Transgenerational Effects of Structural Enrichment in *Danio rerio.*

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Abstract

This thesis examines the potential for the inheritance of environmental information in the highly social teleost fish, *Danio rerio*. I performed a series of studies over four data chapters that examined the effects of varying levels of environmental enrichment (EE) across three generations. I aimed to examine the effects of EE on physiology, brain function and behaviour. To do this, I utilized a combination of video tracking in a novel open tank group shoaling test, larvae movement trials, high resolution imaging of morphology, and adult whole brain gene expression analysis. Taken together, these studies indicate that variation in levels of enrichment is associated with changes in complex behaviours and morphologies, which can be inherited through the paternal germline. The analysis of larvae movement and shape performed here shows that changes in locomotor activity may be established in early development, as subtle changes in developmental timing are found. The results here also show that changes in locomotor activity are stably inherited between generations, whereas changes in social traits are not. Although there were changes in social behaviours within generations, there were no inherited effects of on shoal cohesion. I targeted the expression of two nonapeptides known to be involved in the regulation of social behaviour in fish species, arginine vasotocin (AVT) and isotocin (IT) and found that changes to social traits in the first generation appear to be associated with changes in whole-brain AVT expression. The disparity in the heritability of these two behavioural realms found here is particularly interesting and provides the potential for further investigation.

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Chapter 1: Introduction

In the following chapter I am going to introduce some of the major topics covered in this thesis, starting with how animals respond to changing environmental conditions. I then discuss the role of phenotypic plasticity as a mechanism for short term adaptation to these changing conditions. This occurs at varying timescales, and can be triggered during various stages during development. Plastic responses can be the result of differential regulation of the epigenome and changes in levels of gene expression, and can cause changes in a number of behavioural and physiological systems. I introduce the topic of epigenetic inheritance, and its potential for facilitating this phenotypic adaptation across generations. I discuss how this epigenetic information might be inherited through germline, and that it is still uncertain as to when this effect might arise in different contexts. This represents a major form on non-genetic inheritance, and may play a role in the inheritance of complex traits such as personality, sociality or collective behaviour from parent to offspring. I discuss and review the literature in this field, the challenges and future direction, as well as highlight areas which require further examination.

1.1 Phenotypic Plasticity in Variable Environments

Phenotypic plasticity has been previously defined as the ability of a single genotype to produce more than one phenotype in response to changes in environmental conditions (Schlichting and Pigliucci, 1998). It has been studied extensively in plants as their sessile lifestyle makes dealing with changing environmental factors challenging (Sultan, 2000). Despite this historical focus on plants, phenotypic plasticity has also been studied in a variety of different taxa including birds (Charmantier et al. 2008), insects (Moczek, 2010), and fish species (Schneider et al. 2014), suggesting that it may be ubiquitous among living organisms. A good example of this type of plasticity is the induction of morphological defenses. In the water flea *Daphnia pulex*, the presence of chemical cues (kairomones) from predators can rapidly induce morphological defenses (elongated helmets, neck teeth and tail spines) within a single generation (Tollrian, 1993). This range of phenotypes produced by a given genotype across environmental gradients is commonly referred to as a phenotypic 'reaction norm' (Schlichting and Pigliucci, 1998). Reaction norms are commonly visualized as a straight line,

with an elevation and a slope that describes the change in a phenotypic value across discrete environments or environmental gradients.

Phenotypic plasticity and its role in species diversity and evolution has also been an area of interest for some time (West-Eberhard, 1989). Under some circumstances plasticity can be adaptive. This can occur in heterogenous environments, when future environments are predictable and the cost of plasticity is low (Via et al. 1995). In this sense phenotypic plasticity represents a short-term mechanism for positive adaptation in changing environments. There is also recognition that plastic responses are not always adaptive in this way, and there may also be inherent costs and limits. This has led to the idea that plasticity may be maladaptive under certain circumstances (Murren et al. 2015). One of the most commonly recognized costs of plasticity is the cost of maintaining the biological machinery required for sensing environmental conditions (DeWitt et al. 1998), or the reliability of environmental cues over time (Ghalambor et al. 2007). In circumstances where environmental conditions fall outside of the range of those typically experienced by ancestral populations, this represents a form of 'environmental stress'.

1.2 The Importance of Early-Life Experience

Developmental plasticity refers to the ability of a single genotype to produce a range of phenotypes in response to environmental conditions during development (West-Eberhard, 2003; Uller, 2008; Gilbert, 2004). This concept has given rise to the term 'eco-devo', which describes how organisms develop in real-world environments (Sultan, 2007). There are numerous examples in nature of this type of environmental sensitivity during early life development. The previously mentioned case of inducible morphological defenses in *Daphnia* species is an excellent example. In *Daphnia galeata,* exposure to predator cues during early instar stages can induce changes body length, tail spine and helmet length (Stabell et al. 2003). This was observed when developing individuals were conditioned in water which had previously contained predators fed a diet of *Daphnia galeata*. This suggests that *Daphnia* respond to predators by sensory detection of conspecific alarm signals and predator cues that are present in their environment. Another example can be found in *Bicyclus* butterflies, which exhibit seasonal plasticity in wing patterning (eyespots) and other traits which are associated with survival and reproduction (Brakefield et al. 2007). Environmental temperature during

the larval stage can also act as a major cue for the wet vs dry season developmental trajectories. This results in the creation of eyespots on the wing during the wet season. The eyespots are thought to serve as a visual cue to deflect predator attacks away from vital body parts and may therefore facilitate successful escape behaviour and survival (Olofsson et al. 2010). Another example the is temperature-dependent sex determination observed in turtles at hatching, both in the laboratory as well as in the wild (Bull and Vogt, 1979). In this case warm temperatures produced mostly female progeny, whereas cooler temperatures were mostly male. The evidence across a variety of taxa, as outlined here, clearly demonstrates the importance of environmental conditions in unmasking innate phenotypic variation.

1.3 The Special Role of Behaviour

Behaviour has been previously described to have a 'special role' in plasticity and in the generation of novel phenotypic variation (West-Eberhard, 1989). This is perhaps due to the fact that behaviour is an especially plastic aspect of phenotype and is frequently the first aspect of the phenotype that responds to environmental variation. Behaviour is remarkably flexible across many different domains, evidence of this can be seen in behaviours such as mate choice and mating tactics, predator evasion strategies, and foraging behaviour (Ghalambor et al. 2010). This adjustment of behaviour relies on the interaction between pre-programmed behavioural responses, and an individual's accumulated life experience (or learning) (Mery and Burns, 2010). These have recently been termed developmental, and activational plasticity (Snell-Rood, 2013). Developmental behavioural plasticity refers to the expression of multiple behavioural phenotypes from a single genotype in response to the environment (learning), similar to the classic definition of plasticity. Whereas activational refers to innate behavioural plasticity, or the activation of an underlying gene network as environments are encountered (Snell-Rood, 2013).

Individuals within the same population can fundamentally differ in their behavioural responses in different environmental contexts, or animal 'personality' (Dingemanse et al. 2010). This concept that individuals differ in their average level of behaviour is widely accepted in humans, however, perhaps surprisingly this has only recently been revealed in other animal species. The way in which these behaviours change over environmental gradients or contexts is referred to as a behavioural reaction norm (BRN), which can be

visualized as a straight line with a set elevation and slope. Individuals can differ in their BRN's in both elevation ('personality') and slope ('plasticity') (Dingemanse *et al.* 2010; Dingemanse and Wolf, 2013). There are also inherent costs to maintaining these forms of plasticity. For example a study in the butterfly, *Pieris rapae,* has shown that improved host-finding ability and learning performance is associated with a decrease in lifetime fecundity (Snell-Rood et al. 2011).

1.3.1 Collective Behaviour

Collective behaviour is widespread in nature, in the schooling of fish, flocking birds and in human crowds (Delellis et al. 2014). The principle of self-organization through simple interactions between individuals and their environment seems to explain the emergence of collective behaviour of animal groups and societies (Sumpter, 2006). In teleost fish, synchronized movement through shoaling functions as a mechanism to avoid predation by increasing group vigilance (Pitcher, 1986). In groups of birds, flocking can also function as a mechanism for early detection and warning system (Lazarus, 1979). The evidence therefore seems to point to a common function of group living as a mechanism to promote survival. Alarm substances also represent an ecologically important environmental cue, as they typically indicate the presence of predators. In the schooling fish species *Pristella maxillaris*, individuals exposed to alarm cues alter their responses to other group members by reducing proximity and increasing responsiveness to nearby neighbors (Schaerf et al. 2017).

Recent work suggests consistent differences in individual personality can give rise to differences in collective behaviour (del Mar Delgado et al. 2018; Jolles et al. 2020). For example, within flocks of great tits (*Parus major*), less bold individuals seem to behave more collectively in high density areas, whereas bolder individuals tend to disperse to the periphery of the flock (Aplin et al. 2014). This individual variation in personality and positioning within the flock may therefore affect social foraging and within-patch movement. In three-spined stickleback, *Gasterosteus aculeatus*, individual differences in social proximity and swim speed seem to explain the structure, cohesion, and dynamics of group behaviours (Jolles et al. 2017). This individual behavioural heterogeneity and its effects on group dynamics may also be influenced by ecological and environmental conditions. In such cases, environmental and ecological pressures may alter group level effects which arise from individual heterogeneity.

For example, in sticklebacks it has been shown that individuals are more likely to spend time with a group of conspecifics if that group was located in a region close to their own preferred temperature range (Cooper et al. 2018). This shows that individual variation in the tolerance of temperatures represents an abiotic environmental factor that can indirectly effects group composition through individual preference.

1.4 Epigenetics: A Link Between Environment and Phenotype

Fundamentally, plastic responses arise from regulation at the cellular level that receives and processes environmental signals (Schlichting and Smith, 2002). Recent evidence shows that novel phenotypic variants are the result of complex interactions between physiological, genetic and epigenetic processes (Beldade et al. 2011). The term epigenetics has been used to define this as a bridge between genotype and phenotype through the regulation of gene expression (Goldberg et al. 2007; Jaenisch and Bird, 2003; Bird, 2007). Although in recent times it is most commonly used to refer to biochemical markers which bind to DNA and alter gene expression. These can be broadly categorized into three main mechanisms, DNA methylation, histone modification, and non-coding RNA (ncRNA), all of which do not require a change in DNA sequence (Goldberg et al. 2007; Lee, 2012). They have been proposed as an important way by which the genome responds to environmental conditions, because they are rapid in their response, and are potentially reversible (Kilvitis et al. 2017; Norouzitallab et al. 2019). For example, reversible DNA methylation plays an important role in gene expression through the methylation of CpG islands, typically near or within promoter regions of genes (Jones, 2012). Histone tails can also be reversibly modified in a variety of ways by regulating the accessibility of genes to transcription factors and other transcriptional machinery (Badeaux and Shi, 2013). In this way epigenetic mechanisms of regulation play a key role in a host of biological systems.

1.5 Epigenetic Regulation of Brain Development and Function

The results of epigenetic modifications to DNA have been widely associated with changes to brain function and behaviour (Keverne and Curley, 2008; Powledge, 2011; Jensen, 2014). This is particularly true of early life experience. During prenatal and postnatal periods individuals undergo rapid changes in neuronal organization that can result in long term changes to brain

function (Fagiolini et al. 2009). Although, epigenetic regulation of brain function is not exclusively limited to these critical periods of early development. They are a phenomenon that continues throughout adulthood and plays a role in adult brain function, such as in the process of biological ageing (Liu et al. 2009; Roth, 2012). Indeed, memory function seems to be tightly associated with DNA methylation occurring in neurons in the brain (Holliday, 1999). It is therefore unsurprising that DNA-methyltransferase appears to be highly expressed in the central nervous system of the adult brain (Brooks et al. 1996). Presumably to maintain the specific and precise function of the variety of neuronal cells found in the brain. Previous studies have found that cognitive decline through ageing is associated with a decrease in the expression of the DNA methyltransferase *Dnmt3a2* in the hippocampus, and that replenishing *Dnmt3a2* levels restored cognitive functions (Oliveira et al. 2012).

1.5.1 Learning and Memory

Epigenetic mechanisms of gene regulation have been proposed to play a key role in memory formation and brain plasticity (Woldemichael et al. 2014). Contextual fear conditioning (CFC) describes an association that is made between environmental conditions and a stressor, where removal of the stressor in the same environment will elicit the same fearful response through memory formation. Previous studies in rodents have shown that following CFC induced by an aversive foot shock, two DNA methyltransferases (*DNMT3a* and *3b*) that are essential for *de novo* DNA methylation are upregulated in the hippocampus (Miller and Sweatt, 2007). This suggests that complex interactions between both transcriptional activation and silencing through DNA methylation play a key role in memory formation. Histone modifications have also been linked to memory formation in CFC of the adult brain (Levenson et al. 2004). Here, acetylation of histone H3 in area CA1 of the hippocampus appeared to regulate the early stages of long-term memory formation in a CFC paradigm. In another more recent study, CFC was found to result in a rapid increase in histone H3 phosphorylation, again in area CA1 of the hippocampus (Chwang et al. 2006). Taken together these studies demonstrate that memory formation and consolidation following CFC is regulated by complex combinations of DNA methylation and histone modifications, which are in turn regulated by cell signaling networks.

1.5.2 Sociality

In nature animals frequently organize themselves into dynamic social dominance hierarchies. In the guppy *Poecilia reticulata*, there are well defined dominance hierarchies which dictate an individual's role within the group and reproductive potential (Gorlick, 1976). Recent evidence suggests that epigenetic modifications may be associated with changes in social behaviours (Seebacher and Krause, 2019). The nonapeptides arginine vasopressin (AVP) and oxytocin (OXT) belong to a group of hormones that play a key role in social behaviours (Donaldson and Young, 2008). These peptides and their receptors are well established as regulators of social behaviour across a variety of species, and are highly expressed in a core set of brain structures commonly referred to as the 'social behaviour network' (Goodson, 2005; Veenema and Neumann, 2008). In rodent models, both AVT and OXT have repeatedly been shown to modulate behaviours such as social recognition, anxiety, and avoidance (Bielsky and Young, 2004; Lukas et al. 2012). For example, in male rodents intracerebroventricular administration of an OXTR antagonist has been shown to reduce social exploration through examination of a novel conspecific (Lukas et al. 2011). Here exposure of rodents to social defeat resulted in a loss of social preference and increased social avoidance. This was found to be reversible upon administration of synthetic OXT, a short time before social preference testing. In another study, the effects of social defeat were significantly attenuated by exposure to an AVP receptor antagonist (Litvin et al. 2011). Compared to undefeated animals socially defeated mice exhibited increased anxiogenic-like behaviours towards a novel male conspecific, and effect which was partly attenuated with a AVP receptor antagonist.

1.5.3 Stress

Stressful life events are commonplace for an animal living in the wild. In particular, early life stress has significant neurological and behavioural effects that persists well into adulthood (Hunter, 2012; Stankiewicz et al. 2013). One frequently studied early life stressor that has been shown to affect stress responses throughout life is variations in maternal care and maternal separation (Francis and Meaney, 1999). In rodent models early life stress through maternal separation was shown to induce sustained hyperactivity of the hypothalamus–pituitary–adrenal (HPA) axis (sometimes called the 'stress-axis'), which was characterized by

hypersecretion of the protein hormone corticotropin-releasing hormone (Murgatroyd et al. 2009). Glucocorticoid receptors (GR) are important mediators of stress responses. GR expression has been shown to be associated with variations in the level of maternal care through DNA methylation and histone acetylation (Szyf et al. 2005). Other studies have shown that the methylation of the 'brain-derived neurotrophic factor' gene, at a key regulatory exon, is linked to post-traumatic stress disorder induced by exposure to predators or unstable housing conditions (Miller et al. 2017). Taken together, these studies suggest that stress response can be inherited through non-genetic mechanisms of inheritance such as maternal effects, and through a number of different signalling pathways.

1.6 Transgenerational Epigenetic Inheritance: Parental Effects

The role of epigenetic mechanisms of inheritance has been studied extensively in both plants (Schmitz and Ecker, 2012) and animals (Daxinger and Whitelaw, 2012). Heritable phenotypic variations can be generated by epigenetic modifications in germline cells, a form of so called 'non-genetic inheritance' (NGI) or 'transgenerational plasticity' (Chong and Whitelaw, 2004; Szyf, 2015). This occurs as a result of partial germline epigenetic reprogramming and incomplete erasure of the epigenome between generations, and are often broadly referred to as parental effects (Kappeler and Meaney, 2010; Badyaev and Uller, 2009). A classic example of epigenetic inheritance in mammals is the *agouti* locus in mice (Wolff et al. 1998), where incomplete erasure of an epigenetic modification in the maternal germline results in the inheritance of an acquired phenotype in offspring.

In the past decade, there has been significant interest in these epigenetic inheritance systems and the role they play in evolutionary processes (Bonduriansky and Day, 2009; Jablonka and Raz, 2009).Recent evidence suggests that epigenetic inheritance can affect evolution in a number of ways (Jablonka and Raz, 2009). Traditional Mendelian models of inheritance, which rely on the slow process of genetic evolution, are not always able to react quickly enough for organisms to adapt in highly dynamic environments (Szyf 2015; Bonduriansky et al. 2012). This type of plasticity is favoured when environments fluctuate across generations, when offspring environment is predictable from the parental environment, and the cost of obtaining environmental information and responding appropriately is relatively low (Uller, 2008). These epigenetic mechanisms of inheritance may therefore provide 'medium-term'

adaptive potential in response to changing conditions. A recent example of this has been found in the effects of temperature on body size in marine sticklebacks, where the offspring of mothers reared at specific temperatures were larger when offspring were reared at the same temperature (Shama et al. 2014). In this case, maternal rearing temperature is predictive of offspring rearing temperature and offspring benefit from this information by growing to a larger size, an important indicator of fitness.

1.6.1 Maternal Effects

It has been known for some time that the maternal environment can affect offspring phenotype, so called 'maternal effects' (Mousseau and Fox, 1998). They have been previously defined as the influence of maternal genotype or phenotype on offspring phenotype (Bernardo, 1996). This type of non-genetic inheritance through the maternal lineage has been well studied in a variety of taxa including plants and animals (Mousseau and Dingle, 1991; Bernardo, 1996). A classic example of this is the role of maternal care, which can have profound transgenerational effects on the development of stress responses in offspring (Francis and Meaney, 1999). A now classic series of studies has demonstrated this effect in rodents (Weaver et al. 2004; 2007). Here low levels of maternal licking/grooming (LG) behaviour were found to significantly increase methylation at a GR during development, and were also associated with changes in histone acetylation and transcription factor binding to the GR promoter in offspring. These epigenetic changes in affected individuals also seemed to affect behavioural responses to stress, effects which persisted well into adulthood. Cross fostering of offspring to high LG mothers seemed to reverse the effect on methylation at this region, clearly demonstrating that the epigenetic state was dependent on early life experience and maternal input.

1.6.2 Paternal Effects

Fathers can also have influence over offspring development, these are commonly referred to as 'paternal effects' (Curley et al. 2011). They occur much in the same way as maternal effects do, although they have arguably historically received much less attention. In this case sperm functions as the carrier of information in the form of DNA methylation, histone modifications and non-coding RNAs (Rando, 2012). A classic example of this form of epigenetic inheritance is the exposure of rodents to the endocrine disruptor Vinclozolin during embryogenesis

(Anway et al. 2005; Anway and Skinner, 2008). Here, exposure of gestating female rodents to vinclozolin induced a male adult F1 phenotype that was characterized by decreased spermatogenic capacity and an increased incidence of infertility. Further analyses also demonstrated that these effects on male reproductive fitness were associated with altered methylation in the testes of affected males and in the sperm of both F2 and F3 offspring. This does show that environmental information can be inherited non-genetically through males, although exposure to a major toxin does represent a relatively potent experimental manipulation. In another study, the offspring of male mice fed a low protein diet showed metabolic changes in lipid and cholesterol biosynthesis relative to offspring from fathers fed a standard diet (Carone et al. 2010). Epigenomic analysis of offspring revealed differences in cytosine methylation, which was dependent on paternal diet. Offspring of males fed a low protein diet exhibited elevated expression of numerous genes involved in lipid and cholesterol biosynthesis. In particular, a change in methylation state was observed in *peroxisome proliferator activated receptor Alpha* (*Ppara*), a key lipid regulator. Fathers can also transmit a variety of other factors to subsequent generations, most notably cytoplasmic RNA. Evidence of this can be seen in mice, where early life traumatic stress has been found to alter microRNA (miRNA) expression in fathers and their offspring (Gapp et al. 2014). This was also associated with both behavioural and metabolic alterations in offspring. Sequencing analysis here revealed that several miRNAs were upregulated in sperm from stressed males. miRNA's were also altered in the serum, hippocampus, and hypothalamus of adult F2 mice. Direct injection of sperm RNAs from affected fathers into wild-type oocytes reproduced these effects in the resulting offspring, suggesting a link between phenotypes and the miRNA as potential vectors of transmission.

Fathers may also affect offspring development is through indirect, male-induced, maternal effects (Curley et al. 2011). In such cases, females may dynamically adjust offspring investment dependent on perceived quality of the father. This has been described as the 'compensation hypothesis', where females paired with non-preferred males will increase offspring investment to compensate for paternally inherited disadvantages (Gowaty et al. 2007; Gowaty, 2008). This hypothesis has received some support from empirical studies in birds, where female zebra finches (*Taeniopygia guttata*) lay larger eggs when paired with unattractive males (Bolund et al. 2009). This increase in egg size is presumably a mechanism

to offset the potential shortcomings of a father with lower fitness by increasing maternal investment in offspring. The interacting effects of maternal and paternal effects have also been recently observed in rodent models (Mashoodh et al. 2018). Here adult food restriction in male mice was shown to influence growth rate, behaviour, and hypothalamic gene expression in female offspring. Under natural mating conditions however, females paired with food restricted males exhibited increased postnatal care, and phenotypic effects in offspring were reversed. This study demonstrates maternal compensation via postnatal investment as a result of reduced mate preference.

Paternal effects have also been empirically observed in other taxa. Zebrafish have emerged in more recent years as a useful animal model to study the effects of paternal epigenetic inheritance. This is perhaps due to the finding that in contrast to mammalian models, the sperm DNA methylome is retained in developing zebrafish (Jiang et al. 2013). A recent study in zebrafish has demonstrated that high levels of male competition affects survival and reproduction in offspring (Zajitschek et al. 2014). In this study, male competition was manipulated through pairing either one (low competition) or two males (high competition) with a single female. Examination of sperm traits in experimental males showed that males exposed to low competition had a higher proportion of non-motile sperm and lower decline in sperm motility than corresponding males from high competition. This also appeared to affect offspring fitness, as offspring obtained from high competition males showed higher mortality than offspring from low competition males. Offspring from high competition males also hatched within a shorter time frame than offspring from low competition males. Importantly, offspring were obtained through *in vitro* fertilization, controlling for maternal egg investment which may occur due to maternal assessment of male fitness. Although epigenetic markings in sperm and offspring were not measured here, the robust design of this study indicates that these effects are likely mediated through epigenetic reprogramming in the male germline.

1.7 Epigenetic Inheritance of Acquired Behaviours

Recent empirical evidence suggests that transgenerational non-genetic inheritance can facilitate the transmission of complex behaviours between parent and offspring (Bohacek and Mansuy, 2015). In a recent study, male mice from an inbred strain were tested for their

baseline anxiety-like and exploratory behaviour (Alter et al. 2009). They were subsequently bred with females, the resulting female offspring from highly exploratory males showed similar high exploration phenotypes. Paternal open field behaviour also seemed to predict brain weight of both male and female offspring, although the behavioural effects were only present between fathers and daughters. Another study in mice, previously mentioned here, provides evidence for the inheritance of acquired behavioural states (Gapp et al. 2014). In this example, early life trauma was shown to affect male behavioural responses to stress. This was characterized by shorter latency to enter an open arm in an elevated plus maze and spending more time in illuminated areas in a light dark test. Here F2 mice from affected fathers delayed entry into an open arm and spent more time in illuminated areas, behaviours that show striking resemblance to fathers in the same test.

Other studies have shown that chronic social instability can also transmit social deficits across generations (Saavedra-Rodríguez and Feig, 2013). The results from this study demonstrated that social instability during adolescence induced social deficits that persisted across at least three generations. Specifically, both mothers and fathers transmitted enhanced anxiety and reduced social interaction phenotypes to F1 offspring. Social enrichment through communal rearing during postnatal development has also been shown to induce persistent behavioural changes in offspring across multiple generations (Curley et al. 2009). Here, mice pups were raised either with a single mother or under communal conditions. As adults these mice (F1) displayed increased levels of maternal care towards their own offspring (F2). This in turn had effects on F2 behaviour such as reduced anxiety-like behaviour and a higher frequency of nursing.

1.7.1 Effects of Environmental Enrichment (EE) on Brain Function and Behaviour

Environmental enrichment (EE) through novel objects, increased social interactions, or exercise, has been shown to effect brain function and behaviour in a number of ways. A study published over 30 years ago appears to one of the first indications that the behavioural effects of EE can be transmitted from parents to offspring (Kiyono et al. 1985). In this study pregnant rats were reared in either enriched or impoverished conditions. Following this manipulation male progeny exhibited fewer errors in the Hebb-Williams maze paradigm, an effect which

was also observed in cross fostered male offspring. Thus, this study appears to be the first indication that EE during pregnancy can influence learning behaviour in offspring. Only two years later another study demonstrated that this effect was also present when mothers were exposed to EE before pregnancy (Dell and Rose, 1987). Since then, EE has been shown play a role in alleviating the effects of a variety of brain and CNS disorders (Hockly et al. 2002; Nithianantharajah and Hannan, 2006), reversing the negative effects of prenatal stress on behaviour and HPA reactivity (Morley-Fletcher et al. 2003), as well as improving memory formation and consolidation following fear conditioning (Duffy et al. 2001). Generally, it appears as though the consensus on this subject is that EE has generally beneficial effects on stress reactivity and enhances learning and memory.

Some of these behavioural effects of EE also seem to be transmissible across generations. For instance, a previous study found that EE through novel objects increased social interactions, voluntary exercise, and enhanced long term potentiation (LTP), a form of synaptic plasticity important for learning and memory (Arai et al. 2009). These effects were also present in F1 offspring, even in the absence of offspring EE exposure. Another recent study has demonstrated that EE can prevent other transgenerational effects of paternal trauma on offspring coping behaviour (Gapp et al. 2016). In this case, postnatal trauma through maternal separation and the resulting alterations in coping behaviour in male progeny was found to be preventable by paternal exposure to EE. Behavioural changes in this case were accompanied by changes in methylation of the GR promoter in the hippocampus and decreased DNA methylation in sperm cells. Paternal EE was also associated with reversal of GR expression alterations and DNA demethylation in the hippocampus of the male progeny. Taken together, these studies demonstrate that enrichment has significant transgenerational behavioural effects which may be the result of persistent epigenetic modifications.

There is a growing body of work investigating the effects of EE on brain function and behaviour in zebrafish, a species that has emerged as a promising candidate for understanding the role of EE in neurobehavioural models (Volgin et al. 2018). One of the earlier studies in this field utilizing the zebrafish model investigated the effects of EE in socially isolated adult zebrafish (von Krogh et al. 2010). The results from this study found that socially isolated adult zebrafish housed in enriched environments exhibited higher numbers of proliferating cell nuclear antigen (PCNA) positive nuclei in the telencephalon and hence

increased telencephalic cell proliferation. This study demonstrates that EE can induce functional and dynamic changes in the adult zebrafish brain. Further evidence from recent studies has shown that enriched housing in zebrafish can also have effects on response to chronic stress (Marcon et al. 2018), aggression (Woodward et al. 2019), and shoal cohesion (Sykes et al. 2018). Studies have demonstrated that zebrafish, as well as other fish species, exhibit a preference for structurally complex environments over barren environments, suggesting EE is rewarding for them (Kistler et al. 2011).

1.8 Challenges and Future Directions

Despite the overwhelming evidence for the non-genetic transmission of transgenerational phenotypes, many questions remain regarding the nature and significance of such effects. Critical reviews have suggested that the distinction between context-dependent and germline-dependent epigenetic modifications is important (Crews and Gore, 2014). It has been argued that germline dependent epigenetic modifications are fundamentally different to context-dependent epigenetic modifications, in the sense that a germline epigenetic modification has become independent of the causative factor. This has raised important and lingering questions as to what constitutes a true example of transgenerational epigenetic inheritance. Paternally transmitted transgenerational effects may also be affected by male-induced maternal effects such as female mate choice or female assessment of male fitness (Curley et al. 2011). This can potentially obscure the identification of direct germline epigenetic inheritance, and highlights the importance of studies that investigate parental effects in externally fertilizing species that show no maternal care. In these studies in zebrafish, IVF is utilized to control for unwanted effects of female mate choice and differential resource allocation to offspring from mothers. In such cases, the identification of direct germline inheritance of epigenetic states is much clearer and can be more reliably inferred. Future studies examining these effects should therefore utilize this approach and would benefit by eliminating these confounding maternal effects.

To date there are relatively few studies in zebrafish conclusively linking ecologically relevant environmental variation to epigenetic marks such as DNA methylation or histone modifications, particularly when compared to the extensively studied rodent models. It is clear that further investigation is required here, and in different environmental contexts. For

example, the previously outlined studies examining transgenerational effects of male competition are particularly interesting (Zajitschek et al. 2014, 2017). The epigenetic mechanisms linking paternal and offspring phenotype are yet to be determined, although several potential mechanisms have been proposed. The lack of evidence for the transgenerational effects of EE in zebrafish models is particularly surprising, especially given the recent evidence in zebrafish that EE can have significant effects on a variety of neurological and behavioural traits. Given the evidence for the transmission of EE induced behavioural effects in rodents, further investigation in other species may contribute to understanding these effects, the routes of inheritance, affected phenotypes and how widespread they are.

Chapter 2: A Method for Obtaining and Analysing Adult and Larval Zebrafish Trajectories.

2.1 Abstract

In recent years there has been significant advancement in technologies designed to accurately obtain positional information regarding animal movement. These technologies automatically track individuals within groups of animals whilst accurately maintaining individual identity, enabling individual behaviour to be quantified in group contexts. In this chapter, I describe methods developed alongside automated tracking software to obtain and analyse trajectories of free-swimming zebrafish. Behavioural trials were performed on adult and larval zebrafish using a custom-built testing setup and recorded using a consumer grade camcorder. The open-source tracking software idTracker was used to analyse videos and extract individual positions by frame. In adults, locomotor activity measures obtained were average speed, time spent immobile and proportion of area covered. Social behaviour measures that were obtained were nearest neighbour distance, time spent in proximity to another individual and average path length. For larvae, locomotor activity alone was measured using two metrics: total distance and transitions from stationary to active (movement events). The results obtained were validated and accurately estimated these behaviours, indicating that the methods described here are robust techniques for obtaining behavioural parameters from positional data. This could be used to investigate animal behaviour in a variety of contexts.

2.2 Introduction

The quantification of animal movement in the wild has traditionally relied on excessively expensive GPS-based telemetry systems and animal tagging (Clark et al. 2006; Bouten et al. 2013). These techniques have been essential for studying animals in natural habitats, however in controlled laboratories and research environments, this has traditionally been achieved through human observation. There are intrinsic limitations to human scoring of animal behaviour, perhaps the most obvious is the inherent tendency for human error or observer bias to emerge. This has been addressed through the development of automated video encoding techniques and software solutions for the tracking of moving objects. For

example, early versions of as EthoVision® by Noldus was able to track a single moving individual in a behavioural trial. However, this too had limitations, as the automated tracking systems were unable to distinguish between multiple individuals in a single test. Many of these technologies are now able to track multiple individuals in a group and are commercially available software packages, such as EthoVision® XT by Noldus or ANY-maze®. These are excellent for their ease of use and accuracy, however they can be expensive and are therefore not always a viable option for all researchers interested in animal movement and behaviour.

In light of this there has been a significant effort to develop open-source software packages dedicated to the automated quantification of animal movement (Pérez-Escudero et al. 2014; Pinheiro-da-Silva et al., 2017; Sridhar et al. 2018). The tracking applications Tracktor (Sridhar et al. 2019) and ZebTrack (Pinheiro-da-Silva et al. 2017) are particularly well known. The former is useful its ability to track single individuals in noisy environments, although it can also track multiple objects on a uniform background. The latter uses pre-recorded videos in the MATLAB Image Processing Toolbox environment in order to accurately track multiple free-swimming zebrafish, although it can also be used with other animals. In recent years artificial intelligence based deep learning algorithms have been developed to quantify animal movements and for pose estimation, an example of this is DeepLabCut (Nath et al. 2019). The key strength of this software is the ability to 'learn' from a novel set of training videos or images to obtain highly accurate estimations of animal gait or behaviour. Moreover, these recent software packages also employ image analysis algorithms to determine the identity of individual animals and track this from frame to frame, permitting individuals' location to be reliably determined in group settings. This enables researchers to quantify behaviour in social settings using automated methods, removing many of the limitations associated with human-quantification or with behavioural trials of individuals.

In the past few decades, zebrafish have emerged as an important model organism in the study of behaviour. Studies have shown that traits such as activity level differ on an individual level and are consistent over time, between sexes, and over environmental contexts (Tran and Gerlai, 2013). This suggests that locomotor activity is a component of an individual 'personality' that is remarkably consistent. Zebrafish display a variety of innate social behaviours such as shoaling, aggression and dominance, as well as exploratory behaviour (Spence et al. 2008). In the wild, zebrafish are highly social, and they are typically found in

small shoals (4–12 members). This kind of shoaling behaviour in teleost fishes is primarily a mechanism to avoid predators and for optimal food gathering (Pitcher, 1986). Behavioural studies on zebrafish have culminated in the definition and cataloguing of nearly 200 distinct forms of behaviour in a variety of behavioural paradigms (Kalueff et al. 2013). There has been a particular effort to understand the spatio-temporal strategies of zebrafish exposed to a classic open field paradigm (Godwin et al. 2012; Stewart et al. 2012). The open field is a paradigm traditionally used to measure stress and anxiety-like behaviour and it is performed by allowing animals to freely explore a large open arena over a period of time (Prut and Belzung, 2003). Previous studies have found that zebrafish behavioural strategies in an open field trial are remarkably similar to those observed in rodents, suggesting that strategies may be highly conserved across species. For example, similar to rodents, zebrafish also scale locomotor activity depending on arena size (Stewart et al. 2012).

Automated tracking technologies have been used to study the dynamics of zebrafish shoaling (Miller and Gerlai, 2007; Miller and Gerlai, 2008). From the positional data obtained group level behavioural measures such as velocity, within-group positioning, nearest neighbour distance and inter-individual distances can be extracted. Developing and adult zebrafish are both highly social, and visual access to a group of conspecifics has been shown to be a rewarding stimulus (Al-Imari and Gerlai, 2008). More recently, software tools have also been developed for the automated tracking of groups and individual zebrafish larvae. LSRTrack and LSRAnalyse (Cario et al. 2011; Zhou et al. 2014) have been designed specifically for this purpose. In larvae, these kinds of automated tracking applications have been used to measure differences in larval free swimming behaviour evoked by visual stimulation (Bianco et al. 2011), and changes in light intensity (Schnörr et al. 2012). The open field test has also been adapted for larval zebrafish, which seem to display characteristic patterns of exploratory behaviour such as thigmotaxis in a similar way to adults (Ahmad and Richardson, 2013). The tracking of larvae in groups has also shown that zebrafish develop a shoaling preference (Engeszer et al. 2004), which occurs at around 12 days post fertilization (dpf) (Engeszer et al. 2007; Dreosti et al. 2015).

The examination of animal free movement is interesting from a data analysis perspective. They have a temporal component, which consists of relocations or steps between successive time points. The R software package ‘adehabitat’ was specifically designed to allow the

analysis of animals trajectories in this way (Calenge, 2006). A number of statistical models can be applied that make use of the data contained in animal trajectories in order to quantify movement. The main family of model commonly used to model trajectories is the correlated random walk (CRW) model (Ward and Saltz, 1994; Kareiva and Shigesada, 1983). They also form the basis for many modern spatial analyses of animal movement such as the composite random walk model (Benhamou, 2014), Ornstein–Uhlenbeck diffusion model (Blackwell 1997) and various state space models (Whoriskey et al. 2017). The raw data required for the application of these models is step lengths, the linear vector describing the magnitude and direction of displacement between successive time points. Here I outline custom methods developed using MATLAB which are used for the analysis of coordinate data obtained from idTracker software, where step length data and inter-individual distances (IIDS) are obtained from coordinate data and used to calculate individual locomotion and group cohesion throughout the trial.

2.3 Methodology

2.3.1 Animals and Ethical Considerations

All fish used in this study were housed under standard conditions and no regulated procedures were carried out. All fish were housed and maintained in a flow-through custom-built recirculation system (see Figure 1). Water was maintained at 27°C, pH 7.5 and nitrate, nitrite and ammonia levels were regularly monitored. Fish were housed in 5 litre custom-built housing tanks at a stocking density of 5 fish per litre. For the purposes of the following methodology a single shoal of adult zebrafish (>3 months post-fertilisation) was used in a single group behavioural trial as a validation. For larval testing, all subjects were obtained from natural breeding and larvae were tested as individuals at 120 hours-post-fertilisation (hpf). Larvae were reared in an incubator at 27.5 - 28°C until 72hpf in a petri dish containing multiple pooled clutches in 0.0001% methylene blue (MB) solution. This MB solution was used to prevent the growth of bacteria and fungus in the dish containing embryos. At 72hpf larvae were transferred to a new petri dish containing clean conditioned system water until 96hpf when they were tested as individuals.

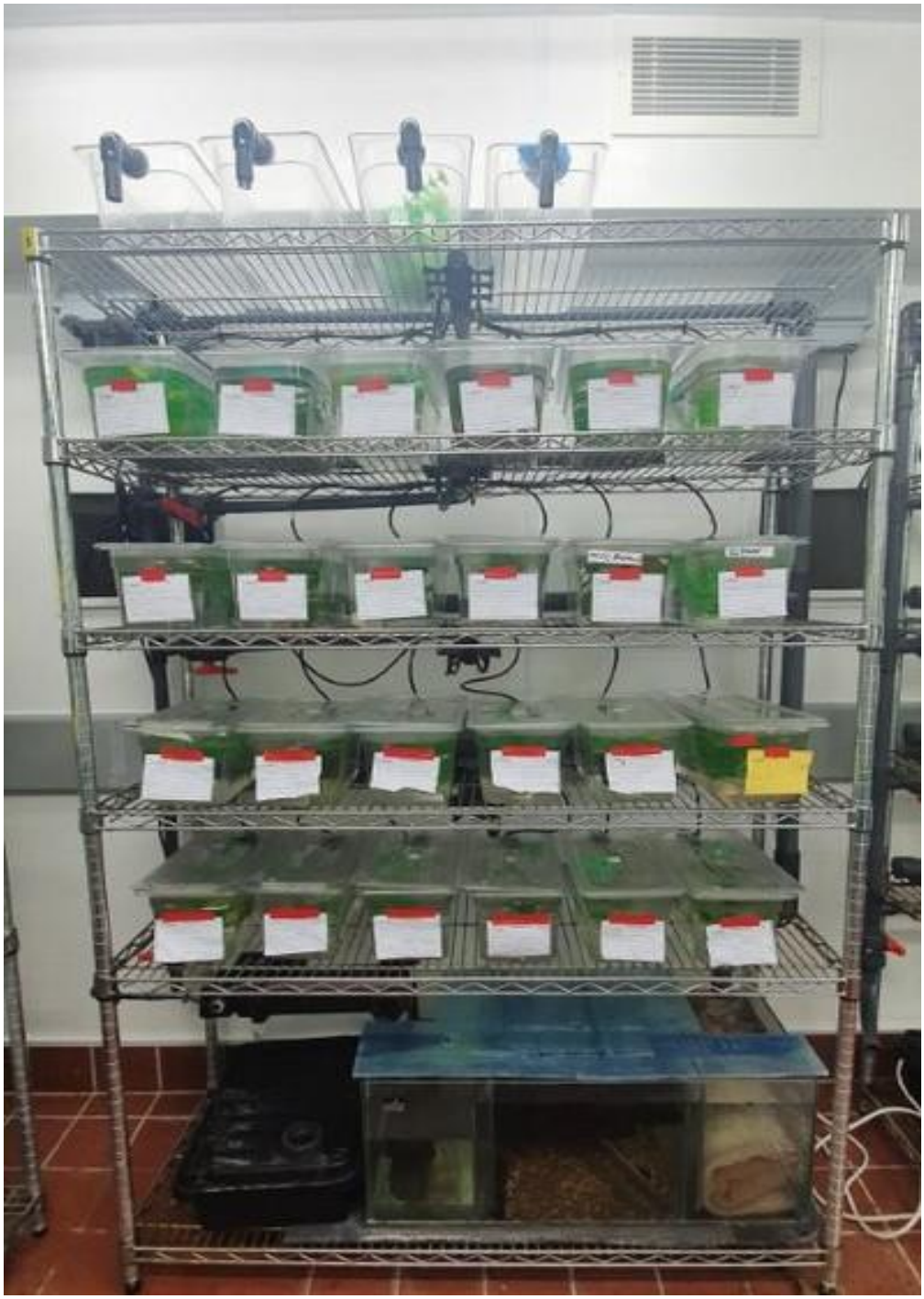

Figure 1. Custom-built recirculating housing unit used for housing of experimental tanks. The large sump consisted of compartments for particulate and biological filtration.

2.3.2 Adult Behaviour

A series of tests were performed as a trial to determine if adults could be successful tracked and coordinates could be obtained. The trial reported here is a demonstration of the results

from the final method used for tracking subjects in experimental chapters. The trial was carried out as a trial inside a 30x30 cm clear acrylic arena filled to 5 cm with clean, conditioned water. This arena was placed on the floor, on top of a MiniSun A1 LightPad to obtain a clear and evenly illuminated background. A tripod and extension arm were used to suspend a consumer camcorder directly above the centre of the arena. The arena and recording setup were enclosed by a floor-to-ceiling length curtain to avoid visual distraction during the test (see Figure 2). The test arena was filled to 5 cm depth with clean conditioned water in order to restrict movement to two dimensions. The movement of a group of ten zebrafish was recorded for a total of 15 minutes. The first 5 minutes of the test was treated as a habituation period and was not tracked. The total length of time that movement of all individuals within the group was tracked was therefore 10 minutes.

2.3.3 Larval Movement

A series of movement assays were performed as a trial to determine if coordinates could be obtained, the trial reported here is a demonstration of the results from the final method used for tracking subjects in later experimental chapters. Five day old larvae were transferred via a pipette from their rearing petri dishes into a black 24-well plate with clear well bases. A single larvae was placed in each well and the larvae was transferred with 0.4 ml of clean conditioned system water. The wells were thus filled to a sufficient depth to allow free swimming of larvae, whilst being restricted to approximately two-dimensions to ensure that the larvae did not swim out of focus during the trial. A total of five larvae per dish were used in the trial. The five larvae were placed in the center most wells on the plate. The 24-well plate was placed on top of a MiniSun A3 light pad in order to evenly illuminate the wells from below and obtain sufficient contrast to allow for tracking. A consumer handheld camcorder was suspended approximately 1m above the plate using a custom build tripod and extension arm setup. The camcorder was positioned at this height to minimise the angle that the center wells were recorded at and so the entire base of the well was visible from above. The plate was positioned directly below the camcorder and positioned in the center of the frame. The camcorder was zoomed in so that the plate filled the screen (see Figure 3 – below). The movement of the five larvae was then recorded for a total of 15 minutes. The first 5 minutes of the test was treated as a habituation period and was not tracked. The total length of time that larval movement was tracked was therefore 10 minutes.

Figure 2. Experimental setup for adult behavioural trials. The entire arena was contained behind a ceiling-to-floor curtain to avoid visual disruption.

2.3.4 Video Editing

The videos obtained during behavioural testing were first converted into a file format which was compatible with the tracking software used. This was done using the open-source video editing software VSDC Video Editor, where MTS files obtained from recording were converted to AVI format. The borders of the video were cropped around the edge of the testing arena and the videos were then opened with idTracker for individual position and trajectory estimation. For the larvae trial, the borders of the video were cropped so that only the centre 6 wells of the plate were retained. The file was then converted from an MTS file format to AVI and exported for tracking.

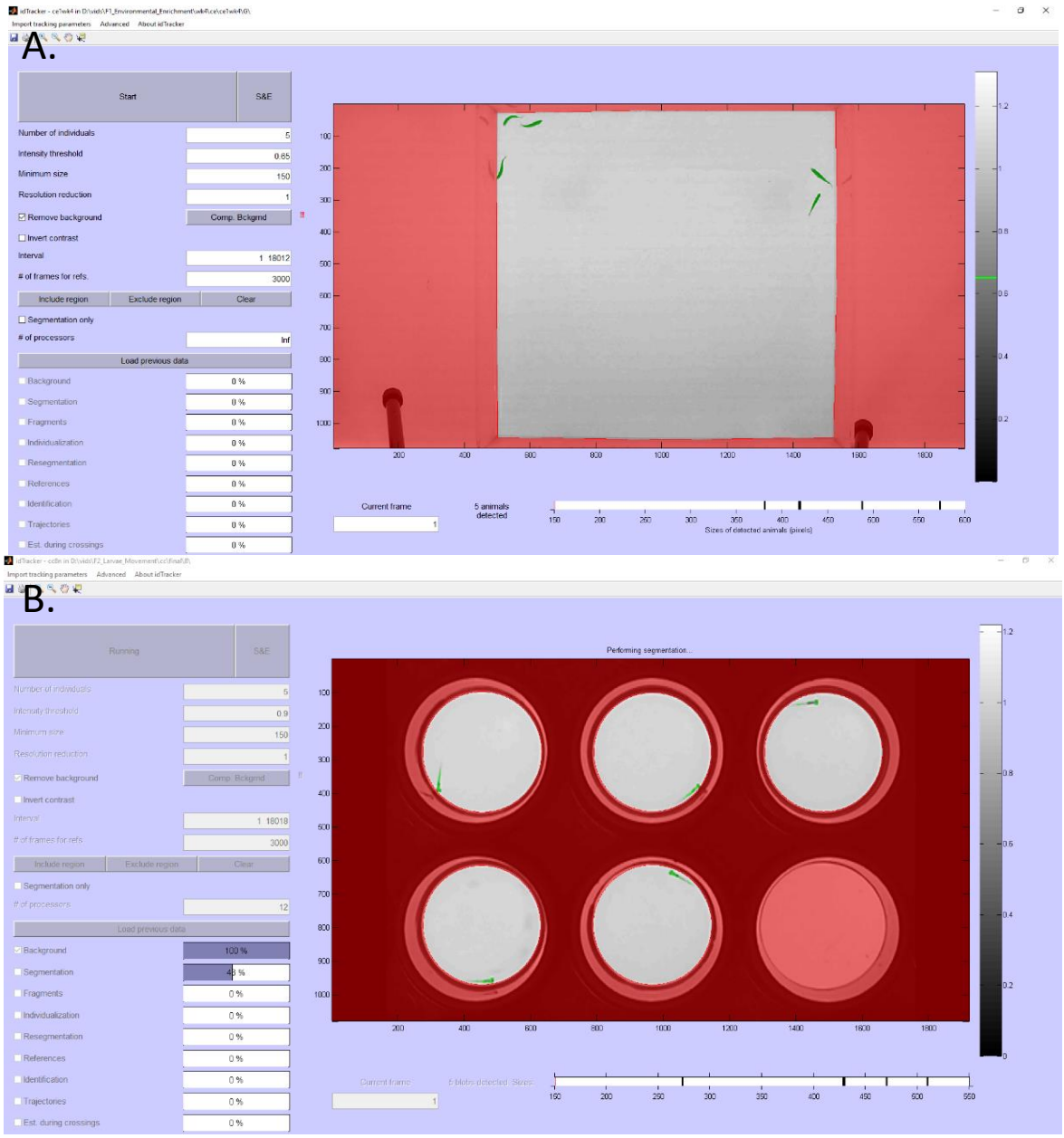

Figure 3. idTracker software windows. The area outside of the test arena (red) was excluded from tracking. The subjects in the arena were detected once they had been shaded green and tracking could be performed. Window from adult trial is shown (A), and larval trial is also shown (B). The red well in the image B did not contain an larvae and so was excluded from tracking.

2.3.5 Obtaining Individual Positional Data with idTracker

The edited and converted videos were then read into idTracker for motion tracking. The tracking area was defined by selecting the base of the arena as the area of interest. The intensity threshold was then incrementally reduced so that only the contrast of the fish bodies against the white background was selected. The appropriate level was confirmed only once the subjects in the test arena were shaded green (see Figure 3 – top). The minimum blob size was reduced to 150 pixels to account for smaller fish and to reduce background noise. In order for idTracker to successfully detect larvae in each well, multiple areas of interest were selected by defining the tracking area as the base of each well. The intensity threshold was then adjusted so that the contrast of each subject in the well was detected as an object. This was confirmed when each larva was shaded green (see Figure 3 – bottom).

2.3.6 Analyzing Adult Movement with Custom MATLAB Scripts

Custom MATLAB scripts were written to analyse the raw coordinate data contained in the individual trajectories. Locomotor activity was measured as the average speed, recorded in centimetres per second (cm/sec), and freezing behaviour as proportion of time spent immobile (sec/min). An individual was 'immobile' if it had travelled less than one body length in any two second time period. Exploration for each individual within the group was measured by calculating the proportion (%) of the total area visited during the test. This was achieved by segmenting the test arena into twenty equally sized quadrants and calculating the proportion of quadrants visited in the entire trial. Social behaviour was measured using three distance-based parameters: average path length, nearest neighbour distance, and time in proximity to another individual. An individual was said to be in proximity to another individual if it was within two body lengths of any conspecific, and was reported as (sec/min). Average path length was defined as the mean distance from a focal individual to every other member of the group in each frame. Nearest neighbour was calculated as the shortest distance between a focal individual and all other individuals in each frame.

2.3.7 Converting to Units

In order to accurately measure behaviours in usable units of measurement (e.g. cm) a conversion factor was calculated from known arena dimensions. In order to do this the

MATLAB Image Viewer application was used to open the first frame of the video. From this application the measure distance tool was used to measure the diagonal distance in pixels from one corner of the arena to the opposite corner. The conversion factor for the video was then calculated as

$$CF = \frac{px}{cm}$$

where the conversion factor was given by the length of the diagonal in pixels divided by the known distance in cm. This gave a conversion factor as the number of pixels per cm. All distance measurements calculated in pixels by default were then converted to cm by dividing by this conversion factor. Average body length of each individual fish in a shoal was calculated in a similar way using MATLAB's Image Viewer application. In this case, frames were obtained which displayed each individual in a straight posture, the length of each subject was measured using the measure distance tool and the average was taken. This value was then used in the following script for all behaviours that utilise body length parameters in their calculation.

2.3.8 Raw Data Format

Raw positional data was stored as XY coordinates for every individual identified in each of an analysed video and was contained within a three-dimensional array of coordinate data (Figure 4). These data comprised two separate matrices representing coordinates on the x and y axis, the third dimension simply refers to the number of matrices in the array, which is in this case two. Inter-individual distances (IIDs) were also stored as a 3-dimensional array structure. In this case, the array comprised a single 2-dimensional matrix for each individual in the trial, where each matrix contained the distances from a single focal individual to all other individuals in the trial. idTracker uses a custom multi-object tracking algorithm that extracts a fingerprint for each object to successfully maintain individual identity, so allowing multiple trajectories to be obtained from a single test. In this case, the raw data is comprised of pairs of coordinates for each individual in each frame of the video. Some frames may be misidentified or out of frame, and these values (NaN) had to be accounted for when calculating endpoint values.

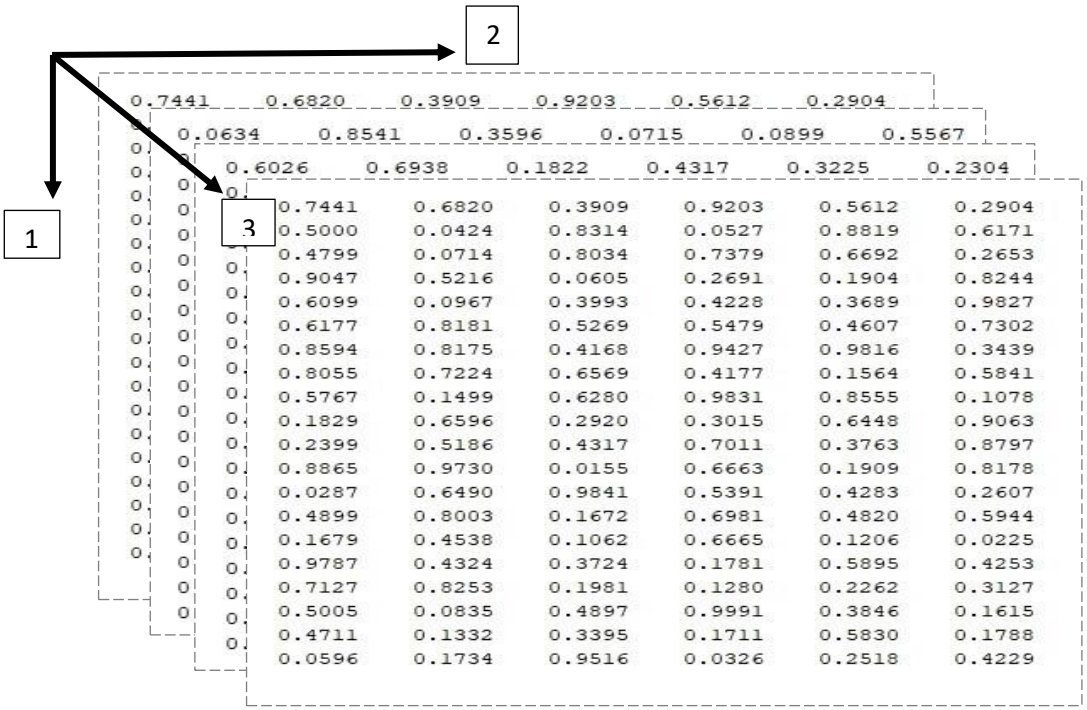

Figure 4. Positional data from idTracker formed matrices that could be stored in an array structure. These arrays consist of 2-dimensional matrices (1,2) stored in an ordered list (3).

2.3.9 Analysing Trajectories

Due to the inherent variation in the data acquired from the tracking software used here (i.e. missed frames), the raw data was first assessed for validity of trajectories for each individual. Where the proportion of the trajectory valid for individual *i* is equal to the total number of frames f minus the total number of missed frames NaN, which is then divided by the total number of frames.

$$p_i = \left(\frac{f - \sum NaN_i}{f}\right)$$

This value was then transformed into the number of seconds and minutes valid for each individual by multiplying the validity value by the total number of seconds s, or minutes m. Distance and speed measures were then normalised using these values to account for the inherent differences in validity of trajectories between subjects. For group measures, only frames in which each individual was successfully identified were used. Therefore, group

measures of behaviour did not need to be standardised in the same way. In this trial there were approximately 2000 missed frames out of the 18000 total frames recorded. The number of missed frames was approximately equal between subjects.

Distance, Mobility and Space Usage

From the raw trajectory array, the two data matrices x and y were defined and used to measure change in position between frames for each subject. This was first calculated as the straight-line displacement between consecutive coordinates in each frame. The absolute distance between successive relocations on both axes was taken, as negative values arising from direction of movement would subtract from cumulative distances and distort the true endpoint values. In MATLAB, the 'diff' function can be used to find the difference between consecutive elements in a matrix or vector along any dimension. This function was used here to find the linear displacement between frames by taking the change in consecutive positions for each column of the x and y coordinate matrices. This resulted in two new matrices, Δx and Δy. These matrices contained all of the relocations between consecutive frames on each axis. A custom function was written, using both Δx and Δy in order to calculate the total distance travelled between two sets of coordinates.

$$d = \sqrt{(\Delta x^2 + \Delta y^2}$$

MATLAB:

```
d=sqrt((diff(trajectories(:,:,1))).^2+(diff((trajectories(:,:,2))).^2));
```

This returns a single matrix d containing the displacement distances between consecutive frames for each individual across the entire behavioural trial. Velocity was reported as distance (cm) per second (s). To calculate this the vector of distances d, for individual d_i was totalled over the length of the trial. The total distance travelled over the trial was then divided by the previously calculated number of valid recorded seconds for the individual.

$$v_i = \frac{\sum d_i}{p_i * s}$$

This value was then simply converted to cm by dividing the pixel value by the conversion factor CF to obtain the final endpoint value for average speed. The resulting distance matrix can also be used for plotting distances over the course of the trial and may be usable in other software packages that rely on step length data.

A subject was considered to be 'immobile' if they had travelled a total distance of less than one body length in a two second time period. As videos were shot at a frame rate of 30 frames per second, the total distance travelled over a two second window was calculated by totaling every 60 values in the distance array d for individual d_i into 'bins'. There is no built-in function in MATLAB to perform this calculation and so a custom function was written into the script:

MATLAB:

```
b(:,j)=arrayfun(@(i)nansum(current(i:i+(fps*2)-
1)),1:(fps*2):length(current)-(fps*2)+1)';
```

The resulting 'time bin' vector b, was then logically indexed to detect two second periods in which the individual had travelled less than a single body length. Where the mobility state of a subject b_i at time t was considered to be 'immobile' if the relocation distance at a given time was less than the mean group body length.

$$b_i(t) = \begin{cases} 2, & if\ b_i(t) < (bl) \\ 0, & else \end{cases}$$

In this case both seconds were considered to be immobile and the value is set to 2. Otherwise, the subject at the given time was considered to be mobile and the value is 0. The average time spent immobile $im\ (\mathrm{sec/min})$, for subject im_i, is the sum of the mobility state vector divided by the total number of valid minutes in the trial for the individual.

$$im_i = \frac{\sum b_i}{p_i * m}$$

To obtain the percentage of area covered, the test arena was first divided into a fixed number of arena zones, z. In MATLAB this was achieved by using the built in 'hist3' function, which creates a bivariate histogram plot representing equally spaced sub-areas. The 'hist3' function

then reads each pair of coordinates in each frame by its location, and places each pair in a bin.

MATLAB:

```
u(:,:,i) = hist3(indxy(:,:,i),[nbins nbins],'CdataMode','auto');
```

This gives a 3-dimensional array containing a map of zones for each individual, where each element of this matrix contained a value for the number of times a zone had been visited. A custom-MATLAB function was written to logically index this matrix. Where the usage state u of a given zone is equal to 1 if the zone has been visited at least once, or equal to 0 if it has not been used at any point.

$$u = \begin{cases} 1, & if\ z > 0 \\ 0, & else \end{cases}$$

The percentage of area covered for a given individual c_i is given by summing the number of zones visited and dividing by the total number of zones, this value was then multiplied by 100 to obtain a percentage value.

$$c_i = \frac{\sum u}{\sum z} \times 100$$

Inter-Individual Distances and Social Behaviours

The XY coordinate data obtained from idTracker was used to calculate inter-individual distances (IID) between all individuals in each frame. IID's were stored in a 3-dimensional data structure, as shown previously in Figure 2, where each individual represents a matrix in the array. Each element in this case represented the linear distance between a focal individual and the other individuals in the trial.

A custom function was written in MATLAB to calculate this array from the raw positional data x an y. Where the inter-individual distance between subject IID_i and IID_j at time t, was calculated by taking the straight-line distance between the two sets of coordinates on each axis.

$$IID(t) = \sqrt{(x_i(t) - x_j(t))^2 + (y_i(t) - y_j(t))^2}$$

The custom-MATLAB function used here performed this calculation between all possible combinations of individuals in the trial.

MATLAB:

```
IID =
sqrt((abs(bsxfun(@minus,trajectories(:,:,1),permute(trajectories(:,:,1),[1 3 2])))) .^2 +
(abs(bsxfun(@minus,trajectories(:,:,2),permute(trajectories(:,:,2),[1 3 2])))) .^2);
```

Before extracting group behaviours, the proximity matrices were trimmed down using a custom MATLAB function so that the resulting matrices contained only frames in which each individual in the shoal's position was successfully obtained. The MATLAB isnan function was used to detect incomplete frames and remove them.

MATLAB:

```
IID(any(isnan(proximities), 2),:) = [];
```

The average path length between the focal individual and other individuals at a given time was calculated by averaging the values in each row of the IID matrices. This can be written as $IID_i(t) = mean(IID_i(t))$. Where the average IID for individual *i* at time *t* is the average of all values in a given row. This yields the vector $avIID_i$ containing the average distances between the focal individual and every other individual at each time point. Average path length was then obtained by again calculating the mean value of this vector, given by $AvP_i = mean(avIID_i)$. Which yields the endpoint value for average path length for a given individual. This value was converted to centimetres by dividing the obtained endpoint value by the previously obtained conversion factor.

Nearest neighbour distance (NND) was calculated as the distance between a focal individual and the closest of all other individuals in the frame. This was given by $nn_i(t) = min\ (IID_i(t))$. This yields the vector nn_i of nearest neighbour distances for the focal individual in each frame of the video. The average of this vector was calculated to obtain the mean nearest neighbour

distance across the whole trial. This is given by $nnd_i = mean(nn_i)$, which yields the endpoint value for nearest neighbour distance for a given focal individual.

Time within two body lengths of another individual was calculated by using logical indexing to transform the IID matrix for each individual into binary values. If the IID between individual *i* and *j* at time t is less than two body lengths, then the proximity value between these two individuals is equal to 1. Otherwise, the proximity value is equal to 0. In MATLAB this can be applied systematically to an entire array. This yields the matrix $prox_i$ for each focal individual containing binary values which indicate whether the focal individual is in proximity to any other individual at a given time.

$$prox_i(t) = \begin{cases} 1, & if\ IID_i(t) < (bl * 2) \\ 0, & else \end{cases}$$

The proximity matrices, $prox_i$, were reduced to vectors containing the sum of the row of the matrix to detect rows with at least 1 interaction. The values in this vector were then logically indexed to find time points where the focal individual was in proximity to any other individual at a given time. Where if the focal individual was detected within two body lengths of any other individual (the summed value of the current row is greater than 0), the proximity value was equal to 1 and equal to 0 if not (the summed value of the row is equal to 0). Time in proximity could then be calculated as

$$tp_i = \frac{\sum prox_i}{p_i * m}$$

The endpoint value for time in proximity, tp, was equal to the sum of the proximity value vector $prox$ for individual $prox_i$, divided by the total number of valid minutes in the trial for a given individual.

Larvae Movement

To extract spontaneous movement data from the recorded videos of the larval movement trials a custom MATLAB script was written. This script calculated the movement of larvae as the straight-line displacement of positional data between frames. This method is similar to the method used for calculating adult movement.

A validity screen was first performed to control for obvious tracking errors such as cross identification between wells, this was confirmed by examination of the plot of trajectories. The distance data was then cleaned by removing values below a certain threshold to account for larvae "drift" observed in the trials, as opposed to active movement. Where any travel distance over a one second period less than 20 pixels was considered noise and set to 0. This value was obtained from manual inspection of the video file against distance data.

The activity level of each larvae was extracted as 'total distance', and the number of transitions from stationary to mobile as 'movement events'. The raw data for larvae movement was stored as a 3-dimensional array identical to the adult movement raw data file. These data were stored as positional data matrices x and y as described previously.

From the positional data, the total distance moved along both axes between frames was given by using the function:

$$d = \sqrt{(\Delta x^2 + \Delta y^2}$$

MATLAB:

```
d=sqrt((diff(trajectories(:,:,1))).^2+(diff((trajectories(:,:,2))).^2));
```

This resulted in a single matrix of distances for each subject in the trial, with each column representing the total distance travelled per frame for a single induvial. The vector d_i was then totalled in 'bins' of 30 frames representing a full one second period. This was done using a custom MATLAB function:

MATLAB:

```
mob(:,i) = arrayfun(@(i)nansum(current(i:i+(fps)-1)),1:(fps):length(current)-(fps)+1)';
```

A logical index was taken of the array of distances in one second bins. In this case mobility state was determined as mobile ($mob_i(t) = 1$) if any movement was detected (distance > 0) and immobile ($mob_i(t) = 0$) if no movement was detected (distance=0):

$$mob_i(f_n) = \begin{cases} 1, & if \ \sum dist\_sec_i(t) > 0 \\ 0, & else \end{cases}$$

Using this logical matrix to determine mobility state, the number of transitions from 0 to 1 in the matrix was determined using the MATLAB 'diff' function.

MATLAB:

```
transitions = sum(diff(mob)>0);
```

For each subject, the sum of this vector gives the total number of transitions from immobile to mobile in the entire trial.

2.4 Results

In the behavioural trial the average body length in pixels for the 10 subjects in this trial was 66.6 pixels, and the average body length of the subjects in centimeters was 1.92cm. The proportion of each trajectory that was valid was approximately equal between all individuals in the trial. The overall average proportion of the trajectories that were valid for all subjects in the trial was ~89%.

2.4.1 Adult Locomotor Activity, Mobility and Exploration

Locomotor activity was calculated as distance travelled (cm) per second over the duration of the trial period. Here distance-per-second data was obtained from raw coordinate data for the entire group. The most frequent relocation distance over a 1 second period was ~5-6cm (see Table 1). An individual was defined as being immobile if they had travelled a distance of less than 1 body length in a 2 second time period. The results here show that all subjects in the test spent a very small proportion of their time (less than 5 seconds per minute) in an immobile state (see Table 1). Exploratory behaviour was measured as the proportion of the total available area that was utilised by an individual during the trial. The results here indicated that the least exploratory individuals explored two thirds of the available space (~66%) and the more exploratory individuals covered as much as 85% of the available space (see Table 1). Most of the exploration was spaced around the periphery of the arena and the centre regions remained unexplored (see Figure 5).

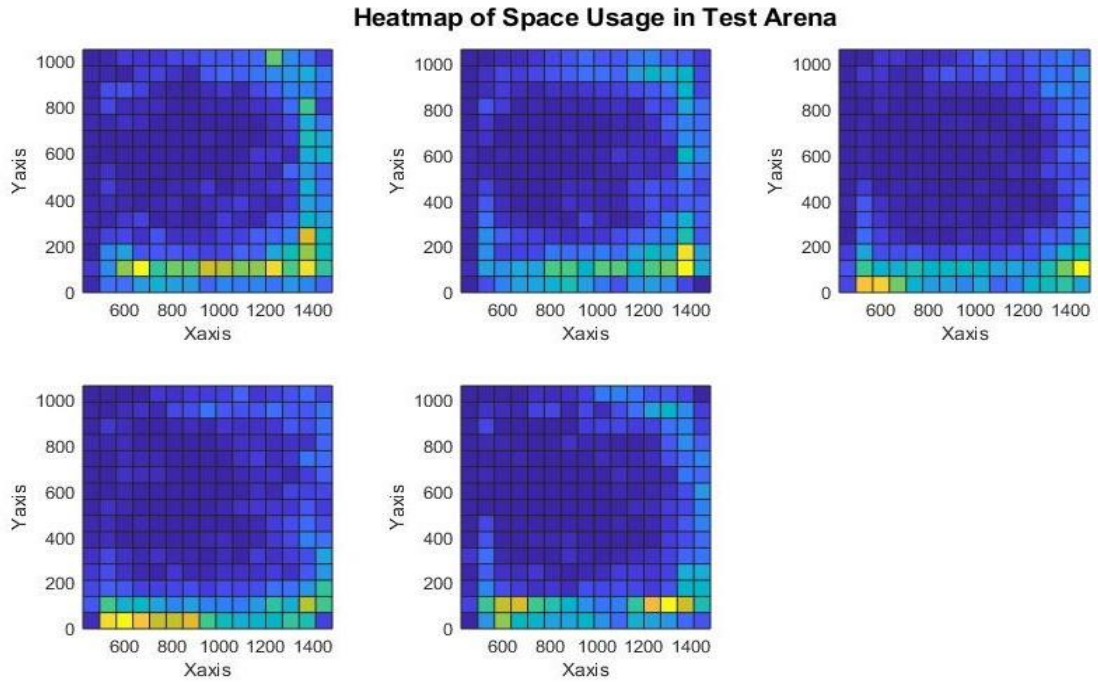

Figure 5. Example heatmaps of exploratory behaviour and space usage of each subject included in a group shoaling trial with five individuals.

Table 1. Endpoint behaviours obtained for each adult subject from an example ten minute group shoaling trial with five individuals.

	BEHAVIOUR					
	Average Speed (cm/sec)	**Time Spent Immobile (sec/min)**	**Area Covered (%)**	**Nearest Neighbour (cm)**	**Time Spent in Proximity (sec/min)**	**Average Path Length (cm)**
1	5.95	2.53	85.33	2.73	50.4	5.39
2	4.97	3.63	83.11	3.15	47.32	5.66
3	5.22	2.16	68.88	1.84	52.15	4.89
4	5.42	2.60	66.66	2.38	52.24	5.20
5	5.43	4.15	76.89	2.33	51.69	5.17

2.4.2 Social Behaviour

Social behaviour was measured by three metrics, nearest neighbour distance, average path length and time within two body lengths of a conspecific. Examining average path length seems to suggest that for the majority of trials all individuals maintained a distance of

approximately 5-6 cm to the other members of their shoal (see Table 1), a distance of roughly 3 body lengths. The average endpoint values for nearest neighbour distance were approximately 2-3 cm, a distance slightly further than a single body length, this was very similar between individuals. It also seems to be the case that individuals spent the majority of their time in proximity to a conspecific throughout the trial, with typically more than 50 seconds per minute being within two body lengths of another individual (see Table 1).

Table 2. Endpoint behavioural values obtained from a single larval movement trial with five subjects.

LARVAL ACTIVITY

	Total Distance (cm)	**Movement Events**
1	18.53	41
2	16.77	22
3	31.96	58
4	54.46	51
5	66.82	45

2.4.3 Larval Movement

Locomotor activity in larvae was measured by two metrics, total distance and movement events. Frame by frame movement was measured as the straight-line displacement between coordinates between successive frames. Total distance was measured as centimetres moved per second, where the sum of these distances was calculated over the course of the trial. Variability in total distance travelled was high between individuals, ranging between approximately 16cm over the 10-minute trial to 66cm over the 10-minute trial (see Table 2). The most active individual therefore moved over three times the distance of least active individual. The number of movement events, or transitions from stationary to active was also variable between subjects. The fewest number of movement events displayed by an individual in the ten-minute trial was 22, whereas the highest was 58, almost three times the

fewest (see Table 2). The least active individual (Subject 2) also displayed the fewest number of movement events (22), whereas the most active individual (Subject 5) did not display the highest number of movement events (45, highest = 58). Indicating that as activity increased, the duration of movement events becomes more variable.

2.5 Discussion

The availability of open source multi-object tracking software such as idTracker (Pérez-Escudero et al. 2014) has enabled the accurate and reliable quantification of animal behaviour in controlled research environments at relatively low cost. The results obtained here from idTracker trajectories supported this, and showed a high degree of reliability as almost 90% of the 18,000 frames for each individual recorded were successfully allocated coordinates. An accuracy of identity score of approximately 97% was also obtained. These results suggest that the relative scale of objects in frame and the overall video quality was sufficient to yield highly reliable tracking results. Here, movement was measured as the straight-line displacement between relocations. This is a typical approach for estimating distance travelled from positional data, although it has been noted that this approach is sensitive to the frequency at which relocations are sampled (Noonan et al. 2019). This is not an issue in this case due to the acceptable frequency at which trajectories were estimated of 30 samples per second. The behaviours outlined here (speed, mobility, exploration, nearest neighbour distance, average path length and time within two body lengths) are commonly used in studies investigating group and individual level behaviours in zebrafish. With good quality trajectories obtained from finely tuned tracking parameters, the behaviours reported here are similar to behavioural values obtained in previous studies, confirming the validity of the methods used here to obtain these behaviours. The approaches outlined here would serve as the basis for more advanced data analysis methods to answer important questions regarding behaviour in a variety of contexts.

The free-swimming adult zebrafish exposed to a group shoaling test here seemed to move at an average speed of approximately 5-6 cm/sec. Given that the average body length of the shoal was approximately 2 cm, this is equal to a speed of between 2-3 body lengths per second. Previous studies have shown that differences in activity levels are consistent between individuals over multiple trials (Tran and Gerlai, 2013). In a study investigating the effects of

caffeine exposure on the average speed of zebrafish, the reported average speed (cm/s) of control groups (exposed to 0mg/L of caffeine) was 5 cm per second (Ladu et al. 2015). These speeds are similar to average speed reported in the behavioural trial utilised here, suggesting that this speed may be a baseline level of activity in zebrafish exposed to a novel tank. Similar to rodents, the level of fear in zebrafish can be measured through novelty-induced freezing. Freezing bouts were uncommon here, as each subject typically spent an average of less than 5 sec/min immobile. A similar study of stress and anxiety like behaviour in adult zebrafish also found that baseline freezing bouts were uncommon (Nema et al. 2016). In this case, zebrafish were observed to display freezing behaviour in only 2-6% of the total recorded time.

Previous studies investigating zebrafish behaviour in an open field paradigm have shown that zebrafish display wall hugging behaviour, or 'thigmotaxis' (Champagne et al. 2010). In zebrafish, increased thigmotaxis is indicative of higher stress levels and is thought to be a coping mechanism for being placed into a novel arena (Stewart et al. 2012). The fish examined in this behavioural also trial seemed to display a high degree of thigmotaxis or wall hugging behaviour. This was demonstrated by the finding that, although individuals explored a high proportion of the test arena, individuals typically spent the highest amount of time in the outer zone of the arena. This also seems to be the case here, as subjects seem to prefer spending time in either one or two corners of the arena relative to other areas. This may however just be an emergent property of two motivations: being social and thigmotaxis, where moving groups will tend to move towards the corners of the arena.

The zebrafish examined in this study displayed a strong tendency to spend time within two body lengths of a conspecific, as demonstrated by the finding that individuals typically spent over 50 seconds per minute within proximity to another individual. This is perhaps unsurprising as zebrafish are known to be a highly social species in the wild (Suriyampola et al. 2016). Previous studies that have quantified shoaling behaviour in zebrafish have shown that within a trial shoal cohesion remained consistent throughout the trial (Miller and Gerlai, 2007; Miller and Gerlai, 2008). Fish within four body lengths of each other have been traditionally considered to be in the same shoal (Hensor et al. 2005). Here, zebrafish are shown to spend most of their time within two body lengths and therefore display a strong shoaling tendency and a high degree of shoal cohesion. Individuals seemed to display an average minimum inter-individual distance of around 2cm, roughly the same length as a single

body length, suggesting that this is a baseline for minimum inter-individual distance. Average path length between shoal members is often used as a measure of sociality and is useful as it incorporates all shoal members with no loss of information (Miller and Gerlai, 2008).

Age in particular seems to be a significant factor in the amount of activity displayed by larval zebrafish. After 4dpf larvae become more active, presumably due to the developmental milestone of swim bladder inflation at this age (Colwill and Creton, 2011). In this case activity levels of larvae aged 5-7dpf were indistinguishable, suggesting that the determination of locomotor activity level occurs during this period. In a previous study, larvae at 5dpf were shown to move and average of 15.2 cm per minute over a 15 minute trial, giving a total distance of 228cm over the entire trial (de Esch et al. 2012). This distance is much further than reported here, although here 24-well plates were used as opposed to 96-well plates. Given that zebrafish larvae have been previously shown to prefer to be close to the edge of wells (Colwill and Creton, 2011), the larger circumference and hence centre region in my test setup may have reduced larval movement into this area and inhibited movement.

Chapter 3: The Effects of Environmental Enrichment on Group Level Behaviours in Adult Zebrafish *(Danio rerio).*

3.1 Abstract

Environmental enrichment (EE) through novel structure and substrates is a key way in which an organism's environment can vary. Current evidence suggests that the level of structural complexity in controlled laboratory housing has significant effects on many aspects of brain function, physiology, and behaviour. Zebrafish are a highly social model species that are frequently used in biological research and display a robust individual and group level behavioural repertoire. Here, the effect of environmental enrichment was investigated by exposing adult zebrafish to either experimentally enriched or standard housing environments for a four-week period. Group level behaviours were then measured using a novel tank group-shoaling test and video capture, subject growth was also measured through imaging. Behavioural trials and body length measurements were performed before and after experimental enrichment. A combination of image analysis and open source multi object tracking software was utilised to obtain data from subjects exposed to both control housing and enriched housing. The results here show that experimental enrichment had significant effects on group level behaviours including locomotor activity and shoal cohesion. However, experimental enrichment had no effect on exploration and a negligible effect on standard body length. Enriched groups displayed significantly increased locomotor activity, decreased freezing behaviour, and a general increase in shoal cohesion. These results suggest that the physical complexity of housing conditions has significant effects on both swimming ability and group dynamics, and is fundamentally important for zebrafish housed in captive environments.

3.2 Introduction

3.2.1 Sources of Environmental Variability

There are many ways in which the environment that can vary within the lifetime of an individual. These changes may be driven by industrial and agricultural activity, or through more natural factors such as seasonality, habitat invasion, or natural destruction (Hoffmann

and Hercus, 2000). The term environmental enrichment (EE) is frequently used to describe changes to specific environmental conditions that enhance biological condition. In this sense, EE has been previously used to describe a factor which improves in the functioning of captive animals in their environment (Newberry, 1995). Some environmental factors are inherently stressful for animals inhabiting them. For example, the presence of predators or limited food availability can be considered environmental stressors. Such environmental conditions constitute a stressor when they cause a reduction in reproductive output or survivability, and when the persistence of these conditions leads to permanent damage (Hoffmann and Hercus, 2000). Increased levels of EE are generally thought to be a positive intervention across an animal's lifespan by promoting naturalistic behaviours in captivity, and by counteracting the negative effects of exposure to stressors (Sampedro-Piquero and Begega, 2016). Indeed, physical structures and substrates appear to provide a more natural environment for captive animals, and novel objects may also provide opportunities for new behaviours to emerge.

3.2.2 Phenotypic Plasticity in Variable Environments

The two main mechanisms that give rise to phenotypic adaptation in changing environments are long term genetic evolution, and short term phenotypic plasticity (Lande, 2009). Phenotypic plasticity has been described as the ability of a single genotype to produce multiple morphologies or behaviours in response to the environment (West-Eberhard, 1989). The phenotypic outcomes observed across different contexts are underpinned at the genetic level. Internal and external environmental signals are integrated into gene networks to produce variable phenotypes (Schlichting and Smith, 2002). Furthermore, plasticity is often involved with ecologically relevant traits such as morphology, physiology, or behaviour, and seems to affect all levels of ecological hierarchies through interactions between species (Miner et al. 2005). It has been suggested that for plasticity to facilitate adaptation it must direct a phenotype to the optimum value, however any movement away from the optimal phenotype may also reduce relative fitness (Ghalambor et al. 2007). There may also be inherent costs of maintaining plasticity for adaptation in novel environments, although these are less well understood (DeWitt et al. 1998; Murren et al. 2015). For example, investment in plasticity can be costly, this is due to the resources required for maintaining additional genetic machinery required for sensory detection of environmental conditions.

3.2.3 Behavioural Plasticity

Behavioural plasticity is a specific form of phenotypic adjustment, it can be broadly classified as either developmental or activational behavioural plasticity (Snell-Rood, 2013). Developmental plasticity is similar to the classical definition of phenotypic plasticity, and refers to the expression of novel behavioural phenotypes from a single genotype (learning). Activational plasticity refers to the activation of pre-existing underlying networks as novel environments are encountered. The range of behavioural responses observed in changing environments is appears to therefore a result of interactions between genetically-determined behaviours and an individual's lifetime experience or learned behaviours (Mery and Burns, 2010). This relationship between behavioural responses to environmental gradients can be quantitatively measured as behavioural reaction norms (Smiseth et al. 2008). Such environmentally induced responses are typically the result of alterations to physiological or neurological processes.

Group level and social behaviours such as social interaction and learning, aggression, and shoaling in fish appear to be particularly plastic in different social contexts (Oliveira, 2012). It is thought that social plasticity in this way is achieved by altering gene expression networks in the brain in response to social information, and that these alterations are orchestrated by changes in the epigenetic states of brain networks (Cardoso et al. 2015; Renn et al. 2008). Social plasticity requires the identification of social information that signal changes in the social environment. For example, in guppies it has been demonstrated that familiarity of conspecifics facilitates social learning (Swaney et al. 2001). Shoaling in zebrafish arises during larval development and individuals continue to shoal from this point onwards. Juvenile and adult zebrafish exhibit distinct shoaling preference for familiar shoals which is remarkably stable throughout their lifetime (Engeszer et al. 2007). In this case, individuals appeared to learn forging behaviours more effectively from familiar conspecifics than from non-familiar conspecifics.

3.2.4 Effects of Structural Enrichment

One of the primary benefits of increased structural complexity for animals is to provide shelter for the avoidance of predators (Morice et al. 2013). However, habitat complexity also affects other behavioural realms such as locomotion, exploration, stress reactivity and sociality. For

example EE has been shown to increase hippocampal neurogenesis, and offset the effects of neuronal ageing (Speisman et al. 2013). Rodents reared in enriched environments have also been shown to exhibit differences in the rate of habituation of locomotor activity and exploration (Varty et al. 2000). Here, high levels of EE reduced habituation of locomotor activity and reduced exploration. Previous studies in rodents have shown that EE also appears to modulate social plasticity and cognition (Gubert and Hannan, 2019). For example, EE has been shown to promote aggressive behaviour in response to a mild stressor in enriched male mice (McQuaid et al. 2012). EE through the addition of novel structures has also been shown to enhance social interactions during dark phases of the day/night cycle in rats (Lambert et al. 2016). Early life EE also increases the likelihood that enriched individuals will become submissive during social encounters later in life in rodents (Cao et al. 2017). In this study male EE rats also appeared to be perceptually more attractive to female rats than non-enriched males. Taken together, these studies in rodent models provide compelling evidence that EE can alter social behaviours such as aggression and social interactions, and may also alter social dynamics in the establishment of dominance hierarchies.

3.2.5 Effects of Structural Enrichment in Fish Models

The effect of structural complexity through physical structures and substrates has been studied in some detail in captive fish species (Näslund and Johnsson, 2016). For fish species housed in captivity, shelters such as pipes, and substrates such as gravel and artificial vegetation, are commonly used forms of structural enrichment. Enriching the environment in this way can affect fish species in a variety of ways including modulating locomotor activity, exploration, aggression, stress reactivity and sociality. For example, gilthead seabream (*Sparus aurata*) reared in enriched tanks have recently been shown to exhibit higher exploratory behaviour in a maze based trial than those housed in standard conditions (Arechavala-Lopez et al. 2019). In juvenile rainbow trout, *Oncorhynchus mykiss*, it has been reported that housing individuals in physically complex environments improves swimming performance and agility over a relatively short period of time (Bergendahl et al. 2017). Where enriched individuals displayed a greater ability to swim along a channel whilst avoiding obstacles. Zebrafish housed in barren environments have also been shown to exhibit increased locomotor activity relative to individuals housed in physically enriched housing conditions (von Krogh et al. 2010).

Structural enrichment in fish also appears to affect social behaviours and group dynamics. In the two-spotted goby, *Gobiusculus flavescens*, EE was found to effect mating behaviour and mating success in a sex dependent manner (Cats Myhre et al. 2012). Here, increased habitat complexity negatively affected encounter and courtship rates in females. In males, increased habitat complexity also resulted in fewer multiple-male courtships. In this case it was suggested that higher levels of structural complexity may reduce mating encounters due to the fact that complex environments make locating, tracking, and assessing potential mates more challenging. EE through structural complexity has also been shown to affect group level behaviours in zebrafish models. A recent study has demonstrated that prior experience of physical enrichment impacts group dynamics in adult zebrafish (Sykes et al. 2018). Here a two week exposure of adult zebrafish to different levels of structural complexity significantly impacted group behaviour. Groups with recent experience of high levels of EE appeared to charge each other more frequently, and formed tighter shoals than groups exposed to less complex environments. These effects were present regardless of the novel context in which behaviour was assayed and did not appear to be a result of differences in activity levels. Other studies in zebrafish have also demonstrated that acute stressors affect shoal density and variation in nearest neighbour distances (Kleinhappel et al. 2019). In this case, groups exposed to acute stressors displayed increased shoal density and reduced variability in nearest neighbour distances.

3.2.6 Aims and Hypothesis.

Structural complexity is a fundamental aspect of an individual's environment that can have significant effects on both physiology and behaviour. This paradigm is well studied in rodent models, and the literature highlights a number of biological functions that are sensitive to the level of structural complexity. While the effects of environmental enrichment on aggression and brain function have been studied in certain fish species, there has been relatively less investigation of the effects of EE on social behaviour or shoaling dynamics. The aim of this study was to investigate the effects of enrichment on group level behaviour and on growth. Adult zebrafish shoals were exposed to a four-week experimental enrichment regime in which experimental groups were exposed to either high or low levels of structural enrichment. Groups were assayed for a range of behaviours in a novel arena group shoaling test both before and after experimental enrichment. I hypothesised that four weeks of environmental

enrichment would have significant effects on mobility, group cohesion and exploration. Based on current evidence, I predicted that high levels of EE for a sufficiently long fixed period would result in reductions in freezing bouts and changes in locomotor activity (von Krogh et al. 2010), as well as an increase in exploratory behaviour (Arechavala-Lopez et al. 2019) and changes in shoal cohesion (Sykes et al. 2018). I also anticipated that these changes in activity, mobility and exploration may also have indirect effects on physiology, growth, and body size.

3.3 Methodology

3.3.1 Animals

All of the fish used were naïve to behavioural testing and imaging prior to testing in this study. Subjects were taken from a single cohort of zebrafish originally obtained as fertilised eggs from multiple clutches, and then raised under identical housing conditions. All fish were housed and maintained in a flow-through custom-built recirculation systems fitted in racks each containing twenty 5L tanks and a sump for constant physical and biological filtration (as described in the previous methods chapter). Fish were fed a standard diet of ground Tetramin flake food for the duration of the study. All groups were fed once daily with TetraMin® Tropical Flakes until all food was consumed within 5 minutes. A consistent, 10/14hr light/dark cycle was maintained, water conditions were maintained at 27°C, pH 7.5 and nitrate, nitrite and ammonia levels were regularly monitored. Non-experimental fish were housed at a stocking density of fewer than 20 fish per tank in accordance with Home Office recommendations. In experimental tanks, fish were housed at a density of 10 fish per tank. Standard housing conditions consisted of a 5L housing tank with a single artificial plant. No Home Office-regulated procedures were carried out in the methods outlined here, and ethical approval for this study was obtained from the Liverpool John Moores University Animal Welfare and Ethics Steering Group.

3.3.2 Experimental Design

Two experimental conditions were utilised in this study, control housing and enriched housing (see Figure 1). The control groups were exposed to standard housing conditions as outlined previously, a standard 5 litre housing tank with a single green artificial plant. The enriched groups were housed in a standard housing tank with gravel substrate, three colours of

artificial plant, with both tunnels and shelters. The artificial vegetation used here were leafy green plants, with either, orange, blue or pink leaves (see Figure 1). The tunnels used here were sections of ¾ inch PVC pipes cut to approximately 5cm in length, a single length of pipe was placed in each enriched tank. Finally, approximately 2cm of gravel was spread evenly across the base of the tank. At the start of this study, a sample of n = 200 mixed sex adult zebrafish were taken at random from a larger population. They were then randomly allocated into new tanks or groups at a density of n = 20 fish per tank. Group size and composition remained consistent throughout the entire experimental period. All tanks were housed on the same recirculating system, and visual contact between enriched and control tanks was removed by using laminated A4 sheets as dividers. All tanks were conditioned in their experimental groups for one week in standard housing conditions to control for any unwanted pre-existing variation. Each experimental group was subjected to two tests, a novel tank group shoaling test and standard body length measurements. Both of these tests were performed on each group at two different timepoints, pre-treatment and post-treatment. Each experimental group was moved into corresponding experimental housing conditions after conditioning and immediately following baseline testing at the start of week 0. Half of the tanks (n = 10) were placed into enriched housing and the other half (n = 10) into control housing. Each tank was exposed to experimental enrichment for four consecutive weeks. At the start of week 5, each tank was removed from experimental housing and tested for the same behaviours as in week 0 (see Figure 2).

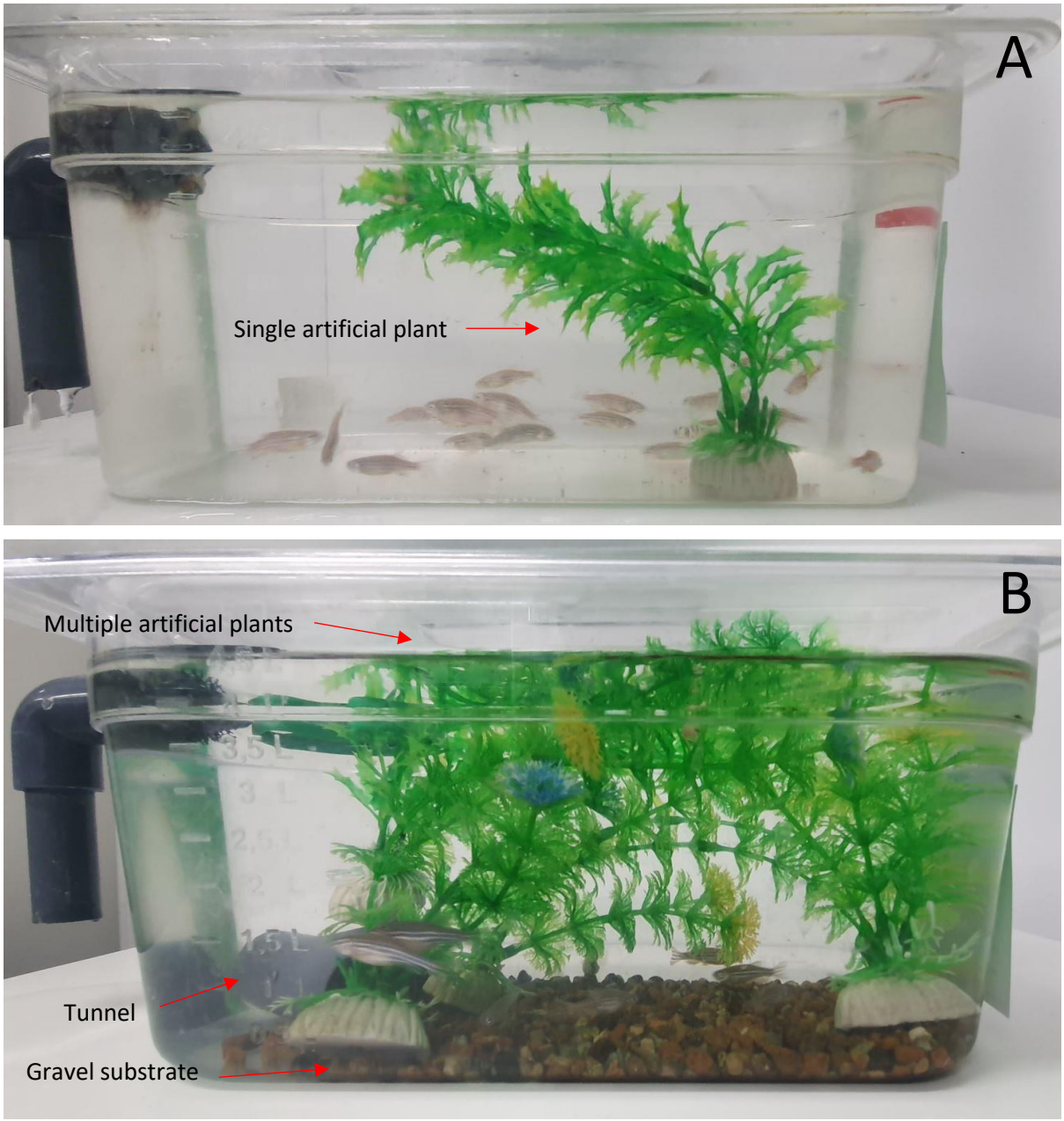

Figure 1. Experimental enrichment conditions. A - Control housing conditions comprising a single artificial plant in a standard housing tank. B - Enriched housing consisting of gravel substrate, a single grey PVC tunnel, and three different colours of artificial plant (orange, blue and pink).

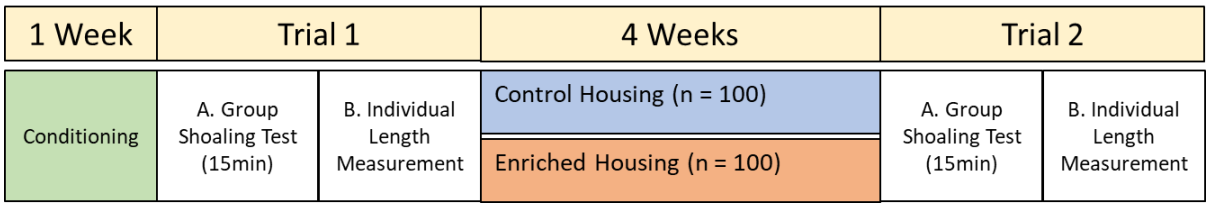

Figure 2. Experimental timeline of enrichment exposure. All groups were conditioned for 1 week in standard housing, which was then followed by trial 1, consisting of a group shoaling test and individual standard length measurement. Experimental enrichment lasted for 4 weeks and consisted of control housing (low enrichment) vs enriched housing (high enrichment). All groups were then immediately tested again in trial 2, which also consisted of a group shoaling test and individual standard length measurement.

3.3.3 Behavioural Tests

Individuals and groups of fish were tested using a novel arena group-shoaling test. The testing arena consisted of a 50x70 cm acrylic tank filled with 5cm of conditioned water. The arena was illuminated from below using an A1 light box (MiniSun LightPad, Minisun, Eccles, UK) to ensure even illumination of the testing arena. The arena and light box were both placed in a larger 2 m diameter circular opaque black tank which was partitioned behind a floor length curtain to prevent visual distractions from outside of the testing arena (as described in the previous methods chapter). The entire arena was recorded from above with a Sony PJ410 Camcorder suspended directly above with a custom-assembled tripod and extension arm. Water in the arena was changed and the tank was cleaned with conditioned water between each trial. Prior to each test, each group was left undisturbed for 5 minutes inside their housing tanks and within the partitioned testing area to reduce stress from transport. Following this, each individual in the experimental group was transferred to the testing arena by netting. The recording was then started manually and the partition curtain was again closed around the test arena for the remainder of the test. The entire test lasted 15 minutes. After 15 minutes, the subjects were removed from the arena and placed back into their respective housing conditions. The first 5 minutes of each trial was regarded a habituation period and not used for the purposes of this study.

The open-source video analysis software idTracker (Pérez-Escudero et al. 2014) was used in order to obtain trajectories for every individual from the video files of behavioural tests. Using

this method, trajectories can be obtained with on average 99.7% correct identities with no propagation of errors (Pérez-Escudero et al. 2014). This software is robust and easy to use and has been previously validated in zebrafish against traditional human behavioural scoring. The raw data obtained here was MATLAB data files, which contained trajectories for every individual stored as coordinate data. Custom MATLAB scripts were developed in order to extract behavioural endpoint values from raw coordinate data and trajectories obtained from idTracker. Each of the behavioural measures were calculated per individual and per frame (~18,000) and averaged across the duration of the test. Standardization of data was performed to control for inherent variation between subjects in tracking accuracy through occasional missed frames. The average body length of all fish in each group was obtained and used for all body length dependent behavioural measures. All behavioural measures were then averaged on a per-tank basis.

Locomotor activity was measured as the average speed of an individual across the entire behavioural trial and was recorded in centimetres per second (cm/sec). Individual freezing behaviour was measured as time spent immobile across the entire trial. An individual was classified as being 'immobile' if it had travelled less than one body length in any two second time period and average time spent immobile was calculated as the proportion of seconds immobile per minute (sec/min). Exploration for each individual within the group was measured by calculating the proportion (%) of the total area covered during the test. This was achieved by segmenting the test arena into twenty equally sized quadrants and calculating the proportion of quadrants that an individual visited in the entire trial. Social behaviour in the group behavioural test was measured using three distance-based parameters: average path length, nearest neighbour distance, and time in proximity to another individual. An individual was said to be 'in proximity' to another individual in a given frame if it was within two body lengths of a conspecific. This was calculated across the entire trial and reported as seconds per minute in proximity (sec/min). Average path length was defined as the mean distance from a focal individual to every other member of the group in each frame. A mean average path length value for each individual was then calculated from all frames across the entire trial and is reported in centimeters (cm). Nearest neighbour was calculated as the shortest distance between a focal individual and all other individuals in each frame. The mean value was then calculated for each subject across the entire trial.

3.3.4 Imaging and Measuring Standard Length

All subjects from each tank used for behavioural testing were then measured for standard body length (BL) The tank of subjects was first transported to the body length imaging apparatus. The tank was left uninterrupted for five minutes to reduce stress from transport. A petri dish filled with clean conditioned water was placed on top of an A3 light box (MiniSun LightPad, Minisun) directly underneath a Sony PJ410 Camcorder at a standardised height from the dish. The petri dish was placed directly next to scale bars (cm) for calibration (see Figure 3). The camcorder was used to capture burst images of subjects in the dish to obtain five images for each individual, which would be used to measure standard length. Pairs of subjects from each tank were placed into the petri dish for image acquisition and then removed and placed into a new tank filled with conditioned water. This was repeated until all individuals in the tank had been imaged, after which subjects were returned to their home tank and replaced in housing racks. All images of subjects were imported into the ImageJ (Schneider et al. 2012) image analysis software for standard length measurements. Each image was calibrated using scale bars (cm) located on either axis of the captured image in each photograph. Calibration consisted of measuring along the x and y axis of the image between the two longest recordable points. Once calibrated, individuals in each image were measured from the tip of the snout to the base of the tail. Mean standard length scores were calculated for each subject and subsequently transcribed into an Excel file for data analysis.

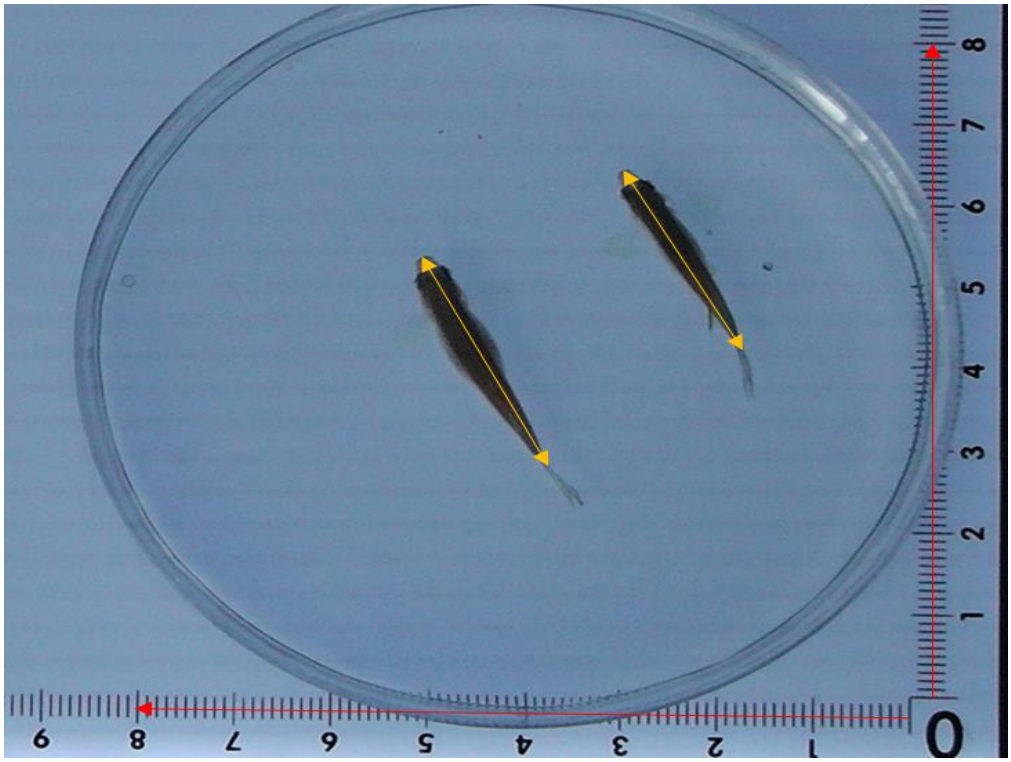

Figure 3. Standard length measurement. In each image, units of measurement (cm) were calibrated using both the x and y axis (red). Then, the distance between the tip of the snout and base of the tail was measured for each specimen (yellow) as standard length (cm).

3.3.5 Data Analysis

A comparison of experimental groups pre- and post-treatment was performed in the R Studio software environment (RStudio Team, 2020) using linear mixed modelling approach for each variable. This analysis was performed using the 'lmer' function in the 'lme4' package (Bates et al. 2015), and was performed on per-tank means as individual level identities could not be retained between trials. The models included two fixed effects: the enriched/standard housing treatment effect, and the pre-/post-treatment trials repeated effect, as well as an interaction term between these two factors. This was used as oppose to a simpler comparison of change between trials in order to ensure there was no significant variation between groups at baseline testing. Tank identity was included as a random effect in the model to account for the repeated sampling of each tank. In order to estimate significance of these terms, type II analysis of variance tests with Wald chi-square tests were performed using the 'Anova'

function from the ‘car’ package in R (Fox and Weisberg, 2018). Due to the large sample size in each model (n = 100) and the application of the central limit theorem, the residuals were assumed to be normally distributed without the need for formal testing. However, the residuals for each model were instead plotted to assess normality and heteroscedasticity. Each model was additionally tested for outlier observations by calculating the probability that Studentized residuals were outliers after applying a Bonferroni correction for multiple comparisons. Each model was additionally tested for outlier observations by calculating the probability that Studentized residuals were outliers after applying a Bonferroni correction for multiple comparisons. In this case criteria for overly influential observations were defined as those with Bonferroni adjusted p values < 0.05. There were no outliers or overly-influential observations in any of the models and therefore no observations were removed.

A scaled and centered principal component analysis (PCA) was performed using all the measured behaviours from the group tests as an exploratory analysis technique. The component analysis was performed using the ‘prcomp’ function from the ‘stats’ core package in R (RStudio Team, 2020). Separate analyses were performed for the pre- and post-treatment data to maintain independence of observations. Components with an eigenvalue exceeding one were extracted for further analysis. Eigenvalues were estimated using Horns Parallel Analysis from the ‘paran’ package in R (Dinno, 2009). Examination of the loadings for each behaviour onto the corresponding component allows for the interpretation of correlated traits in each component. Component scores were plotted for each extracted component with treatment as a grouping factor to visually assess the similarity of the groups at both timepoints in multivariate space.

3.4 Results

3.4.1 Body Length

The analysis of variance performed on the model terms indicated that there was a significant main effect of treatment on body length (ChiSq = 4.43, DF = 1, p = 0.035), however there was no significant interaction (ChiSq = 0.68, DF = 1, p = 0.409), or effect of repeated trials (ChiSq = 2.41, DF = 1, p = 0.120). The results from this model suggested that there was no difference in body length between control groups (2.45 cm) and enriched groups (2.48 cm) pre-

treatment (see Figure 4). Whereas the subjects from enriched tanks (2.54 cm) were longer than those from the control tanks (2.47 cm) post-treatment (see Figure 4).

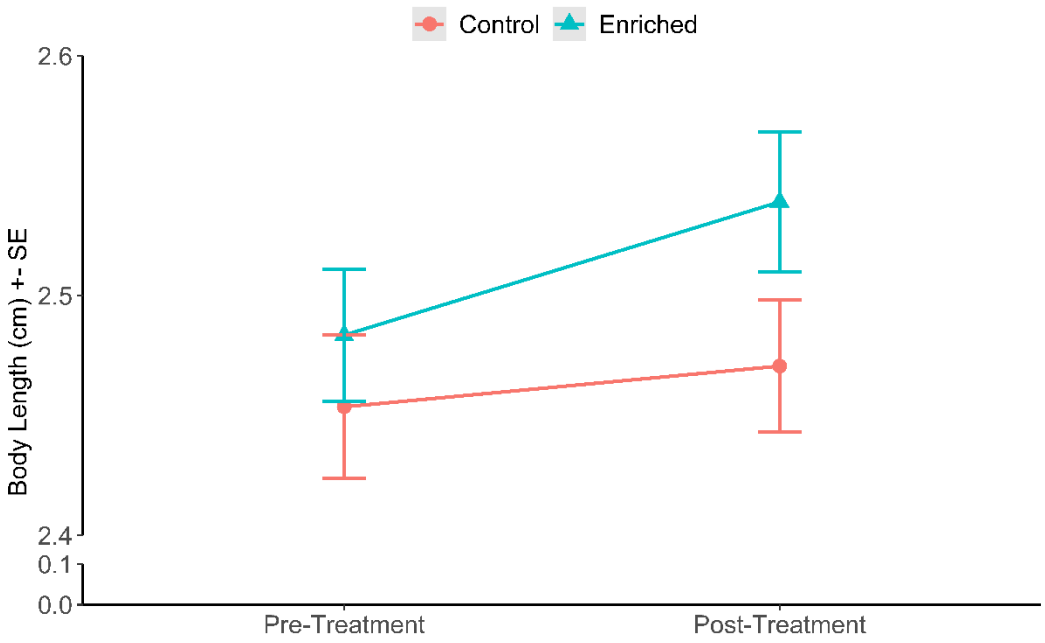

Figure 4. Interaction plot of standard body length (cm) of each group in each test. Mean value of each group in each test are reported with standard errors (SE).

3.4.2 Locomotion, Mobility and Exploration

Average Speed

The estimates obtained from the model for average speed indicated that there was a significant interaction between experimental treatment and trial (ChiSq = 5.03, DF = 1, p = 0.024). Furthermore, there were also significant main effects of both treatment (ChiSq = 14.24, DF = 1, p < 0.001) and repeated trials (ChiSq = 6.77, DF = 1, p = 0.009).

The results from this model showed that enriched groups (13.4 cm/sec) were significantly faster than control (10.6 cm/sec) post-treatment (see Figure 5). However, the enriched groups (11.1 cm/sec) were not significantly faster than control groups (10.4 cm/sec) in the pre-treatment test (see Figure 5). Furthermore, the average speed of control groups did not significantly differ between pre-treatment (10.4 cm/sec), and post-treatment (10.6 cm/sec) tests (see Figure 5). Whereas enriched groups significantly increased their average speed between pre-treatment (11.1 cm/sec) and post-treatment (13.4 cm/sec) tests (see Figure 5).

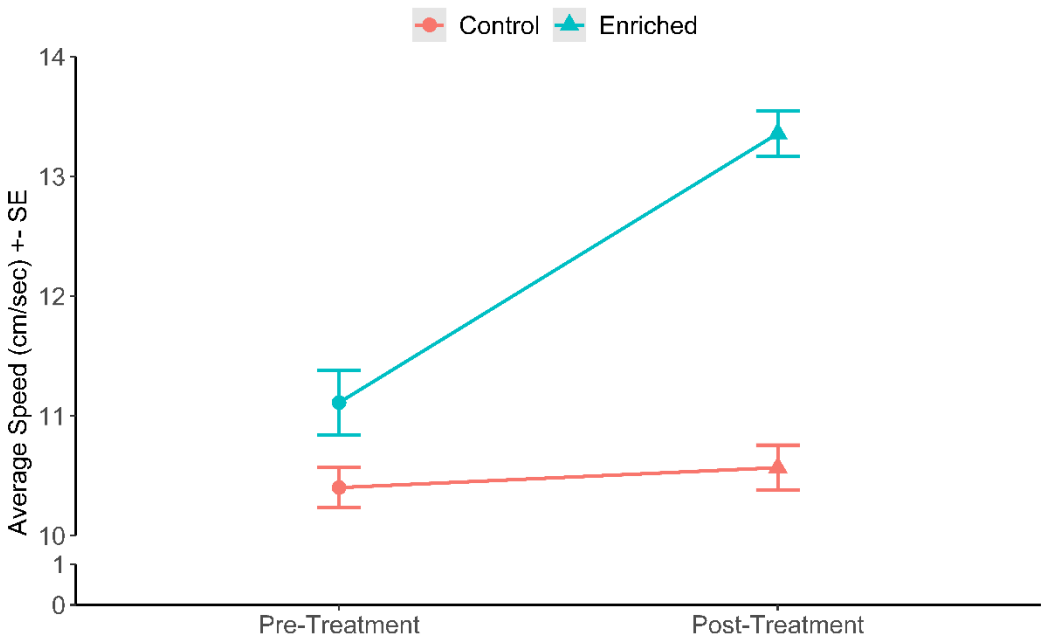

Figure 5. Interaction plot of average speed (cm/sec) of each group in each test. Mean value of each group in each test are reported with standard errors (SE).

Time Spent Immobile

There was a significant interaction between experimental treatment and trial (ChiSq = 6.56, DF = 1, p = 0.010) on time spent immobile. There were also weak main effects of both repeated trials (ChiSq = 3.77, DF = 1, p = 0.052) and experimental treatment (ChiSq = 3.22, DF = 1, p = 0.072).

The results from the model here showed that there was no difference in time immobile between control groups (8.72 sec/min) and enriched groups (8.71 sec/min) pre-treatment (see Figure 6). Whereas the control groups (8.96 sec/min) spent significantly more time immobile than enriched groups (6.91 sec/min) post-treatment (see Figure 6). The results also showed that average time spent immobile of control groups did not differ between pre-treatment (8.72 sec/min) and post-treatment (8.96 sec/min) tests (see Figure 6). Conversely, enriched groups exhibited a significant decrease in their time spent immobile between pre-treatment (8.71 cm/sec) and post-treatment (6.91 cm/sec) tests (see Figure 6).

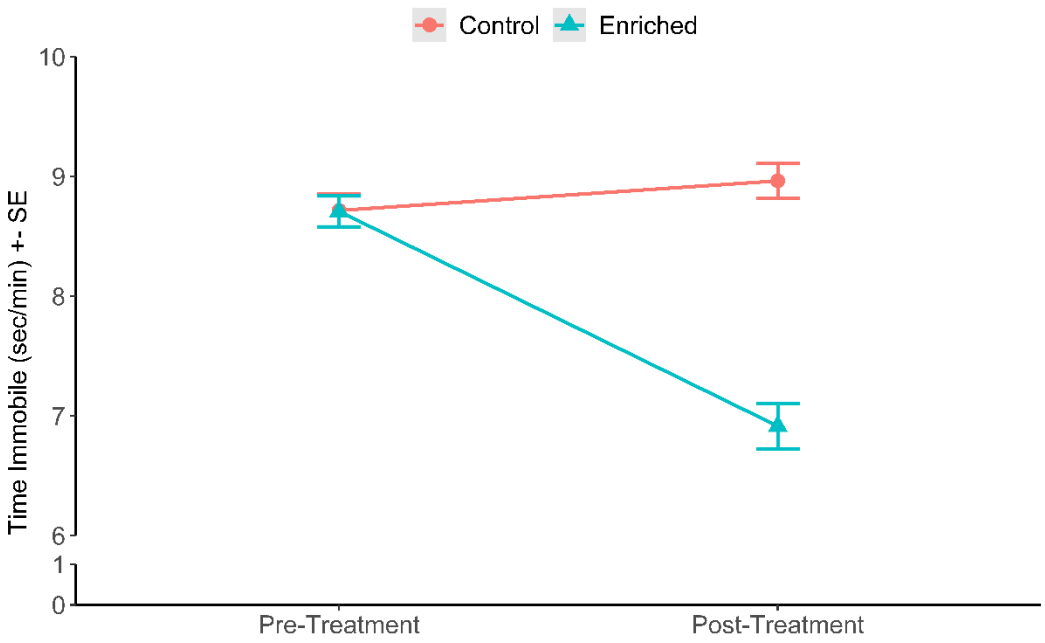

Figure 6. Interaction plot of time spent immobile (sec/min) of each group in each test. Mean value of each group in each test are reported with standard errors (SE).

Area Covered

There were no main effects of treatment (ChiSq = 2.05, DF = 1, p = 0.151) or repeated trials (ChiSq = 0.005, DF = 1,p = 0.939) on the proportion of area covered. There was also no significant interaction between these two main effects (ChiSq = 0.12, DF = 1, p = 0.722, see Figure 7).

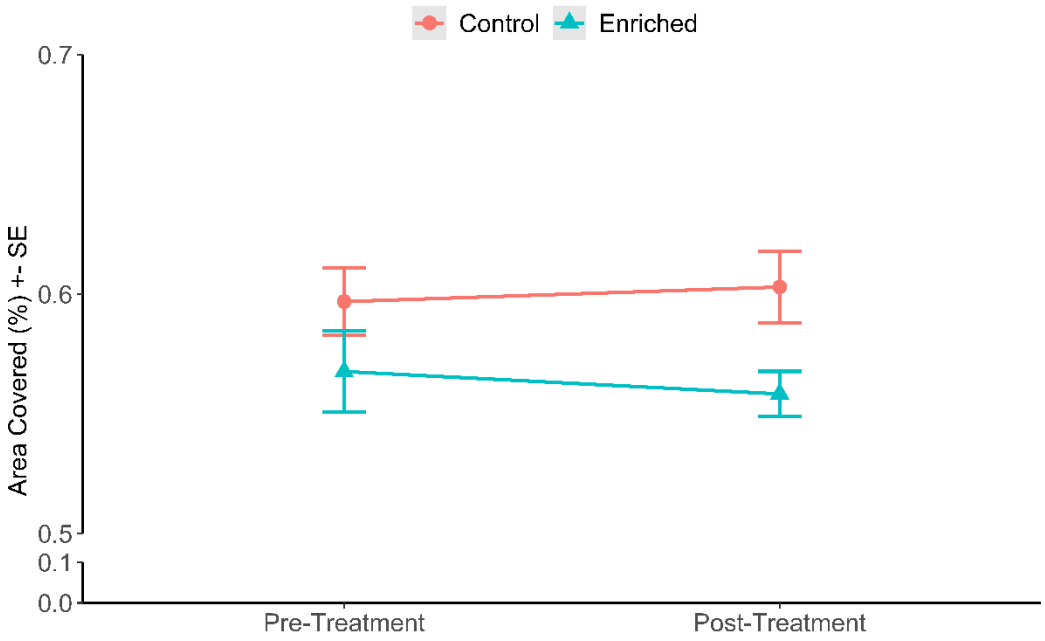

Figure 7. Interaction plot of proportion of area covered (%) of each group in each test. Mean value of each group in each test are reported with standard errors (SE).

3.4.3 Inter-Individual Distance, Nearest Neighbour and Proximity

Average Path Length

There was a significant interaction effect of experimental treatments between trials on average path length (ChiSq = 5.28, DF = 1, p = 0.021). Furthermore, there were also significant main effects of experimental treatment (ChiSq = 7.21, DF = 1, p = 0.007) and repeated trials (ChiSq = 9.82, DF = 1, p = 0.001).

The results from this model indicated that there was no difference in path length between control (35.0 cm) and enriched groups (34.4 cm) pre-treatment (see Figure 8). However, the average path length of control groups (33.8 cm) was significantly longer than enriched groups (27.0 cm) post-treatment (see Figure 8). The results from this model also showed that the average path length of control groups did not differ between pre-treatment (35.0 cm), and post-treatment (33.8 cm) tests (see Figure 8) whereas enriched groups significantly reduced their average path length between pre-treatment (34.4 cm) and post-treatment (27.0 cm) tests (see Figure 8).

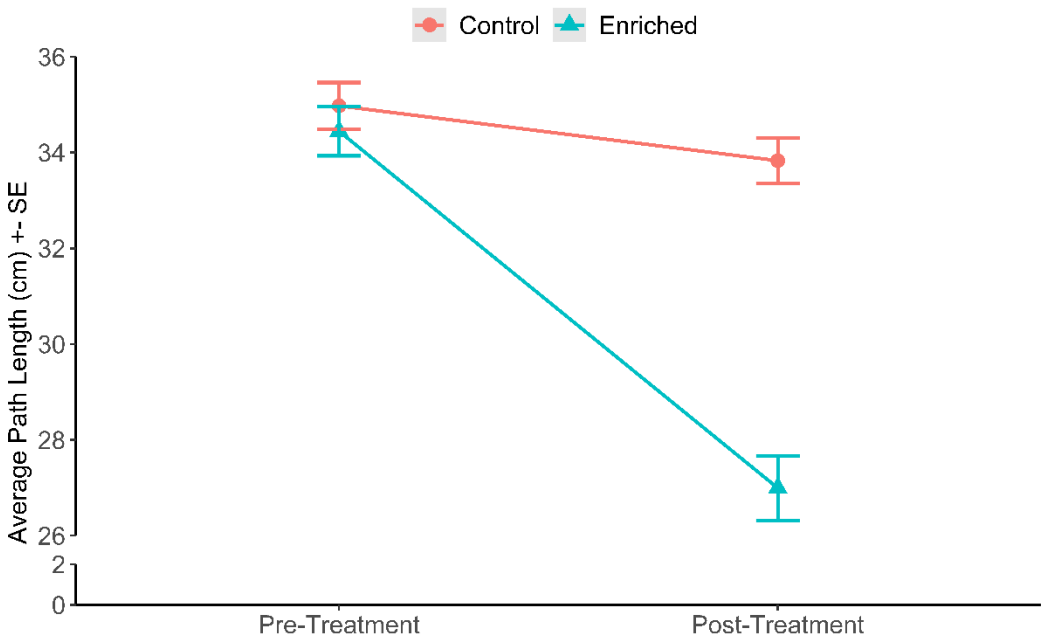

Figure 8. Interaction plot of proportion of average path length (cm) of each group in each test. Mean value of each group in each test are reported with standard errors (SE).

Time in Proximity to a Conspecific

There was also a significant interaction effect of experimental treatment between trials (ChiSq = 5.98, DF = 1, p = 0.014). There was also a significant main effect of experimental treatment (ChiSq = 9.20, DF = 1, p = 0.002) and a large main effect of repeated trials (ChiSq = 24.67, DF = 1, p < 0.001).

There appeared to be no apparent difference in time in proximity between control (29.7 sec/min) and enriched groups (30.6 sec/min) pre-treatment (see Figure 9). However, the enriched groups spent significantly more time in proximity to a conspecific (42.0 sec/min) than control groups (33.5 sec/min) post-treatment (see Figure 9). The model also showed that the time spent in proximity did not differ for control groups between pre-treatment (29.7 sec/min) and post-treatment (33.5 sec/min) tests, whereas enriched groups spent significantly less time in proximity to a conspecific in the pre-treatment test (30.6 sec/min) than in the post-treatment test (42.0 sec/min, see Figure 9).

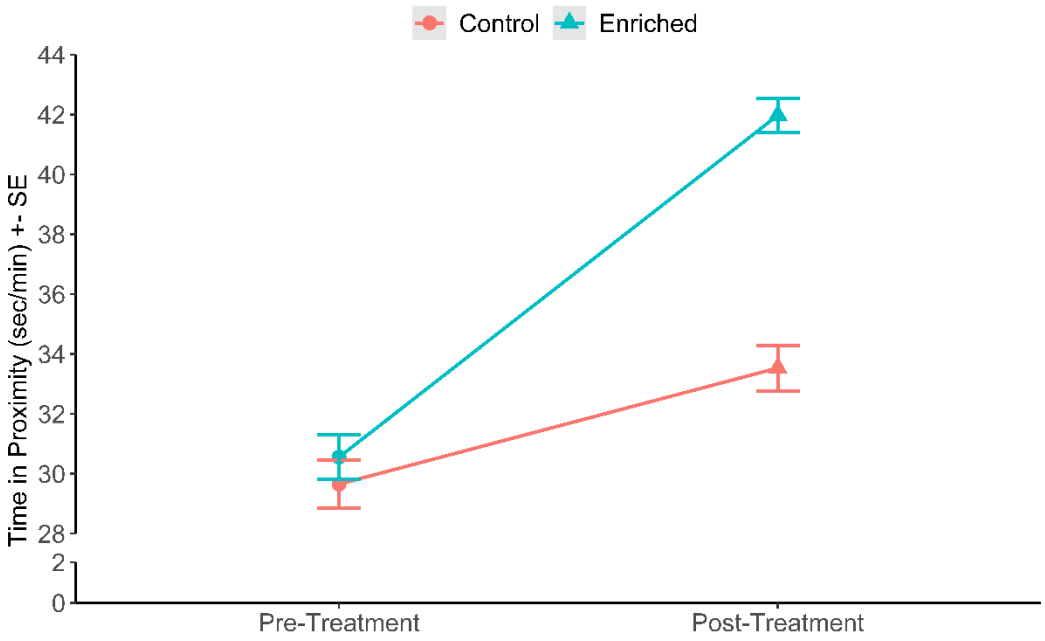

Figure 9. Interaction plot of proportion of time in proximity to conspecific (sec/min) of each group in each test. Mean value of each group in each test are reported with standard errors (SE).

Nearest Neighbour Distance

Estimation of effect sizes for nearest neighbour distance indicated that there were no main effects of experimental treatment (ChiSq = 1.62, DF = 1, p = 0.202) or repeated trials (ChiSq = 2.25, DF = 1, p = 0.133). Furthermore, there also appeared to be no interaction effect of treatment between trials (ChiSq = 0.54, DF = 1, p = 0.463, see Figure 10).

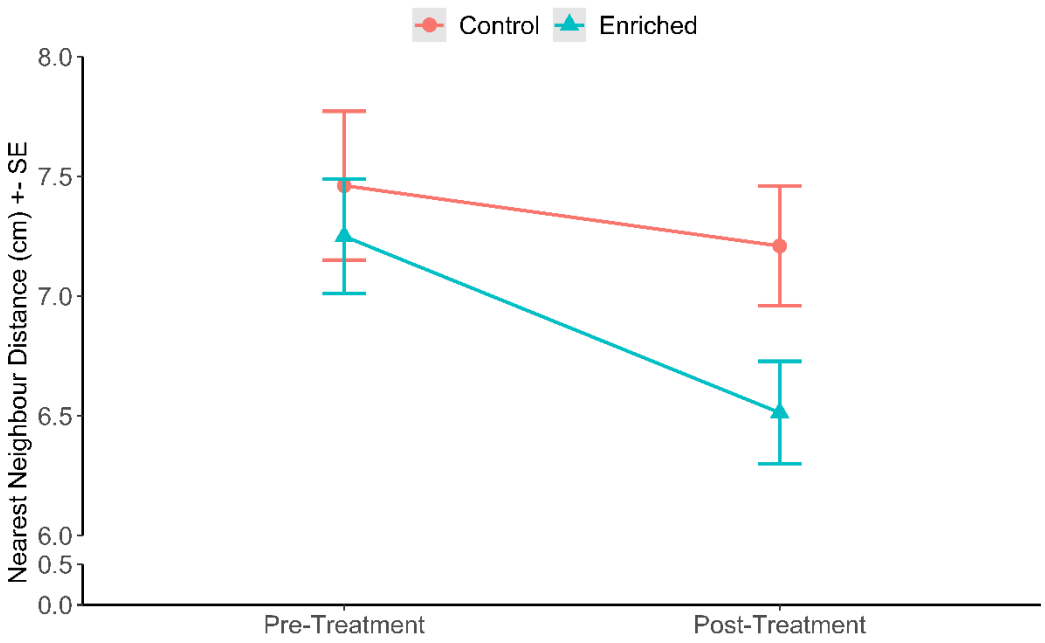

Figure 10. Interaction plot of proportion of nearest neighbour distance (cm) of each group in each test. Mean value of each group in each test are reported with standard errors (SE).

3.4.4 PCA

Pre-treatment

The PCA performed on the tank level data from the pre-treatment behavioural trials gave a total of six novel components, the first two of which cumulatively explained ~67% of the total variation in the data set. The results from the Horn's Parallel Analysis performed on the data from trial 1 indicated that the first two components had adjusted eigenvalues >1 which should therefore be retained for further analysis. PC1 was primarily a measure of social behaviour. In this case, positive scores for PC1 indicated groups that had a longer average path length and nearest neighbour distance and spent less time in proximity to another individual. Conversely, negative scores on this component indicated groups that had shorter average paths and nearest neighbour distances and spent more time in proximity to another individual (see Figure 11 A). PC2 was primarily explaining variation in locomotor behaviour. For PC2, positive scores described groups that displayed increases in both locomotion and time spent immobile, as well as a reduction in exploration. Conversely, negative scores for this component indicated individuals that were more explorative, slower, and spent less time immobile (see Figure 11 A). The plotting of these component scores pre-treatment suggested

that there was very little difference between the groups for either component (see Figure 11 A).

Post-treatment

The component analysis performed on average tank behaviours in the post-treatment behavioural trials gave a total of six novel components, the first two of which accounted for ~66% of total variability in the data. The results of Horn's Parallel Analysis for component retention indicated that only the first component had an adjusted eigenvalue >1 and should be retained for further analysis. However, to maintain a similar approach to that of the results from the first trial, the first two component were retained. PC1 was primarily a measure of speed and proximity. Positive scores in this case indicated subjects which displayed higher locomotor activity and spent more time in proximity to conspecifics. Conversely negative scores described slower swimming groups that also showed reduced group cohesion and hence larger inter-individual distances (see Figure 11 B). PC2 seemed primarily to be a measure of exploration, as proportion of area covered loaded heavily onto this component. Positive scores in this case indicated groups which tended to cover a larger proportion of the available space (see Figure 11 B). The plot of average tank component scores for PC1 and PC2 appeared to reveal a clear separation between control and enriched groups along PC1 (see Figure 11 B). Specifically, enriched groups seemed to score higher along PC1 than control groups. This observation seems to support the finding from the models outlined here, that enriched groups were significantly faster and more cohesive than control groups in the final trial.

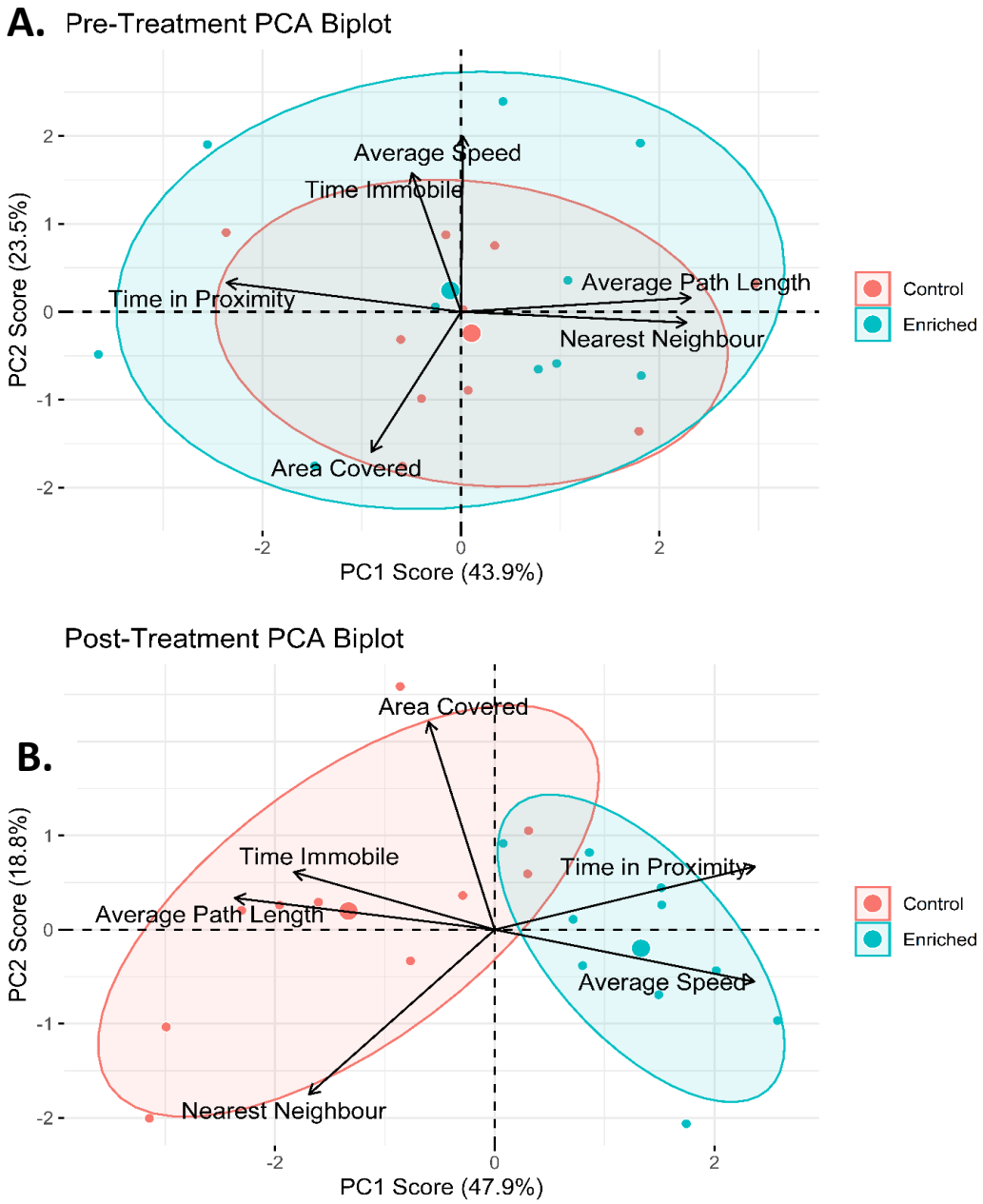

Figure 11. PCA Biplots. Trait loadings onto components are indicated with labelled arrows. Average tank scores for PC1 and PC2 are plotted and grouped by experimental treatment. A.) Scores from pre-treatment test B.) Scores from post-treatment test.

3.5 Discussion

The results from this study suggest that experimental exposure to a highly enriched environment has significant effects on the group level behaviour of captive adult zebrafish exposed to novel tank test. There are two main conclusions that can be drawn from these results. Firstly, a high level of EE has effects on the locomotor activity and mobility of groups in a novel tank test. Secondly, group housing in enriched conditions also produced effects of group cohesion. The finding that enriched groups show increased locomotor activity is interesting, as there appears to be no current literature which suggests that EE would increase baseline locomotor activity in zebrafish models. However, in rodents individuals reared in enriched environments have been shown to exhibit differences in habituation of locomotor activity and exploration (Varty et al. 2000). Here, enrichment reduced the habituation time of locomotor activity, which effectively increases locomotor activity as speed remains higher for a longer period of time. This seems to support the finding here in zebrafish that enrichment increases locomotor activity in behavioural trials, and suggests that the results observed here may be due to differences in habituation of locomotor activity.

Other studies have shown that zebrafish display elevated levels of locomotor activity and high swimming speeds upon exposure to a novel environment (Champagne et al. 2010; Wong et al. 2010). Although, this is thought to be representative of stress and anxiety-like behavioural response, and the decrease of this hyperactivity over time is evidence of habituation. In a recent study in zebrafish, EE did attenuate the effects of unpredictable chronic stress on anxiety-like behaviours and whole-body cortisol (Marcon et al. 2018). However, in this study, non-stressed fish exposed to high levels of EE also appeared more anxious than non-stressed fish kept in barren housing. Here, they suggest that the transition of individuals from an enriched tank to a bare testing tank represents a novel stressor not present for barren housed individuals. It could therefore be possible that the high locomotor activity observed here is in fact a result of experimentally induced differences in habituation and stress responses throughout the duration of the trial. However, I would argue that this difference in locomotor activity observed here in enriched groups is not simply the result of a high stress or anxiety response for several reasons. Firstly, the novelty-based hypothesis seems unlikely, as the marked difference in size and illumination of the test arena compared to both experimental

housing conditions would present an equally novel factor for groups housed under both conditions.

The most convincing evidence that the locomotor response is not simply a stress/anxiety-like response is the significant reduction in freezing bouts observed in highly enriched groups here. Indeed, there is overwhelming evidence in zebrafish that environmentally induced anxiety elicits increased occurrences of freezing bouts (Egan et al. 2009; Bass and Gerlai, 2008). In these studies, increase in freezing bouts was demonstrated in variety of contexts such as white-induced anxiety (i.e. white-light avoidance and the resulting anxiety-like behavioural responses), exposure to predator alarm cues and other anxiolytic compounds such as caffeine. Moreover, some reviews have also suggested that other measures of anxiety in the open field paradigm, such as thigmotaxis (a tendency to stay close to the arena perimeter, or 'wall-hugging' behaviour) and freezing bouts, are more useful to analyse anxiety than measures such as distance, speed or exploration (Maximino et al. 2010). This is because the latter measures are sensitive to confounding effects of locomotor deficits, and therefore other measures such as freezing are required to control for such locomotor effects. The results here therefore appear to highlight the importance of this consideration.

There is evidence that zebrafish housed in captivity, when exposed to higher levels of enrichment, also exhibit enhanced fertility and fecundity (Wafer et al. 2016). This suggests that EE has a positive effect on biological condition, at least with regards to reproductive potential. EE has also recently been demonstrated to have positive effects on larvae survivability and body condition in adults (Lee et al. 2018). Another possibility may be that EE produces better swimmers, as individuals housed in environments with many structures would have to navigate more complex environments and may become better swimmers as a result. This effect has been previously observed in juvenile rainbow trout, where housing fish in complex environments for just one week significantly enhanced an individual's ability to swim along a channel whilst avoiding obstructions (Bergendahl et al. 2017). Another recent study in zebrafish has also demonstrated that exercise-induced contractile activity in adults promotes increased muscle mass and vascularisation (Palstra et al. 2014). Given this evidence, it seems plausible that navigating a complex environment may be more physically challenging, which in turn promotes muscle growth and produces individuals that are simply better and

more agile swimmers. It may therefore be the case, that in promoting improved body condition, here EE also produces individuals that are more physically capable.

The other main finding of this study with regards to group cohesion is that enriched shoals seemed to be much more cohesive in the novel tank test after enrichment. In fish species, group cohesion and shoaling behaviour is primarily a mechanism for the avoidance of predators or to exploit food resources (Miller and Gerlai, 2007). In this sense, shoaling and group cohesion is a key response to external stimuli and stressors. In previous experiments using zebrafish models, an increase in group cohesion has been observed in response to acute stressors such as alarm substances (Speedie and Gerlai, 2008). The observation here that highly enriched groups form tighter shoals is therefore particularly interesting, as the literature would suggest that increased group cohesion is primarily a stress/anxiety-like response. Given the finding here that highly enriched groups seem to be less stressed, the nature of this increase in group cohesion is intriguing. One explanation may be an increase in sociality, as tendency to reduce proximity to conspecifics is a frequently measured metric of sociality in zebrafish studies (Buske and Gerlai, 2011; Dreosti et al. 2015). It therefore seems plausible that these differences in shoal cohesion observed between enriched vs non-enriched groups may be a result of differences in sociality.

Previous studies in rodents have shown that EE may modulate social plasticity and cognition (Gubert and Hannan, 2019). Indeed, EE has been shown to increase aggressive behaviour in response to stressors in male mice exposed to enrichment (McQuaid et al. 2012). Furthermore, EE has also been shown to enhance social interactions during dark phases of the day/night cycle in rats (Lambert et al. 2016). Early life enrichment has also been shown to increase the likelihood that enriched individuals will become submissive during social encounters later in life in rodents (Cao et al. 2017). These studies in rodents seem to support the idea that EE may induce changes in sociality, and may therefore be the cause of changes in shoal cohesion observed in this study. Another possible explanation for this increase in shoal cohesion observed in enriched groups is that it is an adaptive group level response arising from exposure to open water. A previous study in juvenile cod, *Gadus morhua*, has provided compelling evidence for this argument (Salvanes et al. 2007). In this study, groups reared in enriched environments and tested in a complex arena exhibited significantly increased group cohesion compared to groups reared in barren environments. It was argued

here that enriched groups may be more adept at varying their group level behaviour to suit the environment that they find themselves in. In the results I have outlined here, enriched groups may therefore be more adept at tuning their behaviour to environmental conditions than control groups.

In conclusion, the results here demonstrate that a number of group level behavioural phenotypes seem to be plastic in response to a fixed period of experimental enrichment. In line with previous findings in zebrafish, enriched groups exhibited reduced anxiety-like behaviour. However, EE seems to increase locomotor activity of enriched groups and have a negligible effect of exploration, a finding which was not predicted prior to this study and seems to be due to enhanced swimming ability. Furthermore, EE seems to significantly affect group composition through greater shoal cohesion, perhaps due to enriched groups becoming more adept at coordinating group level responses, or alternatively due to enhanced social competence. Taken together, these findings suggest that EE is fundamentally important for the welfare of zebrafish housed in a captivity much in same way as rodent models. This is especially true given the disparity in the UK Home Office and RSPCA requirements for physical enrichment between rodents and zebrafish, with zebrafish currently requiring much less physical enrichment in research environments.

Chapter 4: Transgenerational Effects of Environmental Enrichment on Group Level Behaviour in Adult Zebrafish.

4.1 Abstract

In the previous chapter, a 28-day exposure period to varying levels of EE was shown to affect both swimming speed and shoal cohesion in free swimming groups of zebrafish. The resulting two F0 groups were phenotypically distinct, with enriched groups being faster and more cohesive than the slower moving, and less cohesive, control groups. These F0 fathers were used to examine the interaction between paternal and offspring enrichment exposure. Adult F1 zebrafish obtained from both control and enriched paternal backgrounds were baseline tested at 3 months post fertilisation in order to identify inherited effects of paternal enrichment before testing. The two experimental groups were then split between either high or low enrichment exposure for four weeks and tested again following F1 enrichment. A novel tank group-shoaling test was utilised here on groups of 5 adult zebrafish to assess group level behaviours. I found a significant transgenerational effect of enrichment on F1 locomotor activity (swimming speed), which was detectable in baseline testing and matched the high activity phenotype of fathers. However, social phenotypes (shoal cohesion) did not appear to be inherited in the same way and there was no detectable difference between F1 adults from different paternal backgrounds in baseline testing. There did appear to be some effects of paternal background on social behaviour in the post-treatment trial in these offspring, which were unexpectedly opposite to the observed effect in fathers. These results suggest that while some environmentally-manipulated behaviours (i.e. locomotor activity) seem to be heritable through the male germline, others are not (i.e. social behaviours). The transmission through the male germline under a controlled breeding regime suggests that these effects may be epigenetically inherited.

4.2 Introduction

4.2.1 Phenotypic Plasticity and Nongenetic Inheritance (NGI)

Phenotypic plasticity has been defined as the ability of a single genotype to give rise to more than one phenotype in response to changes in the environment (Schlichting and Pigliucci,

1998). These plastic responses can alter the interactions between an organism and its environment within the lifetime of an individual (Badyaev and Oh, 2008; Ghalambor et al. 2007). They are widespread in nature, and it has been suggested that they can affect numerous levels of ecological organisation through between-species interactions (Miner et al. 2005). There is also evidence that these plastic changes can also be expressed across, as well as within generations (Agrawal et al. 1999). This has given rise to the idea that plasticity may exert transgenerational effects, which could in turn alter the rate or direction of evolution.

The foundations of modern evolutionary theory are built on the classic Mendelian model of inheritance and Darwinian evolution through natural selection. In this model, genes are the primary mechanism of heredity, and the transmission of variation in the genetic code give rise to different phenotypes. However, it has now become clear that this model is incomplete, as recent empirical evidence points to the presence of nongenetic mechanisms of inheritance as an additional heritable factor (Bonduriansky and Day, 2009). By definition, these mechanisms do not involve direct changes to DNA sequence, but rather encompass the transmission of other non-genetic factors. Nongenetic forms of inheritance can influence a variety of traits in a range of organisms, and can allow direct germline transmission of environmentally induced phenotypic variants across generations (Bonduriansky et al. 2012). Nongenetic inheritance (NGI) in this way could provide another mechanism for phenotypic adaptation, and may be an important factor in species persistence in rapidly changing environmental conditions (Bonduriansky et al. 2012).

4.2.2 NGI, Brain Function and Behaviour.

There is plenty evidence for the non-genetic inheritance of altered brain functioning and behaviour (Bohacek and Mansuy, 2015). A classic example of this has been demonstrated in mice and individual variation in maternal licking/grooming (LG) and arched back nursing (ABN) behaviour (Francis et al. 1999). In this study, female offspring of high LG-ABN mothers showed significantly increased licking/grooming of pups in comparison with those of low LG-ABN mothers, clearly suggesting that individual differences in maternal behaviour are transmitted between generations. Importantly, cross fostering revealed that maternal behaviour altered the pattern of transmission across generations, suggesting the inherited

effect here was not genetic in nature. This has since been shown to be associated with levels of cytosine methylation across the *ERα1b* promoter, which appear to be significantly elevated in the adult offspring of low, compared with high LG mothers, suggesting a potential non-genetic mode of transmission between generations (Champagne et al. 2006). There also appears to be evidence in rodents for the non-genetic inheritance of stress-related behaviours. On such study demonstrated that offspring of high LG-ABN mothers had differences in DNA methylation of a glucocorticoid receptor, histone acetylation, and hypothalamic-pituitary-adrenal responses to stress, compared to offspring of 'low-LG-ABN' mothers (Weaver et al. 2004). Importantly, these effects emerged over the first week of life and were reversed by cross fostering, suggesting a clear relationship between epigenetic state, maternal care, and stress reactivity. The persistence of these effects across multiple subsequent generations and parental route for transmission appears to vary. For example, in the previous example of EE reducing the prevalence of repetitive motor behaviour, the observed transgenerational effect was shown to weaken after only a single subsequent generation (Bechard and Lewis, 2016). Clearly, not all of these kinds of effects are inherited in the same way or at the same level.

4.2.3 Paternal NGI

In many species it is usually (but not always) the mother who provide offspring care. However, there is increasing evidence that fathers can have significant effects on offspring development, and that environmentally relevant information can be transmitted through the paternal lineage (Braun and Champagne, 2014; Mashoodh and Champagne, 2019). An example of this paternal inheritance has been found in the effect of the endocrine disruptor, Vinclozolin, on male fertility in rats (Anway et al., 2005). Here, exposure of a gestating female rat to Vinclozolin induced an adult F1 phenotype of reduced spermatogenic capacity. This effect was transmitted through the male germline for 5 generations, and also appeared to correlate with different methylation patterns in the germline. More recently, over 200 small noncoding RNAs have been identified in the F3 offspring of Vinclozolin exposed rats germline, which appear to be differentially regulated and associated with the transcription profiles of the vinclozolin-induced disease phenotypes (Schuster et al., 2016). It has also been recently demonstrated that chronic stress induced by maternal separation results in reduced escape response in male adults exposed to a light/dark test (Gapp et al., 2016). Here F1 male offspring

obtained from stressed males also appeared to exhibit the same reduced escape response in the same test. Another study in rodents also demonstrated that maternal separation can result in increased depressive behaviour in exposed individuals, these same alterations were also found in the offspring of males exposed to maternal separation (Franklin et al., 2010). In this case, a number of genes were found to be differentially methylated around transcription initiation sites in both parents and offspring, suggesting a non-genetic mechanism of inheritance.

4.2.4 Paternal NGI in Zebrafish

When examining transgenerational paternal inheritance, the use of externally fertilising fish species is advantageous over gestating mammalian models such as rodents. The use of externally fertilizing fish species, combined with controlled breeding, can virtually eliminate the effect of confounding factors such as maternally inherited effects and mate choice. Zebrafish have emerged as a growing model organism, particularly so in the field of behavioural neuroscience (Oliveira, 2013). In zebrafish, the paternal, but not maternal, DNA methylome is inherited by early embryos (Jiang et al., 2013). In this case, the maternal DNA undergoes extensive demethylation followed by *de novo* methylation during early development. A recent study has found that the exposure of male zebrafish to varying levels of reproductive competition, not only affected sperm performance in exposed subjects, but also induced lower survival rates in offspring in subsequent generations (Zajitschek et al., 2014). In this case, high competition among males resulted in individuals with faster and more motile sperm. In the resulting offspring, larvae from high competition fathers hatched faster, and showed increased mortality compared to controls. Another recent study from Zajitschek and colleagues has shown that male social status (MSS) and personality can also influence offspring behaviour (Zajitschek et al., 2017). In this case, both MSS and paternal behaviour was shown to be significantly associated with offspring activity, even after manipulation of social status across experimental rounds. Interestingly, both MSS and the modification of social status caused changes in a number of sperm traits including longevity and mobility, suggesting that effects may be mediated by the germline. Taken together, these studies highlight the usefulness of zebrafish models for the examination of paternally mediated transgenerational effects.

4.2.5 NGI and Environmental Enrichment (EE)

Physical complexity is a key way in which an animal's environment can vary. Two of early studies in mice were among the first to observe an effect of EE on brain function and behaviour (Kiyono et al. 1985; Dell and Rose 1987). They demonstrated offspring of pregnant rats exposed to high levels of EE showed reduced number of errors in a Hebb-Williams maze paradigm. These two important studies seem to have given rise to much of the recent work regarding EE and its effects on brain function and behaviour. Recent work in animal models has shown that EE can also exert transgenerational effects on behaviour and brain function. For example, research has shown that exposure of juvenile mice to 2 weeks of EE induced elevated social interactions and voluntary exercise, and enhanced long-term memory potentiation as adults (Arai et al. 2009). The evidence here seems to support the idea that EE can induce transgenerational effects, as enhanced LTP memory was found to be present in the offspring of enriched mothers. Parental mangrove killifish, *Kryptolebias marmoratus*, reared in enriched environments have also been shown to exhibit lower cortisol levels, lower metabolic rates and to be more active and neophobic than those reared in barren environments (Berbel-Filho et al., 2020). In this case, offspring activity and neophobia was found to be determined by the parental environment, and similar methylation patterns were observed in both parents and offspring. Other work has shown that EE can not only itself induce transgenerational behavioural effects, but also reduce the occurrence of other behaviours in offspring. For instance, a recent study documented that exposure of adult male and female rats to morphine resulted in anxiety-like behaviour in their adult offspring (Li et al. 2014). In this case, parental exposure was also associated with increased hippocampal IGF-2 expression in offspring, an effect which was normalized by EE exposure in juveniles. Taken together, these findings suggest that EE has significant effects on brain function and behaviour such as learning and memory.

4.2.6 Aims and Hypothesis

It is clear from the literature and evidence in this field that nongenetic inheritance plays an important role in species persistence in changing environments. Germline nongenetic inheritance is perhaps the most direct mechanism for the inheritance of these effects. Despite the mounting evidence that environmental enrichment through structural complexity can

significantly alter physiology and behaviour, there appears to be little attempt to investigate transgenerational effects of parental exposure to different levels structural complexity. The aim of this study was to investigate the interaction between parental enrichment and offspring enrichment and its effects on group level behaviour. To achieve this, adult zebrafish shoals from two parental backgrounds (described in the previous chapter) were assayed for a range of behaviours in a novel arena group shoaling test both before and after experimental enrichment. Between these trials, adults were split between either high or low levels of structural enrichment for four weeks. I examined the effects of both parental and offspring EE on exploratory behaviour, shoal cohesion, and locomotor activity using a simple group shoaling test. I hypothesized that offspring enrichment would have similar effects to those observed in fathers exposed to high EE. I anticipated that if behavioural phenotypes were inherited from fathers, F1 adults in baseline testing would be faster and more cohesive than those from control paternal backgrounds.

4.3 Methodology

4.3.1 Animal Husbandry and Ethics

All fish were housed and maintained in flow-through custom-built recirculation systems described in the previous chapter. A consistent, 10/14hr light/dark cycle was maintained in the housing room, water conditions were maintained at 27°C, pH 7.5, and nitrate, nitrite and ammonia levels were regularly monitored and kept in accordance with Home Office recommendations. Larvae were fed a controlled diet of ZM Systems™ (Winchester, UK) fry food for up to 3 months post-fertilisation in order to maximise survival and promote normal development. Adult fish (at 3 months post fertilisation) were fed a standard diet of ground Tetramin flake food for the duration of the study. All groups were fed once daily at a pre-determined quantity where all food was consumed within 5 minutes. All subjects used here were naïve to the behavioural testing regime prior to behavioural trials. No Home Office-regulated procedures were carried out in the methods outlined here, and ethical approval for this study was obtained from the Liverpool John Moores University Animal Welfare and Ethics Steering Group.

4.3.2 Experimental Design

In the previous chapter two distinct behavioural phenotypes were identified as a result of experimental enrichment. Groups from enriched housing were faster, less anxious, and more cohesive as a shoal when compared to groups from control housing conditions. The fathers used in this study to obtain F1 offspring were taken directly from the experimental groups described in the previous chapter. The design utilized here consisted of two factors: 1) parental experience and 2) offspring experience. Each of which consisted of two levels, control or enriched. The offspring control and enriched housing treatments used here were the same as the treatments described in the previous chapter. This resulted in a total of four experimental groups representing each possible combination of parental and offspring experience across two generations. The four experimental groups therefore consisted of offspring from control housing paternal backgrounds that were placed as adults into either control housing (Ctrl/Ctrl) or enriched housing treatments (Ctrl/Enr), and offspring from enriched paternal backgrounds that were placed as adults into control housing (Enr/Ctrl) or enriched housing treatment (Enr/Enr).

At the start of this study, a sample of adult male zebrafish (n = 20) were obtained from the experimental treatment groups from the previous study. Half of these males were taken from the control housing treatment and half were taken from the enriched housing treatment. These males were used in a breeding regime to obtain a single clutch of fertilised embryos from each male, from which a random sample of larvae (n = 10) per clutch were used. These clutches of F1 offspring (n = 200) were then reared under standard housing conditions at a density of n = 10 fish per tank until 3 months post-fertilisation. At this point, each clutch of F1 adults were tested using a novel tank group-shoaling test to determine if there was any evidence for the inheritance of different paternal phenotypes, and to obtain baseline measures of behaviour. Immediately following this baseline testing, adult offspring from each clutch were split equally between either control or enriched housing treatments to create the experimental F1 offspring groups. All experimental offspring were then exposed to their respective experimental enrichment regime for a fixed 28-day period. The baseline novel tank group-shoaling test performed prior to experimental enrichment was repeated immediately after the experimental exposure period (see Figure 2). Group size and composition remained the same throughout the entire experimental period. The shoal density of 5 fish per tank

described here for F1 experimental groups differed from the density used in the previous chapter, which utilised a density of 10 fish per tank. This was done to increase the effective sample size based on the number of tanks.

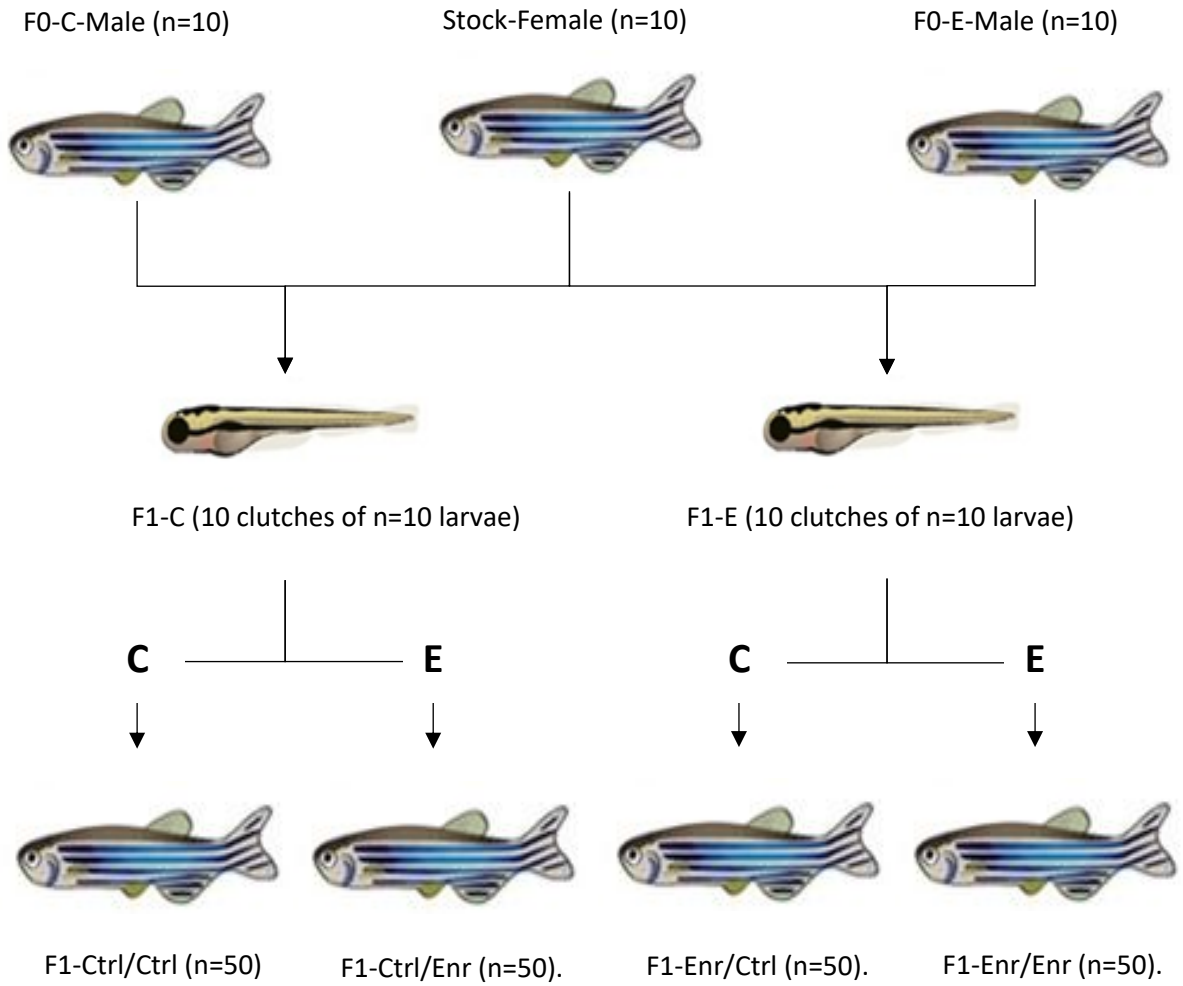

Figure 1. Experimental breeding design. F0 males from either control or enriched treatments were mated with a single group of stock females. The resulting F1 larvae were reared until adulthood and split again between either control or enriched treatment resulting in four F1 experimental groups.

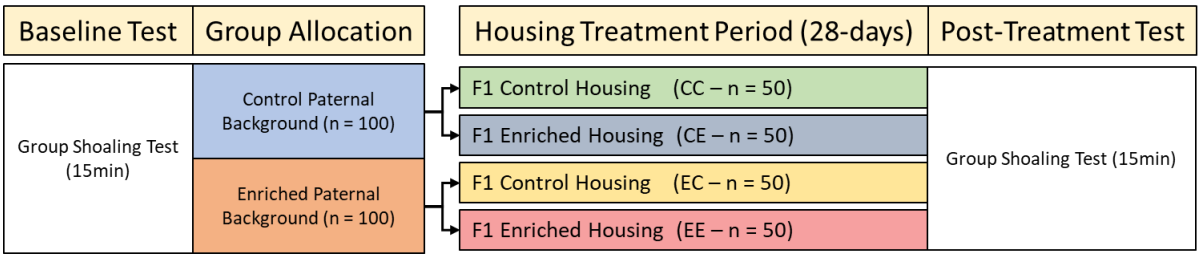

Figure 2. Experimental timeline. F1 adult were baseline tested at 3 months post fertilisation, then split between enriched or control housing conditions for 28 days. The resulting four groups were then tested again post-treatment.

4.3.3 Breeding

Experimental breeding tanks were constructed from an empty housing tank modified with a fine mesh sheet, which was large enough to allow eggs to pass through, and small enough to prevent eggs at the bottom of the tank from being eaten by the breeding adults. Males and females to be crossed were housed separately for 24 hours prior to breeding. In order to control potential variation between the offspring groups, each breeding female was crossed with a single male from a standard housing experimental background and also single male from the experimentally enriched experimental background. In doing so each breeding female was equally represented between both control and enriched F1 offspring. Breeding pairs were moved into their respective breeding tank shortly before the lights came on in the housing facility at 8:30am. The breeding pairs were then left until 1:00pm when they were then removed from the breeding tank. Between 8:00am and 1:00pm the breeding tanks were checked every hour for the presence of fertilised eggs. When fertilised eggs were present in any of these tanks they were immediately removed and processed. Fertilised clutches were immediately cleaned and moved into a petri dish containing 0.0001% methylene blue (MB) solution in conditioned water at a density of 100 eggs per dish. MB was used to reduce the growth of fungus and bacteria in the dishes. These embryos were stored in an incubator at 28°C until hatching at 72 hours post fertilisation (hpf). Each morning for the first 72 hours, the dishes were screened for abnormally developing or dead embryos. After 72 hours, healthy clutches were removed from the methylene blue solution and into standard housing tanks. At 120hpf clutches were reduced to 10 larvae per clutch, the remaining larvae were disposed of to reduce the number of offspring to only the amount required for adult testing.

4.3.4 Behavioural Testing Using A Novel Tank Group-Shoaling Test

All subjects were assayed using a novel tank group-shoaling test to quantify their free-swimming behaviour as groups. The testing suite contained a 40 cm x 40 cm acrylic tank filled with 5 cm of clean water. This shallow depth restricted movement to an approximately 2-dimensional plane. The arena was evenly illuminated from below using a consumer A3 light pad. The arena and light pad were contained behind a floor length curtain to prevent visual distractions from outside of the testing suite. The entire arena was recorded from above with a consumer grade camcorder suspended directly above with a custom-built tripod and extension arm. Water in the arena was changed and the tank was cleaned with conditioned water between each trial. Before each test, the tank to be tested was left undisturbed for 5 minutes within the partitioned testing area to control for stress from transport to the testing suite. Each individual in the tank was then transferred to the testing arena by netting. The recording was immediately started manually and the partition curtain was closed around the test arena for the duration of the test. The entire test lasted 15 minutes, after 15 minutes the subjects were removed from the arena and placed back into their housing tank. The first 5 minutes of each test was regarded as a habituation period to acclimate to the arena and not used for the following analyses. Final test length for each group was therefore 10 minutes long. Video files for each test were converted to AVI format using VSDC Video Editor software and analysed using automated tracking software. Behavioural measures were extracted for each individual in each shoaling group.

Using custom MATLAB scripts a number of endpoint individual and group-based behaviours were extracted from coordinate data for further analysis. The average body length of all individuals within a group was calculated and subsequently used for all body length based measures of behaviour. Two measures of mobility were obtained as average speed (cm/sec) and time spent immobile (sec/min), where an individual was defined as being immobile if it had travelled less than one body length in any two second period. These events were then totalled, and the proportion of time was calculated across the entire trial. Average speed was calculated as the distance travelled between each subsequent one second period in the trial, this was then averaged for the entire trial for each individual. Space usage was estimated as the proportion of the arena covered (%). Briefly, the test arena was divided into equally sized quadrants, the number of quadrants visited at least once in the entire trial was then totalled.

The proportion of quadrants visited out of the total number of quadrants was then calculated and an endpoint value was obtained for each subject. The proximity-based traits obtained here included nearest neighbour distance (cm), average path length (cm) and time within two body lengths of another individual (sec/min). Average path length was defined as the average distance between a focal individual and all other group members in a given frame. The shortest distance between a focal individual and another individual was taken as nearest neighbour distance. An individual was defined as being in proximity to another individual if it was within two body lengths of any other conspecific in a given frame. The proportion of frames 'in proximity' was then calculated across the entire trial. These measures were calculated on a per-frame basis, and averaged across the entire trial for each individual.

4.3.5 Data Analysis

All statistical analyses were performed in the RStudio environment (RStudio Team, 2020). Comparisons of experimental groups within and across tests were performed using a mixed model approach. Models were fitted using the 'lmer' function from the 'lme4' package in R (Bates et al. 2015). These models were performed using per-tank means obtained from individual measurements, as individual identities could not be retained between trials. The models included three fixed factors of parental experience, offspring experience and a repeated trial effect, tank identity was also included as a random factor to account for the repeated sampling of each tank. An interaction term was included for all fixed effects. A type II Wald Chi Square analysis of variance was performed on model estimates to determine the significance of each term in the model. Custom contrasts then were applied to each model using the Kenward-Roger method to obtain significance estimates between experimental groups both within and between trials. Where significant parental effects were observed from model estimates, parental groups within and between trials were compared. Likewise, when significant offspring effects were observed offspring groups were compared within and between trials. Due to the large sample size in each model ($n > 50$) and the application of the central limit theorem, the residuals were assumed to be normally distributed without the need for formal testing. However, the residuals for each model were plotted to assess normality and heteroscedasticity. Each model was additionally tested for outlier observations by calculating the probability that Studentized residuals were outliers after applying a Bonferroni correction for multiple comparisons.

A scaled and centered principal component analysis (PCA) was performed using all the measured behaviours from the group to explore the all behavioural data in 2-dimensional space. The PCA was performed using the 'prcomp' function from the 'stats' core package in R (RStudio Team, 2020). Separate PCAs were performed for both pre- and post-treatment data to maintain independence of observations. Eigenvalues of principle components were estimated using Horns Parallel Analysis from the 'paran' package in R (Dinno, 2009). Components with an eigenvalue exceeding one were extracted for further analysis. Examination of the loadings for each behaviour onto the corresponding component allows for the interpretation of correlated traits in each component. Component scores were plotted for each extracted component with experimental treatment as a grouping factor to visually assess the similarity of the groups at both timepoints in multivariate space.

In the process of importing video files from and SD card to PC, a batch of files containing raw videos of behavioural tests for 2 tanks in each experimental group was found to be corrupted and unrecoverable. The loss of data for these tanks reduced the number of tank units per group to 8. All of the analyses described here were therefore performed on the remaining 8 tanks.

4.4 Results

4.4.1 Locomotion, Mobility and Exploration

Average Speed

With regards to average speed, there was a large significant effect of parental experience (ChiSq = 6.91, DF = 1, p = 0.009) and also a weak, non-significant effect of repeated trials (ChiSq = 3.99, DF = 1, p = 0.046) on average speed. However, there was no significant main effect of offspring experience (ChiSq = 0.002, DF = 1, p = 0.97) and no significant interactions (parental:offspring – ChiSq = 0.16, DF = 1, p = 0.68; parental:week – ChiSq = 1.53, DF = 1, p = 0.22; offspring:week – ChiSq = 1.32, DF = 1, p = 0.25; parental:offspring:week – ChiSq = 1.25, DF = 1, p = 0.26). The comparison of groups by parental condition in the first trial showed that there was a large pre-existing difference in average speed of offspring from control (4.48 cm/sec) and enriched (5.82 cm/sec) paternal backgrounds (estimate = -2.69, DF = 55.7, t = -2.77, p = 0.008, see Figure 3 B). However there was no such difference in speed between

offspring from control (5.55 cm/sec) and enriched groups (6.08 cm/sec) in the post-treatment test (estimate = -1.050, DF = 55.7, t = -1.08, p = 0.285). Furthermore, it appeared as though offspring from control paternal backgrounds increased their average speed significantly between pre-treatment (4.48 cm/sec) and post-treatment (5.55 cm/sec) testing, regardless of offspring experience (estimate = -2.14, DF = 28, t = -2.28, p = 0.029, see Figure 3) whereas offspring from enriched parental backgrounds showed no increase in their average speed between pre-treatment (5.82 cm/sec) and post-treatment (6.08 cm/sec) testing in the same way (estimate = -0.50, DF = 28.0, t = -0.537, p = 0.595, see Figure 3).

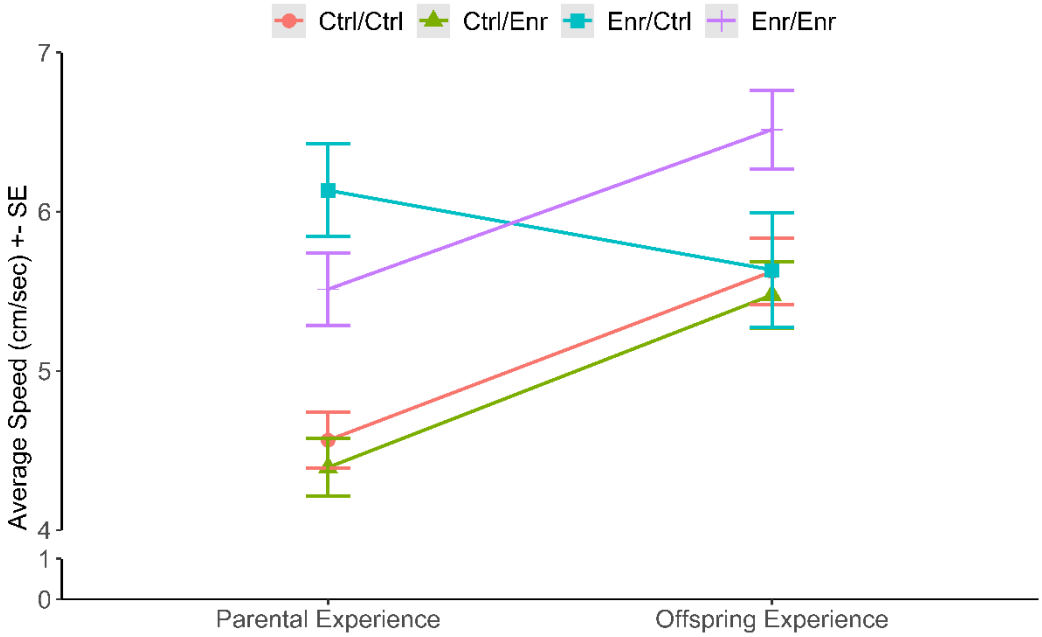

Figure 3. Interaction plot of average speed (cm/sec) of F1 by parental/offspring experience. Mean value of each group (Ctrl/Ctrl; Ctrl/Enr; Enr/Ctrl; Enr/Enr) in each test are reported with standard errors (SE).

Area Covered

For the proportion of area covered, the estimated model terms showed a significant offspring experience by trial interaction (ChiSq = 9.59, DF = 1, p = 0.001), a significant effect of repeated trials (ChiSq = 5.41, DF = 1, p = 0.020) and a weak non-significant effect of parental experience (ChiSq = 3.00, DF = 1, p = 0.083). There was no main effect of offspring experience (ChiSq = 0.08, DF = 1, p = 0.77) and no other significant interactions (parental:offspring – ChiSq = 0.11, DF = 1, p = 0.74; parental:week – ChiSq = 0.05, DF = 1, p = 0.83; parental:offspring:week –

ChiSq = 1.24, DF = 1, p = 0.27). The comparison performed between offspring exposed to different treatments in the first trial indicated that there was a weak difference between control (83%) and enriched (98%) F1 treatment groups (estimate = -0.15, DF = 56, t = -1.980, p = 0.053, see Figure 4). However, offspring control groups (92.5%) explored significantly more than offspring enriched groups (84%) post-treatment (estimate = 0.18, DF = 56, t = 2.399, p = 0.0198, see Figure 4). Furthermore, offspring control groups did not change in their level of exploration between pre-treatment (83%) and post-treatment (92.5%) tests (estimate = -0.04, DF = 28, t = -0.544, p = 0.591, see Figure 4).

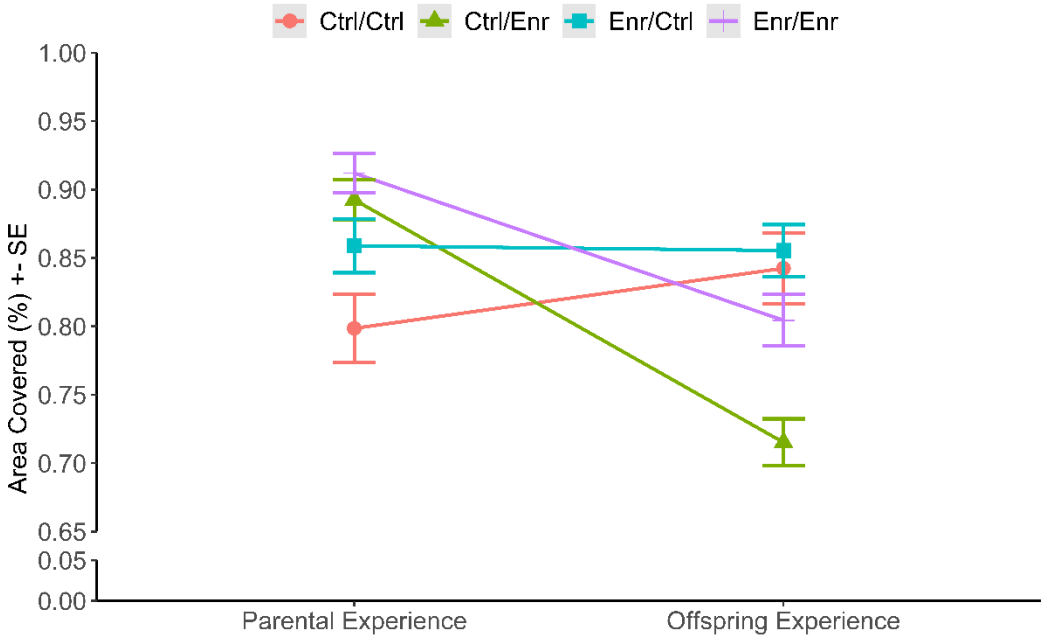

Figure 4. Interaction plot of area covered (%) of F1 offspring by parental/offspring experience. Mean value of each group (Ctrl/Ctrl, Ctrl/Enr, Enr/Ctrl, Enr/Enr) in each test are reported with standard errors (SE).

Time Spent Immobile

The analysis of variances performed on the model terms showed that there were no statistically significant main effects of parental experience (ChiSq = 1.48, DF = 1, p = 0.22), offspring experience (ChiSq = 2.58, DF = 1, p = 0.11) or repeated trials (ChiSq = 0.08, DF = 1, p = 0.77) on time spent immobile. There were also no significant interaction effects (parental:offspring – ChiSq = 0.03, DF = 1, p = 0.87; parental:week – ChiSq = 1.78, DF = 1, p = 0.18; offspring:week – ChiSq = 1.61, DF = 1, p = 0.21; parental:offspring:week – ChiSq = 1.38,

DF = 1, p = 0.24) on time spent immobile (see Figure 5). As no significant main effects or interactions were found, no contrasts were performed.

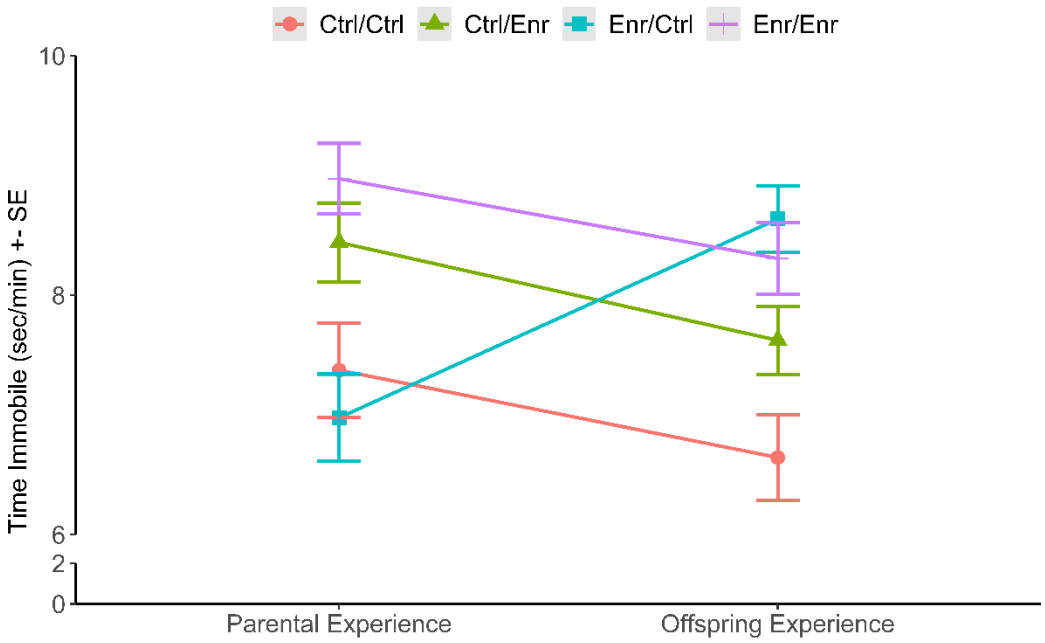

Figure 5. Interaction plot of time spent immobile (sec/min) of offspring by parental/offspring experience. Mean value of each group (Ctrl/Ctrl, Ctrl/Enr, Enr/Ctrl, Enr/Enr) in each test are reported with standard errors (SE).

4.4.2 Inter-individual Distance and Proximity

Average Path Length

With regards to average path length, there was a large significant effect of repeated trials (ChiSq = 40.21, DF = 1, p < 0.001) and of parental experience (ChiSq = 6.49, DF = 1, p = 0.011). However there was no main effect of offspring experience (ChiSq = 0.0001, DF = 1, p = 0.99) and no significant interaction effects (parental:offspring – ChiSq = 2.32, DF = 1, p = 0.13; parental:week – ChiSq = 2.31, DF = 1, p = 0.13; offspring:week – ChiSq = 0.07, DF = 1, p = 0.79; parental:offspring:week – ChiSq = 0.85, DF = 1, p = 0.36). The contrasts performed showed that there was no significant pre-existing difference in average path length between offspring from control (8.78 cm) and enriched (9.56 cm) parental backgrounds pre-treatment (estimate = -2.07, DF = 51.9, t = -1.126, p = 0.265, see Figure 6). However, parentally enriched offspring (14.16 cm) had significant longer average path length than offspring from control parental

groups (11.44 cm) post-treatment (estimate = -5.42,DF = 51.9, t = -2.949, p = 0.005, see Figure 6). Furthermore, offspring from control parents significantly increased their average path length between pre-treatment (8.78 cm) and post-treatment (11.44 cm) tests (estimate = -5.32, DF = 28.0, t = -3.410, p = 0.002, see Figure 6). Parentally enriched groups also significantly increased their average path length between pre-treatment (9.56 cm) and post-treatment (14.16 cm) tests (estimate = -8.673, DF = 28.0, t = -5.558, p < 0.001, see Figure 6).

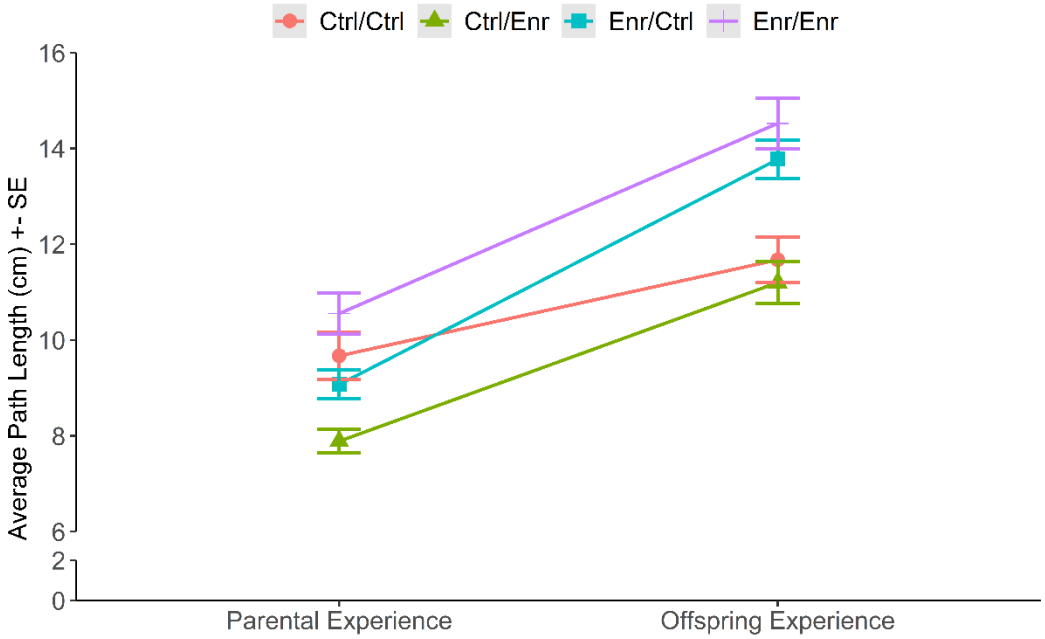

Figure 6. Interaction plots. Average path length (cm) of F1 offspring by parental/offspring experience. Mean value of each group (Ctrl/Ctrl; Ctrl/Enr; Enr/Ctrl; Enr/Enr) in each test are reported with standard errors (SE).

Nearest Neighbour Distance

For nearest neighbour distance, there were significant main effects of parental experience (ChiSq = 5.17, DF = 1, p = 0.023) and repeated trials (ChiSq = 26.67, DF = 1, p < 0.001). There was also a significant parental experience by trial interaction (ChiSq = 5.10, DF = 1, p = 0.024). However, there was no main effect of offspring experience (ChiSq = 0.16, DF = 1, p = 0.69) and no other significant interactions (parental:offspring – ChiSq = 1.84, DF = 1, p = 0.17; offspring:week – ChiSq = 0.26, DF = 1, p = 0.61; parental:offspring:week – ChiSq = 0.26, DF = 1, p = 0.61). There appeared to be no pre-existing difference between offspring from parental control groups (3.99 cm) and parentally enriched groups (4.14 cm) in the pre-treatment test

(estimate = -0.3003, DF = 53.7, t = -0.346, p = 0.731, see Figure 7). However, in the post-treatment test, offspring from enriched parental background (6.17 cm) had a much longer nearest neighbour distance than those from paternal control groups (4.79 cm), regardless of offspring experience (estimate = -2.77, DF = 53.7, t = -3.19, p = 0.002, see Figure 7). Furthermore, offspring from parental control groups significantly increased their time in proximity between pre-treatment (3.99 cm) and post-treatment (4.79 cm) tests (estimate = -1.5885, DF = 28.0, t = -2.054, p = 0.049, see Figure 7). Likewise, offspring from parentally enriched groups also significantly increased their time in proximity between baseline testing (4.14 cm) and post-treatment (6.17 cm) testing, regardless of offspring condition (estimate = -4.0589, DF = 28.0, t = -5.249, p < 0.001, see Figure 7).

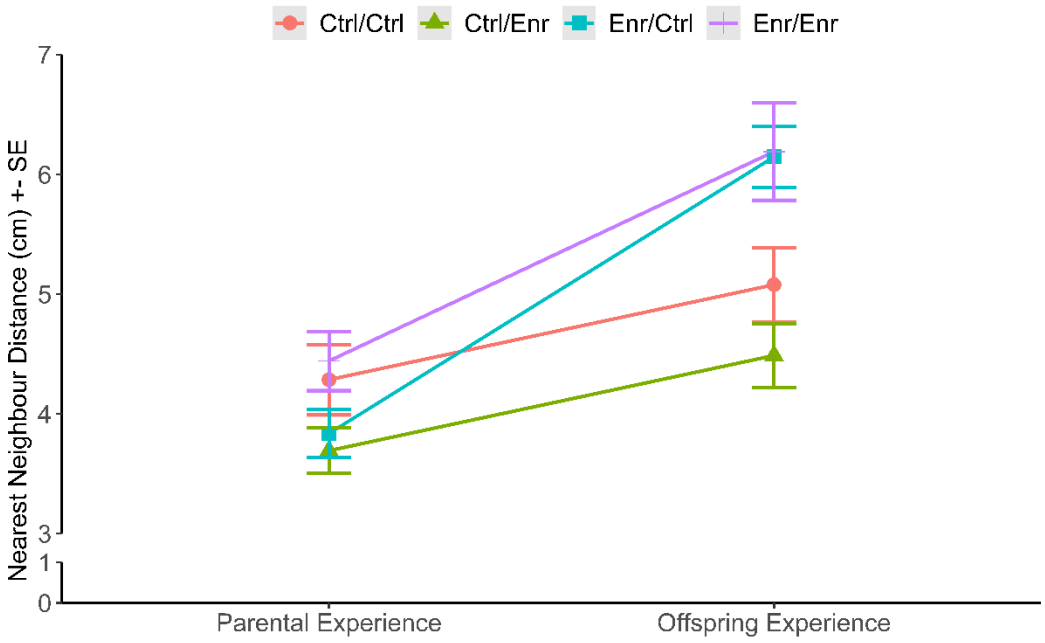

Figure 7. Interaction plot of nearest neighbour distance (cm) of F1 offspring by parental and offspring experience. Mean value of each group (Ctrl/Ctrl; Ctrl/Enr; Enr/Ctrl; Enr/Enr) in each test are reported with standard errors (SE).

Time in Proximity to Conspecific

The analysis of variance indicated that there was a significant effect of repeated trials (ChiSq = 17.23, DF = 1, p < 0.001, see Figure 8), and also a weak parental experience by week effect (ChiSq = 3.22, DF = 1, p = 0.073). However there were no main effects of parental experience (ChiSq = 2.30, DF = 1, p = 0.12) or offspring experience (ChiSq = 0.42, DF = 1, p = 0.51), and no

significant interactions (parental:offspring – ChiSq = 1.04, DF = 1, p = 0.31; offspring:week – ChiSq = 2.5, DF = 1, p = 0.11; parental:offspring:week – ChiSq = 1.09, DF = 1, p = 0.30). The comparison of time in proximity between trials showed that groups spent significantly more time in proximity at baseline testing (42.4 sec/min) than in post-treatment testing (35.08 sec/min) tests (estimate = 29.4161, DF = 28.0, t = 4.151, p < 0.001, see Figure 8). As no other main effects or interactions were found, no further contrasts were performed.

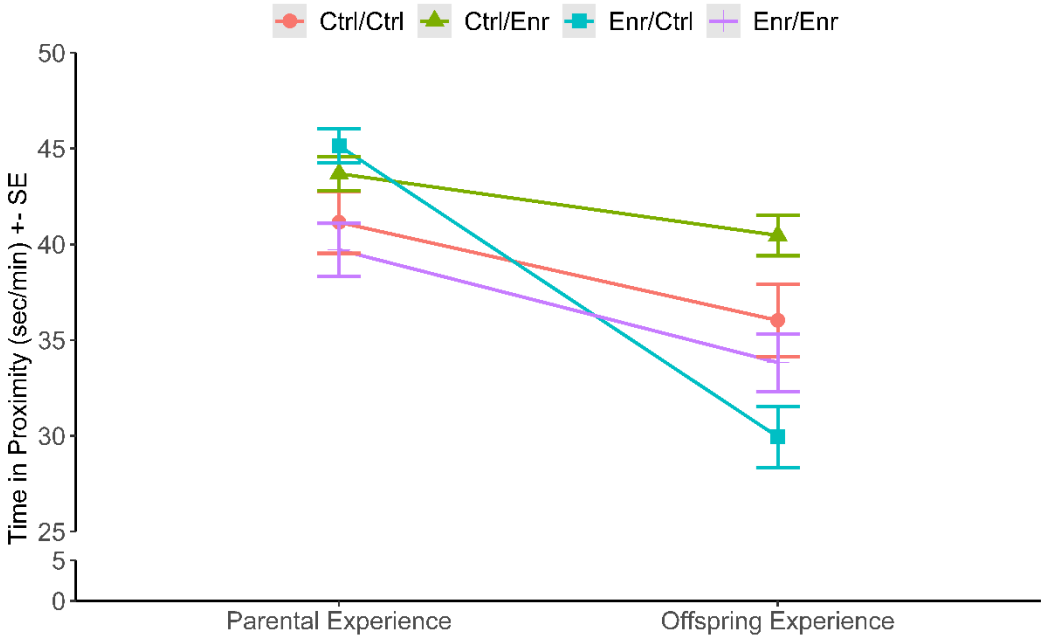

Figure 8. Interaction plot of time in proximity (sec/min) of F1 offspring by parental and offspring experience. Mean value of each group (Ctrl/Ctrl; Ctrl/Enr; Enr/Ctrl; Enr/Enr) in each test are reported with standard errors (SE).

4.4.3 Behavioural Components: PCA

Pre-treatment

The PCA performed on the tank level data from the pre-treatment behavioural trials gave a total of six components, the first two of which explained ~74% of the total variation in the data set. The results from the Horn's Parallel Analysis performed on the data from pre-treatment testing indicated that the first two components had adjusted eigenvalues >1, which should therefore be retained for further analysis. A cut-off value of 0.4 was used to assess which traits loaded heavily onto each component. PC1 was primarily a measure of social

behaviour. This was evident by the observation that nearest neighbour distance, average path length and time in proximity all loaded heavily onto this component. Positive scores for PC1 indicated groups that had a longer average path length and nearest neighbour distance and spent less time in proximity to another individual. Conversely, negative scores on this component indicated groups that had shorter average paths and nearest neighbour distances and spent more time in proximity to another individual (see Figure 9). PC2 was primarily explaining variation in speed and exploratory behaviour. This is evident from the observation that both average speed and proportion of area covered all loaded heavily onto this component. For PC2, positive scores described groups that displayed decreases in both locomotion and exploration, conversely negative scores for this component indicated individuals that were faster and explored more of the available space (see Figure 9). The plotting of these component scores pre-treatment suggested that offspring from different parental backgrounds were separated along PC2, specifically, parentally enriched groups appeared faster and more explorative than controls (see Figure 9). This supports the finding from the models performed in the pre-treatment test, where groups from enriched paternal backgrounds appeared to be significantly faster than controls.

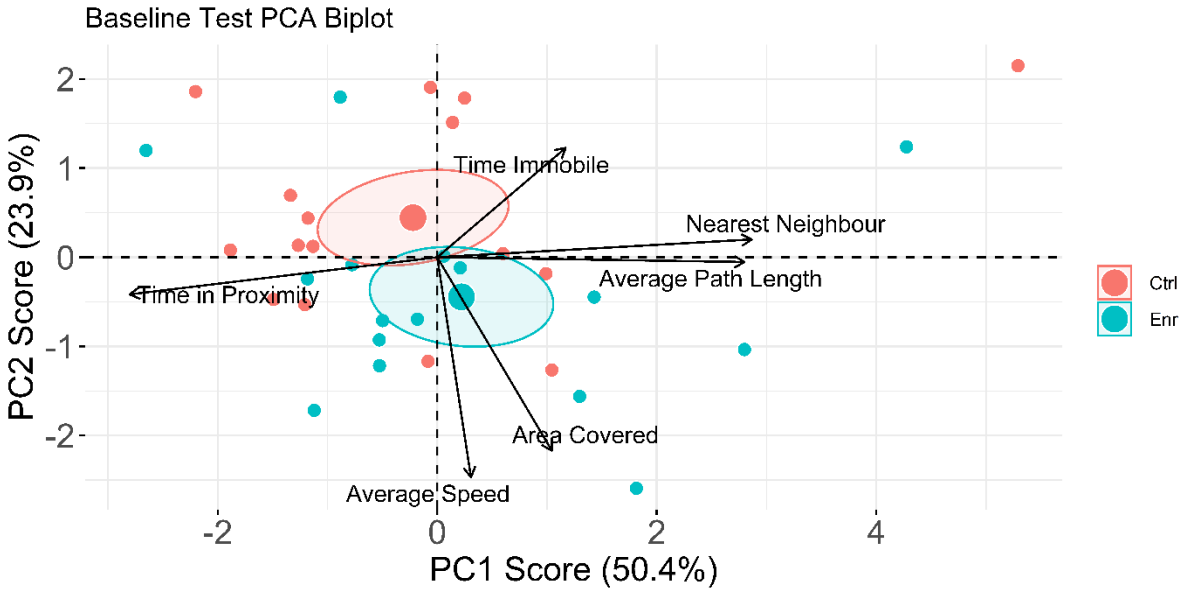

Figure 9. Baseline testing PCA Biplot. Trait loadings onto each component are shown with labelled arrows. The mean component score for each tank and each component are plotted by experimental group.

Post-treatment

The component analysis performed on average tank behaviours in the post-treatment behavioural trials gave a total of six components, the first two of which accounted for ~71% of total variability in the data. The results of the Horn's analysis for component retention indicated that only the first component had an adjusted eigenvalue >1 and should be retained for further analysis. However, to maintain parity with the results from the first trial, the first two component were retained. A cut off value of 0.4 for component loading values was used to assess how traits loaded onto each component. The loadings between trial 1 and trial 2 were largely the same, however, in this case positive PC2 scores indicated increased speed and exploration. The plot of average tank component scores for PC1 and PC2 appeared to reveal a clear separation between control and enriched groups along PC1 (see Figure 10). Specifically, enriched groups seemed to score higher along dimension 1 than control groups, indicating decreased shoal cohesion and reduced proximity in enriched groups. This supported the results from the models fitted to the post-treatment data, where group differences in cohesion emerged based on parental condition in the final trial.

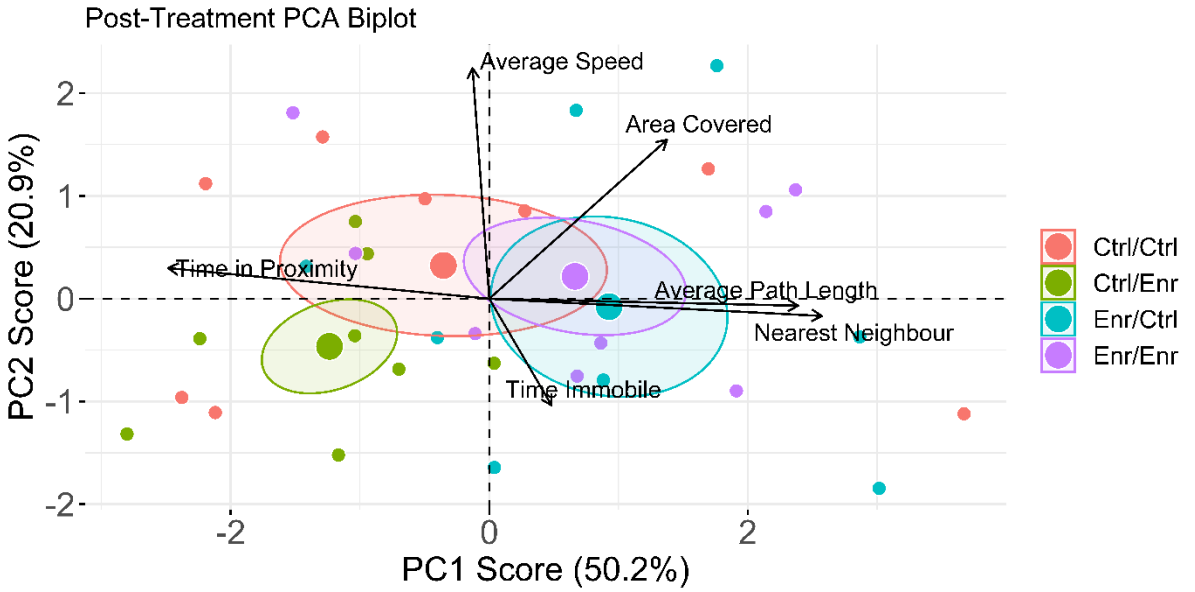

Figure 10. Post-treatment PCA Biplot. Trait loadings onto each component are shown with labelled arrows. The mean component score for each tank and each component are plotted by experimental group.

4.5 Discussion

These results suggest that there is capacity for zebrafish offspring to inherit environmentally induced behavioural phenotypes through the paternal lineage. Indeed, here there appear to be large behavioural differences between experimental groups from different paternal backgrounds, both before and after experimental enrichment in F1 adults. Firstly, there appears to be an inherited effect of swimming speed, which is clearly detectable in enriched F1 adult zebrafish baseline testing. In this case, offspring sired by enriched fathers displayed a high activity behavioural phenotype similar to enriched groups in the parental generation, and was detectable before any experimental manipulation of F1 adults was performed. Second, there was a large difference in shoal cohesion between the F1 adult zebrafish from control and enriched paternal backgrounds post-treatment. Here, groups from enriched paternal backgrounds are less cohesive than offspring from control fathers. However, this effect was not detectable in the first trial and only emerged after repeated exposure to the same behavioural test.

With regards to locomotor activity, the main finding here is that F1 adult offspring from enriched fathers displayed faster swimming speed than offspring from control fathers in the first trial. This is important as effects were observed in the baseline test, before any manipulation of offspring environment, additionally, this is the same effect as was observed in enriched fathers described in the previous chapter. Interestingly, a similar effect has been recently reported in mangrove killifish, where offspring from structurally enriched parents exhibited increased activity levels, regardless of their own environment (Berbel-Filho *et al.*, 2020). In this case all subjects were hermaphrodites and therefore paternal/maternal inheritance could not be determined. Nevertheless, the results from this study seem to support the results found here in zebrafish. The effect on speed here was undetectable in F1 post-treatment testing, and there was no cumulative effect of multiple exposures to enrichment over two generations. It is possible that an upper limit to speed was reached by the offspring of enriched fathers, and therefore further enrichment had little no to effect.

The social traits here did appear to be affected by paternal background in the post-treatment test. This is evident by the reduced cohesion observed in paternally enriched groups in the second round of behavioural testing. This may be a result of paternally inherited differences

in learning and memory. Indeed, if an enhanced memory phenotype were inherited by offspring as a result of paternal enrichment, it would make sense that parental effects were observed only after repeated exposure to the same behavioural test. It has been known for some time that parental EE can induce changes in offspring learning ability and memory retention (Kiyono et al. 1985; Dell and Rose, 1987). Furthermore, there are many recent examples of environmental enrichment affecting memory formation over multiple generations which have been reviewed in depth by (Arai and Feig, 2011). Although, it should be noted that most of these examples are in rodent models, and there appears to be little evidence for the same effect in zebrafish. Although, recent studies in fish species have found that environmental enrichment promotes neural plasticity, cognitive ability and learning behaviour within a single generation (Salvanes et al. 2013; Strand et al. 2010). Nevertheless, there appears to be relatively less evidence for the inheritance of altered learning ability in fish, as there appears to be few studies directly addressing this question. The results here seem to provide an indication that EE may exert transgenerational effects on learning ability and memory in zebrafish. However, it should be noted that, somewhat unexpectedly, the effect on shoal cohesion observed in F1 post-treatment was opposite to the effect observed in F0 fathers in previous chapters.

It is clear from this that the evidence for transgenerational inheritance of environmentally-mediated social behaviours is mixed. Indeed, in this study there is no direct evidence for the inheritance of social behaviours during baseline testing. In another study in zebrafish found transgenerational effects of benzo[a]pyrene exposure to both males and females (Knecht et al. 2017). In this case F0 exposure was associated with hyperactivity and reduced shoal cohesion. This hyperactivity was also observed in the F1 offspring, whereas there was no evidence for the inheritance of altered shoaling behaviour in F1. This is particularly interesting given that this manipulation is far more aggressive and pathological than the one performed here, and yet there is still no evidence for the inheritance of social behaviours. It may therefore be the case that sociality is required to be fluid and plastic within the lifetime of an individual, and it is therefore not surprising that social behaviours are not parentally programmed and inherited. The finding here that swimming speed, but not shoal cohesion, is inherited is particularly interesting. Recent theoretical work has suggested that one of the two main functions of epigenetically mediated transgenerational inheritance is that they carry adaptive

information, where the parental phenotype provides a source of information regarding future selection pressures (English et al. 2015). This transfer of useful information regarding future conditions may be a minimum requirement for adaptive transgenerational plasticity. In results outlined here, it seems plausible that information regarding environmental complexity is far more useful in the context of swimming ability than it is in the context of shoal cohesion. This would make sense, as the ability to navigate complex terrain more effectively could have adaptive benefits including more efficient foraging or enhanced predator evasion. Conversely, forming tighter or less cohesive shoals may provide limited adaptive potential in complex environments. In which case, complex environments provide adequate shelter and protection from threats, and so shoaling cohesion provides little additional benefit to predator evasion.

In conclusion, the results here show a clear transgenerational inheritance of swimming speed based on paternal enrichment conditions, where offspring from enriched fathers display the same high speed phenotype observed in previous chapters. However, there appears to be no such evidence for the inheritance of other traits such as freezing behaviour or shoal cohesion. This disparity in the inheritance of different traits may represent an adaptive transgenerational response where swimming speed has adaptive value in for offspring in enriched environments, whereas shoal cohesion provides little advantage in complex environments with lots of physical shelters. The results also indicate that differences in social traits emerge after repeated trials, suggesting that there may be a parentally inherited effect of learning ability or memory, although there is little evidence in the literature to either support or refute this finding. There are also clear differences in response to EE between F0 and F1 generations, which may be a result of different breeding conditions in different facilities. Nevertheless, there seems to be clear evidence for the inheritance of swimming speed, and due to the controlled breeding design here I suggest that these may plausibly be due to direct germline epigenetic inheritance.

Chapter 5: Effects of Parental and Grandparental Enrichment on Early-Life Morphology and Locomotor Activity in *Danio rerio*.

5.1 Abstract

Recent research suggests that ecologically relevant environmental information can be transmitted non-genetically to subsequent generations. While there is evidence that transmission can occur through both maternal and paternal lineages, many studies have focussed specifically on maternal effects and much less on the role of paternal inheritance. Here I examine the role that paternal experience plays on offspring development over multiple generations in zebrafish. I show that the level of paternal environmental enrichment through structural complexity can affect the development of post-hatching offspring behaviour and morphology over two generations. Over a single generation, high activity phenotypes induced in fathers appear to be transmissible to offspring, who display the same high activity phenotype through spontaneous movement. I show that these effects also persist into a subsequent generation, and that there appears to be a significant interaction between parental and grandparental experience with regards to locomotor activity. Paternal enrichment also induced morphological changes in offspring at the end of the hatching period which may be associated with changes in development rate. Finally, some of these morphological changes appear to accumulate over multiple generations, whereas others appear to be influenced by the stability of environmental conditions across generations.

5.2 Introduction

5.2.1 Developmental Plasticity

In a developmental context, plasticity refers to the fact that in some periods during development, the trajectory of development can be altered by external environmental influences (Pigliucci, 1998) . The timing and duration of these windows can vary considerably between both species and environmental context (Pigliucci, 1998). In recent years the term ecological development, or ‘eco-devo’, has been coined, which refers specifically to how

organisms develop in different environments (Gilbert, 2004). These effects were historically viewed as environmentally generated developmental noise, however it is now regarded as an important character which can itself contribute to adaptation to novel environments. It is clear that developmental plasticity in this way has the potential to be adaptive (Nettle and Bateson, 2015). In the water flea *Daphnia pulex*, the development of inducible defences is triggered by chemical cues (kairomones) released by predators (Tollrian, 1995). Specifically, their life history shifted toward larger body size and higher fecundity. Interestingly, this larger body size appeared to be a trade-off for longer time to reach maturity. Other more recent studies have also found that life-history shifts in age and size in *Daphnia* are also influenced by the presence of predators, by reducing moult number and moult duration (Beckerman et al. 2010). The butterfly, *Bicyclus anynana,* exhibits seasonal polyphenism in wing patterning and other key life history traits such as longevity as a result of environmental cues encountered during pre-adult development (Brakefield et al. 2007). This trait divergence is associated with wet vs dry season variation in natural habitats, and individuals seem to have the capacity to develop either way at fertilization. In these cases of *Daphnia* anti-predator responses and *Bicyclus* wing patterning the plasticity observed during development may plausibly be adaptive, in that they provide a fitness advantage for individuals.

5.2.2 Mechanisms of Developmental Plasticity

The mechanisms that give rise to these new evolutionary targets are diverse, and seem to involve genetic, physiological and epigenetic responses (Beldade et al. 2011). It is increasingly clear that specific genetic factors play a crucial role in environmentally induced developmental trajectories. In some reptile species like turtles, a period of thermosensitivity exists during gonadal differentiation, where sex determination is sensitive to both constant and intermittent changes in temperature (Wibbels et al. 1994). There is evidence for the genetic regulation of this temperature dependent sex-determination in the turtle (Shoemaker et al. 2007). Furthermore, gene expression analysis of *Daphnia*'s morphological response to predator kairomones also shows that a number of genes are significantly upregulated by chemical cues in the postembryonic stage, and may be responsible for subsequent defence morph formation (Miyakawa et al. 2010). In the previously outlined case of wing patterning in *Bicyclus anynana,* studies have shown that the seasonal induction of wing polyphenisms is regulated by hormone (ecdystroid) signalling during a short window of sensitivity during

development (Oostra et al. 2011). Epigenetic regulation of gene expression also appears to play a key role in developmental processes (Jablonka and Raz, 2009). Epigenetic changes do not require a change in DNA sequence, and rely on molecular switches which activate or silence particular genes (Bird, 2007). These kinds of modification typically include DNA methylation, histone modifications and small non-coding RNA (Heard and Martienssen, 2014). In both animal and plants, genome wide epigenetic reprogramming occurs during normal development in the processes of cell differentiation and genomic imprinting (Feng et al. 2010). For example, it has been demonstrated that DNA methylation plays a key role in the environmentally induced phenotypic variation between honeybee castes that results from different developmental experiences (Elango et al. 2009). These epigenetic mechanisms of regulation, in certain cases, may also be inherited by the subsequent generation. This has been described as a type of 'soft-inheritance' which can interact with genetic mechanisms of inheritance and the environment (Richards, 2006). Importantly, this can also be a mechanism by which parents can influence offspring traits.

5.2.3 Parental Effects and Development

Developmental plasticity in offspring frequently involves parental effects (Uller, 2008). For example, in coral reef fish parental condition has been shown to impact offspring early-life history and survival during embryogenesis (Donelson et al. 2008). In this case, good parental condition increased both offspring survival and offspring size at hatching. In an evolutionary context, parentally induced plasticity is favoured when there is fluctuation in environments across generations and offspring conditions are predictable from parental conditions or phenotype (Uller, 2008). Parental effects may be adaptive when there is strong correlation between parental and offspring environment or the offspring environment may be a consequence of parental phenotype. However, parent-offspring conflict may also arise when optimal strategies for parent and offspring are different, and this may result in reduced offspring fitness (Uller, 2008; Marshall and Uller, 2007). Paternal inheritance is a specific form of parental effects that may impact offspring phenotype across multiple generations. These effects are often thought to be transmitted as epigenetic markers in the germline, as fathers contribute little more than genetic material in the sperm and do not typically play a role in offspring rearing in most species (Curley et al. 2011). Consequently, paternal inheritance has received much less attention than maternal effects. The effect of paternal germline

inheritance has, however, been demonstrated in rodents, for example offspring of males fed a reduced protein diet showed increased expression levels of genes involved with lipid and cholesterol biosynthesis (Carone et al. 2010). Interestingly, there were also effects of paternal diet on sperm RNA content in this case suggesting an epigenetic mechanism of transmission. Paternal phenotype may also exert indirect effects on offspring phenotype through paternal influence on maternal investment. This concept has been demonstrated in bird species, where maternal allocation strategies seem to arise from sexual selection of male traits (Horváthová et al. 2012). In this case, females generally invest more into reproduction when paired with attractive males. This has been described as the 'differential-allocation hypothesis' (Burley, 1988).

5.2.4 Inheritance of Acquired Behavioural Phenotypes

There is growing evidence that even in the absence of contact with offspring, fathers can transmit complex behavioural and neurological phenotypes through inherited epigenetic variation in the male germline (Braun and Champagne, 2014). In mice, paternal care has been shown to have significant effects on offspring behavioural development (Frazier et al. 2006). Experimental manipulation by increasing pup retrieving behaviour in fathers seemed to decrease aggressive behaviour in offspring, this effect appeared to be mediated through arginine vasopressin expression in offspring. Paternal deprivation in mice has also been shown to lead to sex-dependent abnormalities in social and reward related behaviours (Bambico et al. 2015), where father deprived male offspring display impaired social interactions with other paternally deprived individuals. The application of the open-field test in an inbred strain of mice has also demonstrated that paternal care can affect open field-activity levels in female offspring (Alter et al. 2009). Chronic social stress through experimentally induced instability of social hierarchies has also been shown to induce social deficits and increased anxiety-like behaviours in offspring, this effect seems to be transmitted through the paternal lineage (Saavedra-Rodríguez and Feig, 2013). In mice, F0 animals subjected to odour fear conditioning before conception were found to produce F1 and F2 offspring with increased behavioural sensitivity to the conditioned odour (Dias and Ressler, 2014).

5.2.5 Plasticity and Inheritance of Body Shape

The inheritance of body size and growth during development has been observed in a range of organisms including birds (Merila and Gustafsson, 1993), a number of aquatic species (Rideout et al., 2004; Green and McCormick, 2005; Evans et al., 2017), and mice (Dunn and Bale, 2009). There is a particularly extensive body of literature regarding body shape plasticity and variation in fish species. A good example of this morphological diversity can be found in fish from the family Cichlidae, which exhibit a particularly high degree of diversity in body shape and morphology (Reddon et al. 2015). This within-generational plasticity in body shape seems to also be affected by environmental factors such as diet, as well as familial inheritance and age (Wimberger, 1992). The effect of diet on body shape and feeding morphology has been shown in other fish species such as the orangespotted sunfish *Lepomis humilis*, where different diets induced changes in jaw morphology within a single generation (Hegrenes, 2001). Other abiotic factors such as temperature also seem to induce plasticity of body shape in fish species, an example of this has been found in zebrafish, where temperature fluctuations during embryonic and larval life stages affects body shape morphology as adults (Georga and Koumoundouros, 2010).

Variation in body shape and size also appear to be inherited across generations. An example of this can be found in the three spined stickleback *Gasterosteus aculeatus*, where the number of vertebrae an individual has and its body size appear to be moderately heritable (Alho et al. 2011). In coral reef fish parental condition has also been shown to affect offspring size at hatching, where good condition in parents increased offspring size at hatching (Donelson et al. 2008). These effects on body size have been shown to be transmissible through the maternal lineage in fish species (Lindholm et al. 2006). In this study, maternal effects on offspring body size were observable in juveniles during development, but seemed to have little effect after sexual maturity in both male and female offspring. In the African cichlid *Lamprologus callipterus* differential growth rates in offspring also appear to be inherited from the previous generation (Wirtz-Ocaňa et al. 2013). In this case growth rates appear to be inherited paternally, male offspring appeared to be susceptible to this variation whereas female offspring did not.

5.2.6 Aims and Hypothesis

In the studies outlined in previous chapters I identified environmentally induced behavioural phenotypes in adult zebrafish, and that some of these acquired traits appeared to be transmissible across generations. Here, I aimed to investigate whether these experimental manipulations affect offspring early-life phenotypes up to 120 hours post-fertilisation (hpf). Larval zebrafish were obtained from controlled breeding regimes in both F1 and F2 generations, the behaviour and whole-body morphology of these larvae were examined in order to identify parental and grand-parental effects on behaviour and body shape. Inheritance through the paternal lineage was targeted by breeding experimental males from experimental backgrounds with a separate cohort of unmanipulated females. This was done to control for confounding maternal effects which may obscure any effects that are a result of paternal germline inheritance. The literature outlined here suggests that paternal experience can significantly affect offspring in a number of ways. I predicted that the behavioural effects observed in the parental and grand-parental adult zebrafish would likely induce behavioural effects in early-life offspring, and that this may also have indirect effects on offspring morphology during development.

5.3 Methodology

5.3.1 Experimental Design

The experimental design for this study consisted of two phases:

1. Breeding from F0 experimental males and testing F1 offspring at 72hpf and 120hpf for shape and movement analysis.
2. Breeding from F1 experimental males and testing F2 offspring at 72hpf and 120hpf for shape and movement analysis.

To begin the first phase of this study, adult male zebrafish from enriched (Enr) and control (Ctrl) housing treatments (as described in the previous chapter) were crossed with a separate cohort of females from standard housing conditions. A single male was taken from each of ten social groups, from each experimental background, and used for breeding. F1 zebrafish larvae were obtained from breeding and used in movement trials. For the morphometric

analysis, F1 zebrafish larvae were mounted and imaged for shape analysis. Breeding was performed with a separate cohort of female zebrafish which were unused in experimental studies. Each of these ten females were bred with a male from each experimental treatment in order to control for maternal effects in the resulting groups (F1-Ctrl and F1-Enr). In the second phase of this study, eight cohorts of F1 larvae from the two experimental groups were reared until adulthood, cohorts were then split again between either control or enriched adult housing conditions for a fixed experimental period of four weeks. The resulting four treatment groups each represented a possible combination of parental or offspring enrichment treatments across two generations (see Figure 1). After this treatment, eight of F1 adult males from each of the four experimental groups (paternal control/enriched × offspring control/enriched) were then crossed with a separate cohort of females from a standard housing background. The resulting F2 offspring from the four experimental groups (F2-Ctrl/Ctrl, F2-Ctrl/Enr, F2-Enr/Ctrl, and F2-Enr/Enr, see Figure 1) were then imaged for morphometric analysis and recorded for behavioural testing.

5.3.2 Animals and Husbandry

The embryos used for this study were obtained from the natural mating of single male and female adult zebrafish. Embryos were incubated for 3dpf at 27.5-28.5°C on a 14/10 h light/dark cycle. Embryos were kept in petri dishes containing 0.0001% methylene blue (MB) solution in conditioned tank water at a maximum density of 100 eggs per dish. Once larvae had hatched at 3dpf, they were then moved to conditioned tank water in petri dishes until 5dpf. Embryos were screened daily using a Leica dissection microscope to remove dead or deformed embryos. Only normally developing embryos were retained for use in this study. At 5dpf larvae were moved into standard housing tanks under standard conditions with reduced water flow until adulthood. Standard juvenile and adult housing conditions in this study consisted of a 5L housing tank with a single form of artificial vegetation (see Figure 1). Larvae were raised on ZM Systems™ fry food diet mixed with fresh system water and injected into the water column in each tank. Juveniles were fed a combination of ZM Systems juvenile food injected into the water column and live *Artemia* nauplii.

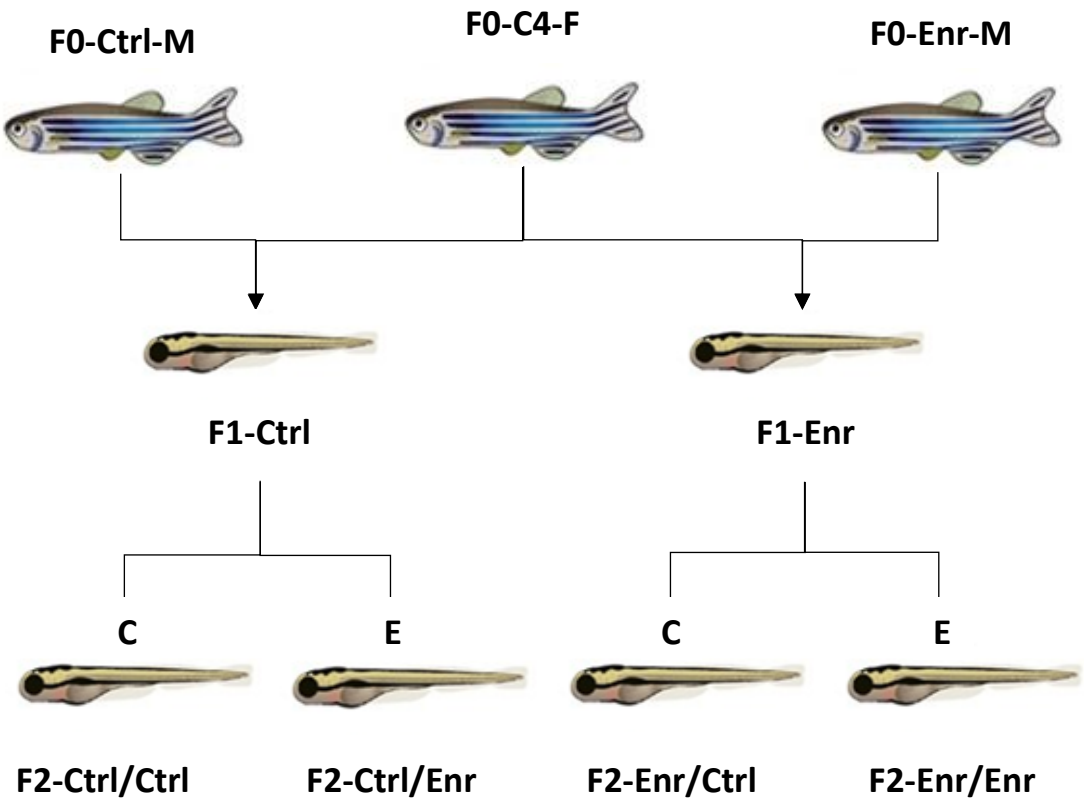

Figure 1. F0 males from control and enriched backgrounds were crossed with a separate cohort of females to obtain F1 from different backgrounds. The two groups were reared until adulthood and then split between either control or enriched conditions. The resulting four experimental groups are then crossed with a separate cohort of females (not shown) to obtain offspring from each

5.3.3 Breeding

Breeding tanks were constructed by suspending a sheet of nylon mesh approximately 5cm below the surface of the water in standard 5l housing tanks. These were kept on the same recirculating water housing racks with biological and particulate filtration described in previous chapters. Mating pairs were moved to a breeding tank approximately 30 minutes before lights on at 8:00am in the fish housing facility. Pairs were then left undisturbed for the first two hours until 9:00am. From this point each breeding tank was periodically checked every hour until 13:00pm for the presence of eggs at the base of the tank. When a sufficient number of eggs were observed in a tank, the mating pair was immediately removed and placed back into their respective housing tanks. Eggs were collected by siphoning eggs from the empty breeding tank through a fine mesh, the mesh was then inverted into a clean petri

dish containing 0.0001% MB solution. The eggs were then transferred again unto another clean dish containing MB to remove any debris that may have transferred into the dish from the mesh.

5.3.4 Larval Imaging

Larval imaging was performed on 72hpf old zebrafish larvae. Imaging was carried out with a Leica M50 dissection microscope (Leica Microsystems, Milton Keynes, UK) fitted with a GXCAM-EYE-5 eyepiece camera (GT Vision, Stansfield, UK) . This was connected in real time to a laptop running the associated GXCapture software for monitoring. For the first generation offspring (F1), a total of 189 images were taken for shape analysis, and in the second generation offspring (F2) a total of 156 images were taken for shape analysis. Larvae were mounted to a microscope well slide using a 3% methylcellulose solution. First, larvae to be tested were moved into a petri dish containing ice water for 5 minutes prior to imaging. Each sample was then moved into a clean slide and excess water was removed with a pipette and towel. A single drop of 3% methylcellulose solution was then dropped directly on top of the larvae from above. Mounted samples were placed underneath a Leica dissection microscope and oriented by using dissection forceps to move the methylcellulose around the sample. This orienting of the sample was done remotely using an eyepiece camera and a connected laptop in order to capture high detail images and monitor the image quality real time. Images were captured in 1920x2560 pixel resolution using GXCapture imaging software. Larvae were always imaged on the lateral side, in full extension and with the body straight (see Figure 2 A). Larvae were not recovered and were euthanized immediately following imaging.

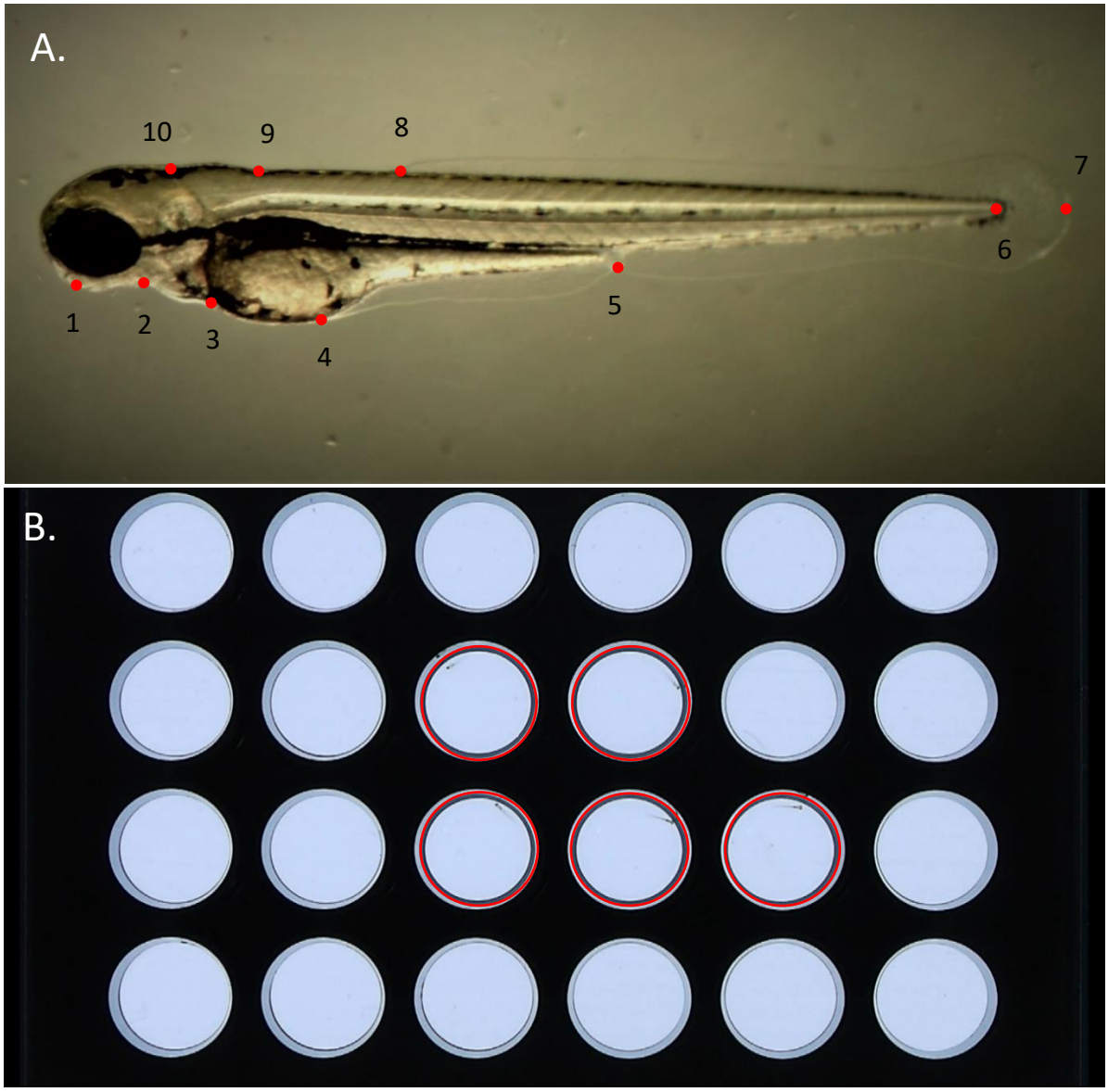

Figure 2. Landmark image (A) showing a single larvae and ten landmark points placed around the body. Video tracking of spontaneous movement (B). Larvae were singly tracked in a well for a total of 10 minutes using idTracker. Center wells in red circles were used in each trial to ensure good well coverage from the recording angle.

5.3.5 Larval Movement

Larvae behavioural trials consisted of a 15-minute test period in which larvae were placed in a black 24-well assay plate with clear flat wells (Figure 2 B). In each plate a sample of 5 larvae from each breeding cross were placed in the centre wells. The assay plate was placed on top of a MiniSun A3 light pad. A consumer camcorder was suspended approximately 1m above

the assay plate and zoomed in to fill the display. This distance was used in order reduce the visibility of the well walls in the frame, this way larvae could be tracked in the entire area of the well. The plate was left undisturbed and recording for the entire 15-minute test. Larvae were recovered from behavioural trials and placed back into standard housing conditions following testing. The first 5 minutes of each trial was regarded as a habituation period and trimmed out of the resulting video using VSDC Video Editor. Larvae were tracked and trajectories were obtained using the open source MATLAB-based application idTracker. A single larva in each of the 5 utilised wells were identified and located in the frame of the video (see Figure 2 B). The coordinates of the centroid for each larva were then tracked across the entire video. A custom-MATLAB script was written to extract the total movement of each larvae between successive frames. The number of transitions from stationary to active was also calculated using a custom function in a MATLB script. A total of n = 80, 120hpf old F1 larvae were used for examination of movement in the behavioural trials. In the second phase of this trial, a total of n = 160, F2, 120hpf old larvae were used in the same behavioural trials.

5.3.6 Data Analysis

Geometric Morphometric Analysis of Body Shape

Digitization of 10 pre-defined landmarks (see Figure 2 A) on all images was performed using tpsDig232 ver. 2.31 (Rohlf, 2005). This configuration of landmarks gave a good estimate of the outline and key features for examination (see Table 1). Landmarks 1-2 were included to estimate lower jaw/snout development, landmarks 3-5 were included to examine changes in yolk sac shape, landmarks 6-7 were used to examine tail fin shape and landmarks 8-10 were included to estimate spine elongation. A TPS list file was created from the images and the ten landmark coordinates on each image using tpsUtil ver 1.76 (Rohlf 2005). Examination of body shape change between groups was performed using morphometric analysis in the tpsRelw32 ver.1.69 (Rohlf, 2005). Using the raw coordinates from all specimens, a 'consensus' configuration of these landmarks was calculated using the generalized orthogonal least-squares Procrustes method of superimposition. Partial warp scores (PWS) for each specimen are then calculated which represent deformations in shape. In tpsRelw32 this is defined as the weight matrix, W. Partial warp scores can then be used as dependent variables in standard statistical tests (Rohlf, 1996). Here they were used in linear mixed effects models (LMM's) to

investigate the effects of experimental manipulation on whole-body shape changes. For F1 offspring, a simple linear model with a single fixed effect of parental condition was fitted to the data. In the F2 offspring a mixed effects model with two fixed factors, parental condition and grand-parental condition, was fitted to the data. In this case a single random factor of parental identity was included. Visualisation of the consensus configuration and the shape changes between groups was performed using MorphoJ (Klingenberg, 2011).

Table 1. List of landmark points (1-10) and a brief description of each point.

LANDMARK	**DESCRIPTION**
1	Tip of the snout
2	Jaw line
3	Heart/yolk sac join
4	Where the fin joins yolk sac
5	Urinary tract
6	Tip of tail
7	Base of fin
8	Where the fin joins the spine
9	End of spine
10	Base of the head

Spontaneous Movement and Activity

In the F1 generation a total of 40 control background and 40 enriched background larvae were tested giving a total of 80 specimens for estimation of movement and activity. In the F2 generation a total of 40 offspring were successfully recorded from each of the four experimental treatment groups resulting in a total of 160 F2 offspring used for behavioural analysis. The two behavioural measures of all subjects (distance moved and movement transitions) were then used as dependent variables in separate negative binomial generalised linear mixed effects models, which were fitted using the 'glmer.nb' function from the 'lme4' and 'MASS' packages (RStudio Team, 2020). This was done to account for the overdispersion

present in the data. In the F1 offspring, paternal condition was included as a single fixed factor, and paternal identity was included as a random effect to account for any similarities between subjects from the same paternal background. In the F2 offspring two fixed factors were included, parental and grand-parental condition, as well as paternal identity as a single random factor. Model residuals were assessed for the presence of outliers. Overdispersion was formally tested to obtain a deviance to degrees of freedom ratio. Summaries of models were obtained as the estimates of effect sizes and their significance. A type II analysis of deviance was performed using all model terms and interactions to assess the significance of each model term.

5.4 Results

5.4.1 F1 Body Shape

A total of 7 principal warps and 16 partial warps describing the total shape variation were extracted from the F1 data set. The first ten partial warps described >95% of shape variation within the dataset and were retained for further examination. In all cases higher scores for a partial warp indicated groups that more closely matched the associated shape deformation (in red), than the consensus shape (in blue, see Figure 3A). Significant effects of parental exposure to experimental enrichment were found in three models. By far the largest effect was observed in scores for PW8 (see Table 2). This accounted for only 2.28% of total shape variation in the data set and seemed to describe the compression of landmarks 2 and 3 and their movement closer together (see Figure 3 A). Larvae from enriched paternal background scored higher on this partial warp relative to controls (see Figure 3 – bottom left) , indicating that the enriched group closely matched the landmark configuration of PW8 (Figure 3 A - red). Larvae from control backgrounds scored negatively on this partial warp, suggesting that they more closely matched the consensus configuration (Figure 3 A - blue). The next largest effect was observed in scores for PW4 (see Table 2). This accounted for 11.46% of total variation in the data set. Similarly to PW8, individuals from enriched parental backgrounds scored significantly higher for PW4 (see Figure 3 – bottom middle). Indicating that enriched more closely matched the red shape in Figure 3 B, whereas control more closely matched the blue consensus configuration. Finally there was also a weak effect of paternal enrichment on PW3, although this was not significant (see Table 2).

Table 2. List of partial warps (1-10), p-values from contrasts between enriched and control backgrounds, test statistic and % of variance explained by each warp. The values in bold indicate significant effects were reported in the model.

PARTIAL WARP	P-VALUE	T-VALUE	% VARIATION EXPLAINED
1	0.11	-1.60	31.15
2	0.39	0.86	17.87
3	**0.10 .**	**1.67**	**16.32**
4	**0.02 ***	**2.40**	**11.46**
5	0.37	-0.90	6.65
6	0.43	-0.79	3.71
7	0.61	-0.51	2.67
8	**< 0.001 *****	**3.51**	**2.33**
9	0.17	-1.37	2.21
10	0.32	0.99	1.81

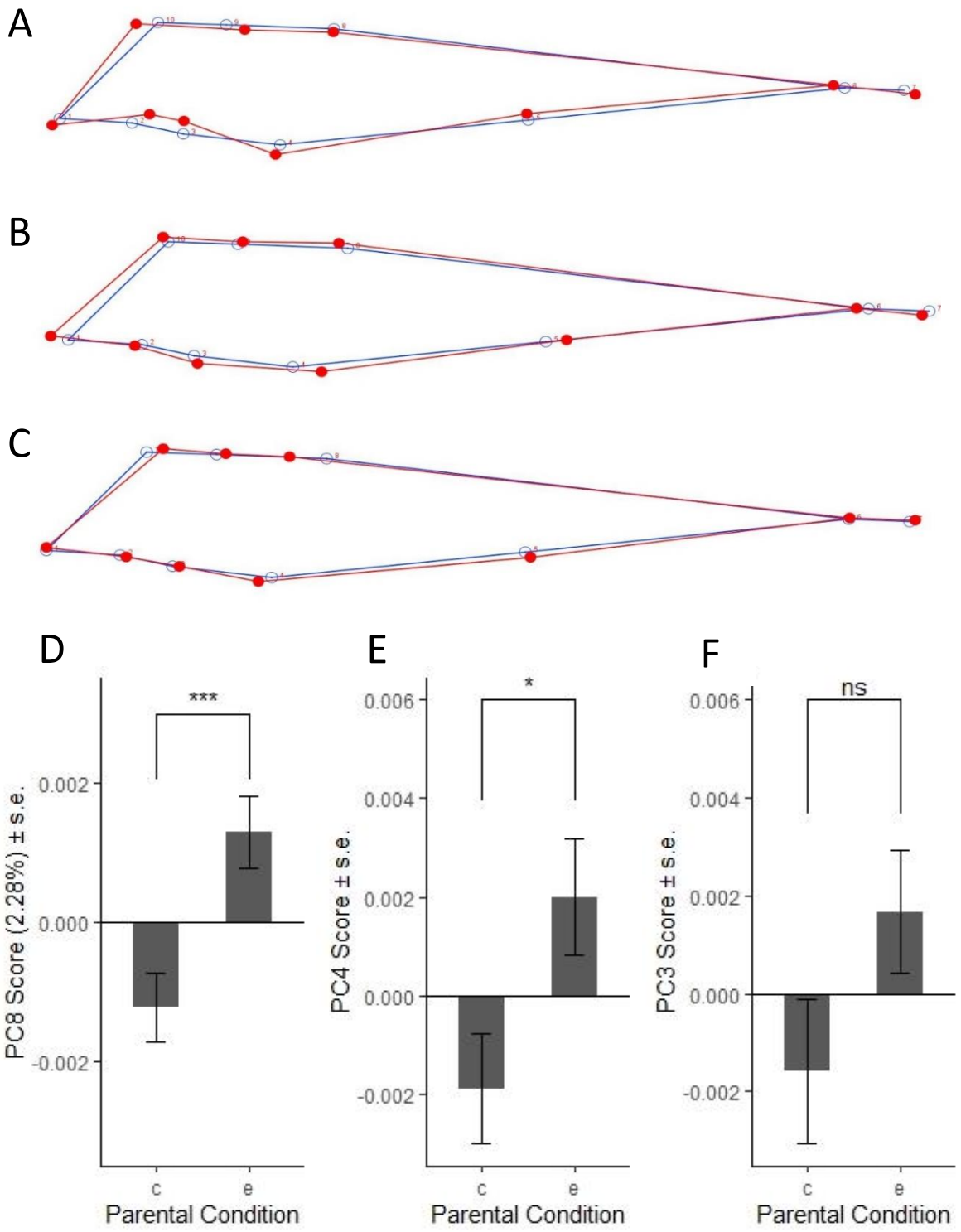

Figure 3. Partial warps 8(A), 4(B) and 3(C) and their associated shape deformations (red), compared to the consensus shape (blue) . Also showing comparison of PW8 scores (D), comparison of PW4 scores (E), comparison of PW3 scores (F).

5.4.2 F1 Locomotor Activity

The model fitted to total distance seemed to sufficiently reduce overdispersion to an acceptable level (deviance to degrees of freedom ratio = 0.84). There appeared to be a large main effect of parental condition on the total distance swam by offspring in the spontaneous movement trial. Offspring from enriched backgrounds and offspring from control backgrounds moved a similar distance, although there was a slight trend for enriched larvae to move farther (estimate = 1.72, error = 1.02, z = 1.69, p = 0.09, see Figure 4 – left). The model fitted to the number of movement events also appeared to successfully account for the overdispersion in the data (deviance to degrees of freedom ratio = 1.003). The model results suggested that there was also a large effect of paternal condition on the number of movement events. Offspring from enriched backgrounds appeared to show a significantly higher frequency of movement events than offspring from control fathers (estimate = 1.92, error = 0.75, z = 2.56, p = 0.01, see Figure 4 – right).

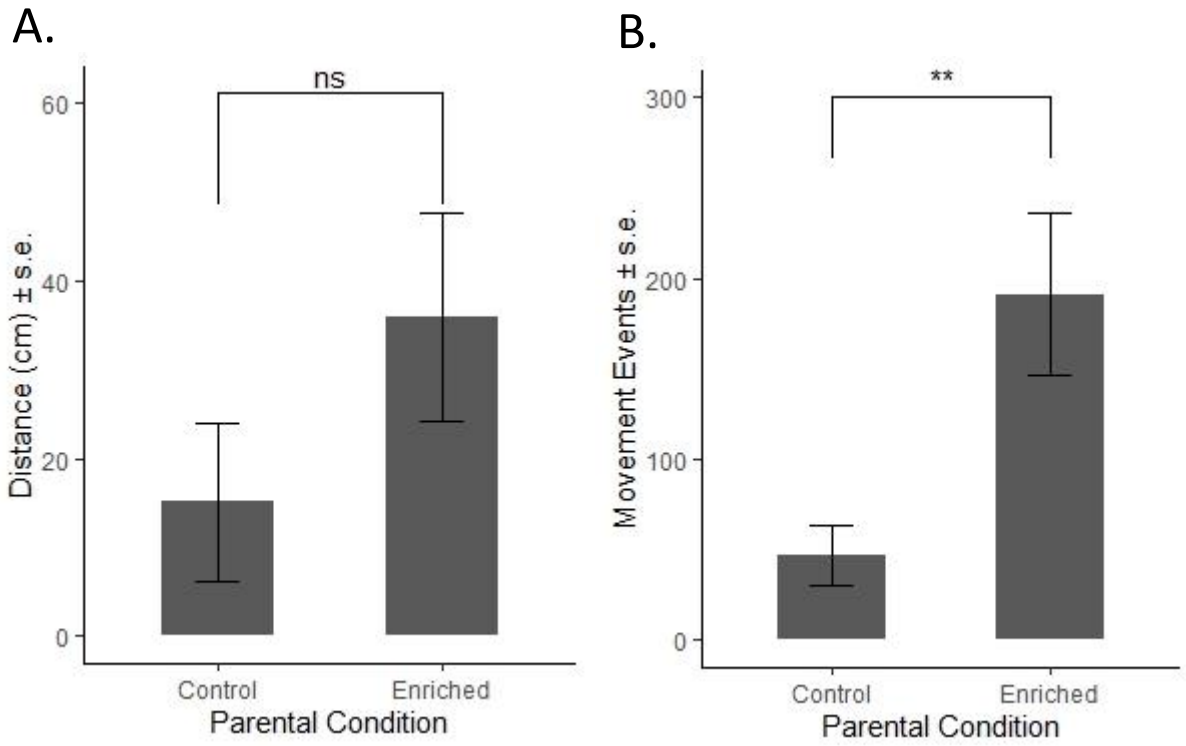

Figure 4. Comparison of spontaneous movement between parental backgrounds. Offspring from enriched paternal backgrounds moved on average significantly further in the trial (A) and also had significantly more average movement events (B).

5.4.3 F2 Body Shape

Like the F1 data set, a total of 7 principal warps and 16 partial warps that accounted for total shape variation were extracted. The first ten accounted for 96.25% of total variation in the data set and were retained for statistical analysis. Two outliers were identified from the model fitted to the F2 specimens, and upon inspection of images I identified a pericardial edema pathology in these two specimens and both were therefore removed from the analyses. Upon removing these specimens, no further outliers were identified. In all cases higher scores for a given shape deformation indicated groups that more closely matched the associated shape deformation (in red), than the consensus shape (in blue, see Figure 5 A).

There was no main effect of parental enrichment or grandparental enrichment on PW1 scores, however the interaction between the two factors was significant (ChiSq = 9.05, DF = 1, p = 0.002, see Table 3). This interaction appeared to be due to differences in individuals whose parents both experienced control conditions but those whose grandparents received either control or enriched housing conditions (t = -3.01, DF = 29, p = 0.005). There were also significant differences between individuals whose grandparents were enriched, but whose parents either experienced control or enriched conditions (t = 2.25, DF = 27.4, p = 0.03). In this case, individuals whose grandparents experienced enriched conditions scored higher than those whose grandparents experienced control conditions (see Figure 9 A), and individuals from enriched grandparental conditions, whose parents experienced control conditions, scored higher than those whose grandparents experienced enriched housing conditions (see Figure 9 A).

Partial warp 5 also showed a significant interaction effect between parental and grandparental experimental conditions (ChiSq = 4.50, DF = 1, p = 0.03, see Table 3). This effect seems to be the result of individuals from both enriched parents and grandparents scoring significantly lower scores than all other groups for this warp (Ctrl/Ctrl, Enr/Enr, t = 3.72, DF = 28.5, p = 0.009; EC-EE, t = 4.72, DF 27.4, p = 0.001; CE-EE, t = 2.09, DF = 26.9, p = 0.05). For partial warp 9 there were main effects of both F1 and F0 condition and there was also a significant interaction (ChiSq= 4.88, DF = 1, p = 0.03, see Table 3). Post-hoc analysis between groups showed that offspring whose parents and grandparents were both exposed to enriched housing scored significantly higher than all other treatment groups (Ctrl/Ctrl,

Enr/Enr, estimate = -0.015, DF = 28.6, p < 0.001; Ctrl/Enr-Enr/Enr, estimate = -0.011, DF = 26.7, p < 0.001; Enr/Ctrl-Enr/Enr, estimate = -0.012, DF = 27.3, p < 0.001, see Figure 9 B), and all other groups differences were non-significant.

Table 3. Results from models performed for each partial warp (PW 1-10). Significant interaction terms are highlighted in bold.

PARTIAL WARP	FIXED FACTORS	CHISQUARE	P-VALUE	% VARIATION EXPLAINED
1	F0	1.51	0.22	38.87
	F1	0.34	0.85	
	F0:F1	**9.05**	**0.002**	
2	F0	3.24	0.07	17.55
	F1	34.15	< 0.001	
	F0:F1	0.44	0.51	
3	F0	0.92	0.34	11.08
	F1	15.44	< 0.001	
	F0:F1	0.04	0.85	
4	F0	6.74	0.009	9.51
	F1	3.16	0.08	
	F0:F1	0.05	0.83	
5	F0	0.71	0.40	5.90
	F1	20.46	< 0.001	
	F0:F1	**4.50**	**0.03**	
6	F0	1.95	0.16	3.87
	F1	0.81	0.37	
	F0:F1	0.37	0.54	
7	F0	4.75	0.03	3.23
	F1	7.10	0.008	
	F0:F1	0.05	0.83	
8	F0	0.26	0.61	2.70
	F1	3.23	0.07	
	F0:F1	0.03	0.86	
9	F0	12.24	< 0.001	1.99
	F1	21.37	< 0.001	
	F0:F1	**4.89**	**0.03**	
10	F0	2.54	0.11	1.54
	F1	11.67	< 0.001	
	F0:F1	1.81	0.18	

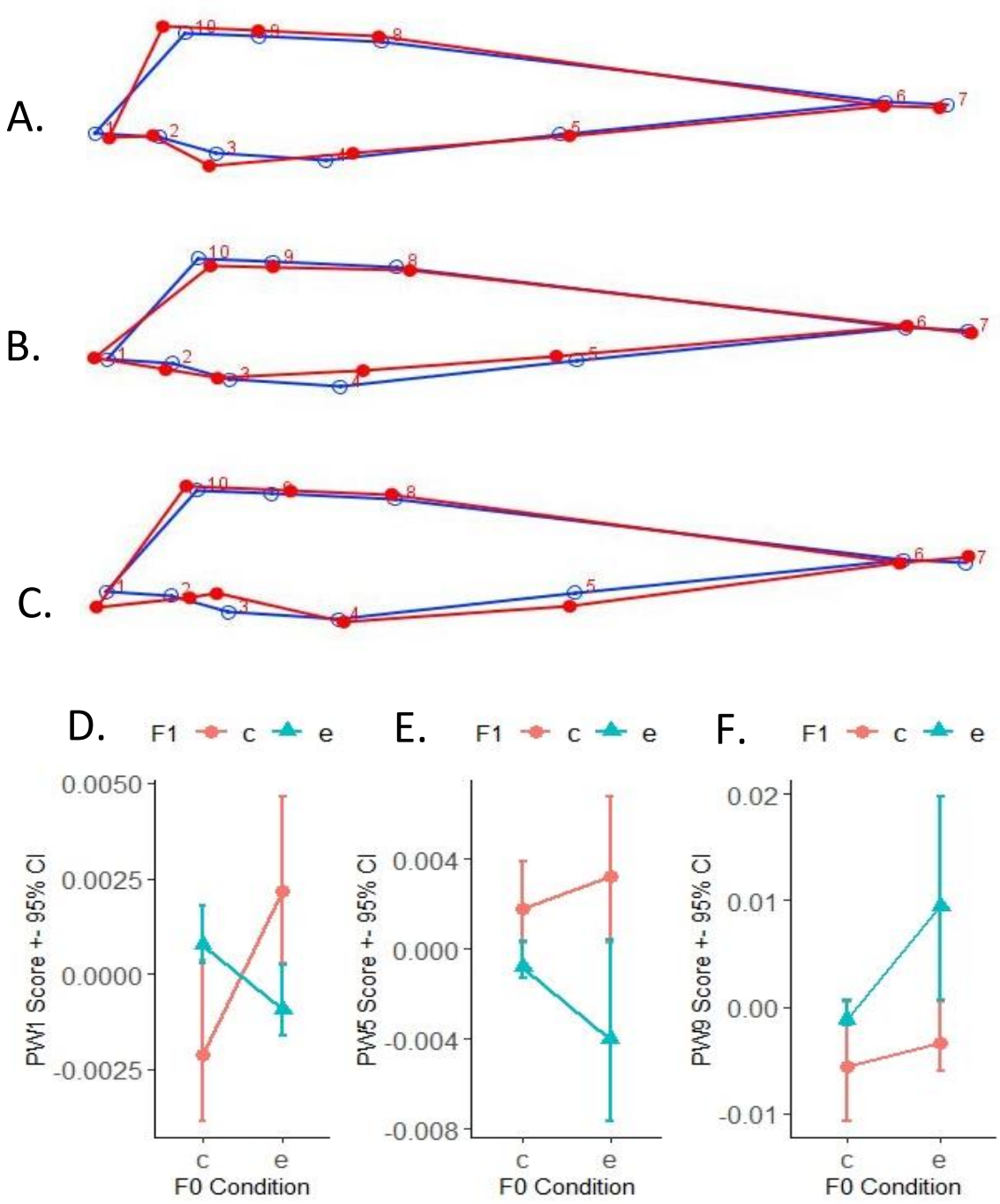

Figure 5. F2 shape deformations that showed significant interaction effects of enrichment between parental and grandparental conditions. Shape deformations for PW1 (A), PW5 (B) and PW9 (C) are shown in red, compared to consensus shape in blue. The scores for PW1 (D), PW5 (E), and PW9 (F) are compared between conditions and generations. In all cases, higher scores indicate groups who more closely match the PW shape rather than the consensus.

5.4.4 F2 Locomotor Activity

For F2 movement, the largest effect was observed in the total distance travelled in the trial. Specifically, there were main effects of both F0 (estimate = 1.85, SE = 0.49, z = 3.78, $p < 0.001$) and F1 (estimate = 1.20, SE = 0.49, z = 2.45, p = 0.015) enrichment, as well as a significant interaction effect (estimate = -1.35, SE = 0.68, z = -1.98, p = 0.048). Post-hoc comparison of experimental groups showed that F2 offspring from control grandparental and parental backgrounds moved significantly less than all other experimental groups. In this case, the largest reduction was relative from offspring of control fathers whose grandparents were enriched (see Figure 6). There was also a significant main effect of F1 (parental) enrichment with regards to movement events (estimate = 0.79, SE = 0.36, z = 2.17, p = 0.03), although there was no effect of grandparental condition (estimate = 0.62, SE = 0.36, z = 1.70, p = 0.09). There was also no interaction between F1 and F0 condition (estimate -0.37, SE = 0.51, z = -0.73, p = 0.47). The largest difference here was between F2 offspring whose parents and grandparents were both enriched and those whose parents and grandparents were both naïve (Ctrl/Ctrl, Enr/Enr). The other main difference was between offspring whose parents

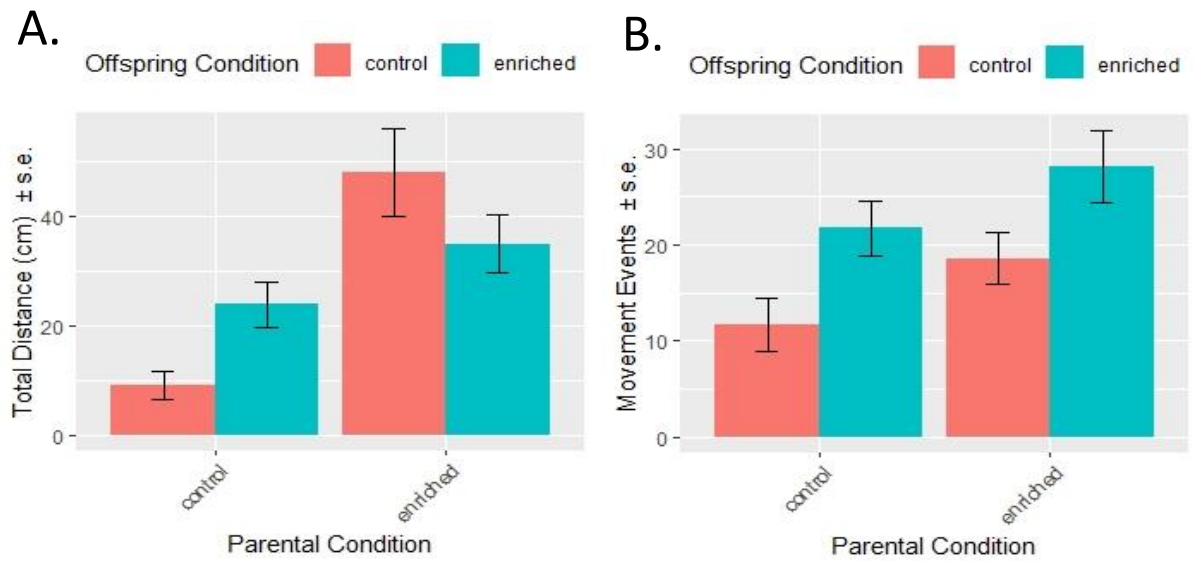

Figure 6. Plots showing differences in locomotor activity in experimental F2 offspring. A.) total distance travelled in a ten minute spontaneous movement test. B.) Number of movement events, or transitions from stationary to active in the same test.

and grandparents were naïve to enrichment, and those whose parents (F1), but not grandparents (F0), were exposed to enrichment (see Figure 6).

5.5 Discussion

The results reported here suggest that acquired adult behaviours can affect offspring early-life development across multiple generations. This included effects on both behaviour and morphology. In adult zebrafish from previous generations, one of the main effects of increased levels of structural enrichment was increased locomotor activity. Specifically, adult zebrafish exposed to high levels of structural enrichment seemed to display higher levels of locomotor activity than adults exposed to basic structural enrichment. That effect seems to translate over a single generation to F1 offspring behaviour as larvae, where offspring from highly enriched backgrounds display a similar high activity phenotype in spontaneous movement trials. These F1 offspring from enriched backgrounds seemed to transition from stationary to active significantly more than offspring from control backgrounds. However, this seems to be largely an effect of the immediate parental generation and much less prominent over multiple generations. This is evident as in F2 testing, there was a detectable significant effect of parental (F1) enrichment, however only a weak effect of grandparental (F0) enrichment. Nevertheless, this high activity phenotype seen in F0 and F1 adults seems to be recapitulated in both F1 and F2 offspring through the number of movement events. This provides a potential link between the adult phenotypes observed in adult behavioural trials and the resulting offspring activity during development.

In F2 offspring, there also appeared to a significant interaction effect between parental and grandparental condition with regards to total distance travelled. This is interesting as there was only a weak effect of paternal enrichment on distance travelled in F1 offspring. Nevertheless, F2 offspring whose fathers or grandfathers were exposed to enrichment showed higher levels of locomotor activity relative to naïve offspring. This suggests that although this particular effect of EE on offspring activity is weak over a single generation, in F2 offspring, a single historical exposure in either generation is sufficient to produce a detectable difference. Moreover, offspring from both enriched fathers and grandfathers showed similar levels of activity to those with only a single historical exposure, suggesting that there is no cumulative effect of a double exposure over multiple generations. Individual

differences in activity levels in adult zebrafish have been shown to be stable over time and environmentally dependent, suggesting that motor behaviour forms a key part of an individual's personality (Tran and Gerlai, 2013). Recent evidence has demonstrated that these differences in personality, and other factors such as social status, play a role in determining offspring activity levels over multiple generations (Zajitschek et al. 2017). These effects of paternal influence on offspring activity observed here may therefore be due to epigenetic mechanisms of inheritance through the male germline. Although, maternal adjustment of egg hormones based on male condition in the short time between meeting and fertilisation cannot be ruled out.

The main effect of paternal enrichment in F1 offspring in terms of morphology was with regards to snout elongation and mouth protrusion. In this case, F1 offspring from highly enriched fathers seemed to show significantly longer snouts and protruding mouths than offspring of fathers from control conditions. This is interesting as during zebrafish embryonic development, the main morphological feature at the end of the hatching period (72hpf) is the protruding snout, during which time the snout elongates to form the mouth (Kimmel et al. 1995). This therefore seems to be a marker of developmental progress during this late hatching stage into juvenile stages. The increased length of the snout in enriched offspring may therefore suggest that they are developing faster than their control counterparts. The largest shape deformation in the F2 offspring (PW1) described a flattening of the face and snout. Here, when grandparents are exposed to high levels of enrichment, additional enrichment of the parental generation produces offspring with increased snout protrusion relative to those offspring whose parents were not enriched.

One particular shape deformation seen in the F1 (PW8), also appeared to be present in F2 (PW9). These two morphologies were strikingly similar, and appeared to represent approximately equal amounts of shape variation in both generations. This shape deformation was associated with an elongation of the snout. This shape deformation also appeared to have cumulative effects across multiple generations. The F1 offspring from enriched backgrounds scored significantly higher on this warp than controls, and in the F2 offspring from enriched fathers and grandfathers again scored significantly higher than all other experimental groups. In previous studies, the effect of parental environment has been shown to produce morphological effects that persist over multiple generations. For example,

hatching temperature has been shown to have an effect on three spined stickleback offspring hatching success, and has been demonstrated over multiple generations (Shama and Wegner, 2014) Parental exposure to Benzo[a]pyrene has been shown to induce body morphology deformities in both F1 and F2 offspring with regards to skeletal and craniofacial deformities (Corrales et al. 2014). Other studies of the multigenerational effects of toxin exposure in zebrafish development have shown that long term parental exposure to some compounds can delay early development of offspring over a single generation (Jantzen et al. 2017). Although clearly here a much less severe and pathological intervention was used, and the results here seem to demonstrate that relatively subtle environmental changes (i.e. enrichment) may also produce subtle shifts in offspring traits.

In conclusion, it appears as though the effects of parental and grandparental enrichment on morphology and locomotor activity seen here are related to a subtle shift in growth and development during the first 5 days post-fertilisation. These effects appear to be transmitted through the paternal lineage, and can produce interactions between the conditions experienced by fathers and grandfathers as adults. The results here show that environmental conditions experienced in later life stages can induce transgenerational effects that persist over multiple generations. These effects appear to encompass subtle changes in both morphology and behaviour within the first 5 days of development. Importantly, an increase in spontaneous movement seemed to link adult activity levels with offspring activity levels between generations. There also subtle changes in morphology which seem to be related to developmental rate. The most interesting finding here is the link between F0 activity and F1 larvae activity, both of which show elevated activity levels relative to control groups. Due to the controlled breeding design utilized, it is likely that these inherited effects are due to epigenetic markers inherited in the sperm, although this has not been conclusively demonstrated here.

Chapter 6: Effects of Environmental Enrichment (EE) Exposure on Whole Brain Nonapeptide Expression in Adult Zebrafish.

6.1 Abstract

In previous chapters, a short period of exposure to EE was found to result in changes in locomotion and grouping behaviour in adult zebrafish, and enriched groups appeared to be more cohesive as a shoal than control groups. In vertebrates the nonapeptides arginine vasotocin (AVT) and isotocin (IT) and their homologues appear to play key roles in regulating social behaviour. Changes in expression of these genes in the brain may therefore be a potential mechanism underlying the differences in grouping behaviour induced by EE. Here, male and female F0 zebrafish exposed to different levels of enrichment were used to compare levels of brain nonapeptide expression between experimental groups. Whole brain RNA was extracted and quantified using RT-PCR, and AVT and IT transcript abundance was measured relative to the housekeeping gene beta-actin (BA). The results here indicate that AVT expression, relative to BA, was significantly reduced in the more cohesive enriched groups relative to controls. There was no difference in IT expression relative to BA between experimental groups, although the overall level of gene expression was higher in enriched groups. There were also no differences in either AVT or IT between males and females. The results therefore suggest that reduced AVT expression is associated with tighter shoal formation in zebrafish, which itself appears to be a result of exposure to higher levels of structural complexity. These results indicate that nonapeptide systems that are thought to regulate social behaviour in fish are sensitive and plastic to the physical / structural environment.

6.2 Introduction

6.2.1 Collective Behaviour

Collective behaviour is found throughout nature. For collective behaviour to emerge, individuals within a group must time and coordinate their behaviour with other group members (Sumpter, 2006). This provides inherent benefits such as reduced predation risk, increased foraging efficiency and improved mating opportunities (Krause et al. 2002). The

maintenance of this group composition and coordination relies on consensus decision making within the group, even in the presence of conflicting interests between members (Deneubourg and Goss, 1989). It has recently been suggested that consistent phenotypic differences in behaviour between individuals within a group (so called 'animal personalities') is a key mechanism underlying the dynamics of collective motion (Jolles et al. 2020). This can occur through differences in leadership, within-group positioning and decision making. This has been empirically demonstrated in sticklebacks (*Gasterosteus aculeatus*), where an individual's social preference and boldness appears to drive group foraging success (Jolles et al. 2017). There is also compelling evidence that group level behaviour can vary depending on environmental conditions. For example, a recent study in the guppy *Poecilia reticulata* has shown that high-predation environments can affect social dynamics and group exploration (Ioannou et al. 2017). Another recent study using sticklebacks has demonstrated that collective behaviour is also modified by the physical environment and food availability (Jolles et al. 2018). In this case, collective behaviour was found to change between open environments, environments with an abundance of food and environments with both food and shelter. Differences were observed in a number of group level traits including group speed, cohesion, and alignment.

6.2.2 Effects of Environmental Enrichment (EE)

A fundamental way in which an organism's environment can vary is through the complexity of the physical environment. For fish species, novel structures and substrates provide a form of environmental enrichment (EE), and can affect a range of behavioural systems including stress, anxiety, aggression and sociality (Näslund and Johnsson, 2016). It has been demonstrated in zebrafish that groups raised in enriched housing environments seem to form tighter shoals than fish raised in barren environments (Sykes et al. 2018). The complexity of the physical environment also appears to significantly affect other social traits such as aggression in zebrafish, as measured by increased attacks made against a mirror image in controlled environments (Woodward et al. 2019). In other fish species, EE has been shown to have further effects on reproductive success, mating behaviour and collective state (Cats Myhre et al. 2012; Rodriguez-Pinto et al. 2020). Taken together, these studies in fish species seem to suggest that social and collective behaviours are sensitive to changes in complexity of the physical environment.

6.2.3 Nonapeptides and Social Behaviour

It is well known that the nonapeptides oxytocin (OXT) and arginine vasopressin (AVP) play a central role in the neurogenetics of sociality, and these mechanisms appear to be highly conserved across species (Donaldson and Young, 2008). Our understanding of the role of AVP and OXT in the regulation of social behaviour has largely been founded on studies in rodents, microtine voles in particular (Keverne and Curley, 2004). For example, central administration of OXT in female prairie voles during mating has been shown to result in a significant preference for the male partner that was present during administration (Williams et al. 1994). In a more recent study, administration of an OXT receptor antagonist in rodents has also shown to reduce social exploration, measured by the rate of interaction with conspecifics, and to prevent social avoidance (Lukas et al. 2011). The administration of OXT in rodents has also previously been associated with enhanced social interactions (Witt et al. 1992) and inhibition of aggression (Harmon et al. 2002). In another early study in the Prairie vole, central administration of an AVP antagonist in males was shown to block the development of selective aggression and partner preference (Winslow et al. 1993). This was further demonstrated via intracerebral injection in rodents of AVP, which has been shown to cause alterations in social memory formation (Le Moal et al. 1987), social recognition (Everts and Koolhaas, 1997), and anxiety-related behaviour (Landgraf et al. 1995). More recent studies have shown that the effect of increased social anxiety induced by social defeat can be attenuated by administration of an AVP receptor (V1bR) antagonist (Litvin et al. 2011).

In contrast to mammals, fish species produce arginine vasotocin (AVT) and isotocin (IT), which are non-mammalian vertebrate homologues of AVP and OXT respectively. Both of these nonapeptides and their associated receptors do seem to be linked to social behaviour in fish species (Godwin and Thompson, 2012). For example, in the zebrafish *Danio rerio*, administration of both AVT and IT neuropeptides has been previously shown to result in dose-dependent increased social preference and reduced fear response (Braida et al. 2012). AVT has also been associated with aggression and dominant-subordinate relationships in zebrafish (Larson et al. 2006), where differences were found in AVT localization in the preoptic area of the brain between dominant and subordinate individuals. Other studies in zebrafish have found that brain AVT expression levels also seem to be associated with a range of social phenotypes, including winner and loser status from opponent interactions, and aggressive

mirror fighters (Teles et al. 2016). In the African cichlid *Astatotilapia burtoni*, aggressive and territorial males have been shown to express higher preoptic area AVT mRNA levels that their non-territorial counterparts (Greenwood et al. 2008). In *A. burtoni* AVT expression has also been linked to the process of social ascent, where males ascending from subordinate to dominant exhibited higher AVT expression compared to already established dominant/subordinate males (Huffman et al. 2015). Similarly, IT also appears to play a role in the regulation of social behaviours such as aggression and social preference in fish species. For example in the cooperatively breeding cichlid *Neolamprologus pulcher,* individuals injected with IT were found to be more responsive to opponent size in a simulated territorial contest, and produced more submissive displays in response to aggression (Reddon et al. 2012). In zebrafish, a recent study has suggested that IT also plays a role in social preference (Landin et al. 2020a): individuals treated with an IT antagonist appeared to exhibit decreased social preference in both adults and larvae, indicating that increased IT results in higher social preference at both life stages.

6.2.4 Sex-Dependent Patterns of AVT/IT Expression

There is also a mounting body of evidence that AVT and IT may be expressed in a sex-dependent manner. In the cichlid fish *Oreochromis mossambicus*, females generally have a larger number of AVT neurons in the parvocellular and magnocellular preoptic area (Almeida and Oliveira, 2015). Furthermore, in the male of this fish species larger AVT neuron cell body appear to be associated with social subordination. AVT is also linked to aggression and social status in other fish species. For example, in a recent study the abundance of gene transcripts for AVT/IT receptors (AVTr/ITr) and both pro-vasotocin and pro-isotocin were quantified in relation to aggression and social status in the pupfish *Cyprinodon nevadensis amargosae* (Lema et al. 2015). They found that ITr expression in the telencephalon was higher in females relative to males, and dominant males exhibited elevated V1a2 vasotocin receptor transcript abundance relative to females. Both of these increases were associated with individual aggressiveness. These findings seem to link nonapeptide expression to social status and aggressiveness in a sex-dependent manner in the brain. This is not the first study linking sex differences in AVT/IT expression to sociality, another study in three-spined stickleback has shown that stocking density also influences brain AVT/IT expression in a sex specific manner (Kleszczyńska and Kulczykowska, 2013). Interestingly, in this case brain IT concentrations

significantly increased along with stocking density, but only in females, and did not change in males. Taken together it seems that social interactions such as aggression are regulated by AVT/IT in a sex-dependent manner, and that both AVT/IT also seem to be responsive to the social environment in a sex-dependent manner.

6.2.5 Epigenetic Regulation of Social Behaviour

There is evidence in other fish species that changes in social behaviour can result from changes to epigenetic regulation of certain genes. For example, in the cichlid fish *A. burtoni,* DNA methylation has been linked to social dominance (Lenkov et al. 2015). In this case, a chemically induced increase in global DNA methylation resulted in a higher likelihood for the treated individual to ascend social rank and become dominant, conversely, lower DNA methylation resulted in individuals less likely to ascend in social rank. Another example in three-spined sticklebacks also demonstrated that exposing individuals to a brief territorial intrusion was sufficient to induce rapid changes to the gene expression, which in turn appeared to be associated with epigenetic changes in chromatin accessibility (Bukhari et al. 2017). Together, these findings appear to suggest that epigenetic mechanisms of gene regulation may be linked to social dominance, and more generally social behaviour. Both AVP and OXT families of nonapeptides do appear to have the potential for epigenetic regulation (Kumsta et al. 2013, Greenwood et al. 2016), suggesting that changes in social behaviours over short time periods may be due to changes in epigenetic markers.

6.2.6 Aims and Hypothesis

There is some evidence that the association between AVT/IT and social behaviour may be less clear in fish species than in rodents. For instance, a study in zebrafish found that that IT signalling regulates novelty recognition, but not social preference (Ribeiro, Nunes, Gligsberg, et al. 2020). It has also been suggested that it may be the case that IT simply regulates basic perceptual mechanisms of biological motion rather than higher-order social recognition in zebrafish (Nunes et al. 2020). A previous study in *N. pulcher* found that peripheral administration of IT decreased the total time spent associating with conspecifics, suggesting that IT may actually inhibit grouping behaviour in this species (Reddon et al. 2014). Furthermore, there is also evidence in zebrafish that IT has no effect on grouping behaviour, which is instead regulated by AVT (Lindeyer et al. 2015). In this case injection of AVT was

actually found to inhibit interactions with a shoal, suggesting that it inhibits pro-social behaviour. It therefore seems to be the case that the proposed role of both IT and AVT as enhancers of pro-social behaviour is overly simplistic and further investigation is clearly required.

The aim of this study was to investigate potential mechanisms underlying the differences in grouping behaviour observed between groups housed in standard and enriched housing in previous chapters. The current literature in this field seems to indicate that these grouping behaviours are regulated by the expression of AVT and IT expression in the brain. It may therefore be the case that differences in AVT and/or IT underpin these group level behavioural changes. Previous work in fish suggests that increased expression of both of these nonapeptides is associated with increased social preference and both AVT and IT have also been associated with a range of social behaviours in fish. However, there is more recent evidence in zebrafish that AVT actually inhibits grouping behaviour, and that IT may have little or no effect on grouping behaviour (Lindeyer et al. 2015). Based on the current literature I predicted that there would be reduced AVT expression in the more cohesive enriched groups relative to controls, with little or no change in IT expression. Finally, sex differences in environmentally-influenced AVT/IT expression are seen other fish species and therefore this was investigated as well. As there is little evidence that IT or AVT differences between males and females exist in zebrafish however, differences between males and females were not predicted.

6.3 Methodology

6.3.1 Experimental Design

In previous chapters, two distinct experimental groups were identified following exposure of groups of F0 adult zebrafish to varying levels of structural complexity. The resulting experimental groups were classified as either enriched or control depending on their previous experience of housing conditions. Significant differences in shoal cohesion and locomotor activity were identified in adults exposed to different levels of environmental enrichment. In this chapter whole-brain nonapeptide expression was measured and compared between F0 adults from either enriched or control housing, this was done to identify a possible neurological mechanism for the previously identified group behaviours. A total of 16 adult F0

fish (8 from control housing and 8 from enriched) were randomly selected from the larger population and were used to quantify whole brain AVT and IT expression. The expression levels of the two nonapeptides arginine vasotocin (AVT) and isotocin (IT) were investigated as potential mediators of the differences observed in social behaviour.

6.3.2 Animals and Husbandry

All zebrafish used in this study were housed and maintained at the Liverpool John Moores University (LJMU) Life Science Support Unit (LSSU). Adult zebrafish were housed in 5 litre tanks in a custom-built recirculating housing system in accordance with Home Office housing guidelines. Water temperature was maintained at 27.5-28.5°C and lighting conditions were maintained on a constant 14/10 h light/dark cycle. All experimental tanks were maintained on a single self-contained system to avoid between-tank variation in water quality parameters. Water quality was monitored and maintained in accordance with Home Office guidelines. All F0 zebrafish were obtained as embryos from the University of Manchester and then reared until adulthood at the LSSU at LJMU, as described in detail in previous chapters. Groups were maintained at a stocking density of 5 fish per litre prior to this study, then at a density of 10 fish per 5 litre tank for the experimental housing phase prior to the start of this molecular study. Adult fish were maintained on a diet of ground Tetramin flake once per day. Half the fish in this study were housed under standard conditions, consisting of a single 5L housing tank containing a single artificial plant. The other half of the fish used in this study were housed under enriched conditions, with multiple colour of artificial plants, gravel and shelter, the details of which are outlined in previous chapters.

6.3.3 Brain Dissection and Preservation

The adult zebrafish used in this study were euthanized by an overdose of pH buffered tricaine methane sulfonate (MS222) at a concentration of 300mg/L through prolonged immersion. Immediately following euthanasia, each fish was decapitated, and brains were dissected in a petri dish containing a 1X phosphate-buffered-saline (PBS) solution. Whole brains were dissected from the skull of each specimen taking care not to damage the brain itself, thus a dissection microscope was used to perform these dissections. Following successful dissection, each brain was immediately transferred into a clean 2ml lock cap Eppendorf tube containing 1X PBS solution, snap frozen in liquid nitrogen and transferred to -80°C freezer for storage.

6.3.4 RNA Extraction, cDNA Synthesis and RT-PCR

Whole brain RNA was extracted and purified using RNeasy Mini Kit from Qiagen (Manchester, UK). Briefly, snap frozen brains were obtained from frozen storage and the tissue was manually homogenized in lysis buffer using a pipette. RNA was eluted and captured using the spin column provided with the kit. The specific protocol outlined by the kit manufacturer was followed, and total RNA was eluted in 30uL of RNase-free water provided in the manufacturers kit. The amount of RNA extracted was quantified using a Thermo Scientific (Loughborough, UK) NanoDrop™ 2000 UV-Vis spectrophotometer. The RNA content of each sample was quantified this way and then subsequently diluted to a concentration of 100ng/uL for cDNA synthesis.

Forward strand cDNA synthesis was then performed using SuperScript™ III Reverse Transcriptase (Thermo Fisher, Altrincham, UK) and oligo dT primers to synthesize cDNA from mRNA transcripts contained within each sample. As per the manufacturer's instructions, 1uL (100ng) of each total RNA sample was combined with 1uL of oligo dT primers, 1uL of 10mM dNTP mix, and then made up to 13uL reaction volume with RNase-free water. This solution was then combined with 4uL of 5X F-S buffer, 1uL 0.1M DTT, 1uL RNAse out (40U/uL) and 1uL of SuperScript™ III Reverse Transcriptase (200U/uL), to a total reaction volume of 20uL in accordance with the manufacturers protocol. cDNA was then synthesized using a Bio-Rad (Watford, UK) T100 Thermal Cycler.

The expression of three mRNA transcripts were quantified. The housekeeping gene beta-actin (BA) was included as a reference gene with which to normalize the expression of other genes, and the expression of two nonapeptides arginine vasopressin (AVP) and isotocin (IT) were also quantified. Custom DNA oligo primers were obtained from Sigma-Aldrich (Gillingham, UK) in order to amplify these three specific transcripts. BA and AVT were obtained from (Pavlidis et al. 2015), and IT primer sequences were obtained from (Wong et al. 2013). Analysis of each primer obtained indicated that there was weak potential for dimer or secondary structure formation. The sequences for the forward and reverse primers for each were as follows:

Table 1. Primer table. Showing gene ID and accession number for each transcript (BA – Beta Actin; IT – Isotocin; AVT – Arginine Vasotocin). Forward and reverse sequences are shown with annealing temperatures (Tm) for each primer, and predicted product size resulting from PCR amplification.

Gene ID	Accession No.	Sequence	Tm (°C)	Product Size (bp)
BA	AF025305	fwd: TGTCCCTGTATGCCTCTGGT	64.6	120
		rvs: AAGTCCAGACGGAGGATGG	64.2	
IT	NM178291	fwd: ATTCGACAGTGTATGCCGTG	63.6	145
		rvs: TCACACGGAGAAGGGAGAAA	64.8	
AVT	NM178293	fwd: TCGTCTGCCTGCTACATCCA	67.0	55
		rvs: TCCGGCTGGGATCTCTTG	66.5	

Two step RT-PCR using these primers was performed on a Qiagen Rotor-Gene Q using a Rotor-Gene SYBR® Green PCR Kit. Reaction mixtures were formulated in accordance with the manufacturer's instructions included with the kit. Briefly, 12.5uL of 2X Rotor-Gene SYBR Green RT-PCR Master Mix was used in a total reaction volume of 25uL to obtain a 1X final concentration. Prior to use in the RT-PCR reaction forward and reverse primers for each gene were diluted to a concentration 10uM, then, 2.5uL of both the forward and reverse primer were added to the total reaction volume of 25uL to obtain a final concentration of 1uM. Finally, 1uL of synthesized cDNA at a concentration of 100ng/uL was added to the reaction mixture, which was then made up to 25uL with RNase-free water. Technical replicates were performed as triplicate 25uL reactions for each sample in each group.

6.3.5 Gene Expression

Triplicate Ct values were obtained for each sample using the same baseline threshold of 0.05. The raw Ct value data was cleaned by calculating the standard deviation (SD) of technical replicates for each sample. In samples where the SD of triplicates exceeded 1, the closest two values were retained and then used to calculate average Ct value for the sample. The log-fold change in expression levels of the two genes of interest, AVP and IT, were calculated relative to the expression of the housekeeping gene BA. This was done using the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008). Briefly, for each sample the difference between the average Ct

value for the gene of interest and BA was calculated to give ΔCt. ΔΔCt was then calculated by taking the difference between each ΔCt value and the average ΔCt of the combined control group. Log-fold change values were then obtained as the $2^{-\Delta\Delta Ct}$ transformation of the ΔΔCt value of a given sample. Statistical analysis of log-fold change gene expression values was performed using RStudio. Due to the fact that log-fold expression values were inherently non-normal, a non-parametric Wilcoxon Rank Sum test was performed to compare average expression levels between the two experimental groups (control vs enriched) for each gene of interest, AVP and IT. A Wilcoxon Rank Sum test was also used to compare average expression values of both AVT and IT between sexes.

6.4 Results

A total of 7 samples had a triplicate standard deviation > 1 (see Figure 1 A), from these samples the closest two replicates were retained and used to calculate the average Ct value as described previously. Both AVT and IT appeared to be positively correlated with BA expression (see Figure 1 B). Average Ct value for each sample by group appeared to show that raw expression level was generally higher in enriched fish for AVT, IT and BA. Although, there was no difference in the raw expression of any of the genes between sexes. The comparison performed between log-fold expression of AVT and IT relative to BA showed that there was a large significant difference between groups in AVT expression relative to BA expression. Specifically, relative AVT expression was significantly reduced in enriched individuals compared to controls ($W = 64$, $p < 0.001$). However, there was no difference between groups in IT expression relative to BA ($W = 40$, $p = 0.430$). Similarly, there was also no difference in log-fold expression of AVT ($W = 33$, $p = 0.958$), or IT ($W = 28.5$, $p = 0.752$) between male and female brains.

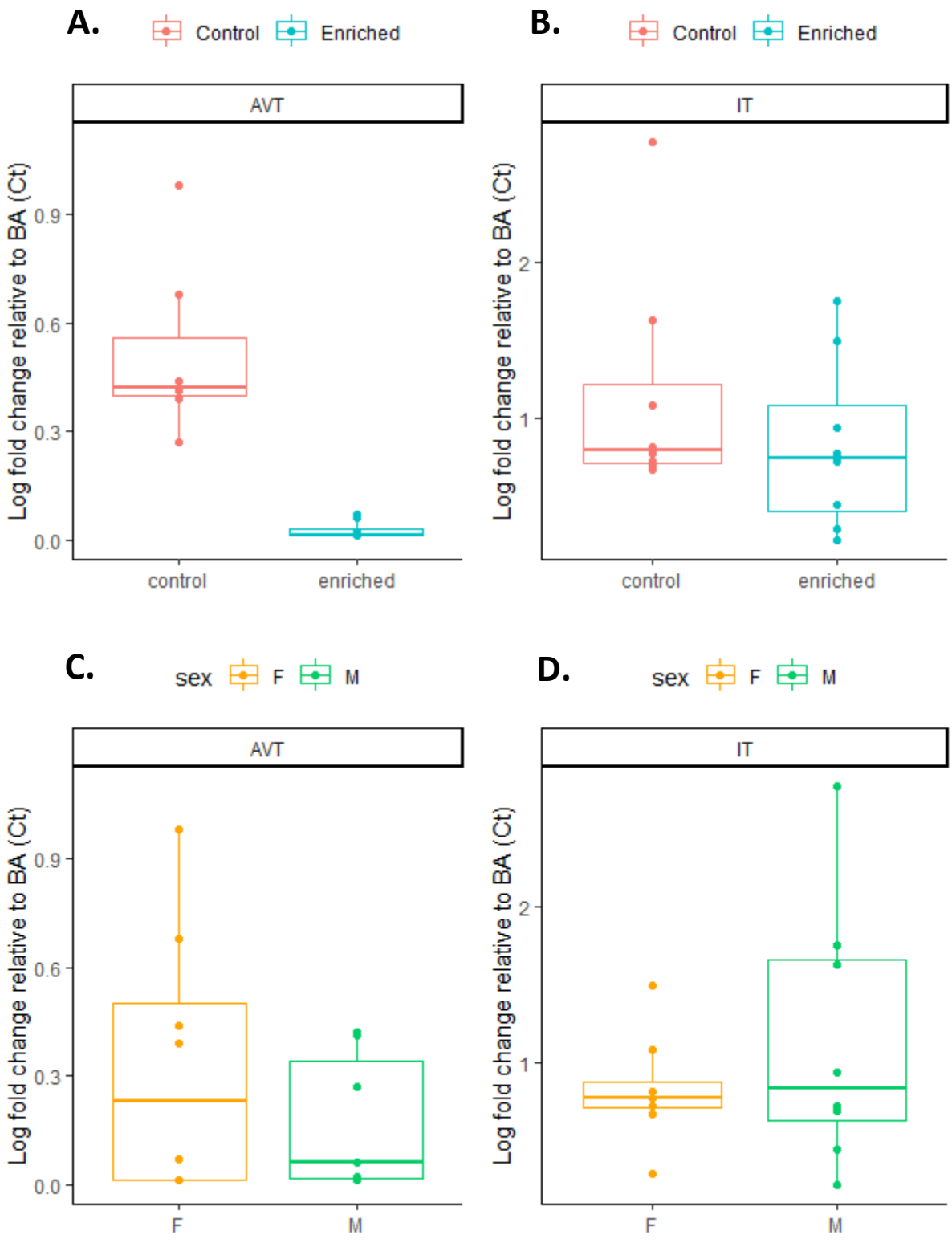

Figure 1. Log-fold change in AVT (A) and IT (B) expression relative to BA. Comparisons are also shown between males (M) and females (F) for both AVT (C) and IT (D).

6.5 Discussion

The results reported here suggest that a short period of enrichment is associated with changes in whole brain nonapeptide levels in adult zebrafish. Interestingly, the findings here also suggest that AVT, but not IT, differs between experimental treatments. More specifically, AVT expression was found to be reduced in individuals exposed to high levels of EE in this study. Interestingly, there are examples of work in zebrafish that support the findings here. For example, administration of AVT has been found to inhibit grouping behaviour, suggesting that AVT inhibits pro-social behaviour in zebrafish (Lindeyer et al. 2015). The findings here seem to support this, as individuals from enriched housing that were more cohesive during the novel tank test displayed lower levels of AVT expression relative those from control housing. This also seems to support the idea that shoaling behaviour and social interactions are discrete components of overall sociality that appear to be differentially regulated by AVT. This contrasts previous work in zebrafish which suggest that both AVT and IT promote social behaviour and reduce fear response (Braida et al. 2012). The results here therefore seem to support the idea that the characterization of AVT and other nonapeptides as enhancers of pro-social behaviour is overly simplistic. It may be the case that these changes in gene expression observed here may be due to epigenetic regulation of AVT, although this was not examined here and further work would be required to test this.

There appeared to be no effect of EE on IT expression, as similar IT expression levels were found between control and enriched groups. Therefore this does not appear to be the driver of group level differences in social behaviour. This was unexpected, as recent studies have associated IT expression levels with social recognition (Landin et al. 2020), and previous studies have shown that IT treated fish are more responsive to social information (Reddon et al. 2012). Given this evidence, it is surprising that there are no differences in IT expression between experimental groups. One possible explanation for this is that IT may actually regulate basic novelty recognition (Ribeiro et al. 2020), or the broad detection of biological motion (Nunes et al. 2020). In which case, IT would be similar between highly cohesive groups and less cohesive groups, if the basic mechanisms of biological motion detection or novelty recognition remain unchanged. This may explain the findings here, and from previous studies which have found no association between IT expression and group cohesion (Lindeyer et al. 2015).

Alternatively, this could be the result of more subtle changes at the neuronal level. In cichlids, differences in social phenotypes among closely related species have been associated with differences in isotocin neuronal phenotypes (Reddon et al. 2017), in this case it was found that highly social species had fewer parvocellular isotocin neurons than less social species. As the authors point out here, whole brain expression capture measures the expression of all isotocin subgroups together, and cannot detect subtle changes in specific types. A recent study performed at Liverpool John Moores University seems to also support this hypothesis (Kelly, 2019). Here the author investigated the number of specific IT neuron types in the pre-optic area of fish exposed to both visual and olfactory stimuli and from different enrichment regimes. In the chapter regarding enrichment, it was shown that fish from enriched housing and significantly more magnocellular IT neurons than control fish. The same increase was not detectable in either gigantocellular or parvocellular IT neuron types. This is particularly interesting as these tests were performed on the same fish used in this study. This is compelling evidence that enrichment exposure in these fish affected expression of IT neuron types differentially, and seems to indicate a subtle shift in neuronal phenotype. It seems likely to be the case that the whole brain expression analysis performed here had insufficient resolution to capture changes in IT neuron types.

In rodent models, a common view is that nonapeptides have a generally positive effect on social behaviour by promoting social recognition and increasing social interactions. For example, OXT has been suggested to facilitate and promote pro-social behaviour, prevent social avoidance and reverse deficits in social behaviour (Lee et al. 2007). Similarly, AVP knockouts in mice have reported resulting impairment in social interaction and social recognition impairment has been associated with blunted AVP release (Lukas et al. 2011). In other taxa however, the role of these peptides is less clear, and appear to contradict these findings in rodent models. For example in zebrafish (*Danio rerio*), injection of vasotocin (AVT) was found to decrease social interactions with a shoal, and isotocin (IT) had no negligible effects on grouping behaviour (Lindeyer et al. 2015). Peripheral administration of IT in the cooperatively breeding fish, *Neolamprologus pulcher,* was also found to decrease the time an individual spent associating with conspecifics (Reddon et al. 2014). This may be due to the fact that *IT* and *AVT* are non-mammalian vertebrate homologues of *OXT* and *AVP* respectively, and so the difference in effects on social behaviour is perhaps unsurprising.

The vasopressin/oxytocin family of neuropeptides are highly homologous but have very different physiological activity and genetic structure, this family arose from multiple genome duplication events and followed by periods of rapid evolution (Hoyle, 1999). They are well characterised as regulators of social behaviour among many species, where variation between species in gene structure can alter peptide receptor distribution, and contribute to differences in social behaviour (Insel and Young, 2000). Due to the high degree of homology, and the range of physiological responses, it appears to be the case that this diversity is due to differences in the signalling systems regulating them (Elphick et al. 2018). Indeed, socially-relevant neuropeptide circuitry is highly plastic, and is an important generator of behavioural diversity (Goodson, 2008). The differences in behaviours observed between species, and across taxa is therefore likely due to changes in this circuitry. The phylogenetic and social diversity of fishes means that major differences in nonapeptide functions between species, particularly when compared to the comparatively tiny rodent taxon, should be expected.

Chapter 7: Discussion

7.1 Overview

The purpose of this research was to examine the effects of environmental conditions across multiple generations, and to identify any potential epigenetically inherited effects and I focussed on the effects of enrichment on behaviour and physiology . The idea here was that these are areas where flexibility in response to experiences as an adult would potentially make these biological systems particularly good candidates for the examination of transgenerational effects. It has been known for some time that behaviour is often the first aspect of an individual's phenotype to respond to external stimuli within a single generation (West-Eberhard, 1989), this short-term behavioural plasticity provides potential for individuals to adapt to changing environmental conditions quickly. More recent work seems to suggest that a host of other traits may also provide adaptive potential in this way. For example, it is now well understood that the induction of morphological defences can also occur over short time periods in both animals and plants (Agrawal et al. 1999). This is a process particularly well studied in *Daphnia* species (Miyakawa et al. 2010; Lüning, 1992). The evidence here seems to indicate that the realms of both behaviour and physiology are responsive to changes in the environment, and over very short time periods.

The inheritance of these short-term effects across generations may provide a mechanism by which longer, medium-term, adaptation can occur. There is substantial evidence for this transmission of parental information in rodent models. For example, one study in mice has shown that the behaviour of female offspring in an open field test was associated with the behaviour of fathers in the same test and before mating, suggesting that offspring open field activity is transgenerationally influenced by paternal activity (Alter et al. 2009). In this case, paternal behaviour also influenced female offspring brain size, and male offspring size at weaning, suggesting that these effects are not limited to simply behaviour. It seems that environmental factors paternal diet can influence offspring metabolism through epigenetic changes in the male germline (Carone et al. 2010). Here, the offspring of male mice fed a low protein diet showed metabolic changes in lipid and cholesterol biosynthesis relative to offspring from fathers fed a standard diet. In another example in mice, parental olfactory experience of a conditioned odor was shown to influence behavioural sensitivity to the same

odor in both F1 and F2 (Dias and Ressler, 2014). Interestingly, a recent study in mice has demonstrated that mothers can modulate paternally inherited effects through factors like male-condition dependent maternal investment (Mashoodh et al. 2018).

In animal species where mothers care for and raise their own offspring, maternal care represents a mechanism by which mothers can influence offspring development non-genetically. Likewise, the provisioning of egg resources or hormones before egg fertilization are also mechanisms by which mothers can influence offspring phenotype. The presence of these kinds of effects makes the examination of germline epigenetic inheritance difficult, as its effects are entangled with other sources of non-genetic inheritance. Animal models which are either externally fertilizing and/or show no maternal care towards offspring are therefore useful in this regard, as mothers in these cases provide little more than the constituents of the egg and genetic material. This is the case for zebrafish, where females show no maternal care towards their offspring. In zebrafish the male is also the carrier of germline epigenetic information, as the female methylome is completely erased in offspring shortly after fertilization (Jiang et al. 2013). To control for maternal inheritance here, experimental manipulations were done with male subjects only, followed by breeding from these male fish in each generation, with a single group of female fish that were used for breeding with the groups of experimental males at each generation. In this way, any observed effects between F1 or F2 groups would be the result of inheritance through the male germline, and likely due to germline epigenetic inheritance.

The design of this study was informed by a series of recent papers examining the effects of paternal experience on offspring phenotype in zebrafish (Zajitschek et al. 2017; 2014). These studies by Zajitschek and colleagues examined the effects of male sperm competition, male personality and social status. In the earlier paper, epigenetically-mediated effects of male reproductive competition were observed on larval offspring performance and survival (Zajitschek et al. 2014): males exposed to high levels of sperm competition produced offspring that hatched over a narrower time frame but exhibited lower survival. In the latter paper, paternal behaviours associated with boldness and social status also appeared to influence offspring activity (Zajitschek et al. 2017). Although, in both cases mechanisms for the transmission of these effects were not investigated directly, and it is still unclear as to how much of the inherited paternal effects are due to paternal genotype. Nevertheless, these

studies seem to suggest that male zebrafish can transmit environmental and ecological information through sperm and epigenetic information, and this can in turn effect offspring phenotypes.

7.2 Evidence for Transgenerational Effects of EE on Adult Locomotor Activity.

I found that varying levels of structural complexity had significant effects on behaviour in fish exposed to a novel tank group shoaling test and that these differences emerged after a relatively short exposure period. Firstly, a high activity phenotype was displayed by enriched F0 groups following experimental manipulation. These effects of enrichment on locomotor activity observed here in zebrafish are interesting, as there is little previous evidence for a similar effect in this species. A similar study in zebrafish has reported that fish from barren housing actually display higher levels of locomotor activity compared to those from enriched housing (von Krogh et al. 2010). However in this case, locomotor activity was defined as the number of turns exceeding 90° per minute, whereas here, activity is measured as the length of the relocations observed between time points. This is important as the former is more indicative of erratic 'zig-zagging' behaviour, and is associated more with anxiety-like behaviour in response to an alarm substances (Speedie and Gerlai, 2008). The reduction of this erratic behaviour in enriched individuals would therefore be expected. Indeed, other studies have shown that zebrafish display elevated levels of locomotor activity and high swimming speeds in behavioural trials, and that this is generally an anxiety-like response upon exposure to a novel environment (Champagne et al. 2010; Wong et al. 2010). However, here EE was shown to produce reductions in freezing behaviour in F0 enriched groups, which is actually suggestive of reduced anxiety. Although there appears to be no recapitulation of this effect here in the F1 generation and there is no evidence for the inheritance of this effect. This is particularly surprising given that decreased freezing behaviour was observed in F0 fathers following enrichment, and there are many examples of environmental factors altering stress related phenotypes in both rodent and fish models (Volkova et al. 2015; Rodgers et al. 2015).

I suggest that this increase in activity observed in enriched groups is therefore likely due to enhanced physical condition, agility and swimming ability. Evidence for this effect of EE has been previously demonstrated in the juvenile rainbow trout, where housing in complex

environments for just one week significantly enhanced an individual's ability to swim whilst avoiding obstructions (Bergendahl et al . 2017). For zebrafish larvae, EE has also been shown to significantly affect survival, growth and behaviour (Lee et al. 2018). Fish from enriched housing conditions displayed higher survival, and a reduction in behaviours associated with anxiety when placed into a new environment. Another study in zebrafish has also demonstrated that exercise-induced contractions in adults promotes increased muscle mass and vascularization (Palstra et al. 2014). It therefore seems plausible that more challenging task of navigating a complex environment here may produce better and more agile swimmers than housing in barren environments.

The results here also show that higher activity induced by EE in the F0 groups was inherited by F1 offspring who also displayed high levels of activity, both as larvae and as adults during baseline testing. The presence of this high activity phenotype in F1 adults, before experimental manipulation, strongly indicates the presence of an inherited effect of paternal condition. One previous study in mice showed that the behaviour of female offspring in an open field test was associated with the behaviour of fathers in the same test and before mating, suggesting that offspring open field activity is transgenerationally influenced by paternal activity (Alter et al. 2009). Although in this case effects were inherited in a sex specific manner, which is likely due to factors such as maternal care. Nevertheless, these results support the findings here that the level of environmental complexity experienced by fathers may also produce effects on offspring phenotype in a similar way. In another example in zebrafish, fathers' social status has been shown to influence offspring activity levels at ten days post-fertilization (Zajitschek et al. 2017). Taken together, these findings seem to suggest that these kind of offspring traits are influenced by paternal experience, with non-genetics effect derived from the social status of the father, or from the paternal physical environment.

7.3 Multigenerational EE Exposure Affects F1/F2 Offspring Morphology and Behaviour.

Multigenerational effects of parental and grandparental enrichment on larval locomotor activity and morphology during the first 5dpf were identified. These effects of parental and grandparental enrichment on offspring appear to provide a potential link between phenotypes observed in both F0 and F1 adult behaviour. Similarly to enriched adult F0 groups,

F1 offspring from enriched parents displayed higher levels of activity through an increased frequency of movement events. There was also a trend for parentally enriched F1 offspring to travel farther than those from control backgrounds. Taken together, these results suggest that parentally enriched F1 offspring display a 'high activity' phenotype similar to their fathers, and during early development. This provides an intriguing link between F0 and F1 adult high activity phenotypes. In F2 offspring, there was again a significant effect of parental condition on the frequency of movement events observed during the spontaneous movement trial. However, in this case there was a weak, non-significant, effect of grandparental condition on movement events. This suggests the effects of EE on this trait is more influenced by the immediate parental experience rather than that of the preceding generation. With regards to total distance travelled there was only a weak effect of parental enrichment, where F1 larvae from enriched fathers travelled slightly farther than their control counterparts. Interestingly, in F2 offspring, there were significant effects of both parental and grandparental experience on total distance travelled. In this case, historic exposure of enrichment, in either generation, significantly increased the total distance travelled in offspring. Exposure in either generation also had an approximately equal effect. This suggests that previous parental or grandparental experience has an effect on offspring swimming behaviour, but only after multiple generations and in a non-cumulative way.

With regards to larval morphology at 72hpf, there appears to be a number of subtle changes in head morphology as a result of parental and grandparental exposure to high level of EE. In F1 offspring from enriched fathers, snout elongation and development of the jaw seems to be enhanced relative to controls, offspring from enriched fathers having a slightly elongated snout and a more defined jaw line. Interestingly, in F2 offspring a highly similar morph was observed to be differentially represented between experimental groups. In this case, offspring whose fathers and grandfathers both experienced enriched conditions were more closely matched to this morph than any other experimental group. This suggests a cumulative effect of EE experience over multiple generations. In both generations enriched individuals displayed elongated snouts compared to controls, suggesting that they may be developing at a slightly different rate. This may also explain differences in locomotor activity observed in F1 and F2 offspring, if parental enrichment produces faster developing offspring, then changes in spontaneous swimming movement during development may be expected. Given that

protrusion of the snout is a key morphological feature at 72 hpf (Kimmel et al. 1995), it seems as though these differences may be due to differences in developmental rate between enriched and non-enriched offspring. It is possible that the inherited effects observed here arise from a shift in developmental rate or timing, which subsequently affected morphology as larvae which then translated into swimming ability as adults.

In previous studies, epigenetic effects of environmental experience in zebrafish have been reported. For example, multigenerational effects of benzo[a]pyrene exposure on zebrafish larvae survival and development have been reported, including developmental deficits and severe deformities (Corrales et al. 2014). However, this study used a highly toxic environmental manipulation, and furthermore, both males and females were exposed and so tracing effects through either lineage is not possible. I used a much more subtle environmental manipulation , and subtler effects are therefore observed. In a more naturalistic example, the differential effects of temperature on thermal plasticity have been observed across three generations in guppies (Le Roy et al., 2017). In this case, transgenerational effects of temperature on swimming performance were large in F1 offspring, which then became more similar over subsequent generations. Here, a similar effect is observed in total distance travelled, where effects of both parental and grandparental condition emerge in F2 offspring, and no effect is observed in F1 offspring. In another example, this time in marine sticklebacks, maternal developmental temperature was found to have cumulative negative effects on hatching success over multiple generations (Shama and Wegner, 2014). I also observed cumulative effects of parental and grandparental enrichment in morphology, defined largely by elongation of the snout and enhanced jaw definition, although a key difference in my findings is that the transmission of these effects was through the paternal lineage.

7.4 Effects of EE on Shoal Cohesion

Another interesting effect of EE on adult behaviour was on sociality and group cohesion. Specifically, a four-week exposure period to high levels of enrichment seemed to result in a significant increase in shoal cohesion. However, the F1 offspring from enriched groups displayed no effect of paternal enrichment during baseline testing, although parental enrichment did effect F1 shoal cohesion in the post-treatment F1 trial. The evidence here

therefore suggests that social traits, unlike locomotor activity, do not appear to be inherited through the male germline in a straightforward manner. Instead, a highly cohesive phenotype is observed in F0 enriched groups following experimental manipulation, and a less cohesive phenotype was observed in F1 paternally enriched groups in the final trial. Furthermore, in baseline testing no effect of paternal enrichment was observed between experimental groups. These effects were observed at adulthood, and were inherited through the male germline.

Previous studies in zebrafish have shown that enrichment can affect social behaviour in a number of ways. It may therefore be the case that these changes in cohesion are a result of changes in sociality. For example enrichment has been shown to affect aggression in zebrafish, where highly enriched individuals displayed a higher frequency of attacks made against their mirror image, and had a tendency to stay close to their reflection rather than avoid it (Woodward et al. 2019). Another recent study in zebrafish has also reported that groups with recent experience of high levels of enrichment also seemed to form tighter shoals than those with experience of basic housing (Sykes et al. 2018). Enriched groups were also found to charge more, suggesting increased aggression, but showed no difference in overall activity. In other fish such as juvenile steelhead trout, it has been demonstrated that enriched housing conditions resulted in juveniles who socially dominated size-matched juveniles from conventional housing (Berejikian et al. 2001; 2000). Studies in other fish species have reported that EE in the early rearing environment generates differences in agonistic behaviour and social interactions (Salvanes and Braithwaite, 2005). Reduced aggressive behaviour in enriched conditions has also been reported in some species of cichlid, for example in both *Amatitlania nigrofasciata* (Barley and Coleman, 2010), and *Tilapia rendalli* (Torrezani et al. 2013).

This is not the only possible explanation for these changes in shoaling cohesion. A previous study in juvenile cod, *Gadus morhua*, has shown that high levels of housing enrichment also increased shoal cohesion in groups exposed to a novel open tank (Salvanes et al. 2007). In this case, it was suggested that enriched groups dynamically adjusted their group cohesion between simple and complex environments, whereas non-enriched groups were less able to coordinate changes in this behaviour between the two environments. This may represent a potentially adaptive effect, as fish gain a significant benefit by forming tighter shoals in open

water (Pitcher, 1986). Experience of environmental complexity might therefore enable fish to be more plastic in their social responses to different environments. While these effects appear similar to those outlined here, there are some key differences. In this example individuals were reared as juveniles in the experimental conditions whereas in my work, fish were moved into experimental housing conditions as adults. However, this does make my results particularly interesting, as they seem to suggest that even relatively brief experience of high levels of enrichment in adult life may produce similar effects on social responses. The idea that enriched groups in this study exhibit higher levels of behavioural flexibility as a result, and that this translates into a more robust group level response to the novel open-water testing arena is an interesting possibility.

This kind of increase in behavioural flexibility may also require a corresponding increase in cognitive functioning. This has been reported in other fish species, in juvenile Atlantic salmon (*Salmo salar*) rearing in enriched housing environments for 8 weeks was shown to promote neural plasticity and cognitive ability (Salvanes et al. 2013). Specifically, individuals reared in enriched conditions showed improved learning ability in a spatial task, and also exhibited upregulated telencephalon expression of NeuroD1, a transcription factor involved in hippocampal neurogenesis. Furthermore, other recent studies have found that some social phenotypes may even rely on neuroplasticity mechanisms that underlie this type of behavioural flexibility (Teles et al. 2016). It may therefore be the case that the changes seen in shoaling behaviour result from differences in cognitive functioning. Although, most models of collective motion show that collective behaviour emerges from a set of simple rules for each individual (Miller and Gerlai, 2012), and may therefore not require that much cognitive ability. Nevertheless, in order to fully test this hypothesis further work would be required, in this case tests of individual cognitive ability (e.g. behavioural flexibility or learning) or brain size measures could be performed to address this question.

7.5 Whole-Brain Isotocin (IT) and Arginine Vasotocin (AVT) Expression

In chapter 3, EE was found to significantly increase shoal cohesion in a novel tank group shoaling trial. In the previous, chapter these highly social F0 adults from enriched conditions

exhibited a significant reduction in AVT expression compared to individuals from standard housing. However there appeared to be no effect of EE on whole brain IT gene expression. Previous work has shown that both AVT, OT and other AVT/OT-like nonapeptides play a role in most aspects of sociality and social behaviours (Goodson, 2013). In the cichlid fish *Astatotilapia burtoni*, AVP/OT signaling pathways have been shown to be important in regulating behavioural interactions in pair-bonding fish (Oldfield and Hofmann, 2011). In this case, treatment with a general AVP/OT receptor antagonist decreased both affiliative behaviour towards mates, and aggressive behaviour to intruders. In goldfish, administration of an AVT antagonist has been shown to inhibit approaches towards visual stimuli of conspecifics (Thompson and Walton, 2004). Suggesting that, at least in this species, AVT inhibits social interaction. Another recent study in zebrafish has also demonstrated that administration of both AVT and an AVT antagonist decreases frequency of social interactions with the shoal. The results here are similar to these studies and appears to support the idea that AVT the role of AVT in regulating social behaviour is more complex than previously thought.

Surprisingly, here IT appeared to not be associated with the highly social phenotype that resulted from enrichment exposure. Given the previously outlines studies it was predicted that there would be a measurable effect of IT between enriched and control groups. In a another recent series of studies in zebrafish, OT receptors were reported to be both regulators of social preference (Landin et al. 2020), and of novelty recognition (Ribeiro et al. 2020).In cichlid fish differences in the sociality of closely related species has been associated with specific IT neuronal phenotypes (Reddon et al. 2017), where highly social species had fewer parvocellular IT neurons compared to less social species. In this case it seems as though IT regulates social behaviour in a subtle way that cannot be detected through whole brain gene expression. This possibility seems to be supported further by findings reported from a Liverpool John Moores University thesis (Kelly 2019). This study investigated the localization and abundance of IT neurons in enriched vs non-enriched adult zebrafish. The results demonstrated that magnocellular IT neurons were significantly more abundant in the brains of enriched individuals, however there was no difference in parvocellular or gigantocellular neurons in the same fish. This suggests that enrichment may effect IT neuron subtypes differently. Given that these were from the same group of fish described in chapters 3 and 6,

this provides convincing evidence that IT is indeed responsive to EE. However, they appear to be expressed in a more subtle way and could simply not be detected using the methods outlined here.

7.6 Conclusions

Considering the studies outlined in this thesis, the findings suggest that housing groups of zebrafish in different levels of enrichment is sufficient to produce effects on behaviour both within and between generations. In general, EE appears to have significant effects on both locomotor activity and social behaviour. However, the inheritance of these two phenotypes appears to be quite different. Within-generations, there are numerous observed effects of EE on adult traits, including locomotor activity, anxiety, exploration and sociality. Between generations EE is found to increase locomotor activity and result in faster swimming offspring, this is predicted to be the result of an enhancement of swimming ability. Social behaviour also seems to be affected by EE exposure, although opposite effects are observed in the two generations studied. In the parental generation EE exposure increased group cohesion, whereas in offspring from enriched fathers were less cohesive as a group. Furthermore, the inheritance of differences in locomotor activity appears to be relatively straight forward: a high activity phenotype is acquired from exposure to high levels of enrichment, which is then also present in F1 adults and larvae, and F2 larvae. This is not a cumulative effect that increases from F1 to F2 larvae however, as upper limits to average speed are presumably reached. Conversely, social traits do not appear to be inherited in the same straight forward manner. Inherited effects on sociality from paternal enrichment in F1 adults are only detectable following repeated exposure to the behavioural trial, and the effects observed in each generation are opposing.

The apparent differences in the heritability of these two aspects of an individual's personality is intriguing. One of the main functions of epigenetic inheritance is to carry adaptive information, where parental experience provides a source of important information regarding the potential conditions an individual will be facing in the future. In this case, it may be that swimming ability has greater utility in a more complex environment than does change in sociality. Additionally, in a complex environment with lots of sources of shelter, behaviour is not only more challenging, but shoaling may also be less useful for the avoidance of stressors.

The results here suggest that swimming ability is established early in life, possibly during the first five days post-fertilisation, and is relatively stable throughout life. If this is the case, it would make sense that this trait would be inherited in a direct manner, especially if it has utility in more complex environments. Conversely, social behaviour appears to be quite plastic throughout the life of the individual and largely depends on the social environment. If an individual must remain socially plastic throughout its life, and the social environment cannot be reliably predicted by the parent in the way that the physical environment can be, it may not be useful to epigenetically program social traits through the germline. This appears to be what I observed here with regards to social behaviours, where generations can vary significantly in their social response to the same factor and there appears to be no inheritance of these traits. Nonetheless, there does appear to be a clear heritable effects of parental enrichment on locomotor activity levels that are transmitted via the male germline.

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