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The Influence of Maternal Care Duration on Offspring Phenotypes in African Cichlids

Tiffany A. Armstrong
B.S. Eastern Washington University, 2014
MRes University of Glasgow, 2015

THIS THESIS IS SUBMITTED IN THE FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Institute of Biodiversity, Animal Health, and Comparative Medicine
College of Medical and Life Sciences
University of Glasgow

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Abstract

The first environment that an individual experiences is created by its parents and, for many species, the female parent has a particularly central role in caring for offspring throughout early development. Through maternal care, mothers can shape how their offspring interact with the environment and increase offspring growth and survival. Maternal care quality, as a measure of number of positive interactions, and duration, as a measure of length of time until individuals are independent, varies among individual females, possibly resulting in variation in offspring phenotypes. Investigating the effects of variation in maternal care quality or duration can provide insights into how and why offspring phenotypic variation develops. Examining maternal care effects can then increase our understanding of phenotypic variation that may affect how individuals interact with and succeed in their environment.

An individual’s morphological development is at least partially influenced by its environment, and morphology can in turn shape the interaction between an individual and its environment. To begin to understand how the maternal environment influences morphological development, I investigated how natural and artificial variation in maternal mouthbrooding duration alters craniofacial shape in four African cichlid species (Chapter Two). Craniofacial shape is an important morphological trait for food acquisition, which can be highly species specific. Using geometric morphometric techniques, I found that craniofacial shape became less convex as maternal care duration decreased, but that this relationship was most pronounced in species with a generalist diet. These findings indicate that duration of maternal care may be related to food acquisition and preferences, which could lead to differential success in unpredictable environments.

Behavioural traits such as boldness and aggression can be important for growth, reproduction and survival. Behavioural development can be influenced by maternal care, which also influences brain development. Brain morphology has been linked to specific behaviours, though it is not understood what role maternal care has in the development of this link. I examined the relationship between reduced maternal mouthbrooding duration and brain anatomy and the relationships among morphological variation in brain volume and behaviours (Chapters Three and Four). I also examined how a reproductively essential trait, aggression between males in a competitive environment, is related to maternal care duration (Chapter Four). Overall, I found that maternal care duration was not directly related to behaviour or brain volume in adult offspring. However, individuals reared under
different maternal care durations exhibited different sets of correlated behaviours. Aggression and inactivity in reduced care individuals was positively associated with the volume of the hypothalamus, while aggression, shyness and lack of exploration in full care individuals was negatively associated with the hypothalamus (Chapter Three). Further investigation of the relationship between aggressive behaviours and the hypothalamus indicated that a greater number of bites per second was negatively associated with the volume of the hypothalamus (Chapter Four). Taken together these results suggest that maternal care duration influences the relationship between the hypothalamus and aggression.

Additionally, I examined the associations among bi-parental presence during early development and aggression in sub-adult offspring (Chapter Five). Parental absence did not have a direct relationship with offspring timidity or fight escalation but did relate to offspring size and offspring size, in turn, was associated with escalation. The results of Chapters Three, Four and Five suggest that maternal care duration may be related to boldness and aggression, though it may be through different mechanisms (i.e. brain anatomy or offspring size).

Furthermore, Chapters Four and Five indicated that individuals with reduced duration of parental care had greater variation in the expression of behaviours than individuals receiving full care. These differences in ranges of phenotypic variation between treatment groups, suggest that increased maternal care duration may restrict phenotypic variation and reduce plasticity within stable environments.

Overall, these results suggest that variation in maternal care is related to variation in offspring development and can alter how individuals interact with their environment. Individual phenotypic variation, in terms of morphology and behaviour, is shaped by some of the earliest experiences individuals have with their environment. The morphological and behavioural variation observed could alter how individuals acquire food, protect resources such as shelter and mates, and could potentially extend to reproductive success. These findings suggest that maternal care, as the first environment offspring experience is exceedingly important to how individuals develop and interact with their environment. Furthermore, variation in maternal care duration among females may serve as pathway through which evolution may occur, due to resulting in phenotypic variation and plasticity in offspring.
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Author’s Declaration

The results presented in this thesis are the culmination of experiments conducted between March of 2016 and May of 2018, supervised by Professor Shaun Killen and Doctor Kevin Parsons. Writing of the thesis took place under guidance from Professor Shaun Killen and Doctor Jan Lindström. This research has not been submitted for any other degree at the University of Glasgow or any other institution. The experiments were conducted by myself, with the exception of video recordings of Chapter Four, which were completed by Iain Hill, and some videos in Chapter Five, which were completed by Mia Lang. Additionally, Iain Hill helped to dissect some of the brains and completed some of the brain imaging. Chapter A-1 was co-written by Alexis Khursigara and Shaun Killen.

Tiffany Armstrong
Chapter One: General Introduction

1.1 PARENTAL CARE BEHAVIOURS AND EFFECTS

Parents influence offspring development and survival in many ways. In fact, parental effects, the influence of a parent’s phenotype on the development of offspring phenotype, play a particularly critical role in the transfer of information about the environment which may increase offspring survival (Badyaev & Uller, 2009; Danchin et al., 2011; Jonsson & Jonsson, 2014). Parental effects often include, but are not limited to, the early transfer of steroids and antioxidants (Badyaev & Uller, 2009), maternal condition and stress (Jonsson & Jonsson, 2014), social influence (van Oers et al., 2005; Danchin et al., 2011), and quality of parental care provided (Royle, Smiseth & Kolliker, 2012). Parental care quality typically refers to the number of positive interactions, which increase the offspring’s survival (Royle et al., 2012). While duration, or the length of time maternal care is provided to offspring before independence, could be considered under quality as longer care durations could result in more positive interactions, it will be referred to as separate throughout this text.

Parental care is widespread in the animal kingdom. Examples of parental care that are perhaps most known include birds and mammals, however, examples can also be found in fish, reptiles, amphibians, insects, arachnids and other invertebrates (Royle et al., 2012). At its broadest definition, parental care can be considered any action that parents take to increase offspring fitness, which would include action such as yolk provisioning or gamete transfers (Clutton-Brock, 1991). However, a narrower definition could be limited to strictly behavioural aspects of parental care (Royle et al., 2012). Types of parental care can vary from simple nest guarding, provision of nutrients, and offspring attendance, to extended care after offspring are independent and assistance with care of the offspring’s offspring (Royle et al., 2012). In this sense, parental care can be defined as behaviour parents perform to increases growth and survival, and thereby fitness, of their offspring (Royle et al., 2012). Parental care, which can be divided into maternal and paternal care, has been documented to impact a variety of traits including: development and survival (Green & McCormick, 2005); offspring size (Lock et al., 2007); sociability (Lévy et al., 2003; van Leeuwen, Mulenga & Chidester, 2014); as well as cognitive function and hippocampal development (Bredy et al., 2003, 2004b).

1.1.1 Parental Care Effects on Offspring Survival and Growth
At its base, parental care serves to increase offspring survival and growth (Gross & Sargent, 1985; Head et al., 2012; Royle et al., 2012). This can be seen both in the short term, with offspring experiencing increased initial survival in the presence of parents, and in the long term, with offspring expressing traits that increase survival later in life (Royle et al., 2012). Initial increases in offspring survival are well studied and result from parents protecting offspring, from predation and infection, and providing nutritional resources (Halffter, Huerta & Lopez-Portillo, 1996; Thiel, 1999; Royle et al., 2012; Klug & Bonsall, 2014). Additionally, adjusting the physical environment in which offspring develop, increasing immune function, reducing desiccation, and increasing oxygenation among other forms of care can all increase offspring survival (Bernardo, 1996). The longer-term impacts on survival include a wider range of parental behaviours, such as those that alter developmental rate to decrease the amount of time offspring spend in life stages with increased predation risk (Klug & Bonsall, 2014), and those that condition social behaviour necessary for survival (Arnold & Taborsky, 2010; van Leeuwen et al., 2014).

Parental presence can have a strong positive effect on the growth of offspring within species. For example in the frog species, *Oophaga pumilio*, tadpoles removed from parents metamorphose earlier but reach smaller mass at adulthood compared to those left with parents (Dugas et al., 2016). This early metamorphosis results in a trade-off of between earlier nutrient independence and maturation but may decrease survival at adulthood (Dugas et al., 2016). In species in which fecundity is strongly related to adult size, such as fish (Duarte & Alcaraz, 1989), decreased size at adulthood could have an add-on effect of reduced reproductive output. In burying beetles *Nicrophorus vespilloides*, larvae in the presence of parents have greater mass regardless of egg size (Monteith, Andrews & Smiseth, 2012). However, in both the frog and burying beetle examples the parents provide food for the offspring, which may increase offspring growth rate. In contrast, male clown fish, *Amphiprion melanopus*, do not provide food to offspring, but spend a considerable amount of time fanning eggs and tending nests, and the time spent doing such has been suggested to have a strong positive impact on the growth of the offspring (Green & McCormick, 2005).

There are relatively few studies of the effects of parental care investment on offspring growth in fish. Despite the fact that recent research has indicated that there are differences in the quality of care of individual male three-spined stickleback, *Gasterosteus aculeatus*, (Stein & Bell, 2012, 2015, 2019), the effects of care quality in this species mostly focuses on offspring survival (McGhee & Bell, 2014; Stein & Bell, 2014) or the costs associate
with paternal care (Deal, Gravolin & Wong, 2016). Similarly, in the paternally caring smallmouth bass, Micropterus dolomieu, studies focus on the effects of paternal care costs on male parents, their energy expenditures (Prystay et al., 2019), and offspring survival (Algera et al., 2017). The lack of available research on the effect of maternal and paternal investment on offspring growth rates, beyond the knowledge that paternal care quality is positively associated with egg size (Sargent, Taylor & Gross, 1987), especially in well studied and recreationally important fish indicates the need for further research.

1.1.2 Parental Care Effects on Offspring Social Interactions

In social animals, the ability of an individual to interact and understand social cues of the group will directly impact the benefits of group living (Chapman, Ward & Krause, 2008; Hesse et al., 2015; Jolles et al., 2015). Individuals in social groups that have a greater desire to remain close to the group may have a greater increase in safety, as well as increased foraging success, over those that stray (Krause & Ruxton, 2002; Brown & Irving, 2014; Ward & Webster, 2016). At the same time, individuals that stray farther from the group have decreased competition for food and resources than those that remain closer (Krause & Ruxton, 2002). Therefore, both social and anti-social behaviours can be beneficial for individual fitness, depending on the social context.

Social behaviours in rodents are shaped by the early social environment, largely from social interactions with their mothers. In rats, Rattus norvegicus, a decrease in maternal licking and grooming can result in a decrease in offspring social (non-aggressive) behaviours (Macrì et al., 2010). Likewise, an increase in maternal licking and grooming (Starr-Phillips & Beery, 2014) and maternal presence (Lévy et al., 2003) in rats can increase offspring social contact (non-aggressive) with sex-matched conspecifics. A similar increase in offspring prosocial behaviours has been observed in prairie voles, Microtus ochrogaster, when maternal contact increases (Perkeybile & Bales, 2015).

Parental care effects on social behaviours are not limited to rodents. Shoaling, for instance, is a complex social behaviour in fishes in which individual shoal members must be aware of the movements of others and make decisions based on those actions (Tien, Levin & Rubenstein, 2004; Chapman et al., 2008). In stickleback, Gasterosteus spp., an increase in the time fathers spend close to fry results in offspring with a stronger shoaling tendency (Kozak & Boughman, 2012). Additionally, when offspring of the African cichlid, Pelvicachromis taeniatus, are raised in isolation, they have reduced shoaling strength (Hesse & Thünken, 2014). However, in the study by Hesse & Thünken (2014) it was
unclear as to whether the reduced shoaling tendency was due to lack of parental care, or lack of social interactions with siblings.

Aggression, or readiness to attack conspecifics (Réale et al., 2007), is ecologically relevant for many species. In this sense, ecologically relevant implies that the behaviours are exhibited in the wild and have implications for an individual’s survival, reproduction, or related to fitness in general. Additionally, while at the individual level aggression is often correlated with boldness resulting it was can be referred to as a behavioural syndrome (Sih, Bell & Johnson, 2004; Réale et al., 2007), throughout the text this term will not be used. The reasoning for this is that the terms relating to animal behaviour, such as behavioural syndromes, personalities, etc. are often described differently by different authors. Thus, to avoid confusion, sets of correlated behaviours across contexts will be referred to simply as correlated behaviours. Further, aggression is not limited to attacks and throughout this thesis will measured through proxies such as displays, chases and bites.

Aggression is often expressed in different contexts, for example, individuals of territorial species that readily attack intruders to defend resources (food, shelter, mates) may also be quicker to attack potential mates (Sih, Bell & Johnson, 2004). An individual that is more aggressive with conspecifics in food competition may also expose itself to injury during disputes (Stamps, 2007). The trade-off of these potential costs of aggression for territorial species is a potential increase in territory size or quality which may result in greater access to mates (Kortet & Hedrick, 2007). However, aggression can be highly plastic and an individual that is highly aggressive in one context may not be in another (Stamps, 2007). Indeed, male African cichlid fish, *Astatotilapia burtoni*, observed by a group of conspecifics alter their aggression, displays and attacks, level depending on the composition (sex, dominance, female reproductive state) of the group of observers (Desjardins, Hofmann & Fernald, 2012). Male *A. burtoni* may be adjusting their aggression levels to reduce future attacks from dominant fish (Desjardins et al., 2012), further highlighting the importance of this type of behaviour on survival and reproduction.

The environment an individual experiences during early development may influence aggression levels later in life. Reduced maternal care quality can result in increased offspring aggression in a range of mammalian species including prairie voles (Perkeybile & Bales, 2015), rats (Menard & Hakvoort, 2007), and Peromyscus mice, *Peromyscus californicus* and *P. leucopus* (Bester-Meredith & Marler, 2003). In addition, house mice, *Mus musculus*, that are weaned early have increased aggression compared to those that receive a natural length of nursing (Kikusui, Takeuchi & Mori, 2004). Similarly, in the
African cichlid, *Neolamprologus pulcher*, removing offspring from their family units at an earlier age results in increased aggression (Fischer et al., 2015). In another African cichlid, *Pelvicachromis taeniatous*, individuals reared in isolation from siblings and parents are more aggressive than those reared with siblings (Hesse & Thünken, 2014). These results demonstrate the importance of the early rearing environment on shaping aggression in individuals which may impact their survival as well as territory and mate acquisition.

### 1.1.3 Parental Care Effects on Offspring Boldness

An individual’s tendency to take risks, described as boldness by Réale et al., (2007), can influence its growth, survival and reproductive success (Biro & Stamps, 2008). Boldness may also be linked to life history events such as survival, as bold individuals may take more risks in the presence of predators and may succumb to early predation (Sih et al., 2004; Wolf et al., 2007; Biro & Stamps, 2008). However, empirical studies have indicated that this may not be a standard rule as bolder big horn rams, *Ovis canadensis*, have a higher survival rate (regardless of mass) than shyer ones (Réale et al., 2009). Meanwhile, captive-reared swift-foxes, *Vulpes velox*, that are bolder have lower survival rates upon wild release (Bremner-Harrison, Proドohl & Elwood, 2004). The differences in findings of boldness effects on survival may be due to which behaviours were used as a proxy for boldness (White et al., 2013), or more directly that boldness effects on survival depend on other environmental factors. Regardless of the directionality of the correlation between boldness and survival, there does seem to be a link between boldness and survival that can vary with context.

Boldness may further influence fitness by affecting an individual’s reproductive success (Biro & Stamps, 2008; Smith & Blumstein, 2010). For example, bold big horn rams, *O. canadensis*, that survive longer have greater reproductive success later in life (Réale et al., 2009). Similarly, bold male largemouth bass, *Micropterus salmoides*, have higher reproductive success than shyer ones (Ballew, Mittelbach & Scribner, 2017). Likewise, positive correlations between boldness and male reproductive success exist in zebrafish, *Danio rerio* (Ariyomo & Watt, 2012), wandering albatross, *Diomedea exulans* (Patrick & Weimerskirch, 2015), and eastern chipmunks, *Tamias striatus* (Patterson & Schulte-Hostedde, 2011). However, in these cases boldness had no effect on reproduction in females (Ariyomo and Watt, 2012; Ballew et al., 2017) or indeed a negative effect (Patrick & Weimerskirch, 2014). In some cases, it is not the absolute level of boldness that may increase reproductive success, as some females may choose males based on their similarity or dissimilarity in boldness to themselves. Female rainbow krios, *Pelvicachromis pulcher,*
are more likely to choose males unlike themselves in terms of boldness, but similar to themselves in consistency of boldness over time (Scherer, Kuhnhardt & Schuett, 2017). In another example, female guppies, *Poecilia reticulata*, prefer to mate with bold males (Godin & Dugatkin, 1996). However, when female and male boldness in guppies are mismatched, there is a decrease in female reproductive success (Ariyomo & Watt, 2013).

Boldness of an individual is determined by multiple factors including genetic inheritance (van Oers *et al*., 2005; Réale *et al*., 2009; Ariyomo & Watt, 2012), maternal inheritance (Wisenden *et al*., 2011), and previous experiences (Bell & Sih, 2007). There is also a role for the early environment in the development of a bold or shy individual. In guppies an unpredictability of food availability during early life leads individuals to be bolder later in life (Chapman, Morrell & Krause, 2010). The complexity of the early environment can also influence boldness, e.g. bull trout, *Salvelinus confluentus*, reared in more complex environments become bolder (Brignon *et al*., 2018). Likewise, recent experience can influence boldness in mosquitofish, *Gambusia affinis*, where individuals that have experienced a predation risk are more likely to leave a shoal to investigate objects (Darby & McGhee, 2019). Recent exposure to an unpredictable food supply also results in bolder individual European seabass, *Dicentrarchus labrax* (Sébastien *et al*., 2016). While maternal care effects on offspring boldness are currently unknown, the effects of the early and recent environment on boldness expression suggest a role for maternal care via the early environment. Given the broad array of life-history traits that interact with boldness, if maternal care influences offspring boldness maternal care quality could have long-lasting effects on offspring fitness.

1.1.4 Parental Care Effects on Exploration

Exploration can be defined as how an individual responds to a novel environment, food, or object (Réale *et al*., 2007) and can be measured in terms of inspection or distances ventured into an unknown region. Exploratory behaviour is at least partially heritable (Winney *et al*., 2018) and can be influenced by the environment (Schuett *et al*., 2013). Exploratory phenotypes may also be likely to emerge when the cost of exploration is low or the benefit is high (Sih *et al*., 2004a; Reader, 2015). The exploratory behaviour of an individual that lives within a group may also be influenced by the behaviour of the group (Reader, 2015). In this case, the group reduces the predation risk, and individuals may benefit from the information of others in the group (Reader, 2015). Exploration may also be related to foraging, as in some fish shoals it appears that hunger plays a role in how exploratory a group will be. For example, shoals of mosquito fish, *Gambusia holbrooki,*
with higher hunger levels will have increased speed when exploring (Hansen, Sch aerf & Ward, 2015). In addition, unpredictability in food availability results in increased exploratory behaviours in zebrafish, *D. rerio* (Holley *et al*., 2014). These relationships among growth, foraging and exploration suggests the importance of exploratory behaviour as an influence on life history traits (Smith & Blumstein, 2010). In rats a lower quality of maternal care results in offspring that are less exploratory (Champagne, 2010). The relationship between the environment and individual exploration suggests that maternal care should influence exploration in offspring which may influence their life-histories and fitness.

1.1.5 Parental Care Effects on Offspring Brain Development

Cognitive abilities of an individual are commonly examined in relation to the size of the whole brain or that of specific brain regions (Bshary, Gingins & Vail, 2014). However, specific regions of the brain are involved with specific aspects of cognition. Here cognition refers to decision making, including social decisions, and foraging among others, as well as memory and spatial recognition (Bshary, Wickler & Fricke, 2002). In mammals for example, spatial cognition and memory are controlled by an interconnected circuit involving the hippocampus, prefrontal cortex and ventral striatum (Floresco, Seamans & Phillips, 1997). Additionally, all vertebrates make social decisions and learn in a similar manner, resulting from a conserved network of nuclei in homologous brain regions (Bshary *et al*., 2014).

Knowing that there are regions of the brain involved in specific functions, research can be tailored to examine the effects of parental care on both behaviours and brain regions. In rats, *R. norvegicus*, maternal care has an impact on the development of the hippocampus and corresponding differences in spatial learning (Bredy *et al*., 2004a). This is a topic that has been covered extensively over the last two decades (examples: Bredy *et al*., 2003, 2004b; Champagne *et al*., 2008; Kwak *et al*., 2008; Mak *et al*., 2013). Rats deprived of maternal care show a decreased complexity in apical dendritic trees of pyramidal cells (the primary cells in the hippocampus and amygdala) within a subregion of the hippocampus and have decreased calretinin, a protein crucial to neuronal survival (Kwak *et al*., 2008). Similarly, rats exposed to increased maternal licking and grooming possessed pyramidal cells that are more complex, which suggests an increase in the number of synapses (Champagne *et al*., 2008; Bagot *et al*., 2009). Additionally, bi-parental care in mice affects offspring in a sexually dimorphic manner. Bi-parentally reared male offspring have increased neurogenesis compared to male offspring reared by a mother only (Mak *et al*.,
While females reared by one or both parents have neurogenesis levels similar to those of males reared by both parents (Mak et al., 2013).

In humans, self-reported higher levels of paternal care results in young-adult offspring with increased grey matter in particular forebrain regions (Narita et al., 2010). Also, in humans, low levels of self-reported parental nurturing of four-year-old children yields hippocampal volumes lower than expected for normal development (Rao et al., 2010). This research indicates that the amount of care allotted to offspring has an effect on brain development and could perhaps influence learning and behaviour. However, it is currently unknown if such trends hold true in other vertebrates beyond mammals.

1.2 BRAIN MORPHOLOGY, FUNCTION, AND PLASTICITY

The localisation theory of brain function posits that major processes are localised to specific brain regions (Tizard, 1959; von der Malsburg, 1994). Evidence for this theory originates from experiments using targeted lesions to the brain, which damage specific regions resulting in altered behaviour or functioning (von der Malsburg, 1994). The preoptic area within the hypothalamus, is one such region thought to be associated with major functions and involved in regulating parental care, aggression and sexual behaviours (O’Connell & Hofmann, 2011). This region has homologs among mammals, birds, reptiles and teleosts, and has been suggested to be part of a conserved social behaviour network in vertebrates (O’Connell & Hoffman, 2011). The existence of such interconnected networks highlights that, while some regions may be primarily involved in specific functions, there are many connections between the regions and functional overlaps.

Within the brain, there is a thought to be a social behaviour neuron network which is involved in regulating parental care and sexual behaviours, mating and reproduction, and communication and aggression (O’Connell & Hofmann, 2011; Fernald & Maruska, 2012). The social behaviour network is also involved with another network known as the ‘mesolimbic reward system’ to create the broader ‘social decision-making network’ (O’Connell & Hoffman, 2011; Fernald & Maruska, 2012). In the African cichlid species, *A. burtoni*, social cues result in rapid changes in gene expression within brain regions associated with the social decision-making network (Fernald & Maruska, 2012). Similar changes in gene expression in relation to social cues have also been seen in zebrafish, *D. rerio* (Teles, Cardoso & Oliveira, 2016). The regions suggested to be involved in the social
decision-making network are those responsible for emotional responses and memory; in mammals this is the hippocampus and amygdala (Bshary et al., 2014). However, in reptiles, birds, and teleosts the homologous regions involved in the social decision-making network are the telencephalon and hypothalamus (O’Connell & Hoffman, 2011; Fernald & Maruska, 2012; Bshary et al., 2014).

Whole-brain volume can also be correlated to specific behaviours. One example comes from a study of 43 African cichlid species from Lake Tanganyika, where females of species which provide female-only parental care have a larger brain volume (Gonzalez-Voyer, Winberg & Kolm, 2009). In addition, larger brains can also be associated with more complex diets (Gonzalez-Voyer et al., 2009), increased problem solving ability (Benson-Amram et al., 2016), increased exploration, boldness (Kotrschal et al., 2014), behavioural flexibility (Kotrschal et al., 2014; van der Bijl et al., 2015), and capacity for spatial navigation (Kotrschal et al., 2015). The ever-expanding research on whole brain volume and behavioural associations, particularly in fish, further indicates the importance of this trait in evolutionary, ecological and behavioural analysis. In addition, these emerging studies indicate that fish are an effective model with which to explore the effects of early life experience on the association between brain development and behavioural characteristics. In this way, investigating the effects of early life experiences, particularly with maternal care, may assist in understanding some of the potential causes for antisocial, or socially deviant behaviours in humans.

The environment experienced by an individual during early development can influence brain morphology. This is seen in rats where quality of maternal care is positively correlated with hippocampal development (see section 1.1.5). In an African cichlid, Neolamprologus pulcher, with bi-parental care, day of isolation from parents and rearing group size both influence the volume of specific brain regions (Fischer et al., 2015). The effect of isolation day in this cichlid is either positive or negative depending on the brain region and rearing group size.

The majority of current understanding on rearing environment effects and brain size come from studies comparing the brains of first-generation captive-bred individuals to their wild counterparts. These comparative studies have indicated that captive individuals have smaller brains than wild individuals (Guay & Iwaniuk, 2008; Burns, Saravanan & Helen Rodd, 2009; Gonda, Hérczeg & Merilä, 2013). In some cases, the reduction in brain volume in captive individuals can be somewhat overcome by altering the captive rearing environment. For example, increasing the structural complexity of the early rearing
environment in rainbow trout, *Oncorhynchus mykiss*, results in an increase in cerebellum volume (Kihslinger & Nevitt, 2006). In Atlantic salmon, *Salmo salar*, increasing environmental complexity in captivity results in an increase in total brain volume (Näslund *et al.*, 2012). In bull trout, *Salvelinus confluentus*, reared in complex captive environments, the total brain volume and cerebellum volume, while larger than in individuals reared in non-complex environments, are smaller than in wild individuals (Brignon *et al.*, 2018). Though captive environmental complexity may be increasing brain size, it may be that brains of individuals reared in captivity may never reach the sizes of the individual’s wild counterparts. However, it is of note that captive rearing results in morphological changes, including variations in the skull (O’Regan & Kitchener, 2005; Hartstone-Rose *et al.*, 2014). These variations suggest that it may not be ideal to compare wild and captive bred specimens, due to differing morphological trajectories. These examples further illustrate the importance of the early environment in shaping the trajectories of individual brain development and growth.

### 1.3 STUDY SPECIES

Fish are an effective system for studying behaviour due to the ease with which their habitat can be manipulated (Kihslinger & Nevitt, 2006). Habitat features such as light, food availability, shelter, temperature, density, and species diversity of tank mates can easily be adjusted in laboratory settings to understand how these features interact with the development of behaviour in fish. Additionally, as fish age their brain continues to grow with no evidence of an age at which brain growth stops (Kihslinger & Nevitt, 2006), and evidence has suggested that brain growth may be impacted by environmental conditions that a fish experiences (see section 1.2). The variables that influence behavioural development show overlap with variables that influence the development of the brain and can be easily manipulated in experiments with fish. Further, fish exhibit great variety in modes of parental care, with 21% of bony fish from 87 families having some form of parental care (Blumer, 1982; Gross & Sargent, 1985). Cichlids are especially notable in this regard, as all cichlid species exhibit some form of parental care (Duponchelle *et al.*, 2008). Care types range from uni-parental care to bi-parental care, bi-parental care with helpers, and male, female or bi-parental mouthbrooding (Barlow, 2000).

To investigate the influences of maternal care on offspring phenotypic development, I examined four species of Lake Malawi cichlids: *Labeotropheus fuelleborni, Tropheops sp.*
“Red Cheek”, *Metriaclima zebra*, and *Dimidiochromis compressiceps* (Fig. 1.1 A-D). To determine if the effects of maternal care extended to a bi-parental care giving species, the Lake Tanganyikan cichlid, *Neolamprologus brevis*, was studied (Fig. 1.1 E). The Lake Malawian species were choosed due to their maternal mouthbrooding behaviour (section 1.3.1), variation of craniofacial shape and associated feeding strategies (section 1.3.2). Hybridisation within African cichlids are fairly common, particularly within the rock-dwelling species of Lake Malawi (Barlow, 2000). In particular, *L. fueleborni*, *T. sp. “Red Cheek” and *M. zebra* are closely related enough to hybridise with each other in the lab (Albertson & Kocher, 2005; Concannon & Albertson, 2015). While *D. compressiceps*, is less closely related as a haplochromine (Liem, 1978), the species size and aggressive tendencies made it well-suited to studies of offspring behavioural responses to maternal care. *N. brevis* was chosen due to the shorter generation time where offspring reach sexual maturity at a smaller size than the mouthbrooding species.

1.3.1 Parental Care Behaviours of Study Species

All cichlids provide parental care and a wide range of care types exist (Barlow, 2000). In Lake Malawi all but one species broods their young in their mouth until they are nutritionally independent, thereby reducing the risks of predation during the early life-stages (Barlow, 2000). In Lake Tanganyika, parental care behaviours include nest guarding, shell spawning, bi-parental mouthbrooding and maternal mouthbrooding (Barlow, 2000). All of these parental care behaviours provide the basis of parental care by increasing the survival of the offspring in the presence of paedophagous adult cichlids (Barlow, 2000).
Figure 1.1. African cichlids used in this thesis. The male *L. fuelleborni* (A) in this image is exhibiting the “orange blotch” colour morph, which is usually common among females. Both male (yellow faces, blue bodies) and females (paler coloured) *Tropheops sp.* “Red Cheek” are presented (B). The male *Metriaclima zebra* (C) represents one of the more muted colour types, as commonly males and females are blue with darker near-black stripes (C). Male *Dimidiochromis compressiceps* are blue as in the image (D), while females are silver. Subadult *Neolamprologus brevis* (E) are much less sexually dimorphic until sexually mature.
1.3.1.1 Mouthbrooding

Maternal mouthbrooding, the most recently evolved form of parental care in fish, has been suggested to be one of the key innovations that allowed for the adaptive radiation in Lake Malawi (Barlow, 2000). This parental care behaviour has independently evolved in several cichlid lineages (Rüber et al., 2004) and in nine other teleost families (Blumer, 1982).

Mouthbrooding involves the incubation of eggs in the buccal cavity, reducing predation on developing young. The length of the incubation period varies among species (Barlow, 2000), and among individuals within species (see Chapter Two). *M. zebra* and *T. sp. “Red Cheek”* may incubate young for up to one month (Martin & Genner, 2009), while *L. fueleborni* has been recorded to incubate for an average of 31 days (Balon, 1985).

Currently, no record of the duration of incubation in *D. compressiceps* has been published. In addition, individual variation in care duration can be influenced by predation, as female *Ctenochromis horei*, in the presence of paedophageous predators have been known to increase the length of incubation periods (Taborsky & Foerster, 2004). Stressed fish may prematurely release embryos, specifically in cases of chasing by other fish or catching with a net (Armstrong, personal observation). This natural variation in incubation periods, and the release of embryos under stress, may result in individual broods with shortened or extended care under natural conditions.

Mouthbrooding is likely to have evolved in response to predation pressure and reduces the time in which vulnerable young are exposed to predators (Barlow, 2000), though additional benefits may result from this form of parental care. One such benefit may stem from the limited space in the buccal cavity resulting in developing eggs and larvae in close proximity to each other during these stages (Fig. 1.2). The physical contact with the walls of the mother’s mouth or with siblings may mimic effects of high levels of maternal licking and grooming in rats. In addition, there is evidence from zebrafish, *D. rerio*, that tactile stimulation reduces cortisol levels (Schirmer, Jesuthasan & Mathuru, 2013). If there is a reduction in cortisol levels due to contact during development, there could be downstream developmental benefits. Though there are a large number of species that exhibit mouthbrooding, very little is known of how this affects the development of young. Currently, the majority of information comes from *Betta spp.*, in which males provide parental care. In *Betta spp.*, females of mouthbrooding species do not produce larger eggs than females of bubble-nest species, nor do respective males spend different amounts of time tending eggs, however, offspring of paternally mouthbrooding *Betta spp.* are larger than bubble-nest species offspring (Rüber et al., 2004). In African cichlids the eggs of mouthbrooding cichlids are often larger, while brood size is smaller than substrate spawning cichlids (Duponchelle et al., 2008). A larger egg often results in a larger offspring, which may leave offspring at an advantage in an environment with gape-limited predators.
1.3.1.2 Bi-Parental Shell-Spawning

Bi-parental care and shell-brooding are common in the Lamprologine cichlids of Lake Tanganyika (Taborsky, 2017). *N. brevis* is one such example: it spawns within shell patches inhabited by multiple females and patrolled by a dominant male (Ota *et al.*, 2012). Females deposit eggs within shells which are then fertilised by either dominant or sneaker males (Ota *et al.*, 2012). The length of time the eggs are incubated in the shell by *N. brevis* has not been documented, but in *L. callipterus* (another Lamprologine obligate shell-spawner) eggs are incubated for 10-17 days before the females abandon the shells until the next spawning event (Sato, 1994; Ota *et al.*, 2012). The behaviour of shell-spawning has the benefit of protecting the vulnerable offspring from the larger predators that are present on the sandy bottoms where the shell patches exist (Brandtmann, Scandura & Trillmich, 1999), in addition to providing refuge for the parents.

While no published observations exist of direct parental behaviours in *N. brevis*, some observations exist for related species. In the Lamprologine shell dweller *L. callipterus*, females remain in the shell with the brood and exhibit tail flicking behaviour that may be to oxygenate the eggs (Sato, 1994). Male *L. callipterus* do not enter the shell after

**Figure 1.2. Tight quarters.** The image shows a partially dissected female cichlid (of unknown species) incubating eggs to illustrate how close together offspring develop inside the mother’s buccal cavity. The buccal cavity, where eggs develop, has been marked with an arrow. Image adapted from Jahani & Chizari (2018).
spawning (Sato, 1994). Similarly, male *N. brevis* are only found above shells, guarding territory from intruding males (Ota et al., 2012). In this sense what is described as biparental care in this species, appears to have a strong divide between the roles of the sexes in providing care to offspring.

1.3.2 Feeding and Craniofacial Shape

African cichlids of the great lakes have evolved to fill virtually every occupiable ecological niche and exploit all available food sources. Differences in food preferences of many species have resulted in craniofacial shapes that exists on a gradient from short jaws and a steep sloped head to long jaws and a gradual-sloped head to an extreme of an upturned lower jaw (Powder & Albertson, 2016; Fig. 1.3). Recent in situ rearing has indicated that the volume and dimensions of the rearing environment can influence the length of the jaw (Hu & Albertson, 2017), which could alter feeding choices. Cichlid jaws have evolved for eating specific diets, for example the long upper jaws and short lower jaws of *L. fuelleborni* allow for a scraping action with the upper jaw to remove algae from rocks as the primary food source (Ribbink, 1990). *Tropheops. sp. “Red Cheek”* has shorter upper jaws than *L. fuelleborni* and short lower jaws for plucking algae from rock surfaces (Martin & Genner, 2009). The wide jaws and bicuspid teeth of *M. zebra* are used for both scraping algae from rock surfaces and suction feeding on plankton from the water column (McKay & Marsh, 1983). As the term rock-dwelling, or *mbuna*, implies all three of these species spend their lives around the rocks of Lake Malawi, often overlapping in territory with other species (Barlow, 2000). The gradual sloping and length of the head of *D. compressiceps* (formerly *Haplochromis compressiceps*), is part of what allows the piscivorous, sand-dwelling cichlid to generate suction during ambush predation events (Liem, 1978). The variation in craniofacial shape amongst these cichlids allow them to exploit different food sources and compete for niches in an environment with the over 700 species present (Barlow, 2000). Extending the results from Hu & Albertson (2017) to the variation that exists in African cichlids both at species and individual levels, there may be a role for maternal mouthbrooding to influence morphological development and therefore offspring resource exploitation.
**Figure 1.3. Gradients of craniofacial shape.** Adaptive craniofacial shapes of a selection of African cichlids from Lake Malawi to represent the gradient of short (left) to long (right) lower jaws, with the most extreme shapes in black boxes. From left to right species are: *Labeotropheus fuelleborni*, *Tropheops* sp. “Taiwan”, *Cynotilapia zebraeides*, *Tyrannochromis nigriventer*, *Aulonocara rostratum*, and *Caprichromis orthognathus*. One of the species, *L. fuelleborni*, was used in the current study as well as two species similar to *Tropheops* sp. “Taiwan” and *Cynotilapia zebra*, respectively. *D. compressiceps* has a longer face than *T. nigriventer* but feeds similarly by suction feeding piscivorous prey. The latter two on the gradient are much more extreme and specialised than *D. compressiceps*. Adapted from Powder & Albertson (2016).

**1.3.3 Cichlid Brains: Regions, Functions and Known Variation**

The main divisions in any vertebrate brain are forebrain, midbrain and hindbrain, but within these further subdivision may take place (Kotrschal, van Staaden & Huber, 1998; Braithwaite, 2005). In cichlid research, it is common to divide the brain into easily identifiable regions: olfactory bulbs and telencephalon (forebrain); optic tectum, cerebellum, hypothalamus, and pituitary (midbrain); and the dorsal medulla (hindbrain) (e.g. Pollen et al., 2007; Sylvester, Pottin & Streelman, 2011; Fischer et al., 2015; Fig. 1.4). These regions are also typical in other vertebrates and share mammalian homologs (Kotrschal et al., 1998; Braithwaite, 2005; O’Connell & Hofmann, 2011; Bshary et al., 2014). While each of these regions has their own primary function (Kotrschal et al., 1998), there are also many connecting pathways (see section 1.2).

In African cichlids, adult brain anatomy is known to vary based on whether fish are *mbuna* (rock-dwelling) or *utaka* (sand-dwelling), which also have different patterns of early development (Sylvester et al., 2011). Additionally, brain morphology differs among the African Great Lakes (Huber et al., 1997), microhabitats (Huber et al., 1997; Pollen et al., 2007; Gonzalez-Voyer & Kolm, 2010), and social organisation (Pollen et al., 2007; Gonzalez-Voyer & Kolm, 2010) as well parental care types (Gonzalez-Voyer & Kolm, 2010). Further, plasticity in the development of cichlid brains has been observed where rearing group size and parental presence result in changes in the relative size of different regions (Fischer et al., 2015). The known variation based on environmental and developmental factors, coupled with the knowledge that fish brains are plastic and continue
to grow throughout their lives (Kihslinger & Nevitt, 2006), suggests that cichlid fish are an excellent system for investigating brain development.

Figure 1.4. Cichlid brain anatomy. Images of the brain of an African cichlid, *Dimidiochromis compressiceps*, from dorsal (A), lateral (B) and ventral (C) views. The dorsal medulla (DM), cerebellum (Cer), optic tectum (OT), telencephalon (Tel), olfactory bulbs (OB), and hypothalamus (Hyp) are indicated with arrows. As the pituitary is difficult to keep intact when removing brains, this is marked with a red dot where it would be located. The regions examined are labelled on each image. The scale bar represents 5 mm.
In African cichlids the forebrain appears to serve a primary spatial memory function, indicated by the relationship between forebrain size and environmental complexity (Kotrschal et al., 1998; Pollen et al., 2007; Gonzalez-Voyer & Kolm, 2010). Additionally, the telencephalon, a major forebrain region, plays a role in social decision-making (Bshary et al., 2014) and monogamous Lake Tanganyikan cichlids have larger telencephala (Shumway, 2010). While it has been difficult to determine a homolog of telencephala in fish with the brain regions of other vertebrates, the ventral pallium of the telencephalon may be homologous with the amygdala in mammals (O’Connell & Hoffman, 2011).

The midbrain of the fish functions for reproduction, social behaviours, and vision. The hypothalamus in fish is also related to the social decision-making network (O’Connell & Hoffman, 2011), houses sex steroid hormone receptors, and when stimulated in midshipman fish, *Porichthys notatus*, results in sound production (O’Connell & Hoffman, 2011). In multispecies studies on Lake Tanganyikan cichlids, the hypothalamus volume was negatively correlated with strength of mating competition (Gonzalez-Voyer & Kolm, 2010) and larger hypothalami were associated with polygynous species (Pollen et al., 2007). In cichlids, the olfactory bulbs are often reduced in a such a way that vision becomes the dominant sense (Kotrschal et al., 1998). Though, within Lake Tanganyikan cichlids the optic tecta volume, the region associated with vision, is negatively correlated with depth at which the fish live (Gonzalez-Voyer & Kolm, 2010).

The hindbrain of fish serves for largely for navigation in complex environments. In a multispecies analysis of Lake Tanganyika cichlids, Pollen et al. (2007) found that there was a positive relationship between cerebellum volume and habitat complexity, possibly arising from the need of increased motor control and sensory integration. Gonzalez-Voyer & Kolm (2010) also found that increased cerebellum volume was associated with depth, sexual selection and habitat type. The association with habitat type is likely due to the relationship between the cerebellum and spatial orientation and motor control (Kotrschal et al., 1998). The dorsal medulla is largely responsible for integrating information from the lateral line (Morita & Finger, 1987). In Lake Tanganyikan cichlids the dorsal medulla has been found to be negatively associated with habitat complexity (Pollen et al., 2007; Gonzalez-Voyer & Kolm, 2010).

### 1.4 GENERAL OUTLINE AND AIMS
The overall goal of this thesis was to investigate the role of maternal care on offspring development, particularly craniofacial morphology, brain anatomy, and adult behaviour. This was primarily done through experimentally manipulating the duration of parental care and rearing the offspring to the required stage to investigate the given trait. The stage required varied, depending on the species and the question, from 16 days to one-year (or more) post fertilisation. Chapter Two focuses on the effects of maternal care, via mouthbrooding, on craniofacial shape in four African cichlid species. Chapters Three and Four focus on only one species of African cichlid and examine how maternal mouthbrooding relates to bold, exploratory, and aggressive behaviours, and brain anatomy in adult offspring (one year or more post fertilisation). Finally, Chapter Five investigates the effects of bi-parental care on bold, exploratory and aggressive behaviours in offspring just reaching sexual maturity (6 months post fertilisation) in an African cichlid.

1.4.1 Chapter Two: Duration of Maternal Care Alters Craniofacial Shape in African Cichlids

Here I examined the effect of reduced maternal care duration on offspring craniofacial shape. Using geometric morphometrics, I examined craniofacial shape variation in four species of African cichlids, *Labeotropheus fuelleborni*, *Tropheops sp.* “red cheek”, *Metriaclima zebra*, and *Dimidiochromis compressiceps*, to investigate three questions: 1) does the duration of maternal care differ between closely related species and individuals?; 2) Does the duration of maternal care influence offspring size?; 3) Does reducing maternal care duration alter craniofacial development and does this vary between species?

1.4.2 Chapter Three: Maternal Care Influences Brain Anatomy and Correlated Behaviours

In this chapter, I investigated how reduced-duration maternal care alters offspring brain anatomy and correlated behaviours into adulthood. I used *D. compressiceps* offspring reared for one year to answer the following questions: 1) how maternal care duration alters brain anatomy, specifically focusing on the dorsal medulla, cerebellum, optic tectum, hypothalamus and telencephalon; 2) how maternal care influences behaviours relating to boldness, exploration and aggression; and 3) if a correlation between behaviour and brain regions results from variation in maternal care duration.

1.4.3 Chapter Four: Maternal Care Duration Influences Brain Shape but not Brain Volume or Behaviour in an African Cichlid

To further analyse the effect of maternal care duration on brain development into adulthood, I examined brain shape variation among adult *D. compressiceps* under different durations of maternal care duration using geometric morphometric techniques. I further analysed aggressive behaviours against both a mirror and a size-matched competitor. I then examined the relationship between brain region volumes and aggressive behaviours.
1.4.4 Chapter Five: The Effect of Bi-Parental Absence on Correlated Behaviours in Offspring

In this chapter I analysed how deprivation of bi-parental care in *Neolamprologus brevis* offspring influences behaviours bold, exploratory and aggressive behaviours. Behavioural assays were completed on fish reared in isolation of parents and on fish reared in the presence of their parents to answer the following questions: 1) is there a relationship between boldness, exploration and aggression in *N. brevis*; 2) are boldness, exploration and aggression influenced by parental deprivation?
Chapter Two: Duration of maternal care alters craniofacial shape in African cichlids

2.1 INTRODUCTION

How an individual interacts with its environment is directly impacted by its morphology (Ricklefs & Miles, 1994) and in turn, and individual’s environment can affect its morphology (Grant & Grant, 1989, 1993; Vanhooydonck et al., 2009; Desrochers, 2010). For many animal species, the first environment that an individual experiences is created or selected by its parents, most often its mother. Parental care serves to increase offspring growth and survival (Clutton-Brock, 1991; Royle, Smiseth & Kolliker, 2012) and maternal care, in particular, can influence other aspects of development including offspring behaviour (Curley, Champagne, Bateson, & Keverne, 2008; Perkeybile & Bales, 2017) and brain development (Curley & Champagne, 2016).

If we accept maternal care as an environmental factor, then there may also be a role for maternal care to adjust how offspring interact with their environment through changes in morphological development. Environmental signals during the early life stages can shape the morphological characteristics of developing individuals. For example, in the water flea Daphnia pulex, predator presence in the maternal environment will induce morphological anti-predator defences in pre-hatched embryos (Boersma, Spaak & De Meester, 1998; Maurone, Suppa & Rossi, 2018). This may be analogous to how the presence of a large predator, Esox lucius, can induce morphological changes in body depth of two prey species of fish, Perca fluviatilis and Rutilus rutilus (Eklöv & Jonsson, 2007). During early development, quality of parental care may positively influence the size of clownfish, Amphiprion melanopus, at metamorphosis from larval stages (Green & McCormick, 2005).

While quality of care, in terms of number and type of interactions, influences offspring growth or survival, there is currently no research indicating how parental care duration, be it maternal or paternal, may influence morphological characteristics of offspring. Though if maternal care duration can alter the development of morphological traits that effect how offspring interact with their environment, this may be a route through which offspring develop to cope with changing or unpredictable environments.

As a means to reduce offspring predation during the most vulnerable life-stages (Barlow, 2000), the parental care behaviour of mouthbrooding has evolved in ten teleost families (Blumer, 1982), including multiple independent occurrences in cichlid lineages (Goodwin,
Balshine-Earn & Reynolds, 1998; Rüber et al., 2004). With many paedophageous cichlids, it has been suggested that mouthbrooding has developed to increase the size at which offspring first encounter predators, so that they may be too large and mobile for easy capture (Barlow, 2000). Indeed, under a risk of predation, female mouthbrooding cichlids will increase the duration of the incubation period, further giving offspring a size advantage against gape-limited predators (Taborsky & Foerster, 2004). Mouthbrooding cichlids typically produce larger eggs and smaller brood sizes than substrate-spawning cichlids (Duponchelle et al., 2008) which may result in larger offspring. However, in the non-cichlid Betta sp., there is no difference in the size of egg between bubble nest building and mouthbrooding species, nor is brood size reduced in mouthbrooders, yet larval offspring from mouthbrooders are still larger (Rüber et al., 2004). In addition to the predation protection that comes from mouthbrooding, these findings from Betta sp. suggest a route for mouthbrooding to increase offspring size or growth rates. It is unknown whether mouthbrooding duration increases offspring growth rates or size, but if such an effect exists it could help to explain why this system of parental care evolved and persists, particularly in the cichlids of the East African Great Lakes. While examples of mouthbrooding can be found in all three major lakes, all of the cichlid fishes of Lake Malawi provide mouthbrooding. If an increase in the duration of maternal care increases offspring growth it also could potentially result in changes in phenotypic trajectories that may lead to phenotypes which could be better suited to variation in environments. As all cichlids provide some form of parental care (Barlow, 2000), it is likely that the differences in care quality or duration among individuals could influence offspring development.

Mouthbrooding cichlids provide a unique system for studying the effects of maternal care because a portion of the brood can be removed before hatching, with a high probability of survival, with mothers continuing to rear the remainder. This facilitates investigation of variation among individual offspring at both natural and reduced maternal care durations. Furthermore, unlike mammals, for which variation in maternal care quality mainly occurs post-partum, mouthbrooding cichlids offer the opportunity to manipulate maternal care duration before offspring are fully developed. The jaws of mouthbrooding cichlid species are adapted to both mouthbrooding (Oppenheimer, 1970) and a variety of feeding behaviours. For example, cichlids from Lake Malawi exhibit a range of craniofacial shapes from short lower jaws and long upper jaws to long lower jaws and short upper jaws, including extremes at both ends of the spectrum (Cooper et al., 2010; Powder & Albertson, 2016). In Lake Tanganyikan cichlids, similar variation in craniofacial shape exists and is expected to have evolved due to constraints on craniofacial shape in food preferences and,
in species which mouthbrood their young, the requirements of holding developing young
(Tsuboi, Gonzalez-Voyer & Kolm, 2015). Due to the importance of craniofacial shape on
feeding and reproductive behaviours, mouthbrooding cichlids can be used to determine the
ability of maternal care duration to alter morphology and thereby effect how offspring
interact with their environment to increase fitness.

Using four species of African cichlids which maternally mouthbrood their young, but feed
in different ways, I examined how maternal care duration influences offspring craniofacial
shape (Fig. 2.1). The first three species are all rock dwelling, with \textit{L. fue}lleborni feeding by
scraping algae (Ribbink, 1990), \textit{Tropheops sp.} “Red Cheek” plucking algae (Reinthal,
1990; Martin & Genner, 2009) and \textit{M. zebra} being generalist plankton and algae feeders
(McKaye & Marsh, 1983). \textit{D. compressiceps} on the other hand, is a sand-dwelling ambush
piscivore (Liem, 1978). Using offspring from these species, I investigated two questions:
1) Are there differences in the natural incubation time among species and females and does
this alter craniofacial shape; and 2) Does artificially reducing maternal care duration alter
craniofacial development, and does this vary among species? I predicted that there would
be variation in the duration of brood care among the species, based on both the size of
females and the size of the brood. I further predicted that extended duration in the smaller
and crowded environment of the mother’s buccal cavity would result in a shorter and more
steeply sloped craniofacial shape in offspring. By artificially reducing maternal care
duration, I aimed to compare how offspring of substrate spawning species, or eggs from
mouthbrooders that may be ejected, to those that receive full duration maternal care. I
expected that offspring which received an extreme reduction in maternal care duration
would have longer and less sloped craniofacial shapes than those that received full
maternal care.

2.2 METHODS

2.2.1 Study Animals and Housing

Male to female ratios and total numbers of animals differed by species within holding
tanks. Two brood stock groups of \textit{L. fue}lleborni were maintained: 1) in a 283 L system
with Fluval Fx4 (Hagen Inc., Quebec, Canada) external canister filters, weekly water
changes to control for nitrate and pH were completed, due to the reduced filtration
capacity, this tank setup housed three females and one male; 2) a 180 L recirculating
system housed a ratio of 1M:3F (total of 12 individuals). \textit{Tropheops sp.} “Red Cheeks”
were kept at 1M:5F with a total of 24 individuals, *M. zebras* were kept at 1M:4F with a total of 15, and both species were maintained in 180 L tanks on a recirculating system. As *D. compressiceps* is larger and more aggressive, thus requiring larger tank dimensions and greater group numbers to suppress aggression, these fish were kept at 2M:6F with a total of 24 individuals and maintained in 283 L tank with Fluval Fx4 (Hagen Inc., Quebec, Canada) external canister filters and weekly water changes to control for nitrate and pH, for the first four broods and for the remaining two broods some of the fish were moved to a 180 L tanks on a recirculating system with a ratio of 1M:7F and a total of 16 fish, with the remaining eight smaller fish placed into a non-brood stock tank. External and internal heating was used to maintain temperatures of 27-29 °C and a 12:12 light/dark cycle was used for all tanks. Fish were fed 3x per day with 1x of each of the following: commercial flake, commercial pellet, or a frozen blended mix of whitefish, prawns, peas, and garlic blended with commercial flake.
2.2.2 Experimental Design

Brood stock tanks were checked daily for evidence of spawning, identifying mouthbrooding females by their swollen buccal cavity. When a female was observed to be holding eggs, she was caught and moved to an empty 180 L tank on a recirculating system, to reduce stress and allow for observation and collection of released offspring. Three days following the spawning event, roughly one half of the brood was removed, by gently
pushing on the lower jaw with a plastic pipette while the female was under water. Removed eggs were placed in a commercially-produced egg tumbler, designed to use compressed air to “bounce” the eggs in a manner similar to what they would experience inside the mother’s buccal cavity. Though removed eggs experienced a potentially more stressful environment due to increased handling and an artificial environment, the isolation from the mother, and reduced proximity to siblings, was the target effect. Egg tumblers allowed handling to be kept to a minimum, to reduce potential stress, though not all stress could be eliminated. However, maternal care ideally reduces stress, so some levels of increased stress are expected in order to allow for comparisons. Females were then observed daily until the first day the brood was released, at which point all juveniles that remained with the mother for the full duration of natural maternal care were collected, euthanised in an overdose of anaesthetic, and measured for standard length (SL; snout to caudal peduncle) using digital callipers.

As morphology may change with individual size, individuals receiving reduced maternal care were collected from the tumbler on the same day as full maternal care siblings, measured for SL and, if the mean was ±0.5 mm of the full care siblings, euthanized in an overdose of anaesthetic. SL was used, rather than age, due to potential differences in rates of development which would in itself effect morphology. I chose to use SL in an effort to minimise differences in morphology that were simply due to different stages in development, rather than changes in the trajectory of development itself. If the mean SL of reduced care individuals was not within ±0.5 mm of the full care siblings, individuals were placed back into the tumbler and measured daily until such a SL was reached. The day of spawning and the day of release of all broods from each female was used to determine the total number of days each female incubated offspring within her buccal cavity.

2.2.3 Sample Preparation and Imaging

Following euthanasia, samples were fixed in 4.0% paraformaldehyde (pH 6.9) for four hours and then transferred to 70% ethanol overnight. Individuals were then stained following the protocol outlined in Walker and Kimmel (Walker & Kimmel, 2007), with the following alterations: pigmentation was bleached using equal parts 6% H₂O₂ and 2% KOH for 15 min over a light source, and excess stain was cleared by successive changes of glycerol from 25%, to 50% to 75% for a period of three-seven days each. Following clearing, individuals were submerged in 100% glycerol and imaged with a Leica M165FC dissecting scope using the attached Leica DFC450C camera (Leica Microsystems, Wetzlar, Germany). Left side lateral images were collected of the head of 25 individuals from each treatment group within each of the four species, for a total of 200 samples.
Figure 2.2. Landmarks on an example *Tropheops sp.* “Red Cheek”. Locations of homologous landmarks, with curves of 6 (A) and 4 (B) sliding landmarks indicated. Landmarks and their associated anatomical location (based on Parsons *et al.* 2011; 2014): 1 – tip of most anterior tooth on the premaxilla; 2 – joint between the nasal bone and the neurocranium; 3 – end of the parietal; 4 – tip of the supraoccipital crest; 5 – ventral edge of the pterotic; 6 – point on eye socket that is most posterioventral; 7 – point on eye socket that is most anterioventral; 8 – posterior end of the ethmoid plate; 9 – where the palatoquadrate meets the retroarticular; 10 – end of the ceratohyal complex.

2.2.4 Data and Statistical Analysis

Differences in natural maternal incubation time were recorded from the full care females to determine if there was an effect of variation of natural incubation time that may occur in the wild on craniofacial shape. This was recorded from a total of 19 broods from the four species: *N*<sub>Tropheops</sub> *sp.* “Red Cheek” = 5; *N*<sub>L. fuelleborni</sub> = 5; *N*<sub>M. zebra</sub> = 5; *N*<sub>D. compressiceps</sub> = 4.

Craniofacial shape differences were examined within and among species by first defining the shape with landmarks. Using the software tpsDig2 2.26 (http://life.bio.sunysb.edu/morph/index.html), 10 static landmarks were placed at homologous locations on all samples and 2 curves consisting of 6 and 4 sliding landmarks
Landmarks were chosen based on previous cichlid craniofacial work by Parsons et al. (2011; 2014).

Preliminary analysis of craniofacial shape changes due to reduced maternal care duration within species was determined by using a discriminate function analysis, however data first had to be transformed through a Procrustes superimposition. A general Procrustes analysis was conducted in CoordGen8 (Sheets, 2014); http://www.philadb.com/an-behav/imp/), which positions each individual onto a common centroid, scales to a shared unit size and rotates to a shared orientation, resulting in the minimisation of any squared differences between homologous landmarks (Rohlf & Slice, 1990). Within each species, the number of broods with reduced maternal care for craniofacial examination were as follows: \(N_{Tropheops. \text{sp. “Red Cheek”}} = 4\); \(N_{L. fuelleborni} = 5\); \(N_{M. zebra} = 5\); \(N_{D. compressiceps} = 4\). For full maternal care the number broods included were as follows: \(N_{Tropheops. \text{sp. “Red Cheek”}} = 4\); \(N_{L. fuelleborni} = 5\); \(N_{M. zebra} = 5\); \(N_{D. compressiceps} = 4\). The total number of individuals examined was 200, with 25 from each species treatment group. All subsequent analysis was conducted using R 3.6.1 (R Core Team, 2019). For each species, the pairings of Procrustes superimpositions of craniofacial shape for full and reduced care individuals were analysed using a discriminate function analysis (DFA) with jack-knifing to estimate correct group classifications using MASS package (Venables & Ripley, 2002). Additional DFAs were conducted to estimate group classifications of craniofacial shape for species (regardless of care duration) and care duration for all samples (regardless of species).

As the DFAs do not allow for the investigation of more than one grouping variable, nor indicate the direction nor magnitude of shape changes in multivariate space, subsequent analysis to determine statistical differences in craniofacial shape, as well as trajectories and magnitudes of shape changes, among and within all species, were conducted in the R package geomorph 3.0.6 (Adams, Collyer & Kaliontzopoulou, 2018). Before any statistical analysis was conducted the data were transformed with a Procrustes superimposition using the function gpagen. From this point the Procrustes coordinates will be referred to as “Shape” and centroid size (a proxy for individual size) will be referred to as “Size” for all subsequent analysis.

Craniofacial shape changes due to incubation time within the natural range of each species were quantified using the \(\text{procD.lm}\) function with 1000 iterations. For this test, Shape of the offspring was the response variable, with Species and Incubation Time (days) as explanatory variables. To visualise the effect of species and incubation time on craniofacial shape, a PCA was conducted using the fitted values (an average shape for each brood)
from the model. The first principle component (PC1) was then used as the y-axis with duration of incubation in days as the x-axis as a plot in ggplot2 (Wickham, 2016). To visualise how shape changes manifested themselves in offspring, the PC1 values for each individual were used as the independent variable in tpsRegr 1.44 to generate thin plate spline deformation grids for each species. For ease of understanding the visualisation of shape changes, a grey shadow of the expected craniofacial shape of the fish was added using the open source program Inkscape 0.92.3 (inkscape.org).

Differences between allometric effects of full and artificially reduced care on craniofacial shape within and among species were examined to determine if size effects on craniofacial shape were different between the two treatment groups. This was investigated due to the differences in incubation times among females and broods, which resulted in offspring sampled at different ages and thus different sizes. Allometry was analysed using the procD.lm function with 1000 iterations, Shape was fit as the response variable with explanatory variables of: interaction of Species and Care Duration, interaction of Species and Size and interaction of Care Duration and Size. Non-significant effects were dropped, and models were compared using the anova function to determine which model fit the data better. To visualise shape effects of care duration, species and size, PC1 scores were extracted and visualised as previously described for the procD.lm function.

Effects of artificially reduced maternal care duration on craniofacial shape was analysed by comparing the direction and magnitude of trajectories of shape changes within species by conducting a pairwise analysis on the interaction of care duration and species. To visualise the changes within each species along PC1 and PC2 based on reduced or full care duration and species, each of these PCs from the allometric model was used as the independent variable in tpsRegr 1.44 to generate thin plate spline deformation grids for each species for the most extreme shapes on either end of the axis (for both PCs), grey shadows were added as previously described.

Plots were generated using gridExtra (Auguie, 2017), Rmisc (Hope, 2013) and ggplot2 3.0.0 (Wickham, 2016).
2.3 RESULTS

2.3.1 Relationship between Incubation Time and Offspring Craniofacial Shape

The duration of natural incubation time varied among species, with *L. fuelleborni* having the longest care duration (21 ± 1.05 SEM days) and *D. compressiceps* the shortest (15.25 ± 0.75 SEM days). *Tropheops sp. “Red Cheek”* and *M. zebra* remained with an intermediate duration (18.4 ± 1.33 SEM & 16.2 ± 1.39 SEM days, respectively).

Brood sizes varied in addition, with *D. compressiceps* having the largest brood (47 ± 7.78 SEM), and *L. fuelleborni* the smallest (18.8 ± 3.08 SEM). *M. zebra* and *T. sp “Red Cheek”* were in the middle with 20.6 ± 2.96 SEM and 20.4 ± 4.09 SEM (respectively).

Variation in incubation time among broods altered craniofacial shape differently within and among species (Table 2.1; Fig. 2.3). Along PC1 fitted values, a higher score corresponded with a more convex craniofacial shape from the top of the head to the end of the upper jaw, while lower scores indicated a more concave shape. Examining general trends within all species, as incubation time increased the shape of the offspring head became more convex, with the exception of *L. fuelleborni* which exhibited a neutral trend.
Figure 2.3. **Relationship between incubation time and craniofacial shape.** Average curvature of the craniofacial shape of offspring incubated for different durations. The line indicates the best fit linear model for each of the sets of points. Three days incubation (black box) was the duration of artificially reduced maternal care duration. Purple points indicate *Labeotropheus fuelleborni*, yellow points indicate *Tropheops sp.* “Red Cheek”, dark blue indicate *Metriaclima zebra*, and light blue indicates *Dimidiochromis compressiceps*.
Table 2.1. Results of multivariate linear regression of craniofacial shape. The relationship between maternal incubation within the natural range for each of the four species as well as effects of artificially reduced duration of maternal care and craniofacial shape. Significant effects (p<0.05) are indicated in bold.

<table>
<thead>
<tr>
<th></th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
<th>$R^2$</th>
<th>F</th>
<th>z</th>
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<td>0.127</td>
<td>0.308</td>
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<td>0.022</td>
<td>0.017</td>
<td>5.185</td>
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<tr>
<td>Species*Incubation Time</td>
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<td>Total</td>
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<tr>
<td><strong>Artificially Reduced Care</strong></td>
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<tr>
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<td>0.308</td>
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<tr>
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</table>
2.3.2 Effects of Artificially Reduced Maternal Care Duration on Offspring Craniofacial Shape

DFA predictions of individuals craniofacial shape within *L. fuelleborni*, *Tropheops* sp. “Red Cheek”, *M. zebra* and *D. compressiceps* species groups were 100% correctly classified according to care type (full vs artificially reduced; Fig. 2.4). When species was ignored, DFA predictions of care duration were 88% (full care) and 89% (reduced care) correctly classified.

The multivariate model results indicated that offspring craniofacial shape was influenced by an interaction between species and offspring size, as well as species and care duration (Table 2.1). The change along PC1 indicates that larger offspring of *Tropheops* sp. “Red Cheek” and *M. zebra* had more convex craniofacial shapes, while *L. fuelleborni* exhibited the opposite trend and *D. compressiceps* exhibited very little change (Fig. 2.5). Allometric changes in shape were not affected by care duration in any species.

Care duration affected craniofacial shape in *M. zebra* ($Z_1=1.77$, $p=0.047$) and *Tropheops* sp. “Red Cheek” ($Z_1=2.80$, $p=0.008$) but not in *D. compressiceps* ($Z_1=-0.98$, $p=0.830$) and *L. fuelleborni* ($Z_1=-1.64$, $p=0.954$). *M. zebra* that received shorter care durations had minimal differences in head curvature but had smaller eyes and shallower lower jaws than full care offspring (Fig. 2.6). *Tropheops* sp. “Red Cheek” offspring with reduced care had less convex head shapes with larger eyes and deeper lower jaws than full care offspring (Fig. 2.6).
A) 

B)
Figure 2.4. Craniofacial shape changes with reduced maternal care as predicted by DFA. Shape changes visualised as the grouping LD1 scores generated by the discriminate function analysis during the grouping part of the analysis, for reduced (blue) and full care (orange) individuals for *Labeotropheus fuelleborni* (A), *Tropheops sp. “Red Cheek”* (B), *Metriaclima zebra* (C), and *Dimidiochromis compressiceps* (D). Grid representations of craniofacial shape are magnified 3x using the most extreme LD1 scores (positive and negative) from each species.
Figure 2.5. Relationship between offspring size and craniofacial shape within treatments and species. The changes along PC1 of craniofacial shape represented as circles for reduced care and squares for full care offspring of *L. fuelleborni* (L.F.), *Tropheops sp.* “Red Cheek” (T.R.C.), *M. zebra* (M.Z.), and *D. compressiceps* (D.C.). Best fit linear relationship among the points is represented by the lines. The most extreme (high and low) craniofacial shapes for PC1 are represented by 3x grid deformations along the y-axis. Purple points indicate *Labeotropheus fuelleborni*, yellow points indicate *Tropheops sp.* “Red Cheek”, dark blue indicate *Metriaclima zebra*, and light blue indicates *Domidiochromis compressiceps*.
Figure 2.6. Changes in craniofacial shape along the first two principal components. Craniofacial shapes represented as circles for reduced care and squares for full care offspring of *Labeotropheus fuelleborni* (purple), *Tropheops* sp. “Red Cheek” (yellow), *Metriaclima zebra* (dark blue), and *Dimidiochromis compressiceps* (light blue). The most extreme (high and low) craniofacial shapes for PC1 and PC2 are represented by 3x grid deformations along the y-axis.
2.4 DISCUSSION

These results show that in species with naturally shorter periods of incubation craniofacial shape is less convex, and that artificially reducing maternal care in species with a generalist diet results in decreasing convexity of craniofacial shape. Due to the strong relationship between craniofacial shape and mode of feeding in African cichlids, this effect of maternal care duration on this aspect of morphology could result in alterations of how offspring feed as they grow into the juvenile and possibly adults life-stages, demonstrating a route by which maternal care may fundamentally affect offspring morphology and the way in which offspring interact with their environment.

Within species, the observed changes in craniofacial shape due to differences in maternal incubation duration, could affect food acquisition and diet later in life. There is a relationship between morphology of the face and jaw and preferred food sources in many species including mammals (Zelditch et al., 2017; Law et al., 2018) and birds (Olsen, 2017), and cichlids are no different in this aspect (Barlow, 2000). However, the morphology of craniofacial shape in cichlids allows some species to be more generalist in their food preferences than other more specialist species (McKay & Marsh, 1983). In the present study, Tropheops sp. “Red Cheek” and M. zebra are both generalist feeders, able to shift diets with seasonal changes in food availability (Martin & Genner, 2009). Perhaps relating to their flexibility in diet, these two species exhibited the largest plasticity in response to both variation in natural incubation duration as well as artificially reducing the duration of maternal care. Under artificially reduced care durations, the curvature of craniofacial shape for Tropheops sp. “Red Cheek” was closer to that of L. fuelleborni, which is a specialist feeder adapted to scraping algae from rocks (Ribbink, 1990). It is possible that such a change in the craniofacial shape may result in Tropheops sp. “Red Cheek” offspring that are better suited to scraping algae than another mode of feeding.

Even small changes in morphology could make one species more suited to particular food sources. For example, L. trewevasae, while being similar enough in morphology to cause common misidentification, differs from L. fuelleborni in feeding mechanics that result in preferences of upper (L. fuelleborni) or under (L. trewevasae) sides of rocks (Ribbink et al., 1983). Thus, the observed changes in craniofacial shape in the current study could plausibly result in shifts in diet and food preferences among offspring.

If offspring from shortened care durations are more suited to feed on a specific food source, the mechanical pressures of feeding in specific manner may continue to drive the
changes in craniofacial shape over the course of development to a more extreme shape found in a species which has adapted to that food source. In cichlids, experimentally altering feeding mechanisms by offering food either from the water column or on a flat surface results in a plastic alteration of craniofacial shape, presumably to better exploit these resources as a result of mechanical strain shaping morphology (Bouton, Witte & J. M. Van Alphen, 2002; van Snick Gray & Stauffer, 2004; Gunter et al., 2013; Parsons et al., 2014). Conversely, longer care durations could allow for individuals of two species to successfully co-exist by niche partitioning of preferred food sources. If lengthened maternal care results in a divergence of offspring craniofacial shape that was shared by two species, which happen to share a niche, this could result in less competition for food and may have encouraged longer care durations to ensure offspring success.

The differences in degree of plasticity in response to differing durations of maternal care duration among the four species in this study may be indicative of their specialist versus generalist feeding tendencies. Both *L. fuelleborni* and *D. compressiceps* have more extreme craniofacial shapes than which are suited to specific modes of feeding. *L. fuelleborni* has been found previously to be less plastic in terms of craniofacial shape than *Tropheops sp. “Red Cheek”* (Parsons et al., 2014), which is in line with the results in the present study. The plasticity of *D. compressiceps* has not previously been quantified, though the results here suggest that they are less plastic than *Tropheops sp. “Red Cheek”* and *M. zebra* in terms of craniofacial shape. It is possible that the reduced plasticity here is the result of being more specialised for specific food types or modes of food acquisition. The strong selective pressures that result in specialised morphology often comes with a loss of genetic diversity that would allow for plasticity in response to environmental changes (van Tienderen, 1997). Just as the plasticity of craniofacial shape in *Tropheops sp. “Red Cheek”* and *M. zebra* may allow for offspring from different durations of maternal care to exploit different aspects of the environment, the reduced plasticity of *D. compressiceps* and *L. fuelleborni* may result in offspring that are more robust to effects of maternal stressors that alter care durations.

Maternal care quality and duration varies among individuals and populations, where females can alter investment into offspring in response to various environmental factors. In the mouthbrooding African cichlid, *Ctenochromis horei*, the duration of maternal incubation can be increased by several days in the presence of paedophageous species (Taborsky & Foerster, 2004). Food availability also has an impact on care duration in the mouthbrooding cichlid, *Simochromis pleurospilus*, where females fed lower quantities of
food before spawning will release their offspring an average of two days earlier (Segers, Gerber & Taborsky, 2011). The incubation times within Tropheops sp. “Red Cheek”, L. fuelleborni, and M. zebra in the present study had a range in which the shortest incubation time was at least five days less than the longest incubation time, which resulted in differences in craniofacial curvature. In terms of increased incubation periods, offspring will often be larger simply due to being older than conspecifics. However, results from the current study suggest that there may also be a morphological effect of increased incubation periods. The effects of different durations of maternal care seen here, may extend to other species as individual variation in care duration between females could be expected. Indeed, variation in the duration of maternal care is also present in other species such as Steller sea lions, Eumetopias jubatus, where the western population has shorter periods of lactation than the eastern population (Maniscalco, 2014). In beluga whales, Delphinapterus leucas, there is also a wide range of times at which weaning occurs in wild populations, on the scope of years, which varies depending on offspring size, female age and environmental factors (Matthews & Ferguson, 2015). Variation in age at weaning is fairly common in mammals, and extends to non-human primates (Harvey & Clutton-Brock, 1985) and humans as well (Blyth et al., 2002). However, whether morphological changes that could alter how offspring acquire food or other resources may result from changes in the duration of maternal care in other species remain unknown.

2.4.1 Conclusion

The plasticity of offspring craniofacial shape in response to differences in maternal care duration resulted in variation of phenotypes in offspring which selection may act on that may result in evolutionary changes in niche partitioning. Curvature of craniofacial shape may allow for offspring to exploit different food sources in an environment that may be harsh or resource poor, allowing for the occupation of different niches. The interaction between morphology and the environment, particularly in terms of diet is well known with examples from many species. Further, changes in craniofacial shape resulting from environmental alteration during development are associated with underlying differences in gene expression (Hu & Albertson, 2017). If differences in maternal care duration, via stress or predator presence, alters craniofacial shape of offspring, this may be a way in which mothers affect how their offspring react to and interact with their environment. In this manner, changes in maternal care duration could buffer for the effects of a resource poor environment through changes in craniofacial shape that allow for the exploitation of new food sources.
Chapter Three: Maternal care influences the relationship between brain anatomy and correlated behaviours

A version of this chapter is in review with Journal of Zoology

3.1 INTRODUCTION

In many animal species, the first environment offspring encounter is created or selected by their mother. Indeed, maternal effects are widely recognised for the non-genetic manner in which they alter the phenotype of offspring (Royle et al., 2012). Maternal effects may occur before or during offspring development (Badyaev & Uller, 2009), or after birth or hatching (Royle et al., 2012). As a subset of maternal effects, maternal care in the most broad interpretation includes behaviours exhibited by mothers that increase offspring growth and survival (Royle et al., 2012).

The environment experienced during early development is known to influence brain size and cognitive capacity later in life (Wallace, 1974; Kihslinger & Nevitt, 2006; Burns, Saravananan & Helen Rodd, 2009; Näslund et al., 2012; Wiper, Britton & Higgs, 2014). In rodents, early environmental enrichment increases neural plasticity and enhances hippocampal development, spatial learning, and memory (Wallace, 1974; Ickes et al., 2000; Simpson & Kelly, 2011; Bardi et al., 2016). Similarly, in salmonids early environmental complexity is positively associated with relative brain size (Kihslinger & Nevitt, 2006; Näslund et al., 2012) and cognitive function (Salvanes et al., 2013). Three-spined stickleback, *Gasterosteus aculeatus*, exposed to complex environments early in life develop larger optic lobes than those reared in an empty tank (Herczeg et al., 2015). Due to these long-lasting environmental effects on brain development, it stands to reason that maternal care, as an early environment, should have a key role in the development of the brain of offspring. Indeed, maternal care quality and duration in female rats, *Rattus norvegicus*, has a strong positive relationship with the development of the specific brain regions of her offspring (Champagne et al., 2008). Somewhat similarly, in a cooperatively breeding cichlid, *Neolamprologus pulcher*, isolation from parents and siblings after the free-swimming stage results in decreased volume of some of brain regions and an increase in others, as well as altering social interactions (Fischer et al., 2015). Despite these findings, previous studies have not examined links between induced variation in brain regions with behaviours on an individual level.

Early experiences can also shape behavioural development in many species, and recently behavioural plasticity has been attributed to the environment in which the individual developed (Urszán et al., 2018). For example, behavioural development in rodents can be related to decreased maternal contact during early life stages resulting in offspring that are
less social (Starr-Phillips & Beery, 2014) and exploratory (reviewed in: Champagne & Curley, 2009), and more aggressive (Menard & Hakvoort, 2007). In an African cichlid, *Pelvicachromis taeniatus*, isolation from parents and siblings during early environment results in offspring that are more aggressive (Hesse & Thünken, 2014). Aggressive individuals may also be more bold, as boldness and aggression are two widely-recognised personality traits that are often positively correlated and thought to play a role in an individual’s survival, growth and/or reproductive success (Biro & Stamps, 2008; Sih & Bell, 2008; Réale et al., 2010; Stamps & Groothuis, 2010). If aggressive individuals with reduced maternal care quality or duration are also more aggressive, this could indicate an additional pathway for maternal care to affect offspring fitness.

Cichlid fishes provide an ideal system for studying maternal care (Barlow, 2000). Maternal mouthbrooding in cichlids has been suggested to be a key innovation that allowed for the evolution of adaptive radiations within the African Rift lakes (Barlow, 2000). Therefore, this form of maternal care warrants investigation as a factor in the development of offspring phenotypes. Some behaviours in cichlids may be determined early as species differences occur during the early patterning stages of brain development (Sylvester et al., 2013). Cichlid, and other teleost, brains are clearly defined and easy to identify into five main regions of known functional significance: telencephalon (forebrain), optic tectum and hypothalamus (midbrain), and cerebellum and dorsal medulla (hindbrain) (Kotrschal et al., 1998). While there is great interconnectivity and communication among brain regions, some major regions are suspected to be largely responsible for performing certain functions. The telencephalon is primarily involved in a social decision making network, which also involves neurons in some other regions (O’Connell & Hoffman, 2012), and areas within this region function similarly to the hippocampus and amygdala in mammals, relating to memory formation and emotional responses (Bshary, Gingins & Vail, 2014). The optic tectum is largely related to integrating visual cues with the central nervous system (Trainor & Hofmann, 2007). A second region involved in the social decision making network, the hypothalamus (O’Connell & Hoffman, 2012), is also involved in reproductive behaviours such as courtship and aggression (Demske & Knigge, 1971; Peter, 1977; Shumway, 2010). The cerebellum is the primary control centre for spatial orientation and movement (Kotrschal et al., 1998) and the dorsal medulla integrates information from the lateral line (Morita & Finger, 1987). Additionally, the hypothalamus, optic tectum, cerebellum and dorsal medulla have been shown to be plastic based on early developmental social environments in another African cichlid (Fischer et al., 2015).

The understanding that specific brain regions, while interconnected, largely function in certain ways allows for the investigation of the effects of maternal care on specific brain regions. In *R. norvegicus*, maternal care quality has a positive relationship with the development of the hippocampus and corresponding differences in spatial learning (Bredy et al., 2004). In teleost brains, areas within the telencephalon correspond to the mammalian
hippocampus (Bshary, Gingins & Vail, 2014). As maternal care has a relationship with the hippocampus and related behaviours (Bredy et al., 2004; Champagne et al., 2008) in R. norvegicus, it could be supposed that in other species which provide maternal care similar relationships could be observed. Furthermore, as maternal care quality in R. norvegicus (Menard and Hakvoort, 2007), prairie voles (Microtus ochrogaster) (Perkeybile & Bales, 2015), and Peromyscus californicus and P. leucopus has a negative relationship with aggression levels, maternal care duration (as a stand in for quality) may have a similar effect in other species with maternal care. As the hypothalamus is related to aggression in teleosts (Demske & Knigge, 1971; Peter, 1977; Shumway, 2010), a decrease in maternal care duration that increases aggression may have a corresponding effect on the hypothalamus. The relationship between boldness and aggression in other species (Huntingford, 1976; Chapman et al., 2011; Favati, Løvlie & Leimar, 2017), could suggest that aggressive individuals with a small hypothalamus are also bolder. Investigation of the relationships among maternal care duration, brain anatomy and bold-aggressive behaviours could provide insight into the development of these plastic traits.

This study aims to investigate whether duration of maternal care has long-lasting influences on offspring brain anatomy and behaviour. The existence of such an affect in offspring of wild parents, could indicate an opportunity for selection to act upon variation in maternal care quality and influence the evolution within populations. The African cichlid, Dimidiochromis compressiceps, is a large, predatory, mouthbrooding cichlid endemic to Lake Malawi (Carleton, Hárosi & Kocher, 2000). D. compressiceps is a sand-dwelling species, in which males construct pits in open water to attract females to spawn (York et al., 2018). Further, the relatively large size of this species results in broods ranging circa 80-120 eggs that are incubated in the buccal cavity of the female for an average of 16 days (16.28 ± 2.36 SEM, N=7; Armstrong et al., unpublished data). The behaviours that are potentially associated with finding a location (exploration/activity), digging a pit and spawning in open water (boldness), and defending the territory from approaching males (aggression) combined with the relatively large brood size and short incubation period lends this species well to lab studies on the effects of these behavioural categories. A larger brood size allows for broods to be split, resulting in full siblings reared under different early environments, which can help to disentangle results that may stem from a genetic effect and those that come from the early rearing environment. Through examination of brain anatomy, we focus on how maternal care influences brain volume in D. compressiceps, specifically on the total brain and regions which have homologs which have been found to have a relationship to maternal care, namely the telencephalon and hypothalamus. Further, we aimed to identify any correlation among boldness, activity, exploration and aggression, the effect of maternal care duration on these behaviours, and determine any relationship with variation in brain region volumes. We hypothesise that reduced maternal care will cause offspring to have decreased overall brain volumes and that the regions relating to social behaviours and aggression, the telencephalon and the
hypothesis, will be smaller than in individuals receiving the full duration of maternal care. We also hypothesise that boldness, exploration, activity, and aggression will be correlated and that individuals receiving reduced maternal care will be bolder, more exploratory, and more aggressive and that these traits will negatively correlate with, due to their relationship to social behaviours, volumes of the telencephalon and hypothalamus.

3.2 METHODS

3.2.1 Study Species, Housing Conditions, and Experimental Design

A population of 24 wild caught *D. compressiceps* were housed in two 118 L tanks with a male to female ratio of 1:3. When fish were identified to be incubating eggs, they were moved to a 31 L tank individually, to reduce stress and allow observations of when broods were released. Broods were split by removing a portion of the brood (amount removed depended on the size of the female to estimate how big the brood may have been; Table S1) at three days post-fertilisation, with one half of the brood placed into a 1500 ml Erlenmeyer flask (reduced duration) with 1000 ml of water from the main filtration system. The other half was left with the mother until they were naturally released (full duration). Flasks were treated with 0.1 ± 0.05 ml of methylene blue for the first week of development to reduce the risk of fungal infection and had aeration maintained through a diffuser. Methylene blue is commonly used to rear fish in artificial environments to reduce fungal infections (Mommer & Bell, 2014; Ribas *et al.*, 2017). Flasks and tanks were maintained at 27-29°C, exposed to a 12h:12h light cycle and placed in a darker corner of the room to more closely resemble the interior environment of the mother’s buccal cavity. All effort was made to minimise differences in the early abiotic environment, such as water quality, temperature and light, to reduce variation that may have stemmed from these factors. Water in the flasks was changed and eggs were checked for viability daily.

When full-duration juveniles were naturally released (17-21 days post fertilisation), the mother was removed and placed back into the stock tank. When reduced-duration juveniles had fully absorbed their yolk (20-25 days post fertilisation), they were moved to individual 31 L tank identical in setup to the housing of the full-duration juveniles, both of which contained a PVC shelter and artificial plant. All fish remained in their respective 31 L tanks until four months of age, at which point they were moved to a 118 L tank. The 118 L tanks contained 5 plastic slabs, 2 half terracotta flowerpots, and 6 PVC pipes to reduce aggression and increase hiding locations. Due to logistic constraints in housing three
families were able to be maintained up to one year of age. Fish were kept in their respective treatment and family groups throughout development.

One week before behavioural assays began for each family, 10 fish from each maternal care duration group were selected at random, anesthetised with benzocaine (0.075 ml benzocaine/L tank water), and tagged with visible implant elastomer (VIE) tags (Northwest Marine Technology, Inc., Washington, USA) for identification. The exception to this was one family, which had 11 adult individuals in each treatment, all of which were used in this study. As all fish in this study were tagged, any behavioural alterations that may have occurred due to the tagging process should be the same in both maternal care duration groups. Behavioural assays for each family were conducted over two days, from 11 AM to 4 PM on each day, on the week the family reached one-year post fertilisation. Brains were collected, after behavioural assays, by euthanising fish in an overdose of benzocaine solution (0.15 ml/L) followed by severing of the spinal cord. Standard length (SL; length from the point farthest forward on the fish’s face to the end of the caudal peduncle) was measured to the nearest mm (± 0.5 mm) using analogue callipers, and fish were weighed to the nearest 0.01 g (± 0.005 g), and then dissected to determine sex and to remove brains, which were stored individually in 4% paraformaldehyde (PFA; pH 6.9).

3.2.2 Brain Anatomical Variation

To assess variation in brain structures, all samples were photographed for morphological measures. Brain samples which had been previously fixed in 4% PFA, were transferred to a small petri dish filled with a 2% agarose base, in which a groove had been carved to symmetrically position the sample. Images were taken using a Leica M165FC dissecting scope and Leica DFC450C camera (Leica Microsystems, Wetzlar, Germany), from dorsal, right lateral and ventral views. During dissection some brains were damaged and could not be adequately measured resulting in, a total of 54 (28 reduced care and 26 full care) brains were measured. Measures of length, width, and height of the total brain as well as the dorsal medulla, cerebellum, hypothalamus, telencephalon and total brain were completed in ImageJ 1.48 (Schneider, Rasband & Eliceiri, 2012). The observer was blind to the maternal care duration and sex of the individual the sample was obtained from. Measurements were completed (Fig. 3.1) and volumes were calculated following the protocol outlined in Pollen et al. (2007), where paired region volumes (telencephalon and optic tectum) are determined using only the right hemisphere (doubling the calculated volume) with the following equations:
Brain volume = \[\frac{((\text{Length} \times \text{Width} \times \text{Height}) \times \pi)}{6 \times 1.23}\]

Structure Volume = \[\frac{((\text{Length} \times \text{Width} \times \text{Height}) \times \pi)}{6}\]

**Figure 3.1. Brain measurement diagram.** Visual representation of measurements used to calculate the total brain volume (A) where length (1), width (2), and height (3) were measured from the entire brain. Volume of each structure was calculated by measuring widths (B), heights (C), and lengths (D) of the dorsal medulla (a), cerebellum (b), optic tectum (c), telencephalon (d) and hypothalamus (e).
3.2.3 Behavioural Assays

Fish completed assays in a single day in the following order: Novel Environment, Novel Object, Shelter Use, and Mirror Aggression. To standardise hunger all fish were fasted for 24 hours and tagged fish were caught from the main tank via dipnet and transferred into one of three 10 L, opaque containers filled with 8 L of water and aerated via compressed air through a diffuser, for holding. After 10 minutes of acclimation to this holding environment, a random fish was selected and transferred to an opaque start box (15 x 11.5 x 17.5 cm), located on the right side within the behavioural arena (90 x 30 cm filled with aquarium water to a height of 12 cm), for 60 s (Fig. 3.2). All assays began by raising this box from a pulley system. At the end of each of the first three assays the start box was lowered, and the fish was returned to the box for 60 s before the next assay. The assays were completed in the same order for all fish due to the need for the fish to have not experience the environment for the first assay. Completing the mirror assay last allowed the fish to spend the longest time in the environment before being exposed to the mirror.

All fish completed assays as follows, however, due to technical errors with recordings final sample sizes differed and are given in the description:

1) The Novel Environment assay determined exploration of an arena to which the fish had never been exposed (reviewed in: Sih et al., 2004b). From this assay the distance travelled, time spent freezing, and mean distance to arena edge were quantified for 5 min. (N_{ReducedDuration} = 30; N_{FullDuration} = 31).

2) Willingness to investigate a Novel Object determined by exposing the fish to the drop of one of two novel objects (either a round 20 x 2 mm metallic washer or a spherical 1.5 mm glass marble) one minute after the start of the assay. The object was dropped in order to determine a startle response, however, as not all fish would have been facing the location of the object equally which could have altered their reaction to the startle, this behaviour was omitted from analysis. The latency to inspect, defined as when the fish oriented itself with and began swimming in a direct line toward the object was quantified. If the fish did not inspect the object within two min after it was dropped, a max time of 120 s was recorded (N_{ReducedDuration} = 30; N_{FullDuration} = 28).

3) The Shelter Use assay, in which the fish could explore the tank or seek refuge (for three min.) in an opaque shelter (15 x 11 x 4.5 cm) placed on the opposite end of
the tank as the start box. Distance travelled and time spent in the shelter were quantified ($N_{\text{ReducedDuration}} = 30; N_{\text{FullDuration}} = 31$).

4) The Aggression assay, in which fish were exposed to their mirror image, lasted for three minutes (Réale et al., 2007). In this assay time of first approach as well as the number of attacks were quantified ($N_{\text{ReducedDuration}} = 30; N_{\text{FullDuration}} = 31$).

Behavioural assays were recorded using a GoPro Hero 4 (GoPro, California, USA) suspended 50 cm over the arena (30 fps, 720 ppi). Distortion, due to the wide-angle lens, was removed using the “barrel distortion correction” plugin ([http://emiliano.deepabyss.org/](http://emiliano.deepabyss.org/)) for VirtualDub 1.10.4 (Lee, 2005) and one frame for every 10 was exported as a JPEG for movement tracking. Movement tracking was completed using the MTrackJ (Meijering, Dzyubachyk & Smal, 2012) plugin for ImageJ 1.48 (Schneider et al., 2012). Distance was calibrated using the known 3 cm length of each of the grid squares, and a point was manually fixed on each frame at the most anterior point of the fish’s head, resulting in a track for each individual in all four contexts. The number of attacks against the mirror were counted manually.
Figure 3.2. Experimental arena diagram. The start box was located on the right side of the tank (A) and was lifted with a pulley at the start of each assay. In the Novel Environment (B) fish were allowed to explore for five minutes, while in the Novel Object assay an object was dropped after one minute and fish had 2 minutes to inspect the object. The shelter (D) was placed in the upper left corner of the tank, before the fish was released from the start box. Similarly, the mirror (E) was placed along the short-left wall while the fish was in the start box.

3.2.4 Statistical Analysis

To minimize potential allometric effects on brain measures, a linear regression with SL as the explanatory variable was completed for total brain volume and the residuals from this model were then used in place of total brain volume for all additional analyses (Fig. 3.3). Similarly, size was controlled for in each of the brain regions, using total brain volume (with the volume of the target region subtracted) as the explanatory variable in linear regressions, with each of the region volumes as response variables. Using total brain volume without the target region helps to control for the correlation between the total brain volume and the target region. The residuals of all of these models were used for remaining
analyses. Determination of the effect of maternal care duration on brain and region volumes was completed using mixed effect linear models, with maternal care duration, and sex, as well as an interaction between the two, as fixed effects. Family was fitted as a random effect. Non-significant variables were dropped during model selection and the best model was selected using AICs following the method outlined in Burnham & Anderson (2002), where a difference of >2 between models indicated support for a better fit of the lower AIC model. Models were generated in the package lme4 (Bates et al., 2015) and lmerTest (Kuznetsova, Brockhoff & Christensen, 2017) and base R 3.6.1 (R Core Team, 2019). Model fits were generated with the package piecewiseSEM (Lefcheck, 2016).

Before a principal component analysis (PCA) was conducted, a Spearman rank correlation matrix, in the package Hmisc v4.2-0 (Harrell, 2019), was completed to determine if behaviours were correlated across contexts. Correlations were conducted using all data, as well as subsets of Full and Reduced Care individuals separately. The correlation matrix for the treatment group subsets differed from both the full data set matrix and each other (Table 3S.1), a PCA was conducted separately on both Full and Reduced Care behaviours using the “prcomp()” function in base 3.6.1 (R Core Team, 2019). This function computes the PCA based on a singular value decomposition of the data matrix, data were scaled and centred during the PCA. Loadings for each trait that were >0.30 were considered to be correlated and therefore more strongly contributing to the variation captured by the component.

Using the PCA score of the first three principal components (the number of components required to reach 65% variation captured; Table S3.1) for each treatment group as response variables, linear mixed effect models were conducted to determine the relationship among correlated behaviours, Sex, SL, and brain region volumes. Sex, SL, total brain volume, telencephalon volume, and hypothalamus volume were fitted as fixed effects with family as a random effect. Model selection was conducted in the manner outlined previously, and all figures were created with ggplot2 (Wickham, 2016) and gridExtra (Auguie, 2017).

3.3 RESULTS

3.3.1 Survivorship

When reduced-duration juveniles were moved from flasks to their respective 31 L tanks, out of 114 juveniles there was a survival rate of 99%. There is no accurate means to
determine survivorship of full-duration individuals before day of release. Over the course of one year, survival rate was 59% for reduced-duration and 60% for full-duration (Table 3.2).

Table 3.1. **PCA loadings and percent variance explained.** Loadings of behavioural traits in Principal Component Analysis for eight behaviours recorded from the four assays, for PCs 1, 2, 3. Loadings above 0.30 were considered to have contributed to that component and are indicated in bold.

<table>
<thead>
<tr>
<th></th>
<th>Inactive-Aggressive</th>
<th>Exploratory-Aggressive</th>
<th>Bold-Non-exploratory</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>REDUCED-CARE</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Distance Travelled (Nov Env)</td>
<td>-0.519</td>
<td>-0.232</td>
<td>0.018</td>
</tr>
<tr>
<td>Time Frozen</td>
<td>0.495</td>
<td>0.163</td>
<td>0.056</td>
</tr>
<tr>
<td>Avg. Distance to Wall (Nov Env)</td>
<td>-0.013</td>
<td>0.282</td>
<td><strong>0.626</strong></td>
</tr>
<tr>
<td>Inspection Latency</td>
<td>0.093</td>
<td><strong>-0.619</strong></td>
<td><strong>0.446</strong></td>
</tr>
<tr>
<td>Total Distance to Wall (Shelter)</td>
<td>-0.427</td>
<td>-0.175</td>
<td>0.210</td>
</tr>
<tr>
<td>Time Inside Shelter</td>
<td>-0.225</td>
<td>0.056</td>
<td><strong>-0.547</strong></td>
</tr>
<tr>
<td>Latency to Approach Mirror</td>
<td><strong>0.450</strong></td>
<td>-0.168</td>
<td>-0.083</td>
</tr>
<tr>
<td>Number of Bite Events</td>
<td>-0.200</td>
<td><strong>0.629</strong></td>
<td>0.236</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>1.771</td>
<td>1.143</td>
<td>1.120</td>
</tr>
<tr>
<td>Proportion of Variance</td>
<td>0.392</td>
<td>0.163</td>
<td>0.157</td>
</tr>
<tr>
<td>Cumulative Proportion</td>
<td>0.392</td>
<td>0.556</td>
<td>0.712</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>FULL-CARE</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Distance Travelled (Nov Env)</td>
<td>-0.550</td>
<td>-0.159</td>
<td>0.157</td>
</tr>
<tr>
<td>Time Frozen</td>
<td>0.546</td>
<td>-0.006</td>
<td>-0.050</td>
</tr>
<tr>
<td>Avg. Distance to Wall (Nov Env)</td>
<td>-0.080</td>
<td><strong>0.465</strong></td>
<td><strong>-0.368</strong></td>
</tr>
<tr>
<td>Inspection Latency</td>
<td>0.309</td>
<td>0.275</td>
<td><strong>0.523</strong></td>
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<tr>
<td>Total Distance to Wall (Shelter)</td>
<td>-0.482</td>
<td>-0.135</td>
<td>0.034</td>
</tr>
<tr>
<td>Time Inside Shelter</td>
<td>-0.035</td>
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<td><strong>-0.389</strong></td>
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<td>Latency to Approach Mirror</td>
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<td>Number of Bite Events</td>
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<td>Standard deviation</td>
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<td>1.177</td>
<td>1.113</td>
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<td>Proportion of Variance</td>
<td>0.350</td>
<td>0.173</td>
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<td>Cumulative Proportion</td>
<td>0.350</td>
<td>0.523</td>
<td>0.678</td>
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**Figure 3.3. Brain volume relationships.** Scatter plots of relationships between dorsal medulla (DM) volume (A), cerebellum (Cer) volume (B), optic tectum (OT) volume (C), and hypothalamus (Hyp) volume (D), and telencephalon (Tel) volume (E) with the total brain volume. The relationship between total brain (Total) volume and standard length (E) is also shown. Full-duration maternal care is represented by orange, while reduced-duration maternal care is represented by blue.
Table 3.2. **Survivorship by brood and age.** Sizes of broods by full-duration maternal care and reduced-duration maternal care at one month of age and one year of age.

<table>
<thead>
<tr>
<th>Brood</th>
<th>Full-Duration Released</th>
<th>Reduced-Duration Removed</th>
<th>Total Brood</th>
<th>Full-Duration 1 Month</th>
<th>Reduced-Duration 1 Month</th>
<th>Full-Duration 1 Year</th>
<th>Reduced-Duration 1 Year</th>
</tr>
</thead>
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<td>Family A</td>
<td>80</td>
<td>33</td>
<td>113</td>
<td>80</td>
<td>33</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>Family B</td>
<td>60</td>
<td>39</td>
<td>99</td>
<td>60</td>
<td>38</td>
<td>37</td>
<td>26</td>
</tr>
<tr>
<td>Family C</td>
<td>40</td>
<td>42</td>
<td>82</td>
<td>40</td>
<td>42</td>
<td>35</td>
<td>30</td>
</tr>
</tbody>
</table>

### 3.3.2 Brain Anatomy

Total brain volume and the volumes of the telencephalon and hypothalamus had no relationship with maternal care (Table 3.3; Fig. 3.4). However, the volume of the hypothalamus was marginally larger in males. Of the total volume of the brain the hypothalami of females comprised 3.948 – 6.432% with a mean of 5.047 (±0.147 SEM), while for males the hypothalami comprised 4.206 – 6.950% of the total volume with a mean of 5.259 (±0.119 SEM). Of the additional brain regions, only the dorsal medulla was significantly smaller in reduced-care individuals (Table 3S.2; Fig. 3S.1).
Figure 3.4. Total and brain region volumes. Plots of residuals (controlled for individual size) of the total brain volume (Total; A), telencephalon (Tel; B), and hypothalamus (Hyp; C). Plots are presented with the x-axis based on the variables left in the model and coloured by care duration; full-duration maternal care in orange and reduced-duration maternal care blue.
3.3.3 Behaviour

In the reduced-care treatment group, the first three principal components captured 71% of the variation, while in the full-care treatment group, the first three principal components captured 68% variation (Table 3.1).

In reduced-care individuals, fish with a higher score on PC1 were inactive and bold. On PC2 and PC3 reduced-care fish with higher scores were exploratory and aggressive, and bold and non-exploratory, respectively. In full-care individuals, a higher score on PC1 indicated individuals were inactive and non-exploratory. On PC2 and PC3, full-care fish with higher scores were aggressive, and shy, non-exploratory, and aggressive, respectively (Table 3.1).

3.3.4 Relationship Between Brains and Behaviour

Linear mixed effects model results indicated that reduced-care individuals with larger hypothalami were more inactive and bolder and those with a larger telencephala were also bolder and non-exploratory. Full-care individuals with larger hypothalami were shyer, non-exploratory and more aggressive. Full-care individuals showed no other significant relationships and the total brain volume had no relationship with any of the PCs in either data subset (Table 3.3; Fig. 3.5). However, after a Bonferroni correction, only the relationship between standard length and the “inactive-bold” behaviours in the reduced-care individuals remained significant.
**Table 3.3. Brain and behaviour linear mixed effect model results.** The effect sizes of the models are given as $R^2_M$ (marginal – fixed effects) and $R^2_C$ (conditional – fixed and random effects), significance ($p<0.05$) is indicated in bold with marginal and weak effects ($p<0.08$) are underlined. The reference groups are reduced-duration maternal care and female.

<table>
<thead>
<tr>
<th></th>
<th>Estimate</th>
<th>Std. Error</th>
<th>d.f.</th>
<th>$t$-value</th>
<th>p-value</th>
<th>Adj p-value</th>
<th>$R^2_C$</th>
<th>$R^2_M$</th>
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</thead>
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<tr>
<td><strong>Total Brain</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Intercept</td>
<td>0.064</td>
<td>11.614</td>
<td>2.133</td>
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<td>0.996</td>
<td></td>
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<tr>
<td>Care Duration</td>
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</tr>
<tr>
<td>Full Care</td>
<td>-6.801</td>
<td>4.400</td>
<td>46.043</td>
<td>-1.546</td>
<td>0.129</td>
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<tr>
<td><strong>Telencephalon</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
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<td>0.658</td>
<td>48.000</td>
<td>0.073</td>
<td>0.942</td>
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<td>&lt;0.001</td>
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<tr>
<td>Care Duration</td>
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<tr>
<td>Full Care</td>
<td>0.107</td>
<td>0.971</td>
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<td><strong>Hypothalamus</strong></td>
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<td></td>
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<tr>
<td>Intercept</td>
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<td>0.429</td>
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<td>-0.612</td>
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<td></td>
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<tr>
<td>Male</td>
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<td>0.314</td>
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<td>1.987</td>
<td>0.053</td>
<td>0.477</td>
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<td>0.041</td>
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<tr>
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<td>0.324</td>
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</tr>
<tr>
<td>Intercept</td>
<td>0.087</td>
<td>0.194</td>
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<td>0.450</td>
<td>0.657</td>
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<tr>
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<td>25.000</td>
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<td>0.013</td>
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<td>-1.233</td>
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<td>1.256</td>
<td>0.220</td>
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<td>0.306</td>
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</table>
Figure 3.5. Relationship between brain and region volumes and behaviour. 
Relationships between each PC1 (A & B), PC2 (C & D) and PC3 (E & F) and the variables remaining the selected model. The size of the point increases with the standard length of the individual. Full-duration is indicated by orange and reduced-duration by blue.
3.4 DISCUSSION

The results of this study indicate that suites of correlated behaviours in *Dimidiochromis compressiceps* may differ as a result of alteration of the early maternal environment. Further, results suggest that an alteration of maternal care duration alters the relationship between brain regions and correlated behaviours. While there was no relationship between sex and the expression of correlated behaviours, there was a marginal relationship between sex and the volume of the hypothalamus. These results indicate that although the brain displays plasticity in response to various environmental factors, the early maternal environment may affect neurological links between brain and behaviour that last throughout life.

Duration of maternal care, or early life experiences appears to shape the expression of correlated behaviours in offspring. In both treatment groups, the largest amount of behavioural variation was captured by a set of correlated behaviours that, in part, related to their activity levels. However, the difference between these two groups is that activity was negatively associated with aggression in the reduced-care individuals, while the full-care individuals exhibited a positive association with activity and exploration. Activity and aggression are often positively correlated in other species (Bell & Stamps, 2004; Kortet & Hedrick, 2007; Chapman *et al.*, 2011) and this positive relationship can increase aggression in an individual due to increased activity resulting in increased interactions between individuals (Sih *et al.*, 2012). The negative relationship between activity and aggression in the reduced-care individuals could then serve as a benefit, as aggressive interactions can be energetically costly and result in injury (Huntingford, Tamilselvan & Jenjan, 2012) and activity has an energetic cost as well. In this sense, the reduced-care fish may be able to conserve energy from reducing activity, decrease potential costs of aggression by lowering interactions, but able to succeed in aggressive competitions or more likely to defend territories, mates or resources. Exploration and activity are also commonly correlated (Adriaenssens & Johnsson, 2013; Moule *et al.*, 2016), resulting in an expected relationship for the full-care individuals. Individuals that are more exploratory could have higher foraging rates, which could help to provide energy for the higher activity levels.

The duration of maternal care also appeared to affect how aggression correlated with other behaviours. In both treatment groups the second largest amount of behavioural variation...
was captured by aggression, however, in the reduced-care groups this was positively associated with exploration. Aggression and exploration have been positively associated in other species (Adriaenssens & Johnsson, 2013), so this finding is not entirely unexpected. Though it is unexpected that reduced-care fish that were bold were also non-exploratory, as there could be some risk associated with exploring a novel object or environment. In this sense, it is not unexpected that the full-care fish which were shy were also non-exploratory. However, shy and non-exploratory full-care individuals were also aggressive, which was unexpected. This set of correlated behaviours could be due to the ecology of the species. As *D. compressiceps* is an open water predatory species (Liem, 1978; Carleton & Kocher, 2001), it may not be necessary for them to be active or exploratory, but as the species digs pits in the sand for spawning (York *et al.*, 2018) and is not a shoaling species, aggression between conspecifics may be common.

The hypothalamus is related to aggression (Demske & Knigge, 1971; Peter, 1977; Shumway, 2010), it is not unsurprising that hypothalamic volume is associated with aggression in the present study. In other species, such as the tree lizard, volume of the hypothalamus was found to be positively associated with aggression levels (Kabelik, Weiss & Moore, 2006). Though there was a marginal difference in the volume of the hypothalamus, regardless of treatment group, and a positive association between aggression and hypothalamic volume it is interesting that there is no relationship between sex and the correlated behavioural sets containing aggression. Further investigating differences in neural densities may assist in teasing apart these differences. The measurement of volume largely focused on the inferior lobes of the hypothalamus (Karoubi, Segev & Wullimann, 2016), which is associated with some aggressive behaviours (Demske & Knigge, 1971). However, other aggressive behaviours are associated with the pre-optic area (Peter, 1977). Additionally, it may be that the mirror test, with the fish’s aggression levels being reinforced and escalated by the “competitor” never backing down may be a less informative test for differentiating aggression levels between the sexes.

The positive relationship between the telencephalon volume and the bold-non-exploratory set of correlated behaviours was unexpected. Telencephalic volume has been found to be associated with activity (Wilson & McLaughlin, 2010), but associations between the telencephalon and risk-taking or boldness are uncommon. However, in the present study boldness was quantified by how far the fish ventured into the open arena, or distance to the
This measure, thigmotaxis, is commonly used in other species to measure anxiety (Simon, Dupuis & Costentin, 1994; Schnörr et al., 2012) and anxiety levels have an association with the hippocampus (Engin & Treit, 2007). In rats (Rattus norvegicus) decreased hippocampal volume is associated with decreased anxiety like behaviours (Kalisch et al., 2006). It may be that the measure of boldness used here is a better representation of anxiety like behaviours, which could explain the relationship to boldness. Future work could investigate this with both additional assays that specifically target boldness, but not anxiety as well as those that target anxiety, but not boldness. As it is specific areas within the telencephalon that are homologous with the hippocampus (Bshary et al., 2014) it would be pertinent to investigate this association with methods that identify neural regions, rather than volume alone.

While the relative volume of whole brains or any of the hypothesised regions did not vary with maternal care duration, the relative volume of dorsal medulla was smaller in reduced-care individuals. This is in line with the findings by Fischer et al. (2015) who found that the relative volume of the dorsal medulla in a Lake Tanganyikan cichlid was influenced by isolation from parents and rearing group size, indicating plasticity of this structure. The dorsal medulla in fish is primarily responsible for the integration of information from the lateral line (Morita & Finger, 1987; Kotrschal et al., 1998). It may be the case that the more densely packed and smaller dimensions of the mother’s mouth may result in a larger dorsal medulla in adult fish, allowing for the integration of a larger amount of lateral line information in the full-duration individuals. However, it should be considered that this brain region is difficult to measure as lines of demarcation are not always readily visible. While the samples were measured blind, human error may have contributed to some of the differences observed. It is also of note that female cerebellum volumes had much less variation than males. This could be related to a finding in Lake Tanganyikan African cichlids where species which provided female-only care had smaller cerebellum than males of those species (Gonzalez-Voyer & Kolm, 2010). If Lake Malawi cichlids show a similar trend, it may be that there are constraints on the size of the cerebellum that reduce plasticity of this region.

While this study provides new insights into how maternal care duration, and the early environment contributes to phenotypic variation, there are a few additional environmental factors that could have contributed. For instance, eggs of the reduced-care groups were reared in lower densities, with methylene blue and had daily handling to change the water.
within the flask. Each of these may have contributed to phenotypic differences that were observed. However, as other studies have found that rearing away from parents and in different group sizes alters behaviours of offspring (Arnold & Taborsky, 2010; Hesse & Thünken, 2014; Fischer et al., 2015b; Hesse et al., 2015), the effect of maternal isolation should have had a stronger role to play in offspring development than other environmental effects. While the sample is somewhat small, the evidence presented here suggests that if sample sizes were increased the differences observed may have been stronger. Though fish always completed the assays in the same order, which may lead to behaviours being not completely independent, the differences in behavioural correlations between the two groups suggests that this may be a minor issue.

In the wild fish that are released before the age of free swimming may not survive. However, the aim here was investigate the most extreme case, while three days post fertilisation is really early, females naturally vary in their incubation times. Investigations on whether a release where fish are mature enough to hide from predators, but not full developed have similar behavioural changes would be an interesting route for future investigations. Additionally, reducing the brood size within the mother’s mouth may have had effects on how full-duration fish developed. It would be interesting to compare reduced brood size fish to those that are not reduced to determine any of these effects. A reduction of brood size may occur in the wild due the predatory nature of Caprichromis orthognathus, a species of Lake Malawi cichlid which uses its head as a battering ram to attack brooding females from below. This ramming results in expulsion of the whole brood or part of the brood, for C. orthognathus to feed on. If some fish either escape or are left in the mother’s mouth to full-term this may result in some of the behaviours observed here.

3.5. Conclusions

Our results indicate that the early environment can affect how behaviours are correlated in a manner that lasts into adulthood. There may also be a relationship between maternal care duration and hindbrain development. Further there is evidence that differences in maternal care duration effects how the volumes of the telencephalon and hypothalamus are related to sets of correlated behaviours. These results further understanding into how variation among individual behaviour is shaped early in development. As care duration varies among females, the results here suggest that this variation may lead to different sets of correlated behaviours among offspring. The variation in correlated behaviours as a result
of the early environment may provide an avenue for which maternal care variation results in different evolutionary trajectories.
Table 3S.1. Correlations among behaviours. Spearman rank correlation matrix of behaviours from the four behavioural assays for all, full-care and reduced-care treatment groups. Significant (p<0.05) correlations are indicated in bold, while marginal (0.05<p<0.08) correlations are underlined.

<table>
<thead>
<tr>
<th></th>
<th>Total Distance (Nov Env)</th>
<th>Time Frozen (Nov Env)</th>
<th>Average Distance to Wall (Nov Env)</th>
<th>Inspection Latency</th>
<th>Total Distance to Wall (Shelter)</th>
<th>Time Inside Shelter</th>
<th>Latency to Approach Mirror</th>
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<tbody>
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<td>Time Frozen</td>
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<td>Avg. Distance to Wall</td>
<td>-0.060  0.680</td>
<td>0.070  0.607</td>
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<tr>
<td>Inspection Latency</td>
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<td>0.190  0.149</td>
<td>0.080  0.563</td>
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<tr>
<td>Total Distance to Wall</td>
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<td>&lt;0.001  1.000</td>
<td>-0.190  0.155</td>
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<tr>
<td>Time Inside Shelter</td>
<td>0.190  0.152</td>
<td>-0.200  0.128</td>
<td>-0.040  0.788</td>
<td>-0.210  0.116</td>
<td>0.190  0.155</td>
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<td>Latency to Approach Mirror</td>
<td><strong>-0.390  0.002</strong></td>
<td><strong>0.310  0.017</strong></td>
<td>-0.220  0.099</td>
<td>0.080  0.574</td>
<td><strong>-0.440  &lt;0.001</strong></td>
<td><strong>-0.260  0.047</strong></td>
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<tr>
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<td>0.120  0.360</td>
<td>0.100  0.465</td>
<td>0.130  0.325</td>
<td>0.130  0.335</td>
<td><strong>-0.260  0.050</strong></td>
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<td><strong>REDUCED-CARE</strong></td>
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<td>-0.050  0.784</td>
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<td>(Shelter)</td>
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<tr>
<td>Time Inside Shelter</td>
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<td>0.030  0.893</td>
<td>-0.170  0.361</td>
<td>0.150  0.421</td>
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<tr>
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<td><strong>0.470  0.009</strong></td>
<td>-0.220  0.250</td>
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<td><strong>-0.550  0.002</strong></td>
<td>-0.190  0.324</td>
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<tr>
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<td>-0.140  0.468</td>
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<td>-0.070  0.712</td>
<td>0.170  0.360</td>
<td>0.040  0.832</td>
<td>-0.340  0.062</td>
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<tr>
<td><strong>FULL-CARE</strong></td>
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<td>Time Frozen</td>
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<tr>
<td>Avg. Distance to Wall</td>
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<td>0.020  0.904</td>
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<tr>
<td>Inspection Latency</td>
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<td>-0.080  0.702</td>
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<tr>
<td>Total Distance to Wall</td>
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<td><strong>-0.710  &lt;0.001</strong></td>
<td>-0.180  0.349</td>
<td>-0.340  0.078</td>
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<tr>
<td>Time Inside Shelter</td>
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<td>-0.110  0.564</td>
<td>-0.130  0.502</td>
<td>-0.250  0.193</td>
<td>0.230  0.245</td>
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<td>Latency to Approach Mirror</td>
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<td>-0.310  0.112</td>
<td>-0.020  0.914</td>
<td><strong>-0.390  0.043</strong></td>
<td>-0.320  0.092</td>
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<td>0.090  0.641</td>
<td>0.230  0.242</td>
<td>-0.190  0.336</td>
</tr>
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</table>
Table 3S.2. Brain volume linear mixed effect model results. The effect sizes of the models are given as $R^2_M$ (marginal – fixed effects) and $R^2_C$ (conditional – fixed and random effects), significance (p<0.05) is indicated in bold with marginal and weak effects (p<0.08) are underlined. The reference groups are full-duration maternal care and female.

<table>
<thead>
<tr>
<th></th>
<th>Estimate</th>
<th>Std. Error</th>
<th>d.f.</th>
<th>t-value</th>
<th>p-value</th>
<th>Adj. p-value</th>
<th>$R^2_C$</th>
<th>$R^2_M$</th>
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<tr>
<td>Intercept</td>
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<td>0.238</td>
<td>3.152</td>
<td>-1.747</td>
<td>0.174</td>
<td></td>
<td>0.234</td>
<td>0.348</td>
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<td>Care Duration</td>
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<tr>
<td>Full Care</td>
<td>0.901</td>
<td>0.223</td>
<td>43.336</td>
<td>4.046</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
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<td><strong>Optic Tectum</strong></td>
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<tr>
<td>Intercept</td>
<td>-1.012</td>
<td>1.290</td>
<td>48.000</td>
<td>-0.784</td>
<td>0.437</td>
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<td>0.025</td>
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<td>Care Duration</td>
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<tr>
<td>Full Care</td>
<td>2.126</td>
<td>1.902</td>
<td>48.000</td>
<td>1.118</td>
<td>0.269</td>
<td>0.807</td>
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<tr>
<td><strong>Cerebellum</strong></td>
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<tr>
<td>Intercept</td>
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<td>0.927</td>
<td>5.559</td>
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<td>0.423</td>
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<td>Care Duration</td>
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<tr>
<td>Full Care</td>
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<td>0.071</td>
<td>0.944</td>
<td>1.000</td>
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<tr>
<td>Sex</td>
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<tr>
<td>Male</td>
<td>1.458</td>
<td>0.795</td>
<td>45.108</td>
<td>1.834</td>
<td>0.073</td>
<td>0.219</td>
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</tbody>
</table>
**Figure 3S.1. Brain region volumes.** Plots of residuals (controlled for individual size) of the dorsal medulla volume (DM; A), cerebellum (Cer; B), and optic tectum (OT; C). Plots are presented with the x-axis based on the variables left in the model and coloured by care duration; full-duration maternal care in orange and reduced-duration maternal care blue.
Chapter Four: Maternal care duration influences brain shape but not brain volume or behaviour in an African cichlid

4.1 INTRODUCTION

Maternal care is widespread across animal taxa with examples from birds, mammals, reptiles, amphibians, fish and many invertebrate classes (Royle et al., 2012). Maternal care influences offspring survival and growth (Clutton-Brock, 1991; Royle et al., 2012), and the quality and duration of maternal care varies naturally among individuals of the same species. While this variation can influence the development of brain anatomy and behaviour in offspring (Curley et al., 2008; Perkeybile & Bales, 2017), the degree to which there exists functional links between brain morphology and behaviour in offspring in response to maternal care remains unknown. In species with maternal care, the first environment that offspring experience is created by their mother and gaining understanding of how maternal care quality affects brain anatomy and offspring behaviour can give insight into how individual variation within species beings to arise during the earliest life-stages.

Maternal care quality can influence the brain development of offspring in a regionally specific manner. In rats, for example, increased maternal care increase the number of synapses in offspring and improved neuronal survival (Champagne et al., 2008; Kwak et al., 2008; Bagot et al., 2009). At a broader morphological scale, offspring of a cooperatively breeding cichlid, Neolamprologus pulcher, that are isolated from parents and siblings show an increased volume of some brain regions but a decrease in other regions (Fischer et al., 2015). While maternal care effects on brain development in fishes is understudied, the general environment during early development does affect fish brain development. For example, early experiences with structurally complex environments result in changes in brain morphology in salmonids (Kihslinger & Nevitt, 2006; Näslund et al., 2012; Chapter One). Taken together, the effects of maternal care duration on brain development in mammals and the effects of the early environment on brain development in fish suggest a pathway for maternal care in fishes to influence brain development of offspring. Variation in brain anatomy could alter how individuals interact with their environment and determining if maternal care duration contributes to variation could give insights into the development of this variation.
Variation in maternal care duration and quality alters aggression in offspring and this effect can depend on offspring sex. For example, male prairie voles, *Microtus orchogaster*, experiencing reduced maternal quality are more aggressive toward conspecifics than those that receive high quality care while aggression in female offspring is unaffected by maternal care quality (Perkeybile & Bales, 2015). Additionally, early weaning in mice, *Mus musculus*, increases aggression in male-male interactions in adult offspring (Kikusui *et al.*, 2004). However, isolation from parents and siblings in *N. pulcher* (Fischer *et al.*, 2015) and *Pelvicachromis taeniatus* (Hesse & Thünken, 2014) result in offspring that are more aggressive than those remaining with their parents and siblings, though it remains unknown whether there is a sex specific effect on offspring aggression with reduced duration or quality of maternal care in fishes. For males of species that hold territories, an effect of maternal care duration or quality on offspring aggression may result in male offspring that are better or worse at defending territories from intruding males.

The maternal care behaviour of mouthbrooding is widespread among cichlid fishes and involves the mother collecting eggs during spawning and storing them in her buccal cavity until the offspring are free swimming and able to feed for themselves (Barlow, 2000). This behaviour protects offspring from predation during the most vulnerable stages of their life (Barlow, 2000). This behaviour also provides a high amount of maternal contact, as eggs are rotated by mouth movements of the mother (Barlow, 2000), resulting in contact with the walls of the buccal cavity as well as with siblings. In rats, the physical contact provided by licking and grooming of the mother results in altered epigenetic landscapes in offspring that in turn result in altered behaviour (Champagne *et al.*, 2008; Danchin *et al.*, 2011). It is possible that the maternal contact provided with mouthbrooding may act in a similar way to licking and grooming in rats facilitate alteration of behaviour and brain anatomy in offspring. African cichlids endemic to Lake Malawi provide an excellent system to investigate maternal care effects as all but one of the 700-1000 species provide maternal care in the form of mouthbrooding (Barlow, 2000).

*Dimidiochromis compressiceps* is a predatory species (Liem, 1978) endemic to Lake Malawi that maternally mouthbroods its young (Carleton, Hárosi & Kocher, 2000). Males attract females by constructing pits in the sand and defending the territory from approaching males (York *et al.*, 2018). In addition, fish will fight over resources such as food, shelters and priority locations in the tank (personal observation). Fish typically reach maturity at six months (Carleton et al. 2000), and brood sizes can be up to 125 offspring (Armstrong et al. unpublished data). After an average of 15 days (mean = 15.25, SD =0.75, N=7) females release developed juvenile fish from their buccal cavity (see Chapter 2). The
territorial behaviour, large broods and maternal care in *D. compressiceps* allow for investigation of the effects of maternal care duration on offspring behaviour. Following the finding that *D. compressiceps* reared under different care durations exhibited different sets of correlated behaviours (Chapter Three), I aimed to further investigate the aggressive response in a competitive scenario in different fish, but from the same families as used previously (Chapter Three). Using adult male offspring reared under two durations of maternal care, I aim to investigate if reduced maternal care duration results changes of brain volume or shape and if there is a relationship between brain volume and aggressive behaviours. Specifically, due the relationship between the telencephalon and social behaviours (O’Connell & Hofmann, 2011), and the hypothalamus on aggressive behaviours (Demski & Knigge, 1971), I predicted that individuals with larger hypothalamic would have increased aggressive behaviours and that the opposite trend would be true for the telencephalon volume. Further, I expected that individuals with a reduction in maternal care would be more aggressive, and when placed in a scenario with an individual of similar care duration aggression. In addition, that this aggression would escalate greater when reduced care duration individuals were in a scenario against another reduced care individual, than when they were against an individual with full duration maternal care. Finally, I expected that individuals reared with full duration maternal care would be less aggressive, and thus aggression would escalate much less when the scenario was two full-duration individuals against each other than when the scenario was comprised of one fish of each care duration treatment.

4.2 METHODS

4.2.1 Study Species and Animal Husbandry

Two populations of 12 *D. compressiceps*, wild caught from Lake Malawi, were housed at a male to female ratio of 1:3 were each housed a 118 L tank. To reduce stress and determine day of brood release incubating females were moved to 31 L tanks the day after spawning. On the third day post-spawning, roughly forty eggs were removed and placed into a 1500 ml Erlenmeyer flask (reduced duration maternal care). Flasks were filled with 1000 ml of filtered tank water and 0.1 ± 0.05 ml of methylene blue to reduce the risk of fungal infection (Mommer & Bell, 2014; Ribas et al., 2017) for the first week of development. To maintain proper aeration and water quality, flasks were aerated with compressed air through a diffuser and water was changed daily. Flasks were placed in a darker corner of the room to reduce stress from light and maintained at 27-29 1° C in a room with a 12h:12h light cycle. After yolk sack absorption, reduced-care juveniles were moved to a 31 L tank
with a plastic plant and a PVC pipe for three months. At three months old the fish were moved to a 118 L tank containing 5 plastic slabs, 2 half terracotta flowerpots, and 6 PVC pipes to reduce aggression during holding. All effort was made to minimise differences in the early abiotic environment, such as water quality, temperature and light, to reduce variation that may have stemmed from these factors. Water in the flasks was changed and eggs were checked for viability daily.

The remainder of each brood was left with the mother until she naturally released them (full duration maternal care; 17-21 days post fertilisation in this study). Full-care juveniles were then left in the 31 L tank with a plant and a PVC pipe shelter until they were three months of age, at which point they were moved to a 118 L tank the same as previously described. All fish were kept in their respective family and treatment groups for the duration of the experiment. Fish reared for the behavioural part of this study were 18 months post fertilisation and were from two families.

One week before behavioural assays began all fish from each family that were over 90 mm in standard length were anesthetised with benzocaine (0.075 ml benzocaine/L tank water) and tagged with visible implant elastomer (VIE) tags (Northwest Marine Technology, Inc., Washington, USA) for identification. Behavioural assays were conducted over two months, from 8 AM to 6 PM on each day. Fish were fasted for 24 hours before each behavioural test. Brains were collected following behavioural assays by euthanising fish in an overdose of benzocaine solution (0.15 ml /L) followed by severing of the spinal cord. Standard length (SL) was measured to the nearest mm (± 0.5 mm), fish were weighed to the nearest 0.01 g (± 0.005 g), and then dissected to verify sex and to remove brains, which were stored individually in 4% PFA (pH 6.9) until imaging.

4.2.2 Behavioural Assays

A total of 13 reduced-care fish and 20 full-care fish were identified visually as male (by vent (Barlow, 2000) and fin shape as well as colouration). In the mirror assay two of these reduced care fish were unable to be used, one due to technical difficulty and another due to mortality not related to the experiment. In the competition assay, one full care fish was unable to be used due to mortality not relating to the experiment.

Aggression against a mirror was conducted in a 90 x 30 x 30 cm tank filled to a 15 cm depth of water. Fish were held in a 20 x 30 x 30 cm isolation area with an opaque divider, a plastic plant and an opaque, half-cylinder, shelter (15 x 11 x 4.5 cm) for 5 minutes to acclimate to the arena. After the isolation time the dividers, plant and shelter were
removed, and the fish was allotted 15 minutes to interact with a mirror located on the opposite end of the tank from where they were isolated. Time to leave the start area, time to first bite, and bite rate (Table 4.1) were determined from videos using SolomonCoder v17.03.22 (Péter, 2017).

Aggression in a competitive environment was determined within rectangular experimental arena (90 x 30 x 30 cm) filled with water to 15 cm depth. Removable dividers were placed in the tank to create isolated 20 x 30 x 30 cm areas on either end of the tank. In the centre of the tank was an opaque half-cylinder shelter (15 x 12 x 6 cm), which served as a refuge and to elicit a competitive interaction. A pair of fish were placed into the tank, each in their own isolation area with an opaque and divider and a plastic plant for two hours, at which point the plant was removed, five minutes later the dividers were removed and the fish were able to interact for 30 minutes. As the plant served as an additional potential cover for the fish, it was removed to give stronger encouragement for the two fish to compete for the sole shelter in the middle of the tank. Plants were initially placed in to add additional cover to reduce stress during acclimation. Interactions were recorded via a GoPro Hero 4 (GoPro, California, USA) suspended above the tank for thirty minutes. Each fish was tested twice, once against a competitor from their own maternal care duration treatment (“Same”) and once against the opposing care duration treatment (“Mixed”). Each fish was naïve to its competitor, size-matched within 5 mm, and the order in which fish were tested and the care duration of the competitor fish was randomised. Following the assay, fish were placed back in their original tanks, the experimental tank was emptied, rinsed and refilled before starting the next trial. Each fish had a minimum of 48 hours between successive trials.

Individual movements of each fish in the competitive environment were tracked with idTracker (Pérez-Escudero et al., 2014) to assist in maintaining identification of individuals. Aggressive and submissive behaviours were manually recorded from the videos in idPlayer using terms and definitions adapted from previous cichlid research (Fernald, 1977; Riebli et al., 2011; Scaia et al., 2018; Table 4.1).

To synthesise behaviours in the competitive arena into a single variable an absolute aggression score was calculated by following equation: (attacks*3)+displays (McCormick, Watson & Munday, 2013; Killen et al., 2014). Where here attacks are the combined variables of Bite and Chase.
Table 4.1. Terms and definitions of aggressive behaviours. Definition of terms used in the mirror assay and terms used for aggressive (display, bite, chase) behaviours in the competition assay.

<table>
<thead>
<tr>
<th>Behaviour</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Start Latency</td>
<td>Time leaving start - time lifting box</td>
</tr>
<tr>
<td>Bite Latency</td>
<td>Time first bite - start latency</td>
</tr>
<tr>
<td>Bite Rate</td>
<td>Total bites/time of first bite to end of video (s)</td>
</tr>
<tr>
<td>Display</td>
<td>Fish either raises its dorsal fin, flares its gills, rapidly moves its body laterally, or any combination of the above</td>
</tr>
<tr>
<td>Bite</td>
<td>Fish contacts the other fish using its mouth.</td>
</tr>
<tr>
<td>Chase</td>
<td>Fish rapidly approaches the other fish and continues to rapidly follow if the other fish swims away. This often coincides with a display from the chasing fish.</td>
</tr>
</tbody>
</table>

4.2.3 Neuroanalysis

A total of 28 brains were collected following behavioural assays, 11 reduced duration and 17 full duration. Individual brain measurements of the length, width and height of the dorsal medulla, cerebellum, hypothalamus, optic tectum, telencephalon and total brain were completed in ImageJ 1.48 (Schneider, Rasband & Eliceiri, 2012; Fig. 4.1). Brain sample sizes differ from the original sample sizes due to damage during dissection and the mortalities which occurred. Volumes were calculated following the protocol outlined in Pollen et al. (2007), where paired structures’ volumes are determined using only the right hemisphere and the following equations are used:

\[
\text{Brain volume} = \frac{(L \times W \times H) \pi}{(6 \times 1.23)}
\]

\[
\text{Structure Volume} = \frac{(L \times W \times H) \pi}{6}
\]

Brain shape was analysed by combining brain images from this study with those previously collected (see Chapter Three) for a total of 60 brains, 27 reduced duration and 33 full duration for dorsal images; ventral images totalled 64 (27 reduced duration and 37 full duration). Data sets were combined due to the need for sample sizes to be greater than the number of landmarks for statistical comparisons of this type of data. Dorsal shape was determined by placing nine fixed landmarks, chosen based on ease of identification, and 40
semi-landmarks to determine the overall shape of the brain (Fig. 4.2A). The cerebellum and telencephalon were outlined with 12 sliding landmarks each, while the optic tectum had 16. Ventral shape consisted only of the hypothalamus and was determined using six fixed landmarks and 28 semi-landmarks (Fig. 4.2B). Landmarks were placed using the software tpsDig2 2.26 (http://life.bio.sunysb.edu/morph/index.html).
Figure 4.1. Visualisation of brain measurements. Length (1), width (2) and height (3) of total brain was measured from dorsal and lateral views (A). Length for dorsal medulla (a), cerebellum (b), right optic tecta (c), right telencephalon (d), and right hypothalamus (e) was measured from the lateral image (B). Width for the dorsal medulla (a), cerebellum (b), right optic tecta (c), and right telencephalon (d) was measured from the dorsal view, while width of the hypothalamus (e) was measured from the ventral view (C). Height of all regions (a-e) were measured from the lateral view (D).
Figure 4.2. Locations of homologous landmarks. Fixed landmarks are indicated on images of dorsal (A) and ventral (B) brain samples by open circles. Fixed landmarks were placed where regions join as these locations were easily identifiable. Curves of sliding landmarks are indicated by solid white lines.

4.2.4 Statistical Analysis

When fish were dissected to verify sex, it was found that five of the fish were female despite showing external characteristics that are typically associated with males. In addition, not every fish was able to be paired with a size-matched competitor. While this still allowed for all fish to be included in statistical analysis of brain anatomy and
aggression against a mirror, competitive aggressive interactions between males and females would vary. Due to this, behavioural data from the competitive environment were subsetted to include only males that were tested twice ($N_{\text{ReducedDuration}} = 11$; $N_{\text{FullDuration}} = 8$). In addition, logistical issues resulted in some fish used in only one pairing, ending in an unbalanced data set that resulted in potentially unreliable statistical outputs. Results of this experiment are therefore more similar to qualitative examination of trends.

Potential allometric effects on brain measures were minimised by linear regression of SL as the explanatory variable and total brain volume as the response variable, then using the residuals from the model as the new volume measure for further analysis. To similarly minimise the effect of total brain volume on each of the regions, the residuals of a linear regression with brain volume (with the volume of the target region subtracted) as the explanatory variable and each of the region volumes as a response variable were used in all further analyses. Regressions and all further analysis were conducted in R 3.6.1 (R Core Team, 2019).

The relationship among maternal care duration, brain morphology, and aggression in the mirror assay was determined using a linear mixed effects model. As start latency was more indicative of a bold behaviour rather than aggressive behaviour, the hypothalamus was not included in this model. Start latency, bite latency and bite rate were each used as response variables, dorsal medulla volume, telencephalon volume and SL were fitted as fixed effects along with the factors care treatment and sex of the individual. Family was fitted as a random effect. Model selection was conducted by dropping the least significant variable and comparing models by Likelihood ratio tests using the “anova” function from base R 3.6.1 (R Core Team, 2019). Models were fitted using the package “lme4” (Bates et al., 2015) and “lmerTest” was used to determine p-values (Kuznetsova et al., 2017). Model fit was determined using the package “piecewiseSEM” to calculate marginal and conditional $R^2$ (Lefcheck, 2016). Final sample sizes: $N_{\text{ReducedDuration}}=10$ (1F;9M); $N_{\text{FullDuration}}=17$ (2F;15M).

The potential effect of the competitor’s care duration on the aggression expressed in the competitive assay was analysed by examining the change in aggression between the “Same” and “Mixed” scenario. A linear mixed effects model with the interaction of care duration and telencephalon volume as well as care duration and hypothalamus volume were fitted as fixed effects along with SL and Family as a random effect was fitted on the change in aggression. Model selection was completed as previously described. Final sample sizes: $N_{\text{ReducedDuration}}=10$; $N_{\text{FullDuration}}=8$. 
Relationship between maternal care duration and variation in start latency, bite latency, bite rate and change in aggression was examined through a Fligner-Killeen homogeneity of variance test. In this test p<0.05 indicates that the variance between treatment groups is significantly different. In addition, differences in variation in bites, chases and displays in the competitive environment were examined.

The relationship among maternal care duration, sex and the volume of the total brain, telencephalon and hypothalamus was determined using linear mixed effect models with family as a random variable, and the interaction of care duration and sex as a fixed effect. Model selection was completed using stepwise elimination of non-significant variables and likelihood ratio tests, as previously described. Final sample sizes: N_{ReducedDuration}=12 (1F;11M); N_{FullDuration}=17 (2F;15M).

The relationship between maternal care duration and dorsal and ventral brain shape was determined using package “geomorph 3.0” (Adams, Collyer, & Kallontzopoulou, 2018). The multivariate shape data was first positioned onto a common centroid, scaled to shared unit size and rotated to a shared orientation with the Procrustes analysis in the package. This new data set was then used as the response variable for the remaining shape analysis, where a multivariate linear model was used with the dorsal and ventral shape of the brain as the response variable and the interaction between care duration and sex as the explanatory variable and 5000 iterations. The Procrustes superimpositions of dorsal and ventral shape were each used as a response variable with the interaction of care duration and sex as the explanatory variable. To visualise shape differences the landmark data was Procrustes superimposed using the software CoordGen8 (Sheets, 2014; http://www3.canisius.edu/~sheets/morphsoft.html), and this new data was set was analysed with a discriminate function analysis (DFA) with jack-knifing to predict group classifications in the “MASS” package (Venables & Ripley, 2002). The resulting predicted values from the DFA were then used an independent variable in tpsRegr (http://life.bio.sunysb.edu/morph/index.html) and the results were magnified 3x.

All plots were generated using the packages “ggplot2” (Wickham, 2016) and “gridExtra” (Auguie, 2017).

4.3 RESULTS

4.3.1 Maternal Care Effects on Total Brain and Region Volumes
Maternal care duration and sex had no association with offspring total brain volume, nor the volumes of the telencephalon or hypothalamus (Table 4.2).

### 4.3.2 Maternal Care and Brain Volume Effects on Behaviour

Maternal care duration did not influence any behaviours in the mirror or competition assays (Table 4.2; Fig. 4.3). Telencephalon volume had a positive relationship with start latency, in the mirror assay but did not affect any other behaviours (Table 4.2; Fig. 4.3A). The hypothalamus had a strong negative association with bite rate (Table 4.2; Fig. 4.3C).

Homogeneity of variance test indicated that there was no difference in the variation between maternal care treatments for start latency ($\chi^2_1 = 0.051$, $p=0.821$), bite latency ($\chi^2_1 = 1.56$, $p=0.211$), or bite rate ($\chi^2_1 = 0.038$, $p=0.845$).

In the competitive environment, variation in neither bites ($\chi^2_1 = 0.146$, $p=0.702$) nor displays ($\chi^2_1 = 0.074$, $p=0.786$) was significantly different between treatment groups (Fig. 4S.1). However, variation in chases ($\chi^2_1 = 8.397$, $p<0.004$; Fig. 4S.1) and the change of aggression with competitor type was marginally greater in the reduced care duration treatment group ($\chi^2_1 = 3.522$, $p=0.061$).
Table 4.2. Results linear mixed effects models for brains and behaviours. Response variables for each model are given in italics. The baseline is reduced duration. The effect sizes of the models are given as $R^2_M$ (marginal – fixed effects) and $R^2_C$ (conditional – random effects). Significant explanatory variables (p>0.05) are given in bold.

<table>
<thead>
<tr>
<th>Response Variables</th>
<th>Estimate</th>
<th>Std. Error</th>
<th>d.f.</th>
<th>$t$-value</th>
<th>p-value</th>
<th>$R^2_M$</th>
<th>$R^2_C$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total Volume</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>-2.209</td>
<td>5.731</td>
<td>3.102</td>
<td>-0.385</td>
<td>0.725</td>
<td>0.009</td>
<td>0.070</td>
</tr>
<tr>
<td>Care Duration</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Full</td>
<td>3.161</td>
<td>6.304</td>
<td>25.047</td>
<td>0.501</td>
<td>0.621</td>
<td>0.034</td>
<td>--</td>
</tr>
<tr>
<td><strong>Telencephalon Volume</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>-0.667</td>
<td>0.882</td>
<td>26.000</td>
<td>-0.756</td>
<td>0.456</td>
<td>0.013</td>
<td>0.184</td>
</tr>
<tr>
<td>Care Duration</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Full</td>
<td>1.098</td>
<td>1.131</td>
<td>26.000</td>
<td>0.971</td>
<td>0.341</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Hypothalamus Volume</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>-0.129</td>
<td>0.503</td>
<td>1.909</td>
<td>-0.256</td>
<td>0.823</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Care Duration</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Full</td>
<td>0.286</td>
<td>0.440</td>
<td>25.023</td>
<td>0.651</td>
<td>0.521</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Start Latency</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.210</td>
<td>0.245</td>
</tr>
<tr>
<td>Intercept</td>
<td>339.509</td>
<td>87.428</td>
<td>3.966</td>
<td>3.883</td>
<td>0.018</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Care Duration</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Full</td>
<td>-107.917</td>
<td>100.182</td>
<td>23.001</td>
<td>-1.077</td>
<td>0.293</td>
<td>44.008</td>
<td>16.677</td>
</tr>
<tr>
<td><strong>Bite Latency</strong></td>
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</tr>
<tr>
<td>Intercept</td>
<td>24.508</td>
<td>8.331</td>
<td>5.125</td>
<td>2.942</td>
<td>0.031</td>
<td>0.079</td>
<td>0.089</td>
</tr>
<tr>
<td>Care Duration</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Full</td>
<td>-15.383</td>
<td>10.228</td>
<td>24.007</td>
<td>-1.504</td>
<td>0.146</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Bites per Second</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.355</td>
<td>--</td>
</tr>
<tr>
<td>Intercept</td>
<td>0.219</td>
<td>0.044</td>
<td>24.000</td>
<td>4.982</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Care Duration</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Full</td>
<td>0.052</td>
<td>0.056</td>
<td>24.000</td>
<td>0.935</td>
<td>0.359</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Hypothalamus</strong></td>
<td>-0.089</td>
<td>0.024</td>
<td>24.000</td>
<td>-3.775</td>
<td>0.001</td>
<td>0.079</td>
<td>0.089</td>
</tr>
<tr>
<td>Change in Aggression</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>86.800</td>
<td>40.950</td>
<td>16.000</td>
<td>2.120</td>
<td>0.050</td>
<td>0.098</td>
<td>--</td>
</tr>
<tr>
<td>Care Duration</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Full</td>
<td>-83.430</td>
<td>61.420</td>
<td>16.000</td>
<td>-1.358</td>
<td>0.193</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 4.3. Association among brain and behaviours representing selected models. Start latency (A) and bite rate (C) shown against the brain region which best explained the behaviour according to the model. Bite latency (B) and change in aggression (D) were not significantly associated with maternal care or behaviour but are shown grouped by care duration. Full care treatment groups are in orange, with reduced care treatment groups in blue. Size of the point relates to the SL of the individuals and lines are representative of the predicted linear association of the behaviour and brain region.
4.3.3 Brain Shape Analysis

Although discriminate function analysis (DFA) classified dorsal brain shape 100% correct based on care duration, there was no statistical difference in dorsal brain shapes between care treatments ($Z_1 = -0.384; p > 0.05$). There was a significant difference in dorsal brain shape based on sex of the individual ($Z_1 = 1.856; p = 0.0276$; Fig. 4.4A), though the DFA misclassified one of 15 females with all 45 males correctly classified (Fig. 4.4A). DFA classifications of ventral brain shape resulted in 100% classification based on both sex ($N_{female} = 18, N_{male} = 46$) and care duration (Fig. 4.4B), and there was a significant interaction of care duration and sex ($Z_1 = 1.668; p = 0.051$; Fig. 4.4B).

The five females which appeared male, were evenly distributed throughout the LD1 scores from the DFA. The bins in which they are located have been marked with a star.
A) 

B)
Figure 4.4. Dorsal and ventral brain shape DFA classifications. The dorsal (A & B) and ventral (C & D) brain shape classifications from the discriminate function analysis (DFA).

Shape of the brain is represented by the linear discriminate (LD1) score from the DFA analysis. The most extreme shapes (3x multiplied) are indicated on their respective graphs with deformation grids in purple for females, yellow for males, blue for reduced care and orange for full care. Stars on plots A and C indicate the bins in which females that appeared male before dissection were placed into.
4.4 DISCUSSION

In this study, reducing the duration of maternal mouthbrooding in Dimidiochromis compressiceps resulted in offspring with a hypothalamus that was more constrained on the horizontal plane. The volume of the hypothalamus was negatively associated with aggression, though maternal care had no effect on the volume of this region. Additionally, female fish had brains that were much rounder from the dorsal view, suggesting that the optic tecta was larger in these individuals than in males. Overall, and in comparison, with Chapter Three, these finding suggest that maternal care effects on behaviour and brain volumes may diminish, and that over time, may be out-weighed the effects of brain morphology on behaviour.

While the total volume of the offspring hypothalamus was not influenced by reducing maternal care duration, the reduction in curvature and horizontal constriction suggest that areas within the hypothalamus may be altered by maternal care duration. The observed relationship between a small hypothalamus and increased aggression could be due to the compartmentalisation of behavioural control within areas of the hypothalamus. For instance, the preoptic area has been associated with courtship, spawning, pair bonding and after-spawning aggression (Demska & Knigge, 1971; Peter, 1977; Shumway, 2010). In the current study, however, the shape and volume of the hypothalamus was determined by measures of the inferior lobes (Karoubi et al., 2016), which is associated with some forms of aggression (Demska & Knigge, 1971). If the inferior lobes of the hypothalamus are smaller because the preoptic area is larger (i.e. if there is a trade-off between the size of subregions within the overall structure of the hypothalamus), then the increase in aggression may not be directly due to the inferior lobes. However, whether this sort of trade-off in volumes of specific areas of brain regions exists is unknown and could be the focus of future work. Additionally in tree lizards, Urosaurus ornatus, the volume of the hypothalamus and aggressive behaviours are positively associated with testosterone levels (Kabelik et al., 2006). If testosterone was driving the aggressive response in D. compressiceps, it may be that testosterone levels do not have an effect of increasing hypothalamic volume in this species. Future work could investigate the links between hormones, aggression and brain region volumes in this species.

The morphological requirements of mouthbrooding result in a sexually dimorphic head shape in African cichlids (Tsuboi et al., 2015). Additionally, in African cichlids head shape constraints are associated with the size of the brain (Tsuboi, Gonzalez-Voyer & Kolm,
If the constraints of head shape have resulted in not necessarily varying volumes in this species, but differences in the shape of the region this could explain some of the variation observed in this study between the sexes. There are also examples of region specific sexual dimorphism in animals from *Drosophila* (Cachero *et al.*, 2010) to humans (Hofman & Swaab, 1989). African cichlids also show sexual dimorphism in the dorsal medulla and cerebellum (Gonzalez-Voyer & Kolm, 2010). In examining the shape differences between males and females in this study, the dorsal medulla and cerebellum appear smaller in females. However, Gonzalez-Voyer & Kolm (2010) determined sexual dimorphism according to correlations between brain regions measures of ecological performance and not by directly comparing the brain regions between sexes. In the present study, the differences in dorsal medulla, cerebellum and optic tecta volumes have resulted in a female brain shape which is larger in the middle as compared to the males. However, whether these differences in brain shape are the result of differences in neural densities between sexes, or differences due to hormonal variation between sexes could be the topic of future research.

The lack of maternal care effects on brain anatomy differs from previous research on *Dimidiochromis compressiceps* (see Chapter Three). However, fish in the current data set were six months older than the previous analysis. Brains of fish grow throughout their entire lives (Kihslinger & Nevitt, 2006) and so growth patterns may have changed across different life-stages. Such a change in brain growth could be indicative of a period in which maternal effects on brain morphology are diluted or disappear at 18 months post fertilisation, the effects of the current environment may begin to outweigh the effects of early life experience on brain development. Because all fish were reared in identical environments at similar densities, it is possible that brain shape may converge at some point in their development. It is also possible that the reduced sample size in the current study as compared to prior work could have contributed to the observed differences between studies.

Similarly, any offspring behavioural differences as a result of maternal care effects that might have been present earlier in life may have subsided before the age of 18 months. Long-term exposure to similar social environments may have resulted in any early effects of maternal care duration being overridden by that of the social environment later in life. Specifically, if aggressive behaviours are affected by brain volume, and brain volumes of fish that received differing duration of care start to converge throughout their lives, any differences in aggression will also be diminished. However, if aggression is not influenced
by maternal care duration there is perhaps another mechanism for the emergence of aggressive phenotypes, possibly serving as buffer against the negative effects of a stressful early environment caused by poor maternal care quality. In male rats, the stress induced by separation from mothers during early development results in increased some forms of aggressive behaviours in adulthood (Veenema et al., 2006), maternal separation does not appear to affect latency to attack or total number of attacks. Similarly, in the present study bite latency and bite rate were not affected by care treatment, which could indicate that these behaviours are not affected by maternal care. For lekking species, such as D. compressiceps, holding territory is essential to spawning success and there is a direct relationship between ability to hold territory and aggression (Mumby & Wabnitz, 2002; Festa-Bianchet et al., 2010). If adult aggression is unaffected by the degree of care that males receive early in life, then maternal care in this species is unlikely to affect mating success in offspring that manage to survive until that life-stage.

When facing actual competitors, there was some evidence that individuals that received reduced care showed greater variation in the change in aggression between competitor types as well as variation in the number of chase events. The varying levels of aggression displayed toward competitors may reflect an ability to more readily adjust to changes in the aggression of competitors. Aggressive responses are energetically costly and have the risk of injury or mortality (Huntingford et al., 2012). An individual that can modulate its aggression in response to the behaviour of its competitor may reduce these risks rather than escalating a fight. The relatively small sample size in this portion of the current study makes it difficult to definitively state the reasons for the observed variation in aggression but this would be an interesting avenue for additional research.

Regardless of maternal care treatment, individuals with a smaller telencephalon tended to leave the starting area earlier, perhaps indicating an increased tendency to take risks. The telencephalon in fish shares homologous neural regions with the hippocampus and is involved with the social-decision-making network in vertebrates (O’Connell & Hofmann, 2011; Bshary et al., 2014). While there is no known association between either the telencephalon or hippocampus and risk-taking or boldness, it is possible that individuals were able to view their reflection from the start area and this apparent social stimuli affected their decision to leave. If we then consider the start latency as a social behaviour, individuals with a larger telencephalon took longer to come to this social decision, though it is unclear why a larger telencephalon would result in increased latency. It is possible that
larger volume does not result in more neurons or better connectivity of neural pathways that result in faster decision making.

4.4.1 Conclusion

The results of this study indicate that maternal care duration may influence aggression in adult offspring via alterations to the shape of the hypothalamus. However, how aggressive behaviours relate to competitive scenarios and resource holding potential needs further investigation. The lack of direct effects of maternal care on brain volumes and behaviours, may indicate that there is a point in life where the plasticity induced by maternal effects is overridden by other forms of environmental effects. There may also be a lack of maternal care effects on certain behaviours that are more canalised in their development, or under the influence of different drivers.
Figure 4S.1. Variation in aggressive behaviours in the competitive environment. The total number of bites (A), chases (B), displays (C), and the aggression score (D) in either the mixed (fish with different care durations) or same (fish with same care durations) scenario. The size of the points relates to the standard length of the individual.
Chapter Five: The effect of bi-parental absence on correlated behaviours in offspring

5.1 INTRODUCTION

Parental care is widespread in the animal kingdom and can take the form of uni- or bi-parental care. Parental care serves to increase offspring growth and survival through parental investment (Clutton-Brock, 1991; Royle et al., 2012). Increased care quality is also known to increase offspring sociality (e.g. Lévy et al., 2003; Starr-Phillips & Beery, 2014) and decrease aggression (e.g. Menard & Hakvoort, 2007). In animals that provide bi-parental care, one sex often invests more into care post fertilisation (Royle et al., 2012). In species where males guard and females provide care, this system may allow for females to invest more into offspring care than when females are the sole care provider. In such a species, the presence of both parents may have effects on offspring behaviours that are important for survival and reproductive success later in life, over those that are abandoned by parents.

Within individual animals, various dimensions of behaviour can be correlated across ecological contexts. In three-spined stickleback Gasterosteus aculeatus, for example, aggression toward conspecifics during the breeding season is positively correlated with boldness in the presence of a predator (Huntingford, 1976). In domestic fowl, Gallus gallus domesticus, aggressive individuals were also bolder (Favati et al., 2017). However, while aggressiveness resulted in a higher social rank in domestic fowl, boldness had no effect on rank (Favati et al., 2017). In contrast boldness in zebrafish, Danio rerio, has a positive effect on social rank (Dahlbom et al., 2011). In mosquitofish, Gambusia holbrooki, boldness may be linked to increase foraging ability (Wilson, Godin & Ward, 2010). Boldness is also positively correlated with time spent foraging in barnacle geese, Branta leucopsis (Kurvers et al., 2009), wandering albatrosses, Diomedea exulans (Patrick, Pinaud & Weimerskirch, 2017), and female African penguins, Spheniscus demersus (Traisnel & Pichegru, 2019). Boldness and exploration are also often linked, which perhaps explains some of the effects of boldness on foraging (Sih et al., 2004a). Indeed, wandering albatross that are more bold are also more exploratory when foraging (Patrick et al., 2017). If individuals that are more exploratory are able to increase their food-intake, they may be able to increase their growth rate, with the trade-off of exposing themselves to increased predation.
Behavioural development can be influenced by genetic and environmental factors. Individual’sboldness, for example, can be genetically inherited (van Oers et al., 2005; Réale et al., 2009; Ariyomo & Watt, 2012) or increased by environmental complexity (Brignon et al., 2018). Predator exposure also has the effect of decreasing aggression (Herczeg, Ghani & Merilä, 2016) boldness (Hellström & Magnhagen, 2016) and exploratory behaviours (Burns et al., 2009). Exploration may also be increased by higher quality maternal care (Champagne, 2010). As aggression, boldness, and exploration may be linked and can be influenced by the environment parental care quality may result in changes in the expression of these behaviours. However, though aggressive, bold and exploratory behaviours may be linked in many species it is currently unknown how parental care quality may alter these links. Because parental care quality can be influenced by the environment the parents inhabit or experience (Royle et al., 2012), environmental changes may result in offspring behavioural changes through parental care quality.

Parental care, through reducing predation or the perceived threat of predation may alter levels of aggression, boldness, or exploration in offspring. In turn this may have an impact on how offspring develop, interact with predators and acquire territories, mates, or food. In the obligate shell-spawning cichlid *Neolamprologus brevis*, males and females coexist during spawning in large shell patches, with many females and a single dominant male (Ota et al., 2012). While females spend time within the shells with the developing eggs, males patrol above the shell patch to fend off potential predators or competing males (Ota et al., 2012). Though *N. brevis*, have a clear division in effort during the brooding process both provide the investment necessary to reduce predation on offspring which may result in behavioural effects in the offspring. In this study, I aimed to investigate the effect reduced parental care quality, by examining the relationship between parental absence aggression, boldness and exploration in sub-adult offspring. I predicted that individuals with reduced parental care would be more aggressive, bold, active, and exploratory.

**5.2 METHODS**

**5.2.1 Study Species and Rearing Conditions**

Four pairs of *N. brevis* (three males were wild-caught from Lake Tanganyika and one was captive-bred and all females were captive bred) were housed in 48 x 26 x 34 cm tanks, on a recirculating filtration system. Tanks were maintained at a pH of 8.7 ± 0.2 and a
temperature of 26 ± 2 °C. Each pair was given two 90° PVC elbows (2 cm diameter opening) with a plastic cap on one end, to serve as simulated shells and spawning locations. PVC elbows were checked daily for the presence of eggs. When eggs were determined to be present, they were either left with the parents (parental presence) until the day the parents allowed the juveniles to leave the shell or were removed to isolate developing eggs from the parents (parental absence). Isolated offspring were moved to a 1 L container with an air stone, placed within a water bath to control temperature. Water was changed daily and checked for juveniles outside of the PVC elbow. When juveniles emerged from the shelter, they were moved to a 5 L tank on a central filtration system and fed powdered commercial flake food three times a day, until they were three months old. At three months fish were moved (kept within their family groups) to 48 x 26 x 34 cm tanks on a central filtration system, where they remained. When fish were six months old, and beginning to sexually mature, behavioural assays began.

5.2.2 Behavioural Assays

Behavioural assays took place inside a 45 x 20 cm tank, which had a 3 x 3 cm grid drawn on the outside of the bottom of the arena and was then painted white along with three external walls. The arena was filled with 8.5 cm of filtered tank water, to reduce any stress from change in water chemistry. The week that fish reached six months post fertilization behavioural assays began by catching fish from their home tank via dip net and immediately transferring them to a transparent container filled with tank water. The transparent container was used to transfer fish to the opaque starting cylinder within the experimental arena without air exposure, when the fish were placed inside the start cylinder the transfer container was inverted on the top of the cylinder to prevent fish from jumping out (Fig. 5.1A). The starting cylinder measured 6 cm in diameter and 10 cm in height and was located on either the left or right side of the experimental arena. Two plastic plants were placed to the left and two to the right of the cylinder, serving as a covered area, and the fish remained within to acclimate for five minutes (Fig. 5.1B). At the end of the acclimation period the cylinder was lifted, and the fish were allowed to explore for 20 minutes – referred to as open field assay from here forward (Fig 5.1C). At the end of 20 minutes the fish was caught with the transparent container, plants were removed, and the opaque cylinder was placed on the opposite end of the arena from the previous location, but in the same configuration (Fig. 5.1A). Fish were transferred to the starting cylinder and left for five minutes. During acclimation a mirror was placed on the opposite end of the arena from the start cylinder (Fig. 5.1D). At the end of acclimation, the cylinder
was lifted, and the fish were allowed to explore for 10 minutes – referred to as mirror assay from here forward. Following the aggression assay standard length (SL) was measured to the nearest 0.1 mm and sex was determined by examining the vent of the fish. Due to technical errors with some of the videos, final sample sizes for the boldness assay were as follows: \( N_{\text{ParentalAbsence}} = 35 \) and \( N_{\text{ParentalPresence}} = 27 \). While sample sizes for the aggression assay were \( N_{\text{ParentalAbsence}} = 35 \) and \( N_{\text{ParentalPresence}} = 25 \).

**Figure 5.1. Diagrams and images of the arena setup.** The boldness arena (A) had plants on either the left or the right side of the arena for shelter allowing the fish to either hide or explore. In the aggression arena (B) the mirror was placed on the side opposite of the plants to reduce any bias in sides.

5.2.3 Video Analysis

Aggression, boldness, and exploration were quantified from the videos of the two assays (Table 5.1). Due to camera issues the mirror assay was shortened to eight minutes so that all videos were the same length.

Aggression was measured by using Solomon Coder 17.03.22 (Péter, 2017) to count the number of times the fish made contact with the surface of the mirror and amount of time between leaving the start area and making first contact. Technical errors in the videos resulted in final sample sizes of \( N_{\text{ParentalAbsence}} = 35 \) and \( N_{\text{ParentalPresence}} = 25 \).

Start latency in the mirror assay was considered a proxy for bold behaviour and was also quantified with Solomon Coder 17.03.22 (Péter, 2017). In addition, boldness was measured from the open field assay by quantifying latency to enter the open, and time spent near the covered area EthoVision XT 13.0 (Noldus Information Technology, Netherlands). Ethovision was also used to determine exploration, distance to the covered area, and activity, velocity when in the open area. Fish that never left the covered area were given a latency and time spent near the covered area score of 1200, as well as zeros for distance.
from cover and velocity. Due to technical errors with some of the videos, final sample sizes for the boldness assay were as follows: $N_{\text{Parental Absence}} = 35$ and $N_{\text{Parental Presence}} = 27$.

**Table 5.1. Definition of behaviours.** Behavioural definitions used for aggression (bite latency and bites per second), boldness (start latency, latency to enter open area, and mean distance to cover), exploration (time near cover) and activity (velocity).

<table>
<thead>
<tr>
<th>Behaviour</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Start Latency</td>
<td>Time of leaving start - time cylinder was lifted</td>
</tr>
<tr>
<td>Bite Latency</td>
<td>Time of first bite - start latency</td>
</tr>
<tr>
<td>Bites per Second</td>
<td>Number of bites / time from first bite to end of video</td>
</tr>
<tr>
<td>Latency to Enter Open</td>
<td>Time left cover - time cylinder was lifted</td>
</tr>
<tr>
<td>Time Near Cover</td>
<td>Time spent within 3 cm of cover</td>
</tr>
<tr>
<td>Mean Distance to Cover</td>
<td>Average distance to cover over 20 minutes</td>
</tr>
<tr>
<td>Velocity</td>
<td>Total distance travelled / time in open</td>
</tr>
</tbody>
</table>

### 5.2.4 Statistical Analysis

A Spearman rank correlation matrix, in the package “Hmisc” v4.1-1 (Harrell, 2018), was conducted to determine if behaviours were correlated across contexts before performing a principal component analysis.

A principal component analysis was conducted to more closely examine the relationship among behaviours across the two contexts. Loadings above the absolute value of 0.30 were considered important to that component. Components which explained a high amount of variation and contained variables that pertained to unique behaviours were examined with a linear mixed effects model. While correlations in the data set which included all individuals was more similar to that of parental absent group than the parental present (Table 5.2) and PCAs were conducted on all three data sets (parental absent and parental present PCAs in Table 5S.1), in order to statistically compare differences in correlated behaviours only the PCA which included all individuals was used for subsequent analysis.

The response variables were the pc scores, with explanatory variables of care treatment, sex, standard length, time of day the assay was conducted (morning, afternoon or evening), and an interaction of care treatment and sex were fitted as fixed effects with brood as a random effect. Model selection occurred in a stepwise down fashion, eliminating non-
significant effects and comparing models using AICs (Burnham & Anderson, 2002). Models were fitted using the package “lme4” (Bates et al., 2015). Model fits were assessed using the package “piecewiseSEM” (Lefcheck, 2016). The effect of parental presence on standard length was also determined using a linear mixed effects model with care treatment, sex and their interaction as fixed effects. Brood was fitted as a random effect and model selection was done as previously described. Where interactions were present, the pairwise comparisons of care treatment effects within the sexes were completed using the “emmeans” package (Lenth, 2019).

The differences of variance between the care treatment groups on PC1, PC2, and SL were determined using a Fligner-Killeen test of homogeneity. The Fligner-Killeen test was used rather than Levene’s test due to the data being zero-heavy and containing outliers. In this test p<0.05 indicates that the data are not homogenous in their variance.

All figures were completed using the packages “ggplot2” (Wickham, 2016) and “gridExtra” v2.3 (Auguie, 2017).

5.3 RESULTS

5.3.1 Behavioural Correlations

All behaviours, except for two, were correlated within and across contexts (Spearman correlations, Table 5.2). When all individuals were included in the correlation analysis, start latency in the mirror assay was positively correlated with latency to leave the covered area in the open field assay. Bite latency in the mirror assay was also positively correlated with latency to enter the open area. Bite rate was also positively correlated with time near the cover, and mean distance to cover, as well as negatively correlated with latency to enter the open area. However, velocity and bites rate were not correlated. When parental present and parental absent treatment groups were compared, correlations and trends among behaviours were different and there were much fewer significant correlations in the parental present treatment group.

5.3.2 Principal Component Analysis of Behaviours

The first principal component captured 56.9% of the variation and was characterised by increased start and bite latency (aggression assay), longer to leave cover, and reduced distance from cover (Table 5.3). Based on PC1 loadings, individuals with higher PC scores
on PC1 were shyer, less likely to initiate aggression, and less exploratory. Thus, PC1 is referred to as timidity, from here forward. The second principal component captured 17.7% of the variation and was explained by increased bite rates, longer time to leave cover, less time near cover, and less distance travelled. Based on PC2 loadings, individuals that had increased aggression, took longer to initiate boldness but maintained it longer and were less exploratory. Therefore, PC2 is referred to as escalation, because individuals with high scores on this component will escalate aggressive interactions even though they are inactive.

In the parental present treatment group, PC1 captured 52.5% of the variation and indicated that individuals with a higher score were bold, exploratory, active, but non-aggressive (Table 5S.1). Meanwhile, PC1 in the parental absent treatment group captured 56.8% of the variation. Individuals with a higher score on this PC were shy and aggressive (Table 5S.1). However, as these PC’s capture similar behaviours, though inverted, in both treatment groups there is a negative correlation between boldness and aggression.

5.3.3 Effect of Parental Presence on Timidity, Escalation and Standard Length

Timidity was not influenced by care treatment or any other explanatory variable measured (Table 5.4; Fig. 2). Escalation was not influenced by care treatment or offspring sex (Table 5.4; Fig. 5.2). However, larger individuals escalated aggression less than smaller individuals (Table 5.4).

Standard length was marginally influenced by an interaction of care treatment and sex (Table 5.4; Fig. 5.3). Specifically, males reared in the absence of parents were larger than males reared with parents ($t_1 = -2.492, p=0.037$). However, there was no difference in SL between females in either care treatment ($t_1 = -1.772, p=0.127$).

Variation in timidity ($\chi^2_1=13.094, p<0.001$) and escalation ($\chi^2_1=13.094, p<0.001$) were significantly higher in the parentally absent groups (Fig. 5.2 A&B). Variation in SL was not significantly different between the parental care treatments ($\chi^2_1=1.595, p=0.207$). However, for males, variation in SL was higher in the parentally absent treatment group ($\chi^2_1=5.882, p=0.015$), while there was no difference between care treatments within the female group ($\chi^2_1=0.154, p<0.695$) (Fig. 5.3).
Table 5.2. Spearman rank correlations. Spearman rank correlation matrix of behaviours from the behavioural assays for all, parental present and parental absent treatment groups. Significant (p<0.05) correlations are indicated in bold, while marginal (0.05<p<0.08) correlations are underlined.

<table>
<thead>
<tr>
<th></th>
<th>Start Latency</th>
<th>Bite Latency</th>
<th>Bites per Second</th>
<th>Latency to Enter Open</th>
<th>Time Near Cover</th>
<th>Mean Distance to Cover</th>
</tr>
</thead>
<tbody>
<tr>
<td>All individuals</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>r</td>
<td>-0.44</td>
<td>-0.55</td>
<td>0.42</td>
<td>0.46</td>
<td>-0.44</td>
<td>-0.35</td>
</tr>
<tr>
<td>p</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.007</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parental Present</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>r</td>
<td>0.13</td>
<td>-0.16</td>
<td>0.06</td>
<td>-0.06</td>
<td>-0.16</td>
<td>0.07</td>
</tr>
<tr>
<td>p</td>
<td>0.534</td>
<td>0.451</td>
<td>0.24</td>
<td>0.764</td>
<td>0.767</td>
<td>0.749</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parental Absent</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>r</td>
<td>-0.66</td>
<td>-0.71</td>
<td>0.59</td>
<td>0.67</td>
<td>-0.57</td>
<td>-0.55</td>
</tr>
<tr>
<td>p</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
Table 5.3. Loadings from the principal component analysis. Behaviours on PC1 indicate a timid-initiative personality on the individuals, while PC2 indicates an aggression escalation-aggression maintenance. Loadings that contributed to the component are indicated in bold.

<table>
<thead>
<tr>
<th></th>
<th>Timidity (PC1)</th>
<th>Escalation (PC2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Start Latency</td>
<td>0.406</td>
<td>-0.298</td>
</tr>
<tr>
<td>Bite Latency</td>
<td>0.410</td>
<td>-0.263</td>
</tr>
<tr>
<td>Bites per Second</td>
<td>-0.200</td>
<td>0.679</td>
</tr>
<tr>
<td>Latency to Enter Open</td>
<td>0.436</td>
<td>0.254</td>
</tr>
<tr>
<td>Time Near Cover</td>
<td>0.453</td>
<td>0.188</td>
</tr>
<tr>
<td>Mean Distance to Cover</td>
<td>-0.401</td>
<td>-0.075</td>
</tr>
<tr>
<td>Velocity</td>
<td>-0.265</td>
<td>-0.525</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>1.996</td>
<td>1.113</td>
</tr>
<tr>
<td>Proportion of Variance</td>
<td>0.569</td>
<td>0.177</td>
</tr>
<tr>
<td>Cumulative Proportion</td>
<td>0.569</td>
<td>0.746</td>
</tr>
</tbody>
</table>
Table 5.4. Results of linear mixed effects models on the first two principal components. Significant explanatory variables \( p<0.05 \) are indicated in bold font and marginal effects \( 0.08<p>0.05 \) are indicated by underline. The effect sizes of the model are given as R2M (marginal – fixed effects only) and R2C (conditional – fixed and random effects), and baselines are parents present and female. Baselines for the models are Female and Parents Present.

<table>
<thead>
<tr>
<th></th>
<th>Estimate</th>
<th>Std. Error</th>
<th>D.F.</th>
<th>t-value</th>
<th>p-value</th>
<th>( R^2_M )</th>
<th>( R^2_C )</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>STANDARD LENGTH</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>26.371</td>
<td>1.499</td>
<td>7.291</td>
<td>17.597</td>
<td>&lt;0.001</td>
<td>0.391</td>
<td>0.839</td>
</tr>
<tr>
<td>Care Treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parents Absent</td>
<td>3.180</td>
<td>1.846</td>
<td>7.450</td>
<td>1.722</td>
<td>0.126</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>2.484</td>
<td>0.593</td>
<td>53.271</td>
<td>4.189</td>
<td>&lt;0.001</td>
<td>0.108</td>
<td>0.408</td>
</tr>
<tr>
<td>Care Treatment*Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent*Male</td>
<td>1.575</td>
<td>0.855</td>
<td>54.047</td>
<td>1.842</td>
<td>0.071</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>TIMIDITY (PC1)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>-0.932</td>
<td>0.747</td>
<td>7.006</td>
<td>-1.247</td>
<td>0.252</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Care Treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parents Absent</td>
<td>1.343</td>
<td>0.925</td>
<td>7.286</td>
<td>-1.452</td>
<td>0.188</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>ESCALATION (PC2)</strong></td>
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Figure 5.2. Relationship among parental absence, timidity, and escalation. Principal component scores for PC1 (Timidity; A) and PC2 (Escalation; B) as it is related to parental absence (blue) or presence (orange). Individuals are represented as filled circles and the mean of each care treatment is indicated with a black square.
**Figure 5.3. Relationship between parental absence and standard length.** Standard length of individuals as it relates to parental presence, represented as blue (absent parents) and orange (parents present) filled circles. The mean of each care treatment is indicated with a black square. Female offspring are in the left panel and males are on the left.

5.4 DISCUSSION

The results here indicate that correlations of individual boldness, aggression and exploratory behaviours are present among individual *Neolamprologus brevis*, which are not under the direct influence of parental presence during early development. Though parental investment may impact expression escalation of aggression indirectly through effects on offspring size. Overall these results suggest that the development of behaviours necessary for growth and reproduction is more strongly influenced by other environmental or genetic factors, which may help to reduce potential negative effects of reduced parental investment.
The increased size of males and females with absent parents in this study may give these individuals a greater chance of success in territorial disputes over smaller individuals. In wild populations of *N. brevis* larger males are more often those that guard shell patches of gravid females (Ota *et al.*, 2012). This is not uncommon in other species, where larger males hold more territory and have greater access to mates or better resource quality (Gabor, 1995; Serrano-Meneses *et al.*, 2007; Caro & Collins, 2010). In nest guarding species females prefer larger males due to their potential increase in success against predators (Côte & Hunte, 1989), or larger territories and better body conditions (Moginie & Shima, 2018). The increase of male size with parental absence in the present study may allow these individuals a way to compete for reproductive opportunities with individuals that had a less stressful start to life. Additionally, in the wild shells in the centre of the patch would be more protected and may be more desirable to females. In this way larger females would potentially be able to outcompete smaller females for these more desirable shells, increasing probability of offspring survival. While in this study I did not look at traits relating to reproduction, it is possible that parentally absent males which may have lower survivability, reach reproductive size early to compete with males who have higher survivability and increased reproductive output at later life stages.

The increase in size of male and female offspring that were reared in parental absence may also be due to compensatory growth resulting from a resource poor upbringing. In this study, I observed both male and female parents bringing food to offspring. Offspring that were reared in parental absence did not have this benefit and ventured from the pipe at an earlier age (often a week or more earlier). While the increased foraging, and decreased competition, of offspring from parentally absent treatment groups may have resulted in larger individuals in the lab setting, in the wild leaving an unattended shell would have a high probability of resulting in mortality. In the lab setting, parentally absent offspring had to forage to get food from the tank floor and expend more energy, which may have altered nutritional availability between care treatments. It is possible that offspring which experienced this poor nutritional environment at early stages experienced some degree of compensatory growth when fish from both treatment groups were placed into the same nutritional environment (Wilson & Osbourn, 1960; Metcalfe & Monaghan, 2001; Ali, Nicieza & Wootton, 2003). However, catchup growth rates often have a trade-off of potentially reducing reproductive output or increasing mortality later in life (Metcalfe & Monaghan, 2001; Ali *et al.*, 2003).
Both timidity and escalation have benefits and costs in the wild. Individuals that are timid or shy may have increased survivability when predation is high, but low foraging rates when predation is low (Sih et al., 2004; Stamps, 2007). Aggressive individuals may be able to defend territories from conspecifics, but run the risk of injury or mortality and energetic costs when engaging in such an interaction (Jaeger, 1981; Marler & Moore, 1988; Makowicz & Schlupp, 2015). Activity and exploration have similar costs and benefits to boldness, in that individuals may have higher predation risk and energy expenditure but have higher foraging success (Sih et al., 2004). In this study individuals from one care treatment were not more likely to be on either end of the spectrum, in either suite. However, parental absence resulted in offspring which existed on both ends of the spectrum, while those reared by parents exhibited much less variation. In this way parental presence may be directing offspring development along a path that is more suited to the current environment. Meanwhile, offspring that are not under the influence of parental presence are more varied in the development of behaviours which could allow for increased survivability in unpredictable environments.

In this study the individuals which escalated aggression the most were the females reared by their parents. In wild populations of *N. brevis*, the highest ranking female has the best located shell in the patch (Ota et al., 2012). As aggression is necessary for holding territory (Yasukawa, 1979; Watson & Milbrandt, 1990; Perrone et al., 2019) and social rank (Favat et al., 2017) parents could be increasing the probability of their female offspring having greater reproductive success through their investment. In a species where females compete for males, *Poecilia formosa*, smaller females were more aggressive and escalated their aggression faster in competitive environments, perhaps to overcome a smaller size (Makowicz & Schlupp, 2015). The escalating aggression observed in the current study may provide a similar opportunity for smaller females to compete for better quality resources in a highly competitive breeding environment. In this way parents may increase their own fitness through providing increased investment into their offspring.

While the variation of both suites of behaviours and standard length was greater in the parentally absent treatment group, the differences between the treatment groups were not significantly different. It is also of note that parental presence results in some different sets of correlated behaviours than parental absence. This may indicate an underlying mechanism for phenotypic development that could buffer against poor quality maternal care. In the present study, offspring were removed from their parents but left to develop with siblings, which could have acted as a social buffer on the development of behaviours.
Indeed, in another social species, *Pelvicachromis taeniatus*, isolation from both siblings and parents during early development resulted in deprecated social abilities (Hesse & Thünken, 2014; Hesse et al., 2015). A similar effect can be seen in human children, where sibling support can help to overcome some of the negative social effects of lack of maternal involvement (Milevsky & Levitt, 2005; McHale, Updegraff & Whiteman, 2012). As *N. brevis* is a social species, it may be the case that behavioural development is influenced by being raised with siblings. Further research into this effect of siblings as a potential buffer for early life stress, could yield insights into behavioural development in other social species, including humans.

5.4.1 Conclusion

The results of this study give insight into the behavioural development of a social species, in which parental investment does not change the mean expression of the behaviour within the population but may be able to influence the range of expression. Variability in parental care is as widespread as parental care itself, and poor parental care can result from resource poor or high stress environments. Variability in offspring phenotypes in such an unpredictable environment may be beneficial for increasing the overall survival of a brood in one such environment. Further, this study indicates that there may be an environmental buffer to poor parental investment, possibly sibling presence during development.

5. Supplemental

**Table 5S.1. Loadings from the principal component analysis.** Loadings that contributed to the component are indicated in bold. Loadings are given for both the parental present and parental absent treatment groups.

<table>
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<td>PC2</td>
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Chapter Six: General Discussion

Throughout this thesis, I examined the effect of parental care on offspring craniofacial shape, brain anatomy and behaviour. To conclude, I will discuss how the research presented here contributes to our knowledge of maternal care effects and provides insight into how early life effects, such as parental care, can influence phenotypic development.

In Chapter Two, results indicated that the duration of maternal mouthbrooding differentially affected craniofacial shape in a species-specific manner. But overall at the species level, shortened maternal care caused a less convex face shape. This change in convexity could result in offspring which exploit different food sources than their parents, especially in highly competitive environments such as the African Great Lakes, where niches are highly partitioned, and species are uniquely adapted to exploit resources in a way that minimises competition. Instances of niche partitioning by morphology can be seen in examples of adaptive radiations such as the Anolis spp. lizards of the Caribbean Islands (Butler, Sawyer & Losos, 2007) and Darwin’s finches on the Galapagos Islands (Grant & Grant, 2002). While ecomorphology, or how the morphology of an individual functions within its ecology, has been studied for its role in evolution for quite some time (Losos, 1990), the role of the maternal environment in shaping the development of functional structures has not been studied.

The classic examples of adaptive radiation and ecomorphology of Darwin’s finches and Anolis spp. perhaps aren’t well suited for the investigation of parental care effects on morphological development. Commonly studied Anolis spp. do not provide maternal or paternal care after offspring hatch (Stamps, 1978; Cox et al., 2011). Though birds provide high levels of parental investment and bills do show morphological plasticity in response to the early environment (Burness et al., 2013), the effect of maternal care quality or duration on bill morphology remains unstudied. The cichlids of the African Great Lakes are a commonly cited example of adaptive radiation, and all of the more than 2500 species provide some form of parental care (Barlow, 2000). The result of this many species is a highly competitive environment, both within and among species. The ease at which fish densities can be manipulated allows for investigation of within species competition effects on maternal care duration. The stress of competition from high density environments may result in females that release offspring early to acquire food sources before others, or later to be larger and more competitive for food sources against smaller offspring. This would
allow for investigation of effects of the competitive environment, within or even between species. Furthermore, broods of African cichlids vary in size among species, and individuals, and larger broods allow for creating full sibling offspring reared in different environments, with adequate sample sizes for investigation of developmental effects. The ease at which the environment both the mother and offspring experience can be manipulated, therefore makes African cichlids particularly suited to studied effects of maternal care duration.

African cichlids have a range of craniofacial shapes, with an extreme overbite as seen in *Labeotropheus fuelleborni*, to the extreme underbite seen in *Caprichromis orthognathus* (Powder & Albertson, 2016). The underbite in *C. orthognathus* serves as a battering ram that the fish used to attack from underneath a mouthbrooding female, forcefully ejecting her brood for the paedophageous predator (Barlow, 2000; Powder & Albertson, 2016). It is interesting that, in Chapter Two, as care duration was decreased in the predatory *Dimidiochromis compressiceps*, the shape of the face began trending toward the concavity observed in *C. orthognathus*. This change in curvature could have resulted in a different food choice in offspring with shorter maternal care durations. If these reduced-care offspring prefer to feed on invertebrates from the water column by attacking from below, it may have begun to strengthen the observed shape towards a more concave extreme. Future work with feeding trials that examine food preferences of individuals within species based on their craniofacial shape could provide insight into this possibility.

Food preferences can be learned by observing conspecifics (Mason, Arzt & Reidinger, 1984; Sherwin, Heyes & Nicol, 2002) and social cues can provide individuals with information on when, where, how and what to eat (Galef & Giraldeau, 2001). In the wild, mouthbrooding African cichlids often care for young even after they are free-feeding, allowing them to venture out of the safety of the buccal cavity to forage and taking them back in when predators are nearby (Barlow, 2000). In one Lake Tanganyikan species, *Tropheus moorii*, mothers take strands of algae into their mouth to allow offspring to feed during the yolk-sac stage of development (Schürch & Taborsky, 2005). In wild situations where mothers shorten their brooding length, any craniofacial changes may not result in differences in food choices if mothers bring their offspring to food patches that don’t match the changes in the shape of the face, but rather match the typical diet for the species. However, as mothers may feed on different foods than offspring, particularly in predatory species, offspring may not be directly copying their mothers to learn feeding mechanisms. For example, if juvenile *D. compressiceps* feed from below prey, rather than suctioning
from in front of them, this may compound any effects of facial concavity by strengthening muscles that further alter the shape of the jaw and face. Feeding trials in which foods are offered in ways that require different mechanisms to obtain food could be used to determine if craniofacial shape rather than socialisation drives feeding style. Continuing these trials over the course of development could also be used to determine if feeding styles further compound craniofacial shape changes. If maternally induced changes in craniofacial convexity results in offspring that have different preferences in food choices, this could indicate a way for maternal care duration to have contributed to the niche occupation and adaptive radiation of African cichlids. The species used in Chapter Two, all had different average maternal care durations and individual females within each species varied around that average. The variation of maternal care duration among individual females may have helped to provide craniofacial modifications that allowed for the exploitation of new niches. However, more investigation is required to explore this theory.

In many respects, cichlid fishes can be used to represent craniofacial variation in humans, because of their gradient of jaw lengths from an overbite to an underbite, with everything in between (Powder & Albertson, 2016). If we expand this to examining how changes in maternal care duration can change craniofacial shape it may be possible to look at the effects of the duration of nursing in mammals or humans. Variation around average nursing times commonly occur in marine mammals (Maniscalco, 2014; Matthews & Ferguson, 2015), non-human primates (Harvey & Clutton-Brock, 1985) and humans (Blyth et al., 2002). Particularly in humans, the duration of breastfeeding can be affected by socio-economic drivers. Employment status and the accommodations available from employers can affect how long a mother breastfeeds her offspring (Hawkins, Magurran & Armstrong, 2007). In addition, lack of confidence in maternal ability that stems from living in poverty can reduce breastfeeding duration (Blyth et al., 2002). These are only a sample of reasons why breastfeeding duration may vary among humans. The mechanics of breastfeeding results in an increase in the musculature of the jaw and results in shaping the oral cavity in a manner that is consistent with the naturally-evolved development, which is disrupted by bottle feeding (Palmer, 1998). Furthermore, genes involved in craniofacial development in humans, such as the Wnt/β-catenin signalling pathway, are highly conserved (Wang, Song & Zhou, 2011) and active in the craniofacial development of African cichlids (Parsons et al., 2014). The changes in cichlid craniofacial shape based on maternal care duration, may allow for the closer examination of these changes in craniofacial shape in humans based on bottle feeding. Using cichlids as a system to
investigate human altered craniofacial shapes could provide a system in which effects and potential treatments could be studied more readily.

The ability of maternal effects to last into adulthood was investigated in Chapters Three and Four. While Chapter Three indicated that this can indeed occur, chapter Four indicated that there may be an expiration date on maternal care effects on brain anatomy and behaviour. However, the smaller sample size in Chapter Four may also contribute to the lack of observed differences in brain volumes or behaviours in the care treatment groups. In both chapters, however, the telencephalon and hypothalamus had significant associations with behaviours, particularly boldness and aggression.

The telencephalon in fish shares homologous neural areas with the mammalian hippocampus (O’Connell & Hofmann, 2011; Bshary et al., 2014) and abnormalities in this region can increase impulsive aggression in mammals (Nelson & Chiavegatto, 2001). However, in neither Chapter Three nor Four was aggression linked with telencephalon volume. The lack of a link between the telencephalon and aggression in this thesis could suggest that volume of a brain region is not the best measure for examining neural density, as densities could differ between subregions associate with specific functions. Future investigation of this relationship through neural staining could perhaps provide insight into the lack of relationship with total telencephalon volume and aggressive behaviours. However, the positive relationship between telencephalon and boldness (Chapter Four) could be related to impulsiveness. The measure of boldness used in Chapter Four, latency to leave the start area in the mirror assay, could have been related to aggression as leaving the start area may have been triggered by seeing the reflection in the mirror. Therefore leaving the start area could indicate a higher level of aggressive impulsiveness in individuals with smaller telencephala.

The effects of maternal care on aggression in a competitive scenario proved difficult to examine and so there remain several questions regarding my findings in this area. Difficulties stemming from aggression within the holding tank resulted in some fish not being able to be used twice and, instead, the use of a sequential pairing system for staging competitive interactions. This system of pairing fish meant that if one fish was used once, its partner was sometimes used twice, and those partners were not all used twice. Additionally, although fish were identified to be male as best as possible, when they were dissected to verify sex, it was determined that some females with male-like external traits had been included in the competitive assays. Being paired with a female may have resulted in a different level of aggression than would have been displayed against a male.
competitor. Because of this, those males were also removed from the analysis. All of these issues resulted in a small data set with aggressive displays that were dependent on the aggression displayed by the competitor. For future work in this area, a resident-intruder design may be a better way to quantify how maternal care duration affects aggression in adult offspring. Another approach may be to test fish against more than one competitor of each treatment. Additionally, there may be merit in providing fish with viewing a video of a similarly sized fish exhibiting a set routine of behaviours (Chabrolles et al., 2017; Wackermannova et al., 2017), which may serve to minimise the effects stemming from variation in competitor behaviours.

Chapter Five examined how isolation from parents during development alters behaviours in a species with bi-parental care. There were no direct effects of parental presence on behaviour, but parental presence influenced the size of offspring which in turn influenced the escalation of aggression. The results also suggested that males were compensating for a poor early life start by increasing growth rates once food availability was equalised between the two treatment groups. However, females reared in isolation which were the smallest individuals may have compensated for a poor life start by increasing aggression. In zebra finches, Taeniopygia guttata, compensatory growth trade-offs have sex-specific effects (Arnold et al., 2007). Neolamprologus brevis appears to also be exhibiting a sex-specific effect when compensating for poor early starts. Notably for this species, males became aggressive at a young age and would chase other fish out of small holes in the tank lids, which may have resulted in selection for larger or more aggressive males within each family. The reduction of brood sizes due to aggression also resulted in fish reared in different densities which may have had some effect on the displayed behaviours. However, aggression within the rearing tanks did not appear to differ between the two treatments but this is something that future studies could examine.

During the course of the experiment I observed both male and female parents spitting mouthfuls of chewed flake food into the mouth of the PVC pipe, presumably to feed growing young. For the young reared in isolation I did not begin feeding them until they ventured from the PVC pipe, at which point food was mixed with water and transferred with a pipette to the bottom of the tank. Young reared in isolation of parents had to expend energy to feed, while young reared with the parents were able to conserve their energy stores and gain additional food. Though young reared by their parents may have competed for food, those reared in isolation of their parents also had competition for food patches. Males also positioned PVC pipes in a location in the tank where the current from the
bulkhead pushed the flow of water into the PVC pipe. Because of this positioning small food particles would often flow into the shell, resulting in the female not needing to move far from the pipe to feed. As females of shell dwelling species often fight for the best shell location, some of this may be due to currents and food opportunities where they do not need to venture far from the shell to feed. This effect of maternal rank on potential resource allocation to offspring is another way in which maternal effects could influence growth of offspring.

In the wild *N. brevis* males patrol shell patches, which allows females to spend time within the shells tending to and guarding eggs (Ota et al., 2012). In addition, males position shells, using their larger teeth and greater body weight to accomplish this task. Though apart from the observed potential food provisioning, males do not directly interact with offspring during their development. With the disparity in time investment to offspring, it would be interesting to examine how offspring reared in a condition which is between parental absence and presence, for example female only or male only care, would behave in the same scenarios. Male guarding of the shell allows the female to invest more time into fanning eggs, removing debris or finding food then if males are removed from the scenario would females adjust the time spent within the shell to be able to guard or forage? Investigating if male absence results in variation in brood quality would be an interesting route to investigate paternal effects on offspring phenotypes.

Overall the results presented in this thesis indicate that variation in maternal care duration results in variation in offspring phenotypes. This variation may allow individuals to adapt to harsh or unpredictable environments. As the effects of maternal care last into adulthood, there may be effects of maternal care variation on reproductive success which could then affect the next generation. Future investigation could examine reproductive success and mate selection in offspring with reduced duration maternal care.

Furthermore, these results indicate that African cichlids provide a system in which maternal care effects can be studied. All African cichlids provide some form of parental care, and examples of every type of parental care can be found within this family (Barlow, 2000). Using species from this family, which have large broods and where environmental variables are easily manipulated would allow for further investigation of the roots of variation. As the first environment any offspring experiences is generated by their parents, using fish as a system to investigate the effects of this early environment allows insight into the variation at the earliest stages. Development of African cichlids is also known with stages clearly defined (Fujimura & Okada, 2007). In fish which develop outside of the
mother or can be easily removed without causing too much undue stress to the mother, these early developmental stages can be easily identified in offspring and variation at these stages can be easily monitored.

Finally, the results here indicate that there is a role for variation in maternal care among females in evolution through the association with varying phenotypes and levels of plasticity. In this way, the maternal environment, as the earliest environment an offspring experiences, has the ability to shape trajectories of populations through morphological and behavioural phenotypes.
References


Appendix

Chapter A.1: Oil exposure alters social group cohesion in fish

This chapter was co-written with Alexis Khursigara, Professor Shaun Killen, Hannah Fearnley, Doctor Kevin Parsons and Doctor Andrew Esbaugh and has been accepted for publication in Scientific Reports. Alexis Khursigara and I completed the experiment and wrote the majority of the manuscript and are both named as co-authors.

A.1.1 INTRODUCTION

Animals living in groups often must coordinate movements with group mates in space or time, with varying degrees of synchronicity\(^1\)\(^2\). This type of social behaviour is particularly important, as group living may be beneficial for predator avoidance, foraging, and reproductive success\(^3\)\(^4\). This is especially true for fish, in which a large range of species spend some or all of their life within a group-living scenario\(^5\)\(^6\). Additionally, group movements allow individuals to reduce the energetic costs of swimming by taking advantage of vortices produced by groupmates\(^7\)\(^9\). In a shoal of fish, the series of individual decisions that result in cohesive collective behaviours are influenced by a range of abiotic and biotic factors, including food availability, water flow rate\(^2\), turbidity\(^10\), predator abundance\(^11\), and a suite of social cues\(^12\). In addition, social behaviours in fish are affected by various forms of anthropogenic environmental disturbance, including human-induced hypoxic episodes\(^13\)\(^14\), ocean acidification\(^15\), and anthropogenic noise\(^16\). Environmental contaminants such as industrial pollutants and heavy metals can also adversely impact fish social behaviour, such as schooling and courtship\(^17\)\(^18\). This is particularly relevant as group decision making, social learning, and group responses to potential threats\(^19\)\(^20\) are all dependent on the behaviours of individual group members, with key individuals often influencing the behaviour of an entire group\(^21\)\(^22\).

One of the most serious forms of anthropogenic disturbances is pollution from the release of petroleum products into aquatic environments\(^23\)\(^24\). Uncontrolled oil spills are the most well-known form of crude oil pollution, which was epitomized by the Deepwater Horizon spill that released over 700 million L of crude oil into the Gulf of Mexico over a period of 84 days\(^25\)\(^26\). However, oil is also routinely released into aquatic habitats via shipping, terrestrial runoff, and dumping. Oil toxicity is driven by a class of chemicals known as polycyclic aromatic hydrocarbons (PAHs), and marine fish are particularly susceptible to
these chemicals. Embryonic exposure can result in craniofacial and cardiac deformities that have been linked to mortality at very low concentrations (μg/L)\textsuperscript{27-31}. In juvenile and adult life stages, acute oil exposure impairs cardiac performance\textsuperscript{32-36}, which leads to reductions in swimming performance, maximum metabolic rate and aerobic scope\textsuperscript{37-40}. Similarly, acute oil exposure can also cause changes in routine metabolism\textsuperscript{41-42}. Importantly, the sublethal effects of an acute exposure event lasting only 24 hours can persist in the animal for multiple weeks\textsuperscript{39-40}, which raises concern about the long-term ecological performance of exposed individuals.

Despite well-known physiological effects of oil pollution, there is surprisingly little known about the negative impacts of oil exposure on fish social behaviours. Behavioural characteristics are crucial when extending organismal toxicology to ecologically relevant population-level effects, as described in the adverse outcomes pathway framework\textsuperscript{43}. Sociability, or an animal’s tendency to interact with conspecifics\textsuperscript{45}, plays an important role in shoal behaviours. Shoals comprised of more social individuals have higher shoal cohesion, though exhibit a reduction in average swim speed and social alignment\textsuperscript{46}. Further, individuals with lower sociability are more likely to swim faster and act as a leader within a school, with these individuals effectively initiating group movements\textsuperscript{46}. Sociability is in turn influenced by individual metabolism with less social animals having higher standard metabolic rates\textsuperscript{47}. Therefore, the previous mentioned negative impacts of oil exposure on metabolism\textsuperscript{37-40} and cardiac performance\textsuperscript{32-34}, could imply a direct pathway for oil exposure to influence shoal cohesion. This was highlighted recently in a study of coral reef fish following acute oil exposure, whereby exposed individuals showed a suite of behavioural changes – habitat usage, thigmotaxis, and basic aspects of shoaling behaviour – that significantly increased predation rates\textsuperscript{48}. Further, sensory abilities, such as vision\textsuperscript{49-50}, olfaction\textsuperscript{51}, and input from the lateral line\textsuperscript{52}, influence shoal cohesion, grouping choices, and coordinated movements. Any change in sociability, sensory ability, or locomotor capacity in any or all group members due to oil exposure could therefore disrupt overall group function with important ecological consequences.

To this end, the current study examined the effects of environmentally relevant levels of crude oil exposure on social behaviour and group cohesion in Atlantic croaker (\textit{Micropogonias undulatus}). This gregarious fish species is prevalent in the Gulf of Mexico and depends on estuarine environments, which can be particularly impacted by oil pollution. Specifically, we aimed to answer the following questions: 1) under acute oil exposure is shoal cohesion, or individual exploratory behaviours, different in an open-field
when compared to non-exposed groups?; and, 2) if a single individual is acutely exposed is the cohesion of the shoal, or the exploratory behaviours of other individuals altered?

A.1.2 RESULTS

A.1.2.1 Oil Chemistry Analysis

HEWAFS from both the low and high concentrations were analysed for 50 individual PAHs. As expected, for both HEWAFs, 2 ring PAHs were highest in abundance (47% and 53% in the low and high concentrations respectively) followed by 3 ring PAHs (41% and 36%), with the remaining being 4 and 5 ring PAHs (Figure A.1.1). Using initial and final concentrations, the average geometric mean (± SEM) for the low concentration was 6.0 ± 0.9 μg l⁻¹ ΣPAH₅₀ and for the high concentration was 32.9 ± 5.9 μg l⁻¹ ΣPAH₅₀.

**Figure A.1.1.** Relative composition of polycyclic aromatic hydrocarbons in the low and high concentration HEWAFs. 50 individual PAHs were measured and are shown on the X-axis. Dashed lines denote subclasses of PAHs.
A.1.2.2 Effects of Oil on Group Behaviour

While fish tended to increase their movement speed over the course of each 15 min trial, individuals in HO groups showed decreased speed of movement as compared to fish in all other treatments (Figure A.1.2 A). Fish in HM groups were closer to the arena wall when compared to all other treatment groups (Table A.1.1). Fish in HO and HM groups showed increased mean neighbour distances (Figure A.1.2 G; Table A.1.1). Within a given treatment there was generally large among-group variation for all behavioural indices, and overall, values for model $R^2_C$ was higher than $R^2_M$ (Table A.1.1, A.1.2).
Figure A.1.2. Responses to high (32.9 ± 5.9 μg l$^{-1}$ ΣPAH$_{50}$) or low (6.0 ± 0.8 μg l$^{-1}$ ΣPAH$_{50}$) oil exposure in juvenile Atlantic croaker in groups of four individuals swimming within an open field (n = 15 groups in total). Groups were composed of either control fish (that were not oil exposed; green); fish exposed to a high concentration of oil (dark blue); fish exposed to a low concentration of oil (light blue); three fish not exposed to oil plus one fish exposed to a high concentration of oil; or three fish not exposed to oil plus one fish exposed to a low concentration of oil. Each data point overlaid on the boxplots represents one fish within a group. Refer to Supplemental Tables 1, 2 and 3 for statistical comparisons among treatments.

A.1.2.3 Effects of Oil-Exposed Individuals on Non-Exposed Individuals within the Same Group

Within LM groups, treated individuals tended to be further from the arena walls than untreated fish (Figure A.1.2 F; Table A.1.3, A.1.4). While there were no differences in distance from the wall between treated and untreated individuals within the HM groups, this is likely because even untreated fish within high mixed groups were closer to the arena.
walls than fish in control groups (Table S2). Untreated fish in HM groups showed increased mean neighbour distances compared to fish in control groups comprised entirely of unexposed fish (Figure 2h; Table S2,S3).

**Table A.1.1.** Results of a linear mixed effects models examining the factors influencing behaviour in groups of fish receiving various levels of oil exposure. For the fixed effect of “treatment”, control groups, which received no oil exposure, are the reference level. Models included individual nested within group as a random effect.

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<td>0.029</td>
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Table A.1.2. Tukey HSD pairwise comparisons for the treatment levels for the models presented in Table A.1.1. HO = high oil; LO = low oil; HM = high mixed; LM = low mixed.

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**Table A.1.3.** Results of linear mixed effects models comparing oil exposed and unexposed within mixed groups, and to fish in control groups in which no fish were oil exposed. For the fixed effect “individual treatment”, control groups are the reference level. Models included individual nested within group as a random effect.

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Table A.1.4. Tukey HSD pairwise comparisons for the treatment levels for the models presented in Table A.1.3. HO = high oil; LO = low oil; HM = high mixed; LM = low mixed.

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<td>1.101</td>
<td>0.829</td>
</tr>
<tr>
<td>LM(exposed)-HM(unexposed)</td>
<td>4.112</td>
<td>0.003</td>
</tr>
<tr>
<td>LM(unexposed)-HM(unexposed)</td>
<td>1.402</td>
<td>0.364</td>
</tr>
<tr>
<td>LM(unexposed)-LM(exposed)</td>
<td>-2.711</td>
<td>0.146</td>
</tr>
<tr>
<td><strong>Mean distance to neighbours (cm)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HM(exposed)-control</td>
<td>0.172</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>HM(unexposed)-control</td>
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<td>&lt; 0.0001</td>
</tr>
<tr>
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</tr>
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<td>LM(unexposed)-LM(exposed)</td>
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<td>0.978</td>
</tr>
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</table>
A.1.3 DISCUSSION

The results here suggest that environmentally relevant oil exposure scenarios cause decreased cohesion in fish social groups. At the highest level of exposure, groups comprised of oil-exposed fish had increased distances between neighbouring fish within groups. This effect was also observed in groups that contained a single high oil-exposed fish in a shoal of four, whereby untreated fish within the same group showed disruptions to normal behaviour and greater distances between neighbours. Overall, these results indicate that exposure to oil pollution in aquatic environments has the potential to negatively affect fish social behaviours and group functioning.

The data presented here clearly demonstrate that an acute oil exposure of $32.9 \pm 5.9 \mu g l^{-1}$ $\Sigma PAH_{50}$ results in a less cohesive shoal and alters overall group behaviour. While acute oil exposure has a well-documented suite of sub-lethal effects on fishes, the severity of sub-lethal endpoints on overall ecological performance can be difficult to ascertain. The concept of ecological death is useful in this context, as it draws an equivalency between immediate mortality and any toxicological impairment that reduces the ability to perform in the environment and produce offspring\textsuperscript{12}. Shoaling for fish is a particularly noteworthy behaviour in this regard as it has been shown to have positive effects in the context of foraging and predator avoidance\textsuperscript{53}. For example, during predator attacks group cohesiveness allows groupmates to react to danger even when they themselves did not observe the attack\textsuperscript{19}, and coordinated movements of prey fish during predator-prey interactions can serve to confuse predators. Any reductions in shoal cohesion and coordination will preclude individuals from experiencing the benefits of group living, and therefore put the individuals at greater risk.

The overall reduced speed of movement among groups of oil exposed fish could also reduce foraging ranges and the likelihood of encountering food while exploring a given environment\textsuperscript{54}. Fish in swimming schools can also position themselves relative to groupmates such that they can reduce their own costs of movement by taking advantage of the vortices produced by other fish within the school\textsuperscript{8,2,55}. Individual fish also prefer specific spatial positions within schools (e.g. front versus back, edge versus centre) in relation to their own physiological and behavioural traits\textsuperscript{56,57}, possibly contributing to the establishment of leader-follower dynamics and formation of characteristic social networks\textsuperscript{58}. Reduced group cohesion may impact the tendency of fish to occupy their preferred or optimal positions within moving groups; however, further work is needed to
examine the extent to which oil exposure may impair the ability of individual fish to occupy their preferred spatial position within social groups.

Individual speed of movement is also a determinant of leadership during directed group behaviours, with individuals being attracted to more active conspecifics and those that display directional, linear movements\textsuperscript{16,59,60}. Reduced spontaneous activity therefore suggests that leadership capacity could be compromised in oil-exposed fish. Interestingly, however, exposed and unexposed fish in the HM groups all displayed decreased distances from the arena wall as compared to fish in other treatment groups. Although their ability to lead may be diminished, it is possible that exposed fish in HM groups were nonetheless altering the behaviour of unexposed groupmates in an emergent manner that is not possible when all fish in a group are oil-exposed. A tendency to remain closer to the wall in an open field test is generally interpreted as a reduction in boldness or risk-taking behaviour\textsuperscript{61}, and it is possible that abnormal behaviour in oil exposed fish elicited increased timidity in unexposed groupmates. This is the opposite effect to previous observations in larval red drum and coral reef species, both of which demonstrated a decrease in anxiety-like behaviour individually\textsuperscript{62} and in groups\textsuperscript{48}. Clearly, additional work is needed to investigate interactions between leader-follower dynamics and resultant levels of risk experienced by groupmates among fish with varying levels of oil exposure within the same social group.

It is notable that the negative effects on shoal cohesion occurred when even a single individual within the group was exposed to oil. It is important to remember that oil spills are heterogeneous events, and that the physiological effects of acute 24 h exposures have been shown to persist over prolonged time scales\textsuperscript{39,40}. As such, it is plausible that in the wild, fish with varying levels of oil exposure will interact as they migrate or otherwise move within their environment. The results here demonstrate that in smaller shoals, the presence of a minority of exposed individuals can disrupt the behaviour of the entire group. In other contexts, it has been observed that the presence of key individuals within a group can have a beneficial effect on group function\textsuperscript{21}. For example, social learning can allow naïve groupmates to more quickly gain knowledge of foraging patches or danger from more informed individuals\textsuperscript{20,59}. The ecological relevance of the influence of oil-exposed fish on the unexposed groupmates warrants further study, but this appears to be an example whereby one individual has a disproportionate effect on the behaviour of the entire group.

It has previously been speculated that individuals with particular behavioural or physiological traits may act as “keystone” individuals that have a disproportionate effect on the behaviour or success of entire groups\textsuperscript{21,58,63}. The current results also suggest that
individual sensitivity or exposure to pollutants, and other adverse environmental conditions, could induce similar effects on social groups stemming from disrupted behaviour in a minority of individuals.

In the current study, groups were relatively small, and so the particular behavioural tendencies of one individual would likely have a large influence on overall group behaviour, even in groups of fish receiving equal oil exposures (and including the control groups). It is notable that for mean neighbour distance, groups of fish receiving high oil exposures (for all fish or one fish within the group) showed greatly increased among-group variation compared to all other group types. Additional research is required to understand how the effects observed in this study scale up to larger group sizes, and whether larger groups have reduced among-group variation in the behaviour they display. In addition, fish social groups often display among-group assortment based on various morphological and possibly physiological characteristics. An interesting area for future work would be to determine how groups of fish with varying phenotypic composition may show differential group-level responses to oil exposure.

The underlying cause for the oil induced behavioural changes is not immediately clear. The established paradigm for oil toxicity in fish is that sensitivity is driven by cardiac malformations caused by 3 ringed PAHs. It is possible that the observed changes stem from altered metabolic characteristics, as a recent study demonstrated that similar oil exposure scenarios significantly reduced maximum metabolic rate and aerobic scope in this species. Prior work has demonstrated that individuals with a higher metabolic rate can be less social, presumably because they prioritize food acquisition over the safety of being in a group; however, such observations have not been extended to aerobic scope. It seems more likely that the mechanism relates to neurological or sensory impairment, both of which have recently been shown to be impacted in yolk-sac larval fish during developmental exposure. This hypothesis was previously posited to explain the changes in anti-predator behaviour observed in coral reef fish species, and the reduced prevalence of thigmotaxis (i.e. anxiety behaviour) in larval red drum. Such developmental effects could impact the ability to detect and respond to shoal-inducing sensory stimuli, or the tendency to generate such stimuli.

In our experimental protocol, we ran control groups first during each experimental day to eliminate risk of oil contamination between trials. It is therefore conceivable that temporal variation in groups or trial order may have contributed to the observed differences among treatments. Several lines of evidence, however, suggest that if such effects occurred, they
are small relative to the direct effects of oil exposure. Firstly, the direction of temporal effects on a given behavioural measure (e.g. movement speed, which increased with time in the control group) was opposite the effects of the oil exposure treatments. This suggests that even if there were temporal effects on behaviour it would have only served to reduce the magnitude of the observed effects from oil exposure. Furthermore, the HO and LO groups were run at the same time of day and yet still displayed differences in behaviour. Individuals in LO groups behaved more similarly to control groups than those in the HO exposed groups, indicating that changes observed in the HO treatment were the result of the exposure level and not time of day or trial order. Additionally, the total time for all trials to be completed was relatively short (e.g. within 2 hours), reducing the potential of a temporal or hunger effect on behaviour. It’s also important to note that in the HM groups, the unexposed individuals behaved differently than the oil-exposed fish within their group, again indicating that exposure level and not time of day or trial order was responsible for the observed changes in behaviour.

In summary, this study demonstrates that oil exposure alters social cohesion in sub-adult Atlantic croaker. Indeed, exposure of a single individual can alter the behaviour of the group. Importantly, the exposure scenarios used here are comparable to ΣPAH\textsubscript{50} concentrations found in the Gulf of Mexico following the Deepwater Horizon spill. Social behaviours are key to foraging, predator-avoidance, migration, and reproduction in most fish species, and so reduction in shoal cohesion is likely to have a range of adverse effects on these aspects of species’ ecology. Although additional work is required to more fully ascertain the mechanisms by which oil exposure alters behaviour, the data presented here provide added support for the recently described phenomenon of oil induced behavioural impairments in fish, while also highlighting novel effects of ecological significance. Future examinations of individual variation in sensitivity to oil exposure in a social context would provide further understanding of the selective effects of oil pollution on fish populations, and the potential implications for evolutionary trajectories. Within-generation effects of pollution on behavioural plasticity, and indeed any anthropogenic stressor, are likely to have consequences for important ecological phenomena such as fish migrations, spawning aggregations, and survival during the juvenile stages, in which fish frequently use shoaling as an anti-predator behaviour.
A.1.4 METHODS

A.1.4.1 Oil Preparation

To test the effects of oil exposure, oil was prepared following previously described standard protocols for high-energy water accommodated fractions (HEWAF)\textsuperscript{28-30}. Briefly, non-weathered oil collected from the source of a Massachusetts pipeline, an appropriate surrogate for Deepwater Horizon source oil, was loaded with seawater (35ppt) at a rate of 1 g per l. Oil was blended in a heavy-duty blender (Waring Commercial, Connecticut, USA) at a low setting for 30 s, and then placed into a Teflon 1 L sieve funnel for 60 minutes. The lower 85% of the WAF was removed and used to generate oil exposures. All HEWAFs were prepared fresh for exposures. Oil was delivered under proper chain of custody and was stored at 4°C until for roughly a year before used.

A.1.4.3 Fish and Exposure

To determine the effect of oil exposure on social behaviours we obtained 200 juvenile Atlantic croaker (9.8-15.1 cm standard length) from a commercial supplier. Fish were acquired in groups of 50 but were randomly split into smaller groups of 25 for acclimation. During acclimation, fish were held in 76 cm x 99 cm tanks, filled to 350 L, at 24 ± 1 °C, and maintained with sterilized seawater (35 ppt) for two weeks before experiments began. Holding tanks were large enough to maintain water quality for groups of 25 or less. To maintain fish at conditions that were consistent to their native environment in the Gulf of Mexico, filtered seawater was piped directly from the Gulf into the lab.

Twenty-four h prior to exposure, 12 fish were randomly selected from each group, anaesthetized with 250 mg l\textsuperscript{-1} of MS222 (buffered with 500 mg l\textsuperscript{-1} NaHCO\textsubscript{3}), weighed, measured for total and standard length, and fitted with either a yellow, orange, green or purple 7 x 6 mm plastic bead for identification purposes. The 12 fish were divided into groups of four, which would be used for the behavioural trials. Selection for groups was random though there was an effort to group together fish with a similar body mass (± 9.0 g). Fish were then allowed to recover (observed for return to equilibrium) for one h in an aerated opaque tank and returned to the original holding tank where they were food-deprived for 24 h prior to oil exposure. The following day the tagged fish were moved to individual aerated, 6.14 L tanks (24 x 16 x 16 cm), which were filled with fresh seawater, and exposed to one of three nominal concentrations of HEWAF (0, 0.7 and 2%) for 24 h.
Concentrations were chosen based on levels recorded shortly after the Deepwater Horizon spill in 2010\textsuperscript{69,72}, and have been used previously on this species\textsuperscript{62}.

The behaviours of the following group compositions were tested: control (\(N = 9\)), low-oil exposure (LO; \(N = 7\)), high-oil exposure (HO; \(N = 8\)), low-mixed exposure (LM; \(N = 7\)) and high-mixed exposure (LM; \(N = 9\)). The mixed groups contained three control fish and one exposed fish of the respective oil dose. Each individual within the group had a unique coloured bead, to allow for individual tracking and identification. A group of four fish was used to allow for the tracking of individuals within the group while also allowing all fish to move freely within the arena without constrained movement.

During the oil exposure protocol, standard water quality parameters were monitored, and seawater samples were taken for PAH analysis at the beginning and end of three exposures for each oil dose. Samples were taken throughout the course of the study. PAH analysis was performed commercially by ALS environmental under extraction protocol EPA 3510C and measurement protocol 8270D SIM. Samples were spiked with fluorine-d10, fluoranthene-d10 and terphenyl-d14 to assess extraction efficiency, with general recovery of >80%, >90% and >90%, respectively. Detection limits ranged from 4.5 – 20.5 ng l\(^{-1}\) depending on the specific PAH. All samples were stored at 4°C and delivered within one week of collection under proper chain of custody.

A.1.4.2 Behavioural Assays

To determine if exposure to oil influenced the exploration or group interactions of Atlantic croaker, the activity of groups of four fish were monitored in open-field tests. The arena for the open field consisted of a circular solid plastic tank with a diameter of 91.5 cm and filled with 7 cm of fresh UV sterilized seawater from the same source used to fill holding tanks. To visually contrast between the fish and the background, a white vinyl covering was used to line the arena. The fish were all caught with a dip net from the exposure tank, released into a 3 L container, where they were rinsed with fresh seawater, and transferred as a group to an opaque cylindrical holding arena (30 cm diameter), located within the centre of the arena. After 10 minutes of acclimation the container was lifted, and fish were allowed to swim freely for 15 min, filmed from above by stationary GoPro Hero 4 (GoPro, California, USA) at 30 frames per second. To avoid contamination, the control group was always released into the arena first, followed by the mixed group and the oil only group last. At the end of each day the arena was drained, rinsed and allowed to dry before being refilled one h before the next assay began. All open field assays were completed within
two h between 9 AM and 12 PM, with no more than 3 groups assessed per day. Owing to logistical constraints, water changes were not done between groups. Importantly, however, we believe that the effects of any olfactory cues accumulating across trials are minimal or non-existent. Firstly, the total time between the first and last trial done each day was relatively short (less than two h), with times that fish were in the arena being equal to only 60 min maximum before the last trial. Secondly, fish were fasted and so no faeces were left in the tanks after testing that could potentially affect fish behaviour. Additionally, there was no difference in the behaviour of the untreated fish in the control and LM groups, indicating that even if there were residual cues, chemical signals, and scents left behind by the previous group, it was not enough to affect the fish in the latter groups. Finally, there were several days in which only two trials were performed, with either a mixed or oil treatment group being performed either first or second during the day. Among these groups, there was no effect of trial order on the behaviour of fish in any treatment (linear mixed effect models, p > 0.50 for all behaviours in all cases).

Videos were analysed using Ethovison (Version 10; Noldus, Wageningen, Netherlands), which was able to track fish based on the unique coloured beads. The following variables were quantified for each fish within each shoal: (1) average speed; (2) average distance from the arena wall; and (3) average mean distance between the focal fish and all other fish within the shoal. All variables were measured continuously throughout each video but then aggregated using mean values within 5 min time bins throughout the trial (see Data Analysis, below).

This research was approved by the Institutional Animal Care and Use Committee of the university at which the research took place (reference number AUP-2015-00147) and followed to the ASAB/ABS Guidelines for the Use of Animals in Research. Of the 200 fish obtained for experimental purposes only two did not survive post anaesthesia, neither of these fish were of the groups exposed to oil.

**A.1.4.4 Data Analysis**

All analyses were conducted using R v. 3.4.0 (R Development Core Team 2017) using the function lmer in package lme4\(^{23}\) for linear mixed effect models, MuMIn 1.9.13 for determining model effect sizes (marginal and conditional R\(^2\)) (**\(^{24}\); http://CRAN.R-project.org/package=MuMIn). All plots were created using the package ggplot2\(^{25}\). An initial set of linear mixed effects models (LMEs) were fitted using restricted maximum likelihood estimation, with a separate model using each of speed, distance from arena wall,
and mean neighbour distance as the response variable. The models also included fish body mass and group exposure treatment (control, HO, HM, LO, LM) as categorical fixed effects. To account for any shifts in behaviour over the course of each trial, videos were split into 3 different bins (5 min each) so that time over the course of the study could be included as a fixed effect. Individual nested within group was included as a random effect. To compare behaviours between exposed and unexposed fish within the mixed groups, and to compare unexposed fish within mixed groups to the control fish, a second set of LMEs were constructed with a given behavioural index as the response variable, fish body mass as a continuous fixed effect, and individual treatment (control, low exposed, low unexposed, high exposed, high unexposed) as a categorical fixed effect, and individual nested with group as a random effect. For all models, interactions between treatment and time were included, but dropped when not significant and the models re-run. Model assumptions were verified by visual examination of residual-fit plots. Significance testing ($\alpha = 0.05$) was employed to provide some indication of the strength of evidence for observed patterns, along with model $R^2$ values. This included marginal $R^2 (R^2_M)$ and conditional $R^2 (R^2_C)$ which indicate the variance explained by fixed factors and by both fixed and random factors, respectively.

A.1.5 REFERENCES


Chapter A.2: Warming alters links among metabolism, brain size, and cognition in common minnow

This chapter was co-written with Doctor Libor Závorka, Doctor Barbara Koeck, Mustafa Soğanci, Doctor Amélie Crespel, and Professor Shaun Killen. For this chapter I was responsible for dissecting, imaging, and measuring brains of the fish used. This manuscript is currently submitted to The American Naturalist.

A.2.1 INTRODUCTION

Mounting evidence suggests that environmental warming can affect cognition of animals (Dayananda & Webb, 2017; Coomes et al., 2019). Effects of climate warming on cognition are expected to be particularly strong in ectotherms because their physiology is very sensitive to ambient temperature (Nati et al., 2016; Pinski et al., 2019). Increased temperatures have, for example, been shown to impair learning in lizards (Dayananda & Webb, 2017), accelerate the onset of age-related learning deficits in fishes (Valenzano, 2006), but also to improve learning capacity in juvenile sharks (Pouca et al., 2019). For ectotherms, acute exposure to warmer temperatures causes an increase in the minimum oxygen uptake rate required to sustain life i.e. a proxy for SMR and decrease in the capacity to deliver oxygen to support aerobic physiological processes above maintenance including activity, digestion, growth and reproduction i.e. a proxy for AS (Sandblom et al., 2014; Nati et al., 2016). When chronically exposed to warmer temperatures, ectotherms are capable of at least partial metabolic thermal compensation, i.e. reducing their SMR and maintaining AS (Stillman, 2003; Sandblom et al., 2014, 2016; Seebacher et al., 2015). However, the effect of changes in oxygen demand and thermal compensation induced by chronic warming on cognitive performance of ectotherms is yet to be understood. This is an important knowledge gap as an increasing number of wild ectotherm populations e.g. fishes are exposed to temperatures above their physiological optimum (Comte & Olden, 2017).

One of the most ecologically important cognitive skills is spatial orientation, which relies on learning and working (i.e. short term) memory of individuals, allowing them to efficiently move through heterogeneous environments composed of different biologically important areas (Hughes and Blight, 1999; Braithwaite, 2005).
Learning leads to the long-term retention of information about cues indicating the location of ‘hot spots’ that are worth revisiting (e.g. patches with frequent reoccurrence of prey). In contrast, working memory drives algorithmic searching which facilitates exploration of environment for resources that are rapidly changing in space and time (Hughes and Blight, 1999). In fishes, the telencephalic-tectal-cerebellar neural complex has been identified as a functional region responsible for spatial learning and working memory (Broglio et al., 2003; Odling-Smee and Braithwaite, 2003a). Previous work also suggests that overall brain size is, in general, positively correlated with cognitive capacity (Reader and Laland, 2002; Kotrschal et al., 2013) and therefore considered an indicator of cognitive skills.

Brain tissue in fish, although representing only about 2% of body mass, is an energetically demanding organ, responsible for 5 to 15% of basal whole-animal oxygen uptake (Nilsson, 1996; Moran et al., 2015). This oxygen demand increases even more during periods of elevated neuronal activity (Shulman et al., 2004). Therefore, the metabolic response of fish to environmental warming may affect brain functioning and size via effects on whole-body oxygen demand and delivery capacity.

Warming has also been shown to increase the expression of energetically demanding behaviours in ectotherms including boldness, activity and aggressiveness (Biro et al., 2009; Briffa et al., 2013). This positive relationship is likely to be limited at extremely high temperatures, which can cause a range of adverse physiological effects, including altered metabolic function and diminished scope for aerobic and neuromuscular performance, which would lead to reduced expression of all behavioural traits (Killen et al., 2013).

Boldness and associated behavioural traits (e.g. activity and aggressiveness) are directly related to life-history trade-offs between resource acquisition and survival (David and Dall, 2016). Previous work suggests that specific cognitive skills related to spatial orientation are often positively associated with boldness (Carazo et al., 2014; Kareklas et al., 2017).

In the present study, we compared aerobic metabolism (i.e. SMR, MMR, AS), with volume of total brain and its functional regions, behavioural traits (i.e. boldness, activity, and aggressiveness), and accuracy of maze solving between common minnows (Phoxinus phoxinus) acclimated for eight months to either: 1) a cool water temperature simulating their current summer climate i.e. 14 °C; or 2) a warm water temperature simulating a severe climate warming scenario (IPCC 2013) i.e. temperature increment by 6 °C to 20 °C. We predicted that: i) warm acclimated fish will have higher SMR, MMR, and similar AS as cool acclimated fish; ii) warm acclimated fish will be more bold, active and aggressive; and iii) warm acclimation will affect size of brain and its functional regions and spatial
orientation, but owing scarcity and ambiguity of previous results we have no predictions regarding the direction of the effects; however, we expected that iv) spatial orientation capacity will increase with increasing brain size and with increasing boldness of individuals.

A.2.2 MATERIAL AND METHODS

A.2.2.1 Fish sampling and housing

In November 2017, young of the year common minnows (N = 80) were collected from the River Kelvin (Glasgow, UK) by dip netting, and transferred to the aquarium facilities of the Institute of Biodiversity, Animal Health and Comparative Medicine (IBAHCM), University of Glasgow. Minnows were randomly split into four groups and housed in four identical rectangular rearing tanks (32 L, 40×40×30 cm, 20 individuals per tank), which contained artificial plants and gravel substrate, and were supplied with UV treated, re-circulating freshwater. Photoperiod followed cycles of 12 hours of light and 12 hours of dark. Since the beginning of the laboratory activity water temperature was 14 °C in rearing tanks 1 and 3 and 20 °C in rearing tanks 2 and 4. Individuals were fed daily until apparent satiation (i.e. fish were given as much feed as they would consume within a 10-minute period) with a mix of frozen bloodworms and aquarium flake food (warm acclimated fish required ~ 50 % more feed than cool acclimated fish). After two months of initial acclimation period (i.e. due to the small capture size), on January 31 and February 1, 2018, all individuals were anaesthetized in a benzocaine solution, measured for fork length to nearest mm and body mass to nearest 0.01 g (fork length: mean ± SD = 65 ± 9 mm; body mass: 2.68 ± 0.55 g). Fish were then tagged with Visible Implant Elastomer (VIE) (Northwest Marine Technology Inc.) with a unique colour combination for individual identification (colour combination of four colours: pink, green, red and white) before being returned to their rearing tanks and left undisturbed until the start of metabolic assays. Fish were monitored daily so that individuals with visibly poor health or condition could be removed. Throughout the study we found no significant difference in body mass of individuals between the temperature treatments, but warm acclimated individuals tended to have greater fork length and had significantly lower condition factor than cool acclimated individuals (Supplementary material A.2.S1).
A.2.2.2 Metabolic Rates and Aerobic Scope

Standard metabolic rate (SMR) and maximum metabolic rate (MMR) of individuals (N = 66) was estimated from rates of oxygen uptake (MO₂) using intermittent flow respirometry (Clark et al. 2013). The metabolic assays were conducted between July 16 and 27, 2018, after eight months of exposure to the temperature treatments. Assays took place in 16 glass chambers (0.0893 L) submerged into a water bath (92 L, 80×40×29 cm), which allows for 16 fish to be measured simultaneously. A peristaltic pump (Masterflex L/S 100 RPM, Cole-Parmer, Vernon Hills, US) was used to create a continuous mixing circuit (0.1 L/min) in the water bath. Bacterial oxygen consumption was kept at minimum by using a UV filter sterilizer and it was evaluated daily before and after the fish were place to the respirometric chambers. Rates of measured oxygen uptake were then adjusted by assuming a linear increase in bacterial metabolism over the time of the assay. A thermostatic reservoir connected to the water bath by a thermoregulator (TMP-REG system, Loligo Systems, Tjele, Denmark) maintained the temperature in the water bath at the acclimation temperature of each treatment (i.e. either 14°C or 20°C). Flush pumps, connected to a timer, flushed oxygenated water through the chambers for 2 minutes with 8 minutes intervals between flushes, during which the oxygen uptake of individuals was measured. Oxygen concentration within each glass chamber was recorded every 2 seconds by a fiberoptic sensor using a 4-channel FireSting O2 system (PyroScience GmbH, Aachen, Germany), which was calibrated in accordance with the supplier’s manual. RespR was used to analyse MO₂ (mg O₂.h⁻¹) data obtained from the Firesting O2 software (Harianto et al., 2019). Oxygen uptake data were corrected for the length and radius of tubing, the volume of the respirometry chambers, and the volume of the fish. Prior to measurement, individuals were fasted for 48 hours and then randomly collected from their rearing tanks by dip nets and transferred to a temporary holding tank (circular bucket 11 L). MMR was measured as the oxygen uptake immediately after an exhaustive exercise protocol where fish were individually chased for two min in a circular tank containing aerated freshwater with temperature corresponding to their treatment (Clark et al., 2013; Killen et al., 2014). SMR was measured as the lowest 10th percentile of oxygen uptake measurements that were recorded over the time the fish were in the respirometers (~18 h overnight) excluding the first 5 hours of measurements during which the oxygen consumption is often elevated (Killen et al. 2014). Aerobic scope (AS) was calculated as the difference between SMR and MMR. After respirometry all individuals were measured for fork length to nearest mm and body mass to nearest 0.01 g. The change in metabolic rates with an increase in
temperature was described by the Q10 indicator, which is derived from the van’t Hoff’s equation (Sandblom et al., 2014; Seebacher et al., 2015):

\[
Q_{10} = \frac{R_2}{R_1} \left( \frac{10}{T_2 - T_1} \right)
\]

Where \( R_1 \) the mean mass specific SMR (i.e. mg O2.h\(^{-1}\) per gram of body mass) of cool acclimated individuals, \( R_2 \) is the mean mass specific SMR of warm acclimated individuals, \( T_1 \) is 14 °C (i.e. cool acclimation treatment) and \( T_2 \) is 20 °C (i.e. warm acclimation treatment). A Q10 for SMR < 2 is indicative of thermal compensation in warm acclimated individuals (Sandblom et al., 2014; Seebacher et al., 2015).

A2.2.3 Behavioural Measurements

Behaviour and spatial cognition of individuals (\( N = 66 \)) was tested from August 2 until August 11, 2018. Every scoring day started at 8:30 AM finishing 7:00 PM. A single batch with a maximum of eight individuals was tested on each scoring day using eight flow-through (~2 L/min) rectangular transparent trial tanks (18 L, 72×46×38 cm, water level 5.5 cm Fig. A.2.1 A) filled with aerated freshwater maintained at the acclimation temperature treatments. Trial tanks were positioned beneath four cameras (HD Webcam C525, Logitech, USA) and lightened by a dispersed dim LED light. Fish were left undisturbed in the starting box of trial tanks for 30 min after introduction to the tank, whereupon the doors on the sides of the starting box were hoisted and the time until fish emerge from the starting box to the barren trial tank was measured for 50 min to evaluate boldness of individuals (Näslund et al., 2015).

Individuals that did not emerge (i.e. 17 individuals out of 66) during this period were assigned the maximum time score i.e. 50 min (3000 s). The starting box was then removed from the trial tank and distance moved in the barren trial tank (i.e. open field test) was recorded over 10 min after 10 min of acclimation following the removal of the starting box to evaluate individual activity (Závorka et al., 2015). After the open field test, a mirror (10×10 cm) was slid in a corner of the wall of the scoring tank and amount of time spend in front of the mirror (i.e. within ~two body lengths) was measured over 10 min after 10 min of acclimation to evaluate response of individuals to conspecifics (Adriaenssens & Johnsson, 2013). Here we consider the response to conspecifics as aggressiveness, however depending on motivation of an individual to approach conspecific in the mirror it can also be considered sociality (non-aggressive social interaction). The distance moved and the time spent in front of the mirror was derived from the video records by placing a grid net of 10×6 equal sized rectangles (rectangle size 6.3×6.4 cm) over the record of trial
tank on a computer screen. Distance moved was measured, following the example of previous studies (Závorka et al., 2015; Näslund et al., 2017), as the number of crossings between rectangles, where each crossing represents a complete passage by an individual over the borderline into an adjacent rectangle. The three behavioural traits were summarized using principal component analysis resulting in a significant first principal component (eigenvalue = 1.68), which explained 56.00% of the total variance in the three behavioural tests and which was negatively correlated with time to emerge from the starting box (R = -0.64, loading = -0.49), and positively correlated with activity (R = 0.78, loading = 0.60) and aggressiveness/sociality towards the mirror (R = 0.81, loading = 0.63). The second and third principal components yield by PCA had eigenvalue < 1 and therefore were not further considered (Supplementary material A.2.S2). The first principal component is thereafter named the “boldness score” and interpreted as a continuum of behavioural types ranging from inactive, unaggressive, and shy individuals (i.e. low values of the score) to active, aggressive, and bold individuals (i.e. high values of the score) (David and Dall, 2016).

Cognitive skills were evaluated in a maze test, which challenges the spatial orientation of tested individuals (Johnsson & Sundström, 2006, Adriaenssens & Johnsson, 2011). The maze test was conducted after the mirror image test, when the starting box was entered back to the trial tank and individuals were gently guided to it by a dip net without being netted or lifted out of water. The maze, which was set to the trial tank while focal fish were in the starting box, was composed of three chambers out of which two were connected by an opening and allowed individual to enter the central chamber and consume bloodworms, which served as a reward (Fig. A.2.1 B). The other chamber was connected to the central chamber by a blocked entrance that allowed the scent of bloodworm disperse through but prevented fish from entering the central chamber. The scent of bloodworms was amplified by placing a bait bag with bloodworms in the central chamber of the maze. This protocol eliminates the potential effect of conditioning (positive or negative) related to the reward scent on performance of individuals in the maze, because odour had the same intensity in both side chambers of the maze. To minimize the effect of lateralization (Bisazza & Brown, 2011), mazes in half of the trial tanks had the correct entrance on left side and in the other half of the trial tanks had the correct entrance on the right side. The correct path to the centre of the maze was marked by gravel pieces as landmarks, which allowed individuals to use allocentric information to navigate in the maze along with the egocentric information (Rodriguez et al., 1994). Fish are known to use both types of spatial information. However, as our focal fish originated from a dynamically changing riverine
environment, which limits the usefulness of allocentric landmarks for navigation (Odling-Smee and Braithwaite, 2003b), it can be assumed that they predominantly relied on egocentric information for navigation. Performance of fish in the maze test was scored in four consecutive trials. Fish were guided back to the starting box at the end of each trial and left there to rest for 20 minutes before the following trial started. We assumed, based on a study by Hughes and Blight (1999), that the resting period between the trials is sufficient for obliteration of working spatial memory. During this period the maze was cleaned from leftover bloodworms and prepared for the following trial. The maze trial started by hoisting the doors and removing the cover of the starting box. Fish performance in the maze was than observed for 80 minutes (4800 s) in each trial. Two variables were recorded in each trial: i) speed of maze navigation i.e. time until the first entry to the central chamber of the maze after emergence from the stating box; ii) accuracy of maze navigation i.e. number of navigation errors until the first entry to the central chamber of the maze. Time until entrance of the central chamber was assigned the highest score (i.e. 4800 s) for individuals which did not reach the central chamber until the end of the trial (49 out of 248 trials evaluated, 16 trials were not evaluated due to a technical failure). The number of navigation errors was measured as the number of compartment swaps before an individual first entered the centre of the maze minus three, which corresponds to the number of compartments to reach the central chamber without detour. To control for feeding motivation of focal fish, their voracity was tested after the last maze trial by recording whether they consumed a set amount of bloodworms (~ 0.1 g) placed in the starting box during 30 minutes.
Figure A.2.1. Diagram of the trial tank for behavioural scoring (a) and maze test (b) view from above. (S) Starting box, (M) mirror, (F) maze chamber with blocked entrance, (R) maze chamber with open entrance, (C) central chamber, (L) landmarks - gravel pieces, (P) petri dish with bloodworms, (B) bait bag with bloodworms, (I) water inlet, (O) water outlet.

A.2.2.4 Brain measurements

Immediately following the completion of the last maze trial, fish were euthanized with an overdose of benzocaine and their final body mass and fork length were measured to the nearest 0.01 g and mm respectively. Heads of fish were removed and fixed in 4% buffered (pH 6.9) paraformaldehyde solution. Brains were then dissected out as described in Gonda et al. (2009) and stored in 4% buffered paraformaldehyde solution until further procedure. Brains were imaged with a Canon EOS 1300D DSLR
camera with EF-S18-55 III lens (Canon, USA) and 13- and 31-mm extension tubes designed for Canon DSLRs (Xit Inc, USA). For each brain sample (N = 55), an image was taken using the dorsal, left lateral and ventral views. Each brain was measured to calculate total volume and the volumes of the dorsal medulla, cerebellum, optic tectum, telencephalon, and hypothalamus. Measurements were completed using ImageJ 1.48 (Schneider et al., 2012) and used to calculate volume with the formulas outlined by Pollen et al. (2007).

A.2.2.5 Statistical analysis

All analyses were conducted in R v. 3.5.3 (R Core Development Team). Effect of acclimation temperature on metabolic rates, volume of brain and its regions were tested by linear mixed effect models LMM (Bates et al. 2015) with rearing tank as a random intercept (categorical variable with four levels), and acclimation temperature (categorical variable with two levels) as fixed factor. Allometric effect was controlled for by including as co-variable the body mass at the time of respirometry for metabolic rates and the final body mass for volume of brain and its regions. Brain regions were in addition controlled for the total brain volume minus for the volume of region used as the dependent variable (Pike et al. 2018). Effects of treatment on boldness score was tested using LMMs with rearing tank and trial tank (categorical variable with eight levels) as random intercepts, acclimation temperature as fixed factor and final body mass and feeding motivation (categorical variable with two levels: bloodworms eaten, or bloodworms not eaten) as co-variable. Cognitive capacity was tested by LMM for time of the first entry and log-transformed number of navigation errors (transformation was used to prevent over-dispersion). Models for cognitive capacity had the same structure as model for boldness score, included in addition trial number as covariable (Supplementary material A.2.S3) and individual’s ID (categorical variable with 66 levels) as a random intercept. We used rearing tank as random intercept to control for pseudo-replication effect despite the fact that this variable had lower number of levels (four) than minimum recommended for random effects (>5 - 6, Bolker et al., 2009). Therefore, models with and without rearing tank as random intercept were compared using AIC and the random factor was kept in the model only if it significantly improved model fit i.e. ΔAIC > 2 (Burnham & Anderson, 2004). Significance of the models was evaluated using ANOVA tables using Type II sums of squares. Model fit was diagnosed by control of distribution of model residuals and association of fitted and residual values. Effect of acclimation temperature on feeding motivation was tested using chi-square test.
To test differences in the covariance structure of phenotypic traits underlying spatial orientation of individuals between temperature treatments, we used a partial least-squares path modelling approach (PLS-PM, plspm package, Sanchez, 2013). All latent variables used in the final model represented a single indicator variable in reflective mode. All variables were controlled for the final body mass. Following variables were included for each individual: SMR, AS, boldness score, total volume of brain, and median number of errors over all maze trials. MMR was not included to the model, because of its high correlation with AS (r = 0.94). Standardized path coefficients were used to evaluate the strength of the causative relationships tested in the model. Significance of path coefficients in the final model was assessed using 95% percentile confidence intervals (CIs) calculated on 200 bootstrap samples (Supplementary material A.2.S4). The final models were performed on a subset of 26 cool acclimated and 25 warm acclimated fish for which no missing values in any considered trait occurred. Structural difference between the final models for two temperature treatments was compared using bootstrap t-test based on 200 bootstraps samples (following recommendations outlined in Sanchez 2013).

A.2.3 RESULTS

Warm acclimated fish had a higher SMR (F1;60 = 23.70; p < 0.001; Fig. A.2.2 A) and MMR (F1;60 = 10.68; p = 0.002; Fig. A.2.2 B) than cool acclimated fish, but AS did not differ between the temperature treatments (F1;60 = 2.77; p = 0.101; Fig. A.2.2 C). SMR (F1;60 = 34.28; p < 0.001), MMR (F1;60 = 32.82; p < 0.001) and AS (F1;60 = 22.23; p < 0.001) were positively correlated with body mass in both temperature treatments. Q10 for mean mass specific SMR between cool and warm acclimated individuals was 1.69. Together, the absence of difference in AS between fish from cold and warm temperature treatments and a Q10 < 2 indicate metabolic thermal compensation of the warm acclimated fish.
Figure A.2.2. Correlations between body mass and (A) SMR, (B) MMR, (C) AS in fish acclimated to warm (red filled circles and solid curves) and cool temperatures (blue empty circles and dashed curves). Areas within the dashed lines represent standard error. Circles represent individual data points.

We found that total brain volume was larger in warm acclimated individuals (F1;55 = 7.21; p = 0.010; Fig. A.2.3 A) and positively correlated with body mass in both treatments (F1;55 = 57.04; p < 0.001). We also found differences of relative volume of specific brain regions in response to acclimation temperature. Volume of the dorsal medulla was larger in warm acclimated fish than in cool acclimated fish (F1;54 = 6.52; p = 0.014; Fig. A.2.3 B), and warm acclimated fish had also a non-significant tendency for larger cerebellum (F1;54 = 3.78; p = 0.057; Fig. A.2.3 C). Volumes of the hypothalamus (F1;54 = 1.589; p = 0.408; Fig. A.2.3 D), optic tectum (F1;54 = 1.59; p = 0.213; Fig. A.2.3 E) and telencephalon (F1;54 = 1.98; p = 0.166; Fig. A.2.3 F) were not affected by acclimation temperature.
Figure A.2.3. Boxplots illustrating differences in volumes of the whole brain and its functional regions of fish acclimated to warm (red) and optimal temperatures (blue). Displayed data are not corrected for body mass of individuals. Box edges represent the median and 25th and 75th percentiles and whiskers cover the 95th percentiles. Filled circles represent individual data points.

Boldness score was not affected by acclimation temperature (F1;52.8 = 1.21; p = 0.276; Fig. A.2.4 A) or feeding motivation (F1;56.6 = 0.79; p = 0.377), but positively correlated with body mass (F1;58.8 = 5.14; p = 0.027). There was no effect of acclimation temperature ($\chi^2 = 2.00$; p = 0.158; Fig. A.2.4 B), body mass ($\chi^2 = 0.05$; p = 0.831) and trial number ($\chi^2 = 2.98$; p = 0.395; Supplementary material A.2.S3) on time until first entry to the central chamber of the maze, but individuals with higher feeding motivation entered the centre of the maze earlier ($\chi^2 = 5.72$; p = 0.017). The
number of navigation errors was higher in fish acclimated to warm temperature ($\chi^2 = 4.24$; $p = 0.039$; Fig. A.2.4 C) and was not affected by body mass ($\chi^2 = 0.29$; $p = 0.588$), trial number ($\chi^2 = 0.71$; $p = 0.871$; Supplementary material A.2.S3) and feeding motivation ($\chi^2 = 0.05$; $p = 0.827$). We found that feeding motivation of individuals was not different between groups of cool and warm acclimated fish ($\chi^2 = 0.194$; $p = 0.660$).

![Figure A.2.4. Boxplots illustrating differences in behaviour and cognition of fish acclimated to warm (red) and optimal temperatures (blue). Box edge represents the median and 25th and 75th percentiles and whiskers cover the 95th percentiles. Filled circles represent individual data points.]

We found differences between cool and warm acclimated fish in the associations of AS and brain volume ($|t\text{-test}| = 1.68$, d.f. = 49, $p = 0.049$). In cool acclimated fish brain volume increased with increasing AS (path coef. = 0.38, 95% CI [0.071; 0.641]), while the association between AS and brain volume was negligible in warm acclimated fish (path coef. = -0.021, 95% CI [-0.399; 0.323]; Fig. A.2.5). There was also a tendency, albeit non-significant, for differences in the association between AS and boldness score between the temperature treatments ($|t\text{-test}| = 1.53$, d.f. = 49, $p = 0.066$). Warm acclimated fish that were bolder also tended to have higher AS (0.310 CI [-0.017; 0.684]), but such pattern was not observed in cool acclimated individuals (-0.038 CI [-0.402; 0.363]). Boldness score and brain volume were not significantly associated with SMR in any of the acclimation conditions.
temperatures (Fig. A.2.5). Number of errors in the maze test was not associated to boldness or brain volume (Fig. A.2.5; Supplementary material A.2.S4).

![Diagram representing the PLS-PM assessing the trait covariance structure in cool (A) and warm acclimated fish (B). The width of arrows connecting boxes representing latent variables is proportional to the value of path coefficient and indicates its positive (black) or negative value (grey). Arrows displayed causal direction of the relationships between latent variables assumed by the model. Significant path coefficients are bold typed. Path coefficients that were significantly different between the two models (14°C and 20°C) are indicated by asterisks.]

**Figure A.2.5.**

A.2.4 DISCUSSION

Our results indicate that warm acclimation can lead to the impairment of spatial orientation in a widely distributed freshwater fish species, the common minnow. Since we observed no improvement of individual performance over the four consecutive maze trials at any acclimation temperature, it is very likely that the impairment of spatial orientation of warm acclimated fish was caused by the deterioration of working spatial memory (Hughes and Blight, 1999) rather than due to differences in spatial learning skills of fish (Braithwaite, 2005). This suggest that warming could negatively affect the algorithmic searching patterns that fish use to search for prey and shelter in the rapidly changing landscapes such as riverine environment or intertidal zones (Barnes & Hughes, 1999; Odling-Smee & Braithwaite, 2003b). In contrast to previous studies showing that brain size is positively correlated to cognitive abilities (Reader and Laland, 2002; Kotrschal et al., 2013), we
found that warm acclimated fish performed more navigation errors despite having a larger brain, including a disproportionately larger dorsal medulla and tendency for larger cerebellum (i.e. the centre of spatial working memory, Durán et al., 2014) than cool acclimated fish. This indicates that the increment of brain volume caused by warm acclimation may not be associated with the expansion of functional brain tissue (e.g. number of neurons, Olkowicz et al., 2016). Path analysis based on individual-level data revealed that the positive association between overall brain volume and AS present in cool acclimated fish was absent in warm acclimated fish. Instead, AS in warm acclimated fish tended to be positively associated with the expression of energetically demanding behavioural traits (i.e. boldness score).

The positive association between brain size and AS found in cool acclimated fish is in accordance with previous studies showing that variation in brain size has a significant impact on the overall oxygen uptake of fishes (Nilsson, 1996; Moran et al., 2015). However, the lack of the association between the brain size and SMR suggest that the basic oxygen consumption is not a limiting factor for brain size. Instead, it may be the availability of oxygen for glucose metabolism during and after peaks of neuronal activity that is the predominant limiting factor for functioning and development of brain in fishes (Soengas and Aldegunde, 2002; Shulman, 2004). In addition, evidence suggests that warm acclimation reduces performance of mitochondria in fish brains cells more profoundly than in other organs, such as the heart (Chung et al., 2017). Therefore, the association between AS and brain size in warm acclimated fish could have been masked by a substantial reduction of the contribution of brain to overall oxygen uptake relative to other organs. Oxygen uptake of the brain in warm acclimated individuals could have been further reduced by their lower energetic status (i.e. lower condition factor despite ~50 % higher food intake than cool acclimated fish), because low energetic status has been shown to substantially reduce glucose oxidation in brain of fishes (Soengas and Aldegunde, 2002).

The larger dorsal medulla in warm acclimated individuals may have also contributed to differences between cool and warm acclimated individuals in oxygen uptake, because it is a brain region responsible for regulation of respiratory functions in fish (Woldring & Dirken, 1951). Our results do not support finding of previous studies that boldness is positively associated to spatial orientation skills (Carazo et al., 2014; Kareklas et al., 2017), because boldness score was not associated to number or errors in any of the temperature treatments. In addition, boldness score did not differ between temperature treatments, which suggest that response to stress of our focal fish from isolation and novel
environment in the maze test trials would be unlikely to cause difference in spatial orientation between the temperature treatments (Conrad et al., 2003; Závorka et al., 2019).

Our study provides novel insight to the mechanistic association between cognition and physiology under a climate warming scenario. We suggest that thermal metabolic compensation to warm temperatures can help individuals to maintain a high capacity for aerobic metabolism (i.e. high AS) to perform an energetically demanding behaviours (e.g. boldness, aggressiveness, and activity) which have a direct effect on foraging, territory defence, and predator avoidance in the wild (Metcalf et al., 2016). However, our findings also indicate that thermal compensation in response to warming may affect brain structure and functioning which can ultimately lead to cognitive impairment. Because it is likely that changes in cognitive capacity induced by warming can affect fitness (Boogert et al., 2018) and ecological interactions in ectotherms (Edmunds et al., 2016), further investigation is needed to fully appreciate the implications of potential climate driven cognitive changes in wild animals. Relative ecological importance of cognitive changes should be compared to other changes in behaviour (Cooper et al., 2018), physiology (Killen, 2014) and their associations (Raffard et al., 2019) induced by warming. While our study demonstrates the effects of warming on the plastic phenotypic response within a single cohort of individuals, long-term transgenerational studies are needed to fully appreciate the chronic effects of warming on animal cognition and adaptive capacity of wild fish and other ectotherms.

A.2.5 REFERENCES


A.2.6 SUPPLEMENTARY MATERIAL

A.2.S1 Body Size and Condition of Focal Fish

Body mass, fork length and condition factor of individuals were measured at three different occasions (i.e. at tagging on 31/01 – 01/02/2018, at metabolic assays on 16/07 – 27/07/2018, and after the behavioural assays at the end of the experiment 02/08 – 11/08/2018). Body mass and fork length were measured to the nearest 0.1 g and mm respectively. Condition factor \( K \) was calculated as:

\[
K = \frac{\text{body mass} \times 10^5}{\text{fork length}^3}
\]

Differences in body mass, fork length and condition factor were tested by linear model with temperature treatment as independent variable. To avoid type I errors associated with multiple comparisons, p-values of models were adjusted for false discovery rate (Benjamini & Hochberg 1995).

We found no significant difference in body mass of individuals between the temperature treatments measured at tagging (\( F1;61 = 1.496; p = 0.678 \)), at the time of the metabolic assays (\( F1;61 = 0.0672; p = 0.796 \)), or at the end of the experiment (\( F1;64 = 0.087; p = \))
We found that warm acclimated fish had significantly larger fork length at tagging (F1;61 = 8.4332; p = 0.0153), but there was no significant difference between the temperature treatments in fork length at the time of the metabolic assays (F1;61 = 2.0366; p = 0.1586), or at the end of the experiment (F1;64 = 3.7176; p = 0.0875; Fig. A.2.S1.1 B). Warm acclimated individuals had significantly lower condition factor than cool acclimated individuals throughout the study (at tagging: F1;61 = 15.852; p = 0.0002; at metabolic assays: F1;61 = 16.634; p = 0.0001; at the end of the experiment: F1;64 = 22.755; p < 0.0001; Fig. A.2.S1.1 C).

Figure A.2.S1.1. Boxplot representing differences between cool (blue) and warm (red) acclimated fish in (a) body mass, (b) fork length, and (c) condition factor as measured at tagging, respirometry and behavioural scoring.

A.2.S1. References

A.2.S2 Co-Variation of Behavioural Traits

Figure A.2.S2.1. (a) Distribution of behavioural traits in individual laboratory tests and their correlations. Note that scores of open field test and mirror image test are square-root transformed. Loadings of behavioural traits to the (b) first and second principal components and (c) second and third principal components of PCA.
We found no significant improvement of individual’s performance (i.e. decrease of navigation errors and time until first entry to the central chamber) over the four consecutive trials of the maze test (Fig. A.2.S3.1). This result is consistent with a previous study on wild brown trout (Salmo trutta), which has shown that individuals did not improve navigation in a maze with same structure as used in our study over the initial four trials (Adriaenssens & Johnsson 2011). This might have been caused by the stress experienced by wild fish such as common minnow, which is also a social species (Magurran and Pitcher 1987), due to isolation in the novel environment of the scoring tank (Niemelä & Dingemanse 2014; Závorka et al. 2019). However, we found that boldness score (i.e. combination of boldness, activity, and aggressiveness) of individuals did not differ between the treatments, which suggests that response to stress from isolation and novel environment was unlikely to cause difference in number of errors between the temperature treatments (Conrad et al. 2003; Sih & Guidice 2012).

**Figure A.2.S3.1.** Boxplot demonstrating differences between the treatments in (a) number of navigation errors and (b) time until first entry to the central chamber in each trial of the maze test. Box edge represents the mean and 25th and 75th percentiles and whiskers cover the 95th percentiles. Filled circles represent individual data points (blue – cool acclimated individuals, red - warm acclimated individuals).
A.2.S3 References


### A.2.S4 PLS-PM Results

**Table A.2.S4.1.** Path coefficients of PLS-PM assessing the trait covariance structure in cool and warm acclimated fish. Indirect paths coefficients are displayed in italic, significant values are displayed in bold. Values correspond to Fig.A.2.5 in the main text.

<table>
<thead>
<tr>
<th>Path</th>
<th>Path coefficient [95 % CI]</th>
<th>Bootstrap t-test of group difference</th>
<th>p value</th>
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</thead>
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<td>AS $\rightarrow$ Volume of brain</td>
<td><strong>0.381</strong> [-0.071; 0.641]</td>
<td></td>
<td><strong>0.049</strong></td>
</tr>
<tr>
<td>ASP acclimated</td>
<td>-0.021</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AS $\rightarrow$ Boldness score</td>
<td>[0.071; 0.641] [-0.399; 0.323]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SMR $\rightarrow$ Volume of brain</td>
<td>[0.113; 0.453] [-0.360; 0.404]</td>
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<td>0.295</td>
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<tr>
<td>SMR $\rightarrow$ Boldness score</td>
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<td>0.394</td>
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<tr>
<td>SMR $\rightarrow$ Number of errors</td>
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<tr>
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<tr>
<td>Boldness score $\rightarrow$ Number of errors</td>
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<td>0.466</td>
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