THE NEURAL REGULATION OF FATHERHOOD, INSIGHTS FROM THE ANEMONFISH *AMPHIPRION OCELLARIS*

BY

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DISSERTATION

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ABSTRACT

Parental care is a fundamental social behavior exhibited ubiquitously in diverse lineages across the animal kingdom, and when present is critical for offspring survival. Parental care represents a conflict: should parents invest in offspring fitness at the consequence of forgone reproductive opportunities? Or should they relinquish their parental duties in pursuit of additional mating events and offspring production? When parents do care for their young, specific mechanisms in the brain facilitate parental behavior. Thus far, the vast majority of research directed at understating the neural control of parental care has focused on females. Female care, or maternal care, is far more common among the common animal research models, and hence our understanding of its proximate regulatory mechanisms is well beyond our comprehension of those underlying paternal care. However, when paternal care is present, it is tantamount to maternal care in its contributions to offspring survival. While some of the mechanisms may be conserved, others may differ as males and females present dramatic sex differences in physiology. Hence, research efforts directed at paternal care are needed in order to understand the neural mechanisms underlying why fathers father. Two promising systems in understanding fathering behavior include nonapeptide signaling processes, and circulating steroid hormones. Nonapeptides and steroid hormones are key modulators of a variety of social behaviors in diverse taxa, and have also been identified as critical in the regulation of parental care. This dissertation aims to uncover how nonapeptide signaling, in concert with steroid hormones, regulate male parental care in the anemonefish *Amphiprion ocellaris*. In the introductory chapter 1, the conundrum of paternal care is presented, and the anemonefish *A. ocellaris* is introduced as a promising model in furthering our understanding of its neural regulation. Chapter 2 establishes *A. ocellaris* as a useful compliment to some of the more classically studied models of paternal care within vertebrates. Sex differences in levels of parental effort and in circulating steroid hormones during high levels of parental care were quantified. Results established that males are the predominant caregivers, spending more time in the nest and displaying a more total parental behaviors compared to females. Additionally, males showed higher levels of the circulating steroid hormone 11-ketotestosterone (11KT), which remains stable throughout the breeding cycle, indicating high androgens are not a hindrance to paternal effort. Females displayed higher circulating estradiol, which peaks 6 days after the eggs are laid, presumably in response to egg production and subsequent spawning events. Chapter 3 explores the role of arginine vasotocin (AVT) and isotocin (IT) signaling during paternal care. Here, using
antagonist specific to each peptide receptor, I demonstrated that blockade of IT signaling dramatically reduced total parental effort in males. Moreover, results suggest this was specific to paternal care, as the antagonist had no effect in aggressive dyadic interactions. Furthermore, I show that blockade of AVT significantly increased paternal care, a surprising finding given how high baseline levels of parental effort are in this species. A previous report found that the AVT antagonist reduced aggression. Consequently, I hypothesize this promotion of paternal care in response to the AVT antagonist may be a result of reduced vigilance and aggression, thereby allowing more effort allotted towards parental care. In chapter 4, I demonstrate that aromatase and IT receptor gene expression are dynamically regulated in response to paternal care, independent of AVT V1aR receptor expression as well as the circulating steroid hormone 11KT. Individuals actively parenting had higher levels of aromatase and IT receptor expression than those not currently parenting. IT receptor expression was higher in males compared to females, a pattern reflective of the sex differences in parental effort. Brain aromatase expression was higher in females compared to males, which I hypothesize is an induced response of higher circulating E2 levels in females. No differences in AVT receptor expression or circulating 11KT were found in contrasting parental contexts. Chapter 5 utilizes a unique aspect of anemonefish behavior, step fathering. In this study naïve males were given a batch of eggs to care for, and following 90 minutes of parental behaviors, paternal brains were examined for the co-localization of IT and the ribosomal protein S6 (an neuronal activation marker), in order to determine the extent to which activation of IT neurons is related to paternal effort. Results indicate that paternal effort is negatively related to the activation of IT neurons within the POA. However, the time step-fathering males spent in the nest was positively correlated with the amount of eggs lost, signifying that males spending a lot of time in the nest may have been eating eggs, and hence were not displaying parental behavior. Furthermore, I have anecdotally noted in previous experiments that males improve as fathers with experience. Therefore, using experience biological fathers, or allowing stepfathers more time to interact with several batches of eggs may yield differing results. Ultimately the efforts of this dissertation provide cumulative evidence supporting a conserved role of IT signaling in the regulation of parental care across taxa, and independent of the sex of the primary caregiver. Findings also suggest AVT signaling as ancillary in the regulation of paternal care, as it may be important in nest defense and vigilance behavior. Moreover, I also show aromatase gene expression is an important aspect of parental behavior, as it is higher in a parental context.
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CHAPTER 1:
INTRODUCTION

1.1 INTELLECTUAL BACKGROUND

Paternal care is a costly investment on the part of the caregiver. The cost of parental care presents a trade-off between current parental investment and opportunities for future reproductive events (Clutton-Brock, 1991; Trivers, 1974). Often, this trade-off differs for each sex. Male care is less common, as males produce a large number of metabolically inexpensive gametes, and generally have higher reproductive success siring as many offspring as possible (Gross, 2005; Kokko and Jennions, 2012; Trivers, 1972a). Thus, paternal care is predicted to occur only when the cost of desertion is high, or future reproductive opportunities are low (Gross and Sargent, 1985).

Conversely, females produce fewer gametes that are larger in size, and generally have higher reproductive success when effort is directed effort towards egg survival (Gross, 2005; Trivers, 1972a). Thus, female parental care is much more common and consequently has received more attention in the literature (Gimpl and Fahrenholz, 2001; Keverne and Curley, 2004; Lee et al., 2009; Olazabal and Young, 2006). Nevertheless, in many of the species exhibiting male parental care, paternal care is even more important than female care for offspring survival (Kleszczyńska et al., 2012; Ripley and Foran, 2010; Rodgers et al., 2006a; Trainor and Marler, 2002).

However, unlike female parental care, there have been few studies examining mechanisms underlying male parental care, in part due to the paucity of adequate parental care study systems. Hence, we know relatively little about the proximate regulation of paternal behavior. Not only has the lack of studies exploring paternal care left much unknown as to how fathering behaviors are regulated, but also in understanding its differential regulation compared to maternal care. While we expect many similarities in the underlying mechanisms of parental behaviors across males and females, there is also reason to expect that parental care may be differentially controlled (Clutton-Brock, 1991; Royle et al., 2012; Trivers, 1972a; Trivers, 1974). In females most of the underlying hormones (estradiol, progesterone) are induced during egg and embryo development, or in mammals associated with milk production (prolactin). In contrast to maternal care, primers for paternal care are more subtle, as no such hormonal or physiological changes occur related to embryo development. Thus the signals must be stimulated by the presence of eggs (as is the case for many fishes), pregnancy, or offspring (in cases of postpartum parental care). Despite the subtly of primers for paternal care, males must undergo a rapid and dramatic behavioral shift from non-parenting to parenting. This dynamic regulation of behavior must also correspond to dynamic
changes in the brain, and how this fathering behavioral transition occurs is of primary concern in the experiments implemented in this dissertation.

1.2. MECHANISMS OF PARENTAL CARE

Parental care is a complex behavioral dynamic regulated by an equally complex number of interactions between many different substrates involved in endocrine regulation and neurotransmitter signaling processes. Across vertebrates and beyond, several hormones have been identified as involved in the regulation of parental care, including: androgens, estrogens, progesterone, prolactin and others. During the process of gestation in females, increased levels of estrogen, progesterone, and prolactin work to prime the brain at parturition and establish the onset of maternal care (Terkel and Rosenblatt, 1968). Several studies have also identified prolactin and estrogens as important in the establishment of male parental care as well. In the California mouse, estradiol (E2) is critical for high levels of paternal care (Trainor and Marler, 2001, 2002), and prolactin has been identified as important to fatherhood in a variety of taxa, including fish (Kindler et al., 1991), birds (Buntin, 1996), and mammals (Gubernick et al., 1993). Conversely, studies have identified testosterone (T) and progesterone as promoting aggression and inhibitory to high levels of male parental care (Schneider et al., 2003; Wingfield et al., 1990b). Together, these data present the extensive and complex role of hormone levels in the regulation of maternal and paternal behaviors.

Analogously to steroid hormones, neurotransmitters are equally important in the promotion and maintenance of parental behaviors, where studies have highlighted several neuromodulators of significance in the regulation of parental behaviors, including: dopamine, serotonin, opioids, galanin, oxytocin, and vasopressin. In rodents, pup interactions facilitate the release of dopamine from the ventral striatum in females (Hansen et al., 1993), and ablation of dopamine neurons decreases maternal effort (Hansen et al., 1991a; Hansen et al., 1991b). Similarly manipulations decreasing serotonin levels in the brain of maternal rats reduces maternal care (Lerch-Haner et al., 2008), and variations in the mu-opioid receptor correspond to variation in maternal attentiveness in female primates (Higham et al., 2011). Additionally, recent work has shown activation of galanin neurons in response to parental behavior in both paternal and maternal rodents (Wu et al., 2014). Furthermore, interactions between neurotransmitter signaling process are also important in our understanding of parental care, where for example, oxytocin levels in the ventral tegmental area work in the
regulation of dopamine levels in the nucleus accumbens during maternal care in rodents (Shahrokh et al., 2010).

The complex interactions of neurotransmitters, steroid hormones, environment, and physiology limit the scope of what is possible to address in a dissertation. Here, I focus on the role of the steroid hormones E2, and 11-ketotestosterone (11KT). 11KT is the predominant bioactive androgen in teleost fishes, and studies within anemonefishes exploring the role of androgens in the regulation of behavior have shown 11KT, but not T, as important in the regulation of behaviors (Godwin, 1994a, b; Godwin and Thomas, 1993). These hormones were chosen because of their highlighted importance in the regulation of parental care (Trainor and Marler, 2002), and also due to methodological reasons; These were hormones I was able to accurately measure via blood draws and implementation of the use of enzyme immunoassays. Similarly, OT and AVT were chosen as they have been repeatedly implicated in both maternal and paternal behaviors. However, questions as to involvement of OT in paternal care, and the specific role of AVT remain unclear. Moreover, previous work in the Rhodes lab had established immunohistochemical procedures for the measurement of AVT and also in the role of AVT during aggressive dyadic interactions. While OT has been identified as important in both maternal and paternal behaviors, how OT signaling works in concert with circulating steroid hormones in a primarily paternal species remained unknown.

1.3. COMPLICATIONS IN PATERNAL CARE STUDIES

Thus far, the vast majority of research exploring the proximate mechanisms involved in the regulation of male parental care has been complicated by the co-occurrence of several different parental behaviors exhibited either within close temporal proximity or simultaneously with one another (Kulczykowska and Kleszczyńska, 2014; Pradhan et al., 2014c; Trainor and Marler, 2001, 2002). For example, in the territorial California mouse, males are highly aggressive defending nests against conspecifics (Frazier et al., 2006). In the seasonally spawning three-spined sticklebacks, males have a distinct nest building, courtship, and parental phases (Páll et al., 2002; Van Iersel, 1953), where first they build nests, then court females, and subsequently care for eggs. Additionally in the blue-banded goby, males simultaneously show aggression towards conspecifics, and court additional females, while also providing egg care (Pradhan et al., 2014c; Rodgers et al., 2006a). This confluence of behaviors complicates the disentanglement of mechanisms specific to parental care as opposed to nest building, nest defense, or courtship.
1.4. STUDY SYSTEM

The anemonefish, *Amphiprion ocellaris* is a useful model for studying paternal care. This species is a predominantly paternal care species in which male care is critical for offspring survival (DeAngelis and Rhodes, 2016). Within *A. ocellaris*, parental behavior is isolated from other confounding co-occurring displays. That is, males are not building nests, fighting off conspecifics in territorial battles, or courting females. While pair bonding and courtship displays do occur in *A. ocellaris*, these behaviors occur months, or even years prior to a reproductive event. Additionally, Male anemonefish display an incredibly high level of care during the egg-rearing period and present behaviors that can easily be quantified in a laboratory setting. Total paternal behavioral acts (the number or nipping and fanning) can reach upwards of 200 counts during a 10-minute observational period, and males spend the vast majority of their time in the nest (DeAngelis et al., 2017b; DeAngelis and Rhodes, 2016). Within reproductively active pairs, females lay eggs every 14 days, and the eggs are then cared for by the male for 8-10 days following egg deposition (DeAngelis and Rhodes, 2016). Additionally, *Amphiprion* species have a lifespan of up to 30 years, 6 times longer than any other pomacentrid, which is attributed to the efficient protection from their host sea anemones (Buston and García, 2007). When eggs are present, anemonefish fathers do little else other than care for their eggs. Given the extraordinary lifespan of anemonefishes, and the constant egg laying of *Amphiprion* females, male anemonefish demonstrate a truly remarkable effort toward parental care throughout their lives.

Furthermore, of particular interest in this species is the naturally occurring behavior of step fathering. When male is removed from a nest (naturally from predation or a storm, and in the lab by male removal or nest introduction), another male will move into the nest and provide high levels of care to the eggs until hatching (Yanagisawa and Ochi, 1986a). This removes the otherwise uncontrollable confounds of nest preparation and sperm release. In trying to understand the neural regulation specific to fathering, it is important to isolate out as many other co-occurring displays as possible. This allows research to be directed specifically at fathering, and hence in step-fathering experiments, we are exceptionally confident that the neural responses measured are specific to the regulation of fatherhood and not other non-target behavioral displays.

1.5. EVOLUTIONARY HOMOLOGY OF REGULATORY NETWORKS

Insights garnered from studies of paternal care within *A. ocellaris*, as well as other complimentary models of paternal care, may be more broadly applicable to vertebrate social
behavior, as groundbreaking recent work suggests that many of the brain regions, neuropeptides, and steroid hormones involved in social behaviors share ancient evolutionary origins, and are remarkably conserved in their form and function (Goodson, 2005; Newman, 1999; O'Connell and Hofmann, 2011; O'Connell and Hofmann, 2012; Robinson et al., 2008). This system of neural regulation has been deemed the 'Social Decision-Making' (SDM) network. The shared homology of this network across disparate evolutionary taxa allows insight to be gained broadly about vertebrate social behaviors by selecting the right study system and experimental design. More specific to this dissertation, by studying predominantly paternal anemonefish, within the context of parental care, and in the absence of complicating confounding factors, research findings may elucidate the underlying mechanisms regulating the neural state of the vertebrate fathering brain.

1.6. NONAPEPTIDES AND PATERNAL CARE

The preoptic area (POA) of the hypothalamus is evolutionarily ancient, and one of the most highly conserved of all brain regions, showing remarkable consistency of neurochemical gene expression patterns across vertebrate lineages (O’Connell and Hofmann, 2012). Two neurochemicals, produced by neurons residing within the POA, and which have been broadly implicated in the regulation of social behaviors across evolutionarily distal taxa are arginine vasopressin (AVP, teleost homolog arginine vasotocin, AVT) and oxytocin (OT, teleost homolog isotocin, IT)(Bales et al., 2004; French et al., 2016; Godwin and Thompson, 2012; Goodson, 2008; Gubernick et al., 1995; Insel and Young, 2000). These neuropeptides provide ideal candidates for initial forays into the underlying proximate regulation of paternal care, as they have been identified as important not only the regulation social behaviors, but also in their important functional role in the facilitation of parental care (Bales et al., 2004; Bosch and Neumann, 2012; DeAngelis et al., 2017a; Kleszczyńska et al, 2012; Olazabal and Young, 2006; Royle et al., 2012; Saito and Nakamura, 2011; Weisman et al., 2012).

The importance of OT signaling in parental care has been established in maternal species, showing that OT release is critical for a variety of physiological changes during parturition, and is subsequently necessary for maternal behaviors critical for offspring survival (Bartz et al., 2010; Francis et al., 2000). However, while evidence suggests a conserved role of OT/IT signaling in species displaying paternal care (Feldman et al., 2010; Gubernick et al., 1995; Kleszczyńska et al, 2012; O’Connell et al., 2012; Saito and Nakamura, 2011), conclusive evidence on its direct role in the promotion of fathering is lacking. This dissertation will elucidate the extent to which OT/IT signaling
is conserved in its promotion of parental care, independent of which sex presumes responsibility of the primary caregiver by conducting experiments exploring multiple aspects of the IT signaling system during high levels of parental care, and in the absence of complicating simultaneously occurring behavioral displays.

In contrast to OT/IT, AVT/AVP has been suggested as more important in the behavioral regulation of males (Insel and Young, 2000). Broadly AVP/AVT signaling has been implicated in male behaviors regulating reproductive success, including parental care. However, the specific function of the AVP/AVT signaling system has thus far been largely species dependent, and regulated by distinct social contexts and group dynamics (Foran and Bass, 1999; Huffman et al., 2015; Insel and Young, 2000; Kleszczyńska et al., 2012; Semsar et al., 2001a; Yaeger et al., 2014a). Hence, this dissertation aims to uncover the role AVT signaling plays in paternal care, again, by the utilization of the anemonefish *A. ocellaris*, where parental care can be isolated out from other confounding behavioral displays.

### 1.7. STEROID HORMONES AND PATERNAL CARE

In order to fully comprehend how nonapeptide signaling processes coordinate behavior, we must also investigate the role of steroid hormones and how their reciprocal interactions with nonapeptide neurons orchestrate the production and release of AVP/AVT and OT/IT. Many of the cells within the POA have co-localized expression of the steroid hormone receptors (Foran and Bass, 1999). Hence, mutual interactions between circulating hormones and the neurochemicals AVT/AVP and OT/IT work in concert in the regulation social behaviors (Insel, 2010; Kline et al., 2011). E2 is a steroid hormone that has been identified as an important regulator of parental care (Rilling and Young, 2014; Trainor and Marler, 2002), and is also inherently critical for OT signaling, where it facilitates the production of OT within POA neurons, and OT receptor binding within the POA (Caldwell et al., 1989; Champagne et al., 2001; Gimpl and Fahrenholz, 2001; Miller et al., 1989; Tribollet et al., 1990). Therefore, in understanding how the OT/IT signaling system works in the promotion and maintenance of high levels of parental care, the role of E2 must also be explored. E2 is produced primarily within the gonads where through circulation it crosses the blood brain barrier (Pardridge and Mietus, 1979), influencing neural activity and ultimately behavior. However, E2 can also be synthesized locally within the brain by the steroidogenic enzyme aromatase, which converts T into E2. Furthermore, aromatase activity in the brain has recently been identified as critical for male parental care in the California mouse (Trainor and Marler, 2002). However, whether
circulating E2 and brain aromatase are important in paternal care across taxa remains unclear. Therefore, this dissertation will explore the functional role of circulating E2 and brain aromatase in the regulation of paternal care within the anemonefish *A. ocellaris*.

Conversely to E2, the role of circulating androgens in parental care is less clear. Androgens are typically thought to be beneficial for challenging social encounters, such as intraspecific competition and nest defense, where they work to promote increased aggression in the individuals involved (Wingfield et al., 1990c). And several studies have identified an inverse relationship between circulating androgens and high levels of paternal care (Hegner and Wingfield, 1987; Pall et al., 2002; Pankhurst and Peter, 2002; Van Roo, 2004). Conversely, other study systems do not support this inverse relationship, and have shown the opposite pattern, demonstrating that high levels of androgens are necessary for parental care (Pradhan et al., 2014c; Trainor and Marler, 2001, 2002). Therefore, I also explore the role of how the bioactive circulating androgen in teleost fishes, 11-ketotestosterone, works to promote or inhibit high levels of parental behavior where co-occurring challenging social encounters are largely absent.

### 1.8. CONCLUSION

This dissertation implements the anemonefish *A. ocellaris* as a promising and complimentary model in understating the neural regulation of parental care, where carefully conducted experiments expand our understanding of how nonapeptide signaling processes and corresponding receptor gene expression, along with steroid hormones, and brain aromatase gene expression work in the promotion and maintenance of fathering behavior.
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CHAPTER 2:
SEX DIFFERENCES IN STEROID HORMONES AND PARENTAL EFFORT ACROSS THE BREEDING CYCLE IN AMPHIPRION OCELLARIS

Abstract
While it has traditionally been viewed that high androgens are a hindrance to male parental care, recent studies in several vertebrate taxa have shown the opposite pattern, where high androgens either co-occur with, or are necessary for high parental investment. These inconsistencies suggest that in order to develop a complete understanding of the role sex steroids play in parental care, it is important to study multiple species with varying life-history characteristics. Anemonefishes of the genera Amphiprion provide a useful compliment to more classically studied systems within vertebrate, and more specifically teleost models of parental care. Therefore, parental behaviors, and blood plasma levels of 11-ketotestosterone (11-KT), estradiol (E2), and cortisol were measured in 5 breeding pairs at three points in the spawning cycle. Males displayed 5.6 fold more parental behaviors and spent 67% of their time in the nest as compared to 12% in females, along with significantly higher levels of 11-KT (males = 0.75 +/- 0.076; females = 0.02 +/- 0.005 ng/ml). Alternatively, females displayed higher levels of E2 (males = 0.09 +/- 0.009; females = 3.65 +/- 0.655 ng/ml) and E2 fluctuated across the breeding cycle with low levels on the day eggs were laid and higher levels as eggs developed. Cortisol tended to be higher in males, and higher in breeders than non-breeders, though these differences were not significant (males = 35.8 +/- 11.01; females = 16.9, +/- 3.58 ng/ml). Results suggest A. ocellaris may be a useful model for studying paternal behavior in the presence of high androgens.

2.1 INTRODUCTION
In many species, across a wide range of taxa, parental care is essential for offspring survival. Parental effort is often divided between the sexes in a sex-specific manner and steroid hormones play a critical role in the regulation of these behaviors (Ball et al., 2002; Champagne et al., 2003; Kelley, 1982; Munakata and Kobayashi, 2010). In most mammals, females are the primary caregivers, where high estrogens and low androgens are associated with high parental investment (Trivers, 1972b). Moreover, numerous studies among birds, mammals, and fishes support an inverse relationship between androgens and parental care in males (Hirschenhauser and Oliveira, 2006; Pall

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et al., 2002; Wingfield et al., 1990a). On the other hand, many teleosts show the opposite pattern with males performing the majority of parental care, and in these species, high parental investment is associated with high androgens and low estrogens.

Within males, variation in circulating steroid hormones have been associated with parental effort, but in different ways depending on the species. For example, in male garibaldi (*Hypsopsis rubicundus*) and also the three-spined stickleback (*Gasterosteus aculeatus*), 11-Ketotestosterone (11-KT; the teleost male bioactive androgen) levels were higher during courtship, and lower during parental care. Similarly, in male damselfish (*Chromis dispilus*), a subset of gonadotropin-releasing hormone (GnRH) mediated androgens was higher during courtship and lower during the egg brooding period (Pankhurst and Peter, 2002). Other species have shown the opposite pattern. In the blue-banded goby (*Lythrypnus dalli*), experienced parental males have higher 11-KT levels than non-brooding males (Rodgers et al., 2006b), and administration of carbenoxolone (CBX), an antagonist that blocks the conversion of 11-keto-androsterone to 11-KT, significantly reduced paternal effort (Pradhan et al., 2014b).

In the bluegill sunfish (*Lepomis macrochirus*) and Plainfin Midshipman (*Porichthys notatus*) relationships between 11-KT, egg care, and embryo care change across the breeding cycle (Knapp et al., 1999). Levels of 11-KT in the bluegill sunfish were highest prior to spawning, then lowered during brood care, before increasing again prior to egg hatching (Magee et al., 2006). Taken together, results suggest that the pattern of sex-specific roles in parental care and underlying steroid hormone levels in teleost fishes is highly species specific and in some species changes across the breeding cycle. Therefore, more species with varied life histories and social dynamics are needed to gain a comprehensive understanding of the relationship between sex steroid hormones and parental care across vertebrate taxa (Amundsen, 2003b).

Anemonefishes, from the genera *Amphiprion* and *Premnas* represent an unusual social system, complementary to other more studied species such as the three-spined stickleback, and blue-banded goby. Anemonefish display monogamous pair bonds between a dominant alpha female and beta male, which form long before mating occurs, and can last for decades (Fricke, 1979; Ross, 1978b). Furthermore, anemonefish are protandrous hermaphrodites. These fish hatch with both ovarian and testicular tissue within the gonads, but during ontological development female gonads change to predominantly ovarian while males retain both testicular and ovarian tissue (Fricke, 1983; Godwin, 1994b). Thus, the protandrous, female-dominant anemonefish provides an interesting contrast to the other protogynous or bi-directional male-dominant species (Rodgers et al., 2006b;
Semsar et al., 2001b), and research in this group may elucidate the dynamic relationship between steroid hormones and parental care.

Among the anemonefish species, *Amphiprion ocellaris* is particularly felicitous for laboratory research. *A. ocellaris* is one of the smallest anemonefishes, and is highly dependent on its protective host sea anemones (Santini and Polacco, 2006). Small size and high host dependence has led to spatial restriction, and consequently small home ranges. While small in comparison to other *Amphiprion* species, they are still large enough to enable blood draws to be easily performed without sacrificing the animal. Together, these conditions enable semi-natural behavior to be observed in the laboratory while at the same time facilitating accurate determination of circulating steroid hormones.

Only a handful of studies have measured sex steroid levels and behavior in mature anemonefish. In field studies of the Cinnamon anemonefish (*A. melanopus*), males were the predominant caretakers, and increased the number of fanning acts, as well as time in the nest over the 8-day incubation period of the eggs (Ross, 1978a). In Clark's anemonefish (*A. clarkii*), males spent over 65% of their time in the nest by day 7 of the incubation period (Yanagisawa and Ochi, 1986b). Finally, both male and female *A. ocellaris* display substantial parental care of the eggs, and male care increases as eggs approach hatching (Madhu, 2006), however this effort was not quantified. In association with this greater caregiving, male Cinnamon anemonefish displayed 3-fold higher levels of 11-ketotestosterone (11-KT), the putative bioactive androgen in *Amphiprion* species, as compared to females. In contrast, estradiol (E2) was 7-fold higher in females than in males (Godwin and Thomas, 1993). In teleost species where spawning is seasonal, such as the plainfin midshipman and catfish, E2 covaries with seasonal changes and/or lunar cycles, and direct, mechanistic studies have established that E2 concentrations directly influence vitellogenesis (Lamba et al., 1983; Sisneros et al., 2004). However, in non-seasonal, continuously spawning species, such as anemonefish, patterns of E2 variation across the spawning cycle are less well understood.

In addition to the sex steroids, circulating glucocorticoid levels are associated with reproductive behaviors across taxa, but few studies in teleosts have characterized the relationship between cortisol and parental care between the sexes (Dey et al., 2010; Magee et al., 2006). In Cinnamon anemonefish, levels of cortisol were reported to be similar in males and females (Godwin and Thomas, 1993). However, animals may not have been collected at a time when the pair was currently caring for a brood. In paternal bluegill sunfish, cortisol was higher in males caring for larger broods than in males with fewer eggs (Magee et al., 2006). Parental care requires increased
metabolism, effort, and attention, and thus may be perceived as a stressful experience. Hence, if anemonefish were sampled during egg tending, cortisol might be expected to display a similar pattern as sunfish, with higher cortisol levels in individuals displaying relatively more parental effort.

The first aim of this study was to characterize sex differences in parental care during egg development in laboratory reared, reproductively active pairs of *A. ocellaris*. We hypothesized that males would display more care than females and that care would increase as eggs develop. The second aim was to characterize sex differences in circulating levels of 11-KT, E2, and cortisol across the breeding cycle. We hypothesized that females would display higher E2 and lower 11-KT. We further hypothesized that actively parenting males would display higher cortisol levels than females, associated with greater parental effort.

2.2 MATERIALS AND METHODS

2.2.1 Animals and husbandry

*A. ocellaris* were obtained from ORA (Oceans Reefs and Aquariums, Fort Peirce, FL) as juveniles and raised in 25-gallon tanks (18”x18”x18”) in pairs until reaching sexual maturity. Tank conditions were set to mimic natural environmental conditions with a temperature of 79°F, photoperiod of 12:12 (lights on at 7:00 am and off at 7:00 pm), pH of 8.2 and specific gravity of 1.025. Length and weight measurements were taken prior to the onset of experimental procedures, and again at the conclusion of this study. Fish were fed twice daily with a variety of fresh, frozen, and dried foods. One clay pot was placed in each tank as surrogate for a host sea anemone. Behavioral observations between pairs in anemones versus those pairs used in this experiment showed no overt behavioral differences (unpublished observations). The fish also deposit eggs in the clay pots, and hence the clay pots serve as the ‘nest’ in behavioral analyses described below. All procedures were approved by the University of Illinois Institutional Animal Care and Use Committee and adhered to NIH guidelines. All measures were taken to minimize the number of fish used as well as the pain and suffering of the animals. The University of Illinois at Urbana-Champaign is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

2.2.2 Experimental Design

Five spawning pairs of *A. ocellaris* were used in this experiment. Pairs were selected based on two factors. First, the size of the male needed to be large enough for repeated blood draws and a
blood collection volume of over 25 µl. In preliminary trials this weight was usually just over two grams. Second, pairs needed to be regularly spawning (spawn period of less than 25 days). Spawning pairs are difficult to produce due to the length of time it takes for sexual maturation (Iwata et al., 2008), and out of more than 20 spawning pairs at the time of this experiment, only five pairs had consistent spawn intervals of under 25 days. Although other pairs in the laboratory were spawning, they were relatively novice in their reproductive behavior. After several spawns, intervals decrease and parenting effort increases as individuals become more experienced (unpublished observation). Eggs are laid in the evening hours in the clay pot and consequently the nest was visually inspected for the presence of eggs every morning prior to any experimental procedures. If eggs were identified, the experiment began that day at ‘day 0.’ Eggs took from 8-10 days after deposition to hatch into the planktonic larval phase at which experimental procedures for that spawn period ended. From the five selected pairs, video recordings were analyzed for behavioral displays at three separate time points in separate spawn periods during egg rearing. Following video recordings, blood draws were performed.

### 2.2.3 Behavior

The behavior of the pairs was video recorded on the day the eggs were laid (day 0), as well as in two additional spawning periods, 3, and 6 days following the spawning event for a five-minute period. Number of parental behaviors as defined by the sum of fanning (fanning the eggs with pectoral fins, or caudal fin), nipping events (cleaning the eggs with the mouth) and duration spent in the nest were recorded for both the male and the female using Jwatcher behavioral event recording software. In addition to parental behaviors, agonistic and affiliative behaviors were recorded, including number of bites on the partner, number of chases (charge toward partner), and submissive displays (quiver display or fleeing form partner).

### 2.2.4 Blood sample collection

The first blood sample collection occurred when animals were not tending eggs, exactly 3 days after the eggs hatched from the previous spawn, and 11 days after the eggs were laid from the previous spawn. The second sample was taken on the day the next batch of eggs was laid (day 0). The third and fourth samples were taken 3 and 6 days following egg deposition. Consecutive spawning events were used unless the previous blood draw had occurred within less than 12 days (a
period previously established to be critical for recovery, unpublished data), in which case the following spawn period was used.

All blood sampling was performed between 2:00 and 3:00 pm in order to control for any diurnal changes in steroid hormone levels. Fish were placed between two heavy paper towels wetted with seawater with only the caudal region exposed. Blood samples were taken from the lateral caudal vein using a 27-gauge heparinized butterfly needle (Terumo Medical Products) mounted on a 1 ml syringe (BD Syringe). As much blood as would freely flow into a 1 ml syringe was drawn (50 µl to 300 µl). The time from which the capture net entered the tank (start time), the time the fish was netted (catch time) and the time final collection was finished (blood time) were recorded for all individuals. Steroid hormones can undergo rapid changes due to stress (Kammerer et al., 2010), hence it is critical to isolate samples quickly, and only blood samples collected within three minutes of catching the fish from the home tank were used.

Immediately following collection, blood was dispelled into a 0.6 ml centrifuge tube and placed on ice until the daily collection was finished (no longer than 10-minutes). Samples were then immediately centrifuged (Eppendorf Centrifuge 5417R) at 4000 rpm for 15-minutes following established protocols (Kidd et al., 2010). The plasma supernatant was extracted with a 100 µl pipet (avoiding the red hematocrit layer), isolated, and stored at -80°C until circulating levels of 11-KT, E2, and cortisol were measured via Enzyme Immunoassay (EIA). All EIA measurements were performed in duplicate, and the average of both technical replicates was used to calculate plasma hormone concentrations.

2.2.5 Validation of EIA kits

EIA kits are only capable of measuring concentrations of the hormones within a specific range. Plasma concentrations of the hormones of interest for *A. ocellaris* were not previously known. Hence, appropriate dilutions of the plasma samples that produce hormone concentrations within range of the kits was established. A pooled plasma sample across individuals was serially diluted, measured for hormone concentration, and then compared to serially diluted standards (Fischer et al., 2014; Mills et al., 2010). The pooled sample (total 600 µl) consisted of blood plasma from at least five individuals from the following groups: non-reproductive juveniles (200 µl), reproductive males and females tending eggs (200 µl), and reproductive males and females not tending eggs (200 µl). This pooled sample was combined this way in order to reflect hormone values unbiased by sex, breeding status, or position in the social hierarchy.
11-KT. For the 11-KT assay (EIA kit from Cayman Chemical, Item No. 582751), a sample containing a known concentration of 1000 pg/ml standard was serially diluted 8 times giving a range of 1000 pg/ml to 7.8 pg/ml. The pooled plasma sample was diluted five times (from 1:1 to 1:256). All samples were measured for 11-KT concentration as indicated by percent bound following instructions in the kits.

E2. For the E2 assay kit (Calbiotech Mouse/Rat Estradiol Kit, Lot No. ESG4324), 6 standards were provided containing known concentrations of E2 ranging from 0 pg/ml to 300 pg/ml. The pooled plasma sample was diluted 5 times (from 1:1 to 1:256). All samples were measured for E2 concentration as indicated by percent bound following instructions in the kits.

Cortisol. For the cortisol assay (Cortisol ELISA kit, Cat. No. ADI-900-071), a sample containing a known concentration of 10,000 pg/ml was serially diluted seven times giving a range of 10,000 pg/ml to 156 pg/ml. The pooled plasma sample was diluted 5 times (from 1:1 to 1:256). All samples were measured for cortisol concentration as indicated by percent bound following instructions in the kits.

2.2.6 Statistical Methods
Data were analyzed using R (3.1.1). P<0.05 was considered statistically significant. Sex differences in body mass and length were analyzed using paired t-tests. Behavioral measures (e.g., total duration in the nest, total number of parental acts, fanning or nipping eggs) were not normally distributed and hence were analyzed for sex differences using the non-parametric Wilcoxon Rank Sum Test. Differences in behaviors across the 3 days of the breeding cycle were analyzed using the Kruskal–Wallis test.

Validation of the EIA kits was accomplished using the following procedure. First, percent bound (as measured using the kits) was plotted against the natural logarithm of the dilution ratios of both standards and the pooled plasma samples. Data points were then analyzed by analysis of covariance to determine the extent to which the two lines (sample and standard) were parallel. The natural logarithm of the dilution ratio was entered as the continuous predictor, and the standard versus pooled sample as the categorical variable. A non-significant interaction was taken to indicate that the slopes were not significantly different from each other, and that the slope of the given standards can accurately predict samples.
Sex differences in steroid hormone concentrations were analyzed using unpaired t-tests. For these analyses, the multiple hormone concentration measures per individual across the breeding cycle were averaged together to generate one number per individual. Samples were also analyzed by repeated measures ANOVA with sample day relative to the spawning event (0, 3, or 6) as a within-subjects factor, and sex as the between-subjects factor. These analyses also include start time, catch time and blood time entered as covariates (one at a time).

To determine how the hormones may have fluctuated across the breeding cycle, plasma hormone concentrations were analyzed using polynomial regression with sample day (0, 3, 6 and 11) and the square of these values entered as continuous predictors. Note that sample day 11 occurred 3 days after eggs from the previous batch hatched.

2.3. RESULTS

2.3.1 Sex differences in body mass, body length and frequency of spawning events

Females were larger than males in both standard length, and weight. Mean standard length of females was 72.2 mm +/- 0.42 (SEM; range 68 to 78 mm) whereas in males it was 55.6 mm +/- 0.56 (SEM; 50 to 62 mm) (t= 5.25, df = 4, P<0.007). Mean weight of females was 7.09 g +/- .405 (6.58 to 7.65 g) whereas in males it was 3.33 g +/- 0.78 (2.75 to 4.23 g) (t= 5.29, df = 4, P<.007). The average number of days between adjacent spawning events for the pairs used in the study was 21.7 (+/- 3.17, SEM), 18.7 (+/- 5.50), 15.7 (+/- 3.21), 13.7 (+/- 3.06), and 14.0 (+/- 2.00).

2.3.2 Sex differences in parenting behavior

None of the behaviors changed significantly across the breeding cycle (day 0, 3 and 6) (all P-values greater than 0.19). However, there was a tendency for males to spend more time in the nest as eggs developed (P=0.19). Collapsed across the breeding cycle, males displayed 5.6 fold more total parental behaviors than did females (W = 156.5, P<0.0003, n = 5, Fig. 1A). Males also spent 9.3-fold more time in the nest than did females (W = 19, P<0.0004, n = 5, Fig. 1B). No aggressive or submissive behaviors were observed in any of the videos.

2.3.3 Validation of EIA kits

For each kit, the relationship between the dilutions (both sample and standard) and percent bound followed a sigmoidal curve suggesting that the upper and lower dilutions were at or exceeded the kits maximum and minimum measurement capabilities. Those points that did not fit the linear portion of the curve were removed.
**11-KT.** Sample dilutions of 1:16 to 1:128 were found to be linear (F= 388.3, df = 1,4, P<0.0001), and parallel to the standard (F=0.187, df = 1,4, P>0.65; Fig. 2A). Hence, for measuring 11-KT in individual *A. ocellaris*, plasma samples were diluted 1:30 (by mixing 7 µl of plasma with 210 µl of assay buffer) to ensure the concentrations would fall well within the measurable range and in the linear portion of the standard curve. The average intra-assay coefficient of variation from the two technical replicates per sample was 4.4 (+/- 0.68 SEM).

**E2.** Plasma dilutions from 1:8 to 1:128 were found to be linear (F=877.7, df = 1,6, P<0.0001) and parallel to the standard curve (F=0.013, df = 1,6, P>0.9; Fig. 2B). Individual *A. ocellaris*, plasma samples were diluted 1:35 (7 µl of plasma mixed with 245 µl of assay buffer) for analysis of sex differences. The average intra-assay coefficient of variation was 7.5 (+/- 1.08 SEM).

**Cortisol.** Plasma dilutions from 1:4 to 1:32 were found to be linear (F=648.9, df = 1,4, P<0.0001) and parallel to the standard curve (F=0.221, df = 1,4, P>0.22; Fig. 1C). Individual *A. ocellaris*, plasma samples were diluted 1:30 (7 µl of plasma mixed with 210 µl of assay buffer) for analysis of sex differences. The average intra-assay coefficient of variation was 6.7 (+/- 0.90).

### 2.3.4 Sex differences in plasma-hormone concentrations

No relationship between start time, catch time, or blood time and the levels of any the circulating hormones were observed in analyses of covariance (all P-values greater than 0.24), hence only results of unpaired t-tests are shown.

11-KT was significantly higher in males than females (t=10.4, df = 9, P<0.0001; Fig 3A). In contrast, E2 was significantly higher in females than males (t=4.9, df = 9, P=0.0008; Fig. 3B). Cortisol was slightly higher in males than females, though this effect was not statistically significant (t=1.88, df = 9, P=0.09; Fig. 3C).

### 2.3.5 Correlations between hormones and behavior across the breeding cycle

Plasma levels of 11-KT and cortisol were not correlated with any of the behaviors, and showed no obvious changes across the breeding cycle (all P-values greater than 0.46). Plasma levels of E2 were not correlated with any of the behaviors (all P-values greater than 0.68). However, E2 levels significantly rose following a spawning event (during egg production) and subsequently fell prior to egg deposition (Fig. 4). This was indicated by significant linear (F=5.5, df = 1,17, P=0.032)
and polynomial \((F=4.6, \text{df} = 1.17, P=0.047)\) coefficients in the polynomial regression of E2 concentration by day in the spawning cycle.

2.4. DISCUSSION

Information on sex differences in parental behavior and bioactive steroid hormones during egg care are missing from the literature for *A. ocellaris*. In this study, documentation of sex differences in parental effort (Fig. 1) and circulating levels of E2, 11-KT, and cortisol in reproductively active male and female *A. ocellaris* were quantified for the first time (Fig. 2-4). In populations of actively breeding pairs, males spend the majority of their lives with eggs present in the nest, and devote an impressive amount of parental effort, continuously fanning and nipping eggs, cleaning them from debris and keeping them free of fungus. During the 3, five-minute behavioral observation periods, males display an average of almost 40 parental behaviors (e.g., nipping or fanning their eggs), and spend nearly 70% of their time in the nest tending the eggs. This level of effort is high in comparison to some teleost species, and more comparable to others. In the Azorean rock pool blenny, males display roughly 2 acts of nest cleaning per 20 minutes (Ros et al., 2004), and in the convict cichlid, paternal acts occur at 12 per 10 minutes (O’Connell et al., 2012). The effort displayed by blue-banded goby and three-spined stickleback males is comparable to the data presented in this study (Pall et al., 2002; Pradhan et al., 2014b); time in the nest and the number of behavioral acts are also high in these species. Females, although not absent in parental care, display few parental behaviors, and spend less than 15% of time in the nest (Fig. 1).

While there were a high number of parental care behaviors, other social interactions were infrequent. Males and females form pair bonds several months before spawning occurs; therefore, there were no agonistic behaviors and only one event of affiliative behavior. During initial pair formation there are many such interactions, but that period was not recorded for this experiment, and previous work has demonstrated that during initial pair formation, high levels of aggression are apparent (Yaeger et al., 2014b). This lack of aggression may provide a useful context for future studies examining the role of androgens in paternal care, without the confounding effects of aggression on circulating androgens.

We hypothesized that the high level of paternal care displayed by males would result in higher levels of cortisol, a hormone critical for stress response and energy mobilization. While, no significant differences in cortisol between males and females was observed in our study, males tended to have higher cortisol values. The lack of significance may be due to the high variability
within females and small sample size of this study. Cortisol levels in field measurements in *A. melanopus* were at around 15 ng/ml in males and females, but around 60 ng/ml in sexually transitional individuals. The lack of sex differences in mentioned field studies might be attributed to the fact that the breeding status of individuals measured was unknown, or non-breeding following female removal. If cortisol levels reflect parental effort, then it should be higher during the egg brooding period. Unlike, E2, and 11-KT, where the pooled species sample approximated an average between males and females, cortisol values were higher in reproductively active males and females in comparison to the pooled species sample (data not shown). This result is consistent with the notion that parenting is metabolically expensive (Nunes et al., 2001). Breeding individuals are expending more energy during parental care than are those without a brood and the trend toward higher cortisol values in males may reflect increased parental effort during the egg brooding period.

Males displayed higher levels of parental effort than females (Fig. 1). Males also showed higher circulating levels of 11-KT and lower E2 relative to females (Fig. 3A and B). The simultaneously occurring high paternal effort and high levels of 11-KT are inconsistent with the hypothesized tradeoff between high androgens and parental care observed in avian species (Wingfield et al., 1990a). However, this result is consistent with recent studies showing high androgens do not disrupt parental care in gobies and mice. In the blue-banded goby, males that were experienced fathers showed higher 11-KT during care than inexperienced males, and when no eggs were present that difference disappeared (Rodgers et al., 2006b). Moreover, in the monogamous California mouse, high androgens were necessary for paternal care (Trainor and Marler, 2001). In these species, males are also defending territories and may show aggression towards conspecifics at the same time that they are caring for their offspring, potentially confounding the relationship between androgens and paternal behavior. In Pradhan et al. (2014), blockade of 11-KT synthesis specifically reduced parental care without affecting other social behaviors such as aggression. These results suggest a direct causal connection between 11-KT and paternal care that is not a consequence of other simultaneously occurring behaviors. Our results in *A. ocellaris* males are consistent with these findings, in that they provide complimentary evidence that high androgens can be associated with high paternal behavior and not aggression or other social interactions.

11-KT has never been measured in adult *A. ocellaris* prior to this present study. In previous research where steroid hormones were measured in *A. ocellaris*, the samples were collected from non-reproductive juveniles (Iwata et al., 2008), or in natural field populations of closely related *A. melanopus* (Godwin, 1994a; Godwin and Thomas, 1993). In laboratory studies exploring the
relationship between circulating steroid hormones and dominance status in juveniles, 11-KT was highest in α, and lowest in β individuals (Iwata et al., 2008). This is an interesting result as the α eventually becomes the dominant female with lower 11-KT levels than β-males, or subordinates.

Results from this study demonstrate that laboratory reared, captive bred *A. ocellaris* display sex differences in hormones in a manner expected from measurements performed in other closely related species from the field. In field studies of another anemonefish, *A. melanopus*, E2 was approximately 7-fold higher in females than in males, a pattern also supported here (Fig. 3B). Opposite to E2, 11-KT was much higher in male *A. ocellaris* than in females (Fig. 3A). The sex difference was larger than that reported for *A. melanopus* (Godwin, 1994a). While 11-KT occurs at comparable levels in *A. ocellaris* and *melanopus* males, it is much lower in *A. ocellaris* females than *A. melanopus* females. However, measurements are still reasonably within range in comparison to all *Amphiprion* species reviewed (Godwin, 1994a, b; Iwata et al., 2012; Iwata et al., 2008; Mills et al., 2010).

In addition to establishing sex differences in circulating E2 and 11-KT, we also wished to determine whether the levels of these hormones fluctuated across the breeding cycle in a manner consistent with other species. E2 measurements from four time points during the spawning cycle suggest that E2 rises following the spawning event during egg production, and then falls prior to the next spawn (Fig. 4). This pattern is consistent with other teleosts. In the Amago salmon, E2 levels rise during vitellogenesis and fall in ovulated fish (Kagawa et al., 1981). The data here also show that spawning periods are variable, and between individuals the trajectory of E2 vacillation does not follow the same chronology. In some females, E2 rises more quickly following a spawning event, making it difficult to discern patterns across individuals. This is further complicated by the fact that our samples were taken across four separate spawn periods, and E2 variation likely occurs within individuals during each cycle. In the future, time course studies will be performed using waterborne hormone analysis (Kidd et al., 2010). This method will allow repeated hormonal measurements over a single spawning cycle, which could provide more accurate insight into the pattern of E2 variation.

Results suggest 11-KT does not change across the breeding cycle in males, which reflects the lack of behavioral changes in this study. However, as stated above, this result may be confounded by measurements across spawning periods, and future studies using waterborne methods could yield different results. Alternatively, in species such as *A. ocellaris* where eggs are almost always present and spawning non-seasonal, 11-KT may not fluctuate as is required for timed spawning events to regulate sperm production (Fine et al., 2004; Malison et al., 1994; Pankhurst and Peter, 2002),
secondary male reproductive behaviors (Knapp and Neff, 2007), and territory defense (Cardwell and Liley, 1991).

In our laboratory, social groups of *A. ocellaris* consist of a dominant α female, subordinate β male, and 0 or 1 non-reproductive individuals. The lack of predation risk, anemone defense, territory defense against conspecifics, and decreased overall group size are all limitations of this current study. However, in natural populations of *A. ocellaris* their host anemones are spatially isolated, and it is very common for there to be only two individuals in a group (Mitchell and Dill, 2005a). Anemonefishes also have unusually long lifespans exceeding 30 years, 6 times higher than similarly related damselfishes, which has been interpreted to be a result of the efficient protection of predation by their anemone hosts (Buston and Garcia, 2007). Thus, the lack of predation risk in this study may reflect natural conditions. Our results establish robust sex differences in steroid hormones and parental care. Given the high suitability of the species as a laboratory model, we believe our findings consistent with sex differences in hormones and behavior seen in the natural environment.

In summary, results demonstrate sex differences in parental behavior and circulating sex steroids in *A. ocellaris*. Males displayed greater parental behavior than females and this sex difference in parental investment occurred concurrently with higher circulating 11-KT and lower E2 in males as compared to females. Results establish *A. ocellaris* as a relevant model in social neuroscience for studying paternal behavior in the presence of high 11-KT and without the confounding influence of aggression and behavioral dominance.
2.5. FIGURES

**Figure 2.1** Males display greater parental effort than females. A. Average total number of combined nips and fans displayed by females (black bars) and males (white bars). B. Average duration spent in the nest expressed as a percentage of total sample time. Standard error bars shown. Letters ‘a’ and ‘b’ denote significant differences.
Figure 2.2 Validation of EIA kits. Percent Bound as measured by the kits is plotted against the natural logarithm of the dilution ratio (expressed as a percentage, i.e., 1:4=0.25). The standard dilutions are shown as open circles, whereas the pooled sample dilutions are shown as filled circles. Linear regression lines are shown. A. 11-KT kit. B. E2 kit. C. Cortisol kit.
Figure 2.3 Sex differences in circulating steroid hormone levels. A. Average concentration of 11-KT in the plasma of the pooled sample (n=1; grey bar), females (n=5; black bars), males (n=5; white bars). B. Same as A for E2. C. Same as A except for Cortisol. Standard error bars for males and females reflect individual variation. Standard error bars for the pooled sample reflect technical replication.
Figure 2.4 Rising and falling E2 levels across the breeding cycle. Concentration of E2 plotted against days after spawning for each of 5 separate females. Each female is represented by a different symbol. A loess smoothing function was applied to connect the data points separately for each female to facilitate differentiating the individuals.
2.6. REFERENCES


CHAPTER 3:
OPPOSITE EFFECTS OF NONAPEPTIDE ANTAGONISTS ON PATERNAL BEHAVIOR IN THE
TELEOST FISH *AMPHIPRION OCELLARIS*

Abstract

The nonapeptides isotocin (IT) and arginine vasotocin (AVT), along with their mammalian homologs oxytocin and arginine vasopressin, are well known regulators of social behaviors across vertebrate taxa. However, little is known about their involvement in paternal care. Here, we measured the effect of an IT and an AVT V1a receptor antagonist on paternal behaviors in the primarily paternal teleost *Amphiprion ocellaris*. We also measured effect of the IT receptor antagonist on aggression in dyadic contests between two non-reproductive fish to assess specificity of the effect on paternal behaviors. Individual differences in levels of paternal behaviors (nips, fanning the eggs, and proportion of the time in the nest) were consistent across spawning cycles when no treatments were administered. The IT receptor antagonist severely reduced paternal behaviors but had no effect on aggression, whereas the AVT V1a receptor antagonist increased paternal behaviors. These results support the idea that IT signaling is crucial for the expression of paternal behavior in *A. ocellaris*. Based on a previous study showing that the AVT V1a antagonist decreases aggression in dyadic contests, we hypothesize that the antagonist enhances paternal behavior indirectly by reducing vigilance and aggression, thereby alleviating effort directed towards other competing behaviors and allowing for the increased expression of paternal behaviors.

3.1. INTRODUCTION

Parental care is a costly investment on the part of the caregiver as it presents a trade-off between current parental investment and opportunities for future reproductive events (Clutton-Brock, 1991; Trivers, 1974). Often, this trade-off is different for each sex. Male care is less common, as males produce a large number of metabolically inexpensive gametes, and generally have higher reproductive success siring as many offspring as possible (Kokko and Jennions, 2012; Trivers, 1972a). Thus, paternal care is predicted to occur only when the cost of desertion is high, or future reproductive opportunities are low (Gross and Sargent, 1985). Conversely, females produce fewer gametes that are larger in size, and generally have higher reproductive success when effort is directed towards egg survival (Gross, 2005; Trivers, 1972a). High rates of maternal care has

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consequently led to a bevy of studies on the evolution and underlying neural regulation of female parental care. However, unlike female parental care, there have been relatively few studies examining mechanisms underlying male parental care, despite the fact that in many species exhibiting male parental care, paternal effort is of equal importance to maternal care, and in some species even more important than female care for offspring survival (Kleszczyńska et al., 2012; Ripley and Foran, 2010; Rodgers et al., 2006a; Trainor and Marler, 2002).

While paternal care is relatively uncommon, teleost fishes are a unique group among vertebrates in which male parental care is the predominant parental care strategy (Baylis, 1981; Magee et al., 2006; O'Connor et al., 2009; Pradhan et al., 2014a). Recent work has presented evidence of a highly evolutionarily conserved social decision-making network. More specifically, the brain regions, neuropeptides, and hormones involved in the regulation of social behaviors share similar pathways across vertebrate taxa (O'connell and Hofmann, 2011). Thus, teleosts present interesting opportunities to gain insight into the regulation of vertebrate parental care (Amundsen, 2003a; Gross and Sargent, 1985; Ridley, 1978). To date, the few species of teleosts where paternal care has been explored present confounding results, which may in be in part due to the often simultaneously occurring social displays such as courtship and territory defense (Kleszczyńska et al., 2012; O'Connell et al., 2012). Hence, the high homology of the underlying circuitry involved in the regulation of social decisions allows insights to be gained broadly about fathering by studying species where high levels of paternal care are exhibited and in which care can be isolated form other confounding social behaviors (Amundsen, 2003a; Goodson, 2005; O'Connell et al., 2012).

The anemonefish, *Amphiprion ocellaris* is a predominantly paternal care species in which male care is critical for offspring survival (DeAngelis and Rhodes, 2016). Their obligate symbiosis with protective host sea anemones and highly monogamous lifestyle has removed opportunities of finding additional mates. (Godwin, 2009; Mitchell and Dill, 2005b). Hence, the substantial effort of parental care does not come at the expense of seeking additional reproductive partners, as in other species where additional mating opportunities are more pervasive. This leads to an increased fitness by directing effort towards current brood survival and enables research to be directed specifically at paternal care in isolation of other confounding social displays. In addition, *A. ocellaris* are particularly well suited for laboratory studies on paternal care due to their small home ranges and adaptability to aquarium conditions (Iwata et al., 2008). They also have a short generation time (1 year) and spawn readily in captivity. Parental behaviors are easily observed and quantified, and sex
differences in parental care have already been established. Males are the predominant caretakers, and spend the majority of their lives caring for eggs (DeAngelis and Rhodes, 2016).

As an initial foray into the regulation of the high levels of paternal care displayed by *A. ocellaris*, the vasopressin and oxytocin systems were logical candidates for exploration. The vasopressin and oxytocin systems are well situated for functioning in parental care as cell bodies containing these neuropeptides reside in the preoptic area of the hypothalamus, an area of the brain well known for regulating reproductive behaviors (Insel and Young, 2000; Kline et al., 2011). The cell bodies and dendrites heavily express sex steroid receptors, and project axons all over the brain, the terminals of which release the neuropeptides onto multiple neuron types (Foran and Bass, 1999). Receptors for these neurochemicals are known to be expressed in crucial brain areas that comprise the social decision-making network such as ventral midbrain, basal ganglia, and hippocampus (O'Connell and Hofmann, 2011). Nonapeptide cells in the brain receive information from the gonads via the blood and relay that information to the rest of the brain for making social decisions related to reproduction (Maruska and Fernald, 2011). While a relationship between these highly conserved neuropeptides and maternal care and other social behaviors has been identified, the extent to which these nonapeptides play a role in paternal care remains unclear.

The neuropeptide oxytocin (OT) has been well studied for its role in female parental care; its release at parturition is a catalyst for an array of behavioral and physiological changes critical for offspring survival (Bartz et al., 2010; Francis et al., 2000). While less understood in males, it has been suggested that OT plays a similar role in paternal behavior. In humans, OT rises in males following contact with an infant (Feldman et al., 2010); in the monogamous California mouse *Peromyscus californicus*, OT levels are higher in expectant fathers (Gubernick et al., 1995); and in teleost *Amatitlania nigrofasciata* blockade of the IT receptor reduced paternal effort (O'Connell et al., 2012). These results support a conserved function of OT in the promotion of parental care.

Like OT, the neuropeptide AVP/AVT has a well-documented role in the regulation of social behaviors across a wide array of vertebrates, and has been suggested to be more important in regulating male social behaviors (Insel and Young, 2000). In teleosts, AVT is broadly implicated in behaviors leading to reproduction, but its specific function varies depending on species and social status (Foran and Bass, 1999; Insel and Young, 2000; Kleszczyńska et al., 2012). More specifically, AVT has been implicated in the regulation of dominance, aggression, and courtship (Huffman et al., 2015; Semsar et al., 2001a; Yaeger et al., 2014a). AVP/AVT is clearly important in a variety of male
social behaviors, but surprisingly few studies have addressed its role in paternal care, and therefore how AVT signaling functions to promote or inhibit male parental care remains unclear.

The goal of this study was to determine the extent to which AVT and IT signaling play a role in the modulation of paternal care in a species where high paternal effort can be isolated in the absence of other confounding co-occurring social behaviors often exhibited in other species. In this study we hypothesize that IT signaling is critical for high levels of paternal care, and thus, blockade of IT signaling will reduce total parental effort. Given that blockade of AVT V1a receptors reduced aggression in A. ocellaris, and the diversity of roles reported for AVT in the regulation of multiple different competing social behaviors, we were not certain how blockade of V1a receptors would affect paternal care.

3.2. MATERIALS AND METHODS

3.2.1 Animals and Husbandry

A. ocellaris, bred in the laboratory from a female obtained from ORA (Oceans Reefs and Aquariums, Fort Pierce, FL), and a wild caught male (location unknown; obtained through the pet trade) were used. Tank conditions for all individuals were set to mimic the natural environment, with a temperature of 79°F, photoperiod of 12:12 (lights on at 7:00 am and off at 7:00 pm), pH of 8.2 and specific gravity of 1.026. Individuals were housed in groups of 2 or 3 in 20-gallon aquariums, and allowed over a year for consistent spawning (spawn period of less than 20 days) prior to the onset of behavioral observations or experimental manipulations. Each tank contained one clay pot (4-inch diameter) to serve as the nest site where the fish deposit their eggs. Lengths and weights were taken one week prior to the start of the experiment and then again at the conclusion of the study. In experiment 1, mean body weight and standard length were 2.97 g (range 1.60 – 5.70 g) and 53 mm (45 – 70 mm) for males and 7.38 g (4.60 – 9.60 g) and 70 mm (46 – 96 mm) for females. In experiment 2, mean body mass and standard length of the males were 2.60 g (range 1.88 – 3.44 g) and 45 mm (38 – 53 mm). Fish used in experiment 1 were approximately 24 months of age, while fish used in experiment 2 were approximately 18 months of age. All fish in experiment 1 had previously been observed caring for a batch of fertilized eggs and were established as reproductively mature. The fish in experiment 2 were non-reproductive males taken from a holding tank, which contained greater than 20 fish (i.e., no reproductive pairs could be established). Adequate measures were taken to ensure minimal pain and discomfort for all animals used in experimental procedures.
3.2.2 Experiment 1: Effects of AVT and IT Antagonists on Paternal Behavior

Baseline measures of parental behavior, sex differences and consistency across breeding cycles

Aquariums that contained reproductive pairs (N=8) were recorded daily for a 10-minute behavioral observation period (between 2:00-3:00 pm daily) over the entire spawn cycle. Behavioral analysis for spawn period 1 (SP1) began on the day the eggs were laid (day 0) and ended when eggs hatched into the larval phases (7-9 days). Parental behaviors were scored using JWatcher behavioral event recording software (Blumstein and Daniel, 2007). The amount of time spent in the nest, as well as the total number of nips and fans were quantified for the reproductive male and female in each aquarium. Nips are defined as mouthing the eggs to keep them clean of debris and fungus, while fanning is the process of using the pectoral and caudal fins to aerate the eggs. Total numbers of parental behaviors (the sum of nips and fans) were analyzed. This procedure was repeated again during spawn period 2 (SP2) to test the extent to which individual variation in behavior was consistent across spawn periods, before starting the pharmacological manipulations.

Pharmacological Manipulations

Only males received the pharmacological manipulations. Following the analysis above, during the subsequent spawn period 3 (SP3), males were restrained by hand and received an intraperitoneal (i.p.) injection (BD syringe, 3 mm, 26 gauge) of 0.9% saline (control) at 10 µl/gram body weight on days 4, 5, and 6 after the eggs were laid (N=8). These days were chosen based on when the greatest rise in parental effort was observed during SP1 and SP2 (see Results section). Immediately after the injection, fish were placed back into their home aquarium. Parental behaviors were recorded as described above, 30-minutes after the injection for a 15-minute duration. Behavioral analysis started 30-minutes post injection to allow fish enough time to recover from handling stress but still within the time window when the antagonists are expected to be at pharmacologically significant levels in the brain (O’Connell et al., 2012; Semsar et al., 2001a).

During spawn period 4 (SP4), males were given an i.p. injection of an IT receptor antagonist, again on days 4, 5, and 6, and behavior scored a similar way (N=8). The oxytocin receptor antagonist desGly-NH2-d(CH2)5[D-Tyr2,Thr4]OVT (a gift courtesy of Dr. Maurice Manning) was administered at a
dose of 0.5 µg/g body weight. This dose was chosen, as it is the minimal dose needed that is known to affect paternal behavior in the closely related cichlid fishes Amatitlania nigrofasciata and Neolamprologus pulcher (O’Connell et al., 2012; Reddon et al., 2014).

Individuals were then allowed 3 spawn periods (approximately 28 days for most pairs) to recover from any effects caused by the IT antagonist injections. Following this period, fish were then given the AVP (V1a) receptor antagonist d(CH₃)₅[Tyr(Me)²]AVP, Manning compound (Fisher Scientific, Waltham, MA, USA) again on days 4, 5, and 6, and behavior scored as described above (N=7). A dose of 3.2 µg/g body weight at a volume of 10 µl/g body weight was used as this dose is known to block V1a receptors in the brain of teleosts, with almost no antidiuretic activity (Guillon et al., 2004). Moreover, this dose was previously found to block aggressive behavior in inter-male contests between A. ocellaris (Yaeger et al., 2014a). Antagonist treatments were given serially rather than counterbalanced, a limitation addressed further in the discussion.

3.2.3 Experiment 2: Effect of IT Antagonist on Paired Aggression Trials

To determine the extent to which the IT antagonist alters aggressive and/or submissive behaviors, we used a previously validated paired aggression test which revealed positive results for the AVT antagonist (Yaeger et al., 2014a). Two non-reproductive males were removed from their home aquariums where they were group housed with approximately 30 individuals, and each was placed into a separate 5-gallon bucket to await their injection. Fish were selected from separate home tanks so that in each trial, individuals had never interacted before and there were no established dominance relationships among individuals tested. Fish were sized matched to within 0.2 grams body weight, and 4 mm in length. One fish was randomly assigned to receive a saline injection of 10 µl/g body weight (N=18), and the other an IT antagonist injection at a dose of 0.5 µg/g body weight (N=18), as described above. After the fish were given their injections, they were placed back into their 5-gallon buckets for a 30-min holding period. Following this 30-minute period, the two size matched fish were simultaneously placed into a 15-gallon aquarium and video recorded for 10 minutes. Aggressive, affiliative and submissive behaviors were scored. Bites and charges (rapid approach with an outstretched operculum) were scored as aggressive. Approaches (non-aggressive encounter), concurrent swimming (swimming within a body length), and touching were scored as affiliative, while quivering (rapid shaking, a submissive behavior in A. ocellaris) and fleeing were scored as submissive.
3.2.4 Statistical Methods

Data were analyzed using R (version 3.3.0 'Supposedly Educational') statistical software (Team, 2013). P < 0.05 was considered statistically significant. To establish consistency of individual differences in paternal behavior across the two baseline spawn cycles, data were analyzed using a linear mixed model (Pinheiro et al., 2014). Total parental behaviors and duration in the nest across all the days were analyzed as a function of spawn period (SP1 and SP2) with each aquarium or pair (as the unique identifier) entered as a random effect in the model to account for the repeated measures between spawn cycles. Significance of the random effect was evaluated using a chi-square log-likelihood test comparing the model with versus without the random effect. Lack of an effect of spawn cycle and a significant amount of variation explained by the aquarium random effect was taken to indicate that the behavior was consistent across spawn periods with repeatable differences in behavior represented by the different pairs. Effect sizes were calculated from the variance components estimated by the mixed models and are indicated as R²*.

Sex differences in parental behaviors across the breeding cycle were analyzed using a linear mixed effects model with day entered as the within-subjects factor, and sex as a between subjects factor. The behaviors were averaged across the SP1 and SP2 to produce one value per day per sex. Behavior of each sex was also analyzed separately using a simpler mixed model with only day entered as the within-subjects factor. Post-hoc, pair-wise differences between means were analyzed using Tukey tests. Effect sizes for model parameters were given as eta². To account for the repeated structure, data were first were analyzed with a one-way ANOVA with tank as a fixed effect (tank was entered as a random effect in the linear model described above). Residuals from this model were then analyzed by a two-way ANOVA and eta² extracted. The effect sizes for paired comparisons were given as Cohen’s D.

The effect of the IT and AVT antagonists on paternal behaviors were analyzed as follows. First, the behaviors (total parental behaviors and duration in the nest) were averaged for each individual on the days fish received injections (days 4, 5, and 6 after the spawning event). These values were then analyzed using a linear mixed model with treatment entered as a fixed effect (4 levels: non-treated control, the saline injection, the IT antagonist, and AVT antagonist) and subject entered as a random effect to account for the repeated measures across the three treatment levels. The non-treated control was the average of both SP1 and SP2 for days 4, 5, and 6 to produce values (for parental behaviors and duration in nest) that were comparable to the saline and neuropeptide
receptor antagonist values. Post-hoc, pair-wise differences between means were analyzed using Tukey tests (Hothorn et al., 2008). Effect sizes were calculated as described above.

For the paired aggression trials, data were not normally distributed and were analyzed using a paired non-parametric Mann-Whitney U test. Total number of aggressive behaviors and submissive behaviors were separately compared, within subjects, between the IT-antagonist treatment relative to the saline control.

3.3. RESULTS
3.3.1 Experiment 1
Baseline measures of parental behavior, sex differences and consistency across breeding cycles

Levels of paternal care and duration in the nest were not significantly different between spawn periods (Fig. 1). However, certain males displayed elevated levels of paternal effort (Fig 1A) and duration in the nest (Fig. 1B) compared to others, which was repeated across both spawn periods. This was indicated by a significant random effect of individual (for total parental behaviors, \(R^2* = 0.69, P<0.001\), and proportion time in nest, \(R^2* = 0.76, P<0.001\)) in the linear mixed models, but no effect of spawn period.

The total numbers of parental behaviors (Fig. 2A) and duration in the nest (Fig. 2B) were significantly higher for males than females. This was indicated by a significant effect of sex in the linear model (total parental effort, \(\eta^2 = 0.51, F_{1,14} = 46.2, P<0.001\); proportion time in nest, \(\eta^2 = 0.65, F_{1,14} = 65.9, P<0.001\)). In both males and females, parental effort and duration in the nest escalated as the days progressed, with the highest levels in the latter half of the breeding cycle, however the pattern was slightly different between the sexes (Fig. 2). This was indicated by a significant effect of day (total parental effort, \(\eta^2 = 0.13, F_{9,106} = 7.7, P<0.001\); proportion time in nest, \(\eta^2 = 0.13, F_{9,106} = 9.5, P<0.001\)) and a significant interaction between day and sex for total effort (\(\eta^2 = 0.07, F_{9,106} = 3.8, P<0.001\)). For proportion of time spent in the nest, there was only a trend for an interaction between day and sex (\(\eta = 0.03, F_{9,106} = 1.8, P=0.084\)). No other main effects or interactions were significant.

Considering males alone, parental effort escalated during the spawning period (Fig. 2A, \(\eta^2 = 0.49, F_{9,53} = 6.0, P<0.001\)), where it was lowest on day 1 and highest on day 8, with the most dramatic increases from days 4 to 5 and 5 to 6. Total parental effort was higher on days 6-8 compared to days 0-4 (all Cohen’s D > 1.4, P<0.05). Days 4-6 were targeted for pharmacological
manipulations in subsequent spawn periods (see below) because this was the period of greatest escalation of paternal behavior. Duration spent in the nest also escalated as the eggs matured (Fig. 2B, \( \eta^2 = 0.48, F_{9,53}=5.8, P<0.001 \)), but the time-course was slightly different. Duration in the nest was high on day 0, and then lowered during days 1-4 before rising again on days 5-8. The largest increases in duration spent in the nest were from days 4 to 5, and from days 6 to 7. Proportion time spent in the nest was significantly higher on days 6-8, than 1-4 (all Cohen’s D > 1.10, P<0.05).

Considering females alone, parental effort (\( \eta^2 = 0.40, F_{9,53}=4.1, P<0.001 \)) and proportion of time in nest also escalated as the spawning period progressed (\( \eta^2 = 0.46, F_{9,53}=5.4, P<0.001 \)). Both maternal effort and time in the nest started high the day the eggs were laid and the following day after, and then lowered on days 2, 3 and 4, until escalating on days 5 through 9. Total parental effort and proportion time in the nest were lower on days 2, 3 and 4 than the other days (all Cohen’s D > 0.85, all P<0.05).

**Effect of nonapeptide antagonists on paternal care and duration in nest**

**Paternal care**

The repeated measures ANOVA indicated significant differences between the treatments for total number of paternal behaviors (\( \eta^2 = 0.57, F_{3,20}=27.8, P<0.001 \); Fig. 3A). Post-hoc analyses of pair-wise differences between means indicated that all pair-wise comparisons were significantly different from each other except saline and no-treatment controls (Cohen’s D = 0.45, P=0.64). The IT antagonist decreased total number of paternal behaviors, whereas the AVT antagonist increased paternal behaviors relative to both the saline (IT-Antagonist, Cohen’s D = 2.28, P<0.001, AVT-Antagonist, Cohen’s D = 0.97, P = 0.0038) and no-treatment controls (IT-Antagonist, Cohen’s D = 5.09, P<0.0001, AVT-Antagonist, Cohen’s D = 1.44, P<0.001). The IT and AVT groups also differed from each other (Cohen’s D = 2.58, P<0.001).

**Duration in the nest**

Duration of time spent in the nest was positively correlated with total paternal behaviors across treatments (\( F_{1,90}= 108.4, P<0.001, R^2 = 0.55 \)). If a father in the nest was not actively caring for the eggs, it was simply swimming in one place in the nest next to the eggs and not otherwise engaging in any other behaviors.

The repeated measures ANOVA indicated significant differences between the treatments for duration spent in the nest (\( \eta^2 = 0.61, F_{3,91}=13.57., P<0.0001 \); Fig. 3B). Posthoc analyses of pair-wise
differences between means indicated that only the IT antagonist differed from the other groups. The IT antagonist significantly reduced duration in the nest relative to the saline (Cohen’s D = 1.6, p=0.001), no-treatment control (Cohen’s D = 2.7, P<0.001), and AVT antagonist (Cohen’s D = 1.6, P<0.001). No other pair-wise differences were significant.

3.3.2 Experiment 2

Effect of IT antagonist on aggression

The IT antagonist had no influence on aggressive or submissive displays compared to saline controls. Individuals treated with the IT receptor antagonist displayed an average of 79.4 (+/- 20.54) aggressive acts, while saline treated animals displayed an average of 133.4 (+/- 32.66) aggressive acts (P=0.083). Individuals treated with the IT receptor antagonist displayed an average of 6.5 (+/- 2.26) submissive behavioral acts while those treated with saline control displayed an average of 10.5 (+/- 4.53) submissive acts. Displays of affiliative behaviors were extremely rare, and due to low occurrence, were not statistically analyzed (data not shown).

3.4. DISCUSSION

While the literature has established that both AVT/AVP and IT/OT are important neuromodulators of social behavior in vertebrates (Foran and Bass, 1999; Goodson, 2008; Goodson and Bass, 2001; Insel and Young, 2000), few studies have addressed the role of these neuropeptides in species that display predominantly male parental care where paternal care can be isolate form other co-occurring behaviors. The majority of the research has focused on female parental care where IT/OT signaling has proven critical for offspring survival (Bales and Carter, 2003; Francis et al., 2000; Olazabal and Young, 2006; Strathearn et al., 2009). Because of the diverse roles that these nonapeptides display in a species and ecological-context specific manner, it is important to explore their role in a primarily paternal care species. This work extends the literature by establishing that IT signaling is critical for paternal care in A. ocellaris. As opposed to IT/OT, the literature on AVT and parental effort is less consistent and more species specific (Foran and Bass, 1999; Kleszczyńska et al., 2012; Semsar et al., 2001a). Here, these data add to the literature by showing that blockade of AVT signaling increases paternal care in a primarily paternal care species (Fig. 3). This is an intriguing result as males of this species already display incredibly high amounts of paternal behavior. The literature has established that in many teleost species, including A. ocellaris, AVT signaling is important in the regulation of dominance, aggression, and nest defense (DeAngelis and
Rhodes, 2016; Greenwood et al., 2008; Kleszczyńska et al., 2012; Semsar et al., 2001a; Yaeger et al., 2014a). We speculate that in *A. ocellaris*, blockade of AVT may increase paternal behavior by reducing attention toward vigilance and nest defense, thereby releasing attentional resources and effort to be directed towards parental care. However, this hypothesis was not directly tested here, and would have to be tested empirically before it can be confirmed. Taken together, these results establish the importance of both IT and AVT signaling as oppositely regulating paternal care in *A. ocellaris*.

3.4.1 IT and Paternal Care

This study demonstrates that IT signaling is critical for high levels of parental effort in a species that exhibits predominantly paternal care in isolation from other simultaneously occurring social behaviors. There was ample reason to suspect that the role of IT signaling could be different in *A. ocellaris* than in other species studied. In the uniparental three-spined stickleback, in which males are the sole caretakers of the eggs, IT does not appear to stimulate paternal behavior. Whole-brain IT levels, measured via high-performance liquid chromatography were highest during courtship and then lowered during parental care. IT was also higher in subordinate males displaying nuptial coloration compared to those not vying for a dominance position and reproductive opportunities (Kleszczyńska et al., 2012). These data suggest that IT is important for territory defense, and social status, but not necessarily paternal effort. However, in sticklebacks, males build nests where they actively defend a territory, court females, and care for broods. In this species males display many different behaviors simultaneously, making it difficult to decouple the underlying neurobiological mechanisms specific to each behavioral phenotype.

In the majority of other species studied where males display parental behaviors, OT\IT signaling promoted paternal care similar to *A. ocellaris*. For example, in the bi-parental common marmoset both low (1.0 µg) and high (5.0 µg) doses of OT decreased the amount of food refusals to offspring in fathering males, implying that increased OT levels make fathers more attentive to their offspring (Saito and Nakamura, 2011). Similarly, in the bi-parental teleost fish, *Amatitlania nigrofasciata*, IT neurons in fathering males displayed increased c-Fos expression, suggesting higher activity of IT positive cells during parental care. Further, blockade of IT signaling in this species reduced total parental effort (O’Connell et al., 2012). Additionally, in the bi-parental California mouse *Peromyscus californicus*, plasma OT levels varied across the reproductive cycle and were higher in expectant fathers than non-expectant fathers. Specifically, plasma OT rose 1 day post
copulation and remained elevated for 15 days (Gubernick et al., 1995). Together, these data suggest that the role of IT/OT signaling in *A. ocellaris* is similar to other vertebrate species independent of the parental strategy employed.

### 3.4.2 IT and Aggression

One possible interpretation of the IT antagonist result on paternal behavior is that the dose that was used non-specifically impaired brain function (e.g., made the animal sick, or generally reduced activity) rather than specifically interfered with neural circuitry, which supports parenting behaviors. However, this non-specificity interpretation seems unlikely given that in separate trials, using the same dose, the IT antagonist had no influence on aggression, submissive or affiliative behaviors during the dyadic contests. If the result was non-specific, the IT antagonist would have been expected to impair behavioral performance in the dyadic contests as well, however, no effect on behaviors was observed. Consistent with data shown here, in the closely related damselfish *Stegastes leucosticus*, IT injections had no effect on the number of aggressive displays directed towards an intruder, while AVT increased aggression in the same experiment (Santangelo and Bass, 2006). Similarly with findings presented here in *A. ocellaris*, these data suggest a conserved role of IT signaling in paternal care and a limited function in aggression and dominance. However, it is important to note that we did not test aggression in the context of parenting. Had males been tested for aggression while also exhibiting parental care by using the introduction of a stranger male, or nest predator, different results may have been observed.

### 3.4.3 AVT and Parental Care

To the best of our knowledge, this is the first study to find that blockade of the AVT V1a receptor increases parental behavior in a vertebrate species. This result was surprising as parental effort is already extraordinarily high in *A. ocellaris*, and therefore it was unclear whether or not it would be possible to further increase paternal effort. Additionally, in previous studies exploring the role of AVT in parental effort the opposite result was found. In meadow voles, a 3 ng dose of AVP increased paternal behaviors in previously non-parental males, but had no effect in experienced males, while an AVP antagonist reduced care (Parker and Lee, 2001), the opposite effect found in this current study. Similarly, in two species of pipefishes, AVT was higher in parenting males compared to those that were not parenting (Ripley and Foran, 2010). It is possible that AVT signaling has a different role in *A. ocellaris* as compared to other species as a result of their unique
life history characteristics. More species encompassing a broad diversity of social systems and parental strategies need to be explored in order to identify the specific function of AVT signaling in the regulation of parental care.

The differences between data presented here, and results from meadow voles and pipefishes could be related to the fact that the form of parental care exhibited by these species differs from those seen in *A. ocellaris*. Meadow voles directly care for live young (Parker and Lee). The social bond is different than what is observed in *A. ocellaris* where males provide care only during egg development. Hence, it is possible that AVT plays an important functional role in the bond between sire and offspring and consequently facilitates paternal care. Pipefishes also display dissimilar parental dynamics to *A. ocellaris*. Pipefishes are the only vertebrates where males possess a brood pouch. Offspring are transferred from the female to a placenta like structure within the male where eggs are reared (Ripley and Foran, 2010). In contrast to *A. ocellaris*, the paternal investment of pipefishes is not high levels of behavioral acts, but high demand of the physiological needs of the offspring. This suggests that AVT may have differing roles in the regulation of paternal behaviors across vertebrate species depending on the type of parental care exhibited, and/or the intensity of other competing social behaviors.

We speculate that in *A. ocellaris*, blockade of AVT receptors may have increased paternal effort indirectly by decreasing attentional resources directed towards vigilance behaviors such as territory and nest defense. AVT signaling enhances aggression in several teleost species. In the blue-head wrasse (*Thalassoma bifasciatus*) and burtoni cichlid (*Astatotilapia burtoni*), AVT signaling is critical for social dominance, where administering an AVT receptor antagonist inhibited dominance ascension (Huffman et al., 2015; Semsar et al., 2001a). Similarly, in *A. ocellaris*, blockade of AVT reduced aggression and led to a higher probability that a fish would display subordinate behavior in a dyadic aggression trial (Yaeger et al., 2014a). In a vigilance task trial given to human males, individuals that received 20 IU of AVP via nasal spray showed increased brain activity to a tone stimulus task compared to the placebo (Fehm-Wolfsdorf et al., 1988), supporting the hypothesis that AVT/AVP signaling is an important response for vigilance behaviors. In this present study, no nest predators or intruders were present, and thus the direct role of AVT in behaviors other than parental care was not tested. Future research on this topic is needed to broaden our understanding of the specific role AVT signaling plays in the regulation of vigilance behaviors in *A. ocellaris*. 
3.4.4 Limitations

One limitation of the AVT antagonist data for evaluating effects on paternal behavior (Experiment 1) is that the treatment occurred after animals had received an acute injection of IT antagonist and it is possible that a prolonged effect of the IT antagonist interacted with the AVT antagonist to produce the effect observed here. Recall that at least 28 days separated treatments which we assumed would be sufficient to wash away IT effects, but we did not evaluate this empirically, and to the best of our knowledge there is no information from the literature to evaluate the likelihood of prolonged interactions of an acute IT antagonist with an AVT antagonist on paternal behaviors. The reason the antagonists were administered serially rather than in a counterbalanced fashion is because it was our intention to establish enough information for a single treatment before moving to the next. The treatments were evaluated within subjects, and each subject had their own spawning cycles that varied over the course of several months, hence it is not as if treatments could be applied to a number of individuals at once. Had we not observed a response to the IT with the first injection, we would have changed the dose before moving to the next treatment. Although it is beyond the scope of the present study to administer the AVT antagonist in absence of the IT antagonist, or in a counterbalanced fashion, we wish to alert the reader to this potential confound in our analysis.

In this current study we used peripheral injections of AVT V1a and IT receptor antagonists which are purported to block receptors in the brain, but where those receptors are located, and how they function to influence the cascade of physiological changes associated with social behaviors was not explored. While studies have identified a variety of brain regions involved in parental care, the pre-optic area of the hypothalamus (POA) has been consistently implicated. Within the POA, AVT and IT neurons project directly to other parts of the brain and to the pituitary, where peptide release is involved in the regulation of gonadal steroid hormones. Therefore, variation and the size and number of AVT and IT containing neurons may influence gonadal steroidogenesis. While peptide release influences production of gonadal steroid hormones, AVT and IT neurons in the brain also contain hormone receptors, and thus circulating levels of gonadal steroids also affect the function of AVT/IT neurons. Paternal behaviors are likely mediated by a reciprocal interaction between the neuropeptides and gonadal hormones and future work is needed to identify the specific molecular cascades that connect AVT/IT signaling to high levels of paternal care exhibited by A. ocellaris males.
3.5. CONCLUSION

We used nonapeptide receptor antagonists to block signaling while monitoring paternal behaviors during egg rearing in *A. ocellaris*. Blockade of IT signaling reduced male parental effort, but had no effect on aggression, submission or affiliative behaviors in separate dyadic trials suggesting that the reduced parental care was not a trivial, non-specific effect of the IT antagonist dose. Conversely, blockade of AVT signaling increased male parental effort. Taken together with previous studies highlighting the role of AVT in aggression, we speculate that AVT blockade may have increased parental effort indirectly by shifting attentional resources away from vigilance and aggression, and thus allowing more effort to be directed towards parental care, though empirical data would need to be collected before the AVT vigilance hypothesis can be confirmed.
Figure 3.1 Consistency of baseline levels of paternal behaviors. A) Box plot of total number of paternal behaviors (sum of nips and fans) displayed by 8 different males over two adjacent spawn periods (SP1 and SP2). B) Consistency of proportion of time spent in the nest. Significant differences were detected between males, but not between spawn periods. The line within the bars represents the median. The purple diamonds between the adjacent bars represents the mean of SP1 and SP2 values.
Figure 3.2 Sex differences and patterns of parental behaviors across the spawning cycle. A) Average total number of parental behaviors (averaged across SP1 and SP2) are plotted per day separately for each sex (males in blue and females in red). B) Proportion of time in the nest is plotted. Loess curve and standard error bars are shown.
Figure 3.3 Effects of IT and AVT V1α receptor antagonists on paternal behaviors. A) Average total number of paternal behaviors ± SE plotted for no-treatment (average of SP1 and SP2), in response to saline injection, IT antagonist, and AVT antagonist. The lines connecting the means correspond to the 8 individual males that were tested. The IT receptor antagonist significantly reduced paternal behaviors whereas the AVT V1α receptor antagonist significantly increased paternal behaviors. B) Proportion of time in the nest is shown. The IT antagonist reduced proportion time in nest relative to the other groups. Different letters indicate means are significantly different from each other by Tukey posthoc test at p<0.05 level.
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CHAPTER 4:
DYNAMIC REGULATION OF BRAIN AROMATASE AND ISOTOCIN RECEPTOR GENE EXPRESSION
IN AMPHIPRION OCELLARIS PAIRS DEPENDING ON BREEDING STATUS
(In Press, Hormones and Behavior)

Abstract
Fathering behavior is critical for offspring survival in many species across diverse taxa, but our understanding of the neuroendocrine mechanisms regulating paternal care is limited in part because of the few primarily paternal species among the common animal models. However, many teleosts display primarily paternal care, and among the teleosts, anemonefish species are particularly well suited for isolating molecular mechanisms of fathering because they perform the behaviors in isolation of many other typically competing behaviors such as territorial defense and nest building. The goal of this study was to determine the extent to which whole brain gene expression levels of isotocin receptors, arginine vasotocin receptors, and aromatase as well as circulating levels of the bioactive sex steroid hormones estradiol (E2) and 11-ketotestosterone (11KT) vary in association with parenting behavior in *Amphiprion ocellaris*. Brain aromatase and IT receptor gene expression were higher in both males and females that were parenting versus not. IT receptor expression was overall higher in males than females, which we interpret is a reflection of the greater parental effort that males display. Aromatase was overall higher in females than males, which we conclude is related to the higher circulating E2, which we hypothesize crosses into the brain and increases aromatase transcription. Results suggest both aromatase and IT receptors are dynamically upregulated in the brains of *A. ocellaris* males and females to support high levels of parental effort.

4.1. INTRODUCTION
Given the diverse and environmentally dependent nature of sociality, individuals are often required to make rapid and dynamic behavioral shifts within their collective societies. As social circumstances and group dynamics vary, individuals must find ways to modify their behavior in order to maximize their fitness within the social structure (Lema et al., 2015; O’Connell and Hofmann, 2011; O’Connell and Hofmann, 2012). Understanding how species and sex specific behaviors are coordinated among, and within individuals is of keen interest in social neuroscience, especially as behavioral variation corresponds to variation within the brain (Goodson, 2008; Lee et al, 2009; Okhovat et al., 2015; Olazabal and Young, 2006). One such example of a stark behavioral
shift is male parental care. In contrast to maternal care, where pregnancy or gravidity induce a suite of physiological and hormonal changes prior to parturition or egg laying, physiological cues to fathers are more subtle. Notwithstanding the physiological subtleties priming fathers to care, fatherhood is often critical for offspring survival. Of course human males father, and this behavior is displayed in a highly variable way. Consequently, fatherhood has recently become a highly socially relevant topic (Bales and Saltzman, 2016; Saltzman and Ziegler, 2014).

One obstacle that has limited our understanding of fathering behavior is the paucity of primarily paternal species among the common animal models. In mammals, male care is rare, and when it does occur, is often accompanied by active courtship and nest building, which may modify the brain prior to parental care (Dulac et al., 2014; Wang et al., 1999; Young et al., 2005). This combination makes disentanglement of the underlying neural substrates for parental behavior challenging. Compared to mammals, male care is more common among teleost fishes, and recent findings in fish models has broadened our understanding of the neuroendocrine regulation of fathering behavior in vertebrates (DeAngelis et al., 2017a; DeAngelis and Rhodes, 2016; Kleszczyńska et al., 2012; Knapp et al., 1999; Kulczykowska and Kleszczyńska, 2014; O'Connell et al., 2012; Pradhan et al., 2014c; Rodgers et al., 2006a).

The nonapeptides arginine vasopressin (AVP) and oxytocin (OT), as well as their non-mammalian homologs arginine vasotocin (AVT) and isotocin (IT), have been implicated as key neuromodulators in a variety of social behaviors, including parental care (Bales et al., 2004; DeAngelis et al., 2017a; Feldman et al., 2010; Gubernick et al., 1995; Insel and Young, 2000; Kozorovitskiy et al., 2006; Kulczykowska and Kleszczyńska, 2014). AVP/AVT and OT/IT neuron cell bodies reside solely within the preoptic area of the hypothalamus (POA), and project widely throughout the brain (Goodson, 2008; O’Connell et al., 2012; Olazábal et al., 2013; Rosenblatt and Ceus, 1998).

Cumulative evidence suggests that the nonapeptide neurons themselves are modulated, in part by steroid hormones, which bind to nuclear receptors and thereby alter gene expression within the cells. For example, in the California mouse, local action of brain aromatase, which converts testosterone (T) into estradiol (E2), is critical for high levels of paternal care (Trainor and Marler, 2001, 2002). One important hypothesized function of elevated brain E2 is upregulation of nonapeptide signaling via interactions with E2 nuclear receptors within nonapeptide neurons, where nuclear receptors alter gene transcription in the nonapeptide neurons in a way that augments their function (Gimpl and Fahrenholz, 2001). In addition to regulating the synthesis of the
nonapeptides within POA neurons, E2 facilitates the production of OT receptors throughout the brain in rats (Tribollet et al., 1990) and increases OT receptor binding in the preoptic area and lateral septum at the onset of parenthood in female rodents (Champagne et al., 2001). Therefore, local conversion of T to E2 within the brain, or E2 from circulation that crosses into the brain appears to play an important role in OT signaling, and in the regulation of parental care in rodents.

Compared to the functional role E2 in the brain, the role of the bioactive androgens in regulating nonapeptide neurons and signaling systems involved in fathering behavior is far less understood. In the blue banded goby, conversion of T to 11KT within the brain is critical for high levels of male parental care (Pradhan et al., 2014c), and in the bluegill sunfish blockade of androgen receptors decreased nest defense (Dey et al., 2010). However, we have previously shown that circulating 11KT did not vary across the breeding cycle in male anemonefish, suggesting a minimal or non-existent role of circulating 11KT in fathering (DeAngelis and Rhodes, 2016). Taken together, the current literature suggests fathering behavior is mediated by increased OT/IT signaling via E2 aromatized from T locally within the brain, with minimal contributions from circulating bioactive sex steroids. However, the extent to which aromatase and IT/OT receptor gene expression are dynamically upregulated in male brains as they transition from inactive to active fathers remains unknown.

In contrast to OT, the functional role of AVP/AVT signaling in the regulation of parental care is less understood. However, some recent studies have shown AVP as an important regulator of fathering behavior, nest defense, and territoriality. In prairie voles, AVP injections into the lateral septum, a brain region known to be involved in mediating behavioral acts related to offspring care, enhanced paternal care (Dulac et al., 2014; Wang et al., 1994), and in the California mouse, AVP mRNA levels in the paraventricular hypothalamic nucleus correlated positively with the number of paternal behaviors displayed (De Jong et al., 2012). Similarly in pipefishes, AVT peptide concentrations are higher in the brains of parenting males vs. non-parenting males (Ripley and Foran, 2010). And in sticklebacks AVT peptide concentrations in the whole brain were highest in aggressive males actively caring for eggs (Kleszczyńska et al., 2012). While these studies suggest AVP/AVT signaling is important in paternal care, the specificity of the relation to offspring care as compared to involvement in aggression and territorial defense is less clear, as multiple behaviors occurred simultaneously in these animal models.

Among teleosts, the anemonefish *Amphiprion ocellaris* presents an exciting opportunity for exploring neuroendocrine regulation of male parental care. *A. ocellaris* lives in relatively small and
simple social groups, where pair bonds and social hierarchies are established long before mating occurs. Therefore, there is no active courtship, nest building or intraspecific aggressive interactions co-occurring during high levels of parental care, enabling the underlying regulatory mechanisms to be more specifically extricated (DeAngelis et al., 2017a; DeAngelis and Rhodes, 2016; Iwata et al., 2012; Iwata et al., 2010). Additionally, comparing brains of males that are actively parenting to males that are not actively parenting allows isolation of the molecular mechanisms regulating paternal care. The goal of this study was to determine the extent to which brain aromatase, AVT V1a receptor, and IT receptor gene expression vary within the context parenting and in relation to circulating bioactive sex steroid hormone levels, E2 and 11KT.

We have recently shown that in A. ocellaris blockade of IT receptors significantly reduces paternal care, whereas blockade of AVT V1a receptors increases fathering behavior (DeAngelis et al., 2017a). In a previous report we also documented that circulating E2 varies over the breeding cycle in females, but not males (DeAngelis and Rhodes, 2016). Therefore, we hypothesized that circulating sex steroids (at least E2 in females), brain aromatase, AVT receptor V1a, and IT receptor gene expression would dynamically change as individuals shift in status between parenting and non-parenting. More specifically, we hypothesized that brain aromatase gene expression levels would increase in response to parental care in males, but still display relatively higher levels in females, due to positive feedback regulation by circulating E2 (Callard et al., 2001; Menuet et al., 2005). Further, we hypothesized that IT receptor gene expression would be higher in individuals actively caring for eggs compared to non-active parents. We expected that IT receptor gene expression would be higher in males than females, a reflection of their higher levels of parental behavior. Finally, we hypothesized that AVT V1a receptor gene expression would show the opposite pattern, lower in parenting individuals and lower in males compared to females, based on the pharmacology result where blockade of V1a increased parenting in males.

4.2. MATERIALS AND METHODS
4.2.1 Animals and Husbandry

All fish used were offspring bred in house from broodstock obtained from ORA (Oceans Reefs and Aquariums, Fort Pierce, FL). Fish were kept in 20-gallon tall aquariums (24” x 12” x 16”) integrated via plumbing to a large circulating filtration system. Conditions were set to mimic the natural environment with a pH between 8.0 – 8.4, temperature range of 79 - 82°F, photoperiod of
12:12 (lights on at 7:00am and off at 7:00pm), and specific gravity of 1.026. All procedures were approved by the University of Illinois Institutional Animal Care and Use Committee.

Fish were kept in either one of two social housing conditions. First, animals were kept in pairs, containing one dominant female and one subordinate male. Each tank containing one 6” terracotta pot, serving as a surrogate host anemone and nesting site. All fish in this social setup had been established as pairs for over 2 years prior to experimental observations. Secondly, juvenile anemonefish were housed in groups of over 30 individuals without a terracotta pot. This housing condition suppresses sexual maturation and inhibits any social structure from forming.

A total of 22 males, 22 females, and 8 undifferentiated group-housed fish were used in this study. The females were on average 7.0 cm (± 0.74 SD) long and had a body mass of 7.6 g (± 0.91 SD). The males had a mean body length of 5.5 cm (± 0.39 SD) and mass of 3.4 g (± 0.88 SD). Finally, the undifferentiated group-housed individuals had a mean body length of 5.3 cm (± 0.31 SD) and mass of 3.48 g (± 0.65 SD). There were no differences in weight or length between males and females of different treatment groups.

4.2.2 Experimental Design

All mature pairs were monitored carefully for a 3-month period and observed spawning events recorded. Pairs that were spawning regularly (spawn intervals of less than 21 days) were deemed ‘Active’ spawners (n=11). Pairs were deemed ‘Non-Active’, if they had not spawned during the 3-month observational time period (n=11). Pairs displaying intermittent spawning events, a spawn period > 21 days were not used in this study. Following the 3-month observation period, and only for actively spawning pairs, parental behaviors were quantified for 7 consecutive days following a spawning event. Additionally, the number of eggs on the day the eggs were laid, and the number of eggs 7 days after the eggs were laid was also quantified. Male and females from actively spawning pairs were then euthanized on day 7, following behavioral analysis and egg counts. No behaviors were quantified for non-active pairs (as no parental care occurred). Additionally, individuals from group-housed conditions (n=8) were also sampled.

4.2.3 Behavioral Quantification

For active pairs, all parental behavioral displays by both males and females were quantified from video recordings taken at 2:00 pm daily through the egg-earing period (7 days). Video recordings were taken for 15 minutes and scored following a 5-minute acclimation period (time
provided for fish to display normal behavior following camera setup in front of their home aquarium) for a total of 10 minutes. Video recordings were scored using Jwatcher Behavioral Event Recording Software (Blumstein and Daniel, 2007). The total number of nips (mouthing of the eggs to keep them clean of fungus and debris) and fans (using pectoral and caudal fins to provide oxygen rich water to the eggs) were scored for each day. The time spent in the nest (proportion of the 10-minute video individuals were in the terra cotta pot) was also scored. The total number of behaviors and time in the nest was averaged per day for statistical analysis.

4.2.4 Blood sample collection

All blood sampling occurred between 2:00 pm and 3:00 pm in order to control for any diurnal changes in hormone levels. Fish were placed between two wetted paper towels with only the caudal region exposed. Blood was then sampled from the lateral caudal vein using a 27-gauge heparinized butterfly needle (Terumo Medical Products) mounted on a 1 ml syringe (BD Syringe). Between 50 μl to 250 μl of blood was collected from each individual. Sample volumes varied due to individual variation in body size, and proficiency of procedure. All blood samples were collected within 2 minutes of removal from the home aquarium (a time previously validated as having no effect on measured hormone concentrations, (DeAngelis and Rhodes, 2016)). Immediately following collection, blood was dispelled into 0.6 ml centrifuge tubes and spun in a chilled centrifuge (4° C, Eppendorf Centrifuge 5417R) at 4000rpm for 15-minutes. The plasma supernatant was then extracted, aliquoted in 20 μl samples, and stored at -80° C until assayed.

4.2.5 Hormonal Measurements

Plasma was assayed using previously validated commercially available enzyme immunoassay kits for E2 (Calbiotech, Lot NO. ESG4324) and 11KT (Cayman Chemical, Item No. 582761). Each plasma sample was diluted 1:30 in assay buffer as done in DeAngelis et al. 2016, prior to analysis following to kit instructions. Subsequent absorbance was read using the Epoch Microplate Spectrophotometer (BioTek Instruments).

4.2.6 RNA extractions and cDNA synthesis

The brain of each fish was dissected out and placed in 5 volumes of RNAlater Stabilization Solution (Thermo Fisher, Cat. No. AM7020) and stored at 4 °C overnight. Brains were then transferred to clean microcentrifuge tubes and stored at -80 °C until RNA extractions were
performed (no longer than 1 month). Whole brain RNA extractions were performed using the RNeasy Lipid Tissue Mini Kit (Qiagen, Cat. No. 74804) following manufactures instructions. RNA concentrations were then quantified using a NanoDrop 2000c spectrophotometer (Thermo Scientific). RNA quality number (RQN) and 28S/18S ratios were determined using a Fragment Analyzer Automated CE System (Advanced Analytical AATI, Table 1) at a concentration of 100 ng/μl. With remaining aliquots, RNA concentrations were normalized to 500 ng/μl prior to reverse transcription. RNA was reverse transcribed using the iScript™ cDNA synthesis kit (Bio-Rad Laboratories, Cat. No. 1708890) following manufactures instructions, in a total volume of 20 μl per reaction. To control for potential genomic DNA contamination, samples were treated with RNase free DNase I (New England BioLabs, Cat. No. M0303S) and negative control reactions were run without the iScript transcriptase.

4.2.7 Primer validation

Primers were designed for mRNA sequences of vasotocin receptor 1A (V1ar) (Genbank #AB669615.1), isotocin receptor (Genbank #AB669618.1), aromatase (cyp19a1b) (Genbank #AB918722.1), and beta actin (Genbank #AB9212201.1) using Primer-BLAST software (NCBI) against sequences specific to A. ocellaris. Primer pair specificity was blasted against the reference genome of the damselfish Stegastes partitus (taxid: 144197), the closest relative of A. ocellaris, where the full genome was available. Primer melting temperatures were set with a minimum of 58 °C, a maximum of 61°C, and an optimal of 60 °C. The maximum number of primers to screen was set at 1000, with primer sizes set between 18 and 27 base pairs, with an optimal at 24 base pairs, and a max Poly-X at 3. Primers were then analyzed for hairpins, and primer dimers using OligoAnlayzer (IDT Technologies) prior to serial dilution validations. Only primers showing hairpin analysis with ΔG (kcal.mole-1) values < 2, and primer-dimer values of ΔG < 6 were tested in serial dilutions. All primers were commercially purchased from IDT technologies (Coralville, IA).

Following cDNA synthesis, samples were diluted in a 4-fold series and checked for optimal efficiencies and a single melt curve. All primers displayed an efficiency between 90-110% and an R^2 value of over 0.96 (Table 2).

4.2.8 Relative gene expression quantification

Gene expression was quantified using the SSo Advanced™ Universal SYBR Green Supermix (Bio-Rad Laboratories, Cat. No. 1725271) and CFX Connect™ Real-Time PCR Detection System (Bio-
Rad Laboratories, Cat. No.1855201). All reactions were run in triplicate. The cycle parameters were 98 °C for 30 s, 95 °C for 10 s, 52-55 °C (depending on primer pair, see Table 2) for 30 s, then plate read and repeated for 40 cycles. Following the 40 cycles, a melt curve was performed with an increase in temperature from 58 °C to 95 °C at 0.5 °C increments, followed by a final plate read.

The cycle threshold (Ct) was calculated automatically in the CFX Connect™ software. Only triplicate reactions with a Ct standard error (SE) under 0.85 were used in statistical analysis. Gene expression data were then computed using the equation of Jemiolo and Trappe (2004), which compares changes in the difference between the Ct of the gene of interest and a reference gene (beta actin), expressed as:

$$\text{Fold change} = 2^{-\Delta\Delta Ct} \quad \text{(Jemiolo and Trappe, 2004)}.$$  

4.2.9 Statistical Methods

Data were analyzed using R (version 3.3.0 ‘Supposedly Educational’) statistical Software (Team, 2013). P < 0.05 was considered statistically significant. To determine effects of parenting status and sex on gene expression, and circulating hormones levels, a two-way analysis of variance (ANOVA) was performed with sex and status as independent factors. Effects sizes for these models are reported as eta². For males, because of the additional treatment group of ‘group housed’, a one-way ANOVA was performed followed by post-hoc pair wise comparisons of means using Fisher protected least squared differences (LSD). All variances of residuals in these models were checked for normality visually, and using Shapiro-Wilks normality tests (P > 0.10). Data were either log transformed to meet normality assumptions, or when data could not be normalized, a non-parametric Kruskall-Wallis test was performed. All specific data transformations are noted in the results section. In examining correlations between measured variables, Pearson’s product-moment correlation coefficients are reported. Within the active spawning group principal component analysis was performed on all the response variables (E2, 11KT, aromatase expression, V1a expression, IT receptor expression, total parental behaviors, and time in the nest), to evaluate the extent to which the groups could be separated along the major axes of variation.
4.3. RESULTS

4.3.1 Parental Behavior

During the 10-minute video grading period, males displayed an average of 81 (± 5.91 SEM) total parental behaviors and spent 77% (± 0.21 SEM) of their time in the nest. Conversely, females displayed an average of 21 (± 3.30 SEM) total parental behaviors and spent only 21% (± 0.03 SEM) of their time in the nest. Both average number of behaviors \( [t_8 = -4.63, P = 0.0009] \) and time spent in the nest \( [t_8 = 12.61, P < 0.0001] \) were significantly different between males and females. Additionally, consistent with previous findings, the proportion of time males spent in the nest, and total number of male behaviors, were positively correlated \( [r_8 = 0.881, P = 0.012] \), and the proportion of time males spent in the nest was positively correlated with the proportion of eggs remaining on day 7 \( [r = 0.760, p = 0.011] \).

4.3.2 Brain aromatase gene expression

Values of aromatase gene expression were log transformed in order to meet Shapiro-Wilks normality criteria. Results from the two-way ANOVA showed a significant effect of parenting \( [\eta^2 = 0.433, F_{1,37} = 28.27, P<0.001] \) and sex \( [\eta^2 = 0.273, F_{1,37} = 28.27, P<0.001] \) on relative aromatase gene expression (Fig. 2A). No significant interaction between sex and parenting was present \( [\eta^2 = 0.733, F_{1,37} = 2.877, P=0.090] \). Overall, females displayed 13% higher aromatase gene expression than males, and individuals that were parenting displayed 20% higher aromatase gene expression than those not actively caring for eggs. Separate analyses by sex showed that females that were actively parenting had 25% higher aromatase relative gene expression than non-parenting females \( [P<0.001] \). Additionally, males that were actively parenting displayed 8% higher levels of relative aromatase gene expression than those that were non-parenting \( [P=0.012] \), although the effect was not as dramatic for males as females (Fig. 2C).

Comparing males across treatments, including group-housed males, results from one-way ANOVA showed a significant effect of parenting on relative aromatase gene expression \( [\eta^2 = 0.54, F_{2,26} = 15.86, P<0.0001] \). Post hoc analyses revealed that actively parenting males showed 14% higher levels of relative aromatase gene expression than non-parenting \( [P=0.019] \), and 41% higher expression than group housed males \( [P<0.0001] \). Additionally, non-parenting males show 31% higher levels of aromatase expression that those in group-housed conditions \( [P=0.0001] \).
4.3.3 Brain isotocin receptor gene expression

Two-way ANOVA showed a significant effect of parenting \([\eta^2 = 0.12, F_{1.37} = 5.37, P=0.026]\) and sex \([\eta^2=0.11, F_{1.37} = 5.57, P=0.024]\) on relative IT receptor gene expression, and no interaction between sex and parenting \([\eta^2=0.01, F_{1.37} = 0.58, P=0.45]\). Overall, males displayed 17% higher IT receptor gene expression than females (Fig. 2B), and individuals currently caring for eggs displayed 19% higher levels than those not actively caring for eggs.

Comparing males across treatments, including the group-housed condition, results from one-way ANOVA showed a significant main effect of treatment \([\eta^2=0.45, F_{2,26} =10.63, P=0.00042]\). Post hoc analyses indicated parenting males show a 22% increase in isotocin receptor expression compared to non-parenting males and a 74% increase compared to group housed controls \([P=0.045, P=0.0001]\). Additionally, non-parenting males show 42% higher relative levels of IT receptor gene expression that those in group-housed condition \([P=0.016]\).

4.3.4 Brain arginine vasotocin receptor (V1a) gene expression

Results from the two-way ANOVA showed no effect of parenting \([\eta^2=0.014, F_{1.37} 0.5286, P=0.4718]\) or sex \([\eta^2=0.0015, F_{1.37} 0.529, P=0.799]\) on relative v1a gene expression (Fig. 2C). Additionally, no interaction between sex and parenting was present \([\eta^2=0.0016, F_{1.37}=0.0616, P=0.8]\). Comparing males across treatments, results from one-way ANOVA showed a significant effect of the group-housing condition \([\eta^2=0.54, F_{1.26} =15.86, P<0.0001]\). Males kept in group-housed conditions show 10 % less relative V1a gene expression than males in the active spawning group \([P<0.0001]\), and also 8% less expression than males kept in the non-active group \([P=0.0001]\).

4.3.5 Circulating estradiol levels

Due to the non-normal distribution (not meeting Shapiro-Wilks criteria despite transformations) of circulating E2 levels in females, a Kruskal-Wallis was used in evaluating the effect of breeding status within females. Reproductive status (active parenting vs. non-spawning) had a significant effect on estradiol levels \([df=1, \chi^2 = 5.644, P = 0.017]\), where females that were actively parenting displayed 2-fold higher levels of circulating estradiol levels than non-active females.

Levels of estradiol within males met normality criteria and there was no significant effect of parenting status on estradiol levels \([\eta^2 = 0.30, F_{2.15} = 3.29, P=0.065]\). Due to limitations in blood sampling of group-housed males, only actively spawning and non-spawning males were compared.
In evaluating the difference between the sexes in circulating levels of estradiol, a Kruskal-Wallis test was used. Overall, females displayed 29-fold higher levels of circulating estradiol than males, consistent with previously reported results \( [\chi^2 = 12.80, P = 0.0003] \) (DeAngelis and Rhodes, 2016).

In females, which displayed higher aromatase gene expression and also higher circulating estradiol, aromatase gene expression and circulating estradiol were positively correlated \( [r_{14} = 0.748, P = 0.0009] \). This correlation was not observed in males \( [r_{12} = 0.126, P = 0.65] \). However, males displayed a positive correlation between circulating 11KT levels and estradiol levels \( [r_{12} = 0.721, P = 0.0024] \), a correlation that did not occur in females \( [r_{14} = -0.402, P = 0.112] \).

### 4.3.6 Circulating 11KT levels

Circulating levels of 11KT in females did not meet normality criteria. Hence, a Kruskal-Wallis test was used to determine the effect of reproductive status on 11KT levels. There was no effect of reproductive status on 11KT levels within females \( [\chi^2 = 2.04, P = 0.153] \). Distributions of 11KT in males met normality criteria and so a one-way ANOVA was used to evaluate the treatment effect. Similarly, within males, there was no effect of parenting status on circulating levels of 11KT \( [\text{eta}^2 = 0.15, F_{1,13} = 2.3, P = 0.152] \). Males displayed 9.3-fold higher 11KT than females \( [\chi^2 = 20.3, P < 0.0001] \), supporting previously published data (DeAngelis and Rhodes, 2016).

### 4.3.7 Principal component analysis of sex differences during parental care

Principal component 1 (PC1) explains 55% of the variance in the measured variables and actively spawning males and females separate out into sex specific groups along this axis (Fig. 4). PC1 is largely influenced by sex differences in circulation hormone levels, aromatase expression, and total parental care. PC1 is positively correlated with total behaviors \( [0.42] \), 11KT \( [0.46] \), and time in the nest \( [0.38] \), and negatively correlated with estradiol \( [-0.43] \) and aromatase expression \( [-0.42] \).

PC2 is largely influenced by the more subtle differences in IT receptor and v1a receptor gene expression. Overall, the analysis presents a clear picture of sex differences in *A. ocellaris* during parental care (Fig 3).

### 4.4. DISCUSSION

Here, we present the first study to our knowledge, showing that brain aromatase and IT receptor gene expression levels differ in actively parenting vs. non-parenting males. Both males and
females displayed increased aromatase and IT receptor gene expression while actively caring for eggs compared to non-active pairs, suggesting both brain aromatase and IT signaling are important in the regulation of parental care in the anemonefish *A. ocellaris*. Our results are consistent with a growing literature suggesting that IT/OT signaling plays a key role in paternal care across multiple species of vertebrates. Several studies have shown that fatherhood often requires an active shift in neurological features in order to support the paternal behavioral repertoire (De Jong et al., 2009; Kent and Bell, 2018; Kim et al., 2014; Wu et al., 2014). We conclude that aromatase and isotocin receptor gene expression vary as a plastic response to the dynamic behavioral shift required for parental care.

4.4.1 Brain aromatase gene expression

Brain aromatase gene expression was higher in both males and females that were actively parenting compared to those that were not parenting (Fig. 2A). This suggests that synthesis of E2 from T within the brain is crucial in the regulation of fathering behavior in males. E2 treatments are known to engender parental behaviors in both male and female rodents (Rosenblatt and Ceus, 1998; Wynne-Edwards, 2001). In the California mouse, conversion of T to E2 within the brain by aromatase is critical for high levels of male parental care, where gonadectomized males treated exogenously with T or E2 displayed greater paternal care than controls, and simultaneous use of an aromatase inhibitor blocked the positive effect of T treatment while having no effect on the rise in care in response to E2 (Trainor and Marler, 2002). These findings implicate brain aromatase activity in paternal care. Our results support this idea, and further establish that the effect is local within the brain, as circulating E2 levels were unrelated to parental status and uncorrelated with brain aromatase levels in the male anemonefish.

Simultaneously higher levels of circulating E2 complicate the explanation for higher aromatase levels in females. In addition to displaying a small number of parental behaviors, they were also likely in a physiological state of producing the next batch of eggs to be laid. Consistent with this idea, circulating E2 levels were higher in actively parenting females compared to non-parenting females. Moreover, within females, circulating estradiol levels and brain aromatase gene expression were highly correlated. E2 nuclear receptors, which bind to the promoter region on the aromatase gene, positively regulate aromatase gene expression (Callard et al., 2001). Therefore, if circulating E2 reflected E2 concentrations in the brain, then it is possible that the E2 increased brain aromatase gene expression through this positive feedback mechanism.
Previously, we have shown that circulating E2 levels vary during the reproductive cycle in female *A. ocellaris*, and that E2 levels are higher on day 6 of the egg brooding period than on the day the eggs were laid or when no eggs were present (DeAngelis and Rhodes, 2016). Taken together, these data are consistent with the idea that the higher E2 in parenting females is related to the physiological state of egg production rather than the small number of parental behavioral acts they perform. Nevertheless, whether high brain aromatase activity in females is related to egg development and/or parental care is unclear, and future work exploring each in possibility in a specific context is needed to fully understand the role of E2 during female parental care in *A. ocellaris*.

Overall our data suggests that within males, local conversion of T to E2 within the brain by aromatase is crucial for high levels of parental care. Unlike many other models in which fathering behavior co-occurs with territorial defense, aggression and nest building, in *A. ocellaris* these other behaviors are largely absent during parental care, strengthening the conclusion that local E2 production is key to fathering behavior specifically. Based on the literature, we hypothesize brain aromatase activity is a precursor to the upregulation of the IT signaling system which promotes paternal care, although the causal relationship remains unclear and will require further work to establish. In females, we propose increased circulating E2 and correlated brain aromatase levels work to promote reproduction and/or parental care.

### 4.4.2 Isotocin receptor gene expression

Results here indicate that IT receptor abundance increased in response to parental care. This is consistent with a growing body of literature highlighting the importance of not only the function of OT/IT neurons, but also the expression of OT/IT receptors throughout the brain. In humans, paternal variation is associated with genetic variation at the OT receptor gene (Feldman, 2012), while in female prairie voles natural variation in OT receptor expression during development in the nucleus accumbens positively correlates with alloparental behavior (Keebaugh and Young, 2011) and OT receptor knockout mice perform fewer maternal behavioral acts than controls (Keebaugh et al., 2015). Moreover, a study in the bi-parental convict cichlid showed that blockade of IT signaling inhibited the rise in paternal effort over the egg incubation period, and that single fathers displayed increased activation of IT neurons within the parvocellular layer of the POA (O’Connell et al., 2012). Additionally, we have previously shown that blockade of the IT receptor dramatically reduced male parental effort in the anemonefish *A. ocellaris* (DeAngelis et al., 2017a). The consistency across
species supports recent work showing that the OT/IT signaling system is highly conserved across evolutionary history in both form, and in the functional way it facilitates parental care (Dulac et al., 2014; Goodson, 2008; O’Connell and Hofmann, 2012; Royle et al., 2012).

During reproduction the female brain undergoes a dramatic reorganization of the OT/IT signaling system (Gimpl and Fahrenholz, 2001). Oestrus and pregnancy provide a physiological mechanism for OT production in females, where in rodents, the hormones of pregnancy are critical for the production of OT receptors in the amygdala and hypothalamus and the onset of parenting (Broad et al., 1999). Results presented here suggest that the male brain also undergoes a transformation of the IT signaling system. Parenting males display higher levels of paternal care, and correspondingly higher levels of IT receptor expression, which we speculate may be a response of increase E2 within the brain as mediated by higher aromatase activity. While we did not explore the causal relationship between circulating E2, brain aromatase activity, and IT signaling, future studies investigating these relationships may yield a more specific understanding of the interplay between IT and brain E2 levels as a function of aromatase activity during paternal care.

4.4.3 AVT receptor gene expression

While the AVT signaling system in the brain has generally been thought of as promoting behaviors in males related to reproductive success, few studies have been able to isolate a role in behaviors directed at offspring care. The lack of a direct relationship between AVT signaling and specific parental behaviors may be due in part to the confounding influences of co-occurring behaviors such as territorial defense and aggression. For example, in the three-spined stickleback fish, AVT peptide levels in the brain were highest in the most aggressive males that were also actively caring for eggs (Kleszczynska et al., 2012). Our results suggest that this difference may be related to the heightened aggression rather than paternal care. In anemonefish males, which display low aggression but high levels of parental care, no differences in AVT receptor gene expression were observed (Fig. 2C). Moreover, our previous report found that blockade of AVT using an AVT V1a receptor antagonist actually increased male parental care, perhaps suggesting an opposite role for AVT signaling in directly suppressing paternal care rather than increasing it. However, in that report, we provided an alternative hypothesis that AVT regulates paternal care indirectly by drawing attention away from offspring care and toward aggression and/or territorial defense. Consistent with this idea, in a different context blockade of AVT reduced aggression (DeAngelis et al., 2017a; Yaeger et al., 2014a). Moreover there is a large literature on the role of AVT in aggression
across species (Foran and Bass, 1999; Kleszczyńska et al., 2012; Kline et al., 2011; Semsar et al., 2001a). This interpretation is consistent with our data, given that in our study no nest defense or vigilance behaviors were displayed, hence no differences in AVT V1a expression should have been observed. Future work is needed to directly test the hypothesis that AVT signaling specifically facilitates vigilance during parental care.

4.4.4 11KT

While it has been traditionally viewed that high androgens are inhibitory to high levels of parental care, this ‘challenge hypothesis’ has less support outside of the avian studies where it originated (Wingfield et al., 1990b; Wynne-Edwards and Timonin, 2007). In these avian studies, males spawn seasonally, and are required to build nests, defend territories and compete for mates prior to reproduction. Hence, high androgens are critical in regulating future reproductive success during this combative pre-reproductive period. However, in other studies, androgens are necessary for parental care. In the California mouse, high T levels are necessary for paternal care (Trainor and Marler, 2001), and male sunfish in better body condition had higher circulating androgens during parental care (Magee et al., 2006). Finally, some species display levels of parental care that are independent of circulating androgen levels, such as the smallmouth bass (Hanson et al., 2009), three spined stickleback (Páll et al., 2002), and our previous work in A. ocellaris in which circulating 11KT were unrelated to breeding status in males (DeAngelis and Rhodes, 2016). The data presented here are consistent with previous results, suggesting that parental care in A. ocellaris is likely independent of circulating levels of the main bioactive androgen, 11KT. On the other hand, it is possible that brain levels are regulated locally rather than from the blood as suggested by a study in blue-banded gobies where intracerebroventricular injections of carboxolene, which blocks the conversion of testosterone to 11KT within the brain, reduced male parental care, highlighting the importance of androgen hormone synthesis within the brain in regulation parental care (Pradhan et al., 2014c). Taken together, it is unlikely that circulating 11KT contributes to fathering, but future work is needed to investigate the possible contributions of local synthesis of 11KT in the brain.

4.4.5 Limitations

One important limitation of this study is that we measured gene expression in the whole brain and thus do not know how the patterns of gene expression might vary in different brain regions. The lack of differences in AVT V1a receptor gene expression between groups, for example,
could be because gene expression increased in certain regions and decreased in others, thus obscuring differences when collapsed. In many of the previous studies showing effects of AVT receptors on social behaviors, AVT receptors were quantified in a brain region specific manner (Kline et al., 2011). On the other hand, the genes we measured, aromatase, IT receptors and AVT receptors are known to be widely distributed throughout the brain (Forlano et al., 2001; Greenwood et al., 2008; Hausmann et al., 1995), and thus, focusing on specific regions could miss the main effects, if certain regions are more sensitive to the social manipulations than others. Future work will explore the regional specificity of the changes in gene expression described herein.

Similarly, the brain is composed of many different types of cells (e.g., neurons, glia, endothelial cells) and we analyzed gene expression from the entire mixture, so we do not know whether the gene expression patterns we found were influenced primarily by variation in one or multiple cell types. For example, evidence suggests aromatase is synthesized in radial glial cells (Forlano et al., 2001; Tong et al., 2009), hence it is likely that the gene expression differences we observed for aromatase reflect changes in radial glial cells or astrocytes. Similarly, the nonapeptide receptors are likely expressed in neurons, but may also be expressed on astrocytes and microglia. Future work is needed to explore the gene expression patterns in a cell type specific fashion to confirm the cellular origin of the signals reported herein.

4.5. CONCLUSION

This is the first report to which we are aware that compared gene expression levels of aromatase, IT receptors, and AVT V1a receptors in the whole brains of actively parenting vs. non-parenting male and female *A. ocellaris*. Additionally, we measured the circulating hormone concentrations of E2, 11KT, and cortisol. Our results indicate that aromatase and IT receptor gene expression are dynamically regulated by parental status, higher during parenting than not parenting in both males and females. Furthermore, while hormone levels varied by sex, and E2 was higher in actively parenting females, parental care in *A. ocellaris* appears to be largely independent of circulating hormonal concentrations. Taken together, our results highlight the importance of IT signaling and brain aromatase activity in the regulation of parental care in the anemonefish, *A. ocellaris*.
### 4.6. TABLES AND FIGURES

**Table 4.1 RNA Quality Table.** Analysis of RNA quality from Fragment-Analyzer showing the RNA quality number (RQN), RNA degradation values as measured by 28S:18S ratios, total RNA concentration for each sample, and RNA purity as measured by 260:280 and 260:230 ratios. Letters next to 'FISH ID' correspond to male (m), female (f), and juveniles (j).

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Table 4.2 Primer sequences and validation parameters. Primer pairs are listed for specific target sequences of aromatase (Genbank #AB918722.1), arginine vasotocin V1a receptor (Genbank #AB669615.1), beta actin (Genbank #AB9212201.1), and isotocin receptor (Genbank #AB669618.1). Melt temp denotes the specific meting temperature set for each reaction. Efficiency and \( R^2 \) denote efficacy of 4-fold serial dilution process in primer validation.

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<th>Melt Temp.</th>
<th>Efficiency</th>
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<td>ATAATGTACGAGCAACTA TCACG</td>
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Figure 4.1 Male effort pays off in terms of egg survival. The x-axis shows proportion of time males spent in the nest during the 10-minute behavioral quantification period. On the y-axis is the proportion of eggs remaining as calculated by counting eggs from photographs taken on the day of spawning, and 7 days after egg deposition. The two variables are significantly correlated ($r_s = 0.760$, $p = 0.011$).
Figure 4.2 Aromatase, IT and AVT V1a gene expression (GE) in male and females as a function of parental status. Relative abundance of aromatase (A), IT receptor (B), and AVT V1a receptor (C) gene expression levels in the context of parenting ('Active') vs. non-parenting ('Non-Active'). Females are represented in red and males are represented in blue. Box plots show the interquartile range (IQR) of each group analyzed with whiskers extending to 1.5X the IQR. Horizontal lines represent medians, and diamonds represent means. Scattered points within each boxplot represent the individual values used to generate each plot. Relative gene expression is shown by dividing each individual value by the mean of the Non-Active males within the gene. Relative gene expression was calculated via the equation: Fold change = $2^{-\Delta \Delta Ct}$, by comparison to the reference gene Beta-Actin.
Figure 4.3 PCA of sex differences during parental care. PCA reveals males and females form distinct clusters, and are sufficiently separated by PC1 alone. The percentages within the parentheses give the variances explained by each component plotted. The ellipsoids represent a 95% confidence interval in the occupied space for each sex, females in red, males in blue. Sex differences in PC space are largely driven by higher aromatase gene expression and E2 levels in females than males, and higher 11KT values, greater time spent in the nest, and more total parental behaviors in males relative to females.
4.7. REFERENCES


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CHAPTER 5:
CORRELATIONS BETWEEN STEP-FATHERING BEHAVIOR AND ACTIVATION OF ISOTOCIN NEURONS IN THE PREOPTIC AREA OF THE HYPOTHALMUS IN AMIPRION OCELLARIS

Abstract
The disentanglement of parental care from other behaviors such as courtship, mating and nest defense that occur either simultaneously, or within close temporal proximity has complicated our understanding of the specific neural mechanisms involved in each behavior independent from one another. Hence, in addressing the regulation of parental care, it is important to isolate out as many confounding behaviors as possible in order to uncover the neural control of parental care specifically. Here, we utilize the unique feature of step-fathering behavior in the anemonefish Amiprion ocellaris in order to address how parenting, independent of mating, is regulated by isotocin neurons. Here we measured the activation of isotocin positive neurons, a nonapeptide well known for its role in parental care, in response to nest substitutions. Nests of naïve fathers were switched out with nests from reproductive pairs containing eggs. We then implemented an immunofluorescent double label of isotocin (IT), and the ribosomal protein S6 within the preoptic area (POA) of the hypothalamus, a brain region widely implicated in parental care. The ribosomal protein S6 is activated in ribosomes undergoing transcription, and is widely used a marker of neural activation. The goal of this study was to determine the extent to which fathering behavior induces activation of IT neurons within the POA. Results were not as expected, and suggest that IT activation is inhibitory to fathering behaviors. The proportion of IT neurons activated was negatively related to the time spent in the nest, and total parental behaviors exhibited. Additionally, after delineating fathers as either ‘good’ or ‘poor’ based on the time spent in the nest, results from Fisher’s exact test show that poor fathers display a higher probability of having an IT neuron activated in comparison to good fathers. However, given that time spent in the nest was positively correlated with egg loss, the opposite pattern of what we typically find, we expect the step fathering behavioral paradigm used was ambiguous, in that it was not clear whether behaviors were indicative of good or poor fathering. From previous studies we have noted that fathers increase their parental effort with experience. Hence, a more suitable design for future experiments may benefit from allowing step fathers to garner more experience prior to neural analysis, or by using experienced biological fathers in a step fathering paradigm.
5.1. INTRODUCTION

Parental care includes a remarkably diverse repertoire of behaviors across the animal kingdom. Egg incubation, nursing, matriphagy, and nest care are all forms parental care that are critical for offspring survival. Parental care is defined as any behavioral act that increases offspring fitness or survival (Royle et al., 2012). Thus far, the vast majority of research exploring the proximate mechanisms involved in the regulation of male parental care has been complicated by the co-occurrence of several different parental behaviors exhibited. For example, in seasonally spawning species such as sticklebacks, males need to find a mate, build a nest and rear offspring during the breeding season; in the blue-banded goby males defend territories and mate with females while also caring for eggs (Kleszczyńska et al., 2012; Rodgers et al., 2006a). The anemonefish *A. ocellaris* provides a useful model in understanding parental behavior in isolation from other confounding co-occurring displays (DeAngelis et al., 2017a; DeAngelis and Rhodes, 2016). *A. ocellaris* is a truly monogamous species, where pair bonds are formed many months if not years prior to a reproductive event. While affiliative pair bonding displays and courtship do occur, these behavioral dances are temporally separated from reproductive events, and consequently paternal care (Buston, 2003). Hence, *A. ocellaris* provides a useful model in which parental care, and of particular interest, paternal care can be explored in isolation from many other confounding behaviors seen in research using more common animal models (Roland and O’Connell, 2015).

Additionally, and of particular interest here, is the naturally occurring behavior of step fathering. Within anemonefish, if a particular male is removed from a nest, another male or non-reproductive subordinate will move into the nest and provide high levels of care to the eggs until hatching (Yanagisawa and Ochi, 1986a). This furthers the isolation of parental care by the removal of the confounding occurrence of mating within close temporal proximity to parenting. Furthermore, we have experimentally induced step-fathering behavior in the laboratory system by placing naïve fathers with novel eggs where males then actively care for non-biologic offspring. Anemonefish are demersal spawners, meaning they deposit their eggs on substrates surrounding the nest. In laboratory systems, terra cotta pots are implemented as surrogate anemone hosts. Hence, eggs can easily be moved from one aquarium to another by removing and replaced the terra cotta pots on which the eggs are deposited.

The isolation of parental care using this step-fathering paradigm provides a useful tool in identifying brain areas that are activated specifically in response to parental care. Within anemonefish, and many other species, parental care requires a dynamic shift in behavioral
phenotype from non-parenting to actively parenting. Presumably, this behavioral shift must also require a dynamic response within the brain. Hence, exploring neuroplasticity and uncovering the cell types that are activated in response to a specific behavioral phenotype are central goals in social neuroscience.

One such approach used in identifying activated neurons that respond and/or facilitate behavioral changes is the ribosomal protein S6, which is phosphorylated in activated neurons (Knight et al., 2012). This phosphorylated S6 protein can then be stained with immunohistochemistry to identify activated brain regions and neurons involved in regulation of a specific behavioral phenotype of interested. While examining neural activation, identification of the specific cell types being activated is tantamount in importance. While several neuropeptides, and a suite of steroid hormones have been identified as involved in the regulation of parental care (Dulac et al., 2014b; Godwin and Thompson, 2012; Goodson, 2008; Insel and Young, 2000; Keverne and Curley, 2004), of particular interest here is the role of isotocin (IT) neurons. IT is a neuropeptide produced from neurons residing in the hypothalamic preoptic area (POA). The POA is a highly conserved brain region across vertebrates, which facilitates fundamental social behaviors ubiquitously distributed throughout the animal kingdom. The POA has been implicated in aggression, pair bonding, courtship, and parental care (Foran and Bass, 1999; Greenwood et al., 2008; Rosenblatt and Ceus, 1998; Saito et al., 2004; Wu et al., 2014; Yaeger et al., 2014a).

Furthermore, the mammalian homolog of IT is oxytocin (OT), and OT, along with the teleost IT has been implicated in the direct regulation of both maternal and paternal care in a wide variety of taxa (Goodson, 2008; O’connell and Hofmann, 2011; O’Connell and Hofmann, 2012). Hence, IT neurons are of keen interest in how they are activated in response to high levels of parental care, and here we implement an immunohistochemical double stain of IT and the rpS6 in order to elucidate the role of activated IT neurons in parental care.

The goal of this study was to determine the extent to which levels of step fathering behaviors are correlated with activation of IT neurons. We hypothesized that males displaying relatively higher levels of step-fathering behavior would display a higher proportion of IT neurons displaying the rpS6 activation marker for three reasons. First, because in chapter 3, we observed that an IT antagonist decreased fathering substantially. Second, because in chapter 4 we observed increased IT receptor gene expression in male fathers as compared to males that were not breeding. And finally third, because of a previous study in convict cichlids which found increased activation of IT
neurons in the magnocellular layer of the POA in single fathers as compared to lone males or fish displaying biparental care (O’Connell et al., 2012).

5.2. MATERIALS AND METHODS
5.2.1 Animals and Husbandry

A total of 16 males were used in this study. The males were on average 4.9 cm (± 0.28 SD), and had a mean mass of 3.0 g (± 0.71 SD). All fish used in this experiment were offspring bred in house from a broodstock female obtained from ORA (Oceans Reefs and Aquariums, Fort Pierce, FL), and a wild caught male (location unknown) obtained through the pet trade. Prior to experimental manipulations, fish were housed in 3 separate tanks at high densities (> 30 individuals) in 20-gallon long aquariums (30 ¼” x 12 ½” x12 ¾”) integrated via plumbing to a large circulating filtration system. We previously determined that these group-housed fish display ovotestes and when removed from group housed conditions and placed with a gravid female, spawn and produce viable eggs within a few weeks.

Males were placed individually into 10-gallon aquariums, containing a sponge filter to providing biological filtration and waste reduction, and a 20-watt heater to match temperature to that of the main housing system. Salinity, pH, and photoperiod were matched to home tank conditions.

Each experimental tank contained one 4-inch terra cotta pot, to serve as a nesting site and territory. Terra cotta pots were the same brand and type used in the main research system where they also serve as territories, and nesting sites for reproductively active pairs. Following individual placement into experimental tanks, males were allowed 6 weeks to acclimate, a time period previously established for normal behaviors to be exhibited (unpublished observations), prior to experimental manipulations.

Environmental conditions were set to mimic the natural environment with a pH between 8.0 – 8.4, temperature range of 79 - 82°F, photoperiod of 12:12 (lights on at 7:00am and off at 7:00pm), and specific gravity of 1.026. All procedures were approved by the University of Illinois Institutional Animal Care and Use Committee.

5.2.2 Experimental Design

In the main housing system, in which reproductively active pairs are kept, pairs were monitored daily for egg laying events. When a reproductive female deposited eggs on the inner
surface of a terra cotta pot, the date was recorded. On the following day, terra cotta pots on which eggs had been deposited were then exchanged with pots inside the 10-gallon aquariums containing lone experimental males. Therefore, each male experienced a manipulation where their nest (the terra cotta pot) transitions from having no eggs present, to having eggs present. Immediately following the exchange of pots without eggs to the pot with eggs, the aquarium was video recorded for 90-minutes. These video recordings were then evaluated for parental behaviors for a 20-minute period, 30 minutes after the recordings began (giving each individual time to recover from the replacement of terra cotta pots).

5.2.3 Behavioral Analysis

Total number of parental behaviors performed by these lone step-fathering males in video recordings were quantified using Jwatcher behavioral event recording software (Blumstein and Daniel, 2007). The total number of nips (mouthing of the eggs, keeping them free of fungus and debris), fans (providing oxygen rich water by waving pectoral and caudal fins over the eggs), and time in the nest (time spent in the terra cotta pot) for each individual was quantified.

Following the 90-minute recordings, each male was then euthanized via rapid cervical transection. Brains were then quickly and carefully dissected out and fixed overnight (18 hours) in 4% paraformaldehyde. Brains were then washed in phosphate buffered saline (PBS) solution and switched into 30% sucrose solution for 3 days, in preparation for sectioning via cryostat and immunohistochemistry. Brains were then sectioned via cryostat at a thickness of 20 μm and mounted on slides prior to immunohistochemical staining procedures.

5.2.4 Immunohistochemistry

A fluorescent double label of the ribosomal protein S6 and IT was then performed with the following procedure. First, slides were fixed in chilled 4% paraformaldehyde in order to keep sections bound to slides. Next, slides were washed for 5 minutes, 2 times in PBS at room temperature. Following PBS washes, sections underwent an antigen retrieval step, where slides were boiled for a total of 12 minutes in citric acid and then washed in PBS again. Slides were then treated with blocking serum (5% normal goat serum and 0.3% Triton X-100 in PBS) for one hour. Next, sections were soaked overnight in primary antibodies. The primary antibody for the rpS6 (rabbit anti-pS6 235/236, Cell Signaling, Cat. #4858) was diluted 1:500, and the primary for IT (mouse anti-OT, clone 4G11, EMD Millipore, Cat. #MAB5296) was used at a dilution of 1:250.
Following over night incubation, slides were washed, incubated in PBS with Triton X, and then incubated in secondary fluorescent antibodies for 2 hours and protected from light. For the primary IT, goat anti-rabbit (Cy3, green) was used, and for the IT primary goat anti-mouse (DyLight 649, red) was used, both at a dilution of 1:500. Finally, slides were then rinsed and coverslipped with Gold Anti-fade with DAPI (blue). Alternate sections omitting the primary antibodies were used as controls.

Following staining procedures, slides were then imaged using a Nikon multiphoton single-molecule fluorescence confocal microscope, using excitatory settings specific to secondary antibodies. Alternate sections from the entire rostro-caudal extent of the preoptic area were analyzed for presence of IT positive (green) cells and co-labeling of the IT cells with the rpS6 (red). Every single IT positive cell was counted and analyzed for presence of the double label by focusing along the z-axis to confirm double labeling. In addition, every section that contained at least one IT cell was imaged and the images were later analyzed using Photoshop CS6. Photoshop CS6 provides intensity levels for green, red, and yellow colors. Cells were examined in each color spectrum independently to determine the average intensity values of each labeled cell in their respective spectrum. Cells that showed an intensity value of over 50 in their respective color spectrum were labeled positively. Cells with intensity values of over 50 in both red and green spectrums were deemed double labeled (Fig 1.).

5.2.5 Statistical Methods

The relation between duration spent in the nest and total number of eggs lost was evaluated using simple linear regression. The relation between parental behaviors (total nips and fans, and duration in nest) and proportion of IT neurons displaying the rpS6 activation marker was evaluated using logistic regression. For these analyses the deviance is shown in place of the F statistic. The individuals were also split arbitrarily into two groups based on the number of parental behaviors displayed and evaluated for differences in the proportion of IT neurons displaying the rpS6 activation marker using a fisher exact test.

5.3. RESULTS

5.3.1 Behaviors

Step fathering behavior was highly variable and it was difficult to confirm that an individual in the nest was actually caring for the eggs as opposed to harming or eating them. Only 4 out of the
16 individuals spent more than 50% of their time in the nest, and the average was 32% (± 25 SD), as compared to 62% reported for experienced fathers (DeAngelis and Rhodes, 2016). Moreover, the duration in the nest was positively correlated with egg loss (Fig. 2a; F1,14=8.168, p= 0.01265), whereas it is normally negatively related (DeAngelis et al. in review), suggesting that the males may have been eating a portion of the eggs rather than caring for them. The total number of nips and fans was highly variable and may have been confounded with egg eating. The mean total number of nips (including an unknown number of egg eating events) and fans combined was 50.9 (± 75.5 SD).

5.3.2 Activation of IT Cells

A total of 188 sections through the POA were analyzed by confocal microscopy for the presence of the IT and rpS6 fluorescent label across all 16 individuals. In these sections, a total of 827 neurons were identified as positively labeled for the fluorescent antibody to IT and therefore were deemed IT-positive. Of these, 301 also displayed the rpS6 fluorescent label (i.e., were double labeled).

The proportion of IT neurons displaying the rpS6 activation marker was significantly negatively related to the total paternal behaviors displayed [Fig. 2b, Deviance1,14 = 183.38, P > 0.0001] and proportion of time spent in the nest [Fig. 2c, Deviance1,14 = 197.43, P = 0.0016]. Individuals were arbitrarily divided into two groups based on whether they displayed greater than 35 total nips and fans toward the eggs (“good”; n=5 individuals), or fewer than 35 nips and fans (2d “poor”; n=11). An average of 25% (± 3.4 SEM) of IT neurons displayed rpS6 activation marker in the “good” fathering group compared to 39% (± 1.9 SEM) in the “bad” fathering group, a difference that was statistically significant [fisher exact test, P < 0.0001]. However, recall that the designation of “good” and “poor” may not accurately reflect the quality of the fathering for the reasons described in the “Behaviors” section above.

5.4. DISCUSSION

To the best of the authors’ knowledge, this is the first study to investigate the correlation between activation of IT neurons and levels of fathering behavior in any species. The results confirm our technical capability to measure activation of IT neurons using the double labeling method with rpS6 in association with fathering behavior. However, the outcome was not consistent with our a priori hypothesis. The number of parental behaviors and the proportion of time spent in the nest were negatively correlated with activation of IT cells, rather than positively correlated as we
expected based on previous research. Furthermore, fathers classified as ‘poor’ showed a significantly higher proportion of activated IT neurons as compared to those fathers classified as ‘good’. We conclude that these results should be interpreted with caution because of the uncertainty in evaluating the quality of the step-fathering behavior. We propose that future studies repeat the experiment using experienced instead of naïve fathers and that instead of evaluating the correlation between degree of fathering and activation of IT neurons we use an experimental manipulation in which half the males are presented with eggs and half not. This experimental manipulation will not be limited by the difficulty of evaluating the quality of the fathering, and instead will focus on the simpler comparison of step-fathering versus no step fathering.

5.4.1 Behaviors

The total number of paternal behaviors present in step fathering males used in this experiment was dramatically less than what he have reported in biological fathers in previous studies (DeAngelis et al., 2017a; DeAngelis and Rhodes, 2016). Moreover, in unpublished observations, we have noted that biological fathers increase their total parental effort over time, meaning that they become better fathers with experience. Hence, here we hypothesize that the stepfathers used in this experiment were not displaying a natural high level of paternal care we expected, and therefore it is not necessarily surprising that the paternal behavioral output was not positively related to the proportion of IT cells activated. Future studies may be better designed by either using more experienced males as stepfathers, or by giving naïve stepfathers more time with several batches of eggs in order to provide them with experience, and hence increase their paternal behavioral output.

5.4.2 Activation of IT cells

The proportion of IT cells that were activated as indicated by co-labeling with the rpS6 within the POA was not positively related to the amount of paternal behaviors exhibited, in fact it was negatively related completely opposing our a priori hypothesis. Previous studies exploring the activation of IT cells in the POA found that distinct regions within the POA showed differing results in IT activation in relation to paternal care. In male convict cichlids, the proportion of IT cells that displayed c-Fos neuronal activation marker was elevated in single fathers as compared to lone males or biparental care, however this difference was only significant in one specific region of the POA, the parvocellular layer. Although the proportion was also higher in the magnocellular layer, the
difference was not significant (O’connell et al., 2012). In this present study, we did not distinguish the different subregions of the POA, which could account for the difference. Hence, it is possible that we may have missed POA region specific differences found in earlier works that were washed out in this current study by whole POA measures. However, that seems unlikely given the negative relation we observed. Future work is needed to explore the role of activated IT neurons in POA specific regions, and other regions of the brain involved in parental care.

5.5. CONCLUSIONS

Overall, this study provides valuable insight into how stepfathering behavior in *A. ocellaris* can be used as an invaluable tool in the dissociation of parenting from mating when interested in the mechanism directly involved in paternal care. Additionally, here we show that the activation marker rpS6 can be readily co-labeled with IT neurons to show IT neuronal activation. Future studies utilizing more experienced stepfathers, an experimental approach to manipulate fathering as opposed to examining only correlations between neuronal activation and degree of fathering, and exploring region specific activation patterns in the POA is needed before we can make any strong conclusions about the role of IT cell activation in fathering behavior in *A. ocellaris*. 
**Figure 5.1 Immunofluorescence of Isotocin and the Ribosomal Protein S6.** Each color was excited independently, showing isotocin positive neurons (IT), neurons positive for the ribosomal protein S6 (rpS6), background DAPI stain labeling all cell nuclei, and all colors (red, green, and blue) excited simultaneously (Combined).
Figure 5.2 Male parental behaviors and proportion of isotocin (IT) activated cells. 2a, proportion of time spent in the nest is positively correlated with egg loss. 2b, total parental behaviors are negatively correlated with the proportion of IT cells activated within each individual. 2c, proportion of time spent in the nest is negatively correlated with the proportion of IT cells activated within each individual. 2d, Total paternal behaviors vs. time spent in the nest is shown, blue line depicts arbitrary division point of ‘good’ vs. ‘poor’ fathers.
**Figure 5.3 Isotocin cell activation in good and poor fathers.** Within each individual, the probability of having an isotocin neuron activated is significantly higher in poor fathers compared to good. Circles represent the mean of each fathering type, line represent the standard error of the mean.
5.7. REFERENCES


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CHAPTER 6:

CONCLUSIONS AND FUTURE DIRECTIONS

6.1 Sex differences in steroid hormones and parental effort across the breeding cycles

6.1.1 CONCLUSIONS

This study highlights the anemonefish *Amphiprion ocellaris* as a promising model in elucidating the proximate regulatory mechanisms governing male parental care. In this species, males are the predominant caregivers, and display an extraordinary amount of time and effort tending to eggs throughout their lifetimes. The parental behaviors exhibited by males include: nips (mouthing of the eggs to keep them free of fungus and debris), fans (providing oxygen rich water), and spending time in the nest (presumably being attentive to egg conditions). Additionally, these behaviors are displayed similarly to what has been reported in natural conditions in closely related species (Godwin, 1994a, b; Godwin and Thomas, 1993), providing a novel and auspicious model in studies of male paternal care.

Here I show that circulating androgens remain elevated in reproductive males independent of whether there are eggs present of not. This provides evidence that contradicts the ‘challenge hypothesis’, where in this species, high androgens are not a hindrance to high levels of male parental effort. Furthermore, this study demonstrates that males display high levels of parental care largely in isolation from challenging social encounters, meaning that males are not defending nests from conspecific intruders, fighting off nest predators, or competing for mating opportunities. This allows parental care to be studied specifically, providing confidence in the extrication of specific mechanism involved.

6.1.2 FUTURE DIRECTIONS

In this study, hormonal measurements were taken on different days in separate spawning periods, and hence measurements were taken several weeks apart. This method was implemented because blood draws cannot be performed daily in such small animals. A possible future direction to better understand the subtleties in hormonal variation during parental care in male *A. ocellaris* would be to measure hormones via water born methods as outlined in Kidd et al. 2010 (Kidd et al., 2010). Due to the respiratory properties of teleost fishes, circulating hormones are released into the water via the gills, and therefore enzyme immunoassays can be utilized to measure hormone
concentrations from the water. Because blood draws are not necessary for this method, hormone measurements can be taken daily, and daily variation measured.

An additional opportunity for future examination engendered by this study involves introducing nest predators and/or territorial intruders. If the challenge hypothesis is indeed relevant in this species, I would expect circulating androgens to increase in response to challenging circumstances. Furthermore, it would also be possible to artificially increase androgen via androgen administration and measure parental care. This would provide insight into whether artificial elevation of androgens reduces parental effort in male *A. ocellaris*.

6.2 Opposite effects of nonapeptide antagonists on parental behaviors in the teleost fish *Amphiprion ocellaris*

6.2.1 CONCLUSIONS

This study provides several intriguing findings. First, I show that male parental behaviors in *A. ocellaris* are consistent among individuals across spawning periods and variable between individuals. More specifically, when measuring the number of nips, fans, and the amount of time spent in the nest, individual males show a similar behavioral repertoire regardless of which spawning period is being observed. Fathers in this species also show a pattern of increasing parental care across the egg-rearing period. Parental care starts at a rate of 150 behavioral acts per 10-minute observation period, then slowly decreases until the 4th day of the egg-rearing period where it then significantly rises from day 4 through day 7 where on day 7 parental care peaks at a rate of 300 behavioral acts per 10-minute observation period.

Additionally, I show that blockade of isotocin (IT) and arginine vasotocin (AVT) signaling using intraperitoneal injections specific to each receptor show opposite corresponding results in parental effort in male *A. ocellaris*. Blockade of IT decreases parental effort while blockade of AVT increases parental effort. Here, I conclude that IT shares a conserved role in the regulation of parental care independent of the sex of the primary caregiver, and adds to the literature showing that IT is remarkably conserved in its role facilitating parental care across vertebrate lineages. Conversely, I conclude that AVT is likely involved in ancillary behaviors related to reproductive success but not specifically to male parental care.
6.2.2 FUTURE DIRECTIONS

This study demonstrates that *A. ocellaris* males present consistent individual variation in parental effort and that this variation can easily and consistently be measured across spawning periods. This provides exciting avenues in exploration of the neural mechanism responsible for this variation. Do males that display higher levels of parental effort also show increased IT signaling? Or conversely lower AVT signaling? Future studies, utilizing larger samples sizes, may elucidate answers to these questions by quantifying IT and AVT cells in the preoptic area (POA) of the hypothalamus and correlating these cell numbers to total parental effort.

Furthermore, this study implemented intraperitoneal injections that presumably blocked IT and AVT receptors throughout the brain. However, where those receptors are located and how they affect down stream regulation of paternal effort remains unknown. Hence, future studies examining the abundance and distribution of IT and AVT receptors in the brain may provide insight into the brain region specific regulation of how this signaling systems work to promote parental care in *A. ocellaris*.

Findings from this study suggest that AVT signaling is inhibitory to parental care, as blockade of AVT significantly increased total parental effort. This was a surprising finding for two reasons: first, parental effort is already extremely high in these fish and a ceiling effect of any pharmacological increase in care was expected. Second, it is unusual to increase targeted behaviors with pharmacological manipulations. This finding provides exciting opportunities for future exploration of how and why blockade of AVT signaling increases parental care. I hypothesize that blockade of AVT reduces vigilance and nest defense, hence allotting more effort to be specifically directed towards tending the eggs. One possible future experiment would be to repeat this study in a field setting, where nest predators are naturally present. If in fact blockade of AVT increases parental care by reduced vigilance, this could easily be quantified in the field. Furthermore, we may also expect blockade of IT to increase vigilance as it reduced parental effort. This would be a powerful study to substantiate the claims that these nonapeptides work in distinct ways to regulation offspring survival.
6.3 Dynamic regulation of brain aromatase and isotocin receptor gene expression in *amphiprion ocellaris* pairs depending on breeding status

6.3.1 CONCLUSIONS

Findings from this work show that whole brain levels of IT receptor and aromatase gene expression increase in the context of parental care. Males actively displaying high levels of parental care have higher expression of IT receptors and aromatase than those that are not currently caring for eggs. These findings substantiate the literature consensus that IT signaling is crucial for high levels of parental care, and also add to a smaller body of evidence showing that aromatase activity may also be important for parental care, particularly in males (Trainor and Marler, 2001, 2002). Furthermore, this work highlights the importance of the interplay between circulating steroid hormones, brain aromatase, and IT signaling processes (Gimpl and Fahrenholz, 2001; Tribollet et al., 1990). If indeed estradiol (E2) in the brain is important for high levels of male parental care, then brain aromatase activity converting testosterone to E2 may be a critical component of male parental care. Moreover, recent studies have highlighted an important relationship between E2 and oxytocin (OT) signaling, where E2 facilitates the production of OT receptors and increases the binding affinity of OT within the POA of the hypothalamus.

6.3.2 FUTURE DIRECTIONS

Future works examining the relative gene expression levels of IT receptors and aromatase in a brain region specific manner may provide insight into which brain regions specifically are involved in the regulation of male parental care. These studies should aim to isolated out specific regions including Several studies have shown differential abundance of AVT and IT receptor gene expression levels which are brain region specific. Using the brain atlas developed within the Rhodes lab, future works may aim to either dissect out brain regions, or alternatively, use brain punches to isolate out these regions in measuring brain regions specific gene expression levels.

Another exciting opportunity for future studies generated by these findings is exploring the interplay of T, E2, aromatase, and IT. While it has been hypothesized that local synthesis within the brain of E2 from T by aromatase is important for parental care, this has never been directly measured. Additionally, if the process of increasing E2 within the brain promotes increases in IT and IT receptor production then future works may aim to quantify the relationship between levels of brain E2 and IT. Therefore, direct quantification of T and E2 within the brain while concurrently blocking aromatase activity with a specific aromatase inhibitor may provide exciting evidence in
understanding the interplay between circulating steroid hormones levels, local synthesis of brain steroid hormones, and IT signaling processes.

6.4 Correlations between step-fathering and activation of isotocin neurons in the preoptic area of the hypothalamus

6.4.1 CONCLUSIONS

This is the first study to use a step-fathering paradigm in order to dissociate the neural substrates of reproduction from parental care. The use of stepfathers is a powerful tool in understanding parental care, as the behavioral act of sperm release may alter the brain prior to parental care experience. Here we show that activation of IT neurons can be quantified via immunofluorescence using a double label of IT and the neural activation marker rpS6. However, the step fathers in this experiment were inexperienced, and did not provide care in a way that either experience fathers, or experienced stepfathers normally do. For example, in biological fathers, time in the nest, as well as nips and fans are positively correlated with offspring survival as measured by egg counts on the day the eggs are laid to the last day eggs are present. In this experiment, stepfathering behavior shows the opposite pattern, where time in the nest was negatively correlated with offspring survival. This is presumably due to stepfathers either eating the eggs, or providing insufficient offspring care.

6.4.2 FUTURE DIRECTIONS

The methodological validation of the immunofluorescent double label of IT and rpS6 provides a powerful tool in understanding how IT neurons responds to high levels of male parental care. However, future works should work to provide experience for naïve stepfathers. Males increase their efficiency of care over time, and therefore either using experience biological fathers in a step-fathering paradigm, or providing naïve stepfathers with multiple batches of eggs in order to garner experience may yield results more consistent with what would be expected. That is, increased IT neuronal activation in relation to high levels of male parental care.

Another exciting opportunity may implement a broader approach to identify the neuronal phenotype of activated cells as measured by the rpS6. Here you can run and initial experiment to identify specific regions of the brain that are activated in response to step-fathering behavior. Subsequent to identifying these brain regions, each region can be dissected out, run through a rpS6 binding affinity column where cells marked with the rpS6 are isolated. Then these cells can be
processes for a bottom up approach with RT-PCR measuring cells of interest, or RNA-Seqencing in order to identify all the genes expressed in these activated neurons. Along with the recent development of the *A. ocellaris* transcriptome these methods provide promising avenues for a more targeted approach in understanding male parental care
6.5. REFERENCES


