Testing the emotional value of facial stimuli using attention bias in Rhesus Macaques, *Macaca mulatta*.

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A thesis submitted in partial fulfilment of the requirements of Liverpool John Moores University for the degree of Master of Philosophy

November, 2015

Abstract

Approximately 2000 rhesus macaques, *Macaca mulatta*, are used annually for biomedical research in the UK, the psychological wellbeing and welfare of these primates is a primary concern. Attention bias is a measure of cognitive bias used to assess whether an individual is stressed. Current methods using attention bias to evaluate the welfare of macaques, use images of conspecifics expressing an aggressive face vs. a neutral face with either opened eyes or closed eyes. Nevertheless, it is commonly acknowledged that direct staring, eyes open, is seen as a threat to macaques. The exact emotional value of these conspecific ‘neutral’ facial stimuli, however, is not known.

In order to refine attention bias testing for its use in primate cognitive research, understanding the exact value of facial stimuli used is important. This study uses attention bias to explore whether ‘eyes open’ vs. ‘eyes closed’ has the same relative threat value, or whether ‘eyes open’ could actually be perceived as aggressive. Three different facial expressions (neutral eyes closed, neutral eyes open and aggressive) of unfamiliar monkeys were presented as paired stimuli to measure attention bias to facial stimuli. Twenty-eight individuals were presented with counterbalanced presentations of the three facial stimuli in order to identify the emotional value of stimuli. Image pairs were presented simultaneously for ~3s. Video footage was blind-coded, frame by frame, for gaze towards stimuli. Behavioural reactions in-trial were coded to highlight any variation in key behavioural reactions. Five minute instantaneous scan samples were conducted post-trial to assess variation in time budget.

Results showed that there was a significantly greater attention bias towards the three facial combinations; the greatest bias was toward the eyes open vs. aggressive facial combination. Other factors including monkey ID, reproductive status, trial, stimulus monkey, previous exposure, age (in months), and matriline were considered within the results. Mixed models were used to also show that age had a significant effect on monkeys' attention bias and previous exposure to stimuli significantly reduced attention bias. In-trial behavioural reactions showed a significantly greater representation of extreme reactions to the eyes open vs. aggressive facial combination compared to all other facial combinations. Post-trial behaviours showed a significantly greater representation of fear/avoid behaviours after the eyes open vs. aggressive compared to the eyes closed vs. aggressive facial combination.

Acknowledgements

Thanks go to Liverpool John Moores University for allowing me to partake on this MPhil and the supportive admin team and supplementary programmes run at the university to help me through this process. I also thank Liverpool John Moores University for the kind bursary given to previous Liverpool John Moores Students who continue into post-graduate study, this contribution helped greatly. I would also like to thank Yorkshire Ladies' Council of Education for their kind supportive grant towards my tuition fees.

Gratitude is given to the entire team at the Medical Research Centre, Centre for Macaques. Particularly to Dr David Farningham and Dr Claire Witham for allowing me to conduct my research at the facility. Acknowledgement is made to the care staff for their vast support, being very generous and understanding the needs of my testing timetable and also helping in training monkeys. Furthermore, thank you to the team at Medical Research Centre, Centre for Macaques for giving me the opportunity to partake in a summer internship which allowed me to train more monkeys for use in the current study.

Praise is given in abundance to all of the monkeys at the Medical Research Centre, Centre for Macaques. You made sure every day was never ordinary, be it through stress, laughter or faeces! I hope you are still entertaining all the staff at the Medical Research Centre, Centre for Macaques and aren't missing the supply of peanuts and raisins too much.

A note of thanks and appreciation is made to my loving family and fiancé. If it were not for the financial support received from them this project would have never been viable. I also thank them and my friends for their support during this period, helping me through the stress and listening to my many monkey stories.

The people I have to thank the most are my supervisory team Dr Emily Bethell and Dr Claudia Mettke-Hofmann. I am grateful for all your support throughout this project.

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Chapter 1

Introduction

Introduction

Approximately 2000 rhesus macaques, *Macaca mulatta*, are used annually for biomedical research in the UK (Home Office, 2010; Prescott, 2010). Procedures used during this biomedical research in most cases is performed without anaesthesia, causing psychological distress (Home Office, 2010). Due to their similar neurology to humans, the psychological wellbeing of these primates in captivity is of primary concern for animal welfare (Animal Procedures Committee, 2002; NC3Rs, 2006; Bateson, 2011).

A current method being developed to assess primate welfare and an animal's psychological state is attention bias (Bethell *et al*., 2012a). Attention bias is the tendency to attend to one type of information over another (MacLeod *et al*., 1986). Attention bias can be used to measure gaze towards opposing information to assess whether an individual is stressed. This technique has been constructed as a novel, non-invasive method to measure stress in macaques (Bethell *et al*., 2012a; C.Kemp pers.comm.). In humans attention bias methods predict vulnerability to stress, but may also be used to identify which stimuli individuals find stressful (MacLeod *et al*., 1986). Current methods for macaques use images of conspecifics expressing an aggressive face vs. a neutral face with either opened eyes or closed eyes (Bethell *et al*., 2012a; C.Kemp pers.comm.). However, the exact emotional value of these conspecific facial stimuli is not known. Therefore, in order to refine attention bias testing for its use in primate cognitive research, understanding the exact emotional value of facial stimuli used is fundamental.

1.1 Cognitive Bias

It is commonly accepted that a person's emotional state is strongly influenced by their allocation of social attention (humans: Bar-Haim *et al*., 2007; Mogg *et al*., 2007 and non-human primates: Bethell *et al*., 2012a; Bethell *et al*., 2012b). This social attention plays an integral role in shaping an individual's social interactions (Mansell *et al*., 1999; Derakshan *et al*., 2007; Yiend, 2010). Recent developments in primate cognitive studies suggest that primates also have this preferential tendency (Bethell *et al*., 2012a; Bethell *et al*., 2012b).

1.1.1 Human research

Cognitive bias, including attention bias, has become well established within human psychological research (MacLeod *et al*., 1986; Mathews, 1990; Beard *et al*., 2012). The cognitive element of emotion, and research regarding this, is key to understanding human psychological wellbeing and underlying rational (MacLeod *et al*., 1986; Mathews, 1990; Mogg and Bradley, 1998). Humans are inclined to process information within their daily life differently dependent upon their emotional state, current mood or personality traits (Gomez and Gomez, 2002). For example cognitive studies within humans have shown that disorders such as anxiety, depression and phobias can be related to cognitive processing (Mathews *et al*., 1989; Eysenck, 1992; Mogg and Bradely, 1998; Bar-Haim *et al*., 2007; Shechner *et al*., 2012; Dawel *et al*., 2015; Pergamin-Aue *et al*., 2015). Bar-Haim *et al.* (2007) through a series of a meta-analytic studies looking at anxiety found that threat-related bias is a robust phenomenon and that anxious individuals are predisposed to be attracted more to negative information. One of the most important cognitive human studies by Williams *et al*. (1996) found that an existing cognitive bias is a strong predictor of vulnerability to the onset of future disorders, further study has shown this can therefore be used as a predictor of distress (Pury, 2002). Therefore welfare research can be used to identify individuals who have cognitive profiles that would suggest they are vulnerable to the development of affective disorders associated with poor psychological wellbeing (Mathews and MacLeod, 2002).

There are many different types of bias, and many different disorders that have a strong connection to these biases, demonstrating how well established the relationship between cognition and emotion is within human research. Humans with high anxiety levels show a bias to aggressive information (Bar-Haim *et al.,* 2007; Eldar *et al*., 2008; Mathews and MacLeod, 2002) and interpretive bias: interpreting ambiguous stimuli more negatively (Mathews *et al.,* 1989; Bar-Haim *et al.*, 2007). Further to this, those with anxiety are also likely to show expectancy bias: apprehending more negative events in the future (MacLeod and Byrne, 1996; Eysenck *et al*., 2006). Despite this vast evidence for threat-related attention bias in anxiety the underlying mechanisms supporting these biases remain largely unclear (Ouimet *et al.*, 2009; Cisler and Koster, 2010; Heeren *et al*., 2013). Depressed humans exhibit memory bias: recalling negative memories (Bradley and Lang, 2000; Eysenck *et al*., 2006; Disner *et al*., 2011; Everaert *et al*., 2012; Eysenck and Mogg, 2014). Phobics also show likewise biases towards threat for phobia-related information (Heinrichs and Hofmann, 2001; Öhman and Mineka, 2001; Bar-Haim *et al*., 2007; Rapee *et al*., 2013; De Voogd *et al*., 2014; Haberkamp and Schmidt, 2015).

1.1.2 Animal research

Harding *et al.* (2004; see also Harding, 2002) developed the use of cognitive bias testing in animals as a measure of psychological wellbeing, combining human cognitive theory and methods with operant conditioning techniques commonly used within animal research. Harding *et al*.'s (2004) work has allowed for the development of cognitive bias research on a variety of non-human animals; rats (Brydges *et al*., 2011; Anderson *et al*., 2013; Papciak *et al*., 2013; Kregiel *et al*., 2015), sheep (Doyle *et al*., 2010; Doyle *et al*., 2011; Sanger *et al*., 2011; Guldimann *et al*., 2015; Vögeli *et al*., 2015), cows (Daros *et al*., 2014), birds (Bateson and Matheson, 2007; Hernandez *et al*., 2015), hamsters (Bethell and Koyama, 2015), pigs (Murphy *et al*., 2015), dogs (Burman *et al*., 2011; Müller *et al*., 2012; Kis *et al*., 2015) and rhesus macaques (Mandalaywala *et al*., 2014; Dubuc *et al*., 2015)

King *et al*. (2010) studied the effects of testosterone on attention and memory for emotional stimuli, results showed that monkeys treated with testosterone had faster response times compared to those treated with oil irrespective of emotional value of stimuli. Although all monkeys in King *et al*.'s (2010) study were sensitive to the emotional value of stimuli, results showed that testosterone had no effect memory or attention. Lacreuse *et al*. (2013) further highlights this trend in macaques' reaction to emotional stimuli. Lacreuse *et al*. (2013) studied the variation in attention bias for social and non-social emotional stimuli in male humans and rhesus macaques, their results showed a shared mechanisms of social attention between both species, both displaying a pattern of attention for threatening faces of conspecifics.

The use of cognitive bias as a measure of psychological wellbeing has also developed in non-human primates. Bethell *et al*. (2012b) used a judgement bias task applying a touch screen and ambiguous stimuli findings supported previous attention bias work in that rhesus macaques were more accurate post health check compared to during an enrichment phase. This suggesting that in the post health check phase there was a greater expectation of a negative outcome compared to during enrichment. Further research by Bethell *et al*. (2012a) using an attention bias test found evidence that shifts in emotion state mediate social attention towards and away from facial cues of emotion in rhesus macaques. Bethell *et al*.'s (2012a) work was the first evidence of this form in non-human primates. Their work shows a novel insight into social behaviour of non-human primates and is a solid foundation for developing our understanding of animal psychological wellbeing.

1.1.3 Vigilant strategies

Studies have shown that anxious individuals react quicker to aggressive information than non-anxious individuals, both in human research (Bar-Haim, 2010; Cisler and Koster, 2010; Shechner *et al*., 2012; Dawel *et al*., 2015; Pergamin-Hight *et al*., 2015) and macaques (Bethell *et al*., 2012a). However, within human literature there are conflicting opinions with regards to attention bias and aggressive stimuli. Eysenck (1992) suggests that individuals have a quicker orientation towards aggressive over neutral information. Alternatively, Chen *et al*. (2002) suggests an avoidance strategy with regards to the aggressive stimuli, yet others propose a vigilant-avoidant strategy, by which after initial vigilance of aggressive information the subject is then avoidant (Garner *et al*., 2006). Bethell *et al*.'s (2012a) work with macaques found that during a period of stress macaques would exhibit a vigilant-avoidant strategy towards the aggressive information, however, during periods of enrichment macaques exhibited continuous attention towards the aggressive information.

1.2 Emotional stimuli

There is little understanding as to the significance of facial expressions used by macaques' and a clearer understanding is needed of the utility of these facial expressions (Hoffman *et al*., 2007). When considering facial expressions and emotional value receiver status is very important for macaques', expressions are not typically categorised as we would categorise human expressions along a positive-negative axis, but rather a dominance-submission axis (Altman, 1962). Therefore making interpretation of facial expressions, particularly with comparison to human literature, unattainable (Hoffman *et al*., 2007).

1.2.1 Stimuli use in attention bias

Work by Bethell *et al*. (2012a) used images of macaques to measure attention bias one showing an aggressive face (a macaque image with eyes open exposing their teeth in a typical threat pose) and one image of a neutral face (a macaque image with eyes open with a vacant expression), however this neutrality is presumed. The emotional content of this facial stimulus is ambiguous in meaning since a staring open-mouthed face is aggressive to macaques (Parr and Heintz, 2009). Current research at the Medical Research Centre, Centre for Macaques, (C.Kemp pers.comm.) is using the same images to measure how a stressful event affects attention bias, however in the neutral image used the eyes are closed. Although both results showed similar findings, the ambiguity of the emotional content of stimuli is questionable, eyes open and direct staring is seen as a threat to macaques as a signal of dominance (Chevalier-Skolnikoff, 1973; Van Hooff, 1976; Machado and Bachevalier, 2006). Original work by Bethell (2009) reflects upon this ambiguity stating that neutral facial stimuli were classed as such as they were less aggressive when presented next to the 'aggressive' facial stimuli. Nevertheless, as highlighted, this expression (direct stare) when shown from a dominant to a subordinate is aggressive to macaques (Chevalier-Skolnikoff, 1973; Van Hooff, 1976; Machado and Bachevalier, 2006).

1.2.2 Direct stare

To present knowledge, no paper has yet reviewed the emotional value of facial stimuli commonly used within macaque research. Facial expressions of macaques play an important role in portraying emotion (Partan, 2002). Macaques have a well-documented repertoire of facial expressions (Partan, 2002; Parr *et al*., 2010). Parr *et al*. (2010) discusses the muscular system of the rhesus macaque face and how this muscular based system displays facial expression. Parr *et al*.'s (2010) findings compare humans and related primate species using systematic, anatomically based techniques using muscle based facial movement to code facial expressions. Parr *et al*.'s (2010) work, commonly known as MAQFACs has led to similar muscular based facial expression analysis of other primate species (Chimpanzees, *Pan*: Vick *et al*., 2007; Gibbons, *Hylobatidae*: Waller *et al*., 2012; Orangutans, *Pongo*: Caeiro *et al*., 2013; Barbary macaques, *Macaca sylvanus,* Julle-Danière *et al*., 2015). However, Parr *et al*.'s (2010) work is based on comparisons and muscular movements and does not consider the emotions that are being portrayed by the facial expressions primates are forming. Most literature focuses only on what can be interpreted as negative (Partan, 2002). What is neutral is based on an opposing image of what we, as humans, would interpret as a typically neutral expression (Cohn and Kanade, 2007). Although human interpretation of neutrality is also highly influenced by sex and dominance (Hess *et al*., 2000). As emotional value of macaque facial expressions are based on a very different scale to humans, from dominant to submissive rather than positive to negative, it is hard to cross compare when studying the emotional content of stimuli and their applicability to studies.

It is not only within attention bias that the emotional value of facial expressions clarifying but also in general within macaque research. Dahl *et al*. (2009) looked at facial processing strategies in both humans and macaques, comparing 'neutral' faces of humans and macaques which were manipulated in a variety of ways (e.g. inverting and blurring images) their results showed similar processing of faces between humans and macaques. Nevertheless, again the 'neutral' face used was of a macaque that had its eyes open staring forward. Micheletta *et al*. (2015a) studied crested macaques (*Macaca nigra*) and looked how they matched facial expressions both using still images and videos, again classing 'neutral' as an open eyed macaque.

Hoffman *et al*. (2007) used monkey faces displaying aggressive, neutral and appeasing expressions with the head and eyes either averted or directed to assess differences in gaze-selective responses in the amygdala, however the neutral face that they used was also a monkey with eyes open. The idea of a macaque facial expression with eyes averted such as that used by Hoffman *et al*. (2007) could be suggested as further emotional facial expression to represent neutrality, although this was not feasible to test within the limits of my project. Nevertheless, the results of Hoffman *et al*. (2007) study suggests that eyes averted gaze was actually arousing, increasing attention, and therefore would suggest that it does not have a neutral emotional value. It is suggested that averted gaze is ambiguous to a monkey and therefore requires further attention for the monkey to evaluate the expression (Davis and Whalen, 2001). Ambiguous or uncertain stimuli direction has been found to increase attention (Whalen, 1998; Holland and Gallagher, 1999); monkeys are more likely to explore the direction of the averted gaze rather than the image (Butterworth, 1991; Langton *et* al., 2000; Deaner and Platt, 2003).

It is a common occurrence within macaque literature to presume neutrality of an open eyed facial stimulus in comparison to a more aggressive facials stimulus. However, the emotional content of these facial stimuli has never been examined. Although the development of attention bias as a welfare tool continues to grow (Bethell *et al.,* 2012a; Bethell *et al.,* 2012b; C.Kemp pers.comm.) the facial stimuli used so far have not been tested for their emotional value to macaques. Therefore in order to continue the development of attention bias as a welfare tool clarity of facial expressions and their emotional content is needed comparing relative perception of emotional content.

1.3 Welfare

1.3.1 Welfare standards

Primates are commonly used for biomedical research in the UK, with approximately 2000 macaques being used annually (European Commission, 2009; Home Office, 2010; Prescott, 2010). A large majority of this research coincides with a risk of psychological distress due to working with conscious animals with no anaesthesia (Novak and Suomi, 1988; Home Office, 2010). Due to their socio-cognitive needs (Brent *et al*., 2011, 2013), this psychological distress caused to non-human primates is a concern for animal welfare (Animal Procedures Committee, 2002; NC3Rs, 2006; Weatherall *et al*., 2006; Nelson and Winslow, 2009; Prescott, 2010; Bateson, 2011). Animals experience subjective emotions; therefore research into the distress suffered and how to accurately measure this is key to animal welfare (Dawkins, 1990 and 2006; Mendl, 2001; Harding *et al.,* 2004).

The UK Home Office guidelines state that 'All animals should be observed daily for signs of illness or injury and observed for psychological well-being by an experienced animal care person…' (Home Office 2011, p4). In practice, however, affordable methods for monitoring psychological wellbeing are limited to noting the absence of 'negative' behaviours, and the presence of species typical behaviours, which can be time consuming (Buchanan-Smith, 2010; Prescott *et al*., 2010).

1.3.2 Current measures of anxiety: developing attention bias in primates

One key aim of animal welfare research is to be able to correctly assess an animal's psychological wellbeing and emotional state (Dawkins, 1990; Mendl and Paul, 2004). It is commonly accepted that emotions evolved as a survival function (LeDoux, 1996; Damasio, 2000; Rolls, 2000). Within human research there are methods that exist to measure both the physical (behavioural, physiological) and psychological (cognitive and subjective) components of emotions, in particularly the study of the psychological components is paramount to understanding and improving human psychological wellbeing (Gray, 1981; Mathews and MacLeod, 2002).

There is, however, argument over the validity of emotion as subject for scientific research and this has hindered research development into animal emotions (*in situ*: Fraser, 2009). Emotions are considered unobservable and therefore deemed unsuitable for scientific study: positivists argue for a separation between science and metaphysics (Rollin, 1990; Fraser, 1999). Positivism led to developments in psychology and in turn behaviourism, influencing pioneers of ethology (Burkhardt, 1997). In non-human animals until recent advancements in cognitive bias welfare was, and often still is, assessed using ‘non-psychological’ components of emotion such as behavioural studies (Prescott *et al*., 2010). Recent studies have developed a way to assess the cognitive component of psychological measures (Bethell *et al*., 2012a; Bethell *et al*., 2012b). Development of cognitive techniques can help improve measures of animal emotions, in turn improving an animal’s welfare and psychological wellbeing (Harding *et al*., 2004; Paul *et al*., 2005).

One current method that has been proposed to address this gap is to use attention bias as a welfare tool to measure stress levels (Bethell *et al*., 2012a). Previous work by Mendl *et al*. (2009) has shown that cognitive bias can be used as an indicator of psychological wellbeing. The end goal of Bethell *et al.* ’s (2012a) project is to create a user friendly, affordable option that is able to measure, predict and improve psychological wellbeing, with wide reaching benefits, developing current behavioural management techniques (NC3Rs, 2006; Rennie and Buchanan-Smith, 2006; Prescott, 2010; Prescott *et al*., 2010), results of my study will help define methods for this.

1.4 Influential factors

As previously stated disorders and stress can influence attention bias in both human (MacLeod *et al*., 1986; Mathews, 1990; Beard *et al*., 2012) and non-human primates (Bethell *et al*., 2012a). However, there are many other factors that can influence an individual's attention bias, furthermore many of these factors can overlap.

Genetics is a highly studied and highly influential factor affecting our cognition. Research on humans show that a number of genes are implicated by attention bias (Posner *et al*., 2007; Fox *et al*., 2009). For example, studies have shown that humans with irregularly shorter copies of the 5-HTTLPR gene have a bias to look towards negative stimuli (Fox *et al*., 2009; Pergamin-Hight *et al*., 2012). Though there is insufficient data to support human research in non-human primates, there is however research that shows how genetic factors, such as 5-HTTLPR, may also influence social attention in non-human primates (Champoux *et al*., 2002; Watson *et al*., 2009; Brent *et al*., 2013).

Research studying attention bias in humans has shown the effect of habituation to stimuli (Wright *et al*., 2001; Amir *et al*., 2009). Habituation is used within animal training to reduce response to negative information or stimuli through repeated exposure (Leussis and Bolivar, 2006), which would suggest that the stimuli used within attention bias could have a similar result. Bradley *et al*. (1998) found that repeated exposure to stimuli effected reactions to emotional stimuli over time, showing a decrease in vigilance.

Human research has also shown the influence of age on attention bias. For example Murphy and Isaacowitz (2008) found that younger people had greater vigilance for emotion salience and negativity preference than older adults. Mather and Carstensen (2003) also found similar results that older adults would remember and be more attracted to positive faces, they justify this due to older adults' having a better emotional well-being than younger adults and older adults also have a tendency to remember positive memories and information more readily. There are many other factors, later discussed, that were controlled for or considered within analysis that could influence vigilance within this current study.

1.5 Reviewing practices and stimuli

Stimuli pairs are used as a measure of attention bias (Bethell *et al*., 2012a) however; viewing preference of stimuli can be influenced by a multitude of factors that should be considered (e.g. Bethell, 2009). For example, symmetry has been shown to increase the attractiveness and viewing preference of an image (in humans: Enquist and Arak, 1994 and in rhesus macaques: Waitt and Little, 2006). Colour also influences viewing preferences dependent on brightness and contrast (in humans: Taylor *et* al., 2013 and in rhesus macaques: Waitt *et al*., 2006; Gerald *et al*., 2006). Further to this, macaques have also been shown to have a viewing preference to brighter colours in comparison to grayscale (Conway and Tsao, 2009). Overall, the cognitive processes influencing viewing behaviour is subject to debate as often studies categorise images and do not consider all of the factors, such as colour and symmetry together, that influence viewing preferences and cognitive processing (Park *et al*., 2010).

1.5.1 Emotional stimuli

Attention bias research both within humans (Fox *et al*., 2002; Wilson and MacLeod, 2003) and macaques (Bethell *et al*. 2012a) has shown the use of facial stimuli as a measure of attention bias and psychological wellbeing. The use of facial stimuli is key henceforth this study's aim is to clarify our understanding of perceived emotional content relative to expressions. Previous studies have shown the success of presenting a macaque image to a macaque, both familiar (Deaner *et al*., 2005; Pokorny and De Waal, 2009; Schell *et al*., 2011) and non-familiar (Adachi *et al*., 2009; Dahl *et al*., 2010; Bethell *et al*. 2012a), for a variety of research methods. Dahl *et al* (2009) showed how macaques interpret facial stimuli through a similar process to humans. Bethell *et al*. (2012a) found macaques would show different viewing strategies towards macaque stimuli dependent upon their stress levels. For example Bethell *et al*.'s (2012a) findings showed that monkeys with heightened stress would show a vigilant-avoidant strategy, being initially vigilant to the stimuli followed by rapid avoidance. With this in mind, stress was controlled for as much as possible during testing. Although monkeys received stressors we could not control for e.g. weekly veterinarian visits, external stressors beyond our control were accounted for when possible, for example avoiding testing on stressful days. Therefore the use of facial images, similar to those used by Bethell *et al*. (2012a), were deemed reliable to test attention bias and the emotional content of stimuli for this current study.

1.5.2 Positive stimuli

A positive stimulus is something that possesses rewarding qualities and is associated with appetitive behaviours (Pessoa *et al*., 2002). A positive stimulus is important after exposure to an aversive image that could cause distress and is important as a psychological reward for the monkey's participation and to encourage them to take part in-trials again (Anderson, 1998).

Previous studies have shown the successful use of social stimuli with macaques; using images of individuals as a stimulating reward for participation in a negative experience (Sacket, 1966; Haude *et al*., 1976; Anderson, 1998; Deaner *et al*., 2005; Watson *et al.,* 2012; Méary *et al*., 2014), in some cases these social stimuli would be selected as a reward over food (Andrews *et al*., 1995). Machado *et al.* (2011) also showed that monkeys would look for longer periods of time at a social stimulus rather than a non-social stimulus due to its high arousal levels. This preference for social stimuli over a food reward is widely acknowledged, research has been conducted to show this in rhesus macaques (Nahm *et al*., 1997; Mosher *et al*., 2011) and chimpanzees (Kano and Tomonaga, 2009; Kano and Tomonaga; 2010; Hirata *et al*., 2010). These studies show that the primate brain is predisposed to process social information (Cheeney and Seyfarth, 1990) and that aspects of social information, conspecifics, can have psychologically rewarding properties (Watson *et al*., 2012).

However, recent research (C.Kemp pers.comm.) used pictures of both familiar and unfamiliar infant macaques as positive social stimuli. Females represented various ranks and ages and all had previously seen infants, however behavioural observations of reactions to stimuli were negative, such as alarm barks and fear grinning. These behaviours are commonly exhibited by macaques to a threat, stressor or towards negative information (Balcombe *et al*., 2004), suggesting that the stimuli Kemp (pers.comm.) used are not positive or rewarding. Research has shown the variation in interest towards infants varies with reproductive status

(Maestripieri and Wallen, 1995; Waitt *et al*., 2007). These negative reactions could be as a consequence of social and reproduction status as infants could be seen as a threat to their hierarchy and social status (Tomasello and Call, 1997). This illustrates that social stimuli are not always rewarding, possibly due to individual differences. Nonetheless, the negative behavioural reactions seen by participants in Kemp's study were predominantly from nulliparous individuals, but did also included some multiparous. Ultimately, the positive reward is important for future participation and welfare of subjects in question (Anderson, 1998) and therefore should be considered in protocol.

Aims

The aim of the proposed research was to evaluate the perceived emotional content of facial stimuli commonly used in rhesus macaque research, *Macaca mulatta*, using an attention bias paradigm. Testing this by presenting three facial expressions; a neutral face with eyes closed, the same neutral face with eyes open and an aggressive face. I anticipate the findings of this study can help to develop methods to assess psychological wellbeing in captive macaques.

Objective 1: To train participating monkeys to station (sit still in the same position) for testing using positive reinforcement and clicker training without the use of restraining measures, all conducted within a monkey's social group (Chapter 3).

Objective 2: Development of new positive stimuli, finding a positive stimulus that could be used as a 'reward' (encouragement) for the participating monkey at the end of each trial (Chapter 4).

Objective 3: Development of an attention bias paradigm to present three facial expressions in three different counter balanced presentations of two images at a time including. Facial expressions include: a neutral face with eyes closed, the same neutral face with eyes open and an aggressive face (Chapter 4).

Objective 4: Supplementary behaviour assessments to support attention bias findings, analysing both immediate communicative response to stimuli and longer lasting effects (Chapter 4).

Chapter 2

General Methods

2.1 Study site

The study took part at the Medical Research Centre, Centre for Macaques, Porton Down, Wiltshire, UK (MRC CFM). MRC CFM breeds rhesus macaques for use in medical research in academic institutions in the UK. The facility is home to 250 macaques. On average the facility breeds 40 juveniles per year. Approximately 30-35 monkeys are supplied per year to scientific studies within the UK (including males, females, adults and juveniles); all other individuals remained within the breeding programme (D. Farningham pers.comm.).

2.2 Participating animals

Twenty-eight female indoor group-housed rhesus macaques, *Macaca mulatta* participated in this study. Animals were housed in breeding groups, participating females came from seven different breeding groups. The groups ranged in size from four (one male: three females) to ten (one male: nine females: Table 2.1), these figures do not include juveniles of weaning age or younger for any group. Not all females in groups participated, due to their willingness to participate or the level of training they received. Reproductive status was assessed through continuous monitoring of menstruation and an annual ultrasound after breeding season. Further to gestational pregnancy a juvenile's independence from their mother was based on behavioural observations rather than age, as some individuals became independent at a younger age (Table 2.1). Only two individuals were nulliparous, Vienna and Wasabi, both the youngest individuals, all other monkeys were multiparous. This was a longitudinal study and therefore age was recorded for the date on which a monkey partook in her first trial. Age ranged from ~30-188 months mean age =120 (Table 2.1). For analysis age was combined into age bins of a continuous increasing rate of 20 months (31-50 months= 2 monkeys, 51-70 months= 2 monkeys, 71-90 months= 5 monkeys, 91-110 months= 5 monkeys, 111-130 months= 2 monkeys, 131-150 months= 4 monkeys, 151-170 months= 2 monkeys, 171-190 months= 6 monkeys).

A proportion of monkeys tested had previously been used in a similar attention bias experiment and therefore received exposure to the facial stimuli used in both experiments. Of the 28 participating monkeys 17 had previously been exposed to the facial stimuli during a test conducted four months prior to the start of this study. During these previous tests monkeys that had previously seen the stimuli received exposure to stimuli during eight testing sessions over a four-month period.

Table 2.1. Table to show the group composition, monkey I.D (name), age in months, reproductive status and previous experience of all monkeys that participated in the study.

Group	Monkey I.D	Age in months (age bin)	Reproductive Status	Previous Experience
1 Mulberry (n= ♂ 1: ♀ 3)	Porsche	103 (91-110)	Nursing	Previously exposed
	Helga	188 (171-190)	Nursing	Previously exposed
2 Dean (n= ♂ 1: ♀ 8)	Hilda	186 (171-190)	None	Previously exposed
	Holly	187 (171-190)	None	Previously exposed
	Spangle	79 (71-90)	None	Previously exposed
	Hazel	186 (171-190)	Nursing	Previously exposed
	Hope	188	None	Previously

		(171-190)		exposed
	Hetty	187 (171-190)	Nursing	Previously exposed
3 Judd (n= ♂ 1: ♀ 5)	Shirley	78 (71-90)	Independent	Previously exposed
	Ocelot	113 (111-130)	Independent	Previously exposed
	Tes	65 (51-70)	Independent	Previously exposed
	Tass	66 (51-70)	Independent	Previously exposed
4 Thorn (n= ♂ 1: ♀ 6)	Mustard	140 (131-150)	Nursing	Naïve
	Vienna	38 (31-50)	None	Naïve
	Wasabi	31 (31-50)	None	Naïve
5 Sol (n= ♂ 1: ♀ 9)	Leah	152 (151-170)	Nursing	Previously exposed
	Meesha	139 (131-150)	None	Previously exposed
	Lala	152 (151-170)	None	Previously exposed
	Melody	140 (131-150)	Independent	Previously exposed
	Libby	148 (131-150)	None	Previously exposed
6 Nodon (n= ♂ 1: ♀ 5)	Shallot	87 (71-90)	None	Naïve
	Rene	102 (91-110)	Nursing	Naïve
	Razz	103	Independent	Naïve

		(91-110)		
	Rhumba	90 (71-90)	None	Naïve
	Robyn	104 (91-110)	None	Naïve
7 Sequel (n= ♂ 1: ♀ 5)	Omelette	112 (111-130)	Nursing	Naïve
	Orlanda	110 (91-110)	Nursing	Naïve
	Ruby	85 (71-90)	Independent	Naïve

2.3 Housing

Each breeding group was housed in a cage room (25.7m X 7.34m) and neighbouring free roaming room (75.42m X 21.55m: fig. 2.1). Access to either of these rooms could be controlled by staff using sliding hatches (fig. 2.1). Each group had visual access to the opposite group when in the cage room. The cage room consisted of three levels; monkeys had access to all levels within the cage room (fig 2.2). Each free roaming room had a large bay window looking outside and a smaller window looking into the facility corridor. The smaller window that looked out into the corridor also had a mirror fixed to the outer wall of the main corridor, the direction and orientation of the mirror could be controlled by the monkeys inside the cage room by a lever, so they could see what was happening in the corridor (fig. 2.1). The free roaming room consisted of various environmental enrichment objects such as ropes, tubing, platforms and a mixture of large wooden and plastic objects that could be used as visual barriers (fig. 2.3).

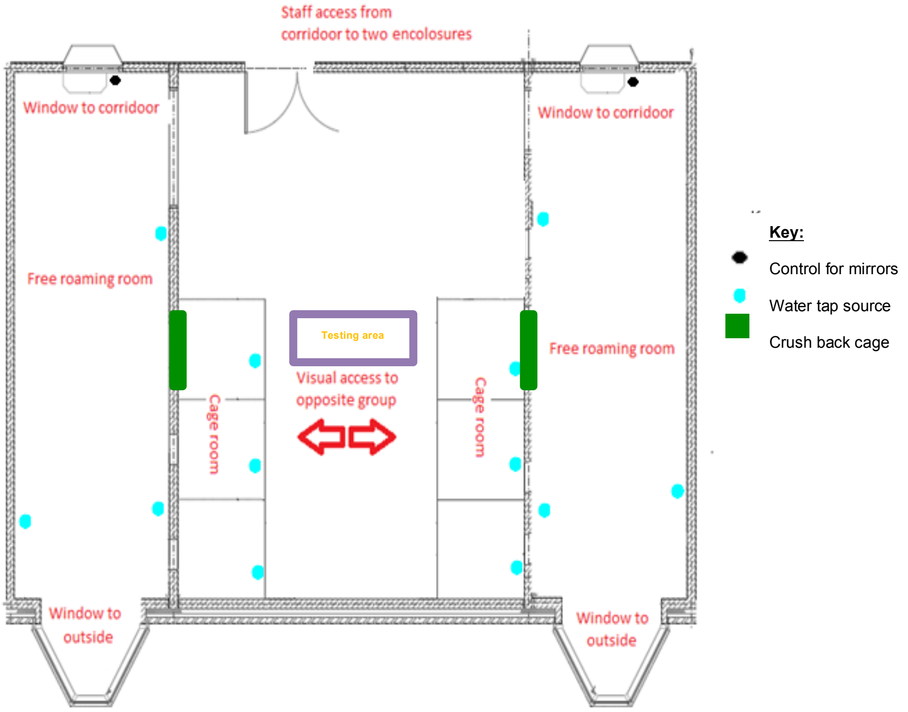

Figure 2.1. Image of housing at CFM, showing cage room and free roaming room, including opposites groups housing**.**

Figure 2.2 shows the different levels within the cage room, separated by wooden slats

Figure 2.3 Photo showing various enrichments within the cage room at MRC CFM including platforms, visual barriers and ropes.

As well as enrichment objects, the free roaming room also consisted of bedding, comprising of wood shavings and straw. The cage room had concrete flooring and levels created by wooden slats. Both rooms were cleaned on a fortnightly basis, by care staff at the facility, removing all faeces and waste matter, washing rooms, sanitizing rooms and changing bedding within the free roaming room.

2.4 Home Office Guidelines

Monkeys' accommodation met all the requirements of the Home Office, providing monkeys with room large enough, in relation to group size, to carry out 'normal' behaviour (NC3Rs, 2006). No animals were singly housed, adhering to guidelines (NC3Rs, 2006). Animals were also provided with various enrichments to encourage natural behaviours and food was spread to encourage foraging as well as the use of foraging related enrichment items, adhering to regulations (NC3Rs, 2006).

2.5 Diet

Animals were fed twice a day Monday–Friday, at 9am and at 2pm. On weekends and bank holidays animals were given one large feed at 9am. Animals were fed a mixture of dry food and one fresh item per mealtime. Dry food included grains, rice and supplement SDS triomunch grains. Fresh items were rotated, including various items such as cabbage, bread, apples, eggs and bananas. Care staff would feed monkeys in the free roaming room, all food was scattered to increase natural behaviours. Animals would also receive 'treats' such as raisins or peanuts for training purposes. Infrequently animals would receive sweeter treats such as cool aid or peanut butter on less frequent occasions such as medication administration; these treats were given primarily in the cage room. As these foods were additional and used for a reward basis the monkey's daily food ration was not adjusted in accordance. Water was available ad-lib from taps in the cage room and free roaming room (fig. 2.1)

2.6 Health

Animal care staff conducted observations throughout the day. Through visual inspections anything of welfare concern, such as any physical injuries or signs of illness and particular changes in an animal's natural behaviour, such as stereotypic behaviours or an increase in aggression, were noted in a daybook. All data from the daybook was collated in a large online database. For severe injuries or illness that required immediate treatment, such as a physical injury requiring stitches, a veterinarian was on call 24 hours with an onsite veterinary room.

Weekly visual inspections by a veterinarian were conducted on Wednesdays. These included animals that had previously undergone treatment or were considered for future treatment. For inspections animals were restrained using the crush back mechanism (figure 2.1). If necessary, small surgical matters such as stitching wounds would be conducted on Wednesdays.

Further to the weekly visual inspections by a veterinarian, each animal received an annual veterinarian check in the summer. This procedure was conducted per group for a weeklong period. The annual veterinarian check comprised of an initial sedated inspection (using ketamine) on a Monday. During this basic health records were made including: bloods, faecal samples, weight, reproductive status and a visual dentistry check. A faecal swab was taken each morning for the following three days. To sedate animals and take faecal swabs animals were restrained in the crush back cage, within the cage room.

2.7 Training

There are two main training methods: operant conditioning (Skinner, 1951) and classical conditioning (Pavlov, 1927). Classical conditioning involves training an animal to associate the trainer (Conditioned Stimuli) with a positive reinforcer (Unconditioned Stimulus) (Pavlov, 1927). This method is said to increase the speed of learning since the trainer and training protocol become associated with positive outcomes. This training reduces stress levels and aversive reactions to the trainer and increases opportunities for learning (Reinhardt, 1997; Waitt *et al*., 2002). Whereas operant conditioning is the process by which an animal learns that performing specific behaviours leads to specific consequences (Reinhardt, 2004; Schapiro *et al*., 2003; Owen and Amory, 2011; Remington *et al*., 2012). The methods used to train monkeys for testing for the current study combine both operant and classical conditioning in a form of training referred to as clicker training.

Clicker training is a training method that relies on individual learning (Skinner, 1951). A secondary reinforcer, a ‘click’, is used to distinguish a particular behaviour, bridging the temporal gap between the behaviour and primary reinforcer (Pryor, 1999, 2009; Williams, 1994). Bridging the temporal gap allows for precise feedback Pryor (1999). Pryor (1999) suggest that the clicker ‘bridges’ this brief moment (temporal gap) between the behaviour and the primary reinforcer by signalling that the primary reinforcer is coming. Clicker training allows behaviours to be shaped so that the click reinforces precisely the spontaneous desired behaviour until it can be modelled by successive approximations to become the desired response (Topál *et al*., 2006).

2.7.1 Training methods

In the present study rhesus macaques were firstly habituated to researcher's presence. This was conducted for two primary reasons a) to minimise influence on behaviour thereby promoting more natural behaviours (Samuni *et al*., 2014) and b) to develop a cooperative relationship increasing success of learning and training (Savastano *et al*., 2003). To measure a suitable level of habituation for a monkey in this study, behaviour was monitored and a record was kept noting reductions in aversive reactions towards the trainer, once these aversive reactions stopped training could progress.

For the proposed methodology clicker training was used to train each an animal to station. Station training involved each animal having its own specific station (figure 2.4). A station was a coloured object that attached to a clip and was secured to the inside of the cage room. Stations were used during every training session.

Figure 2.4 An example of novel objects used as stationing tools attached to clip.

2.7.2 Training protocol

A clicker was used to shape an animal's behaviour. Firstly animals were encouraged to approach the station (fig 2.4) through the presentation of a food reward, once an animal approached the station and as they received their reward a click would be made. The clicker then allowed curious behaviour to the novel object to be encouraged, a click was given any time an animal touched their station. So in turn resulting in an animal eventually holding their specific station, therefore animals would sit still in front of their specific station and hold for the duration of the training session. If a station was moved anywhere within the cage room it was also reinforced that the animal would be expected to move and follow their station around the cage room. Further to learning their specific station each animal was also consistently referred to by their name to reinforce this for aid in testing.

Initially twelve groups were trained including 55 animals suitable for study (not including males or juveniles, although they were also trained). All monkeys reacted to training at a different pace. Due to time constraints not all females trained reached the desired level by testing. Therefore only 28 of the trained 55 monkeys were chosen for participation in this study. These 28 were selected on their response to training, being able to station for approximately five minutes without continuous reinforcement and were well habituated to the apparatus and the notion of revealing stimuli.

A summary of all females trained is presented in table 2.2. The table also justifies whether animals were used in testing briefly explaining limitations, be them external logistical factors or training rate. Table 2.2 further represents the length of training each monkey required to station for testing and the number of sessions this behaviour was reinforced so that monkeys stationed every time. Some monkeys began training earlier in May during my internship at CFM MRC, due to unforeseen circumstances (e.g. monkeys moving facilities) some monkeys trained could not be used for testing. Therefore more monkeys were trained five/six months later than the original monkeys trained in May. All monkeys then received a further four months training during the start of this project and further habituation a month before testing, to another scientist whom would also be carrying out similar testing procedures.

Although some monkeys received more training this did not always affect their training rate, however it did affect the refinement of the skills learnt; monkeys who received more habituation and training stationed better during testing. As was expected, temperament influenced training success, monkeys with cooperative and variable temperaments learnt at a quicker rate, promptly understanding that a click is for a desired behaviour that should be repeated. Aggressive and submissive individuals did not learn as quickly and needed more training sessions to reinforce the concept of stationing.

Table 2.2 represents each monkey that training was attempted with, it gives details of when each monkey began training, how long each monkey took to station (implying their learning rate), how many sessions of training they received up until their first trial began and if they were used for testing and justifies this.

* Levels of stationing: 1- stationed for five minutes, 2- stationed but inconsistent. 3- never stationed.

Monkey	**Commenced of training**	**Temperaments**	**Station progres s ***	**Weeks taken to station?**	**Total Training Sessions received**	**Used for testing? Y/N?**	**If no why?**
Tass	May	Submissive	1	1	101	Yes	N/A

Holly	May	Aggressive	1	1	107	Yes	N/A
Porsche	May	Cooperative	1	1	108	Yes	N/A
Helga	May	Variable	1	2	111	Yes	N/A
Leah	May	Cooperative	1	2	114	Yes	N/A
Omelette	September	Submissive	1	3	54	Yes	N/A
Orlanda	October	Submissive	1	3	54	Yes	N/A
Mustard	September	Aggressive	1	3	79	Yes	N/A
Hope	May	Cooperative	1	3	114	Yes	N/A
Ocelot	May	Variable	1	4	113	Yes	N/A
Lala	May	Cooperative	1	4	121	Yes	N/A
Ruby	October	Aggressive	1	5	54	Yes	N/A
Wasabi	September	Variable	1	5	79	Yes	N/A
Shirley	May	Aggressive	1	5	116	Yes	N/A
Hazel	May	Submissive	1	5	118	Yes	N/A
Hilda	May	Aggressive	1	5	120	Yes	N/A
Meesha	May	Cooperative	1	5	123	Yes	N/A
Spangle	May	Submissive	1	6	124	Yes	N/A
Vienna	September	Aggressive	1	7	79	Yes	N/A
Tes	May	Variable	1	7	125	Yes	N/A
Hetty	May	Submissive	1	11	139	Yes	N/A
Melody	May	Submissive	1	28	123	Yes	N/A
Libby	May	Submissive	1	42	142	Yes	N/A
Razz	Began training with care staff	Cooperative	1	Unknown	73	Yes	N/A
Robyn	Began training with care staff	Cooperative	1	Unknown	73	Yes	N/A
Rhumba	Began training with care staff	Submissive	1	Unknown	73	Yes	N/A
Rene	Began training with care staff	Aggressive	1	Unknown	73	Yes	N/A
Shallot	Began training with care staff	Aggressive	1	Unknown	73	Yes	N/A
Pidray	May	Cooperative	1	1	51	No	Moved to another facility
Polka	May	Submissive	1	1	51	No	Moved to another facility
Paca	May	Cooperative	1	3	76	No	Moved to another facility
Dime	May	Cooperative	1	4	45	No	Moved to

							another facility
Dolly	May	Submissive	1	4	45	No	Moved to another facility
Doreen	May	Variable	1	4	45	No	Moved to another facility
Pamela	May	Variable	1	4	59	No	Moved to another facility
Patricia	May	Aggressive	1	9	76	No	Moved to another facility
Pax	May	Submissive	1	9	79	No	Moved to another facility
Venice	May	Aggressive	2	1	37	No	Aversive reaction to testing
Girl	May	Submissive	2	2	41	No	Partaking in another test at time of testing
Green	May	Submissive	2	2	41	No	Partaking in another test at time of testing
Tanya	May	Aggressive	2	5	121	No	Partaking in another test at time of testing
Lydia	May	Aggressive	2	7	122	No	Inconsistent stationing
Love	May	Variable	2	7	125	No	Inconsistent stationing
Hatty	May	Submissive	2	8	130	No	Insufficient training
Meg	May	Submissive	2	9	130	No	Inconsistent stationing
Sizzle	May	Submissive	2	9	132	No	Inconsistent stationing
Simone	September	Submissive	2	12	67	No	Inconsistent stationing
Thistle	September	Submissive	2	21	67	No	Inconsistent stationing
Lake	September	Submissive	3	Never stationed	33	No	Insufficient training
Pansy	October	Variable	3	Never	40	No	Insufficient

				stationed			training
Tulip	October	Variable	3	Never stationed	40	No	Insufficient training
Pandora	May	Aggressive	3	Never stationed	86	No	Moved to another facility
Hillary	May	Submissive	3	Never stationed	138	No	Insufficient training

2.7.3 Justifying the method

Training monkeys to station, as described above, was beneficial for later attention bias testing. As animals would cooperatively sit still for a training session at their station, it meant there was no need for any restraint measures. Allowing voluntary participation and reducing stress levels compared to more conventional techniques that are known to increase stress levels such as the crush back cage (Laule and Desmond, 1998). Minimising stress levels allowed for ease in cooperation of testing with the equipment and greater reliability of participation in future studies (Clay *et al*., 2009). Minimising stress also allowed greater control over indirect influence on a monkey's attention bias scores (Bethell *et al*., 2012a). External stressful factors that could be controlled for were accounted for by only selecting days for testing when there were least likely to be stressful disruptions

2.7.4 Application of training to testing methodology

To test monkeys, the first monkey to approach the cage room would always be stationed and tested first. Their stationing tool would be positioned, in the testing area (see figure 2.1) and the monkey would then be encourage to come and hold her station and rewarded with a click and food reward for doing so. The testing apparatus was always positioned in the same section of the cage room for consistency, considering light and distance, if necessary for some monkeys the equipment would be raised. All other monkeys, both participating females and unparticipating monkeys, would be stationed throughout the cage room at the same time. The positions in which all other monkeys were stationed would be considered so that testing monkeys could not view the stimuli.

Once monkeys were stationed and rewarded testing would begin. Monkeys would again be rewarded by a click during the trial and at the end of each trial the monkey would receive a larger treat reward e.g. a peanut. Once one monkey had been tested, the monkey’s station would be moved to a new position and the monkey would be rewarded by a click and food reward for following its station to a new position. A new monkey and its station would then be moved to the testing area of the cage room and would be rewarded with a click and food reward for doing so. The same process as before would then be repeated for all animals.

Original methods for the study proposed that behavioural observations of the monkeys would be recorded directly after being exposed to the stimuli, however due to constraints of stationing animals this was not always possible. The length between testing and behavioural observations was minimised to a 45-minute standard when possible. If all monkeys within a group were not tested by this point testing would be paused and behavioural observation conducted for the monkeys tested, testing would then resume for the remaining participating monkeys. Monkeys’ behaviour would be recorded in the same order as they were tested as to minimise any time delay.

Chapter 3

Attention Bias

Introduction

One current method being developed in primates to assess welfare and an animal's psychological state is attention bias (Bethell *et al*., 2012a). Attention bias is the tendency to attend to one type of information over another (MacLeod *et* al., 1986). Attention bias can be used to measure gaze towards opposing information to assess whether an individual is stressed. This technique is being developed as a novel, non-invasive method to measure stress in macaques (Bethell *et al*., 2012a; C.Kemp pers.comm.).

Although the progression of attention bias as a welfare tool continues (Bethell *et al.,* 2012a; C.Kemp pers.comm.), the facial stimuli used so far have not been tested for their emotional value to macaques, only presumed to have a relatively neutral perceived emotional content. Images used consist of an aggressive face against an opposing neutral face. However, the neutral faces used within these studies consist of a frontal macaque face with a vacant in expression with either its eyes open (Bethell *et al.,* 2012a) or eyes closed (C.Kemp pers.comm.). Nevertheless, eyes open and direct staring, when performed from a dominant to a submissive, is seen as a threat to macaques as a signal of dominance (Chevalier-Skolnikoff, 1973; Van Hooff, 1976; Machado and Bachevalier, 2006).

It is a common occurrence within macaque literature to presume an open eyed facial expression has little emotional content when used in comparison to a more aggressive expression (Partan, 2002; Dahl *et al*., 2009; Parr *et al*., 2010; Micheletta *et al*., 2015a), however the emotional content of an open eyed rhesus macaque relative to its perception against other facial expressions has never before been studied. Therefore in order to refine attention bias as a welfare tool, clarification of facial expressions used is necessary.

In this chapter methods used to assess a macaque's reactions to emotional stimuli are described. Firstly the stimuli themselves are discussed, both facial stimuli and non-social stimuli as a reward for the monkey's participation (Cheeney and Seyfarth, 1990; Watson *et al*., 2012). Statistical results of attention bias tests are also presented and discussed.

Methodology

3.1 Stimuli

3.1.1 Facial Stimuli

Facial stimuli comprised of pictures of six adult male rhesus macaques (herein known as ‘stimulus monkeys’). These stimuli were previously collected from monkeys at the Caribbean Primate Research Centre, Puerto Rico in 2006 (Bethell, 2009). Therefore the stimulus monkeys were unfamiliar to the monkeys at MRC CFM.

Each stimulus monkey set consisted of three facial images of the same individual; one image showed a frontal face with a neutral expression and eyes closed (herein known as ‘neutral face eyes closed’), the second image showed the same neutral face with eyes open (herein known as ‘neutral face eyes open’), the third image showed a frontal aggressive expression with eyes open (herein known as ‘aggressive face’). Therefore, for each stimulus monkey there was one ‘neutral eyes closed face’, one ‘neutral eyes open face’ and one ‘aggressive face’ (figure 3.1). The neutral eyes open face and the aggressive faces are original images taken from Bethell (2009), however, the neutral eyes closed face is a photoshopped version of the neutral eyes open face to create an image of a monkey with eyes closed. As access was only available to these stimuli, due to time constraints and subject numbers new stimuli could not be created, stimuli used in testing could only be modified from a larger available data set. Therefore other possible neutral expressions of low emotional content, for example a monkey with eyes averted, could not be tested.

Figure 3.1. Facial stimuli images used for testing. Unknown, conspecific male with the following facial expressions: neutral eyes closed face, neutral eyes open face, aggressive face.

3.1.2 Non-social stimuli

Images of food items were used as non-social stimulus. Six images were used in total: apples, tomatoes, peppers, melons, raisins and peanuts (fig 3.2). These items were chosen due to their familiarity to the monkeys as they were regularly used within the husbandry feeding routine and therefore were presumed to be associated with a positive part of the routine to the monkeys.

Figure 3.2. shows an example of two non-social stimuli photographed at MRC CFM, peppers (left) and tomatoes (right), that were used in testing.

3.1.3 Stimuli Preparation

All digital images were calibrated using photographic standards following Gerald (2001). This was conducted to calibrate digital images based on their scale to fit an A4 frame (iapsonline, 2015) using Adobe® Photoshop® (Adobe.com, 2015). It was important that brightness and luminosity did not differ significantly between the two images in each facial combination pair. Calibrating images is standard practice in human research (Holmes *et al.,* 2008) and has also been shown to be important for primates, particularly rhesus macaques (Waitt and Buchanan-Smith, 2006), so not to cause a bias in gaze that could influence attention bias results. All images were calibrated considering these factors and superimposed on a grey background. For example, a difference in brightness would mean that the stimuli were perceptually different making an image inadvertently more attractive to the subject. This perceptual difference could cause a differential gaze to certain stimuli that could be caused by brightness effects rather than emotional content (Osorio *et al*., 2004).

Stimulus images were printed on high quality image paper using a Konica high chroma printer. The printer used was calibrated at regular intervals to produce similar levels of colour output (Hébert and Hersch, 2014) and the same printer was used each time to eliminate any variation in colour output between printers (Hébert and Hersch, 2014). New stimuli were printed every three weeks and kept in dark conditions when not in use to avoid fading and loss of colour in images overtime (El-Molla *et al*., 2013).

3.2 Apparatus

Figures 3.3, 3.4 and 3.5 show the apparatus used to present pairs of stimuli. Figure 3.3 gives dimensions of the framework. The equipment stood 2020 mm tall (from floor to the top of the pole used to support the sliding framing work) and 1210 mm wide. The framework could be adjusted to two heights depending upon the suitability the participating monkey. Either in the middle level of the cage room, where the stimuli were set 1150 mm above the ground, or the top level of the cage room, where the stimuli were set 1780mm above the ground. All elements of the apparatus were designed to be symmetrical ensuring that there were equal diameters of framing between the stimuli and the edges. Ensuring all elements of the apparatus were balanced was important as not to cause a bias in gaze direction. A slide mechanism (fig. 3.5), attached to both occludes, revealed the two stimuli at the same time.

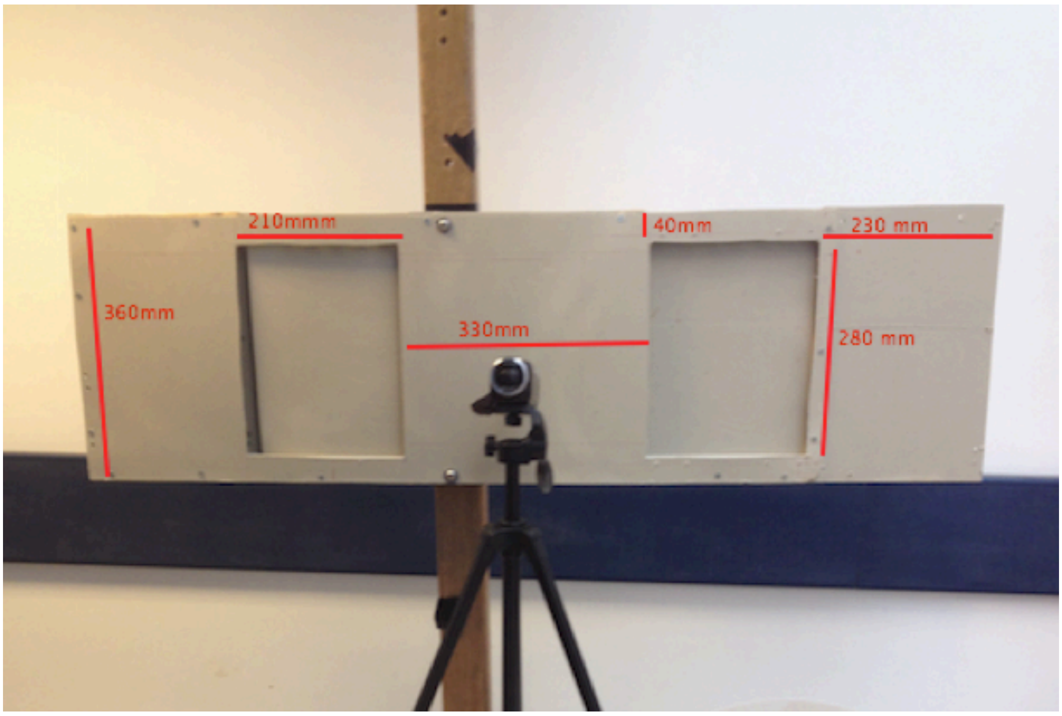

Figure 3.3. Frontal mage of apparatus used to measure attention bias, showing rectangular areas where stimuli were displayed and position of the camera, including measurements in mm.

Figure 3.3. Image of apparatus with stimuli revealed.

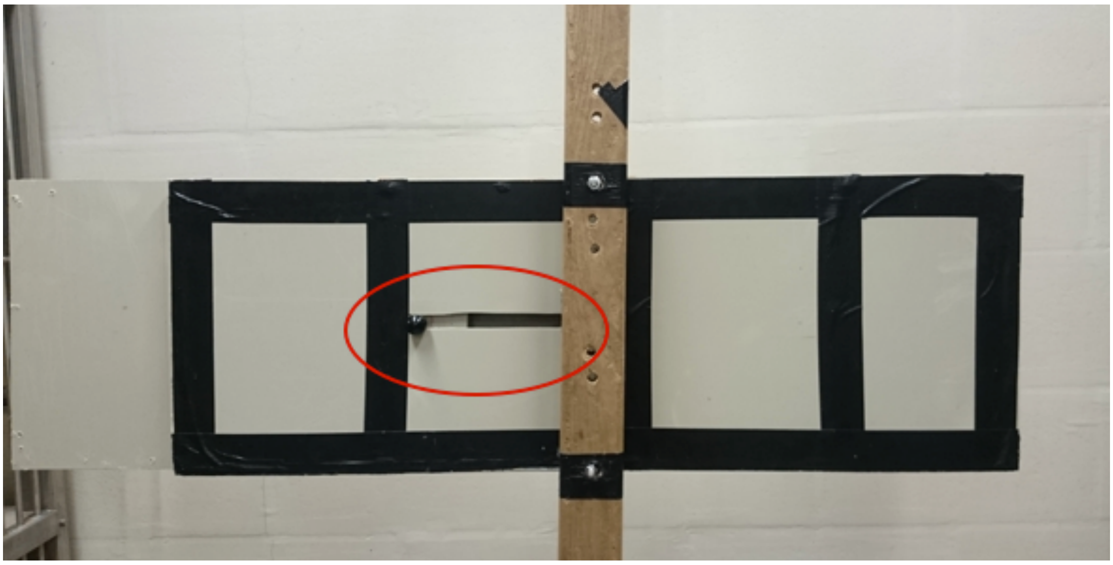

Figure 3.5. Image of apparatus from behind, showing the sliding mechanism that is highlighted with a red ring.

A camera was positioned centrally between the two stimuli in front of the framework to film each monkey's direction of gaze and behavioural reactions to the stimuli. For animals tested on the middle level the camera was positioned on a tripod (figs. 3.3 and 3.4). For animals tested on the higher level this was attached centrally on the wooden post above the frame.

3.3 Experimental Design

3.3.1 Trials

Stimulus monkeys were presented with a counterbalanced presentation of the three emotional facial stimuli (herein known as 'facial combination pairs'). These facial combinations were: neutral face eyes open against an aggressive face (herein known as EO/Ag), neutral face eyes closed against an aggressive face (herein known as EC/Ag) and neutral face eyes closed against a neutral face eyes open (herein known as EC/EO) (figures 3.6a, b and c).

Figure 3.6a an example of facial combination, neutral face eyes open against an aggressive face.

Figure 3.6b an example of facial combination, neutral face eyes closed against an aggressive face.

Figure 3.6c an example of facial combination, neutral face eyes closed against a neutral face eyes open.

Each participating monkey had 12 trials in total. These trials consisted of four trials for each of the three facial combinations. To account for any underlying effect in gaze and side bias, the order in which a monkey saw each facial combination and stimulus monkey was randomised. Predefined presentations were randomly created and given a number from one-twelve (Table 3.1). These presentations were randomly allocated per monkey per trial (Table 3.2).

Table 3.1 shows the allocation of facial combination and stimulus monkey to each presentation.

Presentation	1	2	3	4	5	6	7	8	9	10	11	12
Facial Combination	Ag/ EO	Ag/ EO	EO/ Ag	EO/ Ag	Ag/ EC	Ag/ EC	EC/ Ag	EC/ Ag	EC/ EO	EC /EO	EO/ EC	EO/ EC
Stimulus monkey	4	1	3	5	6	3	1	2	5	2	6	4

Table 3.2 shows which monkey received which presentation per trial 1 -12.

Monkey	**Trial**											
1	10	12	11	3	8	5	9	2	4	7	1	6

2	11	12	2	1	3	5	10	9	7	6	8	4
3	1	3	8	2	7	10	5	6	4	9	11	12
4	5	6	3	7	2	9	11	1	4	8	10	12
5	5	7	1	12	8	10	11	9	2	6	4	3
6	3	2	8	5	11	4	9	7	10	1	5	12
7	1	2	3	6	5	12	4	8	11	10	7	9
8	2	11	1	7	6	4	10	12	5	9	8	3
9	10	7	12	3	8	5	11	9	2	6	4	1
10	3	12	8	2	1	10	11	5	9	5	6	4
11	2	11	3	8	1	12	10	7	4	7	6	9
12	7	8	12	5	2	9	1	11	6	3	10	4
13	12	3	11	4	1	8	5	9	10	6	2	7
14	10	11	12	6	5	4	9	3	8	7	1	2
15	1	3	2	10	7	12	5	6	11	9	8	4
16	3	11	2	1	9	4	5	10	7	12	6	8
17	2	1	3	9	10	5	11	4	12	7	6	8
18	5	7	1	12	8	10	11	9	2	6	4	3
19	11	6	12	5	3	8	2	4	9	10	1	7
20	11	4	6	10	9	8	1	7	3	5	2	12
21	5	9	8	10	7	3	1	4	2	6	11	12
22	4	9	5	7	8	10	12	2	11	6	1	3
23	5	6	8	9	12	3	11	4	1	2	10	7
24	4	5	12	6	10	2	8	3	11	9	7	1
25	5	7	1	12	8	10	11	9	2	6	4	3
26	11	12	2	1	3	5	10	9	7	6	8	4
27	1	9	7	3	8	11	12	2	5	4	10	6
28	11	3	12	1	8	4	5	10	7	2	6	9

Each monkey had one trial a day, two days a week, on alternate weeks for 12 weeks in total (shown in a more detail in Appendix 1). Due to the stress caused by weekly visual inspections by a veterinarian every Wednesday, Wednesdays were not used for testing animals, as not to inadvertently influence attention bias scores.

3.3.2 Attention Bias

Each facial combination was presented and the gaze recorded on video for later coding of attention bias. Directly after the presentation of facial stimuli non-social stimuli were presented to the participating monkey for three seconds. After three second the non-social stimuli remained visible to conduct an orientation phase. This involved tapping on the left side of the apparatus and encouraging the monkey to look left, and the same to the right side. This orientation helped with calibration of gaze direction during blind coding.

This was a double blind study; all facial combinations were previously randomly allocated to the 28 monkeys. The presenter was unaware of the facial combination that they would be presenting and the side of each facial expression while coding. Animals were randomly allocated their presentation order dependent upon when they willingly participated, making the allocation of monkey to presentation order also random.

Every facial combination was presented on four occasions for all stimuli sets (alternating facial expression (aggressive/neutral) twice on the left and twice on the right). In total 336 tests were run, 12 trials were conducted for 28 monkeys.

3.4 Procedure

3.4.1 Testing

Animals participated ‘voluntarily’ in the experiments, making this a self-selecting study and were free to leave and re-enter the test area at any time.

Monkeys were stationed to sit centrally in front of the apparatus and encouraged to gaze centrally, between the two stimuli, by tapping the apparatus framework and calling the participant monkey’s name to get their attention. When the monkey gazed centrally the trial began with the presentation of the facial combination. The presenter announced ‘open’ as a verbal signal to start the video and moved the lever to open the occluders, each stimulus set was presented for three seconds and the participant monkey’s gaze recorded. The presenter then announced ‘closed’ after 3 seconds as a verbal signal that the presentation of stimuli was over and the occluders were shut.

Stationing the participating monkey to sit centrally and encouraging them to look between the stimuli enhanced coding efficiency. Responses from orientation presentations when shown the non-social stimuli allowed an initial calibration to be conducted to determine when monkeys were looking at either stimulus.

3.4.2 Coding

Videos were coded for the direction of gaze using JWatcher Video (Jwatcher.ucla.edu, 2014). Direction of gaze towards the left and right stimuli was coded on a frame-by-frame basis at a frame rate of 30 frames per second, hence recording not only direction but also time spent looking in that direction. The codes used to score the direction are defined in table 3.3.

A third of the trials were coded together by two coders, each coder then coded another third separately. To compare inter observer reliability two thirds of the singularly coded video were double coded. The number of agreements and disagreements for each of the behaviours coded were entered into a matrix and degree of reliability was calculated using Cohen's kappa (k) statistic (Bakeman and Gottman, 1997). Following Bakeman and Gottman's (1997) research, a predetermined kappa value of 0.70 was selected as the criterion level for a good level of agreement between the two coders. Compared codes from both coders attained a Kappa score of 87%, exceeding the criterion level.

Table 3.3. Ethogram showing behavioural categories used to determine direction of gaze

Behaviour	Definition
Left	Eyes orientated to left stimulus (coder's left).
Right	Eyes orientated to right stimulus (coder's right).
Central	Eyes orientated forwards and between the two stimuli.
Away	Head turned away so that both stimuli are outside of peripheral vision in any direction
Away Left	Eyes orientated towards the coders left, image is still in peripheral vision but gaze is not directly on the stimuli.
Away Right	Eyes orientated towards the coders right, image is still in peripheral vision but gaze is not directly on the stimuli.
Away up	Eyes orientated above the stimuli towards the ceiling.
Away Down	Eyes orientated below the stimuli towards the ground.
Extreme Up	Extreme avoidance of the stimuli looking towards the ceiling, chin pointing upwards.
Extreme Down	Extreme avoidance of the stimuli looking down, eyes may be covered by brow ridge.
Out of View	Not possible to determine direction of gaze because either the head or eyes were not visible.

Analysis

3.5 Data Treatment

Due to its wide applications R-Project was chosen to analyse all data (The R Project, 2015). To evaluate attention bias data was pooled for all 28 monkeys. Mean values were used for the three seconds of looking data and discriminated based on various factors to address the aim when analysing data.

3.5.1 Cbind function

In order to control for the overall time that monkeys gazed at stimuli in general (i.e. one individual always looks under half a second) the cbind formula was used to create a new factor (Zuur *et al*., 2009). This function takes into consideration that each individual is different, combining the vectors as columns of a matrix. Therefore cbind in turn represents what is referred to as the attention bias score throughout the results, a relative measure of time spent looking at either stimulus. Within each three seconds trial there were two variables overall time spent looking at the aggressive conspecific face and the overall time spent looking at the presumed to be neutral conspecific face. These two variables were used to create a new response variable (attention bias). The binomial denominator (n) is total looking time at the stimuli: e.g. the total duration in milliseconds of each variable (looking at the aggressive or neutral stimulus) within a specific trial. The cbind formula to create this new response variable is expressed below:

Look at aggressive = total look time at either stimulus-look at neutral

Attention bias <-cbind(look at aggressive, look at neutral)

To represent this data better visually, 0.5 was subtracted from attention bias scores so that in graph form positive values show vigilance towards the stimuli and negative value show avoidance from the stimuli.

3.5.2 Mixed models

Using mixed models allowed us to work with complex data sets (Wang and Goonewardene, 2004; Bolker *et al*., 2009). For the data in question mixed models were beneficial as they allowed us to look at random factors and fixed factors.

A correlation analysis was conducted to show whether there was co-linearity between any variables and which could be included within the general linear mixed model (Dormann *et al*., 2013). A bivariate correlation showed that there was no correlation between any of the variables, r (9) =<0.3.

Within the R software, REML (Viechtbauer, 2010) was used to find the best random effect structure. Candidate models were designed including all possible factors that could explain the data, both random factors and fixed factors, facial combination remained as a fixed factor (Table 3.4). All logical interactions between all factors were formed to create candidate models. All candidate models can be found in Appendix 2.

Table 3.4 shows each variable by the code used in the candidate models, as well as a description of what the variable represents and whether they were random of fixed factors.

Code	Variable
Random Factors	

Group	**Group:** The socially housed group which the monkey was housed, in total there were seven different social groups.
MonkeyID	**Monkey ID:** The personal identification of the monkey.
Matriline	**Matriline:** Line of descent from a female ancestor to descendant.
	Fixed Factors
Reproductive.status	**Reproductive Status:** Monkeys reproductive status at the start of trials, this could include gestational pregnancy or rearing a juvenile.
Trial	**Trial:** Refers to the order of testing e.g. chronological date.
Facial.combination	**Facial Combination:** Which set of facial pairings the monkey saw e.g. EO/Ag, EC/Ag or EO/EC. Facial combinations are listed as 1-6, for each facial pairing it accounts for whether the stimulus was on the left or the right.
Stimuli	**Stimulus Monkey:** The male adult face used in a given test session (six different adult male stimulus monkeys).
Previous.exposure	**Previous Exposure:** Whether the monkey was naïve or had previously seen the stimuli during an experiment conducted four months prior to the start of testing.
Age.mos	**Age in months:** Monkeys age in months, calculated from the date of first testing for that individual.

Running the model select function in the R package assesses relevant contributions of factors and combinations of interactions within the candidate models to find the model that best explains the trend within the data. The test showed that the most suitable model was:

‘Test1<-lmer(cbind ~ (1|Trial) + (1| Matriline/MonkeyID)’

This model had the lowest AICc value that differed from the other models by more than 2 units. This model therefore created the null model containing only random factors and no fixed factors.

3.5.3 Maximum model

Models were created considering the relevance of each possible factor in relation to the aims of this project. The aim of this study was test the influence of facial stimuli on attention bias scores, therefore facial combination was considered the most influential factor over the data set. The following factors were considered that could possible influence the data; age is known to influence social behaviour (Murphy and Isaacowitz, 2008), matriline could show a trend in reactions to stimuli dependent on genotypes (Fox *et al*., 2009; Perez-Edgar *et al*., 2010; Fox *et al*., 2011; Gohier *et al*., 2014), dependent on reproductive status of females social stimuli can be highly arousing influencing viewing strategies (Maestripieri and Wallen, 1995; Schino *et al*., 2003; Waitt *et al*., 2007), stimulus monkeys used may show individual variation that test monkeys find arousing (Schino *et al*., 2003; Holmes *et al*., 2008), finally previous exposure was considered as habituation to a stimuli can reduce the response given over time (Wright *et al*., 2001; Amir *et al*., 2009). Using these aforementioned factors maximum models looked at all possible interactions both between factors and within factors. Therefore models were created to assess the influence of all these factors on monkey's attention bias towards stimuli, in total 76 candidate models were created (see Appendix 2).

The model select function in the MuMIn package (Calcagno and de Mazancourt, 2010) was used to assess 76 candidate models to select the maximum models that best explained the data.

3.6 Statistical analysis

3.6.1 General Linear Mixed Models

In order to test whether the model structure meets the assumptions of normality after running the model select function, the residuals were tested. The deviance from the mean can be seen in Appendix 4. The mean models residuals were normally distributed; therefore general linear models were used to best explain the data. From running 76 suitable general linear models, within the R package using Lme4 and MuMIn packages the results showed a best-fit model could be created. This programme compared all models selecting the model that best represented the trend in the data (the greatest weight) based on relevant contributions of factors and combinations of interactions within all models in comparison to the null.

3.7 ANOVA

To further validate the results, testing the individual significance of each factor within the best-fit model, multiple ANOVAs were conducted using mean data comparing models against each other. For significant data Tukey's post hoc tests were run as a further comparison.

3.8 Results

3.8.1 General Linear Model

Results of the general linear model showed that the best-fit model was:

glmer (cbind~ Previous exposure + AgeMos*Combir + (1|Trial) + (1|
Matriline/MonkeyID), data = dat, family=binomial)

This model had the lowest AICc value and explained 97% of the variation in gaze (Table 3.5). No other models came within 2 AICc points of the model.

Table 3.5 the output of model select function to find the most suitable model showing the three highest weighted models

Fixed factors	Random effects (nested)	Log likelihood	AICc	Delta	Weight
Previous.exposure + AgeMos*Facial.combination	Trial, Matriline/Monkey ID	-53720.67	137698.8	0.00	0.97
ReproductiveStatus + AgeMos*Facial.combination	Trial, Matriline/Monkey ID	-53725.08	107630.9	8.84	0.012
AgeMos*Facial.combination	Trial, Matriline/Monkey ID	-53765.12	107711.0	84.39	0.008

3.8.2 Results of ANOVA

3.8.2.1 Facial Combination

There was a significant difference between facial combination pairs (EO/Ag, EC/Ag, EO/EC), Z=-9.074, *p*=0.008 (fig 3.7). There was a greater significant attention bias towards the EO/Ag facial combination rather than the EO/EC facial combination Z=-33.194, p=0.004. There was a greater significant attention bias towards the EC/Ag facial combination compared to the EO/EC facial combination Z=-39.071, p=0.044. There was a greater significant attention bias towards the EO/Ag facial combination face than the EC/Ag facial combination Z=-18.180, p=0.027. Post hoc tests (figure 3.8) revealed that monkeys had a significantly greater attention bias towards the aggressive face of the EO/Ag facial combination rather than the neutral face $t(107)$=18.680, p=<0.001. Monkeys had a significantly greater attention bias towards the aggressive face of the EC/Ag facial combination rather than the neutral face $t(107)$=21.616, p=<0.001. Monkeys had a significantly greater attention bias towards the eyes open face of the EO/EC facial combination rather than the eyes closed face $t(107)$=22.261, p=<0.001.

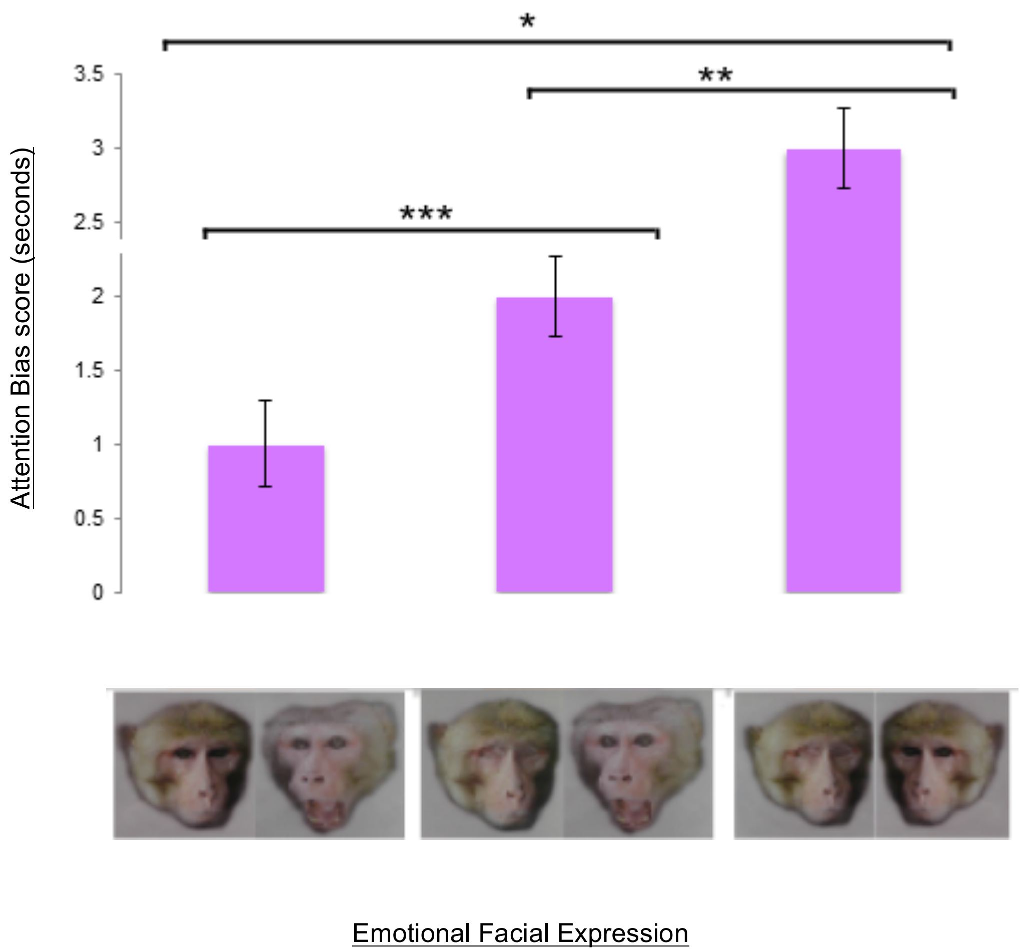

Figure 3.7 graph showing the average time spent looking at the three facial combination pairs (regardless of aggressive or neutral face).

* P = 0.004; ** P = 0.044; *** P = 0.027

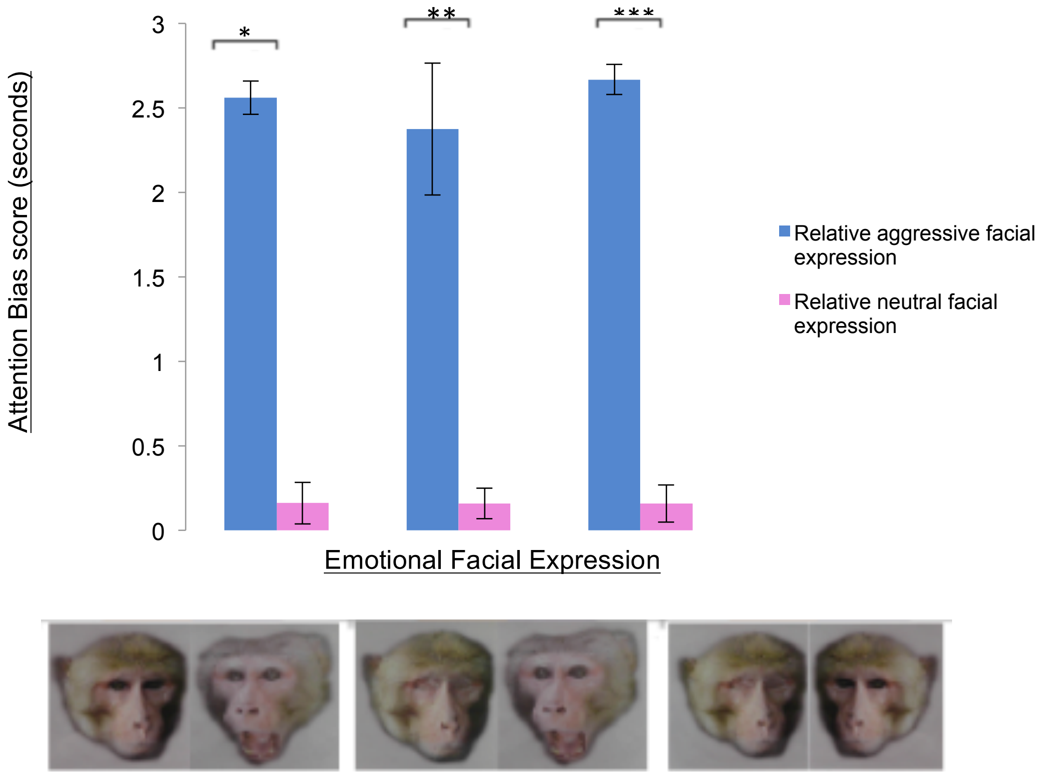

Figure 3.8 graph showing the average time spent looking at the relative aggressive and neutral facial stimuli within each of the three facial combination pairs.

* P = <0.001; ** P = <0.001; *** P = <0.001

3.8.2.2 Age of monkey in months

With regards to monkeys age there was a significant difference between age in months and their attentional bias towards the different facial combinations, Z=5.555, p=0.013 (fig 3.9). There was also a greater significant difference between age in months and ratio of looking data, Z=5.568, p=0.022 (fig 3.8). Post-hoc test revealed that monkeys aged 30-50 months had a significantly greater attention bias towards EO/EC facial combination than any other age group *t*(7)=-2.3840, *p*=0.038. Monkeys aged 71-90 had a significantly greater attention bias towards the EC/Ag facial combination than any other age group *t*(7)=-2.5345, *p*=0.024. Monkeys aged 91-110 months had a significantly greater attention bias towards the E0/Ag facial combination than any other age group *t*(7)=-2.9045, *p*=0.019. The oldest monkeys aged 171-90 months had a significantly greater attention bias towards the EO/Ag facial combination than any other age group *t*(7)=-2.3871, *p*=0.017. There was no significant difference between any other age groups or facial combinations.

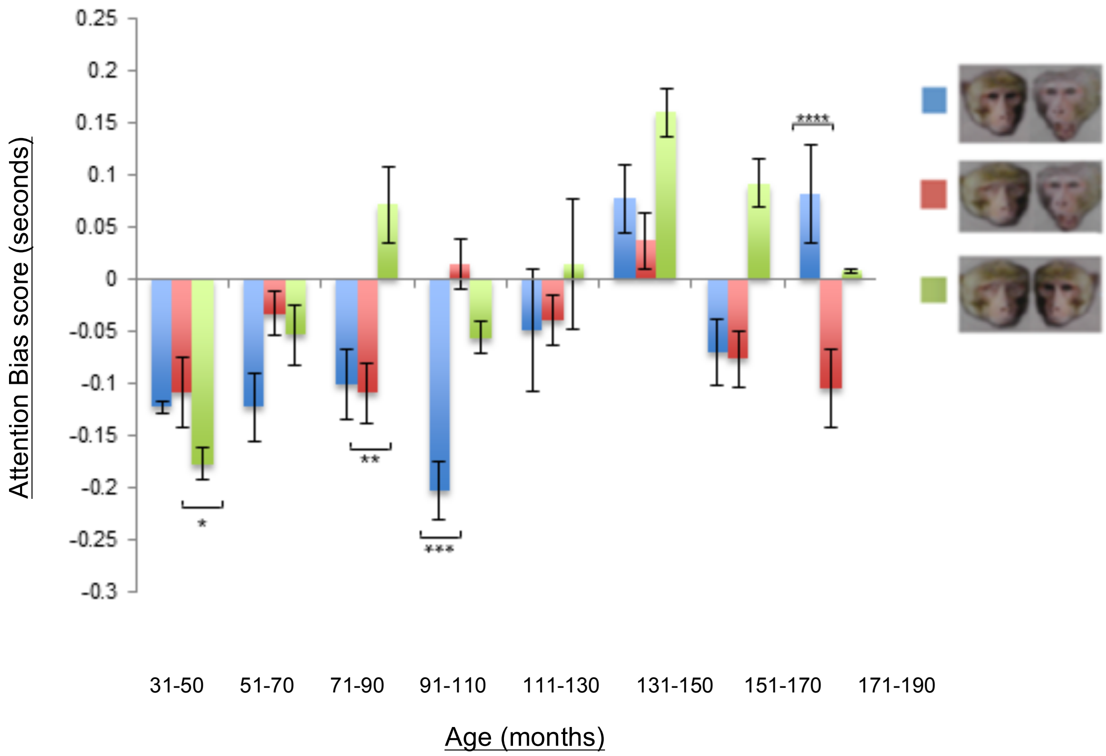

Figure 3.9 graph showing results of a longitudinal age comparison attention bias study, data shows monkeys attention bias dependent upon the stimulus viewed and the monkeys age at the start of testing, positive values show a vigilance towards the stimuli and negative value show an avoidance from the stimuli.

* P = 0.038; ** P = 0.024; *** P=0.019; **** P = 0.017

3.8.2.3 Effect of previous experience

There was a significant difference in the ratio of looking between two groups of monkeys those that had previously seen the facial stimuli and those monkeys that had never seen the stimuli before and were naïve, Z=-5.555, p=0.003, (fig 3.10). Post- hoc tests revealed that monkeys that had never previously seen the stimuli had a significantly greater attention bias toward the EO/Ag facial combination than those that had previously been exposed to the stimuli, $t(2)$=-3.798, p=<0.001 . Those that had seen the stimuli before had a significantly greater attention bias toward the EC/Ag facial combination than those that has never seen the stimuli, $t(2)$=-2.568, p=0.011. Monkeys that had never seen the stimuli before were significantly more avoidant of the EO/EC facial combinations than those that had previously seen it $t(2)$=-3.802, p=0.043.

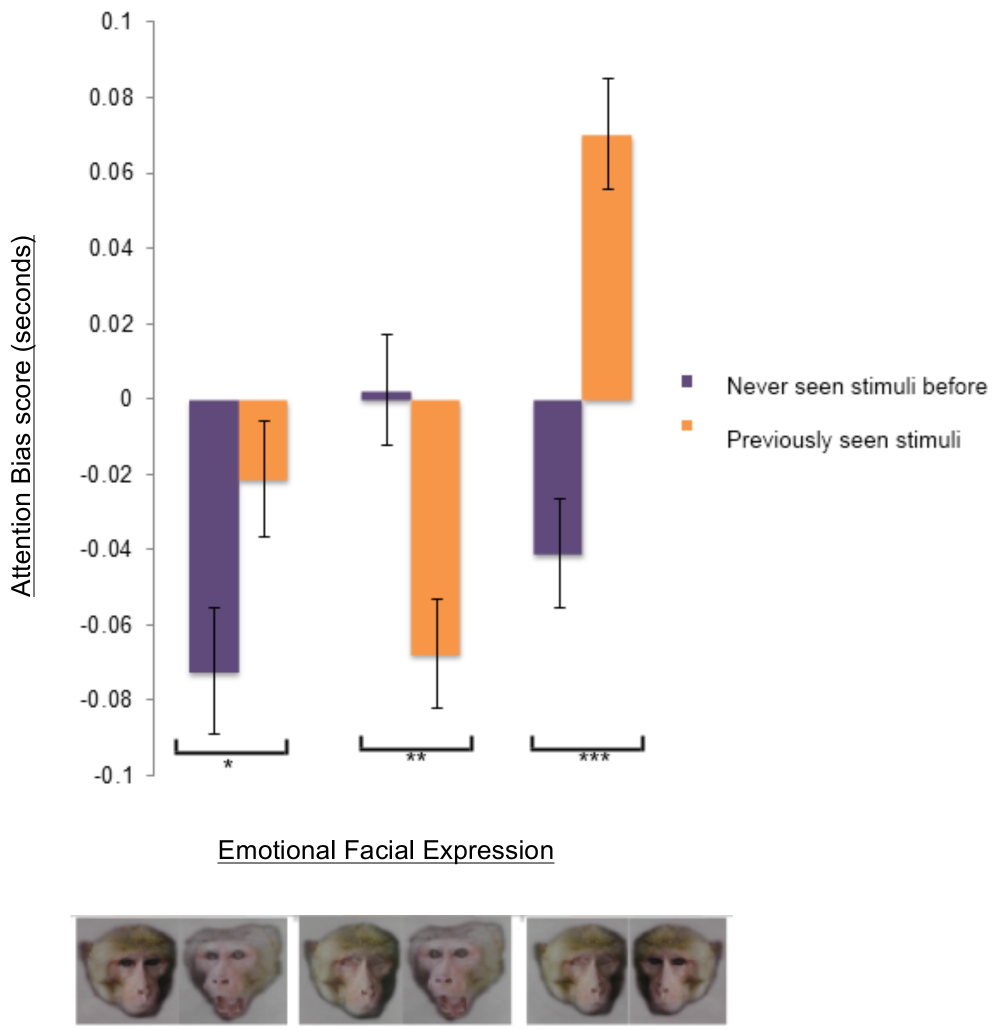

Figure 3.10 graph showing the average time spent looking at the aggressive and neutral face, dependent on monkeys was from the group that had previously seen the stimuli or never seen the stimuli before, positive values show a vigilance towards the stimuli and negative value show an avoidance from the stimuli.

*P= <0.001; **P = 0.011; ***P= 0.043

Discussion

3.9 Emotional facial stimuli

The main aim to assess emotional value of facial stimuli commonly used within macaque research was met through the attention bias paradigm. Although many factors were considered that could have possibly influenced the gaze of monkeys, the main factor to consider that addressed the aim of this study was facial combination. Attention bias results show that there is a difference in attention bias towards different facial combinations; the greatest difference in attention bias was towards the EO/Ag facial combination compared to all other facial combinations. Furthermore, this greater attention bias was shown to be influenced by both age and previous exposure to facial stimuli.

There was a statistical difference between all facial combinations. Therefore showing that this variation in gaze is influenced by the facial pairings. Bethell *et al*. (2012a) previously showed that a monkey's gaze is linked to the emotional value of stimuli. In conclusion, this variation between all three facial combinations can be suggested to be owed to the emotional content of the stimuli used.

Comparing the facial combinations EO/Ag and EC/Ag, monkeys spent significantly more time looking at the EO/Ag combination. This suggests that EO/Ag is a more threatening combination than and EC/Ag. Previous studies have shown that animals are more inclined to look at negative information, both in macaques (Bethell *et al*., 2012a; Bethell *et al*., 2012b) and humans (Ito *et al*., 1998; Segerstrom, 2001; Smith *et al*., 2006), supporting the findings that EO/Ag facial combination has a more threatening emotional value.

For all facial combinations participating monkeys spent significantly more time looking at the more ‘aggressive’ face than the opposing ‘neutral’ face. This implies that whether the facial expression has eyes open or closed has a relative neutral emotional value in comparison to the opposing aggressive face. Research has shown that processing of information, and therefore attention bias, can depend on the emotional content (Pessoa *et al*., 2002). Fox *et al*. (2002) showed that the use of neutral stimuli could reduce attention towards negative information. As there was less attention bias towards EC/Ag this would further suggest that the eyes closed facial stimuli is less emotionally provocative content than an eyes open face as attention bias towards this facial combination was less than the EO/Ag.

Monkeys spent least time looking at the EO/EC facial combination. This illustrates that this combination is less threatening than those with an aggressive face (Ito *et al*., 1998; Segerstrom, 2001; Smith *et al*., 2006; Bethell *et al*., 2012a). For the EO/EC facial combination, monkeys spent more time looking at the neutral eyes open face than neutral eyes closed face. Supporting attention bias research would therefore suggest that the neutral eyes open facial stimuli is more threatening in its emotional content (Bethell *et al*., 2012a). Nevertheless, it could be suggested that the eyes closed face is disconcerting to monkeys as it looks like a monkey blinking, further facial stimuli could be tested as a suitable neutral stimulus.

3.10 Age of monkey in months

Using general linear models allowed inclusion of all possible influencing factors. One factor that was shown to strongly explain the data was age, which can clearly be linked to many other factors. Most groups (cage mates) at the facility were of similar age or genetic background, however group was not found to have a significant effect on the data. Age also, generally, corresponded to previous treatment; older individuals are more likely to have been through more scientific procedures (Rommeck *et al*., 2011), birthing seasons (Beisner and Isbell, 2009) and general alterations in facility management, welfare techniques and standards (Burn and Mason, 2008; Rommeck *et al*., 2011), all of which could affect an animal's predisposition to negative information due to previous negative experience altering their psychological wellbeing. Although age can also coincide with many other factors, there are consistent trends that cannot be faltered within the data showing the strength of the significant impact of age.

Results from comparing ages showed that the youngest individuals, age 30-50 months, looked predominantly at the EO/EC facial combination. This would suggest that the other stimuli combinations were too extreme in their emotional content, containing a high negative emotional value and therefore they looked less (Bar-Haim *et al*., 2010). Implying that these younger individuals are more susceptive to negative stimuli of high emotional value information. Previous research has shown that age is highly influential on our interpretation of negative information, more so when we are young and naïve (Murphy and Isaacowitz, 2008).

A trend can be seen in individuals' aged 71-90 months, compared to other monkeys. Individuals within this age range showed the greatest bias in gaze towards the EC/Ag face combination. All of these individual were nulliparous, nevertheless, reproductive status and history was not a significant factor alone in defining attention bias. Although there is no current research on nulliparous individuals and attention bias, research has shown that interest towards infant stimuli varies with reproductive status (Maestripieri and Wallen, 1995; Waitt *et al*., 2007). Therefore it can be suggested that interest towards adult male facial stimuli may also differ with reproductive status, as a fitness benefit (Schino *et al*., 2003). However, reproductive status was not a significant factor and cannot justify this variation alone. Furthermore there is wide individual variation within this age group and therefore no single factor could explain this variation.

Monkeys' aged 171-190 months also showed a high attention bias towards the EO/Ag facial combination, compared to other monkeys. Interestingly all individuals of this age came from one particular group. In addition this group also had one younger individual aged 79 months who also showed greater significant attention bias towards the EO/Ag. Although group alone showed no significant effect, this consistency between all group members would suggest a particular tendency within this group to increase their susceptibility toward negative information. All members of this group had previously seen stimuli (in: C.Kemp pers.comm.). None of this group was first or second generation relatives, therefore excluding any strong genetic influence (Fox *et al*., 2009; Perez-Edgar *et al*., 2010; Fox *et al*., 2011; Gohier *et al*., 2014). However, this group was nearest to the office and veterinarian room having visible access to both. It is possible that the stress of seeing people and places associated with stressful events could increase anxiety levels (Laule *et al*., 2003). This group also had a male who had been very difficult during training periods, and as a result required a lot of attention, which could have also effected stress levels of participants either through eagerness to perform and participate or the influence of the group's social dynamics (Zweig and Weinshall, 2007).

3.11 Effect of previous experience

There was a corresponding significant difference in attention bias between those that had previously been exposed to the stimuli and those who had never previously seen the stimuli. Out of the 28 monkeys used in this study 17 had previously taken part in a similar experiment (C.Kemp pers.comm.). In this experiment monkeys had seen the aggressive face and the neutral eyes closed face in a similar attention bias paradigm. Previous work using stimuli in attention bias testing has shown the effect of habituation (Wright *et al*., 2001; Amir *et al*., 2009). Habituation reduces response to negative information through repeated exposure (Leussis and Bolivar, 2006).

Statistical analysis revealed monkeys who had not previously seen any of the facial stimuli spent the most time looking at the EO/Ag facial combination compared to any other facial combination, this was significantly greater than those who had already seen the stimuli. This would indicate that although the monkeys previously tested had only seen the EC/Ag facial combination they had become habituated to the use of emotional facial stimuli (Rankin *et al*., 2009). Therefore were not inclined to look as strongly at the negative information (Wright *et al*., 2001; Amir *et al*., 2009).

Monkeys that had previously been exposed to stimuli and those that had not, showed a similar gaze pattern, looking at the EO/EC facial combination for approximately 1.5 seconds. Although monkeys that had not previously seen stimuli looked for significantly longer than those who had. This difference again confirms a habituation to repeated stimuli exposure (Wright *et al*., 2001; Amir *et al*., 2009). However, the similar pattern between both groups of monkeys and the stimuli combination particularly compared to that of EO/Ag facial combination, would again suggest that the EO/EC facial combination has a weaker negative emotional value.

Chapter 4

Behavioural Measures

Introduction

There has been vast progress on advancing new indices of animal welfare (Dawkins, 2004). Nevertheless, it is widely accepted that there is no single method that can be solely used as a measure of welfare (Dawkins, 1980; Broom 1988; Mason and Mendl, 1993). Dawkins (2004) argues that with the wealth of behavioural, biochemical and physiological measures that are now readily available, there is now the problem of integrating them to give a true representation of an animal's welfare. However, Dawkins (2004) further argues that if a measure can assess if an animal is healthy and environmentally satisfied it can assess welfare; behaviour is the most commonly regarded technique for this. For macaques behavioural data is a key component to current measures of assessment (Buchanan-Smith, 2010; Prescott *et al*., 2010).

Within macaque research, behaviour is used as an assessment of welfare (Prescott *et al*., 2010) and also as a measure of their social dynamics (Maestripieri and Hoffman, 2012), which can in turn influence an animal's welfare (Baker *et al*., 2012). The main measure of welfare is the presence or absence of negative behaviours (Prescott *et al*., 2010). These negative behaviours refer to those that highlight signs of stress such as eye poking, pacing, excessive yawning and hair plucking: these groups of behaviours are referred to as stereotypic behaviours (Mason, 2006). However, as well as negative behaviours, behavioural measures can also assess social dynamics through grooming (Sonweber *et al*., 2015), proximity (Gilbert and Baker, 2011) and tension coping mechanisms (Aureli *et al*., 1995; Honess and Marin, 2006). For example, Koyama (2003) showed that within

Japanese macaques, *Macaca fuscata*, matrilineal hierarchy reflected levels of social cohesion in terms of grooming and coalition formation.

Further to conventional behavioural measures, facial expressions are one of the most common communicative systems for macaques (Michelatta *et al*., 2015a) and can therefore be used as an assessment of an animals' state (Parton, 2002). Facial expressions can convey important information to conspecifics about an animal's internal state and possible future behaviour (Leopold and Rhodes, 2010; Waller and Micheletta, 2013).

Behavioural observations were used to assess emotional content of facial stimuli, assessing behaviour both within-trials and post-trials. As behaviours are commonly used to assess an animal's emotional state (Buchanan-Smith, 2010; Prescott *et al*., 2010), these behaviours can therefore be interpreted to imply emotional content of facial stimuli used. More aversive stimuli would be expected to increase behaviours exhibited in time of fear or stress. Macaques are highly expressive with their faces and there are commonly acknowledged emotional states expressed through these facial expressions (e.g. Parton, 2002). These emotional expressions can be used in relation to the stimuli shown to determine reactive emotional state to stimuli. Behavioural data provide important supplementary data to parallel the attention bias findings. The statistical results of the behavioural data are also presented and findings briefly discussed.

Methodology

4.1 In-trial behaviours

Behaviours during trials were coded from the videos recorded during stimuli presentations. Three-second focal animal continuous behavioural observations were conducted. Behaviours were coded using the ethogram defined in table 4.1 using JWatcher software (Jwatcher.ucla.edu, 2014). Behaviours were coded both frame-by-frame (frame rate = 30 frames per second) to code event behaviours and to code state behaviours. For analysis the ethogram (table 4.1) was further categorises the discrete behavioural categories into combined measures, based on previous findings (Parton, 2002). The extreme behaviour category represent those behaviours displayed in time of extreme distress or in threatening situations, representing both behaviours exhibited though fear and to signal dominance. Reactive behaviours included behaviours that are performed as a warning to conspecifics primarily through fear to alleviate the situation. Self-directed behaviour include all behaviours exhibited that are performed to one's self as a consequence of anxiety including stereotypic behaviours. Maintenance behaviours refers to any other general behaviour that does not fit into the above categories.

Table 4.1 Ethogram behaviours during each trial

Behaviour	Definition
	Extreme
Open mouth	Lower jaw dropped so lips form 'o' shape, upper teeth covered.
Fear grin	Lips retracted horizontally to expose teeth, jaws can be together or apart.

Fear grin with lip smack	Lips retracted exposing teeth whilst lips move repeatedly together and apart.
Tense mouth	Mouth is closed, lip corners drawn back to form straight line.
Head movement	Head pulled up in high position or head moved quickly and abruptly up and down.
Flee	Participating monkey quickly exits from the view of camera
	Reactive
Eyebrow raise	Eyebrows move upwards.
Ear flick	Ear movement of any form into a position other than that of comfortable relaxed state; including pointing perpendicularly out from head, retract tightly or a repetitive movement.
Vocalisation	Mouth opens to emit a sound.
Lip smacking	Lips moved repeatedly together and apart; may be audible.
Puckered lips	Lips drawn forward together, cheeks furrowed.
Tongue protrusion	Tongue outside of mouth, may be rhythmical or single movement.
Stare	Direct, prolonged, unwavering look at specific stimuli.
Exit	Participating monkey walks away from view of camera at a calm and steady pace.

	Self-directed
Yawn	Mouth opens widely in stereotyped gaping movement.
Scratch	Grooming of self.
	Maintenance/ other
Interaction with baby	Affiliative behaviour towards infant including grooming and nursing.
Gaze	Eyes relaxed, may be half shut, not looking in any particular direction.
Away	Participating monkey is looking away from the stimuli and facial reactions not visible.
Other	Participating monkey no longer in view of camera or performing behaviour not listed within the ethogram.

4.2 Post-trial behaviours

After a test session, a five-minute focal animal continuous behavioural observation was conducted for the participant monkey using the ethogram defined in table 4.2. The maximum delay between testing and the five minute observation was 45 minutes after exposure to stimuli. Observations were directly inputted into the JWatcher application (JWatcher.ucla.edu, 2014), for analysis bout durations of behaviours exhibited during focal observation were then extracted from the software. The ethogram (table 4.2) further categorises the discrete behavioural categories into combined measures for later analysis. The fear/avoid category includes those behaviours performed most generally through dominance or submission of an individual due to a conflicting or stressful event. Affiliative behaviours refers to those behaviours performed to improve social dynamics between individuals. Self-directed/anxiety behaviours include both personal maintenance and behaviours interpreted to be stereotypic. Maintenance behaviours refers to all other behaviours performed not in the categories above, including standard time budget behaviours. All behavioural repertoires for the ethogram where collected through a selection of previous macaques behavioural work (De Waal and Luttrell, 1989; Van Hooff, 1967; Ostner *et al*., 2008; Bethell, 2009).

Table 4.2 Ethogram stating the behavioural categories

Behaviour	**Definition**
	Fear/avoid
Submissive	Inclined or ready to yield to the authority of another.
Fear grin	Silent bared teeth display. Lips are protracted to reveal teeth and closed or partially open mouth.
Flee	Leaps/moves rapidly away from a stimulus that is potentially aversive. Often accompanied by threat or submissive behaviours.
Displaced	Participant moves as a consequence of other group members' movement.
	Aggressive approach
Aggressive	Behaviour directed towards another individual (monkey or human) including lunging with open mouth or vocalising, staring (with ears often forwards or flicking back and forth), lips protracted to reveal teeth, shaking cage.
Displace	Another individual moves from their position as a consequence of

	participants movements.
Threat	An indication or warning of authority. Monkey raises head and will often move in a bobbing movement emitting a vocalization whilst mouth forms an ‘o’ shape.
	Affiliative approach
Affiliative	Affectionate behaviour performed to increase social bonds with another individual that does not include grooming of any kind or lip smacking e.g. embraced when resting.
Groomed	To be licked, scratched or rubbed by other individual within group.
Grooming	Lick, scratch or rub any part of another individual within the group.
Lip smack	Rapid movement of lips against one and other.
	Self-directed/anxiety
Self-directed	Grooms self, scratches any part of body using hand or foot.
Body shake	Shakes whole body vigorously.
Stereotypic behaviour	All repetitive movements, classed as repetitive once perform more than three times.
Yawn	Involuntary opening of mouth widely and inhale due to tiredness or boredom.
	Maintenance/ Other
Locomotion	Moves from one part of cage to another. Includes fast and slow forms of quadrupedal and bipedal locomotion.

Object	Manipulates object that is not food, including toys (enrichment), cage attachments and ropes.
Vigilant	Visually searches surrounding environment: eyes and/or head move continually with alert posture (sitting or standing upright, often leaning forward with ears pricked up).
Stationary	Passive or sleeping. Includes apparently ‘relaxed’ and ‘depressed’ postures.
Interaction with baby	Actively nursing or readjusting baby’s position. Grooming/ cleaning baby.
Foraging	Actively searching/foraging for food, holding food in hand and placing in mouth or chewing on it.
Sexual contact	Mounting, presentation (sexual display), intercourse.
Out of view	Animal is obscured from view or behaviour exhibited does not fit into any of the above categories.

Analysis

4.3 Data treatment

Behaviours were coded in real time using behavioural codes, however for analysis behaviours were grouped into combined categories (table 41 and 4.2). For in-trial behaviours 20 behavioural codes were combined into four categories (table 4.1), for post-trial behaviours 24 behavioural codes were combined into five categories (table 4.2). For all behavioural data, both observations and video coding, data could not be transformed after attempting multiple transformations (e.g. logarithms, square route and multiplicative inverse) and therefore a non-parametric test was chosen to control for skewed non-normal untransformed data. Median values were used to analyse the data. As the data were combined behavioural reactions to three different facial emotion pairs from the same 28 monkeys a Friedmans test was used to test if there was a significant difference in behaviour categories between facial emotion pairs (Dalgaard, 2008). For those tests that resulted in a significant difference Wilcoxon tests were used to follow up these finding (Dalgaard, 2008).

4.4 Results

4.4.1 In-trial behaviours

There was a significant difference in the combined measure of extreme reactive behaviours exhibited between emotion pairs, X^2= 11.040, *p* = 0.04 (fig 4.1). Further tests showed that there was a significantly greater representation of extreme reactive behaviours exhibited to the EO/Ag than the EO/EC, z = 2.181, p = 0.027. There was also a significantly greater representation of extreme reactive behaviours exhibited to the EO/Ag than the EC/Ag, z= 1.443, p = 0.034. There was no significant difference between EC/Ag and EO/EC, z = 0.738, p =0.069.

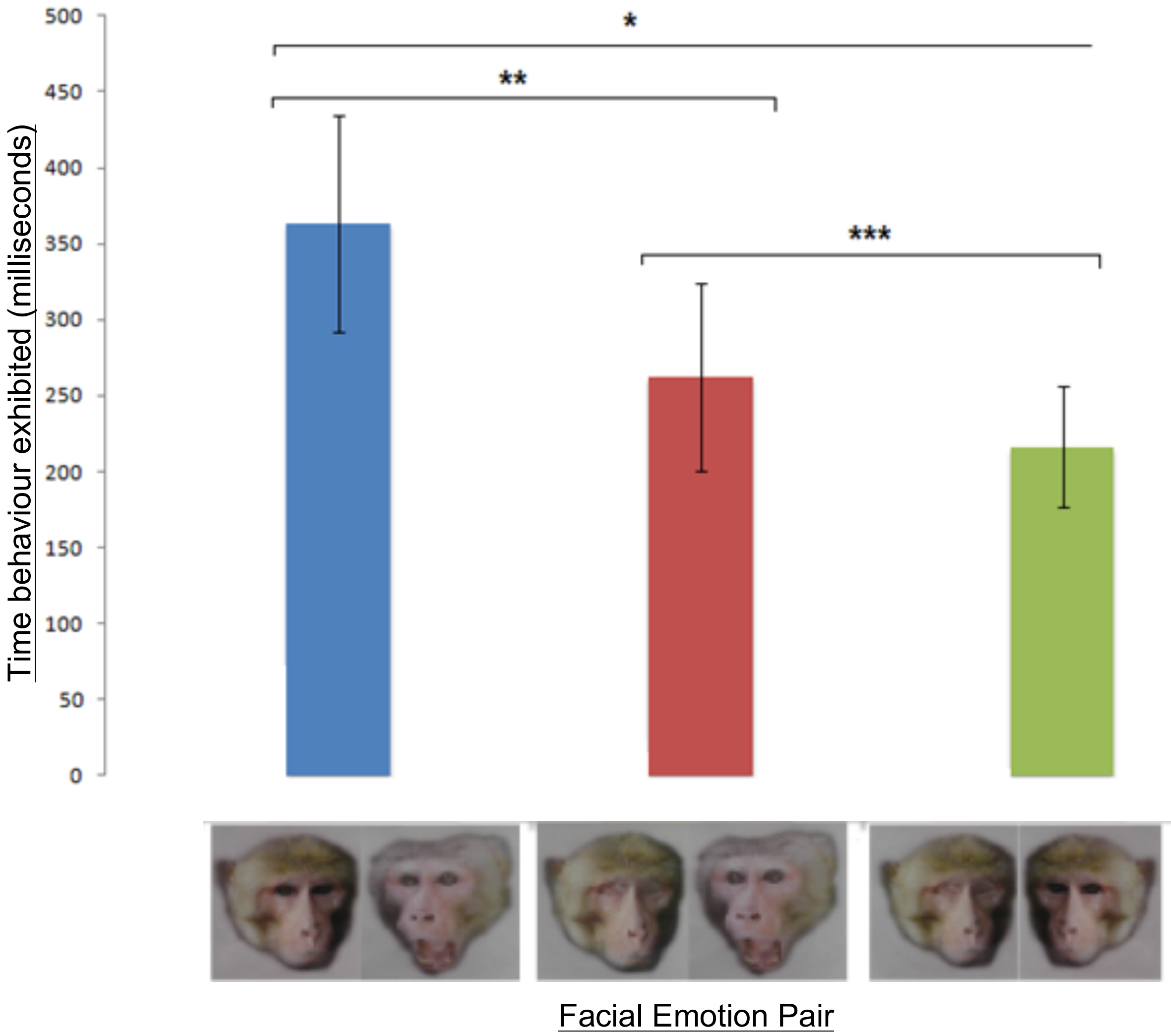

Figure 4.1 graph showing the amount of time spent engaged in extreme behaviours for all three emotional pairs.

*P= 0.027; **P= 0.034; ***P=0.069 (non-significant)

Mild reactive behaviours exhibited did not significantly change between facial emotion pairs, X^2= 3.401, p = 0.18. Stereotypic behaviours exhibited did not significantly change between facial emotion pairs, X^2= 0.216, p = 0.89.

Uninterested/Other behaviours exhibited did not significantly change between facial emotion pairs, X^2= 3.401, p = 0.18.

4.4.2 Post-trial behaviours

There was a significant difference in fear/avoid behaviours exhibited between facial combinations, X^2= 6.180, p = 0.045 (fig 4.2). Further tests showed that there was a significantly greater representation of fear/avoid behaviours exhibited following presentations of the EO/Ag facial combination rather than the EC/Ag facial combination, Z = 0.235, p = 0.032. There was no significant difference between EC/Ag and EO/EC, Z = 1.175, p = 0.054. There was no significant difference between EO/Ag and EO/EC, Z = -0.94, p =0.073.

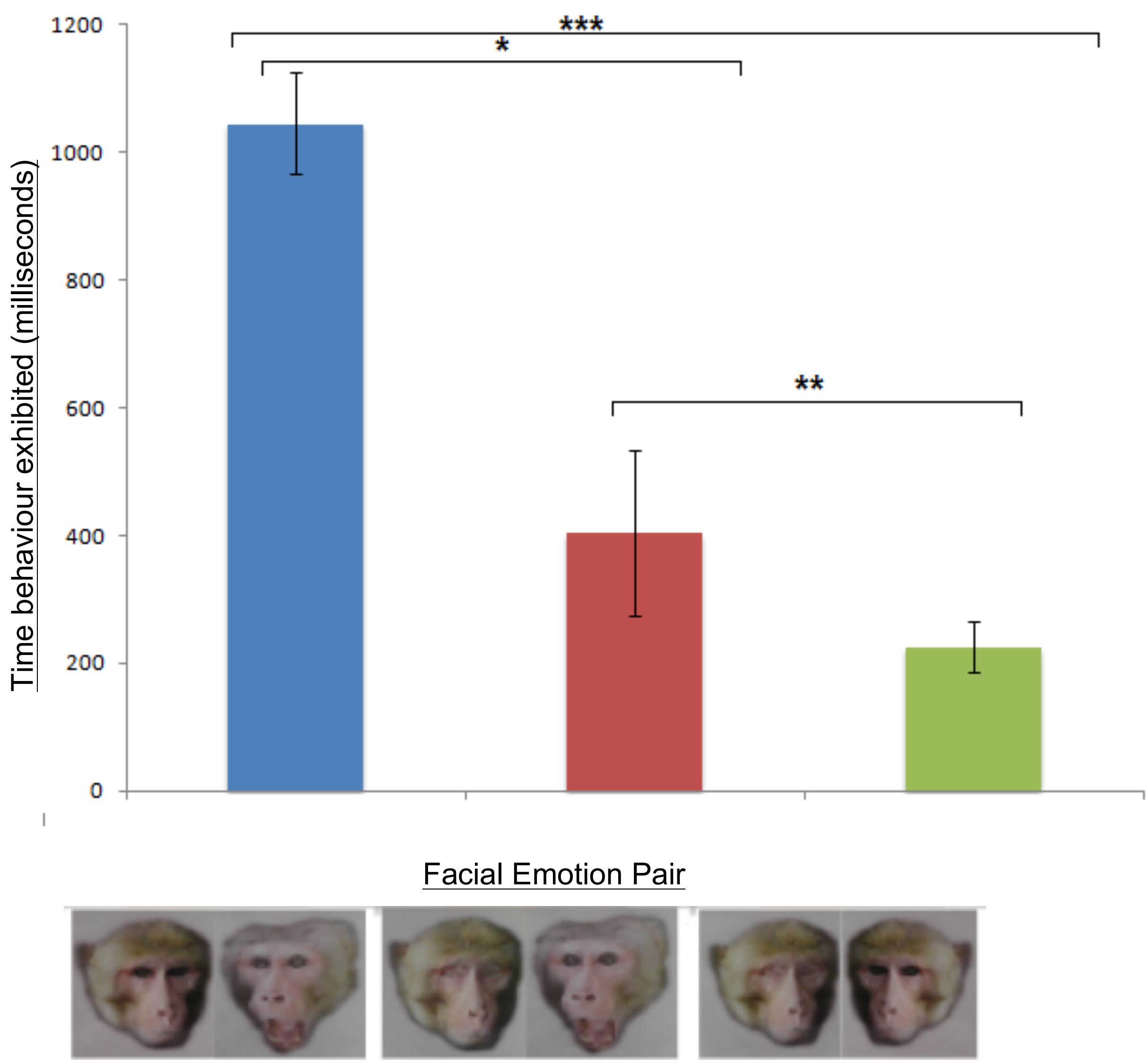

Figure 4.2 graph showing the amount of time spent engaged in fear/avoid behaviours for all three facial emotion pairs.

* P =0.032; ** P = 0.054 (non-significant); *** P =0.073 (non-significant)

There was no significant difference between all other behaviours exhibited after being shown all three facial emotion pairs. Aggressive approach behaviours exhibited did not significantly change between facial emotion pairs, X^2= 3.433, p = 0.18. Affiliative approach behaviours exhibited did not significantly change between facial emotion pairs, X^2= 0.564, p = 0.75. Self-directed behaviours exhibited did not significantly change between facial emotion pairs, X^2= 3.828, p = 0.15. Maintenance behaviours exhibited did not significantly change between facial emotion pairs, X^2= 2.522, p = 0.28.

Discussion

4.5 In-trial behaviours

Behaviours observed during stimuli presentations highlighted a significantly greater representation of behaviours in the extreme reactive behaviour category (e.g. fear grin, head movement (bob), alarm bark). This difference, however, was only seen between two facial combinations: EO/Ag and EC/Ag, as well as EO/Ag and EO/EC. This consistent significant difference between EO/Ag and any other facial combination highlights that the facial combination EO/Ag has a strong threatening value. As there is no difference between EC/Ag and EO/EC it also illustrates that these stimuli sets have less negative emotional content than EO/Ag. Previous work has shown that these behaviours classified as extreme reactive behaviours are shown in cases of severe distress (Balcombe *et al*., 2004) as a reaction to aversive or threatening information (Maestripieri and Wallen, 1995). In conclusion, showing that EO/Ag is more threatening, particularly more than other facial combinations, demonstrating that the neutral eyes open must also hold a threat value as well as the aggressive face or there would be a significant difference between EC/Ag and EO/EC.

As no difference was seen in any other behavioural categories and only seen in the most ‘extreme’ reactive behaviours this suggests that the EO/Ag stimuli had a high negative value. Reactions from other behaviours may have been expected to show a difference in the less threatening facial combinations. However, this lack of a significant difference only further highlight that other facial combinations were less provocative and suggests that stimuli did not arouse viewers as intensely as EO/Ag did (Machado *et al*., 2011).

4.6 Post-trial behaviours

To further support attention bias results of this study, complimentary behavioural data were collected. Behaviour observations conducted after stimuli presentations showed that there was a significant difference in fear/avoid behaviours (submissive, displaced, flee, fear grin, lip smack with fear grin) between EO/Ag and EC/Ag facial combinations, but no other facial combinations. More fear avoid behaviours were exhibited during presentation of EO/Ag. Previous work has shown that fear avoid behaviours are exhibited more frequently under stressful situations or if an animal is threatened (Cooper and Bernstein, 2002; Cooper *et al*., 2007; Gilbert and Baker, 2011). This therefore implies that the EO/Ag has high negative emotional content and is therefore more aggressive than EC/Ag.

There were four other behavioural categories, however, there were no significant differences between any of these categories with regards to facial combination. These other categories included aggressive behaviour, maintenance behaviours, affiliative behaviours and self-directed behaviours. It was expected that the greatest difference in behavioural reaction to a negative stimuli set would be in fear avoid behaviours as the participant monkey would be apprehensive due to previously seeing threatening images (Pritchard *et al*., 2014) it would therefore be expected that they would be more alert to their conspecifics (Gilbert and Baker, 2011). Nonetheless, research would also suggest there should have been a greater representation of affiliative behaviours (e.g. lip smack, grooming and being groomed). Research has shown that when an animal has been subjected to a negative event, group stability is important and can be maintained though affiliative behaviours (Kutsukake and Castles, 2001). No difference in maintenance behaviours shows the need for these behaviours within the monkey's daily time budget.

The lack of difference between facial combination and any other behaviour category other than fear/avoid behaviours could be due to the time delay in behaviours being recorded. As post-trial behaviours were not recorded directly after viewing stimuli it is not possible to say if the behaviours viewed are as a direct result of stimuli viewed. Behavioural reactions as a result of viewing stimuli may have happened directly after testing and therefore not represented within the five minute observations conducted afterwards.

Chapter 5

Discussion

The main argument of the thesis is that current research neglects to consider the emotional value of the commonly used macaque facial stimuli. With recent developments in cognitive biases, which provide objective measures of welfare in animals, and which use neutral-aggressive stimulus pairings, clarification of emotional content of facial stimuli is paramount to the future success of this research.

The general aim was to test the relative emotional value of eyes open versus eyes closed to determine suitable facial stimuli to be used in future rhesus macaque research. This was conducted using an attention bias paradigm to present three facial expressions in counterbalanced presentations; a neutral face with eyes closed, the same neutral face with eyes open and an aggressive threatening face.

5.1 Main findings

Considering all data, both attention bias results and the complimentary behavioural data, it can be clearly affirmed that facial combinations vary in their emotional content. Data throughout all aspects of the results highlight a trend in facial combinations, suggesting EO/Ag to hold the strongest emotional value.

Attention bias findings show a strong statistical difference between facial combinations, the attention bias was significantly greatest towards the EO/Ag facial combination. Previous attention bias research highlights how there is a predisposition to be more vigilant toward threatening stimuli (humans: Ito *et al*., 1998; Segerstrom, 2001; Smith *et al*., 2006 and non-human primates: Bethell *et al*., 2012a) as a survival instinct (LeDoux, 1996; Damasio, 2000; Rolls, 2000).

Monkeys were most vigilant to EO/Ag combination followed by EC/Ag and finally EO/EC. Within human literature it is commonly accepted view that a human's predisposition to information types is a fitness or survival instinct, (Rolls, 2000). Applying this theory to macaques, it would therefore suggest that EO/Ag is the most threatening combination and EO/EC it the least. This study strongly demonstrates that EO/Ag facial combination has the strongest relative emotional value. Continuous patterns between both facial combinations contain the aggressive faces would further suggest that the strong statistical difference shown in the EO/Ag face must be highly influenced by the neutral eyes open face and therefore this facial stimulus must have a strong negative emotional value. Therefore attention bias data clearly shows that the eyes closed facial stimulus is a more suitable neutral stimulus relative to the eyes open facial stimulus.

This study's behavioural data also parallels this trend in emotional content of facial stimuli. Post-trial behaviours showed a significant greater representation of fear avoid behaviours after the presentation of EO/Ag facial combination stimuli compared to EC/Ag, highlighting strong emotional content of this stimuli set. Research has shown this behaviour category is only displayed in times of threat and high stress (Cooper and Bernstein, 2002; Cooper *et al*., 2007; Gilbert and Baker, 2011). As there was no difference in any other behaviours across any other facial combinations. This would prove that, again, this greater representation of behaviours can not only be due to the presence of an aggressive facial expression but must also be somewhat due to the neutral eyes open facial expression.

Behavioural reactions during testing also show a similar pattern. There was a significant difference between extreme reactive behaviours. Extreme behaviours are exhibited as a sign of stress towards a threatening stimulus (Maestripieri and

Wallen, 1995; Balcombe *et al*., 2004; Gottlieb and Capitanio, 2013; Beisner and McCowan, 2014). This significant difference showed a greater representation of extreme reactive behaviours towards EO/Ag compared to EC/Ag as well as a greater representation of extreme reactive behaviours towards EO/Ag compared to EO/EC. This is even clearer evidence than post-trial behaviours reactions, as it shows that there is a consistent difference between EO/Ag and all facial combinations, however not between EC/Ag and EO/EC. This therefore highlights that this trend must be somewhat due to the eyes open face and not just the aggressive face alone.

Evidence from all measures clearly shows the same trend that EO/Ag facial combination has a strong negative emotional content. This pattern exhibited in EO/Ag facial combination can clearly not be due to the presence of an excess of negative emotion and high arousal (Bethell *et al*., 20012b). This consistency clearly highlights that the neutral eyes open facial expression itself can be deemed to have a negative emotional content. If the impact of this facial combination EO/Ag was solely due to the aggressive face a consistently greater significant difference between EO/Ag and EC/Ag would not be so common and both combinations would have a similar emotional value. The use of neutral eyes open facial stimulus as a counter balance to the threatening facial stimulus can be used within attention bias testing, as it still shows a statistical variation between neutral and aggressive faces (Bethell *et al*., 2012a). However, this facial stimulus cannot be deemed as neutral its emotional content. This clarity of emotional content of facial stimuli for a rhesus macaque will help progress animal welfare. With a better understanding of the emotional value of stimuli used, current attention bias methods can be refined; therefore attention bias as a measure of stress can be more reliably measured with appropriate facial stimuli. Measuring attention bias regularly in captive populations can help create a baseline and highlight a heightened stress level in a macaque much sooner than conventional behavioural techniques, thus improving welfare measures.

5.2 Wider Applications

5.2.1 Facial stimuli

As was previously outlined, many studies use macaque facial stimuli with little consideration to the underlying emotional value. The neutral facial stimulus of a macaque is often considered neutral in comparison to what we as humans interpret as neutral (Cohn and Kanade, 2006). To current knowledge, no previous paper has yet reviewed the emotional value of facial stimuli.

MAQFACs is a muscle based facial coding system based on anatomical and muscular movements of rhesus macaques (Parr *et al*., 2010), providing one of the clearest reviews of macaque facial expressions. However, Parr *et al*.'s (2010) work is based on comparisons to human and non-human primates' muscular anatomy and facial expressions and does not consider the emotions that are being portrayed by the facial expressions these muscles are forming. Although the work of Parr *et al*. (2010) inherently describes muscular facial expressions and coding systems and the anatomical applicability of this study is unquestionable. Still, the results of the current study would suggest that a review of facial expressions in general with regard to emotional content should be combined with the work of Parr *et al*. (2010). Clarity of this emotional value of facial expressions from such an esteemed coding system would have direct impacts upon future use of facial stimuli within non-human primate research.

Nevertheless, many studies use a neutral facial stimulus of a macaque with eyes open. Although the stimuli they are using are neutral in respect of perceived emotional content in comparison to their counter expression, there is still a high underlying strong emotionally provocative value. For example, Dahl *et al*. (2009) compared facial processing strategies in humans, using an open eyed face manipulated by inverting and blurring images. The processing strategies discussed in Dahl *et al*.'s (2009) findings are unlikely to change using an eyes closed neutral facial stimulus, macaques are however more likely to have a heightened vigilance to the more emotionally provocative facial stimuli. Michelatta *et al*. (2015a) studied crested macaques (*Macaca nigra*) and looked at how they matched facial expressions both using still images and videos, again classing neutral as an open eyed macaque. However, as Michelatta *et al*.'s (2015a) aim was to explore the ability to discriminate facial expressions and look at visual similarities, their results may be hindered by the high negative emotional content of their neutral macaque facial stimulus. Results of the current study suggest that the open eyed facial stimulus would hold a negative threat value, macaques might discriminate this neutral facial expression differently. Nevertheless, it is important to remember that the macaques used in Michelatta *et al*.'s (2015a) study where zoo housed crested macaques and may therefore interpret emotions differently to the rhesus macaques in this study.

5.2.2 Captive management and welfare

Bethell *et al*. (2012a) provided ground-breaking work using attention bias as welfare tool. However, Bethell *et al*. (2012a) used images of an aggressive monkey against a monkey with open eyes, presuming relatively neutral emotional content levels of the eyes open facial stimulus. Applying the results of this current study, to that of Bethell *et al*. (2012a), would suggest the facial stimuli used were not suitable as a neutral comparison, as the open eyes facial stimulus holds a level of threat value (Chevalier-Skolnikoff, 1973; Van Hooff, 1976; Machado and Bachevalier, 2006). Although Bethell *et al*. (2012a) found significant results that cannot be denied, findings of the current study would suggest that if facial stimuli of a monkey with eyes closed were used instead of eyes open, there would be a greater vigilant avoidance. These results would also present a more true representation of attention bias with regards to facial emotional value. Furthermore, it could also be argued that due the high emotional content of stimuli used in Bethell *et al*.'s (2012a) study monkeys' attention bias could have been as a result of exposure to high emotional content of aversive stimuli rather than the conditions in which the attention bias was measured.

Moreover, current work by Kemp (C.Kemp pers.comm.), does use, what the results of this current study would suggest to be, more appropriate facial stimuli to test attention bias in macaques; using an aggressive face against a neutral face with eyes closed. Presently the results of Kemp's study are still being analysed. Therefore a comparison of the use of stimuli under attention bias conditions as a suitable measure of welfare, comparing results when stressed against results under a period of enrichment, is not currently possible.

Even though a comparison between the findings of Bethell *et al*. (2012a) and Kemp (C.Kemp pers.comm.) is not currently possible, results of the current study in question would suggest that the methods and facial stimuli used by Kemp are preferable as a measure of attention bias and a monkey's welfare. Henceforth, the results of this study are able to develop attention bias methods for the use in captive macaque welfare management. The clarification of suitable methods for the use of attention bias as a welfare tool for macaques will have a significant impact in captive management.

Measuring an animal's psychological wellbeing is a key goal to animal welfare, however until recent developments of attention bias in macaques (Bethell *et* al., 2012b) there have been few methods available to assess psychological processes in animals (Paul *et al.,* 2005). Previous research in macaques (Bethell *et al*., 2012a) as well as research in many other species starlings (Bateson and Matheson, 2007), dogs (Burman *et al*., 2011), hamsters (Bethell and Koyama, 2015), to name but a few, show the use of these, once more commonly used human measures, to be successful cognitive measures in non-human animals too. Development of the attention bias method as a welfare tool (Bethell *et al*, 2012a) is of vital importance due to the psychological distress suffered by macaques through scientific research (Home Office 2010). If an animal has the ability to suffer psychological distress a realistic measure of this is needed (Paul *et al*., 2005).

Primates, particularly macaques, are commonly used for biomedical research in the UK (European Commission, 2009; Home Office, 2010; Prescott, 2010). Procedures are often performed on awake monkeys causing a risk of psychological distress (Novak and Suomi, 1988; Home Office, 2010). As macaques are known to have similar socio-cognitive needs to humans (Brent *et al*., 2011, 2013), this psychological distress caused is a concern for animal welfare (Animal Procedures Committee, 2002; NC3Rs, 2006; Weatherall, 2006; Nelson and Winslow, 2009; Prescott, 2010; Bateson, 2011). Research into measuring this level of distress is improving with the development of attention bias (Bethell *et al*., 2012b), however the clarity of emotional content of facial stimuli provided through this thesis will further help develop this method and therefore aid in animal welfare.

In addition, to being a measure of stress suffered as a result of scientific procedures the development of attention bias methodology in macaques will have wide reaching applications. With future research this methodology can be applied not only for macaques, but also other non-human primate species, both within research facilities and other captive settings as a measure of general wellbeing. Current development of the attention bias methodology also aims to use eye-tracking software to make the attention bias test a more convenient user-friendly hand held tool that can be used by care staff with minimal training.

5.3 Training methods

Training animals to partake in the study was a key element. Training created habituation and a relationship between the animal and trainer, beneficial both for the welfare of the animal and the success of training (Waitt *et al*., 2002; Samuni *et al*., 2014). Training also reduced stress levels compared to other techniques commonly used with laboratory animals such as the push back mechanism (Laule *et al*., 2003; Prescott and Buchanan-Smith, 2003). Although training did encourage animal participation and improve their levels of cooperation through repeated exposure, not all animals could be trained to the same standard due to time constraints of the study. Training participating animals began eight months prior to the start of this project, during a summer internship. These animals were trained in hope of being used for scientific studies. Due to unforeseen circumstances (e.g. injuries, translocation, animals willingness to participate altered by event such as having a baby or moving group), not all of the animals trained could be used in this study. Therefore more animals were trained at the start of September, three months prior to the start of this project, to increase sample size.

As animals were trained at different rates, standards of training were not consistent across all animals. Further to this some animals progressed through the training protocol at a much quicker rate, often due to age; for example younger animals learnt training measures much quicker (Schaprio *et al*., 2003) and exposure of watching other animals be trained (Prescott and Buchanan-Smith, 1999). Although all animals that participated in the study reached a minimum desired level of training standard, due to progress rates some animals received more repeated training sessions, improving their performance much more reliably on command than those who had received less. If training standards were more consistent, it would improve participation rates and reduce stress. Perlman *et al*. (2012) echo the importance of structure and consistency to a training regime for success. Furthermore, Bliss-Moreau *et al*. (2013) notes that understanding individual needs within training is paramount for efficient learning and group composition, in turn reducing stress. Improving training and henceforth testing procedures would have a direct impact on post-trial behaviour observations. This would have allowed behavioural observations to be conducted closer to the presentation of stimuli. Behavioural observations were attempted to be conducted as soon as possible after the trials. However, this was not always possible sometimes-behavioural observations would not be conducted until 45 minutes after the monkeys had seen the facial stimuli, primarily due to monkey participation rates. If behavioural observations could have been conducted immediately after testing results would have had a more realistic reflection on the effect of stimuli exposure on an animal's behaviour and perhaps a greater difference in behaviour repertoire exhibited.

As well as training rates, other factors influenced the success of an animal's performance within testing conditions. To improve welfare conditions staff at the facility regularly trained the monkeys (Prescott *et al*., 2005). However, training performed by facility staff was not always in line with the training protocol and therefore monkeys were receiving different levels of training and for different desired behaviours, which can cause confusion for the animal and reduce likelihood of successful performance of behaviours in future sessions (Perlman *et al*., 2012).

Overall, if all of the above factors were considered and training regimes were run more efficiently, being aware of factors that may influence monkey's participation and mutually agreed training protocol, more animals could have been trained increasing sample size.

5.4 Stimuli

Results of the study clearly show that previous exposure influenced viewing preference, suggesting a habituation to the stimuli (Wright *et al*., 2001; Amir *et al*., 2009). If more facial stimuli were available, the effect of exposure to stimuli could be further tested, the influence of habituation to stimuli and the extent to which this influenced results could be very important for the development of attention bias in rhesus macaques and other species. Another option to test this theory would be to test the animals that received no previous exposure to the stimuli again to see if they show a similar pattern in viewing preferences to that of the previously exposed group. Although the monkeys that previously saw the stimuli took part in a similar style attention bias test (C.Kemp pers.comm.), these results cannot be compared to the current study. This is due to too many differences within the methodology, such as testing during the annual veterinarian check, which may have also influenced viewing preference.

Positive stimuli used were chosen as a result of basic preliminary observations and recordings of animals' reaction to images, using basic behavioural measures to assess macaques' emotional state (Partan, 2002). However, to fully assess the suitability of a positive stimulus for use in future study videos from the current study and that by Kemp (C.Kemp pers.comm.) could be used to compare reactions to stimuli, considering nulliparous individuals. Research shows that sexual status influences viewing preference to infant stimuli (Maestripieri and Wallen, 1995; Waitt *et al*., 2007), preliminary observations highlighted this difference. Although the positive stimuli used were successful at orientation and providing a salient reward for monkeys' participation (Anderson, 1998), finding the most suitable rewarding stimuli will be helpful in the development of attention bias measures as a macaque welfare tool.

The results of this study evidently show a strong negative emotional content of the neutral eyes open facial stimuli. However, as the neutral eyes closed face was created using Photoshop, blurring images to create eyelids to give the impression of eyes closed, the photo does not truly represent a monkey with eyes closed. The images created were compared to photos and videos of monkeys with their eyes closed at MRC CFM to create a likeness in editing. To improve upon this current study, images of monkeys with their eyes closed and counter aggressive and eyes open faces could be collated and compared to further validate these findings.

Further to improving stimuli quality, stimuli collections could also be improved. By expanding the wealth of stimuli available for use in attention bias paradigm tests, we could test other possible ‘neutral’ facial stimuli. As monkeys don’t generally look head on with their eyes closed for more than a few milliseconds when they blink it could be argued that the eyes closed stimuli is therefore not a natural expression monkeys are likely to see and they may perceive it as intriguing or with hesitance of the possible outcome when the stimulus monkey opens their eyes. Averted eyes is another possible expression that could be used as a facial stimulus. Using an averted face looking with eyes to the floor could be seen as a more natural stance of relaxation. However, Hoffman *et al*. (2007) found an averted eyes facial stimulus to be highly arousing to monkeys. Therefore a combination of the two could be suggested with a monkey with eyes closed in an averted stance. Nevertheless, it is a lengthy process to create a large dataset of these facial stimuli and would have to be created using unfamiliar stimulus monkeys to those monkeys that are being tested. Hopefully, improving the accessibility of facial stimuli for use in macaque research can be noted as a necessity from the results of this study.

Further stimuli other than facial expression could also be tested, for example a blurred image of the same facial stimuli could be used as a counterpart so that no emotional expression could be perceived from the stimulus. Other options to improve the ambiguity of facial stimuli could be to test attention bias with objects other than faces. For example a threatening object such as a net used for capturing animals against an impassive object such as a chair.

5.5 External Factors

Working within a breeding facility created many factors that were beyond our control. Staffing issues or external visitors would cause disruption to the animals' daily husbandry routine; previous studies have highlighted how disrupting events such as these can be (Burn and Mason, 2008; Rommeck *et al*., 2011). Therefore the severity of these disruption and possible stress caused was assessed to see if testing was viable or if the high stress levels would be influential over the monkeys' attention bias scores. Furthermore, as animal welfare is a primary concern, all efforts to improve the monkeys' welfare were prioritised. Unfortunately this could cause spontaneous changes to husbandry routines and therefore the testing schedule.

Due to allowing for a sufficient period of animal training and other factors to the timetable such as holidays, staff numbers and key events in the animal husbandry protocol the period of testing was strategically timetabled. Testing could not be carried out past the last date set in the timetable due to key events in husbandry routine that would likely cause high stress levels. Testing was therefore conducted in a relatively short period of time. If testing could have been spaced over a longer period of time, it could have accounted for any habituation to the stimuli over repeated exposure during the testing period. As animals were exposed to facial stimuli repeatedly during a relatively small period of time, it would suggest increased rates of habituation (Wright *et al*., 2001; Amir *et al*., 2009). Furthermore, if testing was prolonged it would have allowed for greater consideration of other external factors such as mating season and birthing season.

5.6 Further direction

5.6.1 Facial stimuli

Further study to clearly define the emotion value of facial expressions could look at individual facial expressions alone. Behaviour observations could be conducted of reactions to individual facial expressions, measuring a single photos emotional content. This would help to highlight individual emotional content, without any possible effect of an opposing facial expression influencing the monkey's gaze. Unfortunately due to the strong effects of habituation, repeated exposure reducing responses to stimuli, these further measures could not be conducted within the realms of this study without over exposing individuals to stimuli (Wright *et al*., 2001; Amir *et al*., 2009) and affecting results.

Studies currently at Marwell Zoo in collaboration with the University of Portsmouth are examining crested macaques, *Macaca nigra,* and the use of facial stimuli. Micheletta *et al*. (2015a) studied facial recognition in macaques looking at familiar and unfamiliar faces, their results showed that they could match facial expressions of familiar monkeys easier. Micheletta *et al*. (2015b) produced similar paper on familiar and unfamiliar face recognition. Although both of these papers show the use of single facial stimuli for macaques to interpret facial expressions, they do not consider emotional values, showing a need for future study.

5.6.2 Rank as a variable

Although rank was recorded it was not included as a possible influencing measure within this study as it was measured subjectively. Measuring rank as a dyadic measure creating a matrix for individuals would have allowed us to investigate the influence of this upon attention bias and interpretation of facial stimuli. Previous studies have shown that rank can influence different underling motivational factors in viewing preferences (Kyes *et al*., 1992), Furthermore, the underlying dominance related to a threat face (Da Wall and Luttrell, 1989) would imply that reactions would vary dependent upon social rank.

5.7 Conclusion

To summarise, the findings of this study did address the primary aim: monkeys were more vigilant to different facial stimuli, suggesting a difference in perception of emotional content of facial stimuli tested. With regards to suitable neutral stimuli, although previous studies have referred to the expression of a monkey with eyes open as neutral (Partan, 2002; Hoffman *et al*., 2007; Dahl *et al*., 2009; Parr *et al*. 2010; Bethell *et al*., 2012a; Micheletta *et al*., 2015a; Micheletta *et al*., 2015b), results of this research conclude this is incorrect. While this image of a macaque may be neutral in respect to an opposing aggressive image, the image of a macaque with open eyes is more negative in respect to an opposing eyes closed image as shown through attention bias and behavioural measures. This result was hypothesised as direct stare is commonly regarded as a threat in macaques (Chevalier-Skolnikoff, 1973; Van Hooff, 1976; Machado and Bachevalier, 2006). Therefore granting studies that have used an open eyed face as neutral facial stimulus (e.g. Dahl *et al*., 2009; Bethell *et al*., 2012a; Micheletta *et al*., 2015a; Micheletta *et al*., 2015b) may still be viable in that this stimulus is less emotionally arousing compared to an opposing aggressive facial stimulus, the term neutral should not be used to describe the emotional content of this facial stimulus. Instead I suggest that a neutral expression with eyes closed is a more appropriate neutral facial stimulus than a neutral face with eyes open. Although further work is needed to compare other possible neutral facial stimulus options.

It is hoped that these findings clarify the emotional content of facial stimuli and the phrase ‘neutral’, used so colloquially, will help improve primate welfare. Attention bias as a welfare tool for macaques, to measure stress, is continuously developing (Bethell *et al*., 2012a; Bethell *et al*., 2012b; C.Kemp pers.comm.). With clarification of suitable stimuli use hopefully this research will help to further develop attention bias use in macaques and lead to further work in the field of primate welfare and attention bias.

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Appendices

Appendices content:

	Page(s)

1)

Timetable showing the order of trials received.

Week Commencing	Monday	Tuesday	Thursday	Friday
09/02/2015	Groups 1 and 2 Trial 1	Groups 3 and 4 Trial 1	Groups 1 and 2 Trial 2	Groups 3 and 4 Trial 2
16/02/2015	Groups 5 and 6 Trial 1	Groups 7 and 8 Trial 1	Groups 5 and 6 Trial 2	Groups 7 and 8 Trial 2
23/02/2015	Groups 3 and 4 Trial 3	Groups 1 and 2 Trial 3	Groups 3 and 4 Trial 4	Groups 1 and 2 Trial 4
02/03/2015	Groups 7 and 8 Trial 3	Groups 5 and 6 Trial 3	Groups 7 and 8 Trial 4	Groups 5 and 6 Trial 4
09/03/2015	Groups 1 and 2 Trial 5	Groups 3 and 4 Trial 5	Groups 1 and 2 Trial 6	Groups 3 and 4 Trial 6
16/03/2015	Groups 5 and 6 Trial 5	Groups 7 and 8 Trial 5	Groups 5 and 6 Trial 6	Groups 7 and 8 Trial 6
23/03/2015	Groups 1 and 2 Trial 7	Groups 3 and 4 Trial 7	Groups 1 and 2 Trial 8	Groups 3 and 4 Trial 8
30/03/2015	Groups 7 and 8 Trial 7	Groups 5 and 6 Trial 7	Groups 7 and 8 Trial 8	Groups 5 and 6 Trial 8
06/04/2015	Groups 3 and 4 Trial 9	Groups 1 and 2 Trial 9	Groups 3 and 4 Trial 10	Groups 1 and 2 Trial 10
13/04/2015	Groups 5 and 6 Trial 9	Groups 7 and 8 Trial 9	Groups 5 and 6 Trial 10	Groups 7 and 8 Trial 10
20/04/2015	Trial Groups 1 and 2 11	Groups 3 and 4 Trial 11	Groups 1 and 2 Trial 12	Groups 3 and 4 Trial 12
27/04/2015	Groups 7 and 8 Trial 11	Groups 5 and 6 Trial 11	Groups 7 and 8 Trial 12	Groups 5 and 6 Trial 12

2)

<u>Development of null model</u>

Test1<-lmer(cbind ~ (1|Group/MonkeyID), data = dat, REML=T)

Test2<-lmer(cbind ~ (1|Matriline/MonkeyID), data = dat, REML=T)

Test3<-lmer(cbind ~ (1|MonkeyID), data=dat, REML=T)

> model.sel(Test1, Test2, Test3)

Model selection table

random df logLik   AICc   delta weight

Test2 MT/MI   3 -1418.544 2843.1 0.00 0.549

Test3 MI 4 -1418.312 2844.7 1.56 0.252

Test1 G/MI   4 -1418.544 2845.1 2.02 0.199

Models ranked by AICc(x)

Random terms:

MI = ‘1 | MonkeyID’

Mt/MI = ‘1 | Matriline/MonkeyID’

G/MI = ‘1 | Group/MonkeyID’

#monkey matriline nested in monkey ID strongest alone strongest so retest with additional values

Test1<-lmer(cbind ~ (1|MonkeyID), data = dat, REML=T)

Test2<-lmer(cbind ~ (1|Group) + (1|MonkeyID), data = dat, REML=T)

Test3<-lmer(cbind ~ (1|Matriline) + (1|MonkeyID), data = dat, REML=T)

Test4<-lmer(cbind ~ (1|Matriline/MonkeyID), data = dat, REML=T)

Model selection table

random df logLik   AICc   delta weight

Test4 MT/MI   3 -1418.544 2843.1 0.00 0.549

Test1 MI   3 -1418.544 2843.1 0.08 0.202

Test3 Mt+MI 4 -1418.312 2844.7 1.56 0.150

Test2 G+MI   4 -1418.544 2845.1 2.02 0.099

Models ranked by AICc(x)

Random terms:

MI = ‘1 | MonkeyID’

Mt = ‘1 | Matriline’

G = ‘1 | Group’

Mt/MI = ‘1 | Matriline/MonkeyID’

 # add more variations nesting matriline within monkey ID

Test10<-glmer(Cbind ~ (1| Matriline/MonkeyID), data = dat, family=binomial)

Test11<-glmer(Cbind ~ (1|Trial) + (1|Matriline/MonkeyID), data = dat, family=binomial)

Test12<-glmer(Cbind ~ (1|Presentation) + (1|Matriline/MonkeyID), data = dat, family=binomial)

Test13<-glmer(Cbind ~ (1|Matriline) + (1|Matriline/MonkeyID), data = dat, family=binomial)

Test14<-glmer(Cbind ~ (1|AgeMos) + (1|Matriline/MonkeyID), data = dat, family=binomial)

Test15<-glmer(Cbind ~ (1|ReproductiveStatus) + (1|Matriline/MonkeyID), data = dat, family=binomial)

Test16<-glmer(Cbind ~ (1| Combir) + (1|Matriline/MonkeyID), data = dat, family=binomial)

Test17<-glmer(Cbind ~ (1| Previous.exposure) + (1|Matriline/MonkeyID), data = dat, family=binomial)

model.sel(Test10, Test11, Test12, Test13, Test14, Test15, Test16, Test17, Test18)

> model.sel(Test10, Test11, Test12, Test13, Test14, Test15, Test16, Test17)

Model selection table

random   df logLik   AICc   delta   weight

Test11 T+Mt/MI   4 -69442.02 138892.2   0.00 1

Test12 Prs+Mt/MI   4 -72379.80 144767.7 5875.57 0

Test16 C+Mt/MI   4 -74264.00 148536.1 9643.97 0

Test10 Mt/MI   3 -74623.23 149252.5 10360.38 0

Test15 PS+Mt/MI   4 -74623.23 149254.6 10362.43 0

Test13 G+Mt/MI   4 -74623.23 149254.6 10362.43 0

Test17 Prv.e+Mt/MI 4 -74623.23 149254.6 10362.43 0

Test14 A+Mt/MI 4 -74623.23 149254.6 10362.43 0

Models ranked by AICc(x)

Random terms:

Prs = ‘1 | Presentation’

Mt/MI = ‘1 | Matriline/MonkeyID’

T = ‘1 | Trial’

C = ‘1 | Combir’

PS = ‘1 | ReproductiveStatus’

G = ‘1 | Matriline’

Prv.e = ‘1 | Previous.exposure’

A = ‘1 | AgeMos

Test18<-glmer(Cbind ~ (1| Matriline/MonkeyID), data = dat, family=binomial)

Test19<-glmer(Cbind ~ (1|Matriline) + (1| Matriline/MonkeyID), data = dat, family=binomial)

Test20<-glmer(Cbind ~ (1|Trial) + (1| Matriline/MonkeyID), data = dat, family=binomial)

Test21<-glmer(Cbind ~ (1|Presentation) + (1| Matriline/MonkeyID), data = dat, family=binomial)

Test22<-glmer(Cbind ~ (1|AgeMos) + (1| Matriline/MonkeyID), data = dat, family=binomial)

Test23<-glmer(Cbind ~ (1|ReproductiveStatus) + (1| Matriline/MonkeyID), data = dat, family=binomial)

Test24<-glmer(Cbind ~ (1| Combir) + (1| Matriline/MonkeyID), data = dat, family=binomial)

Test25<-glmer(Cbind ~ (1| Previous.exposure) + (1| Matriline/MonkeyID), data = dat, family=binomial)

model.sel(Test18, Test19, Test20, Test21,Test22,Test23,Test24,Test25)

Model selection table

random df logLik AICc delta weight

Test20 T+G/MI 4 -69442.02 138892.2 0.00 1

Test21 Prs+G/MI 4 -72379.80 144767.7 5875.57 0

Test24 C+G/MI 4 -74264.00 148536.1 9643.97 0

Test18 G/MI 3 -74623.23 149252.5 10360.38 0

Test25 Prv.e+G/MI 4 -74623.23 149254.6 10362.43 0

Test19 Mt+G/MI 4 -74623.23 149254.6 10362.43 0

Test23 PS+G/MI   4 -74623.23 149254.6 10362.43 0

Test22 A+G/MI   4 -74623.23 149254.6 10362.43 0

Models ranked by AICc(x)

Random terms:

Prs = ‘1 | Presentation’

G/MI = ‘1 | Matriline/MonkeyID’

T = ‘1 | Trial’

C = ‘1 | Combir’

Prv.e = ‘1 | Previous.exposure’

Mt = ‘1 | Matriline’

PS = ‘1 | ReproductiveStatus’

A = ‘1 | AgeMos’

 # use test:

Test21<-glmer(cbind~ (1|Trial) + (1| Matriline/MonkeyID), data = dat, family=binomial)

3)

Candidate models:

Test1<-glmer(Cbind ~ Facial.combination + (1|Trial) + (1| Matriline/MonkeyID), data = dat, family=binomial)

Test2<-glmer(Cbind ~ Stimuli + (1|Trial) + (1| Matriline/MonkeyID), data = dat, family=binomial)

Test3<-glmer(Cbind ~ Face + (1|Trial) + (1| Matriline/MonkeyID), data = dat, family=binomial)

Test4<-glmer(Cbind ~ Previous.exposure + (1|Trial) + (1| Matriline/MonkeyID), data = dat, family=binomial)

Test5<-glmer(Cbind ~ ReproductiveStatus+ (1|Trial) + (1| Matriline/MonkeyID), data = dat, family=binomial)

Test6<-glmer(Cbind ~ Stimuli+ (1|Trial) + (1| Matriline/MonkeyID), data = dat, family=binomial)

Test7<-glmer(Cbind ~ Face + Facial.combination*Stimuli + (1|Trial) + (1| Matriline/MonkeyID), data = dat, family=binomial)

Test8<-glmer(Cbind ~ Face + Facial.combination*Stimuli + (1|Trial) + (1| Matriline/MonkeyID), data = dat, family=binomial)

Test9<-glmer(Cbind ~ Previous.exposure + Facial.combination*Stimuli + (1|Trial) + (1| Matriline/MonkeyID), data = dat, family=binomial)

Test10<-glmer(Cbind ~ ReproductiveStatus + Facial.combination*Stimuli + (1|Trial) + (1| Matriline/MonkeyID), data = dat, family=binomial)

Test11<-glmer(Cbind ~ Facial.combination + ReproductiveStatus*Previous.exposure + (1|Trial) + (1| Matriline/MonkeyID), data = dat, family=binomial)

Test12<-glmer(Cbind ~ Stimuli + ReproductiveStatus*Previous.exposure + (1|Trial) + (1| Matriline/MonkeyID), data = dat, family=binomial)

Test13<-glmer(Cbind ~ Facial.combination + ReproductiveStatus*Stimuli + (1|Trial) + (1| Matriline/MonkeyID), data = dat, family=binomial)

Test14<-glmer(Cbind ~ Previous.exposure + ReproductiveStatus*Stimuli + (1|Trial) + (1| Matriline/MonkeyID), data = dat, family=binomial)

Test15<-glmer(Cbind ~ Facial.combination + Previous.exposure*Stimuli + (1|Trial) + (1| Matriline/MonkeyID), data = dat, family=binomial)

Test16<-glmer(Cbind ~ ReproductiveStatus + Previous.exposure*Stimuli + (1|Trial) + (1| Matriline/MonkeyID), data = dat, family=binomial)

```
Test17<-glmer(Cbind ~ ReproductiveStatus + Facial.combination + Stimuli + (1|Trial) + (1|
Matriline/MonkeyID), data = dat, family=binomial)
Test18<-glmer(Cbind ~ Facial.combination + (1|Trial) + (1| Matriline/MonkeyID), data = dat,
family=binomial)
Test19<-glmer(Cbind ~ ReproductiveStatus + (1|Trial) + (1| Matriline/MonkeyID), data = dat,
family=binomial)
Test20<-glmer(Cbind ~ Stimuli + (1|Trial) + (1| Matriline/MonkeyID), data = dat, family=binomial)
Test21<-glmer(Cbind ~ ReproductiveStatus* Facial.combination + Stimuli + (1|Trial) + (1|
Matriline/MonkeyID), data = dat, family=binomial)
Test22<-glmer(Cbind ~ ReproductiveStatus + Facial.combination + Previous.exposure + (1|Trial)
+ (1| Matriline/MonkeyID), data = dat, family=binomial)
Test23<-glmer(Cbind ~ ReproductiveStatus + Facial.combination*Previous.exposure + (1|Trial) +
(1| Matriline/MonkeyID), data = dat, family=binomial)
Test24<-glmer(Cbind ~ Group + Facial.combination + Previous.exposure + (1|Trial) + (1|
Matriline/MonkeyID), data = dat, family=binomial)
Test25<-glmer(Cbind ~ ReproductiveStatus*Facial.combination + AgeMos + Previous.exposure
+ (1|Trial) + (1| Matriline/MonkeyID), data = dat, family=binomial)
Test26<-glmer(Cbind ~ ReproductiveStatus + Facial.combination + AgeMos+ (1|Trial) + (1|
Matriline/MonkeyID), data = dat, family=binomial)
Test27<-glmer(Cbind ~ ReproductiveStatus + Facial.combination + Previous.exposure*Group +
(1|Trial) + (1| Matriline/MonkeyID), data = dat, family=binomial)
Test28<-glmer(Cbind ~ ReproductiveStatus*AgeMos + Facial.combination + Previous.exposure+
(1|Trial) + (1| Matriline/MonkeyID), data = dat, family=binomial)
Test29<-glmer(Cbind ~ ReproductiveStatus + Facial.combination + Stimuli + Trial + (1|Trial) + (1|
Matriline/MonkeyID), data = dat, family=binomial)
Test30<-glmer(Cbind ~ AgeMos + ReproductiveStatus + (1|Trial) + (1| Matriline/MonkeyID), data
= dat, family=binomial)
Test31<-glmer(Cbind ~ AgeMos + Previous.exposure+ (1|Trial) + (1| Matriline/MonkeyID), data =
dat, family=binomial)
Test32<-glmer(Cbind ~ AgeMos + Facial.combination + (1|Trial) + (1| Matriline/MonkeyID), data
= dat, family=binomial)
Test33<-glmer(Cbind ~ ReproductiveStatus + Previous.exposure + (1|Trial) + (1|
Matriline/MonkeyID), data = dat, family=binomial)
```

Test34<-glmer(Cbind ~ ReproductiveStatus + Facial.combination + (1|Trial) + (1| Matriline/MonkeyID), data = dat, family=binomial)

Test35<-glmer(Cbind ~ AgeMos*ReproductiveStatus + (1|Trial) + (1| Matriline/MonkeyID), data = dat, family=binomial)

Test36<-glmer(Cbind ~ AgeMos*Previous.exposure+ (1|Trial) + (1| Matriline/MonkeyID), data = dat, family=binomial)

Test37<-glmer(Cbind ~ AgeMos*Facial.combination + (1|Trial) + (1| Matriline/MonkeyID), data = dat, family=binomial)

Test38<-glmer(Cbind ~ ReproductiveStatus*Previous.exposure + (1|Trial) + (1| Matriline/MonkeyID), data = dat, family=binomial)

Test39<-glmer(Cbind ~ ReproductiveStatus*Facial.combination + (1|Trial) + (1| Matriline/MonkeyID), data = dat, family=binomial)

Test40<-glmer(Cbind ~ Facial.combination + AgeMos*ReproductiveStatus + (1|Trial) + (1| Matriline/MonkeyID), data = dat, family=binomial)

Test41<-glmer(Cbind ~ Previous.exposure + AgeMos*ReproductiveStatus + (1|Trial) + (1| Matriline/MonkeyID), data = dat, family=binomial)

Test42<-glmer(Cbind ~ Facial.combination + AgeMos*Previous.exposure + (1|Trial) + (1| Matriline/MonkeyID), data = dat, family=binomial)

Test43<-glmer(Cbind ~ Previous.exposure + AgeMos*Facial.combination + (1|Trial) + (1| Matriline/MonkeyID), data = dat, family=binomial)

Test44<-glmer(Cbind ~ ReproductiveStatus + AgeMos*Facial.combination + (1|Trial) + (1| Matriline/MonkeyID), data = dat, family=binomial)

Test45<-glmer(Cbind ~ AgeMos + ReproductiveStatus*Previous.exposure + (1|Trial) + (1| Matriline/MonkeyID), data = dat, family=binomial)

Test46<-glmer(Cbind ~ AgeMos + ReproductiveStatus*Facial.combination + (1|Trial) + (1| Matriline/MonkeyID), data = dat, family=binomial)

Test47<-glmer(Cbind ~ Previous.exposure + ReproductiveStatus*Facial.combination + (1|Trial) + (1| Matriline/MonkeyID), data = dat, family=binomial)

Test48<-glmer(Cbind ~ AgeMos + Previous.exposure*Facial.combination + (1|Trial) + (1| Matriline/MonkeyID), data = dat, family=binomial)

Test49<-glmer(Cbind ~ ReproductiveStatus + Previous.exposure*Facial.combination + (1|Trial) + (1| Matriline/MonkeyID), data = dat, family=binomial)

Test50<-glmer(Cbind ~ Facial.combination + ReproductiveStatus + (1|Trial) + (1| Matriline/MonkeyID), data = dat, family=binomial)

Test51<-glmer(Cbind ~ Facial.combination + Previous.exposure+ (1|Trial) + (1| Matriline/MonkeyID), data = dat, family=binomial)

Test52<-glmer(Cbind ~ Facial.combination + Stimuli + (1|Trial) + (1| Matriline/MonkeyID), data = dat, family=binomial)

Test53<-glmer(Cbind ~ ReproductiveStatus + Stimuli + (1|Trial) + (1| Matriline/MonkeyID), data = dat, family=binomial)

Test54<-glmer(Cbind ~ Facial.combination*Previous.exposure+ (1|Trial) + (1| Matriline/MonkeyID), data = dat, family=binomial)

Test55<-glmer(Cbind ~ ReproductiveStatus + Previous.exposure + (1|Trial) + (1| Matriline/MonkeyID), data = dat, family=binomial)

Tes56<-glmer(Cbind ~ AgeMos*ReproductiveStatus + (1|Trial) + (1| Matriline/MonkeyID), data = dat, family=binomial)

Test57<-glmer(Cbind ~ AgeMos*Previous.exposure+ (1|Trial) + (1| Matriline/MonkeyID), data = dat, family=binomial)

Test58<-glmer(Cbind ~ Previous.exposure + AgeMos*Facial.combination + (1|Trial) + (1| Matriline/MonkeyID), data = dat, family=binomial)

Test59<-glmer(Cbind ~ Facial.combination*Stimuli + (1|Trial) + (1| Matriline/MonkeyID), data = dat, family=binomial)

Test60<-glmer(Cbind ~ ReproductiveStatus*Stimuli + (1|Trial) + (1| Matriline/MonkeyID), data = dat, family=binomial)

Test61<-glmer(Cbind ~ Stimuli + Facial.combination*Facial.combination + (1|Trial) + (1| Matriline/MonkeyID), data = dat, family=binomial)

Test62<-glmer(Cbind ~ ReproductiveStatus + Facial.combination*Facial.combination + (1|Trial) + (1| Matriline/MonkeyID), data = dat, family=binomial)

Test63<-glmer(Cbind ~ Facial.combination + Facial.combination*Facial.combination + (1|Trial) + (1| Matriline/MonkeyID), data = dat, family=binomial)

Test64<-glmer(Cbind ~ Stimuli + Facial.combination*Previous.exposure + (1|Trial) + (1| Matriline/MonkeyID), data = dat, family=binomial)

Test65<-glmer(Cbind ~ Previous.exposure + Facial.combination*Stimuli + (1|Trial) + (1| Matriline/MonkeyID), data = dat, family=binomial)

```
Test66<-glmer(Cbind ~ ReproductiveStatus + Facial.combination*Stimuli + (1|Trial) + (1|
Matriline/MonkeyID), data = dat, family=binomial)
Test67<-glmer(Cbind ~ Facial.combination + Facial.combination*Stimuli + (1|Trial) + (1|
Matriline/MonkeyID), data = dat, family=binomial)
Test68<-glmer(Cbind ~ Facial.combination + Facial.combination*ReproductiveStatus + (1|Trial) +
(1| Matriline/MonkeyID), data = dat, family=binomial)
Test69<-glmer(Cbind ~ Stimuli + Facial.combination*ReproductiveStatus + (1|Trial) + (1|
Matriline/MonkeyID), data = dat, family=binomial)
Test70<-glmer(Cbind ~ Previous.exposure + Facial.combination*ReproductiveStatus + (1|Trial) +
(1| Matriline/MonkeyID), data = dat, family=binomial)
Test71<-glmer(Cbind ~ Facial.combination + Facial.combination*Previous.exposure + (1|Trial) +
(1| Matriline/MonkeyID), data = dat, family=binomial)
Test72<-glmer(Cbind ~ Stimuli + Facial.combination*Previous.exposure + (1|Trial) + (1|
Matriline/MonkeyID), data = dat, family=binomial)
Test73<-glmer(Cbind ~ ReproductiveStatus + Facial.combination*Previous.exposure + (1|Trial) +
(1| Matriline/MonkeyID), data = dat, family=binomial)
Test74<-glmer(Cbind ~ Facial.combination + ReproductiveStatus*Previous.exposure  + Face +
(1|Trial) + (1| Matriline/MonkeyID), data = dat, family=binomial)
Test75<-glmer(Cbind ~ Previous.exposure*Face + AgeMos*Facial.combination + (1|Trial) + (1|
Matriline/MonkeyID), data = dat, family=binomial)
Test76<-glmer(Cbind ~ Previous.exposure + AgeMos*Face + Facial.combination + (1|Trial) + (1|
Matriline/MonkeyID), data = dat, family=binomial)
```

4)

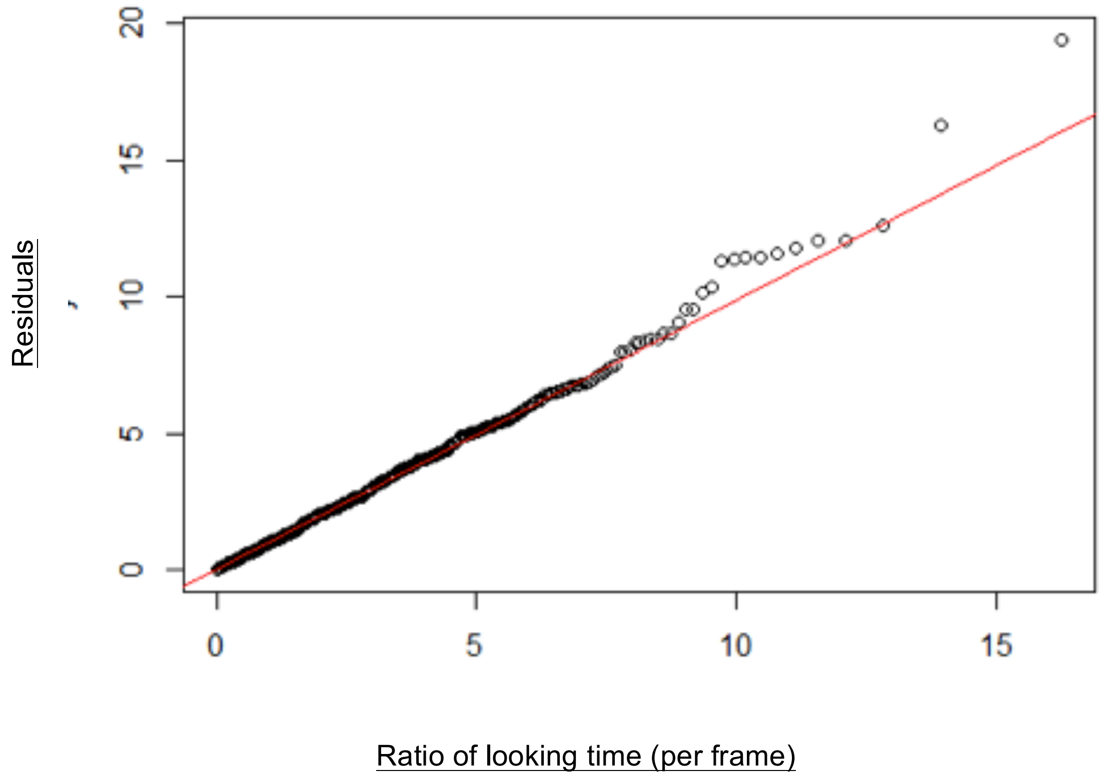

Figure 4. graph showing the residuals of best model