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Understanding mechanisms underlying changes in parental care behaviour in response to perceived paternity in sunfish

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Abstract

Parental care is essential for the survival of many young animals but presents significant costs to the caring parent. To mitigate these costs, parental care systems have evolved to optimize survival and fitness. According to parental investment theory, care allocation is influenced by the offspring's value, which is often linked to their relatedness to the parent. In this thesis, I explore how hormones and gene expression influence parental care, focusing on bluegill sunfish (*Lepomis macrochirus*) and the hybrids they produce with pumpkinseed sunfish (*Lepomis gibbosus*). By manipulating direct and indirect paternity cues – swapping eggs between nests for the former and simulating cuckoldry for the latter – I investigated changes in parental investment via care behaviour. To do so, I examined variations in circulating endogenous 11-ketotestosterone, prolactin, and gene expression. I found that while 11KT levels in bluegill respond to paternity cues, they do not directly regulate parental care behaviour. Rather, these levels seem to indicate preparations for future reproductive events. In contrast, prolactin emerges as a critical hormone in fish parental care, with circulating levels correlating with nurturing behaviour and adjusting in response to perceived paternity. Additionally, I used whole-brain RNA sequencing to determine that gene expression associated with energy transport, immune response, and stress varies in response to paternity perception. Focusing on hybrids, known to provide care despite low genetic relatedness, I found they maintain distinct hormonal profiles and gene expression patterns. Specifically, they exhibit higher prolactin and lower 11-ketotestosterone levels compared to bluegills, pointing to a species-specific regulation of parental care, shaped by evolutionary and environmental factors. Overall, my thesis advances our understanding of parental care regulation in species with male-only care and complex reproductive systems. It underscores the significance of considering a range of endocrine, genomic, and environmental factors in understanding the evolution and maintenance of parental care, thereby enriching our knowledge within evolutionary biology and the neuroendocrine regulation of parental behaviour.

Keywords

Behavioural ecology, parental care, endocrinology, transcriptomics, sunfish, alternative reproductive system

Summary for Lay Audience

Parental care is crucial for the survival of young animals but can be costly for the parent providing care. This thesis delves into parental care, focusing on a species of fish known as bluegill sunfish and their hybrid offspring with pumpkinseed sunfish. Parental male bluegill sunfish provide sole parental care for their brood and can determine which offspring are related to them. They use this information to adjust their level of parental care in response to their paternity. The objective of this research is to determine how hormones and genes influence how parental males adjust their parental care. To do this, I manipulated paternity swapping eggs between nests, or providing a visual cue that the offspring in the nest were not related to the male providing care. I found that a hormone called 11-ketotestosterone responds to cues about paternity. However, it does not seem to directly influence how bluegill care for their young – instead it may prepare them for future reproductive opportunities. In contrast, prolactin, another hormone, plays a crucial role. Prolactin adjusts according to how likely the parental male is to be the true father of the offspring, affecting how much care they give. Furthermore, I used genomic analyses to determine that genes related to energy transport, immune response, and stress vary in response to perceived paternity. Interestingly, I also observed unique traits in hybrid sunfish. Despite their low probability of paternity, they still provide parental care. Their distinct hormonal profiles and quality of parental care suggest a species-specific system of parental care, influenced by their genetic background and environmental factors. Overall, this research challenges traditional views on the regulation of parental care in fish, especially those with intricate reproductive systems. It highlights the importance of considering a range of hormonal, genetic, and environmental factors to understand the evolution and persistence of parental care. This work not only advances our knowledge within evolutionary biology, but also opens new avenues for future research in the neuroendocrine regulation of parental behaviour.

Co-Authorship Statement

This thesis contains modified versions of manuscripts which have been published, submitted, or prepared for submission, for which I am the primary author.

A version of Chapter 2 was published in *General and Comparative Endocrinology* with Timothy Hain (TJHA), Rosemary Knapp (RK), and Bryan Neff (BDN) as co-authors. I contributed to study design, performed sample collection, data analysis, visualization, and wrote the manuscript. TJHA assisted in sample collection and offered editorial comments on the manuscript. RK contributed to study design. BDN funded the project, contributed to study design, provided advice on statistical analysis, and offered editorial comments on the manuscript. Authors retain the right to use and share the article in full or in part in a thesis or dissertation for non-commercial purposes.

A version of Chapter 3 has been submitted for publication with TJHA, and BDN as co-authors. I contributed to study design, performed sample collection, data analysis, visualization, and wrote the manuscript. TJHA assisted in sample collection and offered editorial comments on the manuscript. BDN funded the project, contributed to study design, provided advice on statistical analysis, and offered editorial comments on the manuscript.

Chapter 4 has been prepared for submission, with TJHA, Charlyn Partridge (CP), and BDN as co-authors. I contributed to study design, performed sample collection, data analysis, visualization, and wrote the manuscript. TJHA assisted in sample collection and offered editorial comments on the manuscript. CP contributed to study design, gave advice on statistical analysis, and offered editorial comments on the manuscript. BDN funded the project, contributed to study design, and offered editorial comments on the manuscript.

Chapter 5 has been prepared for submission, with TJHA and BDN as co-authors. I contributed to study design, performed sample collection, data analysis, visualization, and wrote the manuscript. TJHA assisted in sample collection and offered editorial comments on the manuscript. BDN funded the project, contributed to study design, provided advice on statistical analysis, and offered editorial comments on the manuscript.

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List of Abbreviations and Symbols

11KT	11-Ketotestosterone
AKAP9	A-kinase anchor protein 9
bp	Base pair length
CYP11B	Cytochrome P450, family 11, subfamily B, polypeptide 1
CYP17A1	Cytochrome P450, family 17, subfamily A, polypeptide 1
CYP19A1/a/b	Cytochrome P450, family 19, subfamily A, polypeptide 1a/b
CYP21A1	Cytochrome P450, family 21, subfamily A, polypeptide 1
DHEA	Dehydroepiandrosterone
DNA	Deribonucleic acid
ELISA	Enzyme Linked Immunoassay
GNS	N-acetylglucosamine-6-sulfatase
HA1F	Class 2 histocompatibility antigen, F10 alpha chain
HSD3B1	Hydroxysteroid 3ß-dehydrogenase 1
HSD11B2	Hydroxysteroid 11ß-dehydrogenase 2
HSD17B1/3	Hydroxysteroid 17ß-dehydrogenase 1/3
MHC	Major Histocompatibility Complex
PCR	Polymerase Chain Reaction
PDE2A	Phosphodiesterase 2A
qPCR	Quantitative Polymerase Chain Reaction
RNA	Ribonucleic acid
ROA1	Heterogenous nuclear ribonucleoprotein A1
RUSC2	Iporin
SD	Standard deviation
SEM	Standard error of the mean
SNPH	Syntaphilin
USP40	Ubiquitin carboyxl-terminal hydrolase-40

Chapter 1

1 General Introduction

1.1 Parental Care

1.1.1 Evolution of parental care

In 1871, Darwin suggested parental care is likely the foundation of social behaviour in animals, despite little knowledge at the time about how and why parental care evolved (Darwin, 1871). Parental care is ubiquitously present across the animal kingdom yet varies in presence and strategy from species to species. Parental care has been defined as 'any form of parental behaviour that appears likely to increase the fitness of a parent's offspring' (Clutton-Brock, 1991) and has since been expanded to broadly include any parental trait that enhances offspring fitness and is likely to have evolved or is maintained for this function (Smiseth et al., 2013). Under these definitions, parental care includes the allocation of resources to eggs during development, offspring provisioning after hatch or parturition, nest tending and guarding, and even the improvement of offspring reproduction opportunities later in life (Klug & Bonsall, 2014). Parental investment is defined separately as any parental expenditure that benefits the survival and/or fitness of the offspring but reduces the ability of the parent to invest in components of their own fitness, including mate attraction and reproduction opportunities (Klug et al., 2013). Parental expenditure may be time, energy or resources that are allocated to offspring, and typically reduces the survival and/or future reproduction of the parent themselves (Klug et al., 2013).

Given that parental investment increases offspring fitness at a cost to the parent's own fitness, a purely evolutionary perspective suggests that parents should be under selection to avoid providing care for their offspring. There is huge variation in the presence/absence of parental care within and between taxonomic groups and is a central question within evolutionary ecology (Klug et al., 2013). Investment in parental care is expected to be favoured only when the fitness benefits to the parent(s) outweigh the costs associated with care. To mediate the trade-off between the cost and benefit of providing parental care, parental investment theory provides a framework to understand how care is allocated. This

theory predicts parents should assess offspring value and invest more care in offspring that are more reproductively valuable (Trivers, 1972). In an evolutionary context, it is adaptive for parents to invest more in offspring that are more likely to contribute to a parent's overall fitness (Westneat & Sherman, 1993). Therefore, parental investment should increase as offspring value increases (Clutton-Brock, 1991; Montgomerie & Weatherhead, 1988); Figure 1.1). Offspring number and size can typically be assessed fairly easily, with large broods valued over small, and large or otherwise healthy offspring valued over small or sickly offspring (Montgomerie & Weatherhead, 1988; Townshend & Wootton, 1984; Windt & Curio, 1986). If parentage is uncertain, parents should invest more into care for offspring that are likely to be their own (Westneat & Sherman, 1993). Parentage may be determined at an individual level, or as the proportion of offspring in the brood that are the genetic offspring of the parent (Westneat & Sherman, 1993). When females invest in offspring, they are very likely related to their offspring as they are rarely separated from their eggs. Males, however, are often less certain of paternity and thus less likely to invest in providing parental care (Klug et al., 2013). In theory, males should invest more into offspring they are likely to have higher paternity of – whether that paternity is perceived or realized. To determine the relatedness of offspring to a parent, however, requires some form of kin recognition and discrimination (Mateo, 2004).

1.1.2 Recognition mechanisms

Kin recognition was first suggested by Hamilton (1964) where he proposed that relatives are recognized based on phenotypic traits, or by their location close to home. In the context of parental care, parents use kin recognition to allocate care based on parentage, such as parental males allocating care in response to paternity. This allocation of care in response to relatedness, or genetic value, is widespread across taxa including amphibians (Chen et al., 2011), fish (Gray et al., 2008; Manica, 2004; Neff & Gross, 2001), and birds (reviewed in Møller & Birkhead, 1993). Since Hamilton's initial proposition (1964), biologists have described a variety of recognition mechanisms that allow individuals to

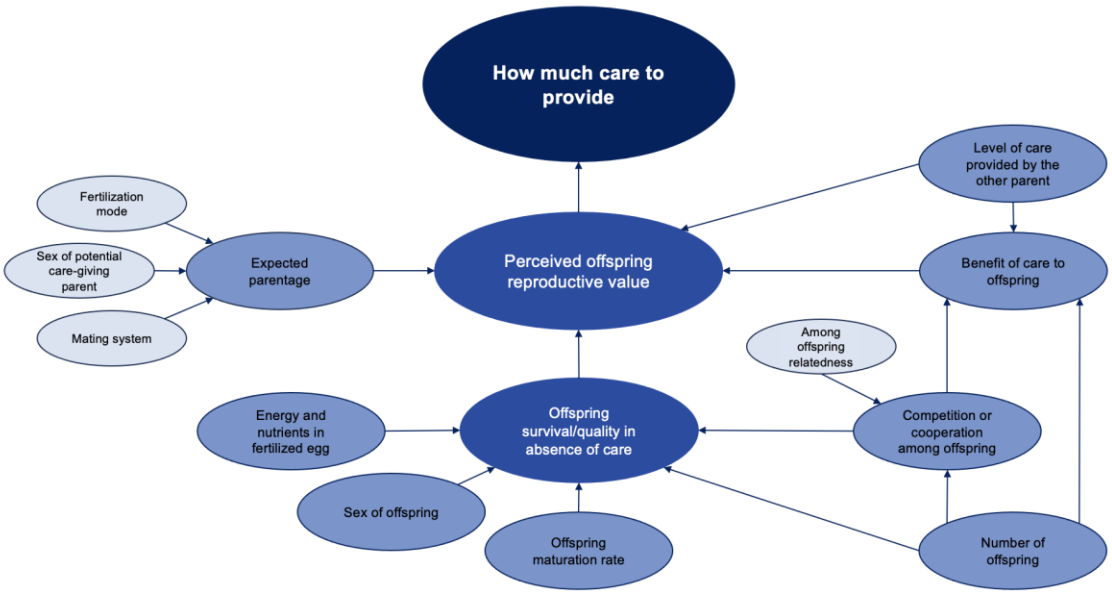

Figure 1.1 Flowchart of factors affecting the allocation of parental care (figure modified from Klug *et al.*, 2012).

direct behaviours to related recipients. In particular, animals can recognize kin directly or indirectly. Indirect kin recognition involves using context-based cues such as location or the number of intrusions by brood parasites to determine the likelihood that individuals they encounter are related (Mateo, 2004). Direct kin recognition occurs when animals use methods including familiarity, or phenotype matching to interpret direct cues of relatedness (Holmes & Sherman, 1982). Familiarity is based on prior association, where individuals remember the phenotype of individuals they have interacted with in contexts normally associated with kinship, and later recognize and treat these individuals as kin (Mateo, 2004). The most common form of kin recognition in species with large broods, including fish, that may not be able to remember each individual they interact with, is phenotype matching (Hain, 2015). This form of recognition occurs when individuals form a 'template' based on the phenotypes of family members encountered during development and use this to determine what related individuals look, smell, or sound like. In species with broods of mixed parentage, individuals may use self-referent phenotype matching whereby the kin template is formed using their own phenotype (Hain & Neff, 2006).

1.1.3 Recognition of offspring

Parents may use one, or a combination of multiple, mechanisms of kin recognition to determine which offspring are related to them. Parental males may use indirect cues to perceive or recognize relatedness, such as the presence of cuckolder males to infer the certainty or uncertainty of their parentage within the nest (Neff, 2003). When using direct mechanisms, parents interpret phenotypic cues from their offspring to recognize or 'realize' parentage. In species with more mobile offspring, parents may recall the phenotype of offspring from their burrows to recognize them after emergence and direct care accordingly (Holmes & Sherman, 1982). Or, in species with large broods, parents may compare their kin template to the offspring in their brood to determine if they are related. From there, parents may invest more parental care into offspring they are related to, to maximize the likelihood of the survival of offspring that may go on to reproduce and propagate the genes of the parent into future generations.

1.2 Mechanisms underlying parental care

Parenting involves the detection and processing of offspring cues, the regulation of parental strategy or 'motivation', and the execution of parental behaviours (Kohl & Dulac, 2018). To facilitate this, parental care necessitates significant physiological changes in the brain and reproductive system (Bridges, 2015; Champagne & Curley, 2013). The predominant understanding of these changes is anchored in the endocrine system (Ball & Balthazart, 2008), however advancements in genomic technologies are helping to understand the roles of genetics in the evolution and expression of parental behaviour.

1.2.1 The endocrine system

The endocrine system is one of the regulatory systems in organisms, in which endocrine glands produce hormones that are transported through the body by blood to reach their target tissues where they bind to receptors to produce a response (Nelson & Kriegsfeld, 2022). Hormones act according to their structure to bind and promote genes or regulate their expression via secondary messengers. Fat soluble steroid hormones bind to protein carrier molecules in the blood, diffuse across the cell membrane, bind to cytoplasmic cell receptors and act as transcription factors. Other hormones like peptide hormones bind to

membrane-bound receptors that initiate pathways and cascades of reactions to activate transcription factors. The hormone concentration and time it circulates in the blood correlates with the intensity and duration of its effects. Once hormones have affected their target cells they become inactivated as they are metabolized or converted to another molecule (Bahrke & Yesalis, 2004; James, 2011). Negative feedback loops are common to regulate hormone concentrations within the body, where the products of the hormone's action inhibit its production either directly or indirectly. For example, in seasonally reproductive birds, the switch from short to long days increases production of gonadotropin hormone-releasing hormone and increases plasma levels of luteinizing hormone. Luteinizing hormone induces testicular development followed by an increase in gonadal steroid hormones such as testosterone that stimulate reproductive behaviour and secondary sex characteristics (Farner & Wingfield, 1980). Testosterone acts on the pituitary and hypothalamus, and signals to decrease the production of gonadotropin hormone-releasing hormone and luteinizing hormone respectively (Nelson & Kriegsfeld, 2022).

Hormones can modulate physiology and behaviour and are strongly associated with the survival and reproduction of an organism. Hormones affect a wide range of behaviours. For example, glucocorticoid hormones are involved in energy metabolism and stress response (Selye, 1937), androgens are involved in social behaviour and reproduction (Farner & Wingfield, 1980), and prolactin is involved in over 300 functions in the body including parental care and immune response (Smiley, 2019; Whittington & Wilson, 2013).

1.2.2 Behavioural endocrinology

Animal behaviour scientists are typically interested in understanding the proximate and ultimate explanations for behaviour. Proximate explanations include mechanisms of how the behaviour occurs while ultimate explanations explore more broad evolutionary questions like why animals exhibit behaviours. To explore more proximate explanations of behaviour, behaviour scientists have recently turned to the rapidly expanding field of behavioural endocrinology. Behavioural endocrinology is the scientific study of the interaction between hormones and behaviour centred on the principles that (1) hormones influence behaviour, and (2) behaviour can influence hormone concentrations (Nelson &

Kriegsfeld, 2022). Hormones may influence behaviour by affecting any or all three interacting components: input or sensory systems, processing or integration systems like the central nervous system, and output systems such as muscles (Nelson & Kriegsfeld, 2022). Each of these components can in turn affect the animal's endocrine state.

Behavioural endocrinology experiments require the assessment and measurement of behaviour in conjunction with the measurement or manipulation of hormones, either in the field or the lab. To establish a causal link between hormones and behaviour experimental results should determine that (1) removal/blockage or elevation of a hormone should stop or increase the actions of the hormone and (2) hormone concentrations and the behaviour in question should be covariant (Silver, 1978). Early studies in endocrinology quantified hormones using radioimmunoassay by using antibodies and purified radiolabeled ligands. More recently, enzyme-linked immunosorbent assays (ELISAs) are used where instead of radioactively tagging antibodies, they are tagged with an enzyme that changes the optical density (colour) of a substrate. To quantify hormones, researchers develop a standard curve of known concentrations that is compared against experimental samples to determine concentrations (Nelson & Kriegsfeld, 2022).

Hormones affect parental behaviour at each component of behaviour including input, processing, and output systems, such that responsiveness to hormones is a requirement of the parental phenotype (Ball & Balthazart, 2008). The endocrine system is involved in the initiation of reproduction, from gonad growth and maturation to reproductive and parental care behaviour (reviewed in Farner & Wingfield, 1980). In particular, sex steroids such as androgens and prolactin have been identified as proximate mechanisms mediating parental care behaviour (Numan & Insel, 2011; Smiley, 2019).

1.2.3 Androgens and parental care

Androgens are a class of steroid that are primarily associated with the stimulation of reproductive traits in males including differentiation of reproductive tracts, secondary sexual characteristics, spermatogenesis, and reproductive behaviour (Borg, 1994). Androgens exert their effects by binding to steroid hormone receptors in the brain (Davey & Grossmann, 2016). Behaviours may respond rapidly to changes in circulating androgen

levels (Steinman & Trainor, 2010). Androgens are produced by a series of reactions that start with cholesterol (Figure 1.2; Borg, 1994). This is converted to pregnenolone which can be converted to progesterone. As the hydrogen is replaced by a hydroxyl group in position 17, progesterone is converted to 17α–hydroxyprogesterone, which in turn is converted to testosterone. Testosterone can be converted to 11β-hydroxytestosterone and 11-ketotestosterone. Androgens act by binding to androgen receptors, specifically or promiscuously, to change the configuration of the receptor, the receptor localization signal is exposed, to allow for translocation into the nucleus where gene expression can be altered (Dehm & Tindall, 2006). Androgens can also rapidly act via non-genomic mechanisms to active cytoplasmic proteins (reviewed in Freeman et al., 2000. In mammals and birds, testosterone is the primary androgen (Borg, 1994; Wingfield et al., 1987), while in most teleost fish, 11-ketotestosterone is often considered to be the main androgen in teleost males (Borg, 1994).

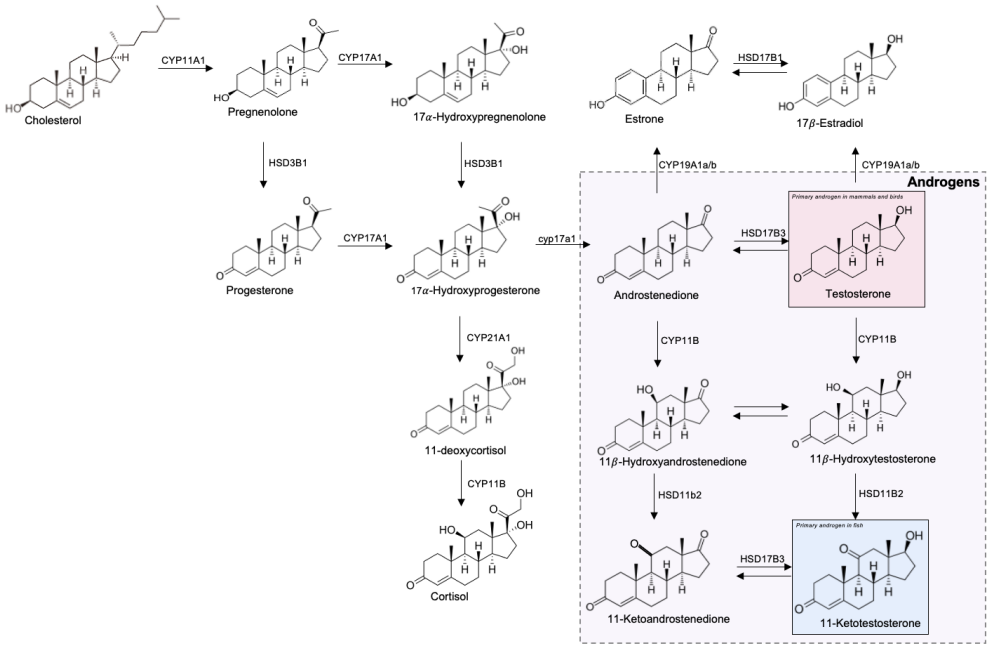

Figure 1.2 Gonadal and steroid hormones and possible conversions (figure modified from Borg, 1994). Purple highlighted region indicates androgen hormone family, with the primary androgen in mammals and bird highlighted in red, and the primary androgen in fish highlighted in blue. Abbreviations are CYP = Cytochrome P450 family, subfamily, polypeptide; HSD = Hydroxysteroid dehydrogenase.

Increases in testosterone increases aggressive behaviours like singing, posturing, and attacking in mammals and birds (Nelson & Kriegsfeld, 2022; Wingfield et al., 1987). High concentrations of androgens are highly correlated with aggressive behaviours (Kuba et al., 2015), and artificially increased concentrations can induce aggressive behaviour (Van Duyse et al., 2002). In fish, testosterone elicits a response consistent with other taxa but at a lower response than 11-ketotestosterone (Moore et al., 2020). Males experimentally increased androgen levels display more aggressive behaviours (Cunha et al., 2019; Rodgers et al., 2012). While androgens are critical to the establishment and maintenance of territories and courtship, high concentrations can suppress parental care and immune response (Wingfield et al., 1990). Consequently, parental males must regulate androgens during parental care to balance nurturing and aggressive behaviours (Wingfield et al., 1990). The challenge hypothesis was proposed by Wingfield et al. (1990) and posits males elevate androgens only in response to a challenge while caring for offspring, rather than maintaining high levels of androgens that may impact nurturing behaviour. Given that parents provide care by nurturing and by defending their offspring, it is clear that androgen-mediated aggressive behaviour is important to parental care.

1.2.4 Prolactin and parental care

Prolactin is a multifunctional polypeptide hormone belonging to a family of hormones including growth hormone and somatolactin (Dobolyi et al., 2020). Prolactin is primarily produced at high levels in pituitary tissues, along with extrapituitary production in several other tissues such as liver, intestines, gonads, muscle, and kidney (Ben-Jonathan et al., 1996). Prolactin is occasionally expressed in tissues that also expressed the prolactin receptor, raising the potential prolactin may be able to act in an autocrine or paracrine manner in addition to endocrine (reviewed in Bole-Feysot et al., 1998). Prolactin secretion is influenced by stimulatory and inhibitory substances including neurohormones, sex steroids, and plasma factors from other tissues (reviewed in Kawauchi et al., 2009). Once prolactin moves to the site of action, it binds to a prolactin receptor via two binding sites. Dimerized prolactin receptors activate a JAK kinase molecule, which phosphorylates STAT transcription factors that dimerize and migrate to the nucleus to bind to promotors and activate prolactin-responsive genes (Bole-Feysot et al., 1998).

Prolactin is ubiquitous in vertebrates with over 300 known functions (Bole-Feysot et al., 1998; Gong et al., 2022) including osmoregulation, growth and development, metabolism, behavioural regulation, immune response, maintenance of water and electrolyte balance, and reproduction. The prolactin gene is conserved across vertebrates containing four well-conserved cysteine residues (K. M. Lee et al., 2006) hypothesized to be responsible for prolactin's common roles, particularly in reproduction and parental care (Manzon, 2002). The name 'pro-lactin' was derived from the idea of a pituitary-derived stimulatory factor that initiated milk development had been identified in the 1920s (Stricker & Grueter, 1928). Prolactin was first identified in birds in the 1930s and is associated with parental care in almost every female bird studied to date (Angelier et al., 2016; Smiley, 2019). In mammals, prolactin is associated with pregnancy, lactation, and parental care after birth in both females and males (Storey et al., 2000). In both mammals and birds, high concentrations of circulating prolactin correlates with increases of parental care behaviours (Dixson & George, 1982; Li et al., 2022; Smiley, 2019; Storey et al., 2000). Studies on the effect of prolactin on fish parental care indicate administration of mammalian prolactin induce parental care behaviour (Blüm & Fiedler, 1965; Cunha et al., 2019), but have not yet established a causal relationship. There are no studies to date that have quantified endogenous prolactin in fish and determined how concentrations covary with parental care behaviour.

1.2.5 Behavioural Genomics

While the neuroendocrine regulation of parental care is relatively well understood, a concept inherent to behavioural endocrinology is the idea that if hormones produce a change in behaviour, there is likely a change in the animal's brain. Moreover, advancements in genomic technologies allow for a more in-depth characterization of changes in the brain and processes in other tissues that facilitate care.

A cornerstone technique of molecular biology is the polymerase chain reaction (PCR) and the subsequent quantitative PCR (qPCR), which is a method used to determine and compare the quantity of gene expression. This fine lens allows researchers to compare single genes at a time. To analyze larger profiles, an earlier technique used to determine relative gene expression during the onset of, or during, a behaviour was DNA or RNA

arrays, which assessed the expression of thousands of genes on a chip or slide using a computer. More recently, RNA sequencing is a relatively new technique that uses next-generation sequencing to determine and quantify all cellular RNA (mRNA, rRNA, tRNA, etc.) in a sample. This allows researchers to analyze the continuously changing cellular transcriptome and identify novel genes and expression patterns. Moreover, these technological advancements in genomic technologies help to understand the role of individual genes in the evolution and expression of behaviour.

1.2.6 Genes and parental care

Genomic studies have determined that alterations in gene expression pertinent to parental care occurs predominantly in the brain. The neural architecture related to this in mammals is well-defined (Dobolyi et al., 2014; Kohl & Dulac, 2018; Zhang et al., 2021). Lesions in the hypothalamic preoptic area impair parental care (Lee & Brown, 2007) and exhibits high expression of receptors for hormones linked to parental care (Numan & Insel, 2011). Genes associated with parental care have also been identified in the hypothalamic-septal region in zebra finch (*Taeniopygia guttata*) and the diencephalon and telencephalon in male three-spined stickleback (*Gasterosteus aceuleatus*), suggesting a full characterization of parental care associated gene expression should not be limited to one area of the brain (Bukhari et al., 2019; Kumari et al., 2022).

Transcriptomic analyses have determined genes expressed during parental care in mammals, birds, and fish are often associated with metabolism, neuromodulatory structure, immune system regulation, and transcription (Bukhari et al., 2019; Duclot et al., 2022; Lynch et al., 2019). However, much of this present has focused on maternal care, reflecting the dominance of this parental care system in mammals and birds. Given the prevalence of paternal care and the different costs associated with male compare compared to female (Klug et al., 2013), it is important to understand the genetic pathways associated with the expression and evolution of paternal care.

1.3 Study Species

I investigated the influence of perceived and realized paternity on parental care behaviour in sunfish. The majority of my thesis focuses on the parental care behaviour of bluegill

sunfish (*Lepomis macrochirus*), along with the parental care provided by the hybrid offspring produced with pumpkinseed sunfish (*Lepomis gibbosus*).

1.3.1 Bluegill sunfish

Bluegill sunfish are a member of the Centrarchidae family and are found in temperate freshwater across North America (Scott & Crossman, 1988). I studied a population of bluegill in Lake Opinicon (44˚34'N, 76˚19'W), which have been studied for their alternative reproductive tactics since the late 1970s (Gross, 1982). The reproductive season lasts from late-May to early-July, during which the 'parental' male morph (Figure 1.3) enters the littoral zone and sweeps the floor substrate with their caudal fin to construct nests in breeding colonies of up to 150 males (Gross & MacMillan, 1981). On spawning day parental males court and spawn with females, then provide uniparental male care to their brood for 7-10 days (Gross, 1982). Males provide nurturing care by fanning eggs within the nest to sweep debris from eggs and flush oxygen over the nest. They circle the nest as a guarding behaviour, which may also increase the movement of water and oxygen over the nest (Côté & Gross, 1993). During the parental care period, parental males forgo their reduce their foraging in lieu of caring for their brood, and may lose up to 10% of their body mass (Magee et al., 2006). The bluegill mating system is highly promiscuous with an average of 25% offspring sired by precocious 'cuckolder' males (Gross & Charnov, 1980). Cuckolder males mature within 1-2 years and use size-based tactics to fertilize eggs in parental male nests. Small cuckolders are called 'sneakers' and conceal themselves in vegetation until they intrude by darting into parental male nests to fertilize eggs (Gross, 1982). As they grow, cuckolders switch tactics and use female mimicry. These 'satellite' or mimic males orbit around the nest and enter when a parental male is spawning with a female, then fertilize the eggs while acting as if they too are spawning with the male (Gross, 1982). Given the potential for low paternity within the nest, parental males are able to discern relatedness to their brood using olfactory cues released by larvae after eggs hatch (Neff & Sherman, 2003). Males adjust their care in response to paternity recognition, where males with higher paternity provide higher quality parental care (Neff, 2003; Neff & Gross, 2001).

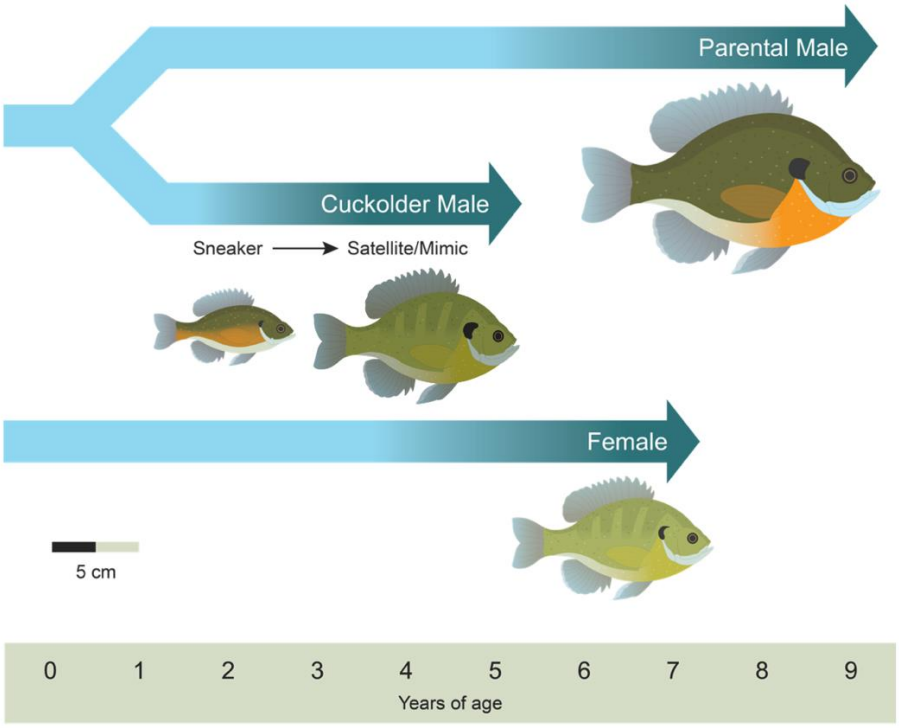

Figure 1.3 Alternative life history tactics of bluegill sunfish (*Lepomis macrochirus*). Figure has been modified from Gross & Charnov (1980). Ages are approximate and may differ among populations.

Similar to bluegill, pumpkinseed sunfish in Lake Opinicon also exhibit alternative reproductive tactics where parental males guard and care for nests while cuckolder males use a sneaking tactic to fertilize eggs (Gross, 1979). On average, pumpkinseed sire close to 78% off the larvae in their nest (Garner & Neff, 2013). 9% of the nest is sired by pumpkinseed cuckolders while the remaining 13% are sired by bluegill cuckolders (Garner & Neff, 2013). When bluegill cuckolders fertilize pumpkinseed nests, they produce hybrid sunfish (Konkle & Philipp, 1992; Figure 1.4). While hybrid males are functionally sterile (Immler et al., 2011), they provide care for offspring. Very little is known about the mechanisms underlying hybrid parental care, including behaviour, kin recognition, endocrinology, and genetics.

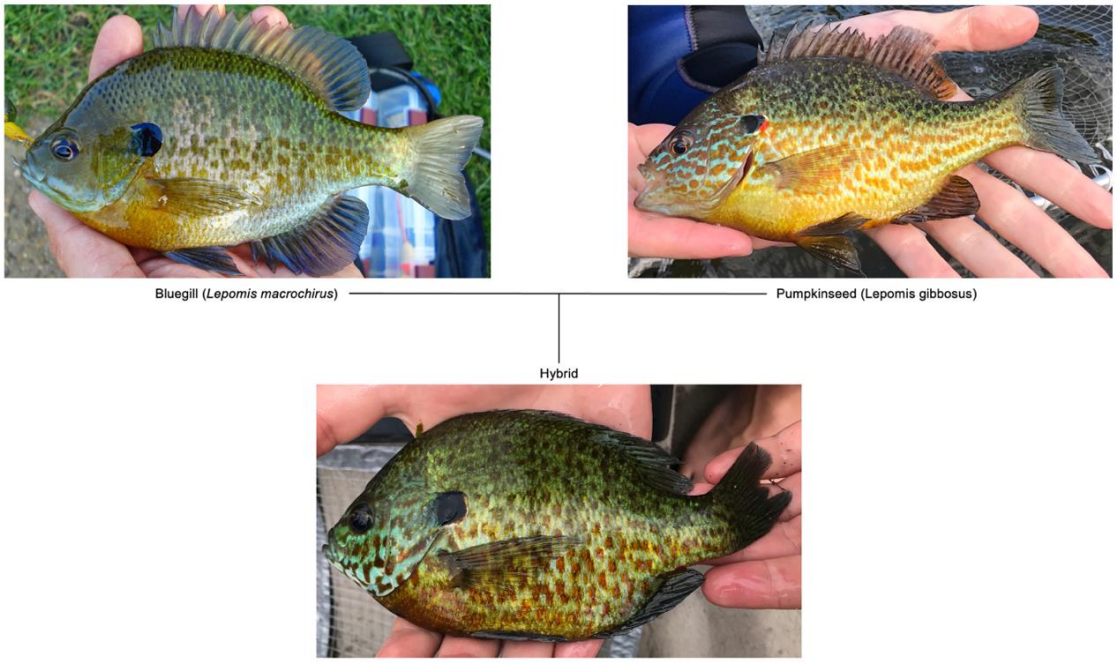

Figure 1.4 Images of bluegill and pumpkinseed sunfish, and their hybrid offspring. Bluegill photo by Nike Brooke. Hybrid and pumpkinseed photos taken by Emma Churchman.

1.3.2 Mechanisms regulating bluegill parental care

Parental males also adjust their care behaviour in response to hormone administration. When implanted with 11-ketotestosterone, bluegill increase the frequency of aggressive nest defensive behaviour (Cunha et al., 2019; Rodgers et al., 2013). Parental males also increase the frequency of aggressive behaviour and decrease the frequency of nurturing behaviour in response to testosterone administration (Rodgers et al., 2012). Similarly, when treated with androgen blocker flutamide, nurturing care increases and aggressive care decreases (Rodgers et al., 2013). When considering paternity, after larvae hatch and bluegill can determine paternity, circulating testosterone and 11-ketotestosterone concentrations are positively correlated with paternity within the nest (Neff & Knapp, 2009). Taken together, androgens are clearly involved in bluegill aggressive behaviour. While 11-ketotestostone is correlated with paternity, and paternity affects parental care, it is possible that 11-ketotestosterone may mediate this change in behaviour.

Prolactin also appears to have a role in bluegill nurturing parental care. Early studies administered bromocriptine, a prolactin inhibitor, and observed a reduction in nurturing behaviour (Kindler et al., 1991). More recently, by increasing circulating prolactin levels via subcutaneous implants, parental males also increase their nurturing behaviour (Cunha et al., 2019). Circulating endogenous prolactin concentrations have only been quantified during the parental care period in female Nile tilapia (*Oreochromis niloticus*; Tacon et al., 2000). Prolactin concentrations varied during the parental care period, however the study did not analyze or link these concentrations to behaviour (Tacon et al., 2000). While bluegill exhibit a positive nurturing response to elevated prolactin, the hormone has not been quantified in response to paternity, nor have any studies explored a causal link to endogenous circulating concentrations and parental care behaviour in fish.

Early work using microarrays to determine how increases in 11-ketotestosterone affect gene expression during parental care in bluegill observed that males with increased 11-ketotestosterone concentrations have reduced expression of immune related genes but were not associated with prolactin gene expression (Partridge et al., 2014). Transcriptome work in bluegill has yet to establish a relationship to paternity and how this is related to parental care.

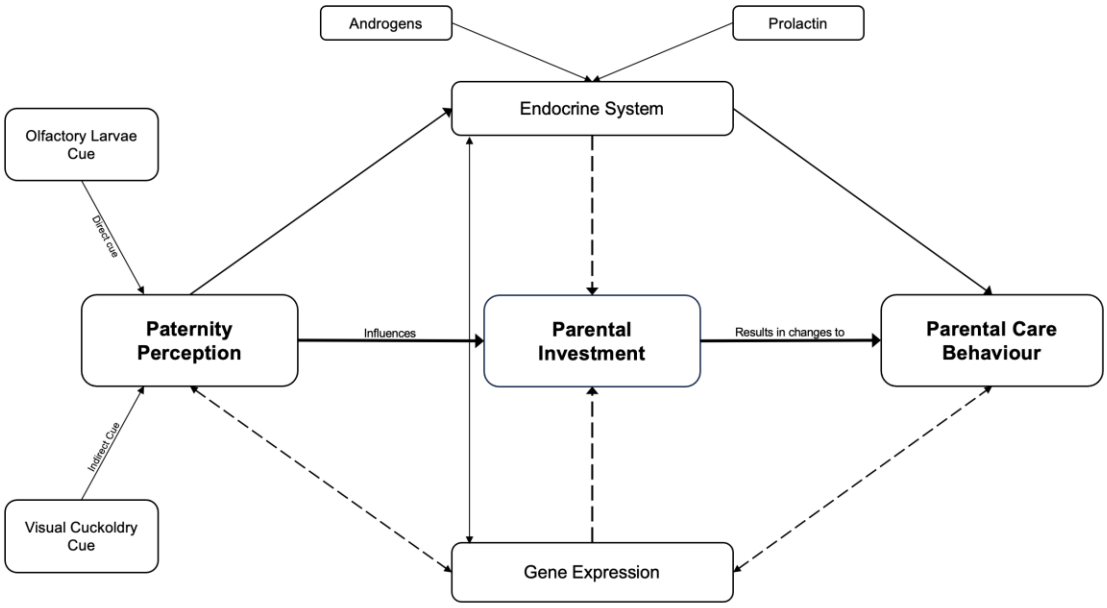

Figure 1.5 Factors influencing adjustments in parental care in bluegill sunfish. Solid lines indicate established relationships, and dotted lines indicate areas that require further investigation.

1.4 Research Objective

The primary objective of this thesis is to determine what mechanisms underlie adaptive adjustments in parental care behaviour in response to paternity in sunfish. My overarching hypothesis was that if 11-ketotestosterone, prolactin, or gene expression varies in response to experimentally manipulated paternity, they may be associated with the adjustment of parental investment via care behaviour provided by parental males. Across three of my data chapters (2-4) I used the bluegill alternative reproductive system to manipulate perceived and realized paternity and analyze each potential proximate mechanism. In my final data chapter, I broadened my scope to hybrid sunfish to determine if these mechanisms have the potential to drive parental care irrespective of paternity. To do this, I used a two-prong approach to fully characterize response to paternity. To manipulate indirect cues of paternity, I experimentally provided cues of cuckoldry on spawning day to increase the uncertainty of paternity within the nest. To manipulate direct paternity, I experimentally lowered paternity within the nest by swapping eggs between nests.

My research had the following objectives:

1) To examine the effect of direct and indirect paternity cues on 11-ketotestosterone concentrations, and how this relates to aggressive behaviour during egg and larvae care (Chapter 2).

2) To characterize circulating prolactin concentrations during the parental care period in fish and correlate endogenous prolactin to parental care behaviour (Chapter 2).

3) To examine the effect of indirect paternity cues on circulating prolactin concentrations during the egg care period (Chapter 3).

4) To examine the effect of direct paternity manipulation on the bluegill transcriptome in response to direct paternity manipulation (Chapter 4).

5) To characterize hybrid parental care behaviour compared to bluegill and pumpkinseed sunfish (Chapter 5).

6) To characterize hybrid 11-ketotestosterone and prolactin profiles during the parental care period (Chapter 5).

7) To examine the effect of direct paternity manipulation on hybrid hormone levels and gene expression in comparison to bluegill and pumpkinseed (Chapter 5).

The collective goal of this work was to identify endocrine and molecular mechanisms that underlie changes in parental care behaviour in sunfish. In doing so, I aim to not only enhance our proximate understanding of adaptive adjustments in parental care, but also contribute to the broader evolutionary narrative of how parental care itself has evolved.

1.5 References

Angelier, F., Wingfield, J. C., Tartu, S., and Chastel, O. (2016). Does prolactin mediate parental and life-history decisions in response to environmental conditions in birds? A review. *Hormones and Behavior*, **77,** 18-29.

Bahrke, M. S., and Yesalis, C. E. (2004). Abuse of anabolic androgenic steroids and related substances in sport and exercise. *Current Opinion in Pharmacology*, **4,** 614-620.

Ball, G. F., and Balthazart, J. (2008). Individual variation and the endocrine regulation of behaviour and physiology in birds: A cellular/molecular perspective. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **363,** 1699–1710.

Ben-Jonathan, N., Mershon, J. L., Allen, D. L., and Steinmetz, R. W. (1996). Extrapituitary prolactin: Distribution, regulation, functions, and clinical aspects. *Endocrine Reviews*, **17,** 639-669.

Blüm, V., and Fiedler, K. (1965). Hormonal control of reproductive behavior in some Cichlid fish. *General and Comparative Endocrinology*, **5,** 186–196.

Bole-Feysot, C., Goffin, V., Edery, M., Binart, N., and Kelly, P. A. (1998). Prolactin (PRL) and its receptor: Actions, signal transduction pathways and phenotypes observed in PRL receptor knockout mice. *Endocrine Reviews*, **19**, 225-268.

Borg, B. (1994). Androgens in teleost fishes. *Comparative Biochemistry and Physiology*, **109C**, 219–245.

Bridges, R. S. (2015). Neuroendocrine regulation of maternal behavior. *Frontiers in Neuroendocrinology*, **36**, 178–196.

Bukhari, S. A., Saul, M. C., James, N., Bensky, M. K., Stein, L. R., Trapp, R., and Bell, A. M. (2019). Neurogenomic insights into paternal care and its relation to territorial aggression. *Nature Communications*, **10,** 4437.

Champagne, F. A., and Curley, J. P. (2013). Genetics and epigenetics of parental care. In N.J. Royle, and P.T. Smiseth (Eds), *The Evolution of Parental Care.* Oxford Academic.

Chen, Y. H., Cheng, W. C., Yu, H. T., and Kam, Y. C. (2011). Genetic relationship between offspring and guardian adults of a rhacophorid frog and its care effort in response to paternal share. *Behavioral Ecology and Sociobiology*, **65,** 2329–2339.

Clutton-Brock, T. H. (1991). *The Evolution of Parental Care*. Princeton University Press.

Côté, I. M., and Gross, M. R. (1993). Reduced disease in offspring: A benefit of coloniality in sunfish. *Behavioral Ecology and Sociobiology*, **33,** 269–274.

Cunha, A. A. P., Partridge, C. G., Knapp, R., and Neff, B. D. (2019). Androgen and prolactin manipulation induces changes in aggressive and nurturing behavior in a fish with male parental care. *Hormones and Behavior*, **116,** 104582.

Darwin, C. (1871). The descent of man and selection in relation to sex, In Charles Darwin, The origin of species and the descent of man (combined volume). *Journal of Anatomy and Physiology*, **5**.

Davey, R. A., and Grossmann, M. (2016). Androgen Receptor Structure, Function and Biology: From Bench to Bedside. *The Clinical Biochemist. Reviews*, **37,** 3-15.

Dehm, S. M., and Tindall, D. J. (2006). Ligand-independent androgen receptor activity is activation function-2-independent and resistant to antiandrogens in androgen refractory prostate cancer cells. *Journal of Biological Chemistry*, **281,** 27882-27893.

Dixson, A. F., and George, L. (1982). Prolactin and parental behaviour in a male New World primate. *Nature*, **299,** 551–553.

Dobolyi, A., Grattan, D. R., and Stolzenberg, D. S. (2014). Preoptic inputs and mechanisms that regulate maternal responsiveness. *Journal of Neuroendocrinology*, **26,** 627-640.

Dobolyi, A., Oláh, S., Keller, D., Kumari, R., Fazekas, E. A., Csikós, V., Renner, É., and Cservenák, M. (2020). Secretion and function of pituitary prolactin in evolutionary perspective. *Frontiers in Neuroscience*, **14,** 621.

Duclot, F., Liu, Y., Saland, S. K., Wang, Z., and Kabbaj, M. (2022). Transcriptomic analysis of paternal behaviors in prairie voles. *BMC Genomics*, **23,** 679.

Farner, D. S., and Wingfield, J. C. (1980). Reproductive endocrinology of birds. *Annual Review of Physiology*, **42,** 457-472.

Freeman, M. E., Kanyicska, B., Lerant, A., and Nagy, G. (2000). Prolactin: Structure, function, and regulation of secretion. *Physiological Reviews*, **80,** 1523-1561.

Garner, S. R., and Neff, B. D. (2013). Alternative male reproductive tactics drive asymmetrical hybridization between sunfishes (*Lepomis* spp.). *Biology Letters*, **9,** 20130658.

Gong, N., Ferreira-Martins, D., Norstog, J. L., McCormick, S. D., and Sheridan, M. A. (2022). Discovery of prolactin-like in lamprey: Role in osmoregulation and new insight into the evolution of the growth hormone/prolactin family. *Proceedings of the National Academy of Sciences of the United States of America*, **119,** e2212196119.

Gray, S. M., McKinnon, J. S., Tantu, F. Y., and Dill, L. M. (2008). Sneaky egg-eating in *Telmatherina sarasinorum*, an endemic fish from Sulawesi. *Journal of Fish Biology*, **73,** 728–731.

Gross, M. R. (1979). Cuckoldry in sunfishes (*Lepomis*: Centrarchidae). *Canadian Journal of Zoology*, **57,** 1507-1509.

Gross, M. R. (1982). Sneakers, satellites and parentals: polymorphic mating strategies in North American sunfishes. *Ethology*, **60,** 1–26.

Gross, M. R., and Charnov, E. L. (1980). Alternative male life histories in bluegill sunfish (*Lepomis macrochirus*). *Proceedings - National Academy of Science, U.S.A.*, **77,** 6937–6940.

Gross, M. R., and MacMillan, A. M. (1981). Predation and the evolution of colonial nesting in bluegill sunfish (Lepomis macrochirus). *Behavioral Ecology and Sociobiology*, **8,** 163–174.

Hain, T. J. A. (2015). *The evolution of kin recognition*. University of Western Ontario, Electronic Thesis and Dissertation Repository, 3431.

Hain, T. J. A., and Neff, B. D. (2006). Promiscuity drives self-referent kin recognition. *Current Biology*, **16,** 1807-1811.

Hamilton, W. D. (1964). The genetical evolution of social behaviour. I. *Journal of Theoretical Biology*, **7,** 1-16.

Holmes, W. G., and Sherman, P. W. (1982). The ontogeny of kin recognition in two species of ground squirrels. *Integrative and Comparative Biology*, **22,** 491-517.

Immler, S., Hamilton, M. B., Poslusny, N. J., Birkhead, T. R., and Epifanio, J. M. (2011). Post-mating reproductive barriers in two unidirectionally hybridizing sunfish (Centrarchidae: *Lepomis*). *Journal of Evolutionary Biology*, **24,** 111-120.

James, M. O. (2011). Steroid catabolism in marine and freshwater fish. *Journal of Steroid Biochemistry and Molecular Biology*, **127,** 167-175.

Kawauchi, H., Sower, S. A., and Moriyama, S. (2009). Chapter 5 The neuroendocrine regulation of prolactin and somatolactin secretion in fish. *Fish Physiology*, **28,** 197-234.

Kindler, P. M., Bahr, J. M., Gross, M. R., and Philipp, D. P. (1991). Hormonal regulation of parental care behavior in nesting male bluegills: do the effects of bromocriptine suggest a role for prolactin? *Physiological Zoology*, **64,** 310–322.

Klug, H., Alonzo, S. H., and Bonsall, M. B. (2013). Theoretical foundations of parental care. In N.J. Royle, and P.T. Smiseth (Eds), *The Evolution of Parental Care*. Oxford Academic.

Klug, H., and Bonsall, M. B. (2014). What are the benefits of parental care? The importance of parental effects on developmental rate. *Ecology and Evolution*, **4,** 2330–2351.

Kohl, J., and Dulac, C. (2018). Neural control of parental behaviors. *Current Opinion in Neurobiology*, **49,** 116-122.

Konkle, B. R., and Philipp, D. P. (1992). Asymmetric hybridization between two species of sunfishes (*Lepomis*: Centrarchidae). *Molecular Ecology*, **1,** 215-222.

Kuba, J., Błaszczyk, B., Stankiewicz, T., Skuratko, A., and Udała, J. (2015). Analysis of annual changes in the concentrations of selected macro- and microelements, thyroxine, and testosterone in the serum of red deer (*Cervus elaphus*) stags. *Biological Trace Element Research*, **168,** 356-361.

Kumari, R., Fazekas, E. A., Morvai, B., Udvari, E. B., Dóra, F., Zachar, G., Székely, T., Pogány, Á., and Dobolyi, Á. (2022). Transcriptomics of parental care in the hypothalamic-septal region of female zebra finch brain. *International Journal of Molecular Sciences*, **23,** 2518.

Lee, A. W., and Brown, R. E. (2007). Comparison of medial preoptic, amygdala, and nucleus accumbens lesions on parental behavior in California mice (*Peromyscus californicus*). *Physiology and Behavior*, **92,** 617-628.

Lee, K. M., Kaneko, T., and Aida, K. (2006). Prolactin and prolactin receptor expressions in a marine teleost, pufferfish *Takifugu rubripes*. *General and Comparative Endocrinology*, *146*(3).

Li, X., Xu, W., Yu, J., Zhang, W., and Wang, H. (2022). Plasma levels of luteinizing hormone and prolactin in relation to double brooding in Great Tit (*Parus major*). *Avian Research*, *13*, 100017.

Lynch, K. S., O'Connell, L. A., Louder, M. I. M., Balakrishnan, C. N., and Fischer, E. K. (2019). Understanding the loss of maternal care in avian brood parasites using preoptic area transcriptome comparisons in brood parasitic and non-parasitic blackbirds. *G3: Genes, Genomes, Genetics*, **9,** 1075–1084.

Scott, W. B., and Crossman, E. J. (1988). Freshwater Fishes of Canada. Oakville (ON): Galt House Publications.

Manica, A. (2004). Parental fish change their cannibalistic behaviour in response to the cost-to-benefit ratio of parental care. *Animal Behaviour*, **67,** 1015–1021.

Manzon, L. A. (2002). The role of prolactin in fish osmoregulation: A review. *General and Comparative Endocrinology*, **125,** 291–310.

Mateo, J. M. (2004). Recognition systems and biological organization: The perception component of social recognition. *Annales Zoologici Fennici*, **41,** 729-745.

Møller, A. P., and Birkhead, T. R. (1993). Cuckoldry and sociality: a comparative study of birds. *American Naturalist*, **142,** 118–140.

Montgomerie, R. D., and Weatherhead, P. J. (1988). Risks and rewards of nest defence by parent birds. *Quarterly Review of Biology*, **63,** 167-187.

Moore, I. T., Hernandez, J., and Goymann, W. (2020). Who rises to the challenge? Testing the Challenge Hypothesis in fish, amphibians, reptiles, and mammals. *Hormones and Behavior*, **123,** 104537.

Neff, B. D. (2003). Decisions about parental care in response to perceived paternity. *Nature*, **422,** 716–718.

Neff, B. D., and Gross, M. R. (2001). Dynamic adjustment of parental care in response to perceived paternity. *Proceedings of the Royal Society B: Biological Sciences*, **268,** 1559–1565.

Neff, B. D., and Knapp, R. (2009). Paternity, parental behavior and circulating steroid hormone concentrations in nest-tending male bluegill. *Hormones and Behavior*, **56,** 239–245.

Neff, B. D., and Sherman, P. W. (2003). Nestling recognition via direct cues by parental male bluegill sunfish (*Lepomis macrochirus*). *Animal Cognition*, **6,** 87–92.

Nelson, R., and Kriegsfeld, L. (2022). *An Introduction to Behavioral Endocrinology, Sixth Edition* (6th ed.). Oxford University Press.

Numan, M., and Insel, T. R. (2011). *The Neurobiology of Parental Behavior*. Springer.

Partridge, C., Rodgers, C. M. C., Knapp, R., and Neff, B. D. (2014). Androgen effects on immune gene expression during parental care in bluegill sunfish (*Lepomis macrochirus*). *Canadian Journal of Zoology*, **93,** 9-13.

Rodgers, C. M. C., Neff, B. D., and Knapp, R. (2012). Effects of exogenous testosterone on parental care behaviours in male bluegill sunfish (*Lepomis macrochirus*). *Ethology*, **118,** 636–643.

Rodgers, C. M. C., Neff, B. D., and Knapp, R. (2013). Androgen-mediated nurturing and aggressive behaviors during paternal care in bluegill sunfish (*Lepomis macrochirus*). *Hormones and Behavior*, **63,** 454-461.

Selye, H. (1937). The significance of the adrenals for adaptation. *Science*, **85,** 247-248.

Silver, R. (1978). The parental behavior of ring doves. *American Scientist*, **66,** 206–215.

Smiley, K. O. (2019). Prolactin and avian parental care: New insights and unanswered questions. In *Hormones and Behavior*, **111,** 114–130.

Smiseth, P. T., Kölliker, M., and Royle, N. J. (2013). What is parental care? In N.J. Royle, and P.T. Smiseth (Eds), *The Evolution of Parental Care.* Oxford Academic.

Steinman, M., and Trainor, B. (2010). Rapid effects of steroid hormones on animal behavior. *Nature Education Knowledge*, **3,** 1.

Storey, A. E., Walsh, C. J., Quinton, R. L., and Wynne-Edwards, K. E. (2000). Hormonal correlates of paternal responsiveness in new and expectant fathers. *Evolution and Human Behavior*, **21,** 79–95.

Stricker, P., and Grueter, R. (1928). Action du lobe antérieur de l'hypophyse sur la monté laiteuse. *Comptes Rendus Des Séances de La Société de Biologie et de Ses Filiales*, **99,** 1978–1980.

Tacon, P., Baroiller, J. F., Le Bail, P. Y., Prunet, P., and Jalabert, B. (2000). Effect of egg deprivation on sex steroids, gonadotropin, prolactin, and growth hormone profiles during

the reproductive cycle of the mouthbrooding cichlid fish *Oreochromis niloticus*. *General and Comparative Endocrinology*, **117,** 54–65.

Townshend, T. J., and Wootton, R. J. (1984). Effects of food supply on the reproduction of the convict cichlid, *Cichlasoma nigrofasciatum*. *Journal of Fish Biology*, **24,** 91-104.

Trivers, R. L. (1972). Parental investment and sexual selection. In B. Campbell (Ed), *Sexual Selection and the Descent of Man: 1871-1971*. Aldine.

Van Duyse, E., Pinxten, R., and Eens, M. (2002). Effects of testosterone on song, aggression, and nestling feeding behavior in male great tits, *Parus major*. *Hormones and Behavior*, **41,** 178-186.

Westneat, D. F., and Sherman, P. W. (1993). Parentage and the evolution of parental behavior. *Behavioral Ecology*, **4,** 66–77.

Whittington, C. M., and Wilson, A. B. (2013). The role of prolactin in fish reproduction. *General and Comparative Endocrinology*, **191,** 123–136.

Windt, W., and Curio, E. (1986). Clutch Defence in Great Tit (*Parus major*) Pairs and the Concorde Fallacy. *Ethology*, **72,** 236-242.

Wingfield, J. C., Ball, G. F., Dufty, A. M., Hegner, R. E., and Ramenofsky, M. (1987). Testosterone and Aggression in Birds. *American Scientst*, **75,** 602–608.

Wingfield, J. C., Hegner, R. E., Dufty, A. M., and Ball, G. F. (1990). The "Challenge Hypothesis": Theoretical implications for patterns of testosterone secretion, mating systems, and breeding strategies. *American Naturalist*, **136,** 829–846.

Zhang, G. W., Shen, L., Tao, C., Jung, A. H., Peng, B., Li, Z., Zhang, L. I., and Tao, H. W. (2021). Medial preoptic area antagonistically mediates stress-induced anxiety and parental behavior. *Nature Neuroscience*, **24,** 516-528.

Chapter 2

2 Parental care behaviour in response to perceived paternity is not mediated by 11-ketotestosterone in bluegill sunfish

A version of this chapter has been published in General and Comparative Endocrinology:

Churchman, E.K.L., Hain, T.J.A., Knapp, R., Neff, B.D. (2023) Parental care behaviour in response to perceived paternity is not mediated by 11-ketotestosterone in bluegill sunfish. *General and Comparative Endocrinology, 343*, 114367.

2.1 Introduction

Parental care is essential to the survival of many young animals. Quality parental care can increase the rate of survival to hatch/birth, growth rate, and even future reproduction later in life (Clutton-Brock, 1991; Klug & Bonsall, 2014). While the benefits of parental care are well-documented, parental care can be costly. Providing care often requires parents to forego potential breeding opportunities, reduce their foraging rate, and can make them more susceptible to predation and parasitism (reviewed by Alonso-Alvarez & Velando 2012). Parental investment theory indicates that these competing factors should result in parents assessing offspring value and then investing more care in high-quality or otherwise more valuable offspring (Trivers, 1972).

Parental investment theory predicts that parents should alter their level of care to reflect the reproductive value of their brood (Trivers, 1972). In an evolutionary context, this response to value is adaptive provided that parents invest more in offspring that are most likely to contribute to the parents' fitness (Westneat & Sherman, 1993). Reproductive value may be based on the number and size of the offspring as well as the relatedness of the offspring to the parent (Westneat & Sherman, 1993). Offspring number and size typically can be assessed fairly easily with large broods valued over small broods and large or otherwise healthy offspring valued over small or sickly offspring (Montgomerie & Weatherhead, 1988). To determine the relatedness of offspring to a parent, however, requires some form of kin recognition and discrimination (Mateo 2004).

Kin recognition mechanisms can allow parents to allocate parental care based on paternity. This allocation of care in response to relatedness, or genetic value, is widespread across taxa, including for example the rhacophorid frog (*Kurixalus eiffingeri*, Chen et al., 2011), scissortail sergeant (*Abudefduf sexfaciatus*, Manica, 2004), sarasins ährenfisch (*Telematherina sarasinorum*, Gray et al., 2008), bluegill sunfish (*Lepomis macrochirus*, Neff & Gross 2001; Neff 2003), and at least 52 species of birds (reviewed in Møller & Birkhead, 1993). Animals can recognize kin directly or indirectly. Indirect kin recognition involves using context-based cues such as location or the number of intrusions by brood parasites to determine the likelihood that individuals encountered are related (Mateo, 2004). For example, in cliff-nesting birds, finding a nestling in one's nest is a strong indication of relatedness because nestlings are unable to move between nests (Cullen, 1957). Bluegill males use the number of sneakers around their nest on the day of spawning to make inferences about their paternity within the nest (Neff, 2003). Direct kin recognition occurs when animals use methods such as familiarity, or phenotype matching to interpret direct cues of relatedness (Holmes & Sherman, 1982). Familiarity is based on prior association, in the sense that individuals remember the phenotypes of individuals they have interacted with in situations normally associated with kinship and later, recognize and treat those individuals as kin (Mateo 2004). Phenotype matching is most common in species with large broods, including fish (Hain, 2015). This form of recognition occurs when individuals form a 'template' of what related individuals look, smell or sound like based on the phenotypes of family members encountered during development (Holmes & Sherman, 1982). These individuals later compare this template to phenotypes of individuals they encounter to determine if they are related (Holmes and Sherman, 1982). In promiscuous species with mixed broods, individuals may use self-referent phenotype matching whereby the kin template is formed using their own phenotype (Hain and Neff, 2006). Specifically, individuals born into broods with mixed relatedness should be more likely to form a self-referent template to determine relatedness of other individuals.

Behavioural endocrinology is a rapidly expanding field with hormones being proposed as a proximate mechanism to mediate parental care behaviour (Numan & Insel, 2011; Smiley et al., 2019). In particular, androgens have been shown to be critical to the establishment and then maintenance of territories and courtship by modulating behaviour. However, increases in androgens can suppress nurturing parental care and immune response, which requires parents

to regulate their androgens during the parental care period (Wingfield et al. 1990). There is a large body of research demonstrating that testosterone in birds mediates aggressive behaviour during the breeding and parental care season. Typically, increases in testosterone increase behaviours like singing, posturing, and attacking (Nelson, 1995; Wingfield et al., 2000). The challenge hypothesis was proposed by Wingfield *et al.* (1990) as a way for parents to balance the trade-off of aggressive behaviour and parental care, by increasing androgen synthesis only in response to challenges to avoid suppressing other forms of parental care and immune response. The challenge hypothesis has since been supported in several taxa including fish, mammals, and reptiles (reviewed in Moore et al., 2020). While the challenge hypothesis broadly explains androgen regulation, much of the research is focused on mammals and birds where testosterone is the primary androgen. When tested in fish, T elicits a response consistent with other taxa, but at a lower response than 11-ketotestosterone (Moore et al., 2020). Unaromatizable 11-ketotestosterone is the active metabolite of testosterone in fishes (Borg, 1994). While fish synthesize 11-ketotestosterone, testosterone, and 11ß-hydroxytestosterone, 11-ketotestosterone is found at the highest levels in the breeding season and has been found to be more effective than testosterone in stimulating secondary sexual characteristics including reproductive behaviour and parental care in many fishes (reviewed in Borg, 1994). Thus, in fishes, 11-ketotestosterone is likely to be the primary androgen, underscoring the nuanced nature of behavioural endocrinology across species.

Bluegill are endemic to North America and have been extensively studied for their alternative reproductive tactics. In bluegill, parental care is performed by males called “parentals” (Gross, 1982). Parental males establish territories within colonies, build nests, court, and spawn with females, and then provide sole care for the offspring by oxygenating eggs, cleaning the nest, and defending the brood from nest predation (Gross, 1982). Parental males sometimes nest and spawn multiple times during the breeding season (68). The bluegill mating system is highly promiscuous, with about 25% of the broods being sired by precocious males called cuckolders (Neff 2001; Neff & Clare 2008; Garner & Neff 2013). In Lake Opinicon, cuckolder males mature at age 2 years and use a sneaking tactic where they hide in vegetation around the nests and dart into nests to fertilize eggs when the parental male is spawning with a female (Gross & Charnov, 1980). At about 4 years of age, cuckolders switch tactic and instead use female mimicry (Gross, 1982). These mimics orbit around the nests like satellites and enter the nest

while the parental male is spawning with a female. “Satellite” males then fertilize the female’s eggs while acting as if they are also spawning with the parental male.

Parental males are able to discriminate between larvae they have sired, and larvae sired by other males. Such kin discrimination happens using both indirect cues of paternity (nest intrusion by sneakers during spawning) and direct phenotype matching of olfactory cues released by larvae after egg hatching (Neff & Gross, 2001; Neff 2003; Neff & Sherman 2003). This ability to discriminate based on paternity leads to differences in parental care: parental males with high paternity provide more aggressive parental care against brood predators than males with low paternity (Neff & Gross 2001; Neff 2003). Prior work has also been able to elicit aggressive behaviour by exposing parental males to exogenous 11-ketotestosterone via subcutaneous implant (Cunha et al., 2019; Rodgers et al., 2012). In these studies, exposure to high concentrations of 11KT resulted in increased aggressive nest defensive behaviour (Cunha et al., 2019; Rodgers et al., 2012). Taken together, 11-ketotestosterone may regulate aggressive behaviour in bluegill during the breeding season, and this aggressive behaviour should vary based on paternity. This, in the current study, my objective is to elucidate the role of 11-ketotestosterone in adaptive adjustments of parental care behaviour in bluegill. I hypothesize the manipulation of perceived paternity will lead to changes in nurturing and defensive behaviour, and the underlying mechanism of these behavioural adjustments is 11-ketotestosterone. I predict males with experimentally reduced perceived paternity will reduce the quality of their parental care and frequency of care behaviours. Furthermore, if 11-ketotestosterone mediates these behaviour changes, I expect males with lower perceived paternity to have lower circulating 11-ketotestosterone concentrations. To test this, I subjected parental males to either a direct paternity manipulation where paternity in the nest was altered, or to a visual manipulation where males perceived the visual cue of nest intrusion by sneakers on the day of spawning. I then measured changes in the circulating concentration of 11-ketotestosterone and parental care behaviour.

2.2 Methods

2.2.1 Species and study site

I studied a population of bluegill in Lake Opinicon (44°34′N, 76°19′W), Ontario, Canada. This 890-hectare lake has been a study site for this species since the mid-1970s (68). In Lake Opinicon, bluegill breed from late May to July. During this time, parental males enter the littoral zone and build nests in colonies of up to 300 males. Parental care lasts between 7 and 10 days, with the eggs hatching around day 3. From 2018-2021 swimmers equipped with snorkelling gear monitored bluegill reproductive behaviour along a 2 km stretch of the littoral zone of the lake. When a colony formed, I tagged each nest with an individually numbered ceramic tile. A single swimmer mapped the colony to record the position of each nest after spawning and assigned each nest an egg score from 1-5 as a proxy of the number of eggs in the nest (Claussen, 1991; Cargnelli and Gross, 1996). This score is based on the percentage of the nest covered in eggs and is highly correlated with the number of eggs and larvae in the nest (Classen, 1991).

2.2.2 Direct Manipulation of Paternity

My objective in this study was to replicate the experimental paternity manipulation of Neff (2003), while adding blood sampling to assess the effect of paternity manipulation on circulating 11-ketotestosterone (11KT) levels. Parental males were paired based on their assigned egg scores the morning after spawning was observed at each colony (day 1; Figure 2.1). The paired males with equal egg scores were then caught one at a time using a dip net and brought to a nearby boat. Nests were covered with a screen to prevent egg predation while the parental male was absent from the nest. I immediately took a 200 µL whole-blood sample from the caudal vein using a 25G needle attached to a 1 mL heparinized syringe. These samples took an average of 85 seconds to collect from the time of capturing the male (range = 21 to 256 seconds) and were used to measure baseline circulating concentrations of 11KT. I then measured total body length (mm) and placed the male in a recovery tank while the nest was manipulated. Both males remained on the boat while their nests were manipulated, and each pair of males were returned to their nests after the manipulation was completed. Each male spent fewer than 10 minutes on the boat, and all manipulations took place between 09:00 –

12:00 EST. The Animal Care Committee at Western University (ACC) approved all procedures performed in this study (AUP #2010-214 and 2018-084).

Following Neff (2003), I assigned each pair of males to one of two treatments: (1) control; or (2) egg manipulation. For the egg manipulation treatment, I swapped about one-half of each male's eggs between the two nests. These swaps were not performed between neighbouring nests to ensure the foreign eggs introduced were unrelated to the focal male. I performed a sham swap in the nests of males assigned to the control treatment, in which I removed and then returned one-half of the eggs to the original nest. This mimicked the disturbance of the egg swap, but not the reduction in paternity.

On day 2, I recorded each male's parental care behaviours by performing a standardized nest defense test between 14:00 – 17:00 EST. I presented a natural egg predator (pumpkinseed sunfish, *Lepomis gibbosus*) in a transparent plastic bag on the border of the parental male's nest and recorded the parental male's defensive behaviour for 1 min using a go-pro camera (Hero 5 and 6, San Mateo, California, USA). Later from the videos I quantified three aggressive behaviours (sensu Neff, 2003): (1) lateral display; (2) opercular flare; and (3) bite.

I monitored nests daily to determine the day of hatch, which was expected on day 3. I performed another nest defense test the day after hatch. Immediately after the test, I collected another blood sample from each parental male, as described above, to measure circulating 11KT concentration. Due to a difference in blood sampling methodology post-hatch in 2018, those samples were not analyzed for 11KT, but the behavioural data from those males were used.

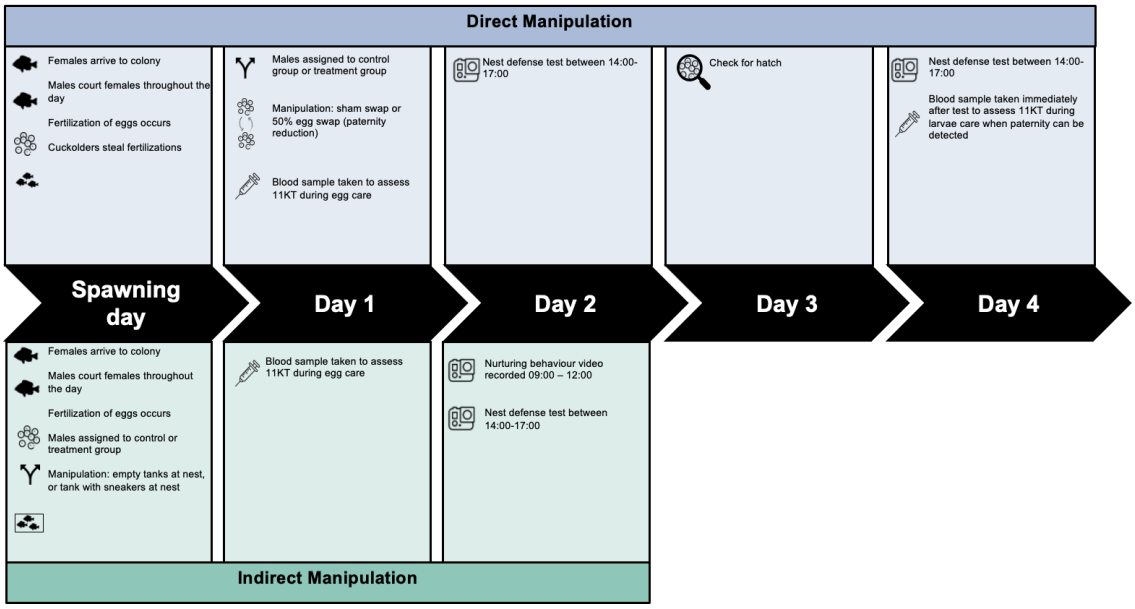

Figure 2.1. Timeline of direct paternity manipulation and indirect perceived paternity visual manipulation on bluegill sunfish (*Lepomis macrochirus*).

Days in blue represent the timeline of the swapped eggs paternity manipulation protocol, and in green represent the indirect manipulation.

2.2.3 Indirect manipulation of perceived paternity

In a second experiment, I manipulated perceived paternity using an indirect cue – the presence of sneakers during spawning. Following Neff (2003), I placed two transparent plastic tanks (20 × 16 × 10 cm) on opposite sides of parental males' nest on the day of spawning (Figure 2.1). I assigned parental males to one of two treatments: (1) control; or (2) sneaker visual cue. I placed two bluegill sneaker males in each tank surrounding the males assigned to the experimental treatment. I left these tanks beside the nest for the duration of the spawning day to present a visual cue of high cuckoldry by sneakers to the parental male. The tanks beside the nests of males assigned to the control treatment remained empty during the day of spawning.

On the day after spawning (day 1), each parental male was caught via dip net and brought to the boat one at a time for initial processing. I measured each male's total body length (mm) and a 200 µL whole-blood sample to measure circulating 11KT. The next morning, I set up GoPro Cameras (Hero 5, 6, or 7) at each parental male's nest. I recorded nurturing behaviour for 30 minutes between 09:00-12:00 EST. I quantified four nurturing behaviours: (1) rim

circling; (2) caudal fan; (3) pectoral fan; and (4) egg consumption (Gross & Macmillan, 1981; Cote & Gross, 1993; Neff 2003). In the afternoon, between 14:00-17:00 EST, I recorded and quantified nest defense parental care behaviour using the same standardized nest defense test from the direct paternity manipulation experiment.

2.2.4 Hormone analysis

I extracted plasma from each blood sample within 8 hours of collection and stored it at -20˚ C for transportation back to the University of Western Ontario. I then used enzyme-linked immunosorbent assay (ELISA) kits (CAT# 582751; Cayman Chemical, Ann Arbor, MI) to determine the concentration of 11KT in the plasma. I ran each sample in triplicate. Concentrations of 11KT were within the range expected based on previous studies of reproductive hormones in bluegill (Neff & Knapp, 2009; Magee *et al.*, 2006).

2.2.5 Statistical analyses

I used R Studio (2015) for all statistical analyses. Degrees of freedom were calculated as the adjusted values as calculated in R. For both the direct and indirect manipulation of perceived paternity experiments, I first used t-tests to determine if there was a difference in egg score or body length between treatments. I used a Shapiro-Wilk test to assess the normality of each behaviour and 11KT concentration. I then used t-tests to determine the effect of experimental treatment on each of the nest defense behaviours (lateral displays, opercular flares, bites), and 11KT concentrations. For the indirect paternity manipulation experiment, I also used t-tests to compare the effect of the treatment on the nurturing behaviours (rim circling, caudal fanning, pectoral fanning, and egg consumption).

I used Spearman's rank correlations to determine if there was a relationship between individual fish 11KT concentration and their nest defense and nurturing behaviours. I compared each treatment separately, and further analyzed each treatment per time point (egg care and larvae care).

2.3 Results

2.3.1 Direct manipulation of paternity

During the egg stage of care, I collected blood from 145 males ($N_{control}$ = 71, N_{swap} = 74), and behaviour observations for 74 males ($N_{control}$ = 32, N_{swap} = 42). The discrepancy between the blood and behaviour sample sizes is largely explained by nest abandonment between the first- and second-day post-spawning. During the larvae care stage, immediately after the nest defense test, I collected blood from 51 males ($N_{control}$ = 22, N_{swap} = 29) and behaviour observations from 65 males ($N_{control}$=29, N_{swap} = 36). The discrepancy between blood and behaviour sample sizes during larval care is explained by video quality and water clarity, along with the coagulation of plasma for some blood samples, which prevented use of the ELISA assay. In 2018, 15 control and 9 swap males abandoned their nests after hatch. In 2020, 12 control and 9 swap males abandoned their nests after hatch. In 2021, 13 control and 8 swap males abandoned their nests after hatch.

Body length and egg score were similar between the control and egg swap treatments. At the egg care stage, there was no significant difference in egg score between the control (2.6 ± 1.1; mean ± SD) and egg swap treatments (2.8 ± 1.0; t_{120} = -0.84, p = 0.40). At the larval care stage, there was no significant difference in the egg score of males who remained after hatch between the control (2.6 ± 1.0) and egg swap treatments (2.8 ± 1.0; t_{120} = -0.84, p = 0.40). At the egg care stage, there was no significant difference in body length between the control (195 ± 10mm) and egg swap treatments (196 ± 12mm; t_{140} = -0.37, p = 0.71). At the larval care stage, there was no significant difference in body length between the control (195 ± 10mm) and egg swap treatments (196 ± 12mm; t_{125} = -0.58, p = 0.56).

There was no significant difference in the number of lateral displays performed by males in the control or swap treatments at the egg (t_{74} = -1.18, p = 0.24) or larval care stages (t_{65} = -0.62, p = 0.54; Figure 2.2A). Similarly, there was no significant difference in the number of opercular flares performed by males in the control or swap treatments at the egg (t_{74} = 0.27, p = 0.79) or larval care stages (t_{65} = -0.75, p = 0.46; Figure 2.2B). There was no significant difference in the number of bites performed by males in the control or swap treatments at the egg care stage (t_{74} = -1.19, p = 0.24). However, after hatch, during the larval care stage, males

in the control treatment performed significantly more bites than males in the experimentally lowered paternity treatment ($t_{65} = 3.38$, $p < 0.01$ Figure 2.2C).

Males in the control and swap treatments at the egg care stage had no significant difference in their 11KT concentration ($t_{57} = 0.74$, $p = 0.46$). However, during the larval care stage, males in the experimentally lowered paternity treatment had significantly higher 11KT than males in the control treatment ($t_{51} = -2.44$, $p = 0.02$; Figure 2.2D). There was also a significant difference in the change in individual 11KT concentrations with control males increasing on average by 5.14 ng/mL and swap males increasing by 19.8 ng/mL ($t_{57} = -3.83$, $p < 0.01$).

There was no relationship between the number of lateral displays performed by parental males in the control treatment and circulating 11KT concentrations (R = 0.21, $p = 0.10$), but there was a positive relationship in the males in the swap treatment (R = 0.22, $p = 0.05$). There was no relationship between the number of opercular flares performed by males and their circulating 11KT in the control treatment (R = 0.058, $p = 0.66$), or the swap treatment (R = 0.064, $p = 0.57$). Similarly, there was no relationship between the number of bites males performed and their circulating 11KT in the control treatment (R = 0.18, $p = 0.16$) or swap treatment (R = -0.12, $p = 0.28$).

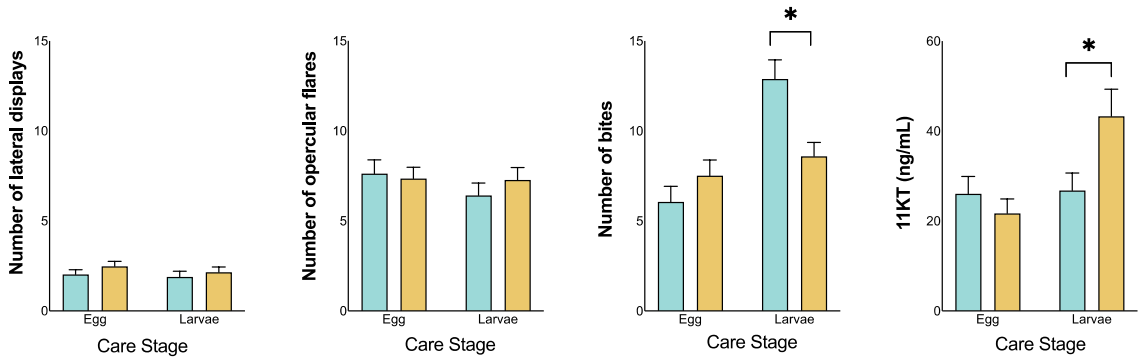

Figure 2.2. Nest defense behaviours (A. Lateral Display, B. Opercular Flare, C. Bite) and 11-ketotestoterone concentration (D) by parental male bluegill (*Lepomis macrochirus*) during the egg and larvae stages in response to a natural predator (Mean ± SEM). Control males (sham swapped eggs) are denoted by blue bars while treatment males with experimentally reduced paternity (swapped eggs) are denoted by orange bars.

2.3.2 Indirect manipulation of perceived paternity

I analyzed the aggressive nest defense behaviour of 62 males ($N_{control}$ = 30, $N_{sneaker}$ = 32), and the nurturing behaviour of 55 males ($N_{control}$ = 26, $N_{sneaker}$ = 29). I collected blood from 56 males ($N_{control}$ = 30, $N_{sneaker}$ = 26). Variation in sample sizes stems from variation in water clarity/video quality, and video quality, and one male from whom blood coagulation in the needle prevented sample collection.

Parental males in the control and sneaker visual treatments were not significantly different from each other in either egg score (control = 2.4 ± 1.1; mean ± SD; treatment = 2.7 ± 1.5; t_{50} = -0.91, p = 0.37) or body length (control = 196 ± 9 mm; treatment 193 ± 13 mm; t_{59} = 1.33, p = 0.19). Based on the analysis of nest defense behaviours, there was no significant difference between the control and sneaker treatments in the number of lateral displays (t_{59} = -0.12, p = 0.90), opercular flares (t_{59} = -0.04, p = 0.97) or number of bites (t_{59} = 1.67, p = 0.09; Figure 2.3). Males in the sneaker treatment performed significantly fewer rim circles (t_{56} = 3.18, p = 0.01), pectoral fans (t_{56} = 2.57, p = 0.02) and egg consumption motions (t_{56} = 3.97, p < 0.01) than males in the control treatment (Figure 2.4). There was no significant difference in the number of caudal fans performed by males in the control treatment relative to males in the sneaker treatment (t_{56} = 0.61, p = 0.55).

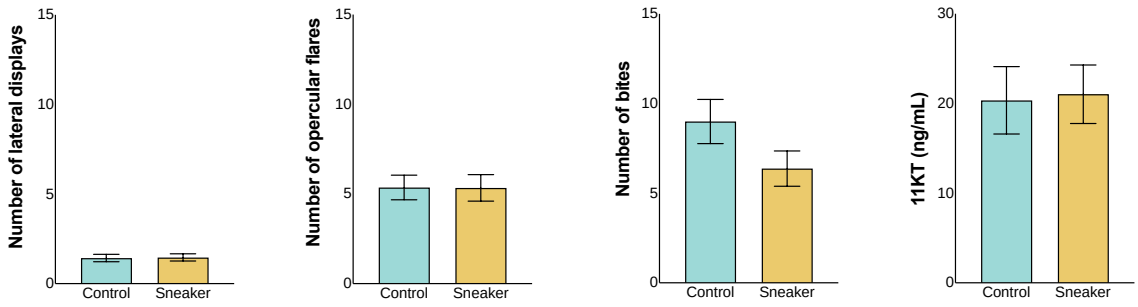

Figure 2.3. Nest defense behaviours (A. Lateral Display, B. Opercular Flare, C. Bite) and 11-ketotestosterone concentration (D) by parental male bluegill (*Lepomis macrochirus)* during the egg stage in response to a natural predator (Mean ± SEM). Control males with higher perceived paternity are denoted by blue bars. Treatment males with lower perceived paternity (sneaker visual cue of cuckoldry) are denoted by orange bars.

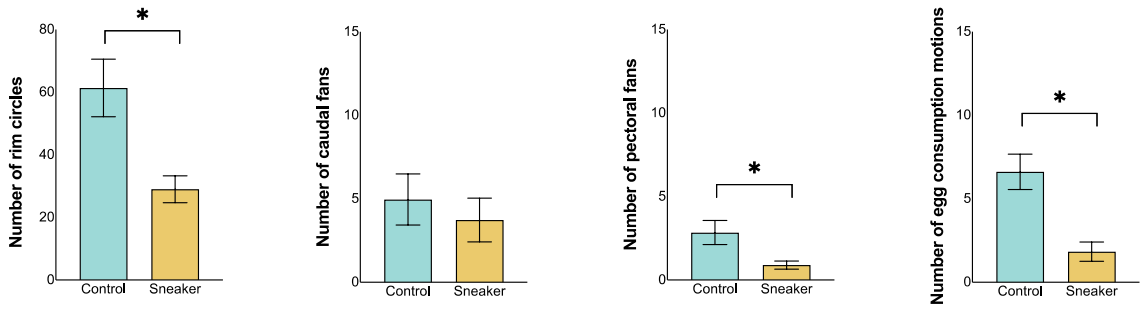

Figure 2.4. Nurturing behaviours (A. Rim circling, B. Caudal fan, C. Pectoral fan, D. Egg consumption) by parental male bluegill (*Lepomis macrochirus*) during the egg stage (Mean ± SEM). Control males with higher perceived paternity are denoted by blue bars. Treatment males with lower perceived paternity (sneaker visual cue of cuckoldry) are denoted by orange bars.

2.4 Discussion

My results show parental males adjust both nurturing and defensive behaviours in response to perceived paternity. Males with higher perceived paternity defended their nests more aggressively and provide a higher quality of nurturing care than those with lower perceived paternity. Indeed, when paternity was directly manipulated by swapping eggs between nests, males with higher paternity more aggressively defended their nests than males with lower paternity. My results support and expand on the experiment by Neff (2003), in which the author also subjected parental male bluegill to indirect and direct reductions in perceived paternity and observed that males with experimentally reduced paternity reduced their level of care. Neff (2003) analyzed overall nest defensive behaviour, which my study expands upon by both supporting increased defensive behaviour by control males, and by analyzing each behaviour separately to determine that biting drives the observed changes. My study also quantified four nurturing behaviours. I determined differences in nurturing parental care are also observable prior to eggs hatching and, in line with Neff (2003), persist after eggs hatch. Taken together, my research replicates and expands upon the behavioural differences by parental males in response to perceived paternity.

My other objective was to determine the role of 11KT in the regulation of parental care behaviour and the possibility of this hormone serving as a mechanism for how parental males

alter their behaviour in response to perceived paternity. The role of 11KT in the regulation of teleost behaviour has been extensively tested using experimental manipulations (Cunha et al., 2019; Rodgers et al., 2012; Ros et al., 2004; Kindler et al., 1991) while the response of naturally circulating plasma concentrations has rarely been examined. The few studies that have quantified plasma 11KT concentrations in response to stimuli yield conflicting results: 11KT increases in response to territorial intrusion in *Sarotherodon galilaeus* and *Sparisoma viride* (Ros et al., 2003; Cardwell & Liley, 1991) but did not increase in *Neogobius melanostomus* or *Acanthochromis polyacanthus* (Sokolowska et al., 2013; Hay & Pankhurst, 2003). At a species-specific level, prior research has demonstrated that bluegill increase their nest defense behaviour when subjected to artificially elevated 11KT delivered via subcutaneous implants (Rodgers et al., 2012; Cunha et al., 2019). My results from the direct manipulation of paternity experiment showed that males with higher paternity actually had lower circulating 11KT concentrations after the eggs hatched. During the egg stage of care, as with males in the visual manipulation of sneakers, there was no difference in circulating 11KT concentration between treatments. Furthermore, circulating 11KT was positively related only to lateral displays in one group of males. All other aggressive defense behaviours were not related to circulating 11KT concentrations. My data thus suggest that 11KT is not responding to changes in paternity or perceived paternity and the observed differences in parental care are regulated by another mechanism. Instead, prior work has shown that males that renest increase circulating androgen concentrations towards the end of the parental care period (Specker & Kishida 2000; Pankhurst & Peter 2002; Magee et al., 2006). Thus, it is conceivable that the elevated 11KT levels in males in our egg swap treatment were associated with renesting potential in response to low paternity.

The difference between our findings and those of previously published work may be interpreted as a difference between response and regulation of 11KT. In prior studies, bluegill had subcutaneous implants inserted to administer varying concentrations of 11KT and implanted males had 60% higher concentrations of 11KT relative to the control males (see Cunha et al., 2019). In these implant studies, bluegill with higher circulating levels of 11KT exhibited more aggressive behaviour, so clearly circulating levels can affect parental behaviour (Rodgers et al., 2012; Cunha et al., 2019). Felix *et al.* (2020) suggest that androgen response varies between individuals and is related to their scope for response (maximum physiological

level – baseline level). In the implant studies, the bluegill 11KT baseline levels were elevated by implantation and the natural scope for response was presumably reduced. In my study, I observed the physiological response to paternity in which 11KT was not directly manipulated, and so changes in hormone concentrations were attributed to their natural baseline and response to paternity cues. In particular, bluegill in my study should have had more flexibility in their androgen concentration changes due to a lower baseline level and thus higher scope for response. In the context of perceived paternity, males do not appear to differentially regulate circulating levels of 11KT during the egg phase, and seemingly only elevate the androgen in response to low paternity once the eggs hatch. This indicates paternity may influence regulation of 11KT in the context of renesting, and not in terms of regulation of aggressive parental behaviour.

Considering my 11KT results contradicted my prediction, I looked further into the relationship between individual circulating 11KT and parental care behaviours. Interestingly, I found no relationship between most of the nest defense behaviours and 11KT concentrations. The one exception was a positive relationship between lateral displays and 11KT concentration for the males in the egg swap treatment. I also found several positive relationships between nurturing behaviours and 11KT concentrations. It is possible that bluegill maintain sufficiently high levels of 11KT that they do not respond to challenges by elevating circulating concentrations. Goymann et al (2019) proposed the "Challenge Hypothesis 2.0" that posits males in promiscuous mating systems are not constrained by a trade-off between nurturing and aggressive behaviours. Rather, males can maintain high levels of androgens during the parental care season without impacting the quality of care. They also suggest that androgen concentrations may be high enough to respond to challenges without requiring any additional elevation in circulating androgen concentrations. My findings support this idea, by providing empirical evidence that male bluegill are not constrained by an androgen-parental care trade-off.

Future research should examine other potential mechanisms underlying adaptive parental care behaviour in bluegill. While males with higher perceived paternity perform a higher quality of parental care, it does not appear that 11KT is the key hormone driving this response. That said 11KT receptors can be activated in less than 10 minutes and may be involved in the observed

differences in behaviour through differences in density or ligand binding efficiency (Goymann et al., 2019; Moore et al., 2020; Borg, 1994). Outside of androgenic activity, we suggest future investigations consider nonapeptides as a potential mechanism to mediate parental care given their role in care modulation (DeAngelis et al., 2020; Cunha-Saraiva et al., 2019) and potential linkage to paternity (Stiver et al., 2019). My study highlights the importance of increasing our understanding of the neuroendocrine and neurogenomic mechanisms that regulate behaviour, as it is clear they are complex and challenging to generalize across taxa.

2.5 References

Alonso-Alvarez, C., and Velando, A. (2012) Benefits and costs of parental care. In: *The evolution of parental care*. Ed: N. J. Royle. Oxford Academic Publishing. Oxford, UK.

Borg, B. (1994). Androgens in teleost fishes. *Comparative Biochemistry and Physiology*, **109C**, 219–245

Cardwell, J.R., and Liley, R. (1991). Androgen control of social status in males of a wild population of stoplight parrotfish *Sparisoma viride* (*Scaridae*). *Hormones and Behavior,* **25,** 1-18.

Cargnelli, L.M., and Gross, M.R. (1996). The temporal dimension in fish recruitment: birth date, body size, and size-dependent survival in a sunfish (bluegill: *Lepomis macrochirus*). *Canadian Journal of Fisheries and Aquatic Sciences*, **53,** 360-367.

Chen, Y. H., Cheng, W. C., Yu, H. T., and Kam, Y. C. (2011). Genetic relationship between offspring and guardian adults of a rhacophorid frog and its care effort in response to paternal share. *Behavioral Ecology and Sociobiology*, **65,** 2329–2339.

Claussen, J. (1991). Annual variation in the reproductive activity of a bluegill population: effect of clutch size and temperature. University of Toronto.

Clutton-Brock, T. H. (1991). *The Evolution of Parental Care*. Princeton University Press.

Côté, I. M., and Gross, M. R. (1993). Reduced disease in offspring: A benefit of coloniality in sunfish. *Behavioral Ecology and Sociobiology,* **33,** 269–274.

Cullen., E. (1957). Adaptations in the Kittiwake to cliff-nesting. Ibis, 99, 275-302.

Cunha, A. A. P., Partridge, C. G., Knapp, R., and Neff, B. D. (2019). Androgen and prolactin manipulation induces changes in aggressive and nurturing behavior in a fish with male parental care. *Hormones and Behavior*, **116,** 104582.

Cunha-Saraiva, F., Balshine, S., Gozdowska, M., Kulczykowska, E., Wagner, R.H., and Schaudelin, F.C. (2019). Parental care and neuropeptide dynamics in a cichlid fish *Neolamprologus caudopunctatus*. *Hormones and Behavior*, **116**, 104576.

DeAngelis, R., Dodd, L., and Rhodes, J. (2020). Nonapeptides mediate trade-offs in parental care strategy. *Hormones and Behavior*, **121**, 104717.

Felix, A.S., Roleira, A., and Oliveira, R.F. (2020). Rising to the challenge? Inter-individual variation of the androgen response to social interactions in cichlid fish. *Hormones and Behavior*, **124**: 104755.

Garner, S. R., and Neff, B. D. (2013). Alternative male reproductive tactics drive asymmetrical hybridization between sunfishes (*Lepomis* spp.). *Biology Letters*, **9,** 20130658.

Goymann, W., Moore, I. T., and Oliveira, R. F. (2019). Challenge hypothesis 2.0: A fresh look at an established idea. *BioScience*, **69**, 432–442.

Gray, S. M., McKinnon, J. S., Tantu, F. Y., and Dill, L. M. (2008). Sneaky egg-eating in *Telmatherina sarasinorum*, an endemic fish from Sulawesi. *Journal of Fish Biology*, **73,** 728–731.

Gross, M. R. (1982). Sneakers, satellites and parentals: polymorphic mating strategies in North American sunfishes. *Ethology*, **60,** 1–26.

Gross, M. R., and Charnov, E. L. (1980). Alternative male life histories in bluegill sunfish (*Lepomis macrochirus*). *Proceedings - National Academy of Science, U.S.A.*, **77,** 6937–6940.

Gross, M. R., and MacMillan, A. M. (1981). Predation and the evolution of colonial nesting in bluegill sunfish (*Lepomis macrochirus*). *Behavioral Ecology and Sociobiology*, **8,** 163–174.

Hain, T. J. A. (2015). *The evolution of kin recognition*. University of Western Ontario, Electronic Thesis and Dissertation Repository, 3431.

Hain, T. J. A., and Neff, B. D. (2006). Promiscuity drives self-referent kin recognition. *Current Biology*, **16,** 1807-1811.

Hay, A.C., and Pankhurst, N.W. (2005). Effect of paired encounters on plasma androgens and behaviour in males and females of the spiny damselfish *Acanthochromis polyacanthus. Marine and Freshwater Behaviour and Physiology*, **38**, 127-138.

Holmes, W. G., and Sherman, P. W. (1982). The ontogeny of kin recognition in two species of ground squirrels. *Integrative and Comparative Biology*, **22,** 491-517.

Kindler, P.M., Bahr, J.M., and Philipp, D.P. (1991). The effects of exogenous 11-ketotestosterone, testosterone, and cyproterone acetate on prespawning and parental care behaviors of male bluegill. *Hormones and Behavior,* **25**, 410-423.

Klug, H., and Bonsall, M. B. (2014). What are the benefits of parental care? The importance of parental effects on developmental rate. *Ecology and Evolution*, **4,** 2330–2351.

Magee, S. E., Neff, B. D., and Knapp, R. (2006). Plasma levels of androgens and cortisol in relation to breeding behavior in parental male bluegill sunfish, *Lepomis macrochirus*. *Hormones and Behavior*, **49**, 598–609.

Manica, A. (2004). Parental fish change their cannibalistic behaviour in response to the cost-to-benefit ratio of parental care. *Animal Behaviour*, **67,** 1015–1021.

Mateo, J. M. (2004). Recognition systems and biological organization: The perception component of social recognition. *Annales Zoologici Fennici*, **41,** 729-745.

Møller, A. P., and Birkhead, T. R. (1993). Cuckoldry and sociality: a comparative study of birds. *American Naturalist*, **142,** 118–140.

Montgomerie, R. D., and Weatherhead, P. J. (1988). Risks and rewards of nest defence by parent birds. *Quarterly Review of Biology*, **63,** 167-187.

Moore, I. T., Hernandez, J., and Goymann, W. (2020). Who rises to the challenge? Testing the Challenge Hypothesis in fish, amphibians, reptiles, and mammals. *Hormones and Behavior*, **123,** 104537.

Neff, B. D. (2001). Genetic paternity analysis and breeding success in bluegill sunfish (*Lepomis macrochirus). Journal of Heredity*, **92**, 111-119.

Neff, B. D. (2003). Decisions about parental care in response to perceived paternity. *Nature*, **422,** 716–718.

Neff, B. D., and Clare, E. A. (2008) Temporal variation in cuckoldry and paternity in two sunfish species (*Lepomis* spp.) with alternative reproductive tactics. *Canadian Journal of Zoology*, **86,** 92-98

Neff, B. D., and Gross, M. R. (2001). Dynamic adjustment of parental care in response to perceived paternity. *Proceedings of the Royal Society B: Biological Sciences*, **268,** 1559–1565.

Neff, B. D., and Knapp, R. (2009). Paternity, parental behavior and circulating steroid hormone concentrations in nest-tending male bluegill. *Hormones and Behavior*, **56**, 239–245.

Neff, B. D., and Sherman, P. W. (2003). Nestling recognition via direct cues by parental male bluegill sunfish (*Lepomis macrochirus*). *Animal Cognition*, **6,** 87–92.

Nelson, R., and Kriegsfeld, L. (2022). *An Introduction to Behavioral Endocrinology, Sixth Edition* (6th ed.). Oxford University Press.

Numan, M., and Insel, T. R. (2011). *The Neurobiology of Parental Behavior*. Springer.

Pankhurst, N.W., and Peter, R.E. (2002). Changes in plasma levels of gonadal steroids and putative gonadotropin in association with spawning and brooding behavior in male demoiselles. *Journal of Fish Biology*, **61**, 394-404.

Rodgers, C. M. C., Neff, B. D., and Knapp, R. (2012). Effects of exogenous testosterone on parental care behaviours in male bluegill sunfish (*Lepomis macrochirus*). *Ethology*, **118,** 636–643.

Ros, A.F.H., Bruintjes, R., Santos, R. S., Canario, A. V. M., and Oliveira, R. F. (2004). The role of androgens in the trade-off between territorial and parental behavior in the Azorean rock-pool blenny, *Parablennius parvicornis. Hormones and Behavior,* **46**, 491–497.

Ros, A.F.H, Canario, A.V.M., Couto, E., Zeilstra, I., Oliveira, R.F. (2003). Endocrine correlates of intra-specific variation in the mating system of the St. Peter's fish (*Sarotherodon galilaeus*). *Hormones and Behaviour,* **44**, 365-373.

Smiley, K.O., Ladyman, S.R., Gustafson, P., Grattan, D.R., and Brown, R.S.E. (2019). Neuroendocrinology and adaptive physiology of maternal care. In: Coolen, L., Grattan, D. (eds). *Neuroendocrine Regulation of Behavior*. Springer.

Sokolowska, E., Kleszczynska, A., Kalamarz-Kubiak, H., Arciszewski, B., and Kulczykowska, E. (2013). Changes in brain arginine vasotocin, isotocin, plasma 11-ketotestosterone and cortisol in round goby, *Neogobius melanostomus*, males subjected to overcrowding stress during the breeding season. *Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology*, **165**, 237-242.

Specker, J.L., and Kishida, M. (2000). Mouthbrooding in the black-chinned tilapia, *Sarotherodon melanotheron* (Pisces: *Cichlidae*): The presence of eggs reduces androgen and estradiol levels during paternal and maternal parental behavior. *Horomones and Behavior,* **38**, 44-51.

Stiver, K.A., Marsh-Rollo, S.E., and Alonzo, S.H. (2019). Neuropeptide manipulation has behavioural and cascading fitness consequences in wild-living fish. *Animal Behaviour*, **157**, 69-76.

Trivers, R. L. (1972). Parental investment and sexual selection. In B. Campbell (Ed), *Sexual Selection and the Descent of Man: 1871-1971.* Aldine.

Westneat, D. F., and Sherman, P. W. (1993). Parentage and the evolution of parental behavior. *Behavioral Ecology*, **4,** 66–77.

Wingfield, J. C., Hegner, R. E., Dufty, A. M., and Ball, G. F. (1990). The "Challenge Hypothesis": Theoretical implications for patterns of testosterone secretion, mating systems, and breeding strategies. *American Naturalist*, **136,** 829–846.

Wingfield., J. C., Jacobs, J. D., Tramontin, A. D., Perfito, N., Meddle, S., Maney, D. L., and Soma, K. (2000). Toward an ecological basis of hormone-behavior interactions in reproduction in birds. In K. Wallen and J. E. Schneider (Eds.), *Reproduction in Context*. MIT Press.

Chapter 3

3 Prolactin modulates changes in parental care behaviour in response to perceived paternity in bluegill sunfish (*Lepomis macrochirus*)

A version of this manuscript has been submitted for publication.

3.1 Introduction

Parental care systems have evolved to optimize offspring survival and parental fitness (Klug & Bonsall, 2014). While beneficial, providing care often comes at a cost for a parent, including the potential for reduced breeding opportunities, decreased time for foraging, and increased vulnerability to predation and parasitism (Alonso-Alvarez & Velando, 2012). Because of these costs, parental investment theory predicts parents will allocate care based on offspring value, whereby offspring that are larger, healthier, and more related to a parent are deemed most valuable (Trivers, 1972). This strategic allocation of care allows parents to invest in higher quality care to offspring that are most likely to increase their fitness by passing on their genes. While such selective care, based on relatedness, has been observed in several fishes (Churchman et al., 2023; Gray et al., 2008; Manica, 2004; Neff, 2003) and over 50 species of birds (Moller & Birkhead, 1993), the mechanisms driving this differential allocation of care remain largely unknown.

Parental care behaviour necessitates significant physiological changes in the brain and reproductive system (Bridges, 2015; Champagne & Curley, 2013; Stiver & Alonzo, 2009). These changes are primarily driven by the endocrine system, such that responsiveness to hormones is a requirement of the parental phenotype (Ball & Balthazart, 2008). Central to this regulation is prolactin, a hormone widely regarded as one of the most important neuroendocrine controls of parental care (Schradin & Anzenberger, 1999). While prolactin was first identified in birds (Riddle et al., 1933), the idea of a pituitary-derived stimulatory factor that initiated milk production had been hypothesized in the 1920s (Stricker & Grueter, 1928) from which the name “pro-lactin” was derived. Prolactin is a multifunctional polypeptide hormone belonging to a family of hormones including growth hormone and somatolactin

(Dobolyi et al., 2020). The family is ubiquitous in vertebrates and prolactin is known to have over 300 functions (Goffin et al., 2002; Gong et al., 2022). The largely-conserved sequence of the prolactin gene is hypothesized to be responsible for its common structure and function across vertebrate taxa, particularly in reproduction and parental care (Manzon, 2002). In mammals and birds, prolactin has been shown to modulate parental care in both sexes, with higher concentrations of circulating prolactin being associated with increases in the expression of parental care behaviours (Chastel et al., 2005; Dixson & George, 1982; Li et al., 2022; Miller et al., 2009; Ouyang et al., 2011; Riechert et al., 2014; Smiley, 2019; Smiley & Adkins-Regan, 2016; Storey et al., 2000). While the prolactin gene originated in the evolutionary ancestors of fishes, the oldest vertebrate taxon, its function in parental care in this taxon is less understood.

Previous studies on prolactin in fishes have focused on the manipulation of prolactin levels through exogenous administration or RNAi to knockdown protein production in target tissues. Early studies that artificially elevated prolactin did so by introducing mammalian prolactin (Blüm, 1968; Blüm & Fiedler, 1965; Lam & Hoar, 1967). These studies showed that administration of mammalian prolactin induce behavioural and histological effects including osmolality and parental care behaviour (Whittington & Wilson, 2013). Purified fish prolactin can now be synthesized, which allows for results from manipulation studies that are more biologically pertinent and reflective of natural physiology (Whittington & Wilson, 2013). Studies that quantify endogenous plasma prolactin activity in fish are limited to a single study. Tacon et al. (2000) quantified prolactin isoforms in plasma from female Nile tilapia (*Oreochromis niloticus*) during their reproductive cycle (Tacon et al., 2000). They found that circulating prolactin varied day to day and suggested that this variation indicates that prolactin is involved in parental behaviour. To date, however, no study has directly linked circulating plasma prolactin concentrations with parental care behaviour in fish.

Bluegill sunfish (*Lepomis macrochirus*) are native to North America and are a focal species in the study of alternative reproductive tactics. Bluegill parental care is performed exclusively by males called “parentals” (Gross, 1982). These males establish territories within colonies, build nests, court, and spawn with females, and subsequently provide sole care for the offspring. In our study population in Lake Opinicon (Ontario, Canada), parental care lasts up to 10 days and has two distinct periods. The first is the egg period (up to 3 days long) where a parental male

actively aerates the eggs by fanning water across the brood, cleans the nest by removing moldy or inviable eggs, and guards the eggs from nest predators. The second is the larval period (up to 7 days long), which primarily involves guarding the hatched offspring from nest predators. The bluegill mating system is promiscuous with some offspring being sired by specialized males called “cuckolders”. Cuckolders fertilize eggs by intruding on spawning events in a parental’s nest, yet provide no care for their offspring (Garner & Neff, 2013; Neff, 2001). In Lake Opinicon, cuckolder males mature at 2 years of age and use a sneaking tactic (“sneakers”), whereby they hide in vegetation around nests and then become visible when they dart into the nest of a parental male to fertilize eggs (Gross, 1982). At about 4 years old, cuckolders switch tactics and instead use female mimicry (Gross, 1982). Mimics hover above the nest, entering while the parental male is spawning with a female, and fertilize eggs while acting as if they are also spawning with the parental male.

Parental male bluegill have evolved the capacity to discriminate between offspring that they sire and those of cuckolder males (Neff & Gross, 2001; Neff & Sherman, 2003). The males make this discrimination using two different mechanisms. During the egg period of care, parental males use the presence of sneakers around their nest during spawning as an indirect cue of paternity — more sneakers lead to lower perceived paternity (Neff & Gross, 2001). After the eggs hatch, parental males use odour cues to directly assess paternity within their brood (Neff & Sherman, 2003). This paternity assessment impacts care dynamics: parental males with high perceived paternity provide greater care compared to males with low perceived paternity (Churchman et al., 2023; Neff, 2003; Neff & Gross, 2001). Furthermore, prolactin has been implicated in modulating parental care behaviours. Specifically, the administration of ovine prolactin has been shown to increase a parental male’s nurturing behaviours (Cunha et al., 2019). However, the endogenous concentration of circulating prolactin has not previously been measured nor has it been linked to the dynamic adjustments in parental care in response to perceived paternity.

In the current study I quantified circulating concentrations of prolactin in plasma taken from parental male bluegill during each day of the parental care period. I expected that prolactin concentrations would be higher during the egg period than the larval period of care because nurturing behaviours (fanning, nest cleaning) are performed almost exclusively during the egg

period. I also targeted colonies to manipulate the perceived paternity of nesting parental males. During the day of spawning, I exposed some parental males to visual cues of sneakers while other males (control group) were not exposed to this manipulation. I subsequently quantified nurturing behaviour and circulating concentrations of prolactin during the egg period of care. I predicted that parental males with lower perceived paternity (those exposed to the sneakers during spawning) would provide less parental care relative to the control males and that males providing less parental care would have lower concentrations of prolactin. My results provide a test of both prolactin's role in parental care in a fish and its role in modulating parental care behaviour in response to perceive paternity.

3.2 Methods

3.2.1 Species and study site

I studied a population of bluegill in Lake Opinicon (44°34′N, 76°19′W), Ontario, Canada. Lake Opinicon is an 890-hectare lake in which researchers have studied this species since the mid-1970s (Gross, 1982). The reproductive and breeding behaviour has been well documented: breeding parental males enter the littoral zone and nest in colonies of up to 150 fish and provide care for up to 10 days, with the eggs typically hatching on day 2 or 3 (Gross, 1982). I monitored reproductive behaviour over a 2 km stretch of Lake Opinicon via swimmers equipped with snorkelling gear. When a colony formed, I tagged each nest with an individually numbered ceramic tile.

3.2.2 Prolactin concentrations during the parental care period

In 2020, I sampled random males within a colony on each day of the parental care period from spawning day (day 0) to day 5 (the last full day of care for this colony). I caught each male via dip net and brought the male to a nearby boat, where I measured their total body length and extracted a 200 µL whole-blood sample from the caudal vein (mean = 61 seconds, range = 33 – 137 seconds, from the time of capture to blood collection). I allowed each male approximately 2 minutes to recover, and then returned him to his nest. I stored blood samples on ice until transport back to shore for processing. I extracted plasma from each blood sample within 8 hours of collection and stored it at -20 ˚C until transportation back to the University of Western Ontario.

3.2.3 Manipulation of perceived paternity

In 2019, a single swimmer mapped the colony immediately after placing the ceramic tiles at nests and assigned an egg score from 1-5 (Cargnelli & Gross, 1996; Claussen, 1991). This score is based on the percentage of egg coverage within the nest and is highly correlated with the actual number of eggs and larvae in the nest (Claussen, 1991). Then, following Neff (2003), parental males were assigned to either (1) a control treatment or (2) a sneaker visual cue treatment (7). I made an effort to assign nests with similar egg scores to each treatment. At the beginning of the day of spawning, I placed two transparent plastic tanks (20×16×10 cm) on opposite sides of each parental male's nest. For parental males assigned to the sneaker visual cue treatment, two sneakers were placed in each tank around the male's nest. The control treatment males' tanks remained empty. All tanks were removed at the end of the spawning day. The day after spawning (day 1), I caught each parental male via dip net and brought the male to the boat for processing for body size and blood sampling while caring for eggs, as described above. On day 2, I recorded nurturing behaviour – see Churchman et al., (2023) for full behavioural results. Briefly, I set up GoPro Cameras (Hero 5, 6, or 7; San Mateo, CA) at each parental male's nest. I recorded behaviour for a continuous 30-minute window between 09:00-12:00 EST and quantified four nurturing behaviours: (1) rim circling; (2) caudal fan; (3) pectoral fan; and (4) nest pecking (Côté & Gross, 1993; Gross & MacMillan, 1981). The first three behaviours move water over the eggs and help to oxygenate them, while nest pecking is a more opportunistic behaviour that involves removing eggs from the nest to prevent the spread of mold (Côté & Gross, 1993; Gross & MacMillan, 1981).

3.2.4 Circulating prolactin concentrations

I used an enzyme-linked immunosorbent assay (ELISA) kit designed with recombinant Atlantic salmon prolactin to determine circulating concentrations of prolactin (Fish Prolactin ELISA Kit MBS700669; MyBioSource Inc., San Diego, CA). The assay was manufactured in an ISO 9001:2015 certified laboratory, and reports and intra-assay precision CV% < 15%, and an inter-assay precision CV% <15%. The laboratory validated the linearity and recovery of the assay during development and production. I ran each sample in duplicate to balance the high plasma demands of the assay with the welfare of the animal during sampling. The ELISA kit standard was designed in µIU/mL, where 1 µIU/mL is approximately equal to a concentration

of 0.047 ng/mL (MyBioSource Inc, personal communication). The Animal Care Committee at Western University (ACC) approved all procedures performed in this study (AUP #2010-214 and 2018-084) and the fish were collected under scientific collection permits from the Ontario Ministry of Natural Resources and Forestry.

3.2.5 Statistical analysis

I performed all statistical analyses using R Studio (RStudio Team, 2015). Degrees of freedom were calculated as the adjusted values within R. All tests were conducted as two-tailed assessments. To analyze the prolactin concentration profiles across the parental care period, I ran an ANCOVA to determine if the concentrations changed over the course of the 5 days. I included body length as a covariate. I then used t-tests to compare the prolactin concentrations on the day of spawning and day 1 of parental care (when eggs were exclusively present in the nest) to the remaining days (day 2-5), when larvae were present in the nest, with Bonferroni correction to account for multiple tests. Next, for the manipulation of perceived paternity study, we first used a Fisher's Exact Test to compare nest abandonment rates between the two treatments. I used t-tests to determine if there was a difference in egg score or body length between treatments. Similarly, I used a t-test to assess the difference in circulating prolactin concentrations between treatments. I assessed the correlation between the four nurturing behaviours using Pearson's r correlation. I normalized each nurturing behaviour via minimum-maximum normalization (Kappal, 2019), then generated an overall nurturing score by scaling and loading the significantly correlated nurturing behaviours with a principal components analysis. I removed three prolactin concentration outliers in accordance with Tukey's fences. I also ran two ANCOVAs to determine the effect of treatment on (1) the nurturing behaviour PCA1 score (2) nest pecking behaviour and included prolactin concentration as a covariate for both. I ran a Spearman's correlation to compare the relationship between prolactin concentration and egg score or body length. For all models, I confirmed the validity of the models by testing for linearity, autocorrelation, homoscedasticity, normality of residuals, and checking for influential observations. All models passed these tests.

3.3 Results

3.3.1 Prolactin concentrations during the parental care period

I collected blood samples from parental male bluegill on the day of spawning (day 0) and each subsequent day until day 5 of the parental care period (Figure 3.1). For this colony, eggs hatched on day 2 and the final full day of care was day 5. I took blood samples from an average of 5 males per day (range = 4 – 7). The body length of these males was 194 ± 10 mm (all means are reported as mean ± SD; range = 176 – 217 mm). Body length did not differ across the sampling days ($F_{1,29} = 3.48$, $p = 0.07$). Average circulating prolactin concentration across all sampling days was 13.7 ± 8.8 µIU/mL (range = 1.95 – 35.3 µIU/mL; Figure 3.1). There was a significant effect of day on prolactin concentration ($F_{1,28} = 4.42$, $p = 0.04$). As expected, prolactin concentrations were higher when eggs were present (day 0 and 1; 19.3 ± 11.2 µIU/mL) versus when larvae were present (day 2-5; 10.8 ± 5.6 µIU/mL; $t_{13} = 2.37$, p = 0.03). There was no significant effect of body length on these prolactin concentrations ($F_{1,28} = 0.91$, $p = 0.35$).

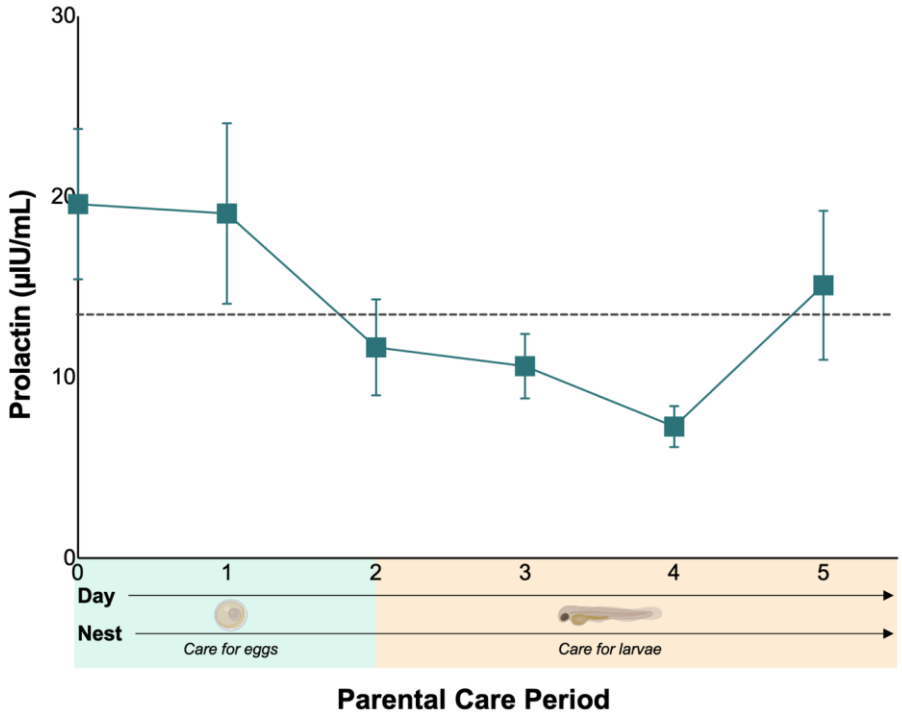

Figure 3.1. Changes in circulating prolactin concentration over the course of the parental care period in bluegill sunfish (*Lepomis macrochirus*). Day denotes the number

of days after spawning (day 0), while the shading denotes the period of parental care with either eggs or larvae present in the nest. Data points represent the mean prolactin concentration per day and error bars denote the standard error of the mean. The dashed line represents the average prolactin concentration throughout the parental care period.

3.3.2 Manipulation of perceived paternity

I manipulated perceived paternity by placing sneaker males around some nests and compared the parental males in these nests to control males that were not exposed to the sneaker male cue. I collected blood from 37 males ($N_{control}$ = 17, $N_{sneaker}$ = 20) and nurturing behavioural observations from 31 males ($N_{control}$ = 14, $N_{sneaker}$ = 17). I was unable to collect behavioural data for six males due to poor video quality. Body length was similar between the control males (197 ± 9 mm) and sneaker treatment males (190 ± 13 mm; t_{33} = 1.63, p = 0.11). Egg scores were also similar between the two treatments (control: 2.2 ± 1.2; sneaker: 2.6 ± 1.3; t_{27} = -0.71, p = 0.48). Four parental males from the control treatment and six parental males from the sneaker treatment abandoned their nest prior to the end of the care period. A Fisher's exact test indicated that there was no significant difference in nest abandonment rate between the two groups (p = 1.0, OR = 0.89 (95% CI = 0.25 – 3.1)).

I measured four nurturing behaviours during the egg period of care: rim circling, caudal fanning, pectoral fanning, and nest pecking. The first three behaviours were correlated and involve moving oxygenated water over the eggs (Table 3.1). Nest pecking involves removing moldy eggs from the nest and was not correlated with the other three behaviours (Table 3.1). I used a principal components analysis to collapse the rim circling, caudal fanning, and pectoral fanning into a single axis (PCA1 had loadings of 0.96, 0.68, 0.18 for the three behaviours, respectively). Parental males in the control treatment performed more nurturing behaviours and nest pecking behaviours compared to males exposed to the sneaker treatment (PCA1: $F_{1,26}$ = 5.36, p = 0.03; Nest pecking: $F_{1,26}$ = 5.76, p = 0.02; Figure 3.2).

Table 3.1. Correlation between individual nurturing behaviours in bluegill sunfish (*Lepomis macrochirus*). Values in the table represent the correlation coefficient R and values that are bolded are significant at $\alpha = 0.05$.

	Rim circling	Caudal fanning	Pectoral fanning	Egg pecking
Rim circling	-	**0.68**	**0.37**	0.14
Caudal fanning	-	-	**0.66**	-0.05
Pectoral fanning	-	-	-	-0.17
Egg pecking	-	-	-	-

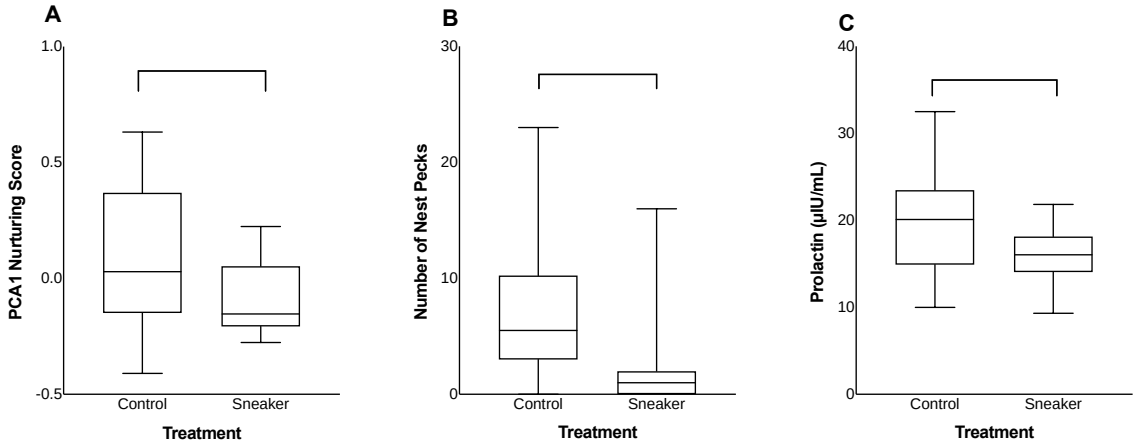

Figure 3.2. Parental care behaviour and prolactin concentrations from two experimental treatments in bluegill sunfish (*Lepomis macrochirus*). Shown are the PCA1 nurturing score (A), nest pecking behaviour (B), and circulating prolactin concentration (C). The treatments comprised a control group (higher perceived paternity) and a group exposed to the visual cue of sneakers around the nest (lower perceived paternity).

Parental males in the control treatment also had significantly higher circulating prolactin concentrations (20 ± 6.0 µIU/mL) as compared to the males in the sneaker treatment (16 ± 3.3 µIU/mL; $t_{26.7} = 2.31$, $p = 0.03$; Figure 3.2C).

I also used an ANCOVA to examine the relationship between circulating prolactin concentrations within each treatment and our behavioural data (Figure 3.2). There was a significant effect of both treatment and prolactin concentration for PCA1 (treatment $F_{1,25} =$

4.82, p = 0.03; prolactin $F_{1,25}$ = 6.78, p = 0.02) and only treatment for the nest pecking behaviour (treatment $F_{1,25}$ = 5.77, p = 0.02; prolactin $F_{1,25}$ = 0.04, p = 0.84). There was no significant interaction between treatment and prolactin concentration for either PCA1 ($F_{1,25}$ = 0.166, p = 0.69) or nest pecking behaviour ($F_{1,25}$ = 1.03, p = 0.32). Finally, males with higher egg scores tended to have higher prolactin concentrations, but this correlation was not significant (r_s = 0.34, S_{27} = 2691.7, p = 0.07) and prolactin concentration was not correlated with body length (r_s = 0.05, S_{33} = 6795.5, p = 0.78).

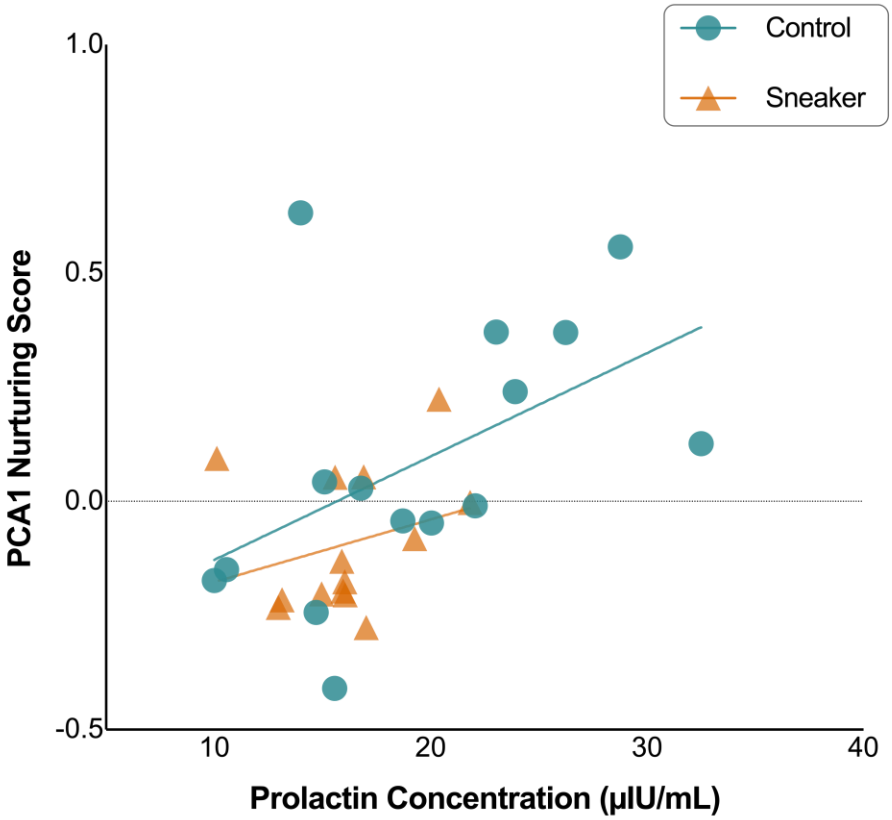

Figure 3.3. Analysis of covariance results for PCA1 nurturing score and circulating prolactin concentration from two experimental treatments in bluegill sunfish (*Lepomis macrochirus*). Shown are the PCA1 nurturing scores for parental males from a control group (green circles) and a group exposed to the visual cue of sneakers around the nest (orange triangles). PCA1 values above 0 indicate higher frequency than average of nurturing behaviour whereas values below 0 indicate a lower frequency than average.

3.4 Discussion

Prolactin is conserved across vertebrates and has its evolutionary origin in the ancestors of fishes (Dobolyi et al., 2020), the basal vertebrate taxon. Although prolactin is well known to affect parental care in mammals and birds (Bachelot & Binart, 2007; Smiley, 2019), no study has linked endogenous concentrations of prolactin to parental care behaviour in a fish. Here, I now show that endogenous concentrations of prolactin are positively associated with parental care behaviour. Moreover, by manipulating perceived paternity, my findings implicate prolactin as a mechanism mediating adaptive changes in parental care behaviour in response to changes in the perceived value of the brood.

Previous research on the behavioural endocrinology of parental care in fishes has focused on androgens and cortisol. Considering prolactin's pivotal role in mammalian and avian parental care, and given the gene's conservation in vertebrate genomes, biologists have recognized the paucity of data showing a similar role in fish parental care (Kindler et al., 1991; Whittington & Wilson, 2013). Previously, exploratory approaches have been largely confined to experimental manipulation of mammalian prolactin and its inhibitors. This research has nevertheless been integral in suggesting that: (1) artificially elevating prolactin levels increases nurturing care behaviour (Blüm & Fiedler, 1965; Cunha et al., 2019; Yada et al., 2004); and (2) increasing levels of bromocriptine (a dopamine receptor agonist that inhibits secretion of pituitary prolactin) decreases nurturing care behaviour (Cunha et al., 2019; Deane et al., 2000; Kindler et al., 1991). My first objective was to bridge this gap by determining the natural circulating concentration of prolactin during the parental care period in bluegill and associating it with parental care behaviour. I sampled bluegill parental males on the day of spawning, while performing care for eggs, and while performing care for larvae. As expected, prolactin concentrations were highest during spawning day, when eggs accumulate in the nest, and during subsequent egg care. Concentrations were lower during the larval period of care, where most of the care involves defending the nest from predators. This pattern mirrors that of mammals and birds, where prolactin concentrations are initially high and decrease over time (Dixson & George, 1982; Garcia et al., 1996; Storey et al., 2000).

Despite the conservation of the prolactin gene across vertebrate genomes, my results suggest that the binding efficiency of fish prolactin to its receptor may be much greater than that of

mammalian prolactin. Cunha et al., (2019) implanted parental male bluegill with two concentrations of ovine prolactin: 1.25 IU/implant and 12.5 IU/implant (Cunha et al., 2019). Given the size of the fish, these concentrations are close to 0.28 IU/mL and 2.8 IU/mL of blood per fish, provided they were full absorbed. Notably, an increase in nurturing behaviour was evident only with the higher dose. In contrast, in our study, circulating endogenous prolactin concentrations remained below 35 µIU/mL, which is several orders of magnitude lower than the ovine prolactin implant concentration used in the aforementioned study. The range of values I detected are more comparable to circulating plasma prolactin concentrations in female Nile tilapia (*Oreochromis niloticus;* 4 – 10 ng/mL (Tacon et al., 2000) vs 0.44 – 1.53 ng/mL for my study when using the conversion of IU to ng units; see Methods). Given the stark difference in circulating concentrations of endogenous prolactin that elicit a behaviour response compared to the required mammalian dose, I expect it is likely the fish prolactin receptor is significantly more sensitive to fish prolactin compared to mammalian prolactin. The prolactin receptor DNA sequence has been characterized in several fishes and is similar in structure to the mammalian receptor with highly conserved functional domains (Breves et al., 2014). Fish prolactin sequences, on the other hand, are less than 40% homologous to mammalian prolactin sequences and lack the N-terminus conserved in other vertebrates (Manzon, 2002; Whittington & Wilson, 2013). I posit the difference in binding efficiencies is due to the subsequent structural variations in the prolactin hormone rather than the receptor. This difference must be considered in the interpretation of work in fishes that is based on responses to administration of mammalian prolactin. Even so, the conservation of core functional domains in prolactin across vertebrates indicates an evolutionary conserved function of prolactin in regulating parental care behaviour.

My second objective was to link circulating concentrations of prolactin to variation in parental care behaviour among individuals. I quantified four nurturing behaviours that are typical in care-giving fishes and determined that parental male bluegill with higher circulating concentrations of prolactin had higher nurturing scores and performed more nest pecking, which is a cleaning behaviour. By associating higher concentrations of prolactin to increased nurturing behaviour I provide evidence of prolactin's regulatory role in bluegill parental care. Furthermore, because these nurturing and nest cleaning behaviours increase survival of the offspring (Kindler et al., 1991), my results implicate prolactin as part of the mechanism

underlying the adaptive adjustments in parental investment in response to assessments of brood value (Alonzo & Klug, 2013). Specifically, while studies of birds and fish consistently demonstrate an association between paternity and paternal care, the mechanisms driving this relationship remain unidentified (Alonzo & Klug, 2013). I manipulated perceived paternity and found that males with lower perceived paternity had lower concentrations of circulating prolactin and provided less care for the offspring in their nest. My findings suggest that paternity perception influences prolactin expression in brain regions governing parental investment. Interestingly, bluegill with higher realized paternity show increased brain expression of phosphodiesterase 2A, a gene responsive to olfactory cues and prolactin (Churchman, unpublished data). Given that bluegill determine paternity through olfactory signals (Neff & Sherman, 2003), a potential gene interaction may be involved in kin recognition and parental care mediated through prolactin. Future research could extend these findings by exploring whether prolactin similarly mediates the relationship between paternity and parental investment in mammals and birds, thereby enhancing our understanding of prolactin's role in adaptive parental care across a broader range of taxa.

In conclusion, by investigating endogenous prolactin concentrations and its association with parental care behaviour in bluegill, my results provide the first data from a fish linking prolactin concentration to parental care behaviour. These data support an evolutionarily conserved function of prolactin in regulating parental care behaviour in vertebrates. Furthermore, by manipulating perceived paternity, my data reveal prolactin as a hormone governing the adaptive changes in parental investment that are made in response to assessments of the fitness value of offspring.

3.5 References

Alonso-Alvarez, C., and Velando, A. (2012) Benefits and costs of parental care. In: *The evolution of parental care*. Ed: N. J. Royle. Oxford Academic Publishing. Oxford, UK.

Alonzo, S. H., and Klug, H. (2013). Paternity, maternity, and parental care. In: *The evolution of parental care*. Ed: N. J. Royle. Oxford Academic Publishing. Oxford, UK.

Bachelot, A., and Binart, N. (2007). Reproductive role of prolactin. *Reproduction*, **133**, 361-369.

Ball, G. F., and Balthazart, J. (2008). Individual variation and the endocrine regulation of behaviour and physiology in birds: A cellular/molecular perspective. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **363,** 1699–1710.

Blüm, V. (1968). Immunological determination of injected mammalian prolactin in cichlid fishes. *General and Comparative Endocrinology*, **11**, 595-602.

Blüm, V., and Fiedler, K. (1965). Hormonal control of reproductive behavior in some Cichlid fish. *General and Comparative Endocrinology*, **5,** 186–196

Breves, J. P., McCormick, S. D., and Karlstrom, R. O. (2014). Prolactin and teleost ionocytes: New insights into cellular and molecular targets of prolactin in vertebrate epithelia. *General and Comparative Endocrinology*, **203,** 21-28.

Bridges, R. S. (2015). Neuroendocrine regulation of maternal behavior. *Frontiers in Neuroendocrinology*, **36**, 178–196.

Cargnelli, L.M., and Gross, M.R. (1996). The temporal dimension in fish recruitment: birth date, body size, and size-dependent survival in a sunfish (bluegill: *Lepomis macrochirus*). *Canadian Journal of Fisheries and Aquatic Sciences*, **53,** 360-367.

Champagne, F. A., and Curley, J. P. (2013). Genetics and epigenetics of parental care. In N.J. Royle, and P.T. Smiseth (Eds), *The Evolution of Parental Care.* Oxford Academic.

Chastel, O., Lacroix, A., Weimerskirch, H., and Gabrielsen, G. W. (2005). Modulation of prolactin but not corticosterone responses to stress in relation to parental effort in a long-lived bird. *Hormones and Behavior*, **47,** 459-466.

Churchman, E. K. L., Hain, T. J. A., Knapp, R., and Neff, B. D. (2023). Parental care behaviour in response to perceived paternity is not mediated by 11-ketotestosterone in bluegill sunfish. *General and Comparative Endocrinology*, **343,** 114367.

Claussen, J. (1991). Annual variation in the reproductive activity of a bluegill population: effect of clutch size and temperature. University of Toronto.

Côté, I. M., and Gross, M. R. (1993). Reduced disease in offspring: A benefit of coloniality in sunfish. *Behavioral Ecology and Sociobiology*, **33,** 269–274.

Cunha, A. A. P., Partridge, C. G., Knapp, R., and Neff, B. D. (2019). Androgen and prolactin manipulation induces changes in aggressive and nurturing behavior in a fish with male parental care. *Hormones and Behavior*, **116,** 104582.

Deane, E. E., Kelly, S. P., Chow, I. N. K., and Woo, N. Y. S. (2000). Effect of a prolactin pharmacological stimulant (sulpiride) and suppressant (bromocriptine) on heat shock protein 70 expression in silver sea bream, *Sparus sarba*. *Fish Physiology and Biochemistry*, **22,** 125-133.

Dixson, A. F., and George, L. (1982). Prolactin and parental behaviour in a male New World primate. *Nature*, **299,** 551–553.

Dobolyi, A., Oláh, S., Keller, D., Kumari, R., Fazekas, E. A., Csikós, V., Renner, É., and Cservenák, M. (2020). Secretion and function of pituitary prolactin in evolutionary perspective. *Frontiers in Neuroscience*, **14,** 621.

Garcia, V., Jouventin, P., and Mauget, R. (1996). Parental care and the prolactin secretion pattern in the King penguin: An endogenously timed mechanism? *Hormones and Behavior*, **30**, 259–265.

Garner, S. R., and Neff, B. D. (2013). Alternative male reproductive tactics drive asymmetrical hybridization between sunfishes (*Lepomis* spp.). *Biology Letters*, **9,** 20130658.

Goffin, V., Binart, N., Touraine, P., and Kelly, P. A. (2002). Prolactin: The new biology of an old hormone. *Annual Review of Physiology*, **64,** 47-67.

Gong, N., Ferreira-Martins, D., Norstog, J. L., McCormick, S. D., and Sheridan, M. A. (2022). Discovery of prolactin-like in lamprey: Role in osmoregulation and new insight into the evolution of the growth hormone/prolactin family. *Proceedings of the National Academy of Sciences of the United States of America*, **119,** e2212196119.

Gray, S. M., McKinnon, J. S., Tantu, F. Y., and Dill, L. M. (2008). Sneaky egg-eating in *Telmatherina sarasinorum*, an endemic fish from Sulawesi. *Journal of Fish Biology*, **73,** 728–731.

Gross, M. R. (1982). Sneakers, satellites and parentals: polymorphic mating strategies in North American sunfishes. *Ethology*, **60,** 1–26.

Gross, M. R., and Charnov, E. L. (1980). Alternative male life histories in bluegill sunfish (*Lepomis macrochirus*). *Proceedings - National Academy of Science, U.S.A.*, **77,** 6937–6940.

Gross, M. R., and MacMillan, A. M. (1981). Predation and the evolution of colonial nesting in bluegill sunfish (Lepomis macrochirus). *Behavioral Ecology and Sociobiology*, **8,** 163–174.

Kappal, S. (2019). Data normalization using median & median absolute deviation (mmad) based z-score for robust predictions vs. min-max normalization. *London Journals Press*, **19,** 39-44.

Kindler, P. M., Bahr, J. M., Gross, M. R., and Philipp, D. P. (1991). Hormonal regulation of parental care behavior in nesting male bluegills: do the effects of bromocriptine suggest a role for prolactin? *Physiological Zoology*, **64,** 310–322.

Klug, H., and Bonsall, M. B. (2014). What are the benefits of parental care? The importance of parental effects on developmental rate. *Ecology and Evolution*, **4,** 2330–2351.

Lam, T. J., and Hoar, W. S. (1967). Seasonal effects of prolactin on freshwater osmoregulation of the marine form *(trachurus*) of the stickleback *gasterosteus aculeatus*. *Canadian Journal of Zoology*, **45,** 509-516.

Li, X., Xu, W., Yu, J., Zhang, W., and Wang, H. (2022). Plasma levels of luteinizing hormone and prolactin in relation to double brooding in Great Tit (*Parus major*). *Avian Research, 13,* 100017.

Manica, A. (2004). Parental fish change their cannibalistic behaviour in response to the cost-to-benefit ratio of parental care. *Animal Behaviour*, **67,** 1015–1021.

Manzon, L. A. (2002). The role of prolactin in fish osmoregulation: A review. *General and Comparative Endocrinology*, **125,** 291–310.

Miller, D. A., Vleck, C. M., and Otis, D. L. (2009). Individual variation in baseline and stress-induced corticosterone and prolactin levels predicts parental effort by nesting mourning doves. *Hormones and Behavior*, **56,** 457-464.

Møller, A. P., and Birkhead, T. R. (1993). Cuckoldry and sociality: a comparative study of birds. *American Naturalist*, **142,** 118–140.

Neff, B. D. (2001). Genetic paternity analysis and breeding success in bluegill sunfish (*Lepomis macrochirus). Journal of Heredity*, **92**, 111-119.

Neff, B. D. (2003). Decisions about parental care in response to perceived paternity. *Nature*, **422,** 716–718.

Neff, B. D., and Gross, M. R. (2001). Dynamic adjustment of parental care in response to perceived paternity. *Proceedings of the Royal Society B: Biological Sciences*, **268,** 1559–1565.

Neff, B. D., and Knapp, R. (2009). Paternity, parental behavior and circulating steroid hormone concentrations in nest-tending male bluegill. *Hormones and Behavior*, **56**, 239–245.

Neff, B. D., and Sherman, P. W. (2003). Nestling recognition via direct cues by parental male bluegill sunfish (*Lepomis macrochirus*). *Animal Cognition*, **6,** 87–92.

Ouyang, J. Q., Sharp, P. J., Dawson, A., Quetting, M., and Hau, M. (2011). Hormone levels predict individual differences in reproductive success in a passerine bird. *Proceedings of the Royal Society B: Biological Sciences*, **278,** 2537-2545.

Riddle, O., Bates, R. W., and Dykshorn, S. W. (1933). The preparation, identification and assay of prolactin—a hormone of the anterior pituitary. *American Journal of Physiology-Legacy Content*, **105,** 191-216.

Riechert, J., Becker, P. H., and Chastel, O. (2014). Predicting reproductive success from hormone concentrations in the common tern (*Sterna hirundo*) while considering food abundance. *Oecologia*, **176,** 715-727.

Rstudio Team. (2015). *R Studio: Integrated Development for R.*

Schradin, C., and Anzenberger, G. (1999). Prolactin, the hormone of paternity. *News in Physiological Sciences*, **14,** 223–231.

Smiley, K. O. (2019). Prolactin and avian parental care: New insights and unanswered questions. In *Hormones and Behavior*, **111,** 114–130.

Smiley, K. O., and Adkins-Regan, E. (2016). Relationship between prolactin, reproductive experience, and parental care in a biparental songbird, the zebra finch (*Taeniopygia guttata*). *General and Comparative Endocrinology*, **232,** 17-24.

Stiver, K. A., and Alonzo, S. H. (2009). Parental and mating effort: Is there necessarily a trade-off? *Ethology*, **115,** 1101-1126.

Storey, A. E., Walsh, C. J., Quinton, R. L., and Wynne-Edwards, K. E. (2000). Hormonal correlates of paternal responsiveness in new and expectant fathers. *Evolution and Human Behavior*, **21,** 79–95.

Stricker, P., and Grueter, R. (1928). Action du lobe antéieur de l'hypophyse sur la monté laiteuse. *Comptes Rendus Des Séances de La Société de Biologie et de Ses Filiales*, **99,** 1978–1980.

Tacon, P., Baroiller, J. F., Le Bail, P. Y., Prunet, P., and Jalabert, B. (2000). Effect of egg deprivation on sex steroids, gonadotropin, prolactin, and growth hormone profiles during the reproductive cycle of the mouthbrooding cichlid fish *Oreochromis niloticus*. *General and Comparative Endocrinology*, **117,** 54–65.

Trivers, R. L. (1972). Parental investment and sexual selection. In B. Campbell (Ed), *Sexual Selection and the Descent of Man: 1871-1971*. Aldine.

Whittington, C. M., and Wilson, A. B. (2013). The role of prolactin in fish reproduction. *General and Comparative Endocrinology*, **191,** 123–136.

Yada, T., Muto, K., Azuma, T., and Ikuta, K. (2004). Effects of prolactin and growth hormone on plasma levels of lysozyme and ceruloplasmin in rainbow trout. *Comparative Biochemistry and Physiology - C Toxicology and Pharmacology*, **139,** 57-63.

Chapter 4

4 Paternity perception and gene regulation in bluegill sunfish (*Lepomis macrochirus*)

A version of this manuscript has been prepared for submission.

4.1 Introduction

Parental care has evolved repeatedly across taxa and falls into several categories: uniparental maternal, uniparental paternal care, and biparental care. Uniparental maternal care involves exclusive maternal investment, whereas uniparental paternal care is characterized by exclusive paternal investment. Biparental care involves contributions from both parents with the distribution of care varying based on offspring requirements and the relative costs and benefits of the care to each parent (Clutton-Brock, 1991). In mammals, uniparental maternal care is the predominant type of care, from which biparental systems have emerged (Reynolds et al., 2002). Contrastingly, biparental care is prevalent in over 90% of avian species (Balshine, 2013) and in fishes, when parental care is observed, uniparental paternal care is more frequent (Gross & Shine, 1981). The provision of parental care is often correlated with enhanced offspring survival, accelerated juvenile growth, and increased reproductive success in adulthood (Klug & Bonsall, 2014).

While parental care increases the survival of offspring and contributes to fitness (Dulac et al., 2014; Reynolds et al., 2002), it is also costly to the parent. The costs can include reduced foraging time, compromised immune function, and lost reproductive opportunities (Alonso-Alvarez & Velando, 2012). Given these costs, parental investment theory predicts care-giving parents will alter their level of care to reflect the value of the brood. This reproductive value can be based on the number and size of offspring along with the relatedness of the offspring to the parent (Westneat & Sherman, 1993). In the case of relatedness, males have been shown to differentially allocate parental care based on perceived relatedness of offspring, yet our understanding of the mechanisms underlying this allocation are limited (Alonzo & Klug, 2013).

Parental care in animals necessitates a sophisticated suite of molecular and physiological changes, particularly impacting neural and reproductive systems (Bridges, 2015; Champagne & Curley, 2013; Stiver & Alonzo, 2009). The predominant understanding of these changes in the context of parental care is anchored in the dynamics of the endocrine system, to the extent whereby hormonal responsiveness is a requirement of the parental phenotype (Ball & Balthazart, 2008). In particular, while the neuroendocrine regulation of parental care is well understood, advancements in genomic technologies are helping to understand the role of individual genes in the evolution and expression of parental behaviour. Transcriptomics, or the analysis of an organism's complete mRNA expression, facilitates the identification of novel genes and expression patterns through RNA-sequencing. Transcriptomic analysis paired with observations of parental care behaviour has the potential to broaden our understanding of the mechanisms governing the allocation of parental care. By conducting transcriptomic analysis of specific tissues such as the brain during parental care periods we can expand our understanding of gene-level behavioural regulation, as well as the potential evolution and adaptation of candidate genes.

Past transcriptomic studies have shown that alterations in gene expression pertinent to parental care predominantly occur within the brain. The neural architecture related to this function in mammals is relatively well-defined (Kohl & Dulac, 2018), with particular emphasis on the hypothalamic preoptic area's role in regulating parental behaviours (Dobolyi et al., 2014; Wu et al., 2014; Zhang et al., 2021). However, parental care associated gene expression is not limited to this area of the brain (Bukhari et al., 2019; Kumari et al., 2022). The bulk of transcriptomic research has been focused on maternal care, possibly reflecting the dominance of maternal care in mammals and biparental care in birds. A recent transcriptomic study in maternal mice determined that expression of neuropeptides and their response pathways are necessary for parental care (Wu et al., 2014). In parental prairie voles, transcriptomic analysis has determined the involvement of processes related to the mitochondria, RNA translation, immune system regulation, and chemokine signaling (Duclot et al., 2022). In birds, transcripts associated with neuromodulatory, structural, and metabolic pathways are up-regulated in parental red-winged blackbirds (*Agelaius phoeniceus;* Lynch et al., 2019). In zebra finches (*Taeniopygia guttata*), similar patterns were noted, as well as enriched dopamine pathways. Transcriptomic studies in fishes have determined shared commonalities at a molecular level

with maternal mammals and suggest the neurogenomic state maintained across pregnancy and post-partum care resembles the neurogenomic state maintained by paternal fish (Bukhari et al., 2019). In parental male three-spined stickleback (*Gasterosteus aculeatus*), transcripts associated with energy metabolism in the brain, modification of the immune system, and transcription are important during parental care (Bukhari et al., 2019).

Bluegill sunfish (*Lepomis macrochirus*) are native to North America and are a well-studied species that exhibit male alternative reproductive tactics. In this species, "parental" males establish and defend territories within breeding colonies, construct nests, court, and spawn with females, and subsequently provide uniparental male care to their brood (Gross, 1982). The bluegill mating system is highly promiscuous with ~25% of offspring being sired by precocious "cuckolder" males (Garner & Neff, 2013; Neff, 2001). Cuckolder males mature early and use a sneaking tactic by concealing themselves in vegetation and intruding into the nests of parental males to fertilize eggs. As they grow, cuckolders switch tactics and instead use female mimicry to orbit around the nest and enter the nest while a parental male is spawning with a female, while acting as if they are also spawning with the male (Gross, 1982). Parental males can discern relatedness to their brood using olfactory cues released by larvae after the eggs have hatched (Neff & Sherman, 2003). This paternity-based recognition influences paternal care, as males with higher relatedness to their brood tend to provide superior care (Churchman et al., 2023; Neff, 2003; Neff & Gross, 2001). Mechanistically, parental males alter their parental care behaviour when exposed to elevated concentrations of hormones including 11-ketotestosterone and prolactin (Cunha et al., 2019; Rodgers et al., 2012). The transcriptomic regulation of paternal care in response to paternity cues has yet to be investigated.

In this study, I experimentally manipulated paternity in the nests of parental male bluegill sunfish and conducted whole-brain transcriptomic analysis to explore the association between gene expression and differences in paternal investment via care behaviour. Although the preoptic area is frequently the focal area in neurogenomic studies of parental care, I chose to examine the entire brain to capture comprehensive changes and pathways occurring throughout the brain. My objective was to determine if there is a discernable difference in gene expression between control males and those with experimentally reduced paternity, and to subsequently

identify candidate genes that may be involved in any changes in paternal behaviour in response to perceived paternity.

4.2 Methods

4.2.1 Bluegill sampling

I collected bluegill via dipnet from Lake Opinicon (44°34′N, 76°19′W), Ontario, Canada in June 2018, 2020, and 2021. The fish used in this study are a subset of those used for a parallel study on the endocrine regulation of parental care in response to perceived paternity and were collected in 2018 and 2021 (see Churchman et al., 2023). Prior to collection, males were exposed to one of two treatments: (1) control; or (2) egg manipulation. For the egg manipulation group, I swapped about one-half of each male's eggs between the two nests the day after spawning. I performed a sham egg swap in the nests of males assigned to the control treatment, in which I removed and then returned one-half of the eggs to the original nest. This mimicked the disturbance of the egg swap, but not the reduction in paternity. I recorded each male's nest defense twice: once while they were caring for eggs (day 2) and once the day after the eggs hatched and the males were caring for larvae (day 4) and scored three behaviours: lateral display, opercular flare, and bite. Across 2018 and 2021, of the 47 parental males who remained on the nest for the five days, 22 were assigned to the control treatment and 25 to the egg swap treatment. I euthanized individuals using clove oil and immediately dissected and stored whole brains in RNAlater (ThermoFisher Scientific; Mississauga, ON, Canada). The total amount of time required for fish capture, euthanasia, dissection, and brain storage in RNAlater was under 10 minutes. I stored the brains in RNAlater at 4 ˚C for 24 hours. They were then flash-frozen and kept in liquid nitrogen until we transported them on dry ice to the University of Western Ontario where they were stored at -80 ˚C. The Animal Care Committee at Western University (ACC) approved all procedures performed in this study (AUP #2010-214 and #2018-084).

4.2.2 cDNA and Transcriptome Assembly, Annotation, and Analysis

I extracted total RNA from whole brains using a standard Trizol (Life Technologies, Carlsbad, CA) extraction protocol (https://tools.thermofisher.com/content/sfs/manuals/Trizol_reagent.pdf). I removed residual genomic DNA from all samples prior to sequencing using a Qiagen

RNAeasy® Cleanup kit. Fifteen samples from each of the control and swap treatments from 2018 were analyzed via transcriptome sequencing (total 30 samples). The remaining 17 samples (7 control, 10 egg swap) collected in 2021 were used for qPCR analysis to verify transcriptome results. I quantified total RNA using a Nanodrop spectrophotometer (ThermoFisher Scientific; Mississauga, ON, Canada). The average A260/A280 of all RNA samples was 1.87 ± 0.09 (mean ± SD).

I sent the 30 transcriptome samples plus an additional 10 samples from parental males in a separate stress-based study to Génome Québec (Montreal, Quebec, Canada) where quality was assessed using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) before cDNA libraries were prepared from the RNA using NEBNext RNA First Strand Synthesis and NEBNext Ultra Directional RNA Second Strand Synthesis Modules (New England BioLabs). The remaining library preparation was completed using NEBNext Ultra II DNA Library Prep Kit for Illumina (New England BioLabs). Libraries were quantified using the KAPA Library Quantification Kits - Complete kit (Universal; Kapa Biosystems). Génome Québec sequenced the libraries using 2 x 125 bp sequencing format on an Illumina Hiseq 2500 (Illumina, San Diego, CA, USA).

Transcriptomes were assembled and annotated by the Canadian Centre for Computational Genomics (C3G) at McGill University. Prior to assembly, the read quality was assessed with FastQC. Nucleotides with a quality score below PHRED = 5 and a minimum read length of 25 were trimmed using Trimmomatic. The reference transcriptome was assembled de novo using Trinity (v. 2.11.0). One representative from each treatment with the highest number of reads was used to construct the combined reference transcriptome. A total of over 249 million paired-end reads were assembled. The fully assembled transcriptome consisted of 371 553 transcripts in total (N50 = 3086 bp, N90 = 373 bp). To determine whether this was an appropriate representation of the brain transcriptome reads from samples not used in the assembly were mapped back to the transcriptome using bowtie-RSEM and >90% of those reads aligned. For gene annotation, Trinotate (v. 3.2.2) was used to identify transcripts via blastx, blastp, signal, TMHMM, RNAMMER, HMMSCAN, and Transdecoder.

I first analyzed differential expression using edgeR in R Studio (RStudio Team, 2015; version 4.2.2, Posit 2022, Boston, MA, USA). I filtered out genes with less than 10 counts per million

in at least 4 samples. I compared expression between treatments using a Fisher Exact test and applied an FDR correction to control for multiple testing (α= 0.05). I identified differentially expressed transcripts as those with an FDR < 0.05. I noted inflated log 2 fold change (log2FC) values from low count values in one treatment. To help with this, I then ran the analysis using DESeq2 (version 1.38.3) in R Studio and calculated log2FC values extracted adjusted p-values. I pre-filtered the data the same way as with edgeR and then compared expression between treatments based on a generalized linear model. Given the similarly inflated log2FC values, I retained the same cut off for differentially expressed genes. In addition, I considered the raw count values of the transcripts to determine if there was a biologically relevant difference.

4.2.3 Quantitative PCR Primer Design and Analysis

Given the limited amount of RNA available for qPCR analysis, I selected eight genes for qPCR that were identified as differentially expressed by both edgeR and DESeq2: *SNPH, AKAP9, GNS, USP40, RUSC2, ROA1, PDE2A,* and *HA1F*. I designed primers to recognize mRNA sequences of using Primer-BLAST software (NCBI; Table 4.1). Reference sequences for the primer design were based on sequences present in the assembled transcriptome. I set primer melting temperatures to a minimum and maximum of 57 ˚C and 63 ˚C respectively. I set the PCR product size to a minimum of 70 and a maximum of 200. I tested the primers against a pooled cDNA sample in a gradient PCR, then by gel electrophoresis to determine the accuracy of the melt temperature and primer product size. I validated primer efficiency using a 1:10 serial dilution curve and a single melt curve. All primers, including the reference gene, displayed an efficiency between 90-110%.

Table 4.1 Primer sequences

Primer	5'-3'	Sequence
Syntaphilin (SNPH)	Forward	TCTCTCTGTCGTCCCAATCT
	Reverse	TCCCTTCCTCTTCACACTCT
A-kinase anchor protein 9 (AKAP9)	Forward	CCTACAGAGCAAAGAGCAAGAG
	Reverse	GCTGTAGGGTGAGGTGTTTAAG
N-acetylglucosamine-6-sulfatase (GNS)	Forward	TTCCACCCACTGCTGTTATG
	Reverse	GAGGTTTGACTGGTGCTCTT
Ubiquitin Carboxyl-terminal hydrolase-40 (USP40)	Forward	ACTCTTCTCCTCGCTCTCTAC
	Reverse	GTTTGTCTGGCTGGTGTTTG
Iporin (RUSC2)	Forward	GTTAGCAGACCGGCAATGA
	Reverse	CTTGTCCATCGTCACCTTCTC
Heterogenous nuclear ribonucleoprotein A1 (ROA1)	Forward	CCCTCACAAAGCAGGAAAT
	Reverse	CTCTGCCACCCTGATTAAAG
Phosphodiesterase 2A (PDE2A)	Forward	CAGCCATCCTTCCCATTC
	Reverse	CGGTTGCTCTCTGTCTAAAG
Class 2 histocompatibility antigen, F10 alpha chain (HA1F)	Forward	CACGATGTTCTGGAGGAAAG
	Reverse	GTCAACACTCATCTGGAAGG
Elongation factor 1-beta (EF1B)	Forward	CGTGGGTTACGGCATCAAGA
	Reverse	GATCTTGTTGAAAGCGGCGA

For the qPCR samples, I synthesized cDNA for the 17 samples using qScript cDNA Supermix (Quantabio; Beverly, MA, USA) from the same amount of RNA per sample and stored the cDNA at -20 ˚C. Due to a low RNA concentration of one sample, I was unable to synthesize cDNA from 1000ng RNA and standardized all samples to the maximum possible concentration of 820ng RNA per reaction (Supplementary Table 1; Appendix B). I used POWER SybrGreen Master Mix (ThermoFisher Scientific; Mississauga, ON, Canada) to amplify cDNA according to the manufacturer's instructions with thermal-cycling conditions configured to an initial 10-minute activation at 95 ˚C then 40 cycles of 15 seconds at 95 ˚C to denature and 60 seconds at 60 ˚C to anneal/extend. Each 12µL qPCR reaction included 2µL cDNA, and 0.4µM forward and 0.4µM of reverse primer. I ran each reaction in triplicate using a QuantStudio 3 real-time PCR cycler (Applied Biosystems; Waltham, MA, USA).

I normalized transcript abundance in two ways: to a reference gene (EF1α) and two internal calibrator samples consisting of pooled cDNA. I used internal calibrator samples in each

run to control for any inconsistencies between qPCR runs. I calculated the relative transcript abundance using the comparative CT method ($2^{-\Delta\Delta CT}$). This compared egg swap-treated samples against the control samples. I then normalized the values by log transforming the relative abundance to obtain logFC values ($\log(2^{-\Delta\Delta CT})$). I compared logFC values between treatments using Welch-corrected t-tests in R Studio.

4.3 Results

4.3.1 Behavioural Results

A full description of behavioural results has been published in Churchman et al. (2023). There was no significant difference in the number of lateral displays, opercular flares, or bites performed by males in the control vs swap treatments during the egg care period (t_{74} = -1.18, p = 0.24; t_{74} = 0.27, p = 0.79; t_{74} = -1.19, p = 0.24). Similarly, there was no significant difference in the frequency of lateral displays or opercular flares between treatments during the larvae care period (t_{65} = -0.62, p = 0.54; t_{65} = -0.75, p = 0.46). However, males in the control treatment bit significantly more frequently than males in the swap treatment during larvae care (t_{65} = 3.38, $p < 0.01$).

4.3.2 Differential Gene Expression

After correcting for false discovery rate, edgeR identified 22116 transcripts with nonzero total read counts of which ten transcripts were differentially expressed (FDR <0.05) between control and egg swap treatments: *GNS, AKAP9,* three isoforms of *SNPH, USP40, RUSC2,* two isoforms of gene "DN4077" that could not be identified by annotation, and another unidentified transcript "DN721" (Table 4.2). DESeq2 identified 22092 transcripts with a nonzero total read count of which 28 were differentially expressed (Figure 4.1; Supplementary Table 1; Appendix B). These transcripts included all the transcripts identified by edgeR (Table 4.2). After correcting with lfcShrink, only transcripts for SNPH and RUSC2 were differentially expressed per log2FC >1 and <-1 and FDR < 0.05. Given the difference in raw count values between treatments (Table 4.2), I considered count differences >150 to be biologically relevant: *SNPH, AKAP9, GNS, USP40, RUSC2, ROA1, PDE2A,* and *HA1F*. The inflated log2FC values may be attributed to zero counts in the control treatment for *SNPH, GNS,* and *AKAP9.*

Among differentially expressed transcripts, *PDE2A* and *HA1F* were expressed at higher levels in control males relative to egg swap males, while *SNPH, AKAP9, ROA1*, and *GNS*, were expressed at higher levels in egg swap males relative to control males. The number of differentially expressed transcripts was too low to perform a statistically powerful GO enrichment analysis.

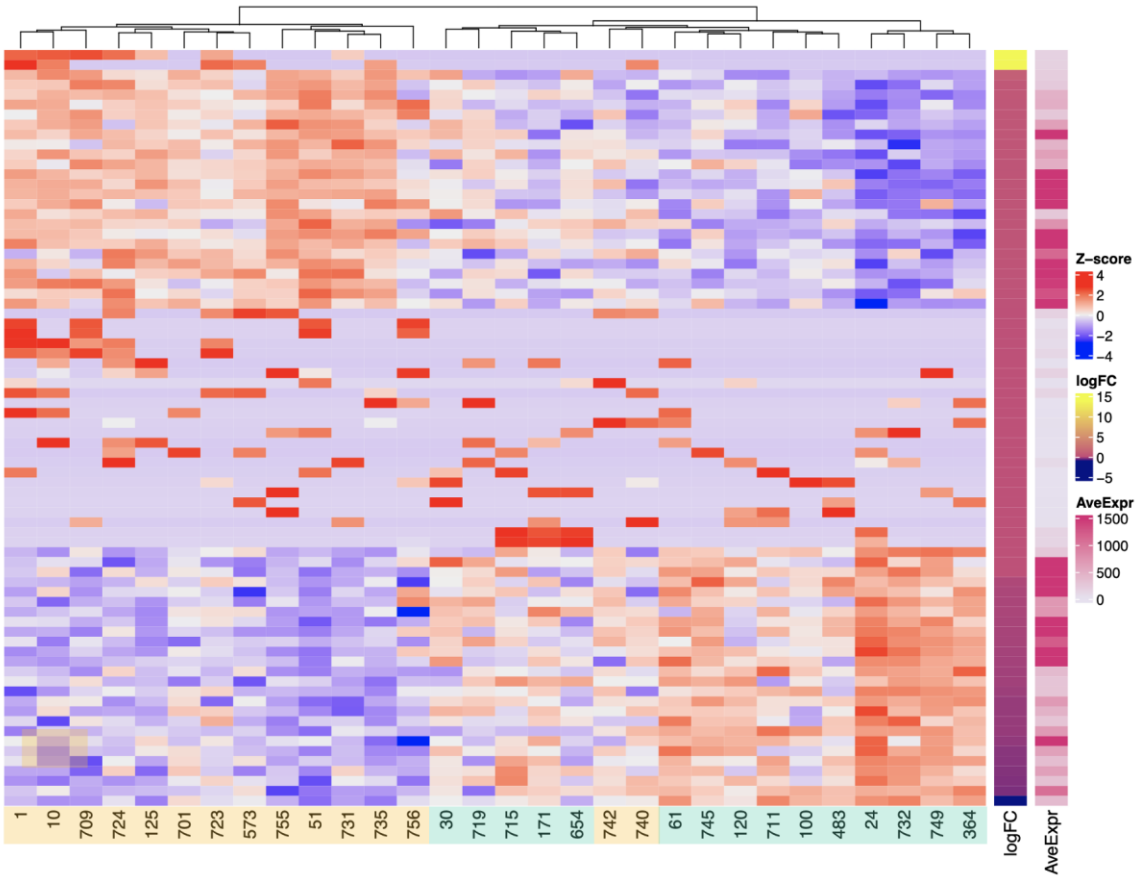

Figure 4.1. Heatmap of differentially expressed transcripts between bluegill parental males with higher paternity compared to parental males with experimentally reduced paternity. Only transcripts differentially expressed at FDR α = 0.05 after false discovery rate correction are included in the heatmap. Each column represents a sample ID (control males are highlighted in blue and the swap males are highlighted in orange. Each row represents a transcript.

Table 4.2. Differentially expressed transcripts between bluegill parental males exposed to either a control treatment (sham swapped eggs) or (2) experimentally reduced paternity (swapped eggs).

Gene ID	Isoform ID	Mean Raw Count		Mean expression	LogFC[1]	Log2FC[2]	Adjusted Log2FC[3]	Adjusted p-value[2]
		Control	Swap					
SNPH	c0_i1_i12	159.87	0	67.74	10.15	25.02	4.26×10^{-7}	<0.001
	c0_g1_i15	281.35	0	117.45	10.95	24.53	3.88×10^{-7}	<0.001
	c0_g1_i20	380.50	0	161.57	11.41	26.22	13.84	<0.001
AKAP9	c0_g1_i6	186.61	0	87.39	10.51	25.37	4.47×10^{-7}	<0.001
GNS	c0_g1_i13	382.99	0	161.02	11.4	26.22	6.02×10^{-7}	<0.001
USP40	c0_g1_i13	96.73	0	46.87	9.62	24.48	5.38×10^{-7}	<0.001
RUSC2	c0_g1_i21	255.12	540.87	384.42	-1.08	-1.08	-0.99	<0.001
ROA1	c0_g1_i14	258.76	102.54	162.54	1.36	10.01	2.84×10^{-7}	0.004
PDE2A	c0_g2_i6	170.13	360.43	261.95	-1.18	-1.18	-1.60×10^{-7}	0.008
HA1F	c0_g1_i4	1.25	276.30	140.38	-7.04	-4.98	1.44×10^{-3}	0.026

[1]Values calculated with edgeR

[2]Value calculated with DESeq2

[3]Adjusted with lfcShrink

4.3.3 Quantitative PCR

After standardizing to EF1A-β, the relative expression did not differ significantly between treatments for any of the 8 genes analyzed by qPCR (Table 4.3; Figure 4.2).

Table 4.3. Quantitative PCR relative expression (log fold change) between bluegill parental males exposed either a control treatment (sham swapped eggs) or (2) experimentally reduced paternity (swapped eggs).

Gene ID	Welch's t-test
Syntaphilin (SNPH)	$t_{12.2} = 1.02$, $p = 0.33$
A-kinase anchor protein 9 (AKAP9)	$t_{13.2} = 1.23$, $p = 0.24$
N-acetylglucosamine-6-sulfatase (GNS)	$t_{12} = 1.24$, $p = 0.24$
Ubiquitin Carboxyl-terminal hydrolase-40 (USP40)	$t_{14.9} = 1.90$, $p = 0.08$
Iporin (RUSC2)	$t_{13.2} = 0.54$, $p = 0.59$
Phosphodiesterase 2A (PDE2A)	$t_{10.2} = 0.63$, $p = 0.54$
Class 2 histocompatibility antigen, F10 alpha chain (HA1F)	$t_{12.5} = 0.13$, $p = 0.90$
Heterogenous nuclear ribonucleoprotein A1 (ROA1)	$t_{12.7} = -0.00$, $p = 1.00$

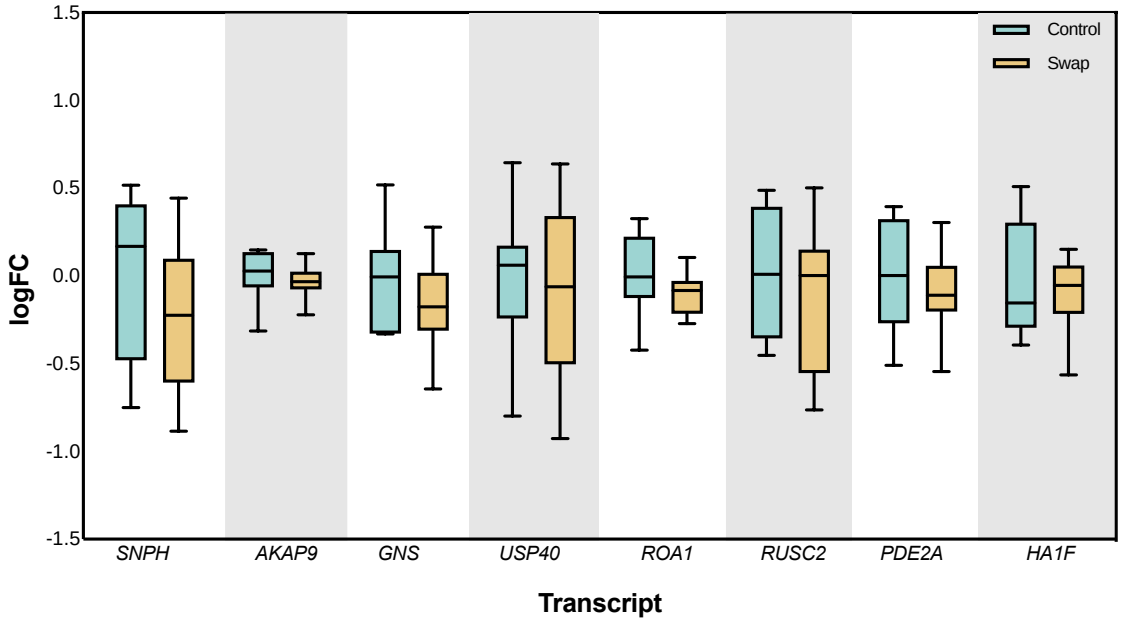

Figure 4.2. Relative differential gene expression between bluegill parental males. Control males (sham swapped eggs) are denoted by blue boxplots while treatment males with experimentally reduced paternity (swapped eggs) are denoted by orange boxplots.

4.4 Discussion

Bluegill sunfish are an excellent model for investigating parental care allocation in response to perceived paternity given their multiple male reproductive tactics, and the presence of a single parental morph that is capable of offspring recognition. In this study, I manipulated the paternity of parental males, assembled, and analyzed their brain transcriptome, and further explored differentially expressed genes via qPCR. Two transcripts were expressed at higher levels in males in the control treatment (high paternity): Phosphodiesterase 2A (*PDE2A*) and class I histocompatibility antigen, F10 alpha chain (*HA1F*). PDE2A is a member of the phosphodiesterase (PDE) family and has a crucial role in the hydrolysis of cyclic nucleotide cAMP (Keravis & Lugnier, 2012; Sadek et al., 2020). Cells use cAMP hydrolysis to finely tune their responses to stimuli including olfactory signals (Breer, 2003; Genovese et al., 2021) and hormones (Nikolaev et al., 2005; Zaccolo et al., 2021). Of particular note is the relationship between the hormone prolactin and PDE activity. A study on the effects of prolactin on cAMP accumulation suggested

prolactin inhibits cAMP accumulation by enhancing PDE activity (Gitay-Goren et al., 1989). It was subsequently observed that prolactin-receptor activity stimulates PDE activity (Fanjul et al., 1993). In a parallel experiment, I determined parental male bluegill with increased perceived paternity have higher concentrations of circulating prolactin (Churchman et al., manuscript submitted for publication). Thus, in the context of this work, I propose males with higher paternity may have increased expression of PDE2A in response to elevated prolactin concentrations. Given that bluegill determine their paternity via olfactory cues produced by larvae (Neff & Sherman, 2003) it is also possible that PDE2A may function as a mechanism through which recognition occurs. Specifically, males with higher paternity have higher levels of PDE2A, which could then be used to regulate cAMP hydrolysis in the response to the olfactory cues produced by the larvae. Taken together it is possible males in the control treatment have higher levels of PDE2A in response to their olfactory stimulated paternity recognition, combined with, or in response to, elevated concentrations of circulating prolactin during parental care.

The transcripts *HA1F* and N-acetylglucosamine-6-sulfatase (*GNS*) were expressed at elevated levels in control treatment males and males in the egg swap treatment respectively. *HA1F*, a part of the major histocompatibility complex (MHC) class I family of genes, plays a crucial role in the immune system (Roche & Furuta, 2015) and has been linked to disease resistance in Atlantic salmon (*Salmo salar;* Grimholt et al., 2003). This finding contrasts with the common trade-off observed between parental care and immune suppression (Bukhari et al., 2019; Carlton et al., 2014; Demas et al., 2012; Duclot et al., 2022; Fedorka, 2014). However, parental behaviours can also increase the parent's risk of disease if their care behaviour exposes them to pathogens (Ganser et al., 2020). Bluegill parental males care for their nest by pecking at surrounding substrate and cannibalizing eggs within their nest that are infected to reduce the spread of fungi and other pathogens (Gross, 1982; Neff & Sherman, 2003). Males with higher perceived paternity engage in more egg pecking behaviour (Churchman et al., 2023), potentially leading to increased pathogen exposure and corresponding heightened expression of *HA1F*. This suggests a possible adaptive immune response, as opposed to broad immunosuppression, with specific pathogen recognition (Magnadóttir, 2006; Uribe et al., 2011). Conversely, *GNS*, an innate immune-response associated lysosomal enzyme crucial in glycosaminoglycan catabolism and

cytokine-binding (Vallet et al., 2022; Yang et al., 2021), showed increased expression in the egg swap treatment males. A previous study has shown down-regulation of immune-related genes during parental care in three-spined stickleback (Bukhari et al., 2019), so given that lower paternity males in the egg swap treatment provide less care (Churchman et al., 2023; Neff, 2003), these males may be able to invest it/maintain a more active innate immune system (Frank, 2002). My data suggest differing immune response strategies between treatments – control males counter pathogen exposure with a *HA1F*-mediated response whereas egg swap males maintain an active innate immune system to due reduced care-giving demands.

Given the role of *GNS* in the catabolism of glycosaminoglycans including heparin, the potential interaction between experimental methods and *GNS* expression warrants further discussion. Fish blood clots very rapidly and extraction techniques must account for this. Standard protocol for blood sampling via the caudal vein is with a heparinized needle (Canada Department of Fisheries and Oceans Canada, 2004), which may subsequently introduce heparin into the bloodstream, potentially influencing *GNS* expression through the catabolism of heparin. However, given that this gene was differentially expressed between treatments and all fish were exposed to the same sampling technique, it seems unlikely that differences in *GNS* expression would be an artifact of our methods. Furthermore, while the data are not available for fish, heparin has a half-life of one to two hours in humans (Cook, 2010). Considering this half-life and the mere ten-minute window between capture, venous puncture, and euthanasia in my protocol, the probability of heparin-induced upregulation of *GNS* expression is low. I therefore consider it to be more plausible the observed differential expression is a response to treatment, rather than an inadvertent consequence of methodology.

Males with experimentally reduced paternity expressed higher levels of Syntaphilin (*SNPH*), A-Kinase Anchor Protein 9 (*AKAP9*), and Heterogenous nuclear ribonucleoprotein A1 (*ROA1*) relative to the control fish. Each of these genes is related to the stress response, which suggests males with lower paternity are under a greater amount of stress during the parental care period. SNPH is involved in mitochondrial movement (Caino et al., 2017; M. Y. Lin et al., 2017). These energy-generating organelles are

transported along neurons to areas of high metabolic demand by specialized proteins including SNPH. When exposed to stressful conditions, this transport system may be damaged and lead to the immobilization of mitochondria (Chang & Reynolds, 2006; M. Y. Lin et al., 2017). While *SNPH* has not been studied in fish, I propose that the expression of *SNPH* transcripts by males in the egg swap treatment could be a response to the stress of low paternity, resulting in a disrupted energy transport pathway within the brain. Given the significant role of the preoptic area in parental care, this region might be particularly vulnerable to diminished energy transport when the parental male is exposed to stress. As a result, I suggest that mitochondrial transport in the brain may influence the allocation of parental care in bluegill.

AKAP9 and *ROA1* were also expressed at higher levels in the egg swap treatment and are both stress-associated transcripts that are integral to physiological stress responses (Colledge & Scott, 1999; Guil et al., 2006; J. W. Lin et al., 1998; Shors & Mathew, 1998; Westphal et al., 1999). In the context of reproduction and parental care, stressed individuals often reallocate resources from current reproduction to survival and future reproduction. The higher *AKAP9* and *ROA1* expression in egg swap males with reduced paternity could signal stress and potentially indicate a shift in resource allocation away from their current brood towards future reproductive investments. Parents should allocate care to the most valuable offspring, and it is possible bluegill males, that assess low paternity in their brood lower their parental care efforts to conserve resources for future reproduction.

Males in the control treatment expressed higher levels of Ubiquitin carboxyl-terminal hydrolase 40 (*USP40*). While not well studied in fish, *USP40* belongs to the ubiquitin carboxyl-terminal hydrolase (UCH) family. Studies on *Cyprinus carpio* and *Monopterus albus* suggest UCHs are important in the processes of nerve degeneration, cellular repair and the development of reproductive cells (Dietrich et al., 2016; Sun et al., 2008). A study in the fish *Oreochromis niloticus* discovered UCH mRNA is expressed in the olfactory bulb and may be involved in chemoreceptive functions (Mochida et al., 2002). Given this, it is plausible that *USP40* may play a role in kin recognition or offspring sensory input in bluegill, potentially showing increased activity when males have higher paternity. Taken together, I suggest males with higher perceived paternity may be more sensitive to

offspring cues, while USP40 may also help to support cellular repair during parental care. *RUSC2* encodes a protein that interacts with a Rab GTPase, which is involved in immunity through the regulation of intracellular trafficking (Bayer et al., 2005; Prashar et al., 2017). Although *RUSC2*'s role in fish immune response has not been examined, its elevated expression level in males in the egg swap treatment compared to those in the control treatment indicate it may be involved in the maintenance of innate immunity during parental care in response to lower paternity and reduced levels of parental care.

In my follow-up qPCR analysis to the transcriptome data, no significant difference was found in gene expression, which I attribute to environmental variation. The qPCR samples were collected in a different field season with notable environmental differences, including a significantly higher ambient temperature during the breeding season (22.7 ˚C vs 27.7 ˚C), which increased water temperature. This temperature increase could result in a 30% increase in metabolic rate for bluegill (Dent & Lutterschmidt, 2003) and may have affected parental care-related gene expression. Regardless, my study highlights the complex genomic responses to paternity and parental care behaviour. I encourage further research to consider the interplay between paternity and parental investment and subsequent effects on both the stress and immune responses. My data implicate differential immune responses for males with high paternity (high parental effort) versus low paternity (low parental effort), rather thana ubiquitous down regulation of immunity with parental care. The data also identify candidate genes that may be involved in kin recognition as well as the reallocation of effort to future reproduction.

4.5 References

Alonso-Alvarez, C., and Velando, A. (2012) Benefits and costs of parental care. In: *The evolution of parental care*. Ed: N. J. Royle. Oxford Academic Publishing. Oxford, UK.

Alonzo, S. H., and Klug, H. (2013). Paternity, maternity, and parental care. In: *The evolution of parental care*. Ed: N. J. Royle. Oxford Academic Publishing. Oxford, UK.

Ball, G. F., and Balthazart, J. (2008). Individual variation and the endocrine regulation of behaviour and physiology in birds: A cellular/molecular perspective. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **363,** 1699–1710.

Balshine, S. (2013). Patterns of parental care in vertebrates. In: *The evolution of parental care*. Ed: N. J. Royle. Oxford Academic Publishing. Oxford, UK.

Bayer, M., Fischer, J., Kremerskothen, J., Ossendorf, E., Matanis, T., Konczal, M., Weide, T., and Barnekow, A. (2005). Identification and characterization of Iporin as a novel interaction partner for rab 1. *BMC Cell Biology*, **6,** 15.

Breer, H. (2003). Olfactory receptors: Molecular basis for recognition and discrimination of odors. *Analytical and Bioanalytical Chemistry*, **377,** 427-433.

Bridges, R. S. (2015). Neuroendocrine regulation of maternal behavior. *Frontiers in Neuroendocrinology*, **36**, 178–196.

Bukhari, S. A., Saul, M. C., James, N., Bensky, M. K., Stein, L. R., Trapp, R., and Bell, A. M. (2019). Neurogenomic insights into paternal care and its relation to territorial aggression. *Nature Communications*, **10,** 4437.

Caino, M. C., Seo, J. H., Wang, Y., Rivadeneira, D. B., Gabrilovich, D. I., Kim, E. T., Weeraratna, A. T., Languino, L. R., and Altieri, D. C. (2017). Syntaphilin controls a mitochondrial rheostat for proliferation-motility decisions in cancer. *Journal of Clinical Investigation*, **127,** 3755-3769.

Canada Department of Fisheries and Oceans Canada. (2004). Canada Department of Fisheries and Oceans animal-user training template: 4.0 blood sampling of finfish.

Carlton, E. D., Cooper, C. L., and Demas, G. E. (2014). Metabolic stressors and signals differentially affect energy allocation between reproduction and immune function. *General and Comparative Endocrinology*, **208,** 21-29.

Champagne, F. A., and Curley, J. P. (2013). Genetics and epigenetics of parental care. In N.J. Royle, and P.T. Smiseth (Eds), *The Evolution of Parental Care.* Oxford Academic.

Chang, D. T. W., and Reynolds, I. J. (2006). Mitochondrial trafficking and morphology in healthy and injured neurons. *Progress in Neurobiology*, **80,** 241-268.

Churchman, E. K. L., Hain, T. J. A., Knapp, R., and Neff, B. D. (2023). Parental care behaviour in response to perceived paternity is not mediated by 11-ketotestosterone in bluegill sunfish. *General and Comparative Endocrinology*, **343,** 114367.

Clutton-Brock, T. H. (1991). *The Evolution of Parental Care*. Princeton University Press.

Colledge, M., and Scott, J. D. (1999). AKAPs: From structure to function. *Trends in Cell Biology*, **9,** 216-221.

Cook, B. W. (2010). Anticoagulation management. *Seminars in Interventional Radiology*, **27,** 360-367.

Cunha, A. A. P., Partridge, C. G., Knapp, R., and Neff, B. D. (2019). Androgen and prolactin manipulation induces changes in aggressive and nurturing behavior in a fish with male parental care. *Hormones and Behavior*, **116,** 104582.

Demas, G., Greives, T., Chester, E., and French, S. (2012). The energetics of immunity: mechanisms mediating trade-offs in ecoimmunology. In G.E. Demas, and R.I. Nelson (Eds), *Ecoimmunology*. Oxford University Press.

Dent, L., and Lutterschmidt, W. I. (2003). Comparative thermal physiology of two sympatric sunfishes (Centrarchidae: Perciformes) with a discussion of microhabitat utilization. *Journal of Thermal Biology*, 28, 67-74.

Dietrich, M. A., Dietrich, G. J., Mostek, A., and Ciereszko, A. (2016). Motility of carp spermatozoa is associated with profound changes in the sperm proteome. *Journal of Proteomics*, **138,** 124-135.

Dobolyi, A., Grattan, D. R., and Stolzenberg, D. S. (2014). Preoptic inputs and mechanisms that regulate maternal responsiveness. *Journal of Neuroendocrinology*, **26,** 627-640.

Duclot, F., Liu, Y., Saland, S. K., Wang, Z., and Kabbaj, M. (2022). Transcriptomic analysis of paternal behaviors in prairie voles. *BMC Genomics*, **23,** 679.

Dulac, C., O'Connell, L. A., and Wu, Z. (2014). Neural control of maternal and paternal behaviors. *Science*, **345,** 765-770.

Fanjul, L. F., Marrero, I., González, J., Quintana, J., Santana, P., Estévez, F., Mato, J. M., and Ruiz de Galaretta, C. M. (1993). Does oligosaccharide-phosphatidylinositol (glycosyl-phosphatidylinositol) hydrolysis mediate prolactin signal transduction in granulosa cells? *European Journal of Biochemistry*, **216,** 747-755.

Fedorka, K. M. (2014). Reproductive and immune system interactions in the context of life history and sexual selection theory. In D. Malagoli, and E. Ottaviani (Eds) *Eco-immunology*. Springer.

Frank, S. A. (2002). *Immunology and Evolution of Infectious Disease*. Princeton University Press.

Ganser, C., Monadjem, A., McCleery, R. A., Ndlela, T., and Wisely, S. M. (2020). Is it best on the nest? Effects of avian life-history on haemosporidian parasitism. *International Journal for Parasitology: Parasites and Wildlife*, **13,** 62–71.

Garner, S. R., and Neff, B. D. (2013). Alternative male reproductive tactics drive asymmetrical hybridization between sunfishes (*Lepomis* spp.). *Biology Letters*, **9,** 20130658.

Genovese, F., Reisert, J., and Kefalov, V. J. (2021). Sensory transduction in photoreceptors and olfactory sensory neurons: common features and distinct characteristics. *In Frontiers in Cellular Neuroscience*, **15,** 761416.

Gitay-Goren, H., Lindenbaum, E. S., and Kraiem, Z. (1989). Prolactin inhibits hCG-stimulated steroidogenesis and cAMP accumulation, possibly by increasing phosphodiesterase activity, in rat granulosa cell cultures. *Molecular and Cellular Endocrinology*, **61,** 69-76.

Gross, M. R. (1982). Sneakers, satellites and parentals: polymorphic mating strategies in North American sunfishes. *Ethology*, **60,** 1–26.

Gross, M. R., and Shine, R. (1981). Parental care and mode of fertilization in ectothermic vertebrates. *Evolution*, **35,** 775-793.

Guil, S., Long, J. C., and Cáceres, J. F. (2006). hnRNP A1 relocalization to the stress granules reflects a role in the stress response. *Molecular and Cellular Biology*, **26**, 5744-5758.

Keravis, T., and Lugnier, C. (2012). Cyclic nucleotide phosphodiesterase (PDE) isozymes as targets of the intracellular signalling network: Benefits of PDE inhibitors in various diseases and perspectives for future therapeutic developments. *British Journal of Pharmacology*, **165,** 1288-1305.

Klug, H., and Bonsall, M. B. (2014). What are the benefits of parental care? The importance of parental effects on developmental rate. *Ecology and Evolution*, **4,** 2330–2351.

Kohl, J., and Dulac, C. (2018). Neural control of parental behaviors. *Current Opinion in Neurobiology*, **49,** 116-122.

Kumari, R., Fazekas, E. A., Morvai, B., Udvari, E. B., Dóra, F., Zachar, G., Székely, T., Pogány, Á., and Dobolyi, Á. (2022). Transcriptomics of parental care in the hypothalamic-septal region of female zebra finch brain. *International Journal of Molecular Sciences*, **23,** 2518.

Lin, J. W., Wyszynski, M., Madhavan, R., Sealock, R., Kim, J. U., and Sheng, M. (1998). Yotiao, a novel protein of neuromuscular junction and brain that interacts with specific splice variants of NMDA receptor subunit NR1. *Journal of Neuroscience*, **18,** 2017-2027.

Lin, M. Y., Cheng, X. T., Tammineni, P., Xie, Y., Zhou, B., Cai, Q., and Sheng, Z. H. (2017). Releasing syntaphilin removes stressed mitochondria from axons independent of mitophagy under pathophysiological conditions. *Neuron*, **94,** 595-610.

Lynch, K. S., O'Connell, L. A., Louder, M. I. M., Balakrishnan, C. N., and Fischer, E. K. (2019). Understanding the loss of maternal care in avian brood parasites using preoptic area transcriptome comparisons in brood parasitic and non-parasitic blackbirds. *G3: Genes, Genomes, Genetics*, **9,** 1075–1084.

Magnadóttir, B. (2006). Innate immunity of fish (overview). *Fish and Shellfish Immunology*, **20**, 137-151.

Mochida, K., Matsubara, T., Kudo, H., Andoh, T., Ueda, H., Adachi, S., and Yamauchi, K. (2002). Molecular cloning and immunohistochemical localization of ubiquitin C-terminal hydrolase expressed in testis of a teleost, the Nile tilapia, *Oreochromis niloticus*. *Journal of Experimental Zoology*, **293,** 368-383.

Neff, B. D. (2001). Genetic paternity analysis and breeding success in bluegill sunfish (*Lepomis macrochirus). Journal of Heredity*, **92**, 111-119.

Neff, B. D. (2003). Decisions about parental care in response to perceived paternity. *Nature*, **422,** 716–718.

Neff, B. D., and Gross, M. R. (2001). Dynamic adjustment of parental care in response to perceived paternity. *Proceedings of the Royal Society B: Biological Sciences*, **268,** 1559–1565.

Neff, B. D., and Sherman, P. W. (2003). Nestling recognition via direct cues by parental male bluegill sunfish (*Lepomis macrochirus*). *Animal Cognition*, **6,** 87–92.

Nikolaev, V. O., Gambaryan, S., Engelhardt, S., Walter, U., and Lohse, M. J. (2005). Real-time monitoring of the PDE2 activity of live cells: Hormone-stimulated cAMP hydrolysis is faster than hormone-stimulated cAMP synthesis. *Journal of Biological Chemistry*, **280,** 1716-1719.

Prashar, A., Schnettger, L., Bernard, E. M., and Gutierrez, M. G. (2017). Rab GTPases in immunity and inflammation. *Frontiers in Cellular and Infection Microbiology*, **7,** 00435.

Reynolds, J. D., Goodwin, N. B., and Freckleton, R. P. (2002). Evolutionary transitions in parental care and live bearing in vertebrates. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **357**, 269-281.

Roche, P. A., and Furuta, K. (2015). The ins and outs of MHC class II-mediated antigen processing and presentation. *Nature Reviews Immunology*, **15,** 203-216.

Rodgers, C. M. C., Neff, B. D., and Knapp, R. (2012). Effects of exogenous testosterone on parental care behaviours in male bluegill sunfish (*Lepomis macrochirus*). *Ethology*, **118,** 636–643.

Rstudio Team. (2015). *R Studio: Integrated Development for R.*

Sadek, M. S., Cachorro, E., El-Armouche, A., and Kämmerer, S. (2020). Therapeutic implications for PDE2 and cGMP/CAMP mediated crosstalk in cardiovascular diseases. *International Journal of Molecular Sciences*, **21,** 7462.

Shors, T. J., and Mathew, P. R. (1998). NMDA receptor antagonism in the lateral/basolateral but not central nucleus of the amygdala prevents the induction of facilitated learning in response to stress. *Learning and Memory*, **5**, 220-230.

Stiver, K. A., and Alonzo, S. H. (2009). Parental and mating effort: Is there necessarily a trade-off? *Ethology*, **115,** 1101-1126.

Sun, J., Shang, X., Tian, Y., Zhao, W., He, Y., Chen, K., Cheng, H., and Zhou, R. (2008). Ubiquitin C-terminal hydrolase-L1 (Uch-L1) correlates with gonadal transformation in the rice field eel. *FEBS Journal*, **275,** 242-249.

Uribe, C., Folch, H., Enriquez, R., and Moran, G. (2011). Innate and adaptive immunity in teleost fish: A review. *Veterinarni Medicina*, **56**, 486-503.

Vallet, S. D., Berthollier, C., and Ricard-Blum, S. (2022). The glycosaminoglycan interactome 2.0. *American Journal of Physiology - Cell Physiology*, **322**, C1271-1278.

Westneat, D. F., and Sherman, P. W. (1993). Parentage and the evolution of parental behavior. *Behavioral Ecology*, **4,** 66–77.

Westphal, R. S., Tavalin, S. J., Lin, J. W., Alto, N. M., Fraser, I. D. C., Langeberg, L. K., Sheng, M., and Scott, J. D. (1999). Regulation of NMDA receptors by an associated phosphatase-kinase signaling complex. *Science*, **285,** 93-96.

Wu, Z., Autry, A. E., Bergan, J. F., Watabe-Uchida, M., and Dulac, C. G. (2014). Galanin neurons in the medial preoptic area govern parental behaviour. *Nature*, **509,** 325-330.

Yang, Q., Zhang, X., Lu, Z., Huang, R., Tran, N. T., Wu, J., Yang, F., Ge, H., Zhong, C., Sun, Q., Zhou, C., and Lin, Q. (2021). Transcriptome and Metabolome Analyses of Sea Cucumbers *Apostichopus japonicus* in Southern China During the Summer Aestivation Period. *Journal of Ocean University of China*, **20**, 198-212.

Zaccolo, M., Zerio, A., and Lobo, M. J. (2021). Subcellular organization of the camp signaling pathway. *Pharmacological Reviews*, **73,** 278-309.

Zhang, G. W., Shen, L., Tao, C., Jung, A. H., Peng, B., Li, Z., Zhang, L. I., and Tao, H. W. (2021). Medial preoptic area antagonistically mediates stress-induced anxiety and parental behavior. *Nature Neuroscience*, **24,** 516-528.

Chapter 5

5 Hormonal and behavioural dynamics of parental care in bluegill (*Lepomis macrochirus*) x pumpkinseed (*Lepomis gibbosus*) hybrid sunfish

This chapter has been prepared as a manuscript for submission.

5.1 Introduction

Alternative reproductive tactics are an important aspect of reproductive behaviour across taxa (Gross, 1996). In several species of sunfish (*Lepomis* spp.), parental males prioritize growth for several years prior to sexual maturation, after which point they use a parental tactic and establish territories, construct nests in colonies, spawn with females and then provide sole care for their brood (Gross, 1982). Smaller "cuckolder" males mature earlier and fertilize eggs by intruding on spawning events in a parental male's nest by initially employing a sneaking tactic ("sneakers") where they hide in vegetation around nests and dart into the nest to fertilize eggs. As they get larger, cuckolders switch tactics and instead use female mimicry ("mimics"), whereby they hover around the nest, then enter the nest while the parental male is spawning with a female by acting as though they too are spawning with the parental male and instead fertilize eggs.

Bluegill (*Lepomis macrochirus*) and pumpkinseed (*Lepomis gibbosus*) sunfish parental males typically sire an average 75-78% of the larvae in their nest in Lake Opinicon (Garner & Neff, 2013). In bluegill, the remaining 25% of larvae are sired by bluegill cuckolders, while in pumpkinseed only 9% are sired by pumpkinseed cuckolders, and the remaining 13% are sired by bluegill cuckolders. When bluegill cuckolders fertilize pumpkinseed nests, they produce hybrid sunfish (Garner & Neff, 2013; Konkle & Philipp, 1992). Hybrid females occasionally spawn in pumpkinseed nests and may backcross with either bluegill or pumpkinseed males (Garner & Neff, 2013). Hybrid males, however, typically have inferior reproductive characteristics and sire <10% of the larvae in their nest (Immler et al., 2011). In the absence of competition, hybrid sperm is fertile, but they are outcompeted by the sperm of either parental species (Immler et al., 2011).

Paternal males can improve their fitness by preferentially investing in care for their own genetic offspring or abandoning broods in which they have sired few young and gain a selective advantage of indiscriminately paternal males (Westneat & Sherman, 1993). Thus, parental investment theory predicts male parental effort to be related to the proportion of the brood that the male has sired (Trivers, 1972). Given that cuckolders provide no care to their offspring in either bluegill or pumpkinseed, it is beneficial for parental males to adaptively adjust their parental care in response to paternity. In pumpkinseed, nest defense during egg care is positively correlated with paternity, however the mechanisms through which the males assess paternity are not known (Rios-Cardenas & Webster, 2005). Bluegill parental males can discriminate between offspring they sire and those of cuckolder males either by (1) interpreting the presence of sneakers around their nest during spawning day as an uncertainty of paternity, or (2) directly assessing olfactory cues released by the larvae to determine paternity. This paternity assessment impacts nurturing and defensive care dynamics during egg and larvae care as bluegill parental males lower the quality of their parental care in response to both perceptions of low paternity (Churchman et al., 2023; Neff, 2003; Neff & Gross, 2001). Hybrids sire very few offspring in their nest yet provide parental care regardless. One potential explanation for this behaviour is a failure to assess paternity, in which case high levels of parental care is their default phenotype. Alternately, there may be a breakdown or upregulation of an endocrine or genomic pathway controlling parental care. Thus, hybrids provide an opportunity to isolate and assess potential mechanisms driving parental care independent of paternity.

Given the importance of behavioural endocrinology to parental care, research into the mechanisms underlying bluegill parental care is typically endocrine focused. As with most taxa, androgens have been proven to increase aggressive nest defense behaviours in bluegill (Cunha et al., 2019; Rodgers et al., 2012). Exogenous administrations of prolactin, colloquially referred to as the hormone of paternity (Schradin & Anzenberger, 1999), have been proven to increase nurturing behaviours in bluegill. More recently, we have proven endogenous circulating concentrations of prolactin correlate with nurturing care in bluegill and are associated with adjustments in care in response to uncertainty of paternity (Chapter 3). It is possible both prolactin and 11-ketotestosterone, the primary androgen in fish (Borg,

1994), regulate changes in parental care in response to paternity and high levels of one or both hormones may drive parental care regardless of paternity in hybrids.

Genomic mechanisms underpinning changes in parental investment in response to paternity have not yet been characterized. However, molecular studies exploring gene expression during parental care suggest there are neurogenomic profiles associated with birds and fish providing parental care and those that do not (Bukhari et al., 2019; Lynch et al., 2019; Partridge et al., 2016). Given the distinct genotypes associated with parental care providing males, it is possible there is a role for genomic regulation of adaptive adjustments in response to paternity. Similarly, in a prior transcriptomic study, bluegill with lower perceived paternity have increased expression of transcripts associated with energy transport in the brain and decreased expression of transcripts associated with immune function (Churchman, unpublished data). Molecular mechanisms regulating parental care have not been established in pumpkinseed, nor hybrids.

The objectives of this study were to:

1. Establish 11-ketotestosterone and prolactin concentration profiles over the parental care period for bluegill, pumpkinseed, and hybrid sunfish, and to compare circulating concentrations of each hormone while fish provide care for eggs.

2. Characterize and compare parental care behaviours across species.

3. Determine if hybrid and pumpkinseed sunfish adjust parental care in response to egg manipulation within the nest, and what mechanisms underlie their behavioural response.

I hypothesize that (1) hybrids will maintain difference circulating concentrations of both hormones compared to bluegill and pumpkinseed; (2) parental care behaviour will differ between species; (3) hybrids will not adjust their level of care in response to nest manipulation in contrast to bluegill and pumpkinseed; and (4) 11-ketotestosterone, prolactin, and gene expression will influence parental care in all species. To that end, I predict higher concentrations of both hormones to be present in hybrids throughout the care period when compared to bluegill to facilitate the persistence of parental care regardless of

paternity. Similarly, I predict there should be no difference in hybrid parental care behaviours in response to egg manipulation within the nest. To test this, I compared hybrids to their paternal and maternal lineages, bluegill, and pumpkinseed sunfish respectively. I established a prolactin and 11-ketotestosterone profile during the parental care period for both bluegill and hybrids. I also subjected parental male bluegill, hybrids, and pumpkinseed to a direct paternity manipulation where paternity in the nest was altered by swapping eggs between nests, and then measured differences in circulating concentrations of 11-ketotestosterone, prolactin, and parental care behaviour.

5.2 Methods

5.2.1 Species and study site

I studied a population of sunfish in Lake Opinicon (44°34′N, 76°19′W), Ontario, Canada. This lake has been a study site for bluegill sunfish since the 1970s (Gross, 1982) and more recently hybrid sunfish within the last decade. In Lake Opinicon, sunfish breed from May to July. Pumpkinseed sunfish start in early May and breed to early July, while bluegill and hybrids begin breeding later in the May/early June until July. During this time, parental males enter the littoral zone. Pumpkinseed build nests separately or in small groups, whereas bluegill build colonies of up to 150 males, some of which include hybrids (Gross, 1982). The stages of sunfish reproductive behaviour typically include (1) “staging” where parental males begin to gather at the nesting site, followed by nest building and then (2) a ”loose to nest” stage where males begin to station and hover at their nest, (3) “tight to nest” where males sit lower and stay closer to the nest, then (4) spawning with females and finally (5) parental care lasts approximately 7 days, with eggs hatching around day 3.

From 2018 to 2021, swimmers equipped with snorkelling gear monitored sunfish reproductive behaviour along a 2 km stretch of the littoral zone of the lake. When swimmers noted males becoming “tight to nest”, I tagged each nest with an individually-numbered ceramic tile.

My first objective was to determine 11-ketotestosterone (11KT) and prolactin concentration profiles over the parental care period. To do this, I took blood samples from random bluegill and hybrid males nesting within colonies on each day of the parental care

period from spawning day (0) to day 5 (the final day of parental care for these colonies – the stage and day of care was confirmed by a single swimmer). I caught each male with a dip net and brought the male to a nearby boat, where I measured their total body length and extracted a 200 µL whole-blood sample via caudal venipuncture (66 ± 21 seconds from time of netting to blood collection; mean ± SD). I allowed each male approximately 2 minutes to recover in a dark water bucket on the boat, and then returned him to his nest. I stored blood samples on ice until transport back to shore for processing. The Animal Care Committee at Western University (ACC) approved all procedures performed in this study (AUP #2010-214 and #2018-084) and fish were collected under scientific collection permits from the Ontario Ministry of Natural Resources and Forestry. Logistically it was not possible to collect enough pumpkinseed to establish a profile as well as conduct behavioural observation and experimental manipulations as described below.

My second objective was to compare parental care behaviour across species and to better understanding the care hybrid and pumpkinseed sunfish provide for their broods. To do so, in 2021, I set up GoPro cameras at each parental male's nest in the morning of day 2 and recorded nurturing behaviour for 30 minutes from 09:00-12:00 EST. I quantified four nurturing behaviours: (1) rim circling, (2) caudal fanning, (3) pectoral fanning, and (4) nest pecking (see Côté & Gross, 1993; Gross & MacMillan, 1981; Neff, 2003). I compared these nurturing behaviours to a historical dataset of bluegill nurturing behaviour collected in 2019 in a parallel experiment (control males from Churchman et al., 2023). I collected nest defense behaviour data while caring for eggs (day 2) and for larvae (day 4). To do so I presented a natural egg predator (pumpkinseed sunfish for bluegill and hybrid nests, and a bluegill for pumpkinseed nests) in a transparent plastic bag on the border of the parental male's nest between 14:00-17:00 EST. I then recorded the parental male's defense behaviour for 1 minute using a GoPro camera (Hero 5 and 6, San Mateo, California, USA). I quantified three defensive behaviours from these videos: (1) lateral display, (2) opercular flare, and (3) bite.

My third objective was to determine if hybrid sunfish adjust parental care in response to paternity as do bluegill. The bluegill used in this part of the study were a subset of those used for a parallel study on the endocrine regulation of parental care in response to

perceived paternity collected in 2018, 2020, and 2021. (see Churchman et al., 2023; and Churchman et al., manuscript submitted for publication). All hybrids and pumpkinseed were collected in 2021. A single swimmer mapped the colonies of bluegill-hybrid sunfish, and individual pumpkinseed nests, immediately after spawning and assigned each nest an egg score from 1-5. This score is based on the percentage of egg coverage within the nest and is correlated with the actual number of eggs and larvae in the nest (Claussen, 1991). Prior to collection, males were exposed to one of two treatments (1) control; or (2) egg manipulation. For the egg manipulation group, I swapped about one-half of each male's eggs between two nests the day after spawning (day 1 of parental care; Figure 5.1). I performed a sham swap in the nests of males assigned to the control treatment, in which I removed and then returned one-half of the eggs to the original nest. This mimicked the disturbance of the egg swap, but not the reduction in paternity. I collected nest defense behaviour data during each period of care: once while providing care for eggs (day 2) and once while providing care for larvae (day 4). To do so, I conducted the standardized aggression test using the natural egg predator as described above. I took blood samples twice to determine 11KT concentrations: once while they were caring for eggs (day 1) while we performed the swap treatment, and once the day after the eggs hatched and males were providing care for larvae (day 4) immediately after the predator presentation. Due to differences in blood sampling timing, the 11KT concentration of 2018 males caring for larvae are not included in this analysis. Furthermore, given that nurturing behaviour is only present during egg care, and prolactin concentrations correlate with nurturing care, I only quantified prolactin concentrations during the egg care period.

In 2021, on the final day of parental care (day 5), I returned to the nesting sites to collect tissue for molecular analysis. To do so, we euthanized individuals using clove oil and immediately dissected and stored whole brains in RNAlater (ThermoFisher Scientific; Mississauga, ON, Canada). The total amount of time required for fish capture, euthanasia, dissection, and brain storage in RNAlater was under 10 minutes. I stored the brains in RNAlater at 4 ˚C for 24 hours. Brains were then frozen at -20 ˚C until transport on dry ice to the University of Western Ontario where they were stored at -80 ˚C.

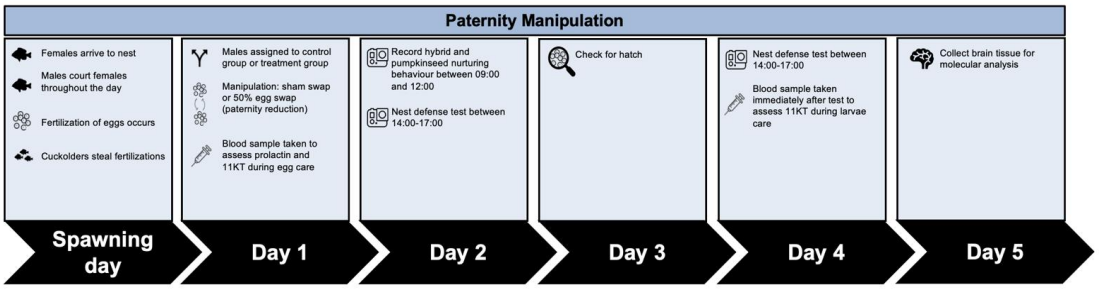

Figure 5.1. Visual representation of methodology.

5.2.2 Hormone analysis

I extracted plasma from each blood sample within 8 hours of collection and stored it at -20˚C until transportation back to the University of Western Ontario. I then used enzyme-linked immunosorbent assay (ELISA) kits to determine the concentration of 11-ketotestosterone (11KT; CAT# 582751Cayman Chemical, Ann Arbor, MI) and prolactin (Fish Prolactin ELISA Kit MBS700669; MyBioSource Inc., San Diego, CA). I ran each 11KT sample in triplicate, and each prolactin sample in duplicate to balance the high plasma demands of the assay with the welfare of the animal during sampling. The 11KT kit was designed in ng/mL while the ELISA kit standard was designed in µIU/mL, where 1µIU/mL is approximately equal to a concentration of 0.047ng/mL (MyBioSource Inc, personal communication).

5.2.3 Quantitative PCR (qPCR) analysis

I extracted total RNA from the whole brain of all 17 sunfish brains collected in 2021 using a standard Trizol extraction protocol (Life Technologies, Carlsbad, CA). We removed residual genomic DNA from all samples using a Qiagen RNeasy Cleanup kit. I quantified total RNA using a Nanodrop One Microvolume UV-Vis Spectrophotometer (ThermoFisher Scientific; Mississauga, ON, Canada). The average A260/A280 of all RNA samples was 2.10 ± 0.04 (mean ± SD). I synthesized cDNA using qScript cDNA Supermix (Quantabio; Beverly, MA, USA) from 820ng RNA per sample and stored the cDNA at -20˚C. Due to a low RNA concentration of one sample, I was unable to synthesize cDNA from 1000ng RNA and standardized all samples to the maximum possible concentration of 820ng RNA per reaction.

I selected and designed primers for eight candidate genes identified by bluegill transcriptome analysis for the same paternity manipulation (Table 5.1): syntaphilin (*SNPH*), A-kinase anchor protein-9 (*AKAP9*), N-acetylglucosamine-6-sulfatase (GNS), Ubiquitin carboxyl-terminal hydrolase-40 (*USP40*), Iporin (*RUSC2*), heterogenous nuclear ribonucleoprotein A1 (*ROA1*), phosphodiesterase 2A (*PDE2A*), and class 2 histocompatibility antigen, F10 alpha chain (*HA1F*). I tested the primers against a pooled cDNA sample in a gradient PCR, then by gel electrophoresis to determine the accuracy of the melt temperature and primer product size. I validated primer efficiency using a 1:10 serial dilution curve and a single melt curve. All primers, including the reference gene, displayed an efficiency between 90-110%.

Table 5.1. Primer sequences for qPCR

Primer	5'-3'	Sequence
Syntaphilin (SNPH)	Forward	TCTCTCTGTCGTCCCAATCT
	Reverse	TCCCTTCCTCTTCACACTCT
A-kinase anchor protein 9 (AKAP9)	Forward	CCTACAGAGCAAAGAGCAAGAG
	Reverse	GCTGTAGGGTGAGGTGTTTAAG
N-acetylglucosamine-6-sulfatase (GNS)	Forward	TTCCACCCACTGCTGTTATG
	Reverse	GAGGTTTGACTGGTGCTCTT
Ubiquitin Carboxyl-terminal hydrolase-40 (USP40)	Forward	ACTCTTCTCCTCGCTCTCTAC
	Reverse	GTTTGTCTGGCTGGTGTTTG
Iporin (RUSC2)	Forward	GTTAGCAGACCGGCAATGA
	Reverse	CTTGTCCATCGTCACCTTCTC
Heterogenous nuclear ribonucleoprotein A1 (ROA1)	Forward	CCCTCACAAAGCAGGAAAT
	Reverse	CTCTGCCACCCTGATTAAAG
Phosphodiesterase 2A (PDE2A)	Forward	CAGCCATCCTTCCCATTC
	Reverse	CGGTTGCTCTCTGTCTAAAG
Class 2 histocompatibility antigen, F10 alpha chain (HA1F)	Forward	CACGATGTTCTGGAGGAAAG
	Reverse	GTCAACACTCATCTGGAAGG
Elongation factor 1-beta (EF1B)	Forward	CGTGGGTTACGGCATCAAGA
	Reverse	GATCTTGTTGAAAGCGGCGA

I used POWER SybrGreen Master Mix (ThermoFisher Scientific; Mississauga, ON, Canada) to amplify cDNA according to the manufacturer's instructions with thermal-cycling conditions configured to an initial 10-minute activation at 95 ˚C, then 40 cycles

of 15 seconds at 95 ˚C to denature and 60 seconds at 60 ˚C to anneal and extend. Each 12µL qPCR reaction included 2µL cDNA, and 0.4µM forward and 0.4µM reverse primer. I ran each sample in triplicate using a QuantStudio 3 real-time PCR cycler (Applied Biosystems; Waltham, MA, USA).

I normalized transcript abundance to a reference gene (EF1) and two internal calibrator samples consisting of pooled cDNA. We used internal calibrator samples in each run to control for any inconsistencies between qPCR runs. We calculated the relative transcript abundance of egg-swap samples against control samples using the comparative CT method ($2^{-\Delta\Delta CT}$). I then normalized the values by log transforming the relative abundance to obtain logFC values.

5.2.4 Statistical Analyses

I performed all statistical analyses using R Studio. Degrees of freedom were calculated by R as the adjusted values. All tests were conducted as two-tailed. Given low sample size due to abandonment, several comparisons were assessed qualitatively rather than statistically and are defined below.

I assessed the effect of species on body length and egg score over all four years with ANOVAs with body length or egg score as the dependent variable and species as the independent variable. I only collected abandonment data for all three species in 2021, and so we used a Chi-Squared test to compare abandonment between species within the one year.

I qualitatively compared the 11KT and prolactin concentration profiles between bluegill and hybrid sunfish. I used an ANOVA to determine the effect of species on circulating prolactin while caring for eggs where prolactin was the response variable and species as the independent variable. I compared the effect of species on circulating 11KT using an ANOVA during egg care with 11KT as the response variable, and the interaction between species and treatment as the independent variable. I compared significant differences using Tukey's HSD test. I qualitatively compared circulating 11KT between species while caring for larvae, considering only the control males. I

used spearman's correlations to determine if (1) prolactin concentrations were associated with parental care behaviours during egg care in hybrids and pumpkinseed; (2) 11KT concentrations were associated with parental care behaviours during egg care in all three species; (3) 11KT concentrations were associated with parental care behaviours during larvae care in bluegill and hybrids, considering only the control males. Pumpkinseed were assessed qualitatively.

I compared four nurturing behaviours between species using ANOVAs to determine the effect of species while caring for eggs: rim circling, caudal fanning, pectoral fanning, and nest pecking. I used ANOVAs to determine the effect of species on three defensive behaviours while caring for eggs: lateral display, opercular flaring, and biting. In all models, individual behaviours were considered the response variable with species as the independent variable. Significant differences were assessed using Tukey's HSD test. I qualitatively assessed the effect of species on these defensive behaviours while caring for larvae by comparing the behaviours of control fish from all three species.

Due to logistical constraints restricting sample size in hybrids and pumpkinseed, I qualitatively compared the effect of experimental paternity treatment on defensive parental care behaviour and 11KT within each species. Lastly, I used Welch's two sample t-tests to compare differences in candidate gene expression between treatments for hybrids and bluegill. I qualitatively compared pumpkinseed expression between treatments.

5.3 Results

5.3.1 Species differences

There was a significant effect of species on body length ($F_{2,170} = 54.15$, $p < 0.001$). Hybrids (215 ± 8 mm; mean ± SD) were significantly longer than bluegill (196 ± 10 mm; $p < 0.001$), but not pumpkinseeds (20 ± 9 mm; $p = 0.36$). Pumpkinseed were also significantly longer than bluegill ($p < 0.001$). There was also a significant effect of species on egg score ($F_{2,165} = 19.87$, $p < 0.001$). Bluegill (3 ± 1; mean + SD) had

significantly higher egg scores than hybrids (1 ± 1; $p < 0.001$), and pumpkinseed (2 ± 1; $p = 0.003$). There was no significant difference between pumpkinseeds and hybrids ($p = 0.68$). In 2021, abandonment did not differ by treatment per species (X^2 (2, 19) = 4.10, $p = 0.13$).

I collected blood samples from a total of 143 males during the parental care period for the prolactin and 11KT profiles ($N_{bluegill} = 130$, $N_{hybrid} = 72$). I quantified prolactin from 69 males ($N_{bluegill} = 32$, $N_{hybrid} = 37$). When comparing prolactin concentrations over the course of the parental care period, hybrids had higher prolactin concentrations than bluegill (Figure 5.2A). I quantified 11KT from 138 males ($N_{bluegill} = 98$, $N_{hybrid} = 40$). When comparing 11KT concentrations over the course of parental care period between bluegill and hybrids (Figure 5.2B), bluegill maintained higher concentrations of 11KT than hybrids.

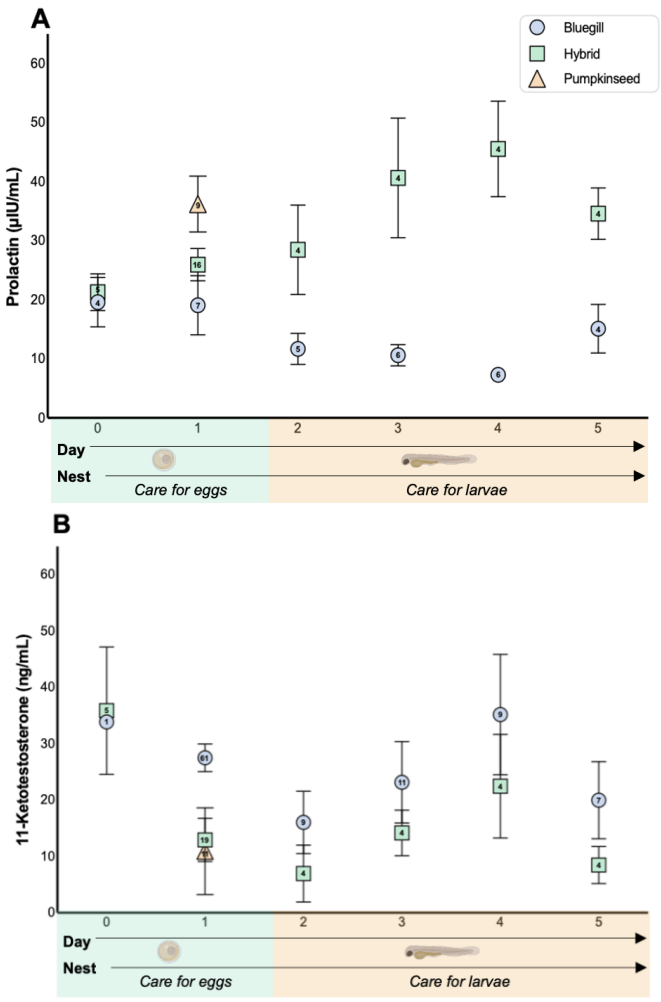

Figure 5.2. Circulating (A) prolactin and (B) 11-ketotestosterone concentrations over the parental care period in bluegill, pumpkinseed, and hybrid sunfish. Day denotes the number of days after spawning (day 0), while the shading denotes the period of parental care with either eggs or larvae present in the nest. Data points represent the mean prolactin concentration per day and error bars denote the standard error of the mean.

There was a significant effect of species on prolactin concentrations during egg care ($F_{2,27}$ = 3.73, p = 0.04; Figure 5.2) There was a significant difference between bluegill and pumpkinseed prolactin concentrations (p = 0.04) but not bluegill and hybrid (p = 0.54) nor hybrid and pumpkinseed (p = 0.14). There was no significant effect of species on 11KT concentrations during egg care ($F_{2,136}$ = 0.91, p = 0.40). Bluegill 11KT concentrations were

higher than hybrid and pumpkinseed during larvae care, while hybrid and pumpkinseed concentrations appeared similar.

There was no significant correlation between prolactin concentrations and hybrid nor pumpkinseed lateral display, opercular flare, bite, rim circling, or nest pecking behaviours (Table 5.2A). There was no correlation between 11KT concentrations and bluegill lateral display, opercular flare, or biting behaviours during egg or larvae care. There were no significant correlations between any hybrid parental care behaviours and 11KT during egg care. There was a significant correlation between hybrid opercular flaring and 11KT while caring for larvae (r = -0.95, p = 0.01), however there were no other significant correlations between hybrid parental care behaviours while caring for larvae and 11KT. There were no significant correlations among any pumpkinseed parental care behaviours and 11KT while caring for eggs. There did not appear to be any relationship among pumpkinseed behaviours and 11KT while caring for larvae.

Table 5.2. Correlation between individual parental care behaviours and circulating 11-ketotestosterone and prolactin concentrations in bluegill, hybrids, and pumpkinseed sunfish during (A) egg and (B) larvae care.

A.

Hormone	Species	Lateral Display	Opercular Flare	Bite	Rim Circle	Nest Peck
11KT	Bluegill	0.13	0.10	0.12		
	Hybrid	-0.02	0.33	0.42	-0.01	0.01
	Pumpkinseed	-0.40	0.07	0.00	0.59	0.42
Prolactin	Hybrid	-0.35	0.20	0.16	-0.14	0.45
	Pumpkinseed	0.31	-0.36	-0.60	0.30	0.10

B.

Hormone	Species	Lateral Display	Opercular Flare	Bite
11KT	Bluegill	0.13	0.11	0.33
	Hybrid	0.45	**-0.95**	-0.80
	Pumpkinseed	N/A	N/A	NEG

***** Correlations were only calculated for control bluegill and hybrid males caring for larvae. Significant correlations are bolded ($\alpha = 0.05$). Pumpkinseed associations while caring for larvae were assessed quantitatively.

There was a significant effect of species on rim circling behaviour ($F_{2,45} = 12.17$, $p = < 0.001$; Figure 5.3) where bluegill performed significantly more rim circling than hybrids ($p < 0.001$) and pumpkinseed ($p = 0.002$). There was no significant difference between pumpkinseed and hybrids ($p = 0.89$). There was a significant effect of species on caudal fanning behaviour ($F_{2,45} = 4.24$, $p = 0.02$) and pectoral fanning behaviour ($F_{2,45} = 5.65$, $p = 0.006$). With the exception of a single hybrid fish, neither hybrids nor pumpkinseed performed any caudal fanning. There was a significant effect of species on nest pecking behaviour ($F_{2,45} = 4.29$, $p = 0.02$), where pumpkinseed performed significantly more pecking than hybrids ($p = 0.01$). There was no significant difference between pumpkinseed and bluegill ($p = 0.11$) nor bluegill and hybrids ($p = 0.34$).

There was a significant effect of species on the number of opercular flares performed while caring for eggs ($F_{2,88} = 3.96$, $p = 0.02$; Figure 5.4), but not lateral displays ($F_{2,88} = 1.93$, $p = 0.15$) nor bites ($F_{2,88} = 1.16$, $p = 0.32$). While caring for eggs, pumpkinseed performed significantly more opercular flares than bluegill ($p = 0.02$). There was no significant difference between hybrids and bluegill ($p = 0.96$) nor pumpkinseed and hybrids ($p = 0.053$). While caring for larvae, there did not appear to be a difference between species in the number of lateral displays performed. Pumpkinseed appeared to perform more opercular flare than bluegill and hybrids, which seemed to flare at similar rates. While caring for larvae, bluegill appeared to bite more hybrids, which in turn bit more than pumpkinseed.

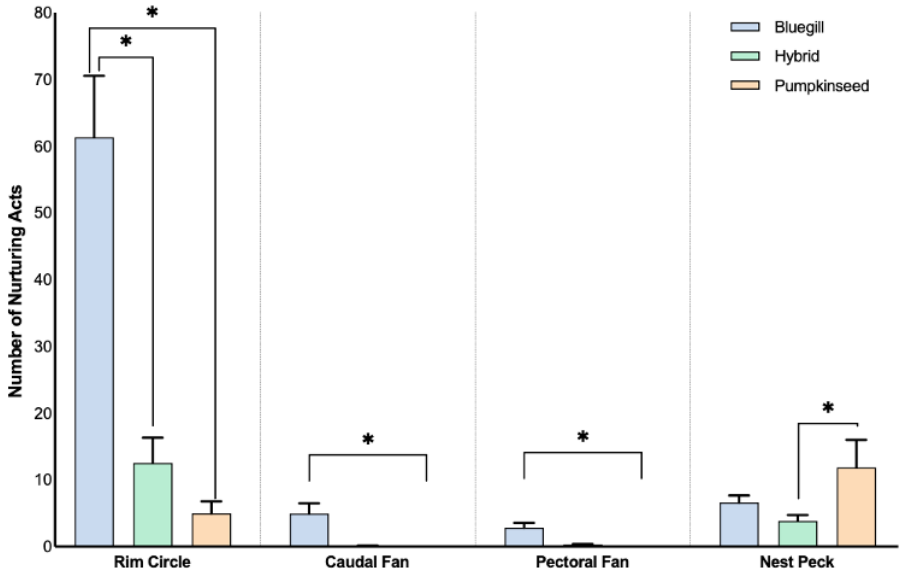

Figure 5.3. Comparison of nurturing behaviours among species of bluegill (BG), hybrid (HY), and pumpkinseed (PS) sunfish while caring for eggs. Significant differences between species per Tukey's HSD test are marked by an asterisk (α = 0.05).

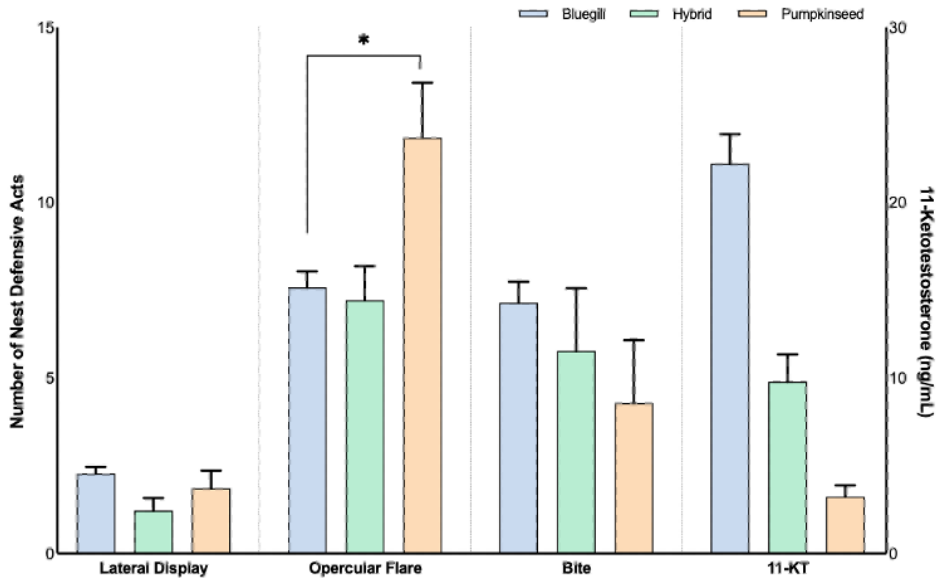

Figure 5.4. Comparison of nest defensive behaviours and 11-ketotestosterone concentration (ng/mL) among species of bluegill, hybrid, and pumpkinseed sunfish while caring for eggs. Significant differences between species per Tukey's HSD test are marked by an asterisk (α = 0.05).

Table 5.3. Mean number of nest defensive behaviours and 11-ketotestosterone concentration (ng/mL) for bluegill, hybrid, and pumpkinseed sunfish while caring for eggs and larvae in each treatment. Males exposed to the experimental swap treatment had 50% of their eggs swapped with another nest. Males in the control treatment were subjected to a sham swap.

		Lateral Display		Opercular Flare		Bite		11KT (ng/mL)	
		Egg	Larvae	Egg	Larvae	Egg	Larvae	Egg	Larvae
Bluegill	Control	2.0 ± 1.4 *N* = 33	1.7 ± 1.7 *N* = 33	7.9 ± 3.9 *N* = 33	6.4 ± 3.9 *N* = 33	6.7 ± 4.7 *N* = 33	12.6 ± 5.6 *N* = 33	23.0 ± 18.5 *N* = 54	26.1 ± 18.7 *N* = 22
	Swap	2.5 ± 1.8 *N* = 42	2.2 ± 1.8 *N* = 41	7.4 ± 4.1 *N* = 42	7.4 ± 4.3 *N* = 41	7.5 ± 5.6 *N* = 42	8.5 ± 4.9 *N* = 41	21.5 ± 16.9 *N* = 56	43.1 ± 32.3 *N* = 27
Hybrid	Control	1.0 ± 1.3 *N* = 6	0.6 ± 0.9 *N* = 5	7.7 ± 1.5 *N* = 6	8.8 ± 3.8 *N* = 5	6.2 ± 5.5 *N* = 6	8.8 ± 5.3 *N* = 5	12.0 ± 6.6 *N* = 10	10.6 ± 11.2 *N* = 6
	Swap	1.7 ± 0.6 *N* = 3	1.0 ± 1.0 *N* = 3	6.3 ± 5.1 *N* = 3	10 ± 1.0 *N* = 3	5.0 ± 6.1 *N* = 3	1.7 ± 1.5 *N* = 3	6.7 ± 5.2 *N* = 7	36.3 ± 32.1 *N* = 6
Pumpkinseed	Control	2.0 ± 1.2 *N* = 5	2.3 ± 1.0 *N* = 4	13.2 ± 4.0 *N* = 5	12 ± 5.6 *N* = 4	5.4 ± 5.2 *N* = 5	3.8 ± 5.8 *N* = 5	2.1 ± 1.1 *N* = 5	10.1 ± 2.3 *N* = 4
	Swap	1.5 ± 2.1 *N* = 2	1.0 ± 0.0 *N* = 2	8.5 ± 2.1 *N* = 2	8.5 ± 6.4 *N* = 2	1.5 ± 2.1 *N* = 2	2.5 ± 2.1 *N* = 2	4.3 ± 2.3 *N* = 5	22.4 ± 14.0 *N* = 2

*Variation in sample size between behaviour and 11KT is due to video quality and plasma coagulation.

5.3.2 Response to paternity manipulation

There did not appear to be any differences between treatments in the circulating 11KT, number of lateral displays, opercular flares, or bites of hybrids caring for eggs. While hybrids cared for larvae, there appeared to be no difference in lateral displays, and opercular flares. Hybrids appeared to have higher circulating 11KT in males assigned to the swap treatment. Similarly, hybrids in the swap treatment appeared to bite more frequently than those in the control treatment. There did not appear to be any differences between treatments in defensive behaviours or 11KT while pumpkinseed cared for eggs.

There was no difference in gene expression between treatments in bluegill for any candidate genes. In hybrids, there was a significant difference in USP40 expression ($t_{7.9}$ = 3.49, p = 0.01), where males in the swap treatment expressed it at lower levels than males in the control treatment. There were no other significant differences in hybrid candidate gene expression between treatments. Pumpkinseed did not express SNPH. Otherwise, there did not appear to be any differences in candidate gene expression between treatments in pumpkinseed.

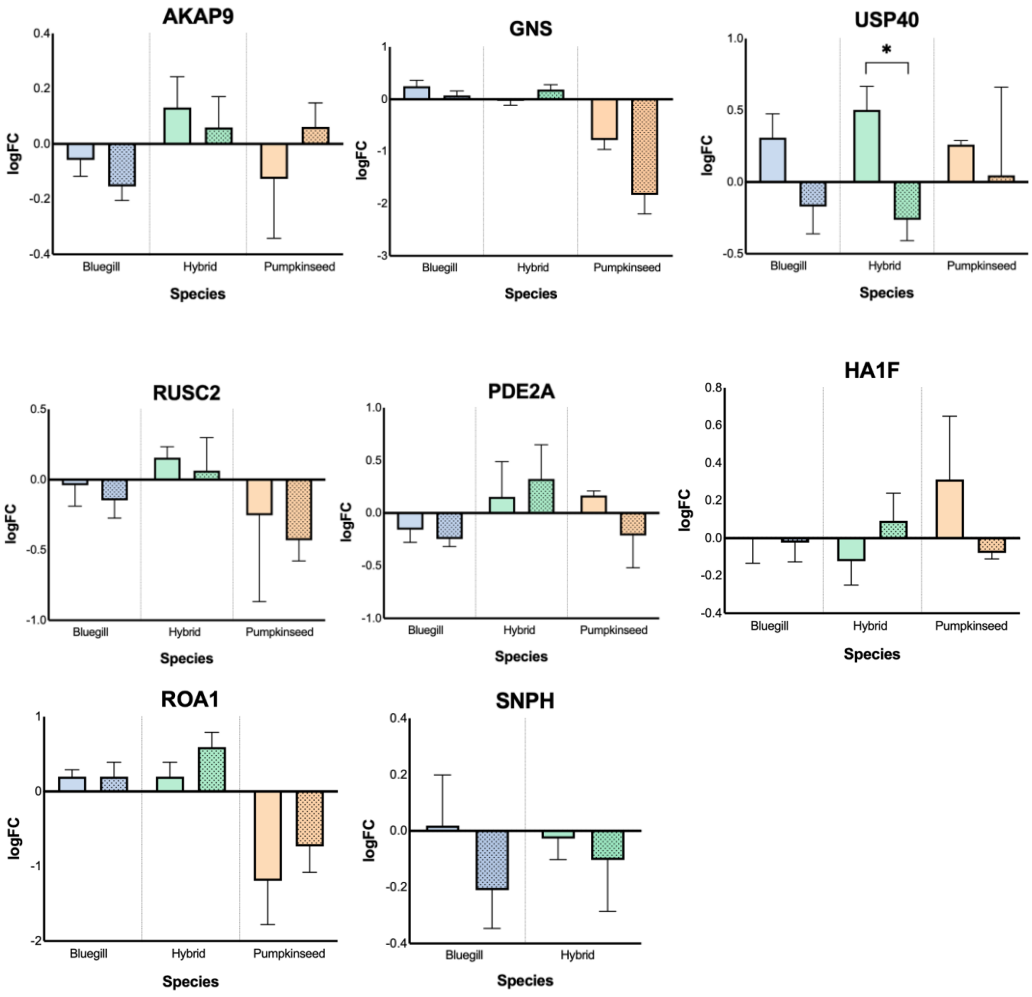

Figure 5.5. Comparison of candidate gene expression (logFC) between treatments in bluegill, pumpkinseed, and hybrid sunfish. Bluegill are denoted by blue, hybrids in green, and pumpkinseed in orange. Solid bars represent the control treatment, and dotted bars represent the swap treatment.

Table 5.4. Differences in candidate gene expression in response to perceived paternity in bluegill and hybrid sunfish. Significant differences are bolded (α = 0.05).

Candidate Gene	Bluegill	Hybrid
Syntaphilin (*SNPH*)	$t_{12.2} = 1.02$, $p = 0.33$	$t_{5.3} = 0.39$, $p = 0.71$
A-kinase anchor protein 9 (*AKAP9*)	$t_{13.2} = 1.23$, $p = 0.24$	$t_{8} = 0.46$, $p = 0.66$
N-acetylglucosamine-6-sulfatase (*GNS*)	$t_{12} = 1.24$, $p = 0.24$	$t_{7.9} = -1.74$, $p = 0.12$
Ubiquitin Carboxyl-terminal hydrolase-40 (*USP40*)	$t_{14.9} = 1.90$, $p = 0.08$	**$t_{7.9} = 3.49$, $p = 0.01$**
Iporin (*RUSC2*)	$t_{13.2} = 0.54$, $p = 0.59$	$t_{4.8} = 0.38$, $p = 0.72$
Phosphodiesterase 2A (*PDE2A*)	$t_{10.2} = 0.63$, $p = 0.54$	$t_{8} = -0.36$, $p = 0.73$
Class 2 histocompatibility antigen, F10 alpha chain (*HA1F*)	$t_{12.5} = 0.13$, $p = 0.90$	$t_{7.8} = -1.10$, $p = 0.30$
Heterogenous nuclear ribonucleoprotein A1 (*ROA1*)	$t_{12.7} = -0.00$, $p = 1.00$	$t_{8} = -1.42$, $p = 0.19$

5.4 Discussion

In line with parental investment theory, bluegill sunfish adjust the quality of their parental care in response to perceived paternity (Churchman *et al*. 2023; Neff 2003). In contrast, hybrid sunfish, resulting from crossbreeding bluegill and pumpkinseed sunfish, are functionally sterile but still exhibit nest care behaviours. Overall, the objective of this study was to characterize circulating prolactin and 11-ketotestosterone (11KT) concentrations during parental care in hybrid sunfish, and to compare the quality of their care and their response to egg manipulation with that of bluegill and pumpkinseed. In particular, I sought to determine what mechanisms drive similarities or differences in their parental care behaviour and in response to perceived paternity.

As was expected, hybrids exhibited a distinct hormonal profile compared to bluegill during parental care. Specifically, bluegill's prolactin levels typically remained below 20 µIU/mL and decreased from spawning day to day 4 of parental care, with a slight increase on day 5. In contrast, hybrids, displayed a nearly inverse pattern, maintaining prolactin levels above 20 µIU/mL, which rose from spawning day to day 4, with a slight decrease in prolactin on day 5. This pattern in bluegill parallels the prolactin trends observed in mammals and birds, where initial high concentrations of prolactin generally diminish over the parental care period (Dixson & George, 1982; Garcia et al., 1996; Storey et al., 2000). However, the absence of a clear link between prolactin levels and parental behaviour in hybrids suggests a more complex mechanism at play in regulating their parental care. In a study on ring doves (*Streptopelia risoria*), more experienced females showed greater sensitivity to prolactin (Wang & Buntin,

1999). This might help explain the observed hormonal differences between bluegill and hybrids. I determined bluegill had higher egg scores than hybrids and suggest if females lay eggs more frequently in bluegill nests compared to hybrids, it is possible that bluegill parental males are also more "experienced" parents than hybrids and are more sensitive to prolactin. Additionally, research on rock doves (*Columba livia*) found that prolactin administration led to increased testes size and higher gonadotropin receptor expression (Farrar et al., 2022). Thus, it is plausible that the elevated prolactin levels in hybrids functions to boost spermatogenesis. Given that hybrid sperm is outcompeted in the presence of any other sperm, high prolactin levels may promote the production of high quantities of sperm rather than high quality (Immler et al., 2011). This trait could also be inherited from their maternal lineage. While comprehensive prolactin profiling for pumpkinseed was not feasible, initial data showed pumpkinseed having higher prolactin levels that bluegill and hybrids the day after spawning. Consequently, hybrids might be genetically predisposed to maintain higher concentration of prolactin than bluegill, which may functionally serve to induce parental care.

Hybrids and bluegill exhibit similar patterns in their 11KT levels throughout the parental care period. Unlike prolactin, however, hybrids consistently displayed lower levels of 11KT than bluegill during this period. A trade-off between nurturing and defensive behaviour has been established in bluegill (Cunha et al., 2019), and that study also showed that administration of prolactin and 11KT increase nurturing and aggressive behaviours, respectively. While a direct trade-off between prolactin and androgens has not been explicitly established, it is plausible that maintaining high levels of both hormones simultaneously is not feasible, which might explain the lower 11KT levels in hybrids compared to bluegill. Interestingly, in hybrids, 11KT levels were inversely related to the defensive behaviour opercular flaring, a finding that contradicts existing research where increased 11KT levels are linked to heightened defensive actions (Cunha et al., 2019). This suggests that hybrids might regulate their defensive behaviour differently from bluegill. Considering the unknown reason for poor hybrid sperm performance, it is possible that issues with their reproductive organs affect their androgen production. Therefore, I suggest hybrids do not regulate their defensive behaviour via 11KT. Dehydroepiandrosterone (DHEA) is an androgen precursor and prohormone and has been proven to be important in the expression of aggression when gonadal androgen synthesis is low in non-breeding birds and mammals (reviewed in Soma et al., 2015). If hybrids are not

physiologically capable of synthesizing high levels of 11KT, or other androgens, then perhaps DHEA is important in the maintenance of hybrid aggression.

During egg care, hybrid sunfish displayed nest defense behaviours that were similar to both their parental species. The notable difference was observed in pumpkinseed, which showed more frequent opercular flaring compared to bluegill, while hybrids demonstrated flaring at a frequency that was intermediate between the two. In the larvae care phase, bluegill engaged in biting more often than pumpkinseed, with hybrids again showing a behaviour level that was intermediate but not distinctly difference from either parent species. These observations indicate that each species adopts distinct nest defense tactics, with hybrids displaying a combination of behaviours from both bluegill and pumpkinseed.

In contrast to their intermediate defensive behaviour, the nurturing behaviour of hybrids was of markedly poor quality. They exhibited fewer nurturing behaviours than either bluegill or pumpkinseed, often just hovering over their nest without active movement. This suggests that, unlike their defensive behaviour, the nurturing behaviour of hybrids is not a blend of their parent species, but rather an inferior form. This lack of nurturing behaviour in hybrids does not correlate with their prolactin levels. This is notable as high prolactin is almost ubiquitously associated with high levels of nurturing behaviour across taxa (Cunha et al., 2019; Dixson & George, 1982; Smiley, 2019; Storey et al., 2000). It is possible that high prolactin levels initially promote a parental care-giving state while females lay eggs in hybrid nests on spawning day, but does not regulate nurturing behaviours. Additionally, hybrids with elevated 11KT levels tended to exhibit less frequent defensive behaviours. My work highlights the complex nature of hormonal regulation in hybrid parental care and suggest that their parenting behaviour is not a straightforward inheritance from their bluegill and pumpkinseed lineages.

In line with previous findings, bluegill with experimentally reduced paternity exhibited a decreased frequency of biting in response to nest predators compared to their control counterparts (Neff, 2003). Pumpkinseed were less tolerant of experimental manipulation, and so with a low sample size after larvae hatched, it is challenging to draw definitive conclusions. My data suggest a similar level of care between both treatments at both stages of care, similar to the response observed by Rios-Cardenas and Webster (2005). They observed no difference in behaviour in response to high or low perceived paternity. Moreover, prior to hatch, hybrids

in both treatments provided a similar frequency of defensive care. After hatch, they appear to provide relatively similar levels of care between treatments as well. There was a slight decrease in biting after hatch in the swap treatment, however this may be attributed to variance in the data. Therefore, as expected, hybrids appear to continue to provide care for offspring regardless of relatedness. Taken together, it is possible that hybrids do not adjust their level of care in response to paternity as a maternally-conserved trait from pumpkinseed. Pumpkinseed nest solitarily, and thus are perhaps less likely to be cuckolded and have a less active kin recognition mechanism. Hybrids may therefore care for unrelated offspring because they do not recognize them as unrelated.

My genetic analysis suggests that hybrids in both treatments express A-Kinase Anchor Protein 9 (*AKAP9*) at high levels than bluegill and pumpkinseed, while pumpkinseed appear to have lower expression of N-acetylglucosamine-6-sulfatase (*GNS*) than bluegill and hybrids. In addition, pumpkinseed did not express Syntaphilin (*SNPH*). There was no differential expression of any candidate genes between treatments in bluegill, while Ubiquitin Carboxyl-terminal hydrolase-40 (*USP40*) was expressed at higher levels in hybrid controls relative to males exposed to the nest manipulation. Due to the logistical challenge of collecting pumpkinseed after two blood samples, my sample size is very low for our molecular analysis. Differences in pumpkinseed gene expression are most likely associated with sample size, rather than a true biological difference. *AKAP9* was upregulated in hybrids and is associated with stress response (Colledge & Scott, 1999; Lin et al., 1998). Hybrids nest in bluegill colonies that evolved as a form of protection during care (Gross, 1982), and it is possible the stress of nesting within a territorial colony of parental males has resulted in increased expression of *AKAP9* in hybrids. Given the low sample size, it is unlikely that this difference reflects a biological response to treatment. Given the low difference in expression (logFC) between the reference gene and the swap hybrid males, it is more likely that the swap males do no express this at a lower level and is a factor of individual variation rather than an effect of treatment. The expression level in the control treatment males relative to the reference gene is closer to the expression levels of bluegill and pumpkinseed. Therefore, I suggest gene expression level in USP40, along with the other candidate genes, is conserved from bluegill and hybrids.

My research underscores multifaceted nature of parental care in hybrid sunfish, revealing how evolutionary lineage and hormone levels intertwine to regulate parental care behaviour. My findings demonstrate that while hybrids exhibit traits inherited from their bluegill and pumpkinseed lineages, their parental care behaviours and physiological responses, particularly in relation to prolactin and 11KT, are distinct and complex.

5.5 References

Alonso-Alvarez, C., and Velando, A. (2012) Benefits and costs of parental care. In: *The evolution of parental care*. Ed: N. J. Royle. Oxford Academic Publishing. Oxford, UK.

Borg, B. (1994). Androgens in teleost fishes. *Comparative Biochemistry and Physiology*, **109C**, 219–245.

Bukhari, S. A., Saul, M. C., James, N., Bensky, M. K., Stein, L. R., Trapp, R., and Bell, A. M. (2019). Neurogenomic insights into paternal care and its relation to territorial aggression. *Nature Communications*, **10,** 4437.

Churchman, E. K. L., Hain, T. J. A., Knapp, R., and Neff, B. D. (2023). Parental care behaviour in response to perceived paternity is not mediated by 11-ketotestosterone in bluegill sunfish. *General and Comparative Endocrinology*, **343,** 114367.

Claussen, J. (1991). Annual variation in the reproductive activity of a bluegill population: effect of clutch size and temperature. University of Toronto.

Colledge, M., and Scott, J. D. (1999). AKAPs: From structure to function. *Trends in Cell Biology*, **9,** 216-221.

Côté, I. M., and Gross, M. R. (1993). Reduced disease in offspring: A benefit of coloniality in sunfish. *Behavioral Ecology and Sociobiology*, **33,** 269–274.

Cremer, S., Armitage, S. A. O., and Schmid-Hempel, P. (2007). Social Immunity. *Current Biology*, **17**, R693–R702.

Cunha, A. A. P., Partridge, C. G., Knapp, R., and Neff, B. D. (2019). Androgen and prolactin manipulation induces changes in aggressive and nurturing behavior in a fish with male parental care. *Hormones and Behavior*, **116,** 104582.

Dixson, A. F., and George, L. (1982). Prolactin and parental behaviour in a male New World primate. *Nature*, **299,** 551–553.

Farrar, V. S., Flores, L., Viernes, R. C., Ornelas Pereira, L., Mushtari, S., and Calisi, R. M. (2022). Prolactin promotes parental responses and alters reproductive axis gene expression, but not courtship behaviors, in both sexes of a biparental bird. *Hormones and Behavior*, **144**, 105217.

Fefferman, N. H., Traniello, J. F. A., Rosengaus, R. B., and Calleri, D. V. (2007). Disease prevention and resistance in social insects: Modeling the survival consequences of immunity, hygienic behavior, and colony organization. *Behavioral Ecology and Sociobiology*, **61**, 565-577.

Garcia, V., Jouventin, P., and Mauget, R. (1996). Parental care and the prolactin secretion pattern in the King penguin: An endogenously timed mechanism? *Hormones and Behavior*, **30**, 259–265.

Garner, S. R., and Neff, B. D. (2013). Alternative male reproductive tactics drive asymmetrical hybridization between sunfishes (*Lepomis* spp.). *Biology Letters*, **9,** 20130658.

Gross, M. R. (1982). Sneakers, satellites and parentals: polymorphic mating strategies in North American sunfishes. *Ethology*, **60,** 1–26.

Gross, M. R. (1996). Alternative reproductive strategies and tactics: Diversity within sexes. *Trends in Ecology and Evolution*, **11,** 92-98.

Gross, M. R., and MacMillan, A. M. (1981). Predation and the evolution of colonial nesting in bluegill sunfish (*Lepomis macrochirus*). *Behavioral Ecology and Sociobiology*, **8,** 163–174.

Hubbs, C. L., and Hubbs, L. C. (1933). The increased growth, predominant maleness, and apparent infertility of hybrid sunfishes. *Papers of the Michigan Academy of Science, Arts and Letters*, **17**, 613–641.

Immler, S., Hamilton, M. B., Poslusny, N. J., Birkhead, T. R., and Epifanio, J. M. (2011). Post-mating reproductive barriers in two unidirectionally hybridizing sunfish (Centrarchidae: *Lepomis*). *Journal of Evolutionary Biology*, **24,** 111-120.

Konkle, B. R., and Philipp, D. P. (1992). Asymmetric hybridization between two species of sunfishes (*Lepomis*: Centrarchidae). *Molecular Ecology*, **1,** 215-222.

Lin, J. W., Wyszynski, M., Madhavan, R., Sealock, R., Kim, J. U., and Sheng, M. (1998). Yotiao, a novel protein of neuromuscular junction and brain that interacts with specific splice variants of NMDA receptor subunit NR1. *Journal of Neuroscience,* **18,** 2017-2027.

Lynch, K. S., O'Connell, L. A., Louder, M. I. M., Balakrishnan, C. N., and Fischer, E. K. (2019). Understanding the loss of maternal care in avian brood parasites using preoptic area transcriptome comparisons in brood parasitic and non-parasitic blackbirds. *G3: Genes, Genomes, Genetics*, **9,** 1075–1084.

Montgomerie, R. D., and Weatherhead, P. J. (1988). Risks and rewards of nest defence by parent birds. *Quarterly Review of Biology*, **63,** 167-187.

Neff, B. D. (2003). Decisions about parental care in response to perceived paternity. *Nature*, **422,** 716–718.

Neff, B. D., and Gross, M. R. (2001). Dynamic adjustment of parental care in response to perceived paternity. *Proceedings of the Royal Society B: Biological Sciences*, **268,** 1559–1565.

Partridge, C. G., MacManes, M. D., Knapp, R., and Neff, B. D. (2016). Brain transcriptional profiles of male alternative reproductive tactics and females in Bluegill Sunfish. *PLoS ONE,* **11**, e0167509.

Rios-Cardenas, O., and Webster, M. S. (2005). Paternity and paternal effort in the pumpkinseed sunfish. *Behavioral Ecology*, **16,** 914-921.

Rodgers, C. M. C., Neff, B. D., and Knapp, R. (2012). Effects of exogenous testosterone on parental care behaviours in male bluegill sunfish (*Lepomis macrochirus*). *Ethology,* **118,** 636–643.

Schradin, C., and Anzenberger, G. (1999). Prolactin, the hormone of paternity. *News in Physiological Sciences,* **14,** 223–231.

Shykoff, J. A., and Schmid-Hempel, P. (1991). Parasites and the advantage of genetic variability within social insect colonies. *Proceedings of the Royal Society B: Biological Sciences*, **243,** 55-58.

Smiley, K. O. (2019). Prolactin and avian parental care: New insights and unanswered questions. In *Hormones and Behavior*, **111,** 114–130.

Soma, K. K., Rendon, N. M., Boonstra, R., Albers, H. E., and Demas, G. E. (2015). DHEA effects on brain and behavior: Insights from comparative studies of aggression. *Journal of Steroid Biochemistry and Molecular Biology*, **145,** 261-272.

Storey, A. E., Walsh, C. J., Quinton, R. L., and Wynne-Edwards, K. E. (2000). Hormonal correlates of paternal responsiveness in new and expectant fathers. *Evolution and Human Behavior*, **21,** 79–95.

Trivers, R. L. (1972). Parental investment and sexual selection. In B. Campbell (Ed), *Sexual Selection and the Descent of Man: 1871-1971*. Aldine.

Wang, Q., and Buntin, J. D. (1999). The roles of stimuli from young, previous breeding experience, and prolactin in regulating parental behavior in ring doves (*Streptopelia risoria*). *Hormones and Behavior*, **35,** 241-253.

Westneat, D. F., and Sherman, P. W. (1993). Parentage and the evolution of parental behavior. *Behavioral Ecology*, **4,** 66–77.

Yang, Q., Zhang, X., Lu, Z., Huang, R., Tran, N. T., Wu, J., Yang, F., Ge, H., Zhong, C., Sun, Q., Zhou, C., and Lin, Q. (2021). Transcriptome and metabolome analyses of sea cucumbers *Apostichopus japonicus* in Southern China during the summer aestivation period. *Journal of Ocean University of China*, **20**, 198-212.

Chapter 6

6 General Discussion

Parental care has an important role in the development, growth, and eventual success of many species. However, despite the benefits of healthier offspring that are more likely to reproduce and pass on the parent's genes, the care is energetically, physically, and reproductively costly to the parent. Consequently, parents may adjust their care to selectively provide, and invest in, higher quality care to offspring that are related, and therefore more valuable, to them. Several hypotheses have emerged that indicate the endocrine system is critical in parental care regulation, and this may interplay with genomic pathways that influence care. In my thesis, I bridge the gap between what is understood about mechanisms that broadly regulate parental care behaviour, and behavioural responses to paternity. The overall goal of my research was to assess changes in parental care behaviour in response to perceived paternity and to identify potential proximate mechanisms that regulate parental care behaviour. To do this, I worked primarily with bluegill sunfish to analyze potential mechanisms and explored the role of hormones and candidate genes in care-giving behaviour irrespective of paternity in hybrid sunfish. In doing so, my thesis contributes to our understanding of how parental investment behaviour evolved and is maintained in systems with variable parentage.

6.1 Summary of findings

6.1.1 11-Ketotestosterone responds to recognition of paternity but does not regulate parental care behavioural response

The role of androgens in the regulation of male reproductive and aggressive behaviour is a cornerstone of behavioural endocrinology. Increases in androgens correlate positively with aggressive behaviour during the reproductive season (Farner & Wingfield, 1980), and experimental administration can induce aggressive behaviours (Hau et al., 2000; Rodgers et al., 2012). While high concentrations of androgens can suppress nurturing behaviour and are often considered detrimental to parental care (Wingfield et al., 1990), it is important to note that parental care can be exhibited as aggressive behaviour, particularly in the defense of offspring (Møller & Nielsen, 2014). Similarly, offspring defense correlates positively with offspring value (Redondo & Carranza, 1989). In chapter 2, I examined the role of 11-

ketotestosterone (11KT), the primary androgen in fish, in regulating parental care behaviour in response to indirect and direct cues of paternity in bluegill. I show that bluegill with higher paternity within their nest increase aggressive defense behaviour, but this is not correlated with circulating 11KT concentrations. Similarly, I show that bluegill with a higher perception of paternity perform more nurturing behaviour, but that this is also not regulated by 11KT. Interestingly, 11KT was higher in males with experimentally lowered nest paternity, suggesting that these males may be preparing to renest rather than perform higher quality care for lower value offspring. The possibility of males increasing 11KT concentrations as they recognize a brood of low genetic value should be tested further by examining physical and reproductive correlates, as well as the timing of the next nesting attempt. Regardless, my findings suggest that 11KT concentrations respond to paternity but does not regulate the behavioural response to paternity.

6.1.2 Prolactin regulates parental care behaviour and responds to paternity perception

Although the critical role of prolactin in parental care has been proven in mammals and birds via correlations with behaviour and experimental manipulations (Smiley, 2019; Storey et al., 2000), the role of prolactin in fish has not been established. Experimentally administered prolactin has been shown to influence parental care behaviour (Cunha et al., 2019). However, empirical evidence linking endogenous circulating prolactin in fish to parental care behaviour is missing. While both fish and mammalian prolactin can induce a response to increase nurturing behaviour in fish, very little is known about how these doses reflect endogenous levels. In chapter 3, I establish circulating endogenous prolactin is a key hormone in fish parental care and is involved in behavioural responses to perceived paternity.

I quantified circulating prolactin on each day of the parental care period from spawning day to day 6 of care, which is when larvae start to leave the nest. Similarly to mammals and birds, prolactin concentrations were highest while eggs accumulate in the nest and during subsequent care, then lowered after eggs hatched and nurturing care subsided (Dixson & George, 1982; Smiley, 2019; Storey et al., 2000). I also determined that the highest measured concentration was substantially lower than prior manipulation work (Cunha et al., 2019; Páll et al., 2004). One of the challenges of behavioural endocrinology is measuring the concentrations that elicit

response, as animals are often sensitive to very low concentrations of circulating endogenous hormones (Nelson & Kriegsfeld, 2022). My work suggests that bluegill exemplify this concept, and that future work manipulating prolactin in fish should account for natural circulating concentrations to ensure accurate biological responses are captured.

For a hormone to be causally linked to a behaviour, manipulation of a hormone should elicit a response, and circulating concentrations should correlate with the behaviour. Prior work has established that bromocriptine, a prolactin inhibitor, decreases nurturing care in bluegill (Cunha et al., 2019; Kindler et al., 1991). In parallel, prolactin administration increases nurturing care (Cunha et al., 2019). My data provides the foundation for a direct link, as I establish circulating prolactin concentrations correlate positively with nurturing behaviour. Taken together, my work solidifies the role of prolactin in fish parental care.

Moreover, I manipulated an indirect cue of uncertainty of paternity on spawning day by presenting a visual cuckoldry cue to the parental male. Prolactin concentrations were lower in fish with lower perceived paternity, and higher in fish that appeared to have not been cuckolded. Fish with higher perceived paternity also provided more nurturing care. My data suggests that parental males lower their quality of care when it is likely they are caring for a nest with low paternity, and this adjustment may be mediated by prolactin.

While the role of prolactin has been clearly established in mammals and birds, my data provides a foundation that prolactin also regulates fish parental care. Further, parental investment theory predicts that parents invest more in offspring that are of higher fitness value (Trivers, 1982), and my data provides evidence that prolactin may mediate this adjustment in care. Overall, it is clear: prolactin is critical for the maintenance of parental care behaviour and mediates how parents allocate care.

6.1.3 Perceived paternity influences energy transport and immune-related gene expression

The predominant understanding of the regulation of parental care behaviour typically rests within endocrinology. However, parental care behaviour requires changes from a sophisticated suite of physiological systems outside of the endocrine, including neural pathways and gene expression (Ammari et al., 2023; Champagne & Curley, 2013). Prior research in mammals,

birds, and fish have determined that genes involved in metabolism, immune response, transcription, and enriched dopamine pathways are important in the establishment and maintenance of parental care (Bukhari et al., 2019; Kumari et al., 2022; Wu et al., 2014). While many species alter care in response to parentage, very little is known about how this occurs and how gene expression is involved in the adjustment of behaviour. In chapter 4, I show that genes associated with energy transport and immune response vary in response to direct recognition of paternity.

I adjusted direct paternity cues by experimentally lowering paternity within the nest by swapping eggs between parental males and compared the gene expression of these males to males with 'sham' swapped nests with eggs that had been disturbed but not replaced via transcriptome analysis. I determined that males with higher paternity expressed one transcript at a lower level than males with experimentally manipulated paternity: syntaphilin (*SNPH*). *SNPH* is associated with mitochondrial movement in the brain (Lin et al., 2017). Higher expression of *SNPH* has been linked to damaged mitochondrial transport systems under stressful conditions (Chang & Reynolds, 2006; Lin et al., 2017). It is therefore possible that providing care to offspring that are less related to a parental male may be a stressor, and this gene is upregulated in males with lower paternity as energy transport to areas of the brain that regulate parental care behaviour are disrupted. Contrastingly, males with higher paternity expressed one gene at a higher level than males with reduced paternity: class I histocompatibility antigen, F10 alpha chain (*HA1F*). *HA1F* is associated with pathogen resistance in Atlantic salmon (*Salmo salar*; Grimholt et al., 2003). Parental males with higher paternity engage more frequently in parental care and are consequently more frequently exposed to pathogens and fungi within their nest (Neff & Sherman, 2003). I propose that *HA1F* is upregulated in males that are providing higher quality care, as an adaptive immune response. Taken together with my behavioural results in chapter 2, bluegill males with higher paternity are more likely to provide higher quality care, which may result in an upregulation of *HA1F*, while the disruption of mitochondrial movement via high *SNPH* expression may impact parts of the brain that involved in parental care, and account for the lower quality of care provided by males with reduced paternity. My work highlights the intricate system of immune, metabolic, and behavioural pathways that influence parental care behaviour, and how these may be involved in response to paternity.

6.1.4 Hybrid Parental Care: Defining care behaviour and potential underlying mechanisms

When bluegill cuckolders fertilize eggs in pumpkinseed nests, they produce hybrid offspring. While little is known about the alternative life histories of hybrids, when they mature and enter a reproductive season larger males defend nests in bluegill colonies and care for offspring. Contrary to parental investment theory that proposes males allocate care accordingly to relatedness (Trivers, 1972), hybrids are considered sterile (Garner & Neff, 2013) yet continue to provide care for, and invest in, offspring. Hybrids therefore provide a unique opportunity to examine mechanisms underlying adjustments in care by determining if these mechanisms are capable of driving parental care regardless of paternity.

To test this, I conducted the same direct manipulation of swapping eggs between nests. However, I hypothesized care behaviour to be similar between control and swap treated males if they provide care regardless of paternity. Further, I expected to see a difference in hormone profile during the parental care period relative to bluegill and pumpkinseed, but not between treatments. Similarly, I expected the expression of candidate genes that were associated with differences in perceived paternity to be similar between treatments. In chapter 5, I show that hybrids exhibited distinct hormonal profiles from their bluegill and pumpkinseed parental lineages, with notably higher prolactin levels that did not correspond to increased nurturing behavior, suggesting a complex regulation of parental care. These hybrids also had lower concentrations of 11KT during the parental care period. However, prolactin concentrations did not correlate with individual nurturing behaviours, and is unlikely to be the primary proximate explanation for the level of nurturing care hybrids provide. Similarly, 1KT concentrations did not correlate with defensive behaviours while caring for eggs, nor most lateral displays or bites while caring for larvae. Interestingly, in control males caring for larvae, 11KT was negatively correlated with the frequency of opercular flares. Thus, there is likely a mechanism other than 11KT regulating aggression in hybrids. Behaviorally, while hybrids showed a blend of defensive behaviors from both parent species, their nurturing behavior was significantly inferior, indicating a complex, non-linear inheritance of parenting traits. In terms of response to paternity cues, bluegill altered their behavior with reduced paternity by reducing care quality, a response not observed in pumpkinseed. The lack of behavioural response in pumpkinseed is consistent with prior work that manipulated perceived paternity (Rios-

Cardenas & Webster, 2005) but may be attributed to low sample size. Hybrids did not appear to respond to nest manipulation, which was in line with my hypothesis. Logistic constraints associated with nest abandonment impacted my sample size for molecular analysis. While it is possible that differential expression reflects biological relevance but may be attributed to individual variation rather than variation among species or between treatments. Preliminarily, hybrids appeared to express higher levels of stress-related gene A-Kinase Anchor Protein 9 (*AKAP9*; Colledge & Scott, 1999) compared to bluegill and pumpkinseed, while pumpkinseed appeared to express lower levels of immune response-associated gene N-acetylglucosamine-6-sulfatase (*GNS*; Yang et al., 2021) than bluegill and hybrids. It is possible hybrids are under stress while expending resources and providing care for offspring they cannot derive genetic benefits from. Differences in pumpkinseed *GNS* expression are most likely associated with low sample size. Moreover, hybrids in the control treatment group appeared to express Ubiquitin Carboxyl-terminal hydrolase-40 (*USP40*) at higher levels than males in the swap treatment. However, *USP40* expression by hybrids in the swap treatment was not substantially lower than expression of the reference gene, which suggests my results are more likely due to variation between the individuals I analyzed rather than a pattern associated with a response to the swap treatment.

Overall, my study suggests there are complex interactions between environmental factors and inherited traits underlying hybrid parental care. While hybrids do not adjust their parental care behaviour in response to egg manipulation, they have unique hormonal profiles compared to their parental lineages. My findings suggest hybrids are subject to distinct pressures, possible due to their sterility and stress associated with the demands of parental care. While I provide insights into the dynamics of hybrid parental care, further investigation is needed to fully understand hybrid behaviour and the proximate mechanisms that regulate it.

6.2 Contributions to the field and future directions

My thesis has advanced our understanding of parental investment by determining how key hormones like prolactin and 11KT are involved in the adjustment of care behaviour in response to paternity, as well as identifying candidate genes that may have a role in this regulation.

6.2.1 Shifting perspective: the regulation of care by prolactin highlights the importance of fish in the evolution of parental care

Prolactin belongs to a gene family comprising of prolactin, growth hormone, and somatolactin, and is a key regulatory molecule across vertebrates (Dobolyi et al., 2020; Freeman et al., 2000). The presence of prolactin in ancient chordate species before the first vertebrate genome duplication is a marker of its long evolutionary history. It is well known that the whole genome duplication occurred 3 times during vertebrate evolution: (1) at the transition from chordates to vertebrates, (2) at the transition from agnathans to gnathostomes, and (3) after divergence of the teleost lineage (Dobolyi et al., 2020). Changes in prolactin sequences throughout vertebrate genome evolution align with major adaptive events, including freshwater adaptations and the development of lactation in mammals, suggesting its role in these pivotal evolutionary transitions (Dobolyi et al., 2020). By establishing the role of prolactin in fish parental care, my research implies that the origins of parental care may trace back to fish as the basal vertebrate species. This may represent a paradigm shift in the study of parental care, which is traditionally taxonomically biased towards birds and mammals (Stahlschmidt, 2011). Fishes, with their remarkable diversity in parental care strategies – accounting for variations in care-giving parents, allocation between parents, and methods of care (Gross & Sargent, 1985) – should be recognized as a critical taxon for understanding the evolution of this behaviour.

In addition to confirming prolactin's regulatory role in fish parental care, my work explores its involvement in care allocation in response to paternity cues. This finding suggests a more complex role for prolactin, potentially influencing how parental care systems are shaped and evolve in response to various ecological and evolutionary pressures. While prolactin is known to initiate parental care behaviour (Smiley, 2019), the observed variation in care in response to different paternity cues indicates that prolactin may also dictate the nature and extent of care provided. Therefore, my thesis posits a proximate mechanism for the evolutionary shifts and adaptations in parental care systems, highlighting how changes in prolactin levels could mediate these transitions to optimize offspring care for maximum fitness benefits. Future research should, thus, consider prolactin as a proximate mechanism to understand the evolution and loss of parental care systems, offering a more comprehensive view of the evolutionary dynamics of parental care across the animal kingdom.

Interestingly, although hybrids maintain high concentrations of prolactin throughout the parental care period, this hormone does not correlate with nurturing behaviour. Despite a low likelihood of genetic relatedness to the offspring, hybrids display parental care behaviours, suggesting an underlying hormonal mechanism operating independently of direct genetic dies. Initially, I hypothesized that elevated prolactin levels would drive and regulate sustained parental care in these species. However, while hybrids do exhibit high prolactin levels, these do not seem to influence nurturing behaviours as observed in bluegill. While prolactin is known to induce caregiving behaviour in mammals (Bridges, 2020; Smiley et al., 2022) and birds (Angelier et al., 2016; Smiley, 2019), the specific role of prolactin in inducing and regulating parental care in fish remains less understood. My findings suggest that while high prolactin concentrations may induce a caregiving state in hybrids, they may not regulate specific nurturing behaviours. It is plausible a larger sample size might support the expectation that prolactin should regulate nurturing behaviours. However, I suggest it is more likely that hybrids, due to their inferior nurturing care compared to their parental lineages, may not engage in sufficient functional care to be regulated by prolactin. Although I did not assess the success of hybrid nests, it is conceivable that, as I hypothesized, high prolactin levels during the reproductive season trigger a caregiving state in hybrids, but that they may lack the ability or learning to effectively care for eggs. Therefore, in this context of naïve parental behaviour, prolactin's role in regulating nurturing behaviour would be irrelevant. This suggests a more complex relationship between hormonal regulation and caregiving behaviour in species with alternative reproductive strategies and hybridization, warranting further exploration to understand the nuances of hormonal influence in parental care and investment.

6.2.2 Rethinking androgens: 11KT has a complex role in the regulation of parental care in systems with alternative reproductive strategies

The prevailing view in behavioural endocrinology posits androgens as key drivers of male reproduction and aggressive behaviour. This theory is supported by evidence suggesting a direct correlation between higher androgen levels and increased (Farner & Wingfield, 1980; Nelson & Kriegsfeld, 2022). However, my findings present a complex scenario. While high androgen levels have been traditionally viewed as counterproductive to parental care (Wingfield et al., 1990), my findings indicate that 11KT does not directly impact parental

behaviour, particularly in relation to paternity. Instead, my research suggests that although androgens are essential for parental behaviours in California mice (*Peromyscus californicus;* Trainor & Marler, 2001), and increased 11KT levels can induce aggression in bluegill (Cunha et al., 2019; Rodgers et al., 2013), the role of endogenous circulating 11KT may have a more nuanced role. My findings support the notion that 11KT is more involved in regulating reproductive behaviour and the distribution of resources across successive broods, rather than directly facilitating parental behaviour.

In bluegill, I observed that increased perceived paternity does not correspond with elevated 11KT levels, which contrasts both heightened bluegill defensive behaviours in response to elevated paternity, and established androgen theory. My results suggest that 11KT may regulate parental male preparation for future reproductive opportunities, particularly in response to reduced paternity. This challenges the binary perspective on androgens, suggesting instead that they have a more complex role within the realm of parental care, particularly in the context of aggressive, defensive parental behaviour. Additionally, while the difference in androgenic regulation between alternative reproductive morphs has been explored, it primarily explores the differences between morphs (Knapp, 2003) rather than how androgen regulation adapts in response to the presence of alternative male morphs. I noted an increase in aggressive behaviour in parental males with higher perceived paternity, but significantly lower 11KT levels than males with reduced paternity and did not correlate with behaviour. This contradicts the expected positive relationship between androgens and aggression (Nelson & Kriegsfeld, 2022). It raises the possibility that in species with alternative reproductive morphs, 11KT might have a broader function, namely in the regulation of reproductive investment across different broods, as opposed to primarily regulating behaviour with a brood. Initially, I hypothesized that 11KT levels would be a determinant in behaviour in response to perceived paternity, with the expectation that aggressive parental care would be positively associated with circulating 11KT.

Instead, perhaps 11KT is positively correlated with the reallocation of resources that may be spent on defending a nest towards stimulating reproductive characteristics such as spermatogenesis in preparation for future reproductive opportunities and offspring. Indeed, increased 11KT levels can induce complete spermatogenesis in fish (Schulz, 2003), and these

levels tend to rise prior to spawning or renesting (Cargnelli & Gross, 1996; Magee et al., 2006; Specker & Kishida, 2000). This highlights the need for future studies to examine the role of 11KT across species, and to consider the temporal regulation of 11KT in species with alternative morphs outside of the comparison between morphs.

Studying hybrid sunfish, which provide care for eggs regardless of paternity, offers valuable insights into the role of androgens in parental care. When measuring 11KT over the parental care period, I observed that hybrids have lower circulating 11KT than bluegill for the duration of the period despite maintaining similar levels of aggressive behaviour. This implies a potential morphological or physiological impairment in the gonads that leads to compromised fertility and a reduction in androgen production. Interestingly, this deviates from the typical positive correlation between aggression and androgens documented in most species (Nelson & Kriegsfeld, 2022). This suggests an alternative mechanism regulates aggression in hybrid parental care. Given the importance of non-gonadally dependent dehydroepiandrosterone in the maintenance of aggression in non-breeding birds and mammals (Soma et al., 2015), it is possible this prohormone is involved in the hybrid regulation of aggression.

Overall, the results of my thesis pertaining to 11KT underscores the complexity of hormonal regulation and emphasizes the need for a broader understanding of androgen functions beyond their conventional roles. My results challenge existing theories in behavioural endocrinology and indicate future research should explore the diverse role of androgens across species and reproductive contexts. The variations in 11KT levels observed in my study, along with their potential implications in parental care behavior, suggest that we should reconsider the binary perspective on androgens. This calls for a more comprehensive approach that considers the ecological and evolutionary contexts in which these hormones operate.

6.2.3 Beyond hormones: stress and immune-response related genes in parental care dynamics

Kin recognition, which starts with sensory detection and neural processing, suggests that the brain is likely involved, at least in part in regulating behavioural adjustments in response to paternity. My results provide evidence that gene expression in bluegill varies in response to paternity and support the notion that parental care is not driven only by endocrine factors, but

interplays with genetic changes. Using transcriptome analysis, I found that caring for less genetically related offspring may act as a stressor and affect brain energy transport and management. Several transcripts expressed in higher levels by males in the swap treatment are associated with physiological stress response. This provides evidence that expending resources on unrelated offspring may stress parental males. Moreover, when stress is high, Syntaphilin (*SNPH*) has been proven to disrupt energy transport to the brain by interrupting mitochondrial movement (Caino et al., 2017; Lin et al., 2017). If energy transport to areas of the brain associated with care behaviour is impacted, then this expression pattern may act as a potential mechanism underlying changes in care in response to paternity. In addition, my results provide evidence that males who provide higher quality care for the offspring have evolved mechanisms to support better adaptive immune function, likely as an adaptive response to the increased exposure to pathogens and fungi in the nesting environment. Such a correlation indicates a potential evolutionary adaptation that highlights the role of the immune system in paternal investment strategies.

I used qPCR to analyze several candidate genes I initially identified as differentially expressed using RNAseq. Transcript expression patterns suggest hybrids express *AKAP9* at higher levels than bluegill and pumpkinseed, and that pumpkinseed express *GNS* at lower levels than bluegill and hybrids. However, due to logistical challenges of sampling hybrids and pumpkinseed, my sample size was too low to definitively determine if these differences are species-specific or simply differences in individual expression. Similarly, while *USP40* was differentially expressed between control and swap-treated hybrid males, it is challenging to determine if this is a biological response to manipulation, or individual variation. If the expression patterns I observed in *AKAP9*, *GNS*, and *USP40* are accurately reflective of biological differences, this suggests stress and immune response are key factors in parental care. My preliminary evidence suggests that a more exhaustive comparative analysis among hybrids, bluegill, and pumpkinseed could yield important information about how hybrids provide care. It may also help to understand how the strategies optimized to balance the costs and benefits of parental care are inherited from different care systems and enhance our understanding of the evolutionary trade-offs and adaptations in parental care mechanisms.

In my qPCR analysis of bluegill, I found no significant genetic differences between treatments within each species. While it is possible this is due to environment differences, or low sample size, it is possible that the differentially expressed transcripts I initially identified as part of my transcriptome analysis may have been due to inflated log2FC values. This may stem from the majority of samples in the swap treatment that did not express these genes. Subsequent secondary analyses, implemented to correct for this inflation suggests the majority of initially identified differentially expressed transcripts were falsely identified. Rather than differentially expressed between treatments, these transcripts more likely reflect individual variation. This highlights the inherent challenges in molecular ecology, particularly when integrating techniques like RNAseq to address ecological questions. These methods have become popular over the past decade but often grapple with discerning meaningful data amidst the 'noise' inherent in highly variable wild populations. Gene expression within individuals can fluctuate based on factors like time of day, developmental or life history stage, and can vary between tissues, cells, or even within cells of the same tissue type (Aubin-Horth & Renn, 2009; Birnbaum et al., 2003; Francesconi & Lehner, 2014; Whitehead & Crawford, 2006). In non-model organisms, the challenge intensifies in distinguishing genes that vary between individuals from those that genuinely vary due to treatment effects.

I chose to sequence the entire bluegill brain to capture the most comprehensive picture of changes in response to perceived paternity possible. In this sense, my goal was to determine if genes were differentially expressed during (A) the sensing of offspring relatedness, (B) the processing of behaviour adjustments, and (C) changes in behaviour itself. However, sequencing the entire brain can introduce an overwhelming amount of "noise", making it challenging to identify genes that are expressed in response to treatment. Additionally, RNAseq is biased towards highly transcribed genes (Łabaj et al., 2011), potentially obscuring more subtle gene expression patterns. The combination of individual variability, the sheer volume of transcripts expressed in the whole brain, and the potential biases in RNAseq likely contributed to the absence of differential expression between treatments observed in my study. Future research in this area may benefit from comparatively sequencing sections of the brain, and using methods that precisely isolate tissues from those regions to effective capture nuanced patterns of expression.

Lastly, my analysis identified several unannotated transcripts. While RNAseq can identify previously uncharacterized transcripts, non-model organisms like bluegill often lack extensive annotation and functional data (Pavey et al., 2012). Despite comparing my sequences to homologs in closely related species, it is possible that there are functional differences that are not accounted for. This presented a challenge in my research, raising the possibility that transcripts I found to be differentially expressed are key in the adjustment of behaviour in response to perceived paternity, yet remain unidentified. As such, the expansion of databases and repositories for gene annotation will be crucial in future research efforts to deepen our understanding of gene expression in species like bluegill.

6.2.4 Paternity beyond genetics: hybrids provide care regardless of paternity

One objective of my thesis was to determine why hybrid males provide care for offspring they are unlikely to have sired. However, to conclusively ascertain whether their response to experimental manipulation differs from that of bluegill, it was necessary to evaluate their reaction using the same experimental protocol. Given their effective sterility and likely zero paternity within nests (Garner & Neff, 2013; Immler et al., 2011), I hypothesized that hybrids would exhibit similar levels of care between treatments. My thesis built upon previous research by Neff (2003) which demonstrated that bluegill reduce care quality in response to perceived paternity, by replicating the behavioural response, and analyzing each parental care behaviour individually. I determined that bluegill with higher paternity more aggressively defend their nest by biting more after larvae hatch. While I observed a slight increase in biting in the hybrid control males, there was substantial variance in behaviour. Thus, it is unlikely this is a response to treatment, and rather individual variation in behaviour. There were no other differences between hybrids in the control and swap treatments in circulating hormone concentrations, or behaviours. It therefore appears hybrids provide similar quality of care regardless of egg manipulation. However, it is worth noting that hybrids do not provide similar quality of nurturing care to bluegill or pumpkinseed. While hybrid aggression is similar to bluegill and pumpkinseed, I observed markedly fewer nurturing behaviours. Future studies should consider the success of hybrid nests.

Given that hybrids provide care irrespectively of relatedness, but that the quality of care is poor, this raises the question of why they provide care. While possible, hybrids do not appear to nest solitarily. Rather they seem to nest in bluegill colonies. The most likely explanation for this is that hybrids nest in colonies to attract females to lay eggs in their nest. If no other sperm is present in the nest, they may be able to fertilize the eggs (Immler et al., 2011). While colonial nesting confers protection from predators (Gross, 1982) – parental male sized fish in Lake Opinicon are unlikely to be predated upon, thus it is possible, but unlikely that hybrids nest in colonies to avoid predation. Alternately, sites selected for colonial nesting should be optimal in terms of resources, protection, disturbance, and environmental conditions. It is possible hybrids nest in colonies and provide some level of care such that they can reside in an optimal environment within the lake. While little is known about bluegill nest site selection, wind and wave protection and dissolved oxygen levels appear to influence nesting sites (Gosch et al., 2006; Stahr et al., 2013). Therefore, perhaps hybrids nest in colonies to identify and reside in optimal lake locations. It is also possible, that hybrids nest in colonies to obtain some other benefit of group-membership. By nesting in colonies, hybrids may benefit from social facilitation and learning whereby hybrids may improve their nesting or defense strategies by observing other parental males.

In summary, the nesting behavior of hybrids in bluegill colonies presents an intriguing aspect of their reproductive strategy. Despite providing subpar parental care, nesting within bluegill colonies could be driven by various potential benefits. These benefits range from attracting females to acquire eggs, residing in an optimal environment with favorable conditions like protection and resource availability, to gaining advantages from group membership such as social facilitation and learning. Future research should consider these possibilities to develop a better understand the complexities of hybrid nesting behavior. This understanding could shed light on broader ecological and evolutionary questions about hybrid species and their reproductive strategies in natural habitats.

6.3 Conclusions

The objective of my thesis was to explore the dynamics of parental care behaviour in sunfish, particularly focusing on how adjustments in parental investment occur in response to paternity. My results suggest that while traditional views on hormones like 11KT and prolactin hold true

in certain contexts, their roles are far more complex and nuanced in regulating parental care behaviour – especially in species with complex reproductive systems like sunfish. My work contributes to our understanding of the mechanisms underlying parental care behaviour and parental investment, but also leads to new areas of interest for future work. My results call for further exploration into the endocrine, genetic, and environmental factors influencing parental care across species, and emphasizes the importance of considering a wide range of factors – from molecular to ecological – in understanding the evolution and persistence of parental care. Overall, my thesis advances our knowledge of parental care behaviour in fish, challenging existing theories and introducing new perspectives on the neuroendocrine regulation of these behaviours. This ultimately paves the way for future studies to delve deeper into the evolutionary biology of parental care, potentially transforming our understanding of this fundamental aspect of life across the animal kingdom.

6.4 References

Ammari, R., Monaca, F., Cao, M., Estelle Nassar, P. W., Del Grosso, N. A., Lee, M., Borak, N., Schneider-Luftman, D., and Kohl, J. (2023). Hormone-mediated neural remodelling orchestrates parenting onset during pregnancy. *Science*, **382**, 76–81.

Angelier, F., Wingfield, J. C., Tartu, S., and Chastel, O. (2016). Does prolactin mediate parental and life-history decisions in response to environmental conditions in birds? A review. *Hormones and Behavior*, **77,** 18-29

Aubin-Horth, N., and Renn, S. C. P. (2009). Genomic reaction norms: Using integrative biology to understand molecular mechanisms of phenotypic plasticity. *Molecular Ecology*, 18, 3763-3780.

Birnbaum, K., Shasha, D. E., Wang, J. Y., Jung, J. W., Lambert, G. M., Galbraith, D. W., and Benfey, P. N. (2003). A Gene Expression Map of the Arabidopsis Root. *Science*, **302,** 1956-1960.

Bridges, R. S. (2020). The behavioral neuroendocrinology of maternal behavior: Past accomplishments and future directions. *Hormones and Behavior*, 120, 104662.

Bukhari, S. A., Saul, M. C., James, N., Bensky, M. K., Stein, L. R., Trapp, R., and Bell, A. M. (2019). Neurogenomic insights into paternal care and its relation to territorial aggression. *Nature Communications*, **10,** 4437.

Caino, M. C., Seo, J. H., Wang, Y., Rivadeneira, D. B., Gabrilovich, D. I., Kim, E. T., Weeraratna, A. T., Languino, L. R., and Altieri, D. C. (2017). Syntaphilin controls a mitochondrial rheostat for proliferation-motility decisions in cancer. *Journal of Clinical Investigation*, **127,** 3755-3769

Cargnelli, L.M., and Gross, M.R. (1996). The temporal dimension in fish recruitment: birth date, body size, and size-dependent survival in a sunfish (bluegill: *Lepomis macrochirus*). *Canadian Journal of Fisheries and Aquatic Sciences*, **53,** 360-367.

Champagne, F. A., and Curley, J. P. (2013). Genetics and epigenetics of parental care. In N.J. Royle, and P.T. Smiseth (Eds), *The Evolution of Parental Care.* Oxford Academic.

Chang, D. T. W., and Reynolds, I. J. (2006). Mitochondrial trafficking and morphology in healthy and injured neurons. *Progress in Neurobiology*, **80,** 241-268.

Colledge, M., and Scott, J. D. (1999). AKAPs: From structure to function. *Trends in Cell Biology*, **9,** 216-221.

Cunha, A. A. P., Partridge, C. G., Knapp, R., and Neff, B. D. (2019). Androgen and prolactin manipulation induces changes in aggressive and nurturing behavior in a fish with male parental care. *Hormones and Behavior*, **116,** 104582.

Dixson, A. F., and George, L. (1982). Prolactin and parental behaviour in a male New World primate. *Nature*, **299,** 551–553.

Dobolyi, A., Oláh, S., Keller, D., Kumari, R., Fazekas, E. A., Csikós, V., Renner, É., and Cservenák, M. (2020). Secretion and Function of Pituitary Prolactin in Evolutionary Perspective. *Frontiers in Neuroscience*, **14,** 621.

Farner, D. S., and Wingfield, J. C. (1980). Reproductive endocrinology of birds. *Annual Review of Physiology*, **42,** 457-472.

Francesconi, M., and Lehner, B. (2014). The effects of genetic variation on gene expression dynamics during development. *Nature*, **505,** 208-211.

Freeman, M. E., Kanyicska, B., Lerant, A., and Nagy, G. (2000). Prolactin: Structure, function, and regulation of secretion. *Physiological Reviews*, **80,** 1523-1561.

Garner, S. R., and Neff, B. D. (2013). Alternative male reproductive tactics drive asymmetrical hybridization between sunfishes (*Lepomis* spp.). *Biology Letters*, **9,** 20130658.

Gosch, N. J. C., Phelps, Q. E., and Willis, D. W. (2006). Habitat characteristics at bluegill spawning colonies in a South Dakota glacial lake. *Ecology of Freshwater Fish*, **15**, 464-469.

Grimholt, U., Larsen, S., Nordmo, R., Midtlyng, P., Kjoeglum, S., Storset, A., Saebø, S., and Stet, R. J. M. (2003). MHC polymorphism and disease resistance in Atlantic salmon (*Salmo salar*); facing pathogens with single expressed major histocompatibility class I and class II loci. *Immunogenetics*, **55,** 210-219.

Gross, M. R. (1982). Sneakers, Satellites and Parentals: Polymorphic Mating Strategies in North American Sunfishes. *Ethology*, **60,** 1–26.

Gross, M. R., and Sargent, R. C. (1985). The evolution of male and female parental care in fishes. *Integrative and Comparative Biology*, **25**, 807-822.

Hau, M., Wikelski, M., Soma, K. K., and Wingfield, J. C. (2000). Testosterone and year-round territorial aggression in a tropical bird. *General and Comparative Endocrinology*, **117**, 20-33.

Immler, S., Hamilton, M. B., Poslusny, N. J., Birkhead, T. R., and Epifanio, J. M. (2011). Post-mating reproductive barriers in two unidirectionally hybridizing sunfish (Centrarchidae: *Lepomis*). *Journal of Evolutionary Biology*, **24,** 111-120.

Kindler, P. M., Bahr, J. M., Gross, M. R., and Philipp, D. P. (1991). Hormonal Regulation of Parental Care Behavior in Nesting Male Bluegills: Do the Effects of Bromocriptine Suggest a Role for Prolactin? *Physiological Zoology*, **64,** 310–322.

Knapp, R. (2003). Endocrine mediation of vertebrate male alternative reproductive tactics: The next generation of studies. *Integrative and Comparative Biology*, **43,** 658-668.

Kumari, R., Fazekas, E. A., Morvai, B., Udvari, E. B., Dóra, F., Zachar, G., Székely, T., Pogány, Á., and Dobolyi, Á. (2022). Transcriptomics of Parental Care in the Hypothalamic-Septal Region of Female Zebra Finch Brain. *International Journal of Molecular Sciences*, **23,** 2518.

Łabaj, P. P., Leparc, G. G., Linggi, B. E., Markillie, L. M., Wiley, H. S., and Kreil, D. P. (2011). Characterization and improvement of RNA-seq precision in quantitative transcript expression profiling. *Bioinformatics*, **27**, i383-391.

Lin, M. Y., Cheng, X. T., Tammineni, P., Xie, Y., Zhou, B., Cai, Q., and Sheng, Z. H. (2017). Releasing Syntaphilin Removes Stressed Mitochondria from Axons Independent of Mitophagy under Pathophysiological Conditions. *Neuron*, **94,** 595-610.

Magee, S. E., Neff, B. D., and Knapp, R. (2006). Plasma levels of androgens and cortisol in relation to breeding behavior in parental male bluegill sunfish, *Lepomis macrochirus*. *Hormones and Behavior*, **49**, 598–609.

Møller, A. P., and Nielsen, J. T. (2014). Parental defense of offspring and life history of a long-lived raptor. *Behavioral Ecology*, **25**, 1505-1512.

Neff, B. D. (2003). Decisions about parental care in response to perceived paternity. *Nature*, **422,** 716–718.

Neff, B. D., and Sherman, P. W. (2003). Nestling recognition via direct cues by parental male bluegill sunfish (*Lepomis macrochirus*). *Animal Cognition*, **6,** 87–92.

Nelson, R., and Kriegsfeld, L. (2022). *An Introduction to Behavioral Endocrinology, Sixth Edition* (6th ed.). Oxford University Press.

Páll, M. K., Liljander, M., and Borg, B. (2004). Prolactin diminishes courtship behaviour and stimulates fanning in nesting male three-spined sticklebacks, *gasterosteus aculeatus*. *Behaviour*, **141**, 11–12.

Pavey, S. A., Bernatchez, L., Aubin-Horth, N., and Landry, C. R. (2012). What is needed for next-generation ecological and evolutionary genomics? *Trends in Ecology and Evolution*, **27,** 673-678.

Redondo, T., and Carranza, J. (1989). Offspring reproductive value and nest defense in the magpie (*Pica pica*). *Behavioral Ecology and Sociobiology*, **25,** 369-378.

Rios-Cardenas, O., and Webster, M. S. (2005). Paternity and paternal effort in the pumpkinseed sunfish. *Behavioral Ecology*, **16,** 914-921

Rodgers, C. M. C., Neff, B. D., and Knapp, R. (2012). Effects of Exogenous Testosterone on Parental Care Behaviours in Male Bluegill Sunfish (*Lepomis macrochirus*). *Ethology*, **118,** 636–643.

Rodgers, C. M. C., Neff, B. D., and Knapp, R. (2013). Androgen-mediated nurturing and aggressive behaviors during paternal care in bluegill sunfish (*Lepomis macrochirus*). *Hormones and Behavior*, **63,** 454-461.

Schulz, R. W. (2003). Endocrine Regulation of Spermatogenesis in Teleost Fish. *Annual Review of Biomedical Sciences*, **5**, 57-68.

Smiley, K. O. (2019). Prolactin and avian parental care: New insights and unanswered questions. In *Hormones and Behavior*, **111,** 114–130.

Smiley, K. O., Brown, R. S. E., and Grattan, D. R. (2022). Prolactin Action Is Necessary for Parental Behavior in Male Mice. *Journal of Neuroscience*, **42,** 8308-8327.

Soma, K. K., Rendon, N. M., Boonstra, R., Albers, H. E., and Demas, G. E. (2015). DHEA effects on brain and behavior: Insights from comparative studies of aggression. *Journal of Steroid Biochemistry and Molecular Biology*, **145,** 261-272

Specker, J.L., and Kishida, M. (2000). Mouthbrooding in the black-chinned tilapia, *Sarotherodon melanotheron* (Pisces: *Cichlidae*): The presence of eggs reduces androgen and estradiol levels during paternal and maternal parental behavior. *Horomones and Behavior*, **38**, 44-51.

Stahlschmidt, Z. R. (2011). Taxonomic chauvinism revisited: Insight from parental care research. *PLoS ONE*, **6**, e24192.

Stahr, K. J., Kaemingk, M. A., and Willis, D. W. (2013). Factors associated with bluegill nest site selection within a shallow, natural lake. *Journal of Freshwater Ecology*, **28**, 283-292.

Storey, A. E., Walsh, C. J., Quinton, R. L., and Wynne-Edwards, K. E. (2000). Hormonal correlates of paternal responsiveness in new and expectant fathers. *Evolution and Human Behavior*, **21,** 79–95.

Trainor, B. C., and Marler, C. A. (2001). Testosterone, paternal behavior, and aggression in the monogamous California mouse (*Peromyscus californicus*). *Hormones and Behavior*, **40**, 32-42.

Trivers, R. L. (1972). Parental investment and sexual selection. In B. Campbell (Ed), *Sexual Selection and the Descent of Man: 1871-1971*. Aldine.

Whitehead, A., and Crawford, D. L. (2006). Variation within and among species in gene expression: Raw material for evolution. *Molecular Ecology*, **15**, 1197-1211.

Wingfield, J. C., Hegner, R. E., Dufty, A. M., and Ball, G. F. (1990). The "Challenge Hypothesis": Theoretical implications for patterns of testosterone secretion, mating systems, and breeding strategies. *American Naturalist*, **136,** 829–846.

Wu, Z., Autry, A. E., Bergan, J. F., Watabe-Uchida, M., and Dulac, C. G. (2014). Galanin neurons in the medial preoptic area govern parental behaviour. *Nature*, **509,** 325-330.

Yang, Q., Zhang, X., Lu, Z., Huang, R., Tran, N. T., Wu, J., Yang, F., Ge, H., Zhong, C., Sun, Q., Zhou, C., and Lin, Q. (2021). Transcriptome and Metabolome Analyses of Sea Cucumbers *Apostichopus japonicus* in Southern China During the Summer Aestivation Period. *Journal of Ocean University of China*, **20**, 198-212.

Appendices

Appendix A. Animal Use Protocol

PI :	Neff, Bryan
Protocol #	2018-084
Status :	Approved (w/o Stipulation)
Approved :	07/01/2018
Expires :	07/01/2022
Title :	Behavioural and molecular ecology of fishes

Table of Contents

Protocol Introduction

The questions on this page activate specific sections within the AUP form.

Note that species selection is part of this introductory page

Does this AUP involve teaching?

Yes ○ No ◉

Is the animal work on this project shared by another Animal Care Committee?

Yes ◉ No ○

Will you be using hazards?

Appendix B. Chapter 4 Supplementary Materials

Supplementary Table 1. Differentially expressed transcripts between bluegill parental males with higher paternity compared to parental males with experimentally reduced paternity. Only transcripts differentially expressed at FDR = 0.05 after false discovery rate correction are included in the heatmap.

Transcript ID	Mean Expression	log2FC	log2FC SEM	W-stat	p-value	Adjusted p-value
TRINITY_DN2384_c0_g1_i20	161.57	26.22	2.02	12.95	2.28E-38	5.02E-34
TRINITY_DN4077_c0_g1_i16	167.29	26.28	2.28	11.54	8.53E-31	9.42E-27
TRINITY_DN37119_c0_g1_i13	161.02	26.22	2.30	11.41	3.61E-30	2.66E-26
TRINITY_DN4077_c0_g1_i1	136.88	24.88	2.37	10.50	8.46E-26	4.67E-22
TRINITY_DN721_c0_g1_i8	142.36	-26.80	2.93	-9.14	6.35E-20	2.80E-16
TRINITY_DN6355_c0_g1_i8	88.18	-26.14	2.93	-8.91	5.02E-19	1.85E-15
TRINITY_DN2384_c0_g1_i15	117.45	24.53	2.83	8.66	4.77E-18	1.44E-14
TRINITY_DN3950_c0_g1_i6	87.39	25.37	2.93	8.65	5.21E-18	1.44E-14
TRINITY_DN2384_c0_g1_i12	67.74	25.02	2.93	8.53	1.49E-17	3.65E-14
TRINITY_DN1632_c0_g1_i13	46.87	24.48	2.93	8.34	7.16E-17	1.58E-13
TRINITY_DN1139_c1_g1_i7	21.81	-24.24	2.93	-8.26	1.42E-16	2.85E-13
TRINITY_DN2690_c0_g1_i2	33.74	24.04	2.93	8.20	2.50E-16	4.60E-13
TRINITY_DN2496_c0_g2_i7	15.38	-23.77	2.93	-8.10	5.43E-16	8.83E-13
TRINITY_DN4212_c0_g1_i22	15.18	-23.76	2.93	-8.10	5.60E-16	8.83E-13
TRINITY_DN933_c0_g1_i3	11.65	-23.40	2.93	-7.97	1.53E-15	2.25E-12
TRINITY_DN691_c9_g1_i3	11.29	-23.35	2.93	-7.96	1.73E-15	2.39E-12
TRINITY_DN122590_c0_g1_i1	8.14	-22.91	2.93	-7.81	5.83E-15	7.15E-12
TRINITY_DN2077_c3_g1_i5	27.33	22.91	2.93	7.81	5.72E-15	7.15E-12
TRINITY_DN330_c0_g1_i2	7.77	-22.85	2.93	-7.79	6.94E-15	7.66E-12
TRINITY_DN933_c0_g1_i4	14.31	22.84	2.93	7.79	6.91E-15	7.66E-12
TRINITY_DN523_c0_g2_i4	12.85	22.57	2.93	7.69	1.43E-14	1.51E-11
TRINITY_DN12149_c0_g1_i10	10.51	22.41	2.93	7.64	2.19E-14	2.20E-11
TRINITY_DN329_c0_g1_i16	15.75	22.38	2.93	7.63	2.40E-14	2.30E-11
TRINITY_DN743_c0_g1_i26	8.81	22.17	2.93	7.55	4.23E-14	3.89E-11
TRINITY_DN24409_c0_g1_i21	384.42	-1.08	0.20	-5.31	1.07E-07	9.47E-05
TRINITY_DN917_c0_g1_i14	162.54	10.01	2.21	4.53	5.80E-06	4.27E-03
TRINITY_DN2458_c0_g2_i6	261.95	-1.17	0.27	-4.35	1.35E-05	8.31E-03
TRINITY_DN1001_c0_g1_i4	140.38	-7.05	1.76	-4.00	6.27E-05	2.56E-02

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Churchman, E. and MacDougall-Shackleton, S.A. (2021) Leptin does not influence migratory behaviour in white-throated sparrows (*Zonotrichia albicollis*). *PeerJ*, 13584

Verma, S., Young, S., Kennedy, T., Carvalhana, I., Black, M., Baer, K., **Churchman, E.,** Watner, A., Allan, A., Louie, A., Palma, D., and Breadner, D. (2023). Detection of Circulating Tumor DNA after Stereotactic Ablative Radiotherapy in Patients with Unbiopsied Lung Tumors (SABR-DETECT). *Clinical Lung Cancer, 23,* https://doi.org/10.1016/j.cllc.2023.11.013

Select Conference Presentations:

Churchman, EKL. & Allan, A**.** (2023). Establishment of an oncology biospecimen lab at the London Health Sciences Centre. Canadian Cancer Research Conference, Halifax, Nova Scotia, Canada. [Poster].

Churchman EKL, Hain TJA, Knapp R, & Neff BD (2020). Perceived paternity alters parental care behaviour in bluegill sunfish (*Lepomis macrochirus*). Society for Integrative and Comparative Biology, Austin, Texas, USA. [Oral – Invited Speaker]

Churchman EKL, Hain TJA, Knapp R, & Neff BD (2019). Perceived paternity alters parental care behaviour in bluegill sunfish (*Lepomis macrochirus*). Canadian Conferences for Fisheries Research, London, Ontario, Canada. [Poster]