLook~
            z.Tukey.Time + z.Tukey.AlopeciaScoreHC + ReproStat +
            as.factor(MAOA) + as.factor(DRD4Haplotype), #OPRM1 + AVPR +  +  as.factor(OXTRHaplotype), #HTTLPR +
TPH2 + STIN + as.factor(HTR2A) +
            random=~animalID+animal, ped=p, data=a, nitt=501000,burnin=1000,thin=500, verbose = FALSE,
prior=prior2))#>1hr to run

autocorr(TLm2$VCV)
# , , animalID
#
# animalID     animal      units
# Lag 0      1.00000000 -0.31264393 -0.05219361
# Lag 500   -0.04101144 -0.01762616 -0.01423182
# Lag 2500   0.01159860  0.01423116  0.04397680
# Lag 5000   0.04139265  0.04210763  0.00667710
# Lag 25000  0.05310351 -0.06738931  0.01602647
#
# , , animal
#
# animalID     animal      units
# Lag 0     -0.312643932  1.000000000 -0.05247561
# Lag 500    0.064245015  0.045395046 -0.02331923
# Lag 2500   0.058319130 -0.050264202 -0.04815538
# Lag 5000  -0.029405869 -0.065608186  0.02506723
# Lag 25000  0.001272406  0.008149561  0.07142487
#
# , , units
#
# animalID     animal      units
# Lag 0     -0.05219361 -0.052475608  1.00000000
# Lag 500    0.06031962  0.001890066  0.02954804
# Lag 2500  -0.01114990 -0.037027341  0.02902097
# Lag 5000   0.02701877 -0.043078416 -0.03047092
# Lag 25000  0.05104424  0.038420628  0.02563831

par(family = "sans",mfrow = c(1,1))#this code specifies number of rows and columns to display the graphs in

#CHECK PLOT OF RESIDUALS
plot(TLm2)

save(TLm2, file = "TLm2.rda")
load("TLm2.rda")
summary(TLm2)
# post.mean  l-95% CI  u-95% CI eff.samp pMCMC
# (Intercept)                43.3935  18.8152  67.1498   1107.7 0.004 **
# z.Tukey.Time                1.5893   0.4239   2.8232   1000.0 0.008 **
# z.Tukey.AlopeciaScoreHC    -2.4390  -4.8463  -0.1124   1000.0 0.046 *
# ReproStatImplanted         -5.8424 -19.5969   6.1566   1077.3 0.338
# ReproStatMaleBreeding      -8.9967 -18.5932   1.3973    772.7 0.092 .
# ReproStatNurse             -5.2544 -13.1442   2.0615    813.2 0.176
# ReproStatPregnant          -8.7354 -16.3966  -0.8983    831.7 0.020 *
# ReproStatWeanerGroup        9.5549  -2.8975  21.9236    736.8 0.138
# as.factor(MAOA)55           3.2717  -7.1033  13.6768    846.6 0.534
# as.factor(MAOA)57           1.0730  -6.7128   8.9549   1000.0 0.802
# as.factor(MAOA)56           4.5472  -4.8343  15.2165   1000.0 0.342
# as.factor(MAOA)67           2.0316  -5.9288  10.5339   1000.0 0.600
# as.factor(MAOA)77           0.3366  -8.5665   9.5605   1000.0 0.948
```

```
# as.factor(DRD4Haplotype)1.1    1.5436 -21.7897  23.1545   1107.2 0.882
# as.factor(DRD4Haplotype)1.2    7.2217 -16.2621  30.2817   1099.3 0.542
# as.factor(DRD4Haplotype)1.3    4.6803 -19.5826  26.6114   1197.4 0.708
# as.factor(DRD4Haplotype)1.4    1.9591 -26.1944  30.3182   1000.0 0.888
# as.factor(DRD4Haplotype)2.2    7.2614 -13.6253  32.8513   1102.8 0.558
# as.factor(DRD4Haplotype)2.3   23.7270  -1.2860  49.2935   1000.0 0.054 .
# as.factor(DRD4Haplotype)2.4   24.8088  -1.6986  52.5279   1108.8 0.068 .

#Oxytocin
a$OXTRHaplotype<-as.factor(a$OXTRHaplotype)
a$OXTRHaplotype<-relevel(a$OXTRHaplotype, ref="1.3")
table(a$OXTRHaplotype)
# 1.3 1.2 1.1 2.2 2.3
# 46 254  49 202  83

TLm3<-(MCMCglmm(Tukey.TotalLook~
              z.Tukey.Time + z.Tukey.AlopeciaScoreHC + ReproStat +
                AVPR + as.factor(OXTRHaplotype), #OPRM1 +#as.factor(MAOA) + as.factor(DRD4Haplotype),HTTLPR + TPH2
+ STIN + as.factor(HTR2A) +
            random=~animalID+animal,  ped=p,  data=a,  nitt=501000,burnin=1000,thin=500,  verbose  =  FALSE,
prior=prior2))#>1hr to run

autocorr(TLm3$VCV)
# , , animalID
#
# animalID      animal       units
# Lag 0     1.000000000 -0.404794472 -0.108226861
# Lag 500   -0.002436063  0.032949869 -0.009771069
# Lag 2500  -0.004440866 -0.026483057 -0.010609770
# Lag 5000  -0.028696189  0.024010278  0.047748300
# Lag 25000  0.063747036  0.001211987 -0.043627050
#
# , , animal
#
# animalID     animal       units
# Lag 0     -0.40479447  1.00000000  0.030336195
# Lag 500   -0.03693568 -0.01722477  0.004993820
# Lag 2500   0.01994269  0.02507466 -0.012020142
# Lag 5000   0.02850171 -0.02781513  0.000243237
# Lag 25000 -0.04293012  0.01425227  0.052683215
#
# , , units
#
# animalID      animal       units
# Lag 0     -0.108226861  0.030336195  1.000000000
# Lag 500    0.052572786 -0.056503923 -0.072580494
# Lag 2500   0.008493177  0.038137879  0.033652557
# Lag 5000  -0.058086828  0.020483116  0.004124464
# Lag 25000 -0.062239351 -0.005552293  0.017198128

par(family = "sans",mfrow = c(1,1))#this code specifies number of rows and columns to display the graphs in

#CHECK PLOT OF RESIDUALS
plot(TLm3)

save(TLm3, file = "TLm3.rda")
load("TLm3.rda")
summary(TLm3)
# post.mean l-95% CI u-95% CI eff.samp  pMCMC
# (Intercept)                48.6560  39.5827  59.2863    767.0 <0.001 ***
# z.Tukey.Time                1.6357   0.5018   2.7718   1000.0  0.002 **
# z.Tukey.AlopeciaScoreHC    -1.4942  -4.0900   0.9565    903.1  0.268
# ReproStatImplanted         -9.3445 -20.7493   3.3598   1000.0  0.132
# ReproStatMaleBreeding      -3.6640 -12.9150   4.7051   1000.0  0.436
# ReproStatNurse             -5.8393 -14.6575   2.5061   1000.0  0.172
```

```
# ReproStatPregnant          -8.9294 -17.8534  -1.0135   1000.0  0.044 *
# ReproStatWeanerGroup         7.0391  -5.4118  18.1714   1000.0  0.256
# AVPRAB                     0.1661  -8.4879   8.9393   1000.0  0.976
# AVPRAC                     2.3447 -16.2079  23.1368   1000.0  0.804
# AVPRBB                     2.3302  -5.1250   8.9445   1128.3  0.482
# as.factor(OXTRHaplotype)1.2   0.9002  -9.3619  12.0797   1000.0  0.864
# as.factor(OXTRHaplotype)1.1   1.6276 -11.2753  14.4809    890.4  0.780
# as.factor(OXTRHaplotype)2.2   1.6267 -10.6649  12.4820   1000.0  0.798
# as.factor(OXTRHaplotype)2.3   2.8959  -8.1869  16.2273   1095.5  0.628

#Opioid
a$OPRM1<-as.factor(a$OPRM1)
a$OPRM1<-relevel(a$OPRM1, ref="CG")
table(a$OPRM1)
# CG  CC  GG
# 82 528  24

TLm4<-(MCMCglmm(Tukey.TotalLook~
            z.Tukey.Time + z.Tukey.AlopeciaScoreHC + ReproStat +
            OPRM1, #+AVPR + as.factor(OXTRHaplotype)#as.factor(MAOA) + as.factor(DRD4Haplotype),HTTLPR + TPH2 +
STIN + as.factor(HTR2A) +
          random=~animalID+animal,  ped=p,  data=a,  nitt=501000,burnin=1000,thin=500,  verbose  =  FALSE,
prior=prior2))#>1hr to run

autocorr(TLm4$VCV)
# , , animalID
#
# animalID       animal       units
# Lag 0      1.00000000 -0.3462923031 -0.054026052
# Lag 500   -0.03103235  0.0005551777  0.002353348
# Lag 2500   0.05980034  0.0249557711  0.017277533
# Lag 5000  -0.07442467  0.0174739932  0.004886453
# Lag 25000  0.02378792 -0.0279476809  0.008377583
#
# , , animal
#
# animalID     animal       units
# Lag 0     -0.34629230  1.00000000 -0.070310555
# Lag 500   -0.02439045  0.02652285  0.021887445
# Lag 2500   0.01979098  0.02532708 -0.066136140
# Lag 5000   0.04207829  0.02422373 -0.035820631
# Lag 25000 -0.02956055 -0.01851006  0.009233819
#
# , , units
#
# animalID       animal       units
# Lag 0     -0.05402605 -0.0703105552  1.000000000
# Lag 500   -0.02520283 -0.0054964875 -0.057750908
# Lag 2500   0.07638928 -0.0530419035 -0.017470075
# Lag 5000  -0.05080830  0.0006911657 -0.008864572
# Lag 25000  0.03566833 -0.0024150502  0.025614915

par(family = "sans",mfrow = c(1,1))#this code specifies number of rows and columns to display the graphs in

#CHECK PLOT OF RESIDUALS
plot(TLm4)

save(TLm4, file = "TLm4.rda")
load("TLm4.rda")
summary(TLm4)
# post.mean l-95% CI u-95% CI eff.samp  pMCMC
# (Intercept)             48.2185  40.3949  56.1963   1000.0 <0.001 ***
#   z.Tukey.Time             1.6273   0.3635   2.6172   1000.0 <0.001 ***
#   z.Tukey.AlopeciaScoreHC   -2.1009  -4.3472   0.3488   1000.0  0.086 .
# ReproStatImplanted       -8.1814 -19.3522   1.7114   1000.0  0.122
```

```
# ReproStatMaleBreeding    -3.2791 -10.5724   4.5906   1000.0  0.402
# ReproStatNurse          -4.7310 -11.8411   1.5501    910.3  0.190
# ReproStatPregnant       -7.8485 -13.7859  -0.1287    883.2  0.014 *
#   ReproStatWeanerGroup      2.5474  -8.9266  13.2105   1000.0  0.646
# OPRM1CC                 2.4599  -4.0103   8.3138   1000.0  0.424
# OPRM1GG                 17.1564   4.7007  30.2496   1000.0  0.014 *
#   ---
################################################################
####################AGG#########################################
################################################################

a$STIN<-relevel(a$STIN, ref="SS")
table(a$STIN)
# SS  LL  SL
# 99 365 170

a$OPRM1<-as.factor(a$OPRM1)
a$OPRM1<-relevel(a$OPRM1, ref="CC")
table(a$OPRM1)
# CC  CG  GG
# 528  82  24

a$MAOA<-as.factor(a$MAOA)
a$MAOA<-relevel(a$MAOA, ref="55")
table(a$MAOA)
# 55  66  57  56  67  77
# 47 136 194  67 132  58

a$HTR2A<-as.factor(a$HTR2A)
a$HTR2A<-relevel(a$HTR2A, ref="3.3")
table(a$HTR2A)
# 3.3 3.4 1.1 1.2 1.4 2.2 2.4
# 8  12 148 263  52 101  50

a$DRD4Haplotype<-as.factor(a$DRD4Haplotype)
a$DRD4Haplotype<-relevel(a$DRD4Haplotype, ref="2.4")
table(a$DRD4Haplotype)
# 2.4 3.4 1.1 1.2 1.3 1.4 2.2 2.3
# 16   8 124 270  19   8 155  34

a$AVPR<-as.factor(a$AVPR)
a$AVPR<-relevel(a$AVPR, ref="BC")
table(a$AVPR)
# BC  AB  AC  BB
# 97 153   8 376

a$OXTRHaplotype<-as.factor(a$OXTRHaplotype)
a$OXTRHaplotype<-relevel(a$OXTRHaplotype, ref="1.3")
table(a$OXTRHaplotype)
# 1.3 1.2 1.1 2.2 2.3
# 46 254  49 202  83

AGGAll<-(MCMCglmm(sqrt.Agg~
              Treatment*WeanEarlyR + z.Tukey.AlopeciaScoreHC + ReproStat + z.Tukey.GroupSizeAdults +
              HTTLPR + TPH2 + STIN + as.factor(HTR2A) + as.factor(MAOA) + OPRM1 + AVPR + as.factor(DRD4Haplotype) +
as.factor(OXTRHaplotype),
            random=~animalID+animal, ped=p, data=a, nitt=501000,burnin=1000,thin=500, verbose = FALSE,
prior=prior2))#>1hr to run

autocorr(AGGAll$VCV)
# , , animalID
#
# animalID     animal      units
# Lag 0     1.000000000 -0.07345506 -0.05019820
# Lag 500   0.057900116  0.02964016  0.03296290
```

```
# Lag 2500  -0.003368678  0.03472893 -0.00502610
# Lag 5000   0.007022996  0.01399256 -0.06795569
# Lag 25000 -0.002116662  0.04039774  0.07738793
#
# , , animal
#
# animalID      animal       units
# Lag 0     -0.073455059  1.000000000 -0.016183865
# Lag 500   -0.019982748  0.006302643  0.029233173
# Lag 2500   0.040700282  0.032912563  0.046879103
# Lag 5000  -0.010336036  0.004023706 -0.009934304
# Lag 25000  0.003232883 -0.035516694 -0.010843487
#
# , , units
#
# animalID      animal       units
# Lag 0     -0.050198196 -0.01618387  1.000000000
# Lag 500    0.030257548 -0.01297646  0.016223963
# Lag 2500   0.022908096  0.02130415  0.008798829
# Lag 5000  -0.009407428  0.06949287 -0.089794041
# Lag 25000  0.036831213 -0.04543708 -0.025653679

par(family = "sans",mfrow = c(1,1))#this code specifies number of rows and columns to display the graphs in

#CHECK PLOT OF RESIDUALS
plot(AGGAll)

save(AGGAll, file = "AGGAll.rda")
load("AGGAll.rda")
summary(AGGAll)
# post.mean  l-95% CI  u-95% CI eff.samp pMCMC
# (Intercept)                -15.00827 -64.69511  27.95727    819.9 0.550
# TreatmentStress              0.38699  -1.60780   2.52827   1000.0 0.724
# WeanEarlyRYes               -3.80917 -11.56536   4.15313   1000.0 0.338
# z.Tokey.AlopeciaScoreHC      -1.85652  -4.86556   1.53315   1000.0 0.216
# ReproStatImplanted           -5.57868 -20.19942   9.63717   1000.0 0.454
# ReproStatMaleBreeding        -0.76395 -12.11151  11.31341    894.5 0.868
# ReproStatNurse               -3.66170 -10.73249   4.17257   1000.0 0.342
# ReproStatPregnant            -4.80014 -12.68105   3.43992   1000.0 0.246
# ReproStatWeanerGroup          3.63508  -9.36467  18.52901   1000.0 0.608
# z.Tukey.GroupSizeAdults      -2.87983  -5.81082  -0.04084   1000.0 0.048 *
# HTTLPRSL                      3.31537  -2.86252   8.58575   1000.0 0.260
# HTTLPRSS                      3.66644  -5.85037  12.87242    869.1 0.446
# TPH2SL                       10.21817  -9.36452  32.45190   1000.0 0.316
# TPH2SS                       11.63226  -8.13547  29.31226   1043.5 0.224
# STINLL                       1.03638  -7.48817   8.18069   1000.0 0.824
# STINSL                       2.66011  -4.49742  10.58010   1000.0 0.504
# as.factor(HTR2A)3.4          8.17490 -15.24458  32.14518    859.9 0.512
# as.factor(HTR2A)1.1          6.87702 -11.57792  28.08446    904.5 0.482
# as.factor(HTR2A)1.2          7.44682 -12.34838  25.43136   1000.0 0.476
# as.factor(HTR2A)1.4         12.85809  -8.63771  37.05551   1000.0 0.274
# as.factor(HTR2A)2.2          6.40456 -13.58969  25.06826   1000.0 0.532
# as.factor(HTR2A)2.4          9.75584 -11.29996  29.91951    838.2 0.362
# as.factor(MAOA)66            4.29549  -6.26984  15.33045   1091.3 0.452
# as.factor(MAOA)57            5.52639  -7.34372  19.01928   1000.5 0.402
# as.factor(MAOA)56            3.16832 -12.27634  18.44694   1000.0 0.652
# as.factor(MAOA)67            4.66213  -8.43967  15.65123   1000.0 0.440
# as.factor(MAOA)77            2.27515 -11.86377  16.33404   1177.1 0.766
# OPRM1CG                      1.48441  -6.40762  11.56735   1000.0 0.742
# OPRM1GG                      5.14344  -8.88321  20.54359   1000.0 0.452
# AVPRAB                       1.66167  -6.00809   9.84733   1000.0 0.696
# AVPRAC                       3.08602 -13.16421  18.06677   1000.0 0.686
# AVPRBB                       5.31684  -1.58424  12.17494   1000.0 0.120
# as.factor(DRD4Haplotype)3.4    11.10975 -31.13445  51.16197   1000.0 0.576
# as.factor(DRD4Haplotype)1.1     3.99846 -16.74777  26.10875   1000.0 0.748
```

```
# as.factor(DRD4Haplotype)1.2    6.92293 -15.52424  27.61446   1000.0 0.542
# as.factor(DRD4Haplotype)1.3    9.90760 -13.31909  38.41948   1000.0 0.466
# as.factor(DRD4Haplotype)1.4    9.86717 -25.71028  44.51751    851.4 0.544
# as.factor(DRD4Haplotype)2.2    9.03268 -11.31818  33.46984    910.5 0.432
# as.factor(DRD4Haplotype)2.3    9.32717 -12.11511  28.78155   1000.0 0.382
# as.factor(OXTRHaplotype)1.2    1.30175  -9.62174  11.50972   1000.0 0.802
# as.factor(OXTRHaplotype)1.1    2.45355 -12.85394  18.65325    994.5 0.762
# as.factor(OXTRHaplotype)2.2    6.67764  -4.38491  19.69584   1000.0 0.270
# as.factor(OXTRHaplotype)2.3    4.64469 -10.76046  19.33896   1000.0 0.548
# TreatmentStress:WeanEarlyRYes  -4.26383 -10.36409   1.47222    785.7 0.164

#####serotonin#####
a$STIN<-relevel(a$STIN, ref="SS")
table(a$STIN)
# SS  LL  SL
# 99 365 170


a$MAOA<-as.factor(a$MAOA)
a$MAOA<-relevel(a$MAOA, ref="55")
table(a$MAOA)
# 55  66  57  56  67  77
# 47 136 194  67 132  58

a$HTR2A<-as.factor(a$HTR2A)
a$HTR2A<-relevel(a$HTR2A, ref="3.3")
table(a$HTR2A)
# 3.3 3.4 1.1 1.2 1.4 2.2 2.4
# 8  12 148 263  52 101  50

a$TPH2<-relevel(a$TPH2, ref="LL")
table(a$TPH2)
# LL  SS  SL
# 17 487 130

a$HTTLPR<-relevel(a$HTTLPR, ref="LL")
table(a$HTTLPR)
# LL  SL  SS
# 306 275  53

AGGm<-(MCMCglmm(sqrt.Agg~
        Treatment*WeanEarlyR + z.Tukey.AlopeciaScoreHC + ReproStat + z.Tukey.GroupSizeAdults +
        HTTLPR + TPH2 + STIN + as.factor(HTR2A) + as.factor(MAOA), #+ OPRM1 + AVPR + DRD4Haplotype+
OXTRHaplotype,
        random=~animalID+animal, ped=p, data=a, nitt=501000,burnin=1000,thin=500, verbose = FALSE,
prior=prior2))#>1hr to run

autocorr(AGGm$VCV) #all ok  <0.1 @10000 lag

# , , animalID
#
# animalID      animal      units
# Lag 0      1.0000000000 -0.139882739 -0.04876208
# Lag 500    0.0015230779 -0.019060237 -0.01685576
# Lag 2500   0.0378061385  0.069495724 -0.03285428
# Lag 5000  -0.0028174088 -0.007834903  0.00938128
# Lag 25000  0.0009200038 -0.026778421  0.02730440
#
# , , animal
#
# animalID     animal      units
# Lag 0     -0.13988274  1.00000000 -0.03587847
# Lag 500    0.04791137 -0.03440284  0.04445457
# Lag 2500   0.03650327 -0.01717243  0.05061267
# Lag 5000  -0.01396188 -0.02911554 -0.02498418
```

```
# Lag 25000 -0.04749544 -0.02254195 -0.02943058
#
# , , units
#
# animalID       animal       units
# Lag 0     -0.048762084 -3.587847e-02  1.00000000
# Lag 500    0.026891927  5.147936e-02  0.03950404
# Lag 2500  -0.034382555 -1.662024e-02  0.03410917
# Lag 5000  -0.004004486 -1.315253e-02 -0.03968377
#Lag 25000 -0.008066787  7.977129e-05  0.01187911

par(family = "sans",mfrow = c(1,1))#this code specifies number of rows and columns to display the graphs in

#CHECK PLOT OF RESIDUALS
plot(AGGm)

save(AGGm, file = "AGGm.rda")
load("AGGm.rda")
summary(AGGm)#to check sample size

# post.mean  l-95% CI  u-95% CI eff.samp pMCMC
# (Intercept)                  9.51631 -11.20701  27.47200   1000.0 0.378
# TreatmentStress              0.16609  -2.16280   2.11181   1000.0 0.866
# WeanEarlyRYes               -0.11988  -5.70086   5.40932   1000.0 0.968
# z.Tukey.AlopeciaScoreHC      -2.20085  -4.38366  -0.08985   1000.0 0.046 *
#   ReproStatImplanted          -6.68060 -14.73467   1.97078   1460.8 0.138
# ReproStatMaleBreeding         1.60166  -6.59384   7.97142   1000.0 0.632
# ReproStatNurse               -1.38031  -7.24638   4.47909    889.2 0.638
# ReproStatPregnant            -2.32139  -9.00803   3.50377    907.7 0.472
# ReproStatWeanerGroup          8.78652  -0.29747  18.19893   1000.0 0.060 .
# z.Tukey.GroupSizeAdults      -2.15936  -4.28687  -0.29496   1000.0 0.022 *
# HTTLPRSL                      2.51250  -1.38667   6.38449   1000.0 0.202
# HTTLPRSS                      3.98754  -1.78805  11.00797   1127.7 0.210
# TPH2SS                        3.72753  -7.99416  15.65584    898.0 0.536
# TPH2SL                        2.59870  -9.03934  15.17993    906.5 0.688
# STINLL                       1.77465  -4.07441   7.12902   1000.0 0.514
# STINSL                       1.27057  -5.32642   7.30154   1000.0 0.718
# as.factor(HTR2A)3.4           3.36525 -16.23162  22.36115   1127.3 0.748
# as.factor(HTR2A)1.1           2.01125 -14.10632  16.58504   1000.0 0.800
# as.factor(HTR2A)1.2           0.30863 -15.43437  14.35845   1000.0 0.964
# as.factor(HTR2A)1.4           5.20106 -11.80975  21.77238   1000.0 0.582
# as.factor(HTR2A)2.2           1.45786 -14.74513  17.20573   1000.0 0.868
# as.factor(HTR2A)2.4           3.50440 -13.02423  18.69045   1215.7 0.686
# as.factor(MAOA)66             6.20421  -2.57919  14.08974   1000.0 0.152
# as.factor(MAOA)57             5.29929  -3.35973  13.35959   1000.0 0.216
# as.factor(MAOA)56             5.65969  -2.90760  14.24927   2163.5 0.222
# as.factor(MAOA)67             6.02959  -2.47511  13.59046   1000.0 0.146
# as.factor(MAOA)77             5.65711  -4.85585  16.23023   1000.0 0.290
# TreatmentStress:WeanEarlyRYes  -4.46527 -10.08102   1.47493   1000.0 0.138
#####dopamine#####

a$MAOA<-as.factor(a$MAOA)
a$MAOA<-relevel(a$MAOA, ref="57")
table(a$MAOA)
# 57  55  66  56  67  77
# 194  47 136  67 132  58

a$DRD4Haplotype<-as.factor(a$DRD4Haplotype)
a$DRD4Haplotype<-relevel(a$DRD4Haplotype, ref="1.1")
table(a$DRD4Haplotype)
# 1.1 2.4 3.4 1.2 1.3 1.4 2.2 2.3
# 124  16   8 270  19   8 155  34

AGGm2<-(MCMCglmm(sqrt.Agg~
          Treatment*WeanEarlyR + z.Tukey.AlopeciaScoreHC + ReproStat + z.Tukey.GroupSizeAdults +
```

```
            as.factor(MAOA) + as.factor(DRD4Haplotype), #+ OPRM1 + AVPR  + OXTRHaplotype + HTTLPR + TPH2 + STIN + HTR2A,
          random=~animalID+animal, ped=p, data=a, nitt=501000,burnin=1000,thin=500, verbose = FALSE, prior=prior2))#>1hr to run

autocorr(AGGm2$VCV) #all ok  <0.1 @10000 lag

# , , animalID
#
# animalID     animal      units
# Lag 0     1.000000000 -0.06867824 0.03111217
# Lag 500   0.020743780  0.03170300 0.01801477
# Lag 2500  0.006176830  0.08920531 0.02266120
# Lag 5000  0.003178717  0.01936604 0.05307967
# Lag 25000 0.001394553  0.01629819 0.06114001
#
# , , animal
#
# animalID       animal       units
# Lag 0     -0.068678239  1.0000000000 -0.044219228
# Lag 500   -0.002035296  0.0238742393 -0.008278796
# Lag 2500   0.015526884  0.0667777985 -0.003317819
# Lag 5000   0.015991748  0.0008612937 -0.014729446
# Lag 25000 -0.015998164 -0.0085904665 -0.047236293
#
# , , units
#
# animalID      animal      units
# Lag 0      0.031112167 -0.044219228 1.00000000
# Lag 500   -0.060660536 -0.001494397 0.01234460
# Lag 2500  -0.070943213  0.008684816 0.01908931
# Lag 5000   0.008010469 -0.044537567 0.02765820
# Lag 25000 -0.010670528  0.017477636 0.02129545

par(family = "sans",mfrow = c(2,2))#this code specifies number of rows and columns to display the graphs in

#CHECK PLOT OF RESIDUALS
plot(AGGm2)
save(AGGm2, file = "AGGm2.rda")
load("AGGm2.rda")
summary(AGGm2)#to check sample size
# post.mean  l-95% CI  u-95% CI eff.samp  pMCMC
# (Intercept)                 21.11141  15.30822  27.76370   1000.0 <0.001 ***
#   TreatmentStress             0.35802  -1.70176   2.42510   1000.0  0.750
# WeanEarlyRYes               1.07792  -3.97275   6.20103   1000.0  0.686
# z.Tukey.AlopeciaScoreHC       -1.74419  -3.44168  -0.01828   1000.0  0.044 *
#   ReproStatImplanted          -4.15138 -13.14360   4.28432   1000.0  0.338
# ReproStatMaleBreeding         -3.33565 -10.97718   3.92068   1000.0  0.404
# ReproStatNurse              -2.98722  -7.99117   1.56578   1337.3  0.244
# ReproStatPregnant           -4.06906  -9.22328   1.36213   1000.0  0.112
# ReproStatWeanerGroup          5.59314  -2.89795  13.26342   1000.0  0.182
# z.Tukey.GroupSizeAdults       -1.14022  -2.99219   0.64777   1000.0  0.218
# as.factor(MAOA)55             0.25159  -8.30240   8.39890   1000.0  0.970
# as.factor(MAOA)66             0.79887  -4.81082   6.02655   1000.0  0.764
# as.factor(MAOA)56             3.28706  -2.70644   9.49214   1000.0  0.282
# as.factor(MAOA)67             1.48883  -2.92810   5.78331   1000.0  0.490
# as.factor(MAOA)77             0.41665  -6.05361   7.52950   1000.0  0.882
# as.factor(DRD4Haplotype)2.4   10.12648  -0.97055  23.02054   1000.0  0.112
# as.factor(DRD4Haplotype)3.4    0.35481 -14.50714  14.43834    853.5  0.976
# as.factor(DRD4Haplotype)1.2    3.07751  -1.32497   7.03304   1000.0  0.138
# as.factor(DRD4Haplotype)1.3    1.01830  -8.27724  10.39201   1224.0  0.850
# as.factor(DRD4Haplotype)1.4    2.51516  -9.09005  16.01284   1000.0  0.692
# as.factor(DRD4Haplotype)2.2    2.12320  -3.37329   6.62454   1000.0  0.400
# as.factor(DRD4Haplotype)2.3    8.37054  -0.42990  19.86360   1000.0  0.100
# TreatmentStress:WeanEarlyRYes  -4.50382 -10.23490   0.82662   1000.0  0.122
```

```
####opioid####
a$OPRM1<-as.factor(a$OPRM1)
a$OPRM1<-relevel(a$OPRM1, ref="CG")
table(a$OPRM1)
# CG  CC  GG
# 82 528  24

AGGm3<-(MCMCglmm(sqrt.Agg~
              Treatment*WeanEarlyR + z.Tukey.AlopeciaScoreHC + ReproStat + z.Tukey.GroupSizeAdults +
             OPRM1, #+ AVPR  + OXTRHaplotype + HTTLPR + TPH2 + STIN + HTR2A + MAOA + DRD4Haplotype,
            random=~animalID+animal, ped=p, data=a, nitt=501000,burnin=1000,thin=500, verbose = FALSE,
prior=prior2))#>1hr to run

autocorr(AGGm3$VCV) #all ok  <0.1 @10000 lag
# , , animalID
#
# animalID      animal       units
# Lag 0     1.000000000 -0.099997320 -0.009337553
# Lag 500    0.018964091 -0.040292748 -0.019569706
# Lag 2500  -0.021630450 -0.009140804  0.012680452
# Lag 5000   0.007225526  0.017659083 -0.035330721
# Lag 25000  0.006999709 -0.020294937  0.032754218
#
# , , animal
#
# animalID      animal       units
# Lag 0     -0.099997320  1.000000000  0.051538631
# Lag 500   -0.011634752  0.007817308  0.002397884
# Lag 2500   0.009491958 -0.005732205  0.019888076
# Lag 5000   0.001551055 -0.065866476  0.024537272
# Lag 25000 -0.032759108 -0.029836949 -0.026944333
#
# , , units
#
# animalID      animal      units
# Lag 0     -0.009337553  0.051538631  1.00000000
# Lag 500    0.023997660 -0.032704143  0.01296015
# Lag 2500  -0.014519648 -0.023492847 -0.02918435
# Lag 5000   0.011106223 -0.017505141  0.04898609
# Lag 25000 -0.049594308  0.001498468 -0.01010907

par(family = "sans",mfrow = c(2,2))#this code specifies number of rows and columns to display the graphs in

#CHECK PLOT OF RESIDUALS
plot(AGGm3)
save(AGGm3, file = "AGGm3.rda")
load("AGGm3.rda")
summary(AGGm3)

# post.mean  l-95% CI  u-95% CI eff.samp  pMCMC
# (Intercept)                 22.55667  17.48702  28.03125   1000.0 <0.001 ***
# TreatmentStress              0.23244  -1.72770   2.20446   1126.1  0.832
# WeanEarlyRYes                0.67011  -3.16614   5.19995   1000.0  0.768
# z.Tukey.AlopeciaScoreHC     -1.50543  -3.13675   0.02617    763.2  0.060 .
# ReproStatImplanted          -5.08034 -11.34606   1.61463   1000.0  0.120
# ReproStatMaleBreeding       -1.66294  -6.38411   3.93739    917.5  0.492
# ReproStatNurse              -2.79277  -7.56577   1.71577   1000.0  0.212
# ReproStatPregnant           -3.70739  -8.32512   0.71809   1000.0  0.116
# ReproStatWeanerGroup         3.54742  -4.14398  10.72418   1000.0  0.360
# z.Tukey.GroupSizeAdults     -1.68095  -3.14171  -0.22021   1000.0  0.032 *
# OPRM1CC                      1.85113  -2.03719   6.52250   1000.0  0.406
# OPRM1GG                      6.24906  -1.85474  14.73456   1000.0  0.158
# TreatmentStress:WeanEarlyRYes  -4.62168 -10.21980   1.02634    747.3  0.110
```

```
#####Oxytocin######
a$AVPR<-as.factor(a$AVPR)
a$AVPR<-relevel(a$AVPR, ref="BC")
table(a$AVPR)
# BC  AB  AC  BB
# 97 153   8 376

a$OXTRHaplotype<-as.factor(a$OXTRHaplotype)
a$OXTRHaplotype<-relevel(a$OXTRHaplotype, ref="1.3")
table(a$OXTRHaplotype)
# 1.3 1.2 1.1 2.2 2.3
# 46 254  49 202  83

AGGm4<-(MCMCglmm(sqrt.Agg~
              Treatment*WeanEarlyR + z.Tukey.AlopeciaScoreHC + ReproStat + z.Tukey.GroupSizeAdults +
             AVPR  + as.factor(OXTRHaplotype), #OPRM1 +HTTLPR + TPH2 + STIN + HTR2A + MAOA + DRD4Haplotype,
            random=~animalID+animal, ped=p, data=a, nitt=501000,burnin=1000,thin=500, verbose = FALSE,
prior=prior2))#>1hr to run

autocorr(AGGm4$VCV) #all ok  <0.1 @10000 lag
# , , animalID
#
# animalID      animal       units
# Lag 0     1.0000000000  0.01458854 -0.014472594
# Lag 500   0.0140736347 -0.01465998  0.015331295
# Lag 2500  0.0007640829 -0.06561341 -0.004860605
# Lag 5000  0.0052392466 -0.08180937  0.009536288
# Lag 25000 0.0407078814  0.01992752 -0.007549483
#
# , , animal
#
# animalID      animal      units
# Lag 0      0.014588535  1.000000000 -0.01489377
# Lag 500    0.045449373 -0.015837251  0.04599929
# Lag 2500  -0.051977258  0.068396402  0.05506224
# Lag 5000   0.002817825 -0.009308885  0.01611046
# Lag 25000  0.012642698  0.059834014 -0.01029794
#
# , , units
#
# animalID      animal       units
# Lag 0     -1.447259e-02 -0.014893770  1.000000000
# Lag 500   -4.227897e-07  0.004899351  0.018068568
# Lag 2500  -4.708669e-02 -0.039436533  0.002687095
# Lag 5000  -5.018010e-02 -0.039698412  0.023833055
# Lag 25000  4.935677e-02 -0.031841632 -0.015871351

par(family = "sans",mfrow = c(2,2))#this code specifies number of rows and columns to display the graphs in

#CHECK PLOT OF RESIDUALS
plot(AGGm4)

save(AGGm4, file = "AGGm4.rda")
load("AGGm4.rda")
summary(AGGm4)

# post.mean  l-95% CI  u-95% CI eff.samp  pMCMC
# (Intercept)                 20.71467  15.12389  27.28390   1177.0 <0.001 ***
#   TreatmentStress              0.32794  -1.91199   2.40244   1000.0  0.754
# WeanEarlyRYes                0.38647  -4.09844   4.88646    903.1  0.856
# z.Tukey.AlopeciaScoreHC       -1.27996  -2.96406   0.26984   1000.0  0.130
# ReproStatImplanted           -6.05607 -12.96831   1.90575   1000.0  0.106
# ReproStatMaleBreeding         -2.58229  -7.85962   2.88540   1000.0  0.366
# ReproStatNurse               -4.46545  -9.53141   1.24665   1000.0  0.112
# ReproStatPregnant            -5.14217 -10.49789   0.02508   1000.0  0.062 .
```

```
# ReproStatWeanerGroup           4.26765  -4.14727  11.34676   1000.0  0.282
# z.Tukey.GroupSizeAdults       -2.05630  -3.83879  -0.41996   1000.0  0.022 *
# AVPRAB                         1.15616  -3.85844   6.48050   1000.0  0.650
# AVPRAC                         2.56348  -8.49272  14.64230   1000.0  0.674
# AVPRBB                         2.70872  -1.65566   6.31410   1000.0  0.234
# as.factor(OXTRHaplotype)1.2    2.20917  -3.78213   9.04510   1000.0  0.500
# as.factor(OXTRHaplotype)1.1    3.93756  -3.64767  12.43861   1132.3  0.328
# as.factor(OXTRHaplotype)2.2    3.83109  -2.86702  11.52608   1000.0  0.322
# as.factor(OXTRHaplotype)2.3    3.84914  -4.33328  11.16512   1000.0  0.320
# TreatmentStress:WeanEarlyRYes -4.59949 -10.06856   1.05285   1000.0  0.098
```