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The Role of Oxytocin on Social Behavior Associated with the Formation of a Social Pair-Bond in the Socially Monogamous Convict Cichlid (Amatitlania nigrofasciata)

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We are submitting a thesis written by Christopher Garcia entitled "The Role of Oxytocin on Social Behavior Associated with the Formation of a Social Pair-Bond in the Socially Monogamous Convict Cichlid *(Amatitlania nigrofasciata)*". We recommend acceptance in partial fulfillment of the requirements for the degree of [redacted]

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The Role of Oxytocin on Social Behavior Associated with the Formation of a Social Pair-Bond in the Socially Monogamous Convict Cichlid (*Amatitlania nigrofasciata*).

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Christopher Garcia

Abstract

The mechanisms for monogamy have evolved several times throughout history across various taxa in accordance with selective pressures. In vertebrates, monogamy is facilitated by the formation and the maintenance of social pair-bonds between mates. Social pair-bonds are a form of selective attachment that require complex neurobiological pathways in order to develop and continue. These neurobiological pathways are often regulated by neuroendocrine mechanisms, such as the release of the two neuroendocrine nonapeptides, oxytocin and arginine vasopressin, in specific parts of the brain or body. These neuroendocrine peptides play a big role in social and sexual behaviors. In prairie voles (*Microtus ochrogaster)* they influence affiliation toward mates and aggression toward conspecifics. Although, oxytocin can affect such physiological processes in the body as parturition and heart rate, as well as social behaviors such as affiliation, mate-defense, and social recognition, these effects are largely species-specific. In prairie voles, oxytocin primarily affects female social behaviors but not those of males'. In other species, however, that is not necessarily the case. For example, in zebra finches (*Taeniopygia guttata*) and convict cichlids (*Amatitlania nigrofasciata)* exogenous oxytocin agonists or antagonists appear to influence social pair-bond formations in both males and females. Agonists tend to promote social pair-bonds while antagonist delay or prevent social pair-bonds.

I, therefore, hypothesized that endogenous oxytocin levels in monogamous convict cichlids would increase as a social pair-bond develops and would decrease after the paired mates are separated. To test this hypothesis, I collected plasma samples from 12 male and 12 female convict cichlids before a social pair-bond developed, during the

pair-bond, and after the pair was separated. The plasma was analyzed for oxytocin levels using ELISA kits specific for freshwater fish. I also hypothesized that as a social pair-bond developed, affiliation between the pair members would increase and aggression would decrease as compared to when they were first introduced. To test this hypothesis, behavior was recorded using 10 min. focal samples using a convict cichlid ethogram. The results from the hormonal analysis revealed that although oxytocin levels did change, they did not do so in accord with my hypothesis. I found a huge variability in baseline oxytocin levels, which is not uncommon, and that may have been a factor in these results. I did find elevated oxytocin levels during the separation stage of the experiment, which may suggest that the oxytocin levels observed were due to other reasons. One reason could be due to timing, as blood collection took place 1 to 3 days after separation, which could mean that the oxytocin levels actually reflect those of a pair-bonded individual. The other more likely reason for the oxytocin levels observed could be that the presence of the offspring significantly increased or maintained oxytocin levels high. This would explain why there was no significant difference in oxytocin levels between one, two or three days of separation in the females, who stayed with the offspring, but there was a significant decrease in oxytocin levels between 1 and 3 days of separation in the males, who were separated from both the female and the offspring.

The results from the behavioral observations went largely as expected except for bouts of affiliative behavior in males, which did not significantly differ between the phases of social pair status. This was largely resolved when affiliative bouts were combined with reproductive bouts to form a whole reproductive cycle data point. In that case male affiliative + reproductive behavior did increase significantly one day prior to

the pair-bond phase/spawn day as compared with introduction day oxytocin levels. There was no correlation between affiliative, aggressive, and reproductive bouts of behavior observed and the oxytocin levels recorded, when the categories of behaviors were tested individually. There was, however, a partial correlation between affiliative + reproductive behavior and oxytocin levels in males, when social pair status was controlled for, although only about 13.5% of the variation in affiliative + reproductive behavior in males was explained by the oxytocin levels. There was also a correlation between affiliative + reproductive behavior and oxytocin levels in females, both when social pair status was controlled for and when it was not. However, only about 16.6% of the variation in affiliative + reproductive behavior in females was explained by the oxytocin levels. Therefore, it seems that oxytocin may influence social pair-bond formation in convict cichlids through several different mechanisms not restricted to affiliation, such as social recognition, increased sociality, or the interactions with other hormones. It is also possible that the peripheral oxytocin levels observed do not reflect those in the central nervous system and thus does not play a role in the social behaviors observed, although there is some evidence to suggest that peripheral oxytocin can affect the central nervous system through indirect pathways (Grippo *et al.,* 2012; Valstad, 2017).

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Introduction

Monogamy

There are many mating strategies among vertebrate species. These strategies vary greatly across taxa and differing vertebrate lineages. Most mammals species tend to be polygynous, which occurs when one male mates with or has access to more than one female (Aloise King *et al.,* 2011) and only about 3% of mammals are monogamous (Reichard and Boesch, 2003). On the other hand, about 90% of birds are, in fact, monogamous (Reichard and Boesch, 2003; Aloise King *et al.*, 2011; Kvarnemo, 2018). Monogamy is also frequently observed in fish species, although at a lower rate than in birds. It is, however, highly prevalent among cichlid fish (Oldfield and Hoffman, 2011).

Not all forms of monogamy are the same and it is important to distinguish between the three types of monogamy in order to understand the social behavior behind them. Social monogamy is the most common type and is simply the sharing of a living arrangement between males and females, such as a territory or nesting site, without any inference of reproduction. Sexual monogamy means a male and a female appear to form an exclusive sexual relationship for at least one breeding cycle. Genetic monogamy is similar to sexual monogamy, but DNA analysis can confirm that the members reproduce exclusively with each other (Reichard and Boesch, 2003).

All mating systems, especially monogamous ones, have an important temporal factor included. The length of time monogamous partnerships last varies significantly across taxa. Most monogamous species are socially monogamous and form pair-bonds for only one or two reproductive cycles, such is the case for most song birds (Petrie and

Kempenaers, 1998). Very few species actually form pair-bonds for life, as sexual and genetic monogamy are very rare in nature (Reichard and Boesch, 2003). Even in seemingly sexually monogamous species, there tends to be a significant level of extra-pair copulations among many individuals (Griffith *et al.*, 2002; Petrie and Kempenaers, 1998).

Among vertebrate species, polygamy, but especially polygyny, tends to be the most common mating pattern (Kvarnemo, 2018). Males tend to increase reproductive success with each additional mate and DNA analysis has shown that many putatively monogamous species actually engage in extra-pair copulations (Griffith *et al.*, 2002). Despite this, the obvious presence of monogamous systems throughout several taxa indicates that there must be a benefit to forming a monogamous pair.

Monogamy arises for any of at least three reasons. First, it can emerge when a female has difficulty or cannot successfully rear her young without the help of a mate and thereby benefits from biparental care. Biparental care in this case is beneficial for the reproductive success of both the male and female because the more a male contributes to the parental care of his offspring, the likelier the pair is to successfully rear the young to independence (Reichard and Boesch, 2003).

Second, monogamy can arise when the distribution of resources is not uniform across space, thereby necessitating the formation and defense of territories. It is easier for a female to defend a territory if she forms a relationship with a male, especially a large one. If females are a limiting resource, then it becomes beneficial for the male to follow the spatial distribution of the females (Reichard and Boesch, 2003).

Third, monogamy can emerge due to mate choice. If there is little variation in the quality of available males, females can benefit from mating with any unmated male. However, if there is large variation in the quality of males, there is a benefit to females for mate guarding. Mate-guarding females can secure the parental care from the male for their own offspring. If parental care is a resource, then both males and females can benefit from securing their mate by fending off potential intruders, especially during the early stages of pair formation (Reichard and Boesch, 2003; Kvarnemo, 2018).

Social Pair-Bonds

In general, monogamous mating systems rely on the formation of social pair-bonds between adults. These bonds are species-specific and usually begin with a proximity-seeking stage between members of a species. For many species, especially birds, the bond formation usually continues with a courtship stage wherein members, primarily females, assess the prospective partner's qualities (Klatt and Goodson, 2013; Pedersen and Tomaszycki, 2012). During this stage, a preference toward a specific individual can develop in some species. Often, it is the act of mating itself that induces a pair-bond to form with a specific individual (Johnson and Young, 2015). Notably, these bonds are a form of selective attachment, with similar neurobiological pathways to those in parent-offspring attachments but involving two adult mates instead. In many cases this bond is also reinforced by the stress and anxiety caused by the separation from the partner (Tabbaa *et al.*, 2016; Johnson and Young, 2015).

In some species, social pair-bonds can develop without a complex or lengthy courtship ritual, instead relying on subtler forms of mate quality assessment. For instance,

in many rodents such as in the common brown rat, *Rattus norvegicus*, copulatory behavior can be treated as courtship communication (Dewsbury, 1988). Species can also develop social pair-bonds through extended periods of increased affiliative behaviors between two individuals (Tabbaa *et al.,* 2016) and/or increased aggressive behaviors toward intruding conspecifics, such as in the monogamous species, the prairie vole, *Microtus ochrogaster* (Insel *et al.,* 1998; Oldfield and Hoffman, 2011; Johnson and Young, 2015). In prairie voles, extended cohabitation has been observed to also induce social preferences and bonds under laboratory settings (Cho *et al.,* 1999), although these preferences may not necessarily include sexual preferences (Young *et al.,* 2011).

The time frame necessary for a pair-bond to form can also differ significantly between species, sometimes even between individuals within a species. Some species can form pair-bonds within hours, while others can remain in courtship for several days before actually forming a pair-bond (Johnson and Young, 2015). Often times, but not always, the duration and complexity of a courtship behavior can predict how long the pair-bond will last, with simple, short, or lekking courting behaviors resulting in only brief periods of bonding. Complex or extended courtships, in contrast, occur most often in monogamous birds, not only for mate quality assessment but also to stimulate and coordinate reproductive physiology (Wachtmeister and Enquist, 2000).

These cases of complex *vs* simple pair-bonding behaviors relating to the length of a pair-bond, are also found in mammals, fishes, and insects. For instance in the monogamous and biparental cichlid, *Neolamprologus callipterus*, androgen levels during the courtship stage tend to be higher than those of the closely related but polygynous, *Lamprologus callipterus,* and thus the monogamous species has higher degree of

aggressive response toward intruders during courtship. The polygynous species, *L. callipterus,* which uses a biparental system, has a higher androgen level and a higher degree of response toward intruders than does *Tropheus moorii,* a polygynous cichlid with only maternal care for the offspring during courtship, but has a lower androgen level and aggressive response than does the monogamous and biparental *N. callipterus*. Polygynous lekking species with only maternal care, such as the cichlid, *Pseudosimochromis curvifrons*, have the lowest amount of androgen levels and the lowest degree of response toward intruders during courtship (Hirschenhauser *et al.*, 2004).

In many cases, such as in the prairie vole, social pair-bonds do not only result in selective partner preference formations but also in selective targets of aggression (Williams *et al.* 1992). In fact, in some species, such as those who are naturally socially affiliative or nonaggressive toward conspecifics like the prairie vole, it is the levels of aggression that changes as a social pair develops. Affiliative behaviors remain constant only for the paired mate (Insel and Hulihan, 1995; Tabbaa *et al.*, 2017). This increase in aggressive behaviors toward intruding conspecifics is often associated with mate-guarding (Insel *et al.*, 1998). An additional behavior associated with social pair-bonds is the biparental care of young (Young *et al.,* 2011).

Hormonal Influences On Pair-Bonds

Much of our understanding of social pair-bonds and attachments, and the neurobiological mechanisms behind them, have come from extensive investigations of the socially monogamous prairie vole (Tabbaa *et al.*, 2016; Johnson and Young, 2015;

Insel *et al.,* 1998; Carter, 2017; Hurleman and Sheele, 2010). This species develops partner preference after only 24 hours (Johnson and Young, 2015) The prairie vole is closely related to the montane vole, *Microtus montanus*, but unlike the prairie vole, the montane vole is polygynous which allows for comparative analyses between the two species (Insel *and* Shapiro, 1992; Insel *et al.,* 1998). Through such studies, several factors have been implicated in the process of pair-bond formation, beginning with the approach between individuals, which is facilitated in some species, such as in mice, *Mus musculus*, and in humans, *Homo sapiens*, by the neuropeptide hormone, oxytocin (OT), by reducing the behavioral and neuroendocrine responses associated with social stress (Crawley *et al.,* 2007; Ditzen *et al.,* 2009). Another neuropeptide hormone commonly involved in social and reproductive behaviors is arginine-vasopressin (AVP).

Both OT and AVP play major roles in the regulation of complex social behaviors including the formation of social pair-bonds, parental care, sexual behavior and aggressive behaviors (Insel and Young, 1998; Carter, 2017). The role of these peptides in facilitating social and reproductive behaviors have largely been conserved across both invertebrate and vertebrate taxa. However, the mechanisms and behaviors by which theses neuropeptides affect social and reproductive behaviors are diverse and species-specific and sex-specific. For example, OT can facilitate socio-sexual behaviors in females by promoting egg laying or parturition and lactation, depending on the species (Johnson and Young, 2015). Based on studies of prairie vole brains, it seems that the distribution and concentration of the oxytocin receptors (OTR) and arginine-vasopressin receptors (V1aR) throughout the brain, along with the regional expression of the neuropeptides themselves, cause the variation in social and reproductive behaviors

observed in many species and individuals (Johnson and Young, 2015; Liu and Wang, 2003; Insel and Shapiro, 1992).

The OT-AVP pathway is very old and homologs for these peptides have apparently existed for about 700 million years (Hurlemann and Scheele, 2015). The peptides consist of nine amino acids each and differ only by two amino acids. They both originated from the same ancestral gene that produced vasotocin, an ancient homolog that differs from OT and AVP by one amino acid. Today, vasotocin is found in fish, reptiles and other vertebrates, including mammal fetuses (Tabbaa *et al.*, 2016; Carter, 2017). OT and AVP are largely produced in the paraventricular nuclei and the supraoptic nuclei by magnocellular neurosecretory cells (Ross *et al.*, 2009) and are released from various regions of the brain, such as the nucleus accumbens, ventral pallidum, or the lateral septum (Johnson and Young, 2015). These peptides are involved in maintaining behavioral and physiological homeostasis (Johnson and Young, 2015; Liu and Wang, 2003; Carter, 2017). They can also be produced by the pituitary gland and released throughout the body to act as hormones promoting lactation and parturition (Hurlemann and Scheele, 2015) and affecting the uterus, testes, digestive system, kidneys, or the thymus (Carter, 2017).

The dopamine pathway and its involvement in the reward system of the brain can also mediate pair-bond formation. Mating increases the release of dopamine in the nucleus accumbens, which in turn can help promote a partner preference (Johnson and Young, 2015). The activation and interaction of dopamine receptors and OT receptors in the nucleus accumbens have been observed to be essential for pair-bond formation in prairie voles. The distribution of both of these receptors within the nucleus accumbens,

among other parts of the brain, may determine the differences in pair-bond formation observed between individuals and between monogamous and polygynous species (Liu and Wang, 2003). In the case of monogamous species such as the prairie vole, OT tends to concentrate in the lateral portion of the amygdala, the prelimbic cortex, the stria terminalis, the midline of the thalamus, and the nucleus accumbens. In contrast, in the polygynous montane vole, OT concentrates in the lateral septum, cortical nucleus of the amygdala, and the hypothalamus (Insel and Shapiro, 1992).

Pair-bonding can also be regulated by OT through its effect on social recognition, given the proximity of OT receptors to areas of sensory attention and processing (Johnson and Young, 2015). For example, in humans, a polymorphic OT receptor allele found in autistic children can be used to predict the ability to recognize familial faces (Skuse *et al.*, 2014; Young, 2015).

After pair formation, the maintenance of pair-bonds is facilitated by other factors such as mate guarding. The social buffering effects of a mate during stressful events helps keep partners together (Johnson and Young, 2015). Other factors involved in pair maintenance are the negative effects associated with social pair loss, such as the release of stress hormones or the expression of depressive-like behaviors, such as those observed in prairie voles during the separation from their partners (Johnson and Young, 2015; Insel and Young, 1998; Grippo *et al.*, 2007).

As noted earlier, OT can interact with dopamine or AVP to influence social behaviors, but OT can also play a huge role in social behaviors. Its effects are highly variable among species and are often sex-specific (Hurlemann and Scheele, 2015). In the

context of social experiences and social recognition, its effects can also vary between individuals (Johnson and Young, 2015). OT usually mediates social behaviors such as social pairing, reproduction, social recognition, aggression related to defense, and it helps reduce stress to permit an individual to interact with other members of its species. (Heinrichs and Domes, 2008; Hurlemann and Scheele, 2015). In prairie voles, OT facilitates partner preference formation in females but not males (Cho *et al.,* 1999). In nature, the release of OT during mating is what ultimately induces partner preference formations in female prairie voles. However, in laboratory settings, the act of mating itself is not necessary for the development of partner preference in female prairie voles, if cohabitation with a male partner is accompanied with the central infusion of OT (Williams *et al.,* 1994). The same partner preference can develop in male prairie voles through the central infusion of AVP accompanied by the presence of a female partner (Insel and Hulihan, 1995).

In male prairie voles, AVP plays the same role that OT does in females. AVP increases partner affiliation and targeted aggression toward intruders (Insel *et al.,* 1998; Cushing and Carter, 2000; Liu and Wang, 2003). In prairie voles, stressful events such as social isolation or partner separation can disturb OT concentrations which in turn lead to changes in behavior and physiology. Social stressors, in prairie voles, can lead to increases in heart rate, heart rate variability, the hyperactivation of the hypothalamic-pituitary-adrenal axis, which results in autonomic imbalances and neuroendocrine disruptions of the hypothalamic paraventricular nucleus, particularly the increase in corticotropin releasing hormones; it may also cause a sympathovagal imbalance while at rest (Grippo *et al.,* 2007; Grippo *et al.,* 2012). OT can help reduce these stressful and

anxiety-like behaviors if a social pair mate is present (Grippo *et al.*, 2007; Johnson and Young, 2015). The peripheral administration of OT can also buffer the autonomic responses associated with stress and anxiety in prairie voles, such as increased heart rates and heart rate variability, but does not necessarily affect their expressed outward behavior (Grippo *et al.*, 2012). Although in prairie voles, the effects of OT and AVP are sex dependent (Cho *et al.,* 1999), OT can affect both sexes in similar ways in other species, such as convict cichlids and zebra finches, *Taeniopygia guttata* (Oldfield and Hoffman, 2011; Klatt and Goodson, 2013).

In contrast with prairie voles, in zebra finches OT antagonists affect both males and females by increasing latency to pair and reducing courtship behaviors, pair formations, and pair stability (Klatt and Goodson, 2013; Pedersen and Tomaszycki, 2012). Another study, this one involving the monogamous convict cichlid, *Amatitlania nigrofasciata*, found that an OT antagonist administered peripherally reduced affiliative behavior in males toward their potential mates and reduced aggressive behaviors to potential intruders during pair-bond formation. The same OT antagonist, however, had no effect on affiliative or aggressive behaviors once a pair-bond was already established. Therefore, in convict cichlids, OT plays an important role in the formation of, but not the maintenance of, pair-bonds (Oldfield and Hoffman, 2011).

The Present Work

For this thesis, I looked into the neuropeptide regulation of social pair-bonds in the convict cichlid. The complex social behavior of the socially monogamous convict cichlid is an ideal subject to use for studying social behaviors. It is small and robust, and

its behavior, neuroendocrinology, and physiology have been widely studied (Schmitter-Soto, 2007), so there is vast data relating to the fish that can be used to compare with my own findings. In addition, the physiology of laboratory and wild populations has not been found to be significantly different (Van Breukelen *et al.*, 2017).

This species typically forms a monogamous pair-bond for breeding season (seasons based on fluctuations in rainfall) in the wild, although it can be several breeding seasons in laboratory settings, and both the male and the female provide parental care for their eggs and free-swimming young. A Breeding pair typically forms in about four to six days. The fry typically take three days to emerge from their eggs after being spawned and take another three days to develop their fins in order to swim. The young typically stay with their parents for four to six weeks before becoming fully independent. Depending on breeding sites and food availability, the parent cichlids may breed again during the same breeding season. Males are more likely to breed with a new females after a brood cycle is complete as females take longer to replace their gametes than males (Wisenden, 1995).

Social pair-bonds between male and female convict cichlids form as an advantage to better defend the brood from intruders and predators (Gagliardi-Seeley and Itzkowitz, 2008). Both pair members defend their brood and, in the absence of the male, the female may be unable to care for the young by herself and may lose them. A study by Bockelman and Itzkowitz in 2008 revealed that the prolonged courtship associated with the social pair-bond formation of the convict cichlid is directly associated with the production of gametes. Courtship is necessary for the female to produce eggs, but if the male is lost close to the time of spawning, such as when a male disappears due to predation, the female can quickly look for a new male partner and spawn as if courtship

had already taken place. If no new social pair-bond forms, however, she does not spawn, suggesting that the presence of a male partner is necessary for egg deposition (Bockelman and Itzkowitz, 2008). Since the brood depends on bi-parental care, the male also has a choice of the female he mates with, including leaving after pair formation if the brood does not seem viable (Itzkowitz *et al.*, 2003). For the most part however, because parental care is a resource for this species, it may be to the advantage of the male to remain with and care for his mate and his brood to increase his reproductive success rate (Reichard and Boesch, 2003).

The neurobiological mechanisms for social attachments and social pair-bonds have been mostly studied in humans, the prairie vole, and a few primates, birds, and cichlids. Still, the results from many of those studies reveal an incomplete picture about the role of OT in social behavior. There are disagreements about whether OT concentrations in the peripheral system are even correlated with the OT concentrations in central nervous system (Valstad *et al.*, 2017). Therefore, we need further studies on the role of OT in social behavior across various taxa, to compare distant and closely related phylogenies, and to offer further insight into the emergence of complex social behaviors.

Measuring circulating OT levels can be an informative tool for studying social behavior. Specific social circumstances may trigger the release of OT (Crockford *et al.*, 2014). Despite debate on the correlation between peripheral nervous system OT and central nervous system OT levels, experiments administrating OT agonists and antagonists across different species reveal that OT, whether in the central nervous system or in the peripheral nervous system, can significantly affect social behaviors.

In this study, I investigated the role that endogenous or circulating OT may have on the formation and the loss of social pair-bonds in the socially monogamous convict cichlid fish. Specifically, my questions were:

1. Do circulating oxytocin levels in convict cichlids change in response to social pair-bond formation and social pair loss?

    - My working hypothesis was that circulating OT levels would increase significantly during pair-bond formation and decrease significantly after social pair loss. This is supported by the release of OT observed in prairie voles after mating and the facilitation of partner preference formation following the use of OT agonists in the prairie vole (Insel *et al.*, 1998). It also seems that most monogamous species have higher numbers of OT and AVP receptors in the brain than promiscuous and polygynous species do (Johnson and Young, 2015). A reduction in OT following social pair loss is supported by the stress and anxiety observed in socially isolated prairie voles, which can be prevented by repeated peripheral or central administrations of OT (Williams *et al.*, 1994; Cushing and Carter, 2000; Grippo *et al.*, 2012; Hurlemann and Scheele, 2015)

2. If the circulating oxytocin levels do change with social pair-bond formation and loss, do oxytocin levels in the plasma correlate with changes in the rates of expression of affiliative or aggressive behaviors?

    - My working hypothesis was that as circulating OT levels increase, affiliative bouts of behavior would increase significantly and aggressive bouts of behavior would decrease significantly. This is supported by the

fact that OT agonists in prairie voles tend to increase affiliative behavior during the partner preference formation stage and OT antagonists produce the opposite effect, reducing the likelihood of producing a partner preference (Cho *et al.*, 1999). In the monogamous cotton-top tamarin (*Saguinus oedipus*), within-species variation in OT was related to variation in affiliation and sexual behavior for both males and females (Snowdon *et al.*, 2010). OT's effect on affiliation is also observed in convict cichlids given an OT antagonist that reduced affiliative behavior during the pair-bond formation stage (Oldfield and Hoffman, 2011).

3. Does a female convict cichlid act in an affiliative or aggressive manner with her former mate, when reunited with him, after a social pair loss?

- My working hypothesis was that, after the disruption of the social pair, a female convict cichlid would act aggressively toward the male if reintroduced to him. This would signal a loss of the social pair-bond between the former pair members because there is a disruption in the ongoing sequences of behaviors putatively at the heart of maintaining the pair-bond. Additionally, the female would be expressing a targeted aggression toward the male normally expressed toward intruding conspecifics (Mackereth and Keenleyside, 1993; Oldfield and Hoffman, 2011).

Materials and Methods

Animals

Adult, reproductively capable *A. nigrofasciata* were purchased from the pet trade (Petsmart/Aquatica) and housed in a laboratory at Winthrop University, Rock Hill, SC. The cichlids were initially housed individually in 37-liter tanks, each with a 115mm diameter flower pot to serve as a breeding site/shelter, since appropriate breeding sites have been found essential for reproduction in this species (Wisenden, 1995; Gumm and Itzkowitz, 2007). Additionally, each tank was provided with about 5 mm of gravel on the bottom, which the cichlids could dig or clear out of their nesting sites. The cichlids were kept at a constant 28-30°C, with light and dark cycles averaging 12 hours' light ±2 and 12 hours' dark ± 2, to simulate tropical day and night cycles. Mean body size for males was 5.13g, SD=1.39 and the mean body size for females was 4.48g, SD= 1.62. They were fed cichlid pellets (Life Spectrum cichlid formula), blood worms, or brine shrimp depending on availability once a day. All males and females were in the black "barred" color phase when first introduced to the tank, but coloring patterns occasionally changed to the cryptic phase after handling. Coloration later changed to parental patterns if they subsequently paired up. Females tended to be more brightly colored with an orange or pink abdomen. All procedures were approved by the Winthrop University Institutional Animal Care and Use Committee (IACUC). After use, animals were given away to interested pet owners by way of online advertisement.

Experimental Design

The cichlids were housed individually, separate from other cichlids at first to identify sex and then to measure mass. To prevent flailing while the mass was being recorded the cichlids were anesthetized first using Tricaine Methanesulfonate (MS-222; Western Chemical, inc., CAS #886-86-2). About 1L of warm water from the cichlid's tank was placed into a separate large holding beaker, in which 350mg of MS-222 were added per liter of water. One cichlid at a time was placed into the beaker until the fish turned on to its side and stopped flailing, signaling sedation. Anesthetization usually took about 5 mins ± 2 mins depending on the individual's size, with larger cichlids taking more time to become fully sedated. At the same time, the first blood samples were collected from each cichlid for subsequent OT analysis. This process is further explained in the hormone measures segment of this thesis. After measurement, the cichlid was returned to its tank to safely recover.

Due to a limited number of fish tanks available only eight cichlids were used at a time, four males and four females, to make four social pairs. This process was thus repeated three separate times, to produce 12 pairs (males, n=12; females, n=12) that were used for the main experiment. Additional cichlids were used that did not pair up, became ill, or died and thus were not used in the experiment. During the first two rounds of the experiment, males tended to be larger than females, but due to low cichlid availability in the pet stores during the last round, it was the females ($\bar{X}$ = 5.65g, 3.6-7.7g) that tended to be slightly larger than the males ($\bar{X}$ = 4.5g, 3.3-5.7g). In the wild, females do tend to prefer larger males due to them being better able to secure and defend breeding sites (Wisenden, 1994; 1995), but size has been shown to not matter if there is no other male

with which to compare. Females will routinely pair with smaller males if they cannot see other larger males (Gagliardi-Seeley et al., 2009). Since breeding sites were provided, there was no need for the males to secure a new site, so the experiment was carried out despite size differences. Still, size was noted, and cichlids were paired with individuals of similar size.

The size of each fish was noted and one male was subsequently placed into the same tank as a female cichlid of similar or slightly smaller size. Behavior was observed each day until social pair formation could be observed. The tanks had cardboard barriers between them to prevent comparisons and interactions between neighboring cichlids and to allow paired tank mates to focus on each other instead. If cichlids acted too aggressively with each other during observations, they were separated by a clear unpigmented plexiglass partion in the center of the tank after observations to prevent direct attacks, but allow them to be in sight of each other. The plexiglass was removed permanently if no direct attacks occurred during observations.

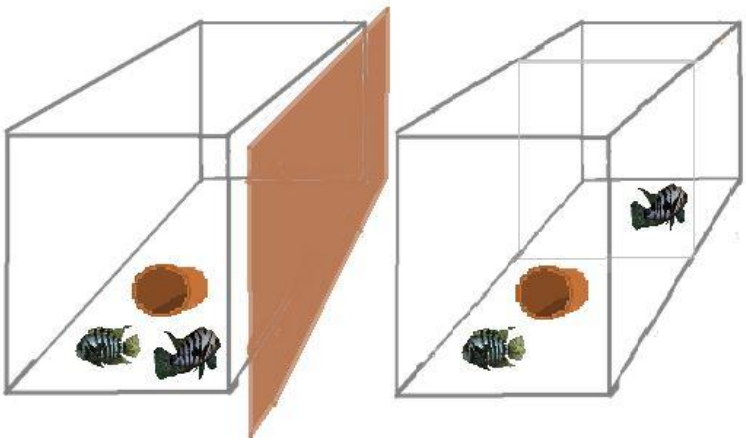

Figure 1. Basic aquarium setup between two adjacent tanks. There is carboard barrier between tanks to obstruct vision between tanks. There is a clear partition in some tanks to separate aggressive fish after observations so as to not cause bodily injuries. Same sex cichlids were also placed in partitioned holding tanks prior to experimentation to avoid conflict.

Behavioral Observations

Behavioral observations for each cichlid started on the day the male was first introduced to a female (Day1). Following introduction, focal males and females were observed for 10 minutes each day throughout pair-bond formation and observations stopped when the pairs were separated. Focal animal sampling (Altmann, 1974) was used to record and score social behavior based on an ethogram of convict cichlid behavior developed by Oldfield and Hoffman (2011). Using this method, a score of one meant one bout of activity was performed. A bout of activity was defined as any behavior described by the following ethogram performed by itself or in a consecutive sequence, within the observation period. A bout stopped when another behavior was performed or no activity was performed for more than one second.

Social behaviors were divided into three categories, affiliative behavior, aggressive behavior and reproductive behavior. The target of the behavior was noted as either the other fish or occasionally the observer. Four behaviors were identified as aggressive. Biting (BI) was when a fish made physical contact with another fish with its mouth open. If biting was left to continue for a prolonged period, it could have led to physical damage on one or both fish, so fish were separated if biting continued for more than 5 minutes. Charging (CG) was when a cichlid rapidly approached another cichlid and was mostly distinguished from "approach" (defined below under affiliative behavior) by context and by the reaction of the other cichlid in the tank. If the target cichlid flinched or swam away rapidly, the behavior was considered a charge. Chasing (CS) was defined as the rapid following of a fish by another fish throughout the tank, sometimes including rapid changes in direction and sometimes involving physical contact if they

caught up. Synchronized attacks (SA) were also recorded although very rarely as they would require the presence of an intruder. Since the tanks did not have visual access to each other, the cichlids rarely needed to synchronize an attack.

Affiliative behaviors included lateral displays (LD), frontal display (FD), tailbeating (TB), circling (CI), greeting (GR), approach (AP), and affiliative bite (AB). Lateral display is when a cichlid showed either its right side or its left side as it remained stationary next to another cichlid and erected its fins. In a frontal display a cichlid oriented to another face to face with its mouth open and its body in a sigmoidal shape. Tailbeating occured when a cichlid either approached another cichlid and used its tail to send a water current toward another cichlid or remained in place parallel to another cichlid and rapidly beat its tail in a sinusoidal fashion. Circling occured when a cichlid swam around its nesting site and its partner or spawn. An approach was simply when a cichlid reduced the distance between itself and another cichlid in a non-aggressive manner or context. It was sometimes difficult to completely distinguish an approach from an aggressive behavior because the target cichlid would react differently at times, either ignoring the approach or seeming to avoid it despite social pair status. This mostly happened with females whom reciprocated most approaches but occasionally swam away from seemingly non-threatening approaches from the male. In general, if a cichlid fled or avoided the approaches, it was considered a charge and thus an aggressive behavior. If the approach was accepted and/or reciprocated, then the behavior was considered an approach and thus an affiliative behavior. An affiliative bite occurred when a cichlid lightly touched another cichlid with its mouth. The mouth can be opened or closed and can be distinguished from an aggressive bite by the reaction of the target cichlid. In this

case, the receiving cichlid did not flee nor flinch but instead remained stationary or reciprocated the behavior. Sometimes, this behavior resembled a kiss when both cichlids performed it at the same time.

The third type of behavior recorded was reproductive behavior, which included dig (DI), quiver (QU), skim (SK), nip-off (NO), and fan (FN). A bout counted as a dig if the cichlid moved the gravel with its mouth or body, resulting in a pit or mount being formed. A quiver occured when the cichlid twitched or shivered rapidly in place. A skim occurred when a cichlid slowly passed by and rubbed its body, specifically its genital papilla, against its spawning site, usually in preparation for spawning. A nip-off took place when the cichlid picked up debris at or near the spawning site in an apparent cleaning fashion. Nip-off also included mouth-brooding behaviors such as when a parent cichlid picked up its fry or eggs with its mouth for transport. Fanning which occurred after spawning, was when a parent pushed water with its pectoral fins against its eggs to oxygenate them. For the most part, only females performed this behavior.

Behaviors were observed each day until pair-bond formation could be determined. Pair-bond formation usually begins when a female starts courting a specific male and attacks neighboring females. A pair-bond is formed when a male stops being receptive to the courtships from other females and instead begins to attack them (Mackareth and Keenleyside, 1993). This usually occurs after 4 days in a captive setting where the cichlids cannot go elsewhere. However, because the tanks in this experiment were shielded from one another, cichlids could really only target each other and not any other cichlids. Since there was no interaction with neighboring cichlids, determining when a pair-bond formed was difficult through behavior observations alone.

Another way pair-bond formation can be determined is by observing the coloration in the female that changed to show it was physiologically ready to spawn (Oldfield and Hoffman, 2011). Both male and female convict cichlids have black or dark gray stripes contrasting with gray or light gray stripes. The intensity of these colors can change slightly in males; however, males do not display the bright colors found in the female's abdomen. Additionally, the intensity of these colors can change much more drastically in females. Female convict cichlids display 3 different coloration phases. The first is dull or cryptic coloration, which as the name describes are subdued with an olive colored background interrupted by black or grey stripes. Females in this phase are usually non-breeding and tend to remain hidden from view. The second is the black phase, in which the female's background color is mostly black or very dark gray, but contrasts with a very bright abdomen, which is usually bright yellow and orange with some blues near the tail. Females in this phase are ready to mate and are actively engaged in affiliative behaviors including courting males. These females are also usually the most aggressive against intruders. The final phase is the parental coloration which is shown after a pair-bond has formed and the female has or is ready to spawn. In this phase, females display contrasting black and white vertical stripes on their sides and retain some amount of bright coloration on their abdomen. This coloration, identifies the female as brood-guarding and no longer available to other males (Wisenden, 1995). Thus, a female was considered to be in a social pair-bond when her coloration changed to a brightly colored abdomen; her abdomen became swollen, indicating readiness to spawn, and she performed certain affiliative or reproductive behaviors vigorously (Oldfield and

Hoffman, 2011). Alternatively, if these behaviors and changes in coloration were missed, spawning presented a definite confirmation of social pair-bond formation.

The color changes in males are not nearly as drastic as they are in females and they have not been observed in the black coloration phase before (Wisenden, 1995). Therefore, coloration in males for this experiment was not noted.

Once a social pair-bond was confirmed using one or more of the above criteria and the second blood collection was performed (details given in the next section), mates were kept together and behavioral observations continued for seven more days to give the cichlids time to recuperate before they were separated. After separation, behavioral observations ceased for one, two, or three days (four per treatment) and the third and final blood collection was performed. The cichlids were separated for different amounts of time to test for any effects that days of separation might have had on OT concentrations. The number of days cichlids were separated were chosen at random. During the third phase, the female was put into a second experiment where she had the opportunity to form a social pair-bond with a different male. This experiment is described in further detail in its own section. After the completion of the second experiment the first male was re-introduced to the female and the behavior was recorded for a final behavioral data point referred to as “re-introduction phase”.

Experiment 1. Hormone Measures

Prior to introducing pair members to each other, blood samples from each cichlid fish were collected to measure the basal OT reading. The cichlids were first anesthetized in a separate container using 350 mg of MS-222 per liter of water. After the cichlid

turned to its side and stopped flailing for a few seconds, which marked full anesthetization, it was quickly moved over an illuminator on a stereo microscope (Wesco Indu Vu 3000 Series) set to 6.5x magnification to have a better view of its veins and arteries. A wet paper towel was placed underneath it and just over its head to cover its eyes. This provided a soft substrate on which the subject could not easily slip as well as providing a material with which to hold on to the subject more easily. The blood was harvested from the caudal vein of the fish close to the caudal fin using a heparinized 27 ½ gauge 0.5mm tuberculin syringe (Beckton Dikinson). The syringe was heparinized just prior to usage with a solution made from heparin sodium salt from porcine intestinal mucosa (Sigma-Aldrich, CAS# 9041-08-1). The heparin solution was made in accordance with the product information sheet, by mixing 50mg of the heparin sodium salt for every mL of distilled water. The syringe was washed with heparin and allowed to sit for 15 seconds before the heparin was flushed out.

Between 25-40 µL ($\bar{X}$ = 32 µL) of blood was collected from each cichlid depending on its size and difficulty in obtaining the blood. This amount was in accordance with the IACUC guidelines for repeated blood collection from the same individual within a 30-day span (Parasuraman, 2010). The cichlids were gently placed back into their respective tanks. The extracted blood was quickly placed into a heparinized 1mL centrifuge tube on ice. The centrifuge tubes were heparinized similarly to the syringes. The blood samples were centrifuged at 4,000 RPM for 15 minutes. The centrifugation separates the blood plasma, which floats at the top, from the red blood cells, leukocytes, and platelets by way of a density-based cell separation. The resulting

supernatant plasma (10-25µL depending on sample size) was pipetted into sterilized 0.5mL Eppendorf tubes and stored at -80 °C for later analysis.

Blood samples were extracted from each cichlid in three different phases using the process stated above. The first blood sample collected was the baseline hormonal reading. The second blood sample was collected after it was determined that the cichlids were in a pair-bond and/or had spawned and was to measure OT levels in convict cichlids during the pair-bond phase. The final sample extraction occurred during a separation phase, in which cichlid pair members were separated from their partners. During the separation phase the male was placed in a separate tank. The female remained in her home tank with her nest and brood. Blood collection occurred 1, 2, or 3 days after separation to see whether the number of days after separation would affect OT levels. Ideally, there would have been a fourth blood collection after former mates were re-introduced to each other following being separated for several days, but this could not be performed without causing significant harm to the fish (due to its size) and going against IACUC guidelines for multiple blood sample collections. Specifically, I could not take more than 1.5% of the cichlid's body weight within two weeks. In this case, a little less than 1% body weight of blood ($\bar{X}$ = 0.032mL) was collected for each sample, spread over a three week period on average.

Pharmacology

Plasma samples were analyzed using a commercially available 96 well-plate Enzyme-linked Immunosorbent Assay kit (ELISA) for fish oxytocin (MyBiosource, Cat# MBS042790). The ELISA was performed in accordance with the manual provided with

the ELISA kit. This kit has a detection range of 6.25pg/mL-200pg/mL. The specificity for OT is close to 100% and there is no significant cross-reactivity with other hormones although there is a < 0.01% cross-reactivity with arginine-vasopressin and cross-reactivity between OT and all its analogues is currently not known. The intra-assay coefficient of variability (Standard deviation/mean x 100) and the inter-assay coefficient of variability for this assay was less than 15%.

The OT ELISA kit works by using a monoclonal anti-oxytocin antibody and a horseradish peroxidase (HRP) conjugate reagent. The samples, the buffer and the HRP-conjugate reagent are incubated together in pre-coated wells for 1 hour. OT from the samples compete with the HRP conjugate reagent for the anti-oxytocin antibody sites. After an hour, the mixture is disposed of and the wells are washed with a wash solution provided by the kit. Two chromogen solutions are added and incubated for 15 minutes to react with the HRP enzyme. As the substrates reach with the HRP, the solution becomes blue. Finally a stop solution is added to each well to stop further reactions and the solution turns from blue to yellow.

Some sample volumes were only 10µL of plasma so to reduce intra-assay variation and to keep samples as equal as possible only 10µL of plasma from each sample was used. This kit did not allow for the dilution of samples, as the concentration gradients of the standards already covered the range of undiluted original samples and diluting samples might have led to concentrations outside the kit's detection range. Therefore, I could not dilute samples and because sample sizes were already small, the analysis could not be run in duplicates. The optical density (O.D.) was read twice at 450nm using an ELISA reader to get a mean absorbance. The standard concentration provided by the kit

was subtracted from the mean O.D. observed to get an adjusted value. The O.D. was inversely proportional to the OT concentrations since OT from the samples compete with the HRP-conjugate reagent for anti-oxytocin antibody sites. The adjusted absorbance values were plotted against a standard curve created based on the O.D. of the standard concentrations. The position of the adjusted absorbance values relative to the standard curve was used to calculate actual OT concentrations.

Experiment 2. Behavior For Second Pair Formation.

Seven days after the separation of paired mates and the completion of the blood sample collecting phase, females convict cichlids were re-paired with new males, that did not take part in the first experiment, to provide them the opportunity to form a new social pair-bond and, ideally, ensure the loss of their first social pair-bond. The new males were for the most part of similar size to the females but because of a lack of availability of large males in the pet market, some of the females were paired with smaller males. There was no issue with this for the most part, as females do occasionally pair with smaller males in the wild when there is no larger male with which to compare them (Gagliardi-Seeley et al., 2009). The same focal sampling method used in the first experiment was used for this experiment. Affiliative, aggressive and reproductive behavior were observed for males and females until the pair could be determined to be in a social pair-bond.

Once a social pair-bond was established, the new male was temporarily separated from the female and the male from the first pair-bond was re-introduced to the female. Behavior observed during this re-introduction was added to the first experiment as re-introduction phase behavioral data. After the behavior was recorded for this phase, the

male was removed from the female and the new male, with which the female had most recently formed a pair-bond, was re-introduced. All healthy cichlid pairs were given away to interested caretakers by way of online advertisements.

This experiment was initially carried out to attempt to induce the social pair loss of the female and her first paired mate and to analyze the behavior and OT concentrations of the cichlids after the subsequent re-introduction of these pair members. I then intended to test for any differences in behavior and hormonal levels between the introduction phase and the re-introduction phase. However, due to the size of the cichlids, a 4^{th} blood sample collection would potentially severely harm the fish, so the hormonal analysis of this part of the experiment was not performed. Only behavioral data were analyzed.

Upon further analysis, these data also allowed for a comparison between the behavior observed with blood sampling included and the behavior without blood sampling included throughout each phase. However, it is important to note that the females had undergone blood sample collections prior to the start of the second experiment. Not all pairs in this experiment spawned despite prolonged housing, decreased aggression, and constant affiliation. Therefore, the data point, pair day, in several cases was determined by high levels of affiliation for at least six days (the average time it took most cichlids to spawn) and low levels of aggression. On only one occasion did a female cichlid refuse to form a new pair-bond even after a second attempt at pair formation with yet another male.

Statistical Analysis

All statistical analyses were performed using IBM SPSS Statistics version 25 software. Statistical significance was set at $p \leq 0.05$. Normality was tested using Kolmogorov-Smirnov tests and homogeneity of variance was tested using Levene's test. OT levels between the three phases sampled (N=24, 12 males and 12 females) were tested using a repeated measures ANOVA with gender as a between-subjects factor. Since blood samples were collected after one, two, or three days of separation, with four pairs per day, the effect days of separation had on OT concentrations was measured using a one-way ANOVA, performed for males and females separately. If an ANOVA demonstrated a difference, a Bonferroni post-hoc test was subsequently performed to pinpoint which groups differed from on another.

The data used to measure behavior within each phase were single-day data points. Initially, I intended to compare bouts of aggressive, affiliative, and reproductive behavior only for introduction day, pair day, and re-introduction day. However, it has been observed that affiliative behaviors are highest during the pair formation stage and are reduced or partially replaced by reproductive behaviors after spawning (Oldfield and Hoffman, 2011); therefore, I included data from one day prior to spawning as single day data points.

Behavioral data were, for the most part, not normally distributed even after several transformations, therefore a Friedman's non-parametric ANOVA by ranks test was used (N=12). This test is a repeated measures design test for non-parametric data and was performed on males and females separately. A Friedman's non-parametric ANOVA

by ranks test was used for male affiliative behavior, female affiliative behavior, male aggressive behavior, female aggressive behavior, male reproductive behavior and female reproductive behavior. A univariate ANOVA was used for each category of behavior to test for differences in behavior due to gender (N=24).

Once spawning commenced, it was possible that some affiliative behaviors would no longer be performed and might instead be replaced by reproductive behaviors (Oldfield and Hoffman, 2011) Since both of these behaviors are necessary for the completion of a reproductive cycle, affiliative and reproductive behaviors were combined and differences due to phase were analyzed using separate tests. Male combined affiliative and reproductive behavior data were normally distributed so differences in behavior across the phases of social pair status were analyzed using a repeated measures ANOVA (N=12). Female combined affiliative and reproductive behavior data were not normally distributed even after several transformations so a Friedman's non-parametric ANOVA by ranks test (N=12) was used to test for differences in behavior due to phases in the reproductive cycle.

To assess whether there was any correlation between the circulating OT levels and the behaviors observed, two different correlation tests were used. Because most of the behavioral data were not normally distributed, a non-parametric Spearman's correlation (N=12) was used to test for a correlation between OT levels and male affiliative behavior, male aggressive behavior, female aggressive behavior, male reproductive behavior and female reproductive behavior. Female affiliative behavior data were normally distributed after a log transformation so a Pearson's test (N=12) was used instead to check for correlations between the stated behavior and OT levels. Correlations

between male affiliative and reproductive behavior combined *vs* OT levels and female affiliative and reproductive behavior combined *vs* OT levels were also tested for using a Pearson's test correlation (N=12) after a normal distribution was achieved using a log transformation.

Statistical Analysis: Experiment 2

None of the behaviors for the second males and females were normally distributed so a Friedman's non-parametric ANOVA by ranks test (N= 24)was used just as in the first experiment. Three phases within each category of behavior were used as data points, unlike the 4 used in the first experiment. The phases used were introduction day, one day prior to pairing, and pair day. There was no re-introduction phase for this experiment so that day was not included. Just as in the first experiment, the categories of behavior tested were male affiliative, male aggressive, female affiliative, female aggressive, male reproductive, and female reproductive. Combined male affiliative and reproductive behavior were analyzed for this experiment but because they were normally distributed after a square root transformation, a Repeated Measures ANOVA (N=12) was used for this behavioral category. Female combined affiliative and reproductive behavioral data required no transformation as data were already normally distributed. Hormones were not analyzed for this experiment, so no correlations tests were performed for this experiment.

Results

Experiment 1: OT Levels

According to the repeated measures ANOVA, there was a significant change in OT concentrations across the phases of social pair status in both male ($F = 8.131$, $df = 2$, $p = 0.002$, Fig. 2 and 3) and female convict cichlids ($F = 9.064$, $df = 2$, $p = 0.001$, Fig. 2 and 3). Mauchly's test of sphericity showed that sphericity had not been violated ($\chi 2 = 4.363$, $p = 0.113$), which means that the variances of the differences of all combinations of levels are equal. Although there was a slight increase in mean OT levels between introduction day and pair day (Fig. 2) a Bonferroni post-hoc test revealed no significant difference in OT levels between the day the cichlids were introduced and pair day ($\bar{X}$ = 63.36pg/ml SD = 2.23pg/ml *vs* $\bar{X}$ = 66.47pg/ml SD = 1.47pg/ml, $p = 0.396$, Fig. 3). The difference instead lay in the day the cichlids were separated ($\bar{X}$ = 77.164 SD = 1.58pg/ml). Circulating OT levels were significantly higher after separation than on introduction day ($p = 0.0003$) and pair day ($p = 0.001$) as seen in Figure 3. The repeated measures ANOVA also showed there was no significant difference in OT levels due to gender, $F(1, 22) = 1.508$, $p = 0.232$.

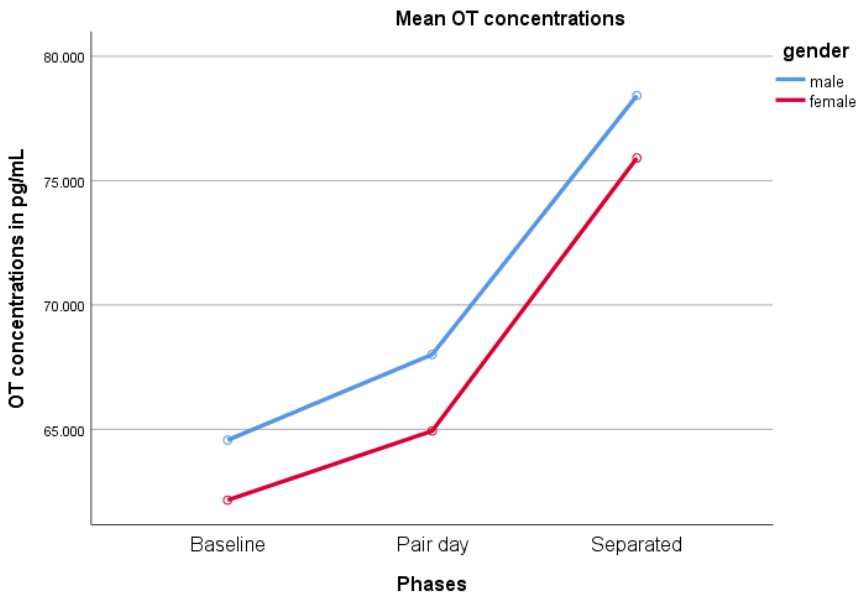

Figure 2. OT concentrations in pg/ml during 3 different phases, starting with baseline, pair day, and separation day. Male and female OT levels were not significantly different from each other.

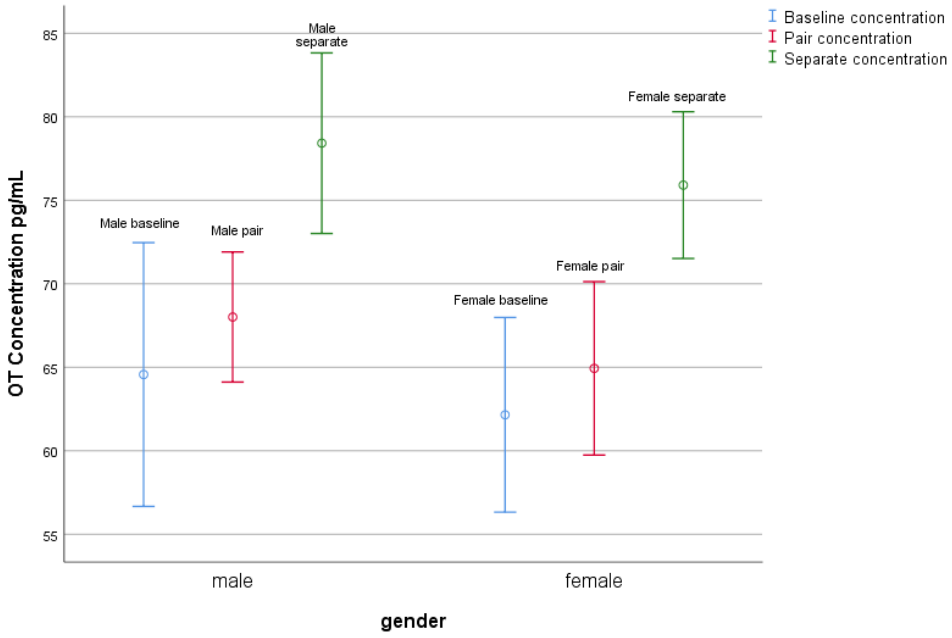

Figure 3. Means and 95% confidence intervals comparing male and female variation in mean OT levels in pg/ml during 3 different phases. Phases analysed include introduction (basal concentrations), pair day (while members were in an established pair), and separated (1, 2, or 3 days of separation from partner). OT concentrations during the separation phase were significantly higher than baseline and pair day concentrations. There was no significant difference due to gender.

The one-way ANOVA used to test whether one, two, or three days of separation had any effect on OT levels revealed that days of separation did indeed have a significant effect on OT levels in males ($F = 5.30$, $df = 2$, $p = 0.030$, Fig. 4), who were isolated, but not in females ($F = 0.668$, $df = 2$, $p = 0.5360$, Fig. 5) who stayed in the tank with their offspring. A Tukey HSD post-hoc test revealed that after three days of separation, OT levels in males were significantly lower than on one day of separation ($\bar{X}$ = 68.90pg/ml SD = 3.68pgml *vs* $\bar{X}$ = 84.05pg/ml SD = 4.73pg/ml, p= 0.024, Fig. 4) but did not differ significantly from two days of separation ($\bar{X}$ = 78.52pg/ml SD = 5.23pg/ml, p = 0.174). One day of separation did not significantly differ from 2 days of separation (p = 0.433). Males on the third day were highly variable in their OT levels when compared to one and two days of separation (Fig. 4). Females in contrast, were highly variable throughout all days of separation, in particular those tested on the second day, which had OT levels that overlapped most of the range seen for all cichlids during the separation phase (Fig. 5).

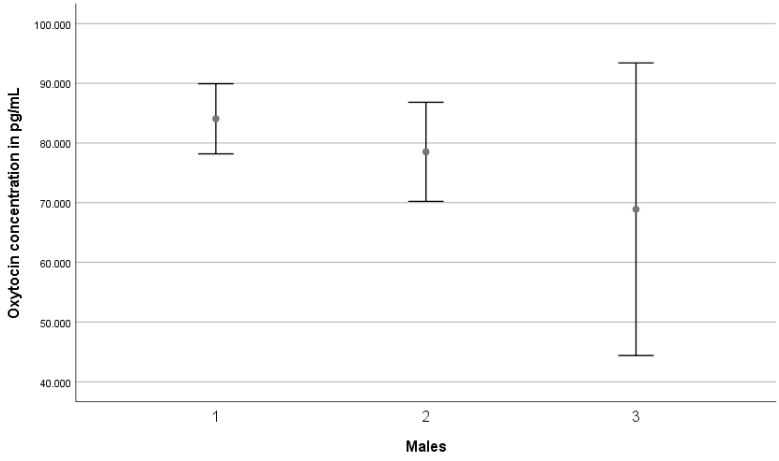

Figure 4. Means and 95% confidence intervals showing variation in mean OT levels in pg/ml in males after 1, 2, or 3 days of separation from female partner. 1 day of separation was significantly different from 3 days of separation. 1 day separated was not significantly different from 2 days separated. 2 days separated was not significantly different from 3 days separated.

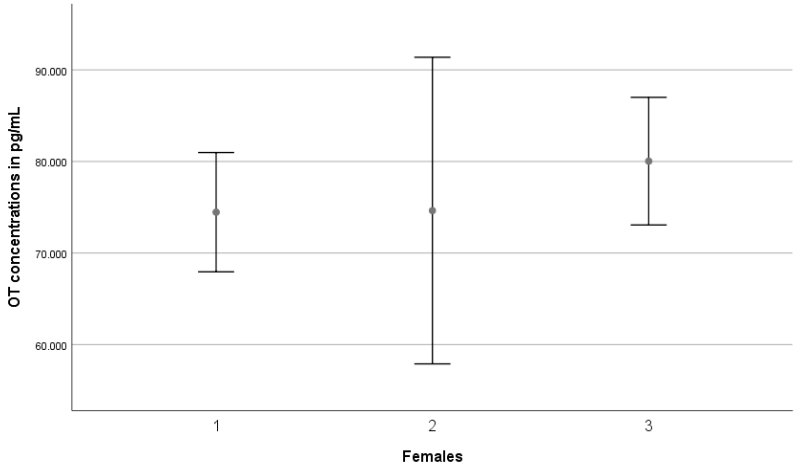

Figure 5. Means and 95% confidence intervals showing variation in mean OT levels in pg/ml in females after 1, 2, or 3 days of separation from its male partner. Females remained with their offspring. There was no significant difference between days of separation.

Male Aggressive Behavior

Behavioral data were for the most part highly variable for males and females, with affiliative behavior being the most variable. There was, however, some significant change between the phases of social pair status, depending on the category of behavior. A Friedman's Non-parametric ANOVA by ranks test showed that male bouts of aggressive behavior were significantly different across the phases of social pair status ($\chi^2 = 22.68$, df = 3, $p < 0.001$, Fig. 6). A Bonferroni post-hoc test revealed that male aggressive bouts significantly decreased from the day of introduction to one day prior to pair day ($\bar{X}$= 3.46 *vs*. $\bar{X}$ = 1.67, p = 0.004) and to pair day ($\bar{X}$ = 3.46 *vs*. $\bar{X}$ = 1.67, p = 0.004) (Fig. 6). Aggressive bouts of activity on the day males were re-introduced were not significantly different from those on introduction day ($\bar{X}$ = 3.21 *vs*. $\bar{X}$ = 3.46, p = 0.635) but were significantly higher than those one day prior to pair day ($\bar{X}$ = 3.21 *vs*. $\bar{X}$ = 1.67, p = 0.021) and those on pair day ($\bar{X}$ = 3.21 *vs*. $\bar{X}$ = 1.67, p = 0.021) (Fig. 6). One day prior to

pair day and pair day were not significantly different from each other ($\bar{X}$= 1.67 *vs.* $\bar{X}$= 1.67, p= 1.0)

Female Aggressive Behavior

The same Friedman's test showed that female bouts of aggressive behavior were also significantly different across the phases of social pair status (χ^2 = 27.24, df = 3, p < 0.001, Fig. 7). A Bonferroni post-hoc test revealed that female aggressive bouts decreased significantly from introduction day to one day prior to pair day ($\bar{X}$ = 3.17 *vs*. $\bar{X}$ = 1.58, p= 0.016) and to pair day ($\bar{X}$ = 3.17 *vs*. $\bar{X}$ = 1.67, p= 0.027) (Fig. 7). Aggressive bouts in females during re-introduction did not differ significantly from those observed during the introduction day ($\bar{X}$ = 3.58 *vs*. $\bar{X}$ = 3.17, p = 0.429), but did differ significantly from those observed one day prior to pair day ($\bar{X}$ = 3.58 *vs*. $\bar{X}$ = 1.58, p = 0.001) and those on pair day ($\bar{X}$ = 3.58 *vs*. $\bar{X}$ = 1.67, p = 0.002) (Fig. 7). Aggressive behavior displayed one day prior to pair day was not significantly different from aggressive behavior displayed on pair day ($\bar{X}$ = 1.58 *vs*. $\bar{X}$ = 1.67, p = 0.847, Fig. 7).

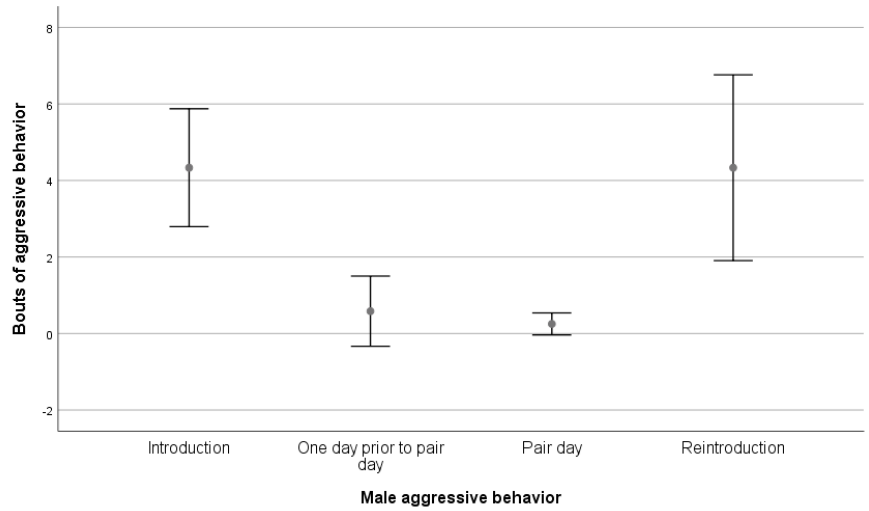

Figure 6. Means and 95% confidence intervals showing variation in mean bouts of male aggressive behavior across phases of social pair status. One day prior to pair day and pair day were significantly lower than introduction day and reintroduction day. Introduction day was not significantly different from introduction day. One day prior to pair day and pair day were not significantly different from each other.

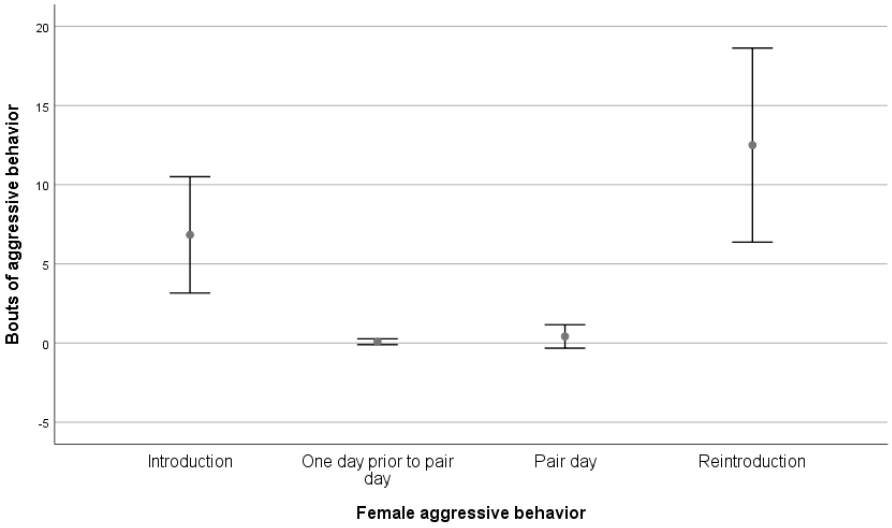

Figure 7. Mean and 95% confidence intervals showing variation in mean bouts of female aggressive behavior across the phases of social pair status. One day prior to pair day and pair day were significantly lower than introduction day and reintroduction day. Introduction day was not significantly different from introduction day. One day prior to pair day and pair day were not significantly different from each other.

Male Reproductive Behavior

Friedman's non-parametric ANOVA by ranks tests showed that bouts of reproductive behavior by males were significantly different across the phases of social pair status ($\chi 2 = 19.74$, df = 3, $p < 0.001$, Fig. 8). A Bonferroni post-hoc test, however, revealed that most of the difference lay at only one point, where reproductive behavior one day prior to pair day was significantly higher than re-introduction day reproductive behavior ($\bar{X} = 3.67$ *vs* $\bar{X} = 1.62$, $p = 0.001$, Fig. 8). There was no significant difference in bouts of reproductive behavior between introduction day and pair day ($\bar{X} = 2.38$ *vs*. $\bar{X} = 2.33$, $p = 0.937$), and introduction and reintroduction day ($\bar{X} = 2.38$ *vs*. $\bar{X} = 1.62$, $p = 0.928$) (Fig. 8). Male bouts of reproductive behavior one day prior to pair day and pair day were not significantly different from each other ($\bar{X} = 3.67$ *vs*. $\bar{X} = 2.33$, $p = 0.068$, Fig. 8). Male reproductive behavior one day prior to pair day was not significantly different from introduction day ($\bar{X} = 3.67$ *vs*. $\bar{X} = 2.38$, $p = 0.086$)

Female Reproductive Behavior

Friedman's non-parametric ANOVA showed that reproductive behavior in females significantly differed between the phases of social pair status ($\chi^2 = 32.11$, df = 3, $p < 0.001$, Fig. 9), differing between many more data points than males did. Bouts of reproductive behavior one day prior to spawning were significantly higher than introduction day ($\bar{X} = 3.50$ *vs*. $\bar{X} = 1.58$, $p = 0.002$, Fig. 9). Pair day bouts of reproductive behavior rate were also significantly higher than introduction day behavior bouts ($\bar{X} = 3.50$ *vs*. $\bar{X} = 1.58$, $p = 0.002$, Fig. 9). Bouts of reproductive behavior on re-introduction day were significantly lower than those on one day prior to pair day ($\bar{X} = 1.42$ *vs*. $\bar{X} =$

3.50, $p < 0.001$) and those on pair day ($\bar{X} = 1.42$ *vs.* $\bar{X} = 3.50$, $p < 0.001$) (Fig. 9). Bouts of reproductive behavior between introduction day and re-introduction day were not significantly different ($\bar{X} = 1.58$ *vs.* $\bar{X} = 1.42$, $p = 0.752$, Fig. 9). Bouts of reproductive behavior one day prior to pair day were not significantly different from pair day (3.50 *vs.* 3.50, p=1.0, Fig. 9)

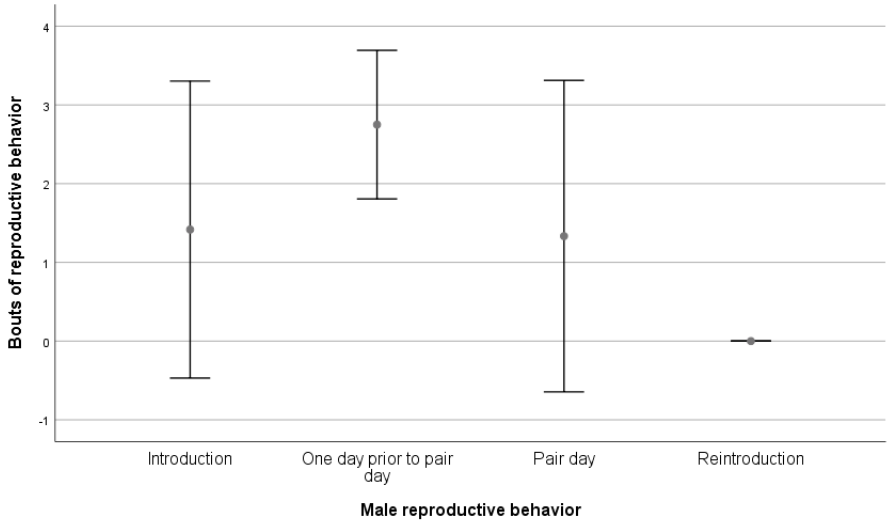

Figure 8. Mean and 95% confidence intervals showing variation in mean bouts of male reproductive behavior across the phases of social pair status. Bouts of reproductive behavior one day prior to pair day were significantly higher than on introduction day. All other days were not significantly different from each other.

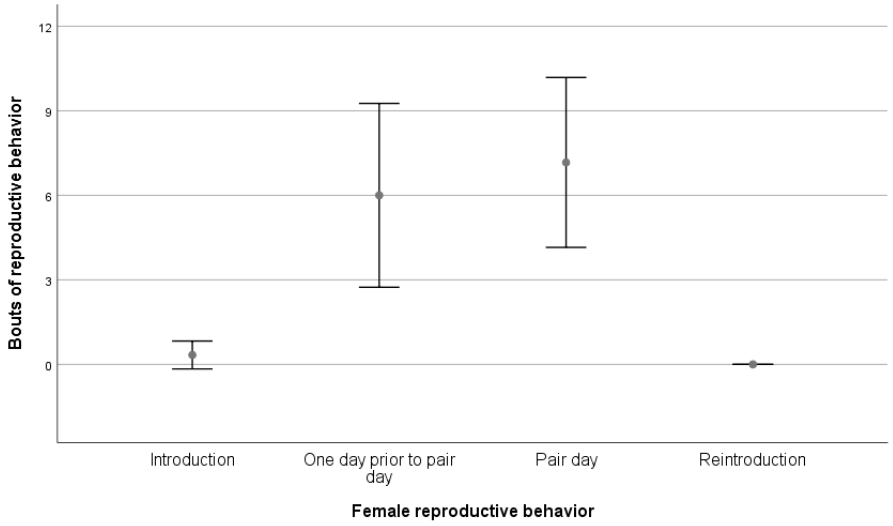

Figure 9. Mean and 95% intervals showing variation in mean bouts of female reproductive behavior across the phases of social pair status. One day prior to pair day and pair day were significantly higher than introduction day and reintroduction day. Reintroduction day was not significantly different from introduction day. One day prior to pair day and pair day were not significantly different from each other.

Male Affiliative Behavior

Affiliative behavior in males did not significantly change across the phases based on Friedman's non-parametric ANOVA by ranks ($\chi^2 = 5.95$, df = 3, p = 0.114, Fig. 10). Affiliative behaviors in males remained highly variable from initial introduction to the female through being separated and re-introduced. Affiliation did slightly increase on average one day prior to pair day and on pair day when compared to the day they were first introduced but the differences were not significant and affiliation varied by individual (Fig. 10).

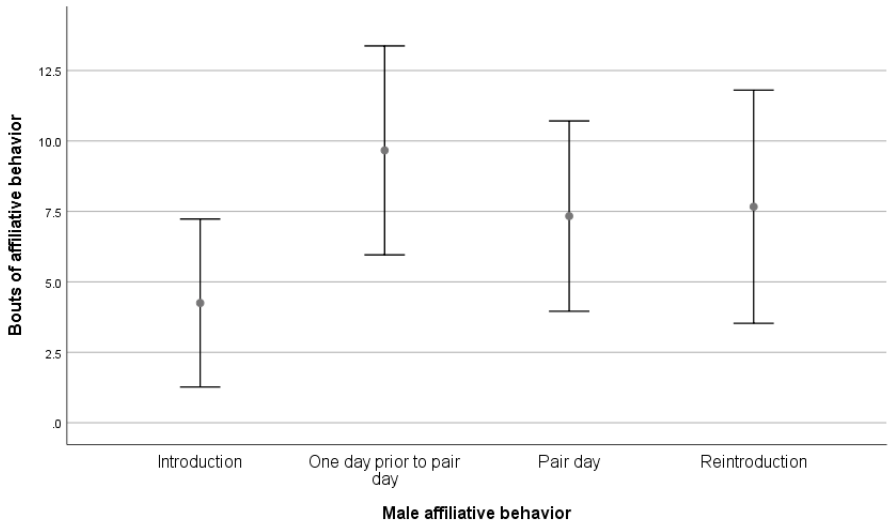

Figure 10. Means and 95% confidence intervals showing variation in mean male bouts of affiliative behavior across the phases of social pair status. There was high variability and no significant difference between any phase.

Female Affiliative Behavior

Female affiliative behaviors were also highly variable but according to Friedman's ANOVA by ranks there was a significant difference in behavior across the phases of social pair status ($\chi^2 = 15.36$, df = 3, p = 0.002, Fig. 11). A Bonferroni post-hoc test revealed that bouts of affiliative behavior one day prior to pair day were significantly higher than those on reintroduction day ($\bar{X} = 3.25$ *vs.* $\bar{X} = 1.54$, p = 0.007, Fig. 11). Pair day bouts of affiliative behavior were also significantly higher than those on reintroduction day ($\bar{X} = 3.12$ *vs.* $\bar{X} = 1.54$, p = 0.016, Fig. 11). Females displayed a slight increase in affiliation one day prior to pair day and on the pair day when compared to introduction day but it was not significantly different as introduction day behavior was highly variable ($\bar{X} = 3.25$ *vs.* $\bar{X} = 2.08$, p = 0.161 and $\bar{X} = 3.12$ *vs.* $\bar{X} = 2.08$, p = 0.289 respectively, Fig. 11). There was no significant difference between introduction day and

reintroduction day bouts of female affiliative behavior ($\bar{X}$ = 2.08 *vs*. $\bar{X}$ = 1.54, p = 0.304, Fig. 11).

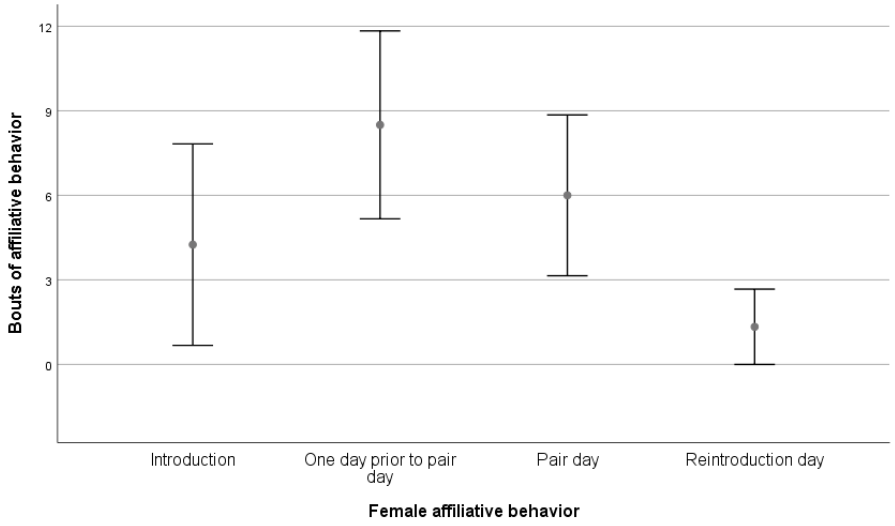

Figure 11. Means and 95% confidence intervals showing variation in mean female bouts of affiliative behavior across the phases of social pair status. Bouts of affiliative behavior one day prior to pair day and on pair day were significantly higher than those on reintroduction day. No other day was significantly different from each other.

Male Affiliative + Reproductive Behavior

When affiliative behavior was combined with reproductive behavior, the data for males were normally distributed so a Repeated Measures ANOVA was used for that case only. Mauchly's test of sphericity showed that sphericity had not been violated (χ^2 = 5.971, df = 5, p = 0.311), so the variances of the differences of all combinations of levels of the variable are equal. The repeated measures test revealed that there was a significant difference across the phases of social pair status (F = 3.829, df = 3, p = 0.019, Fig. 12). A Least Significant Difference post-hoc test revealed bouts of affiliative behavior performed one day prior to spawning were significantly higher than on introduction day

($\bar{X}$ = 14.5 ± 2.30 bouts *vs* $\bar{X}$ = 5.67 ± 1.65 bouts, p = 0.011) but were not significantly different from any other day (Fig. 12).

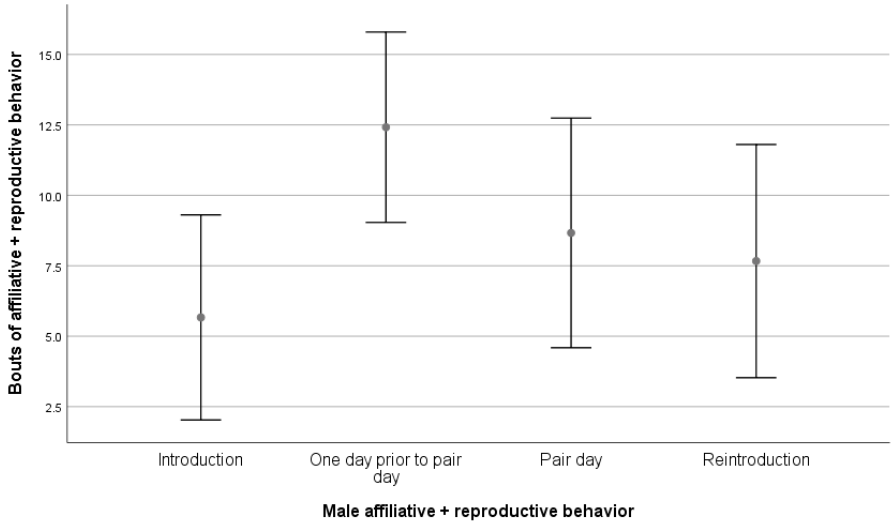

Figure 12. Means and 95% confidence intervals showing the variation in combined affiliative and reproductive behavior in males across the phases of social pair status. Bouts one day prior to pair day were significantly higher than those on introduction day. No other day was significantly different from each other.

Female Affiliative + Reproductive Behavior

Combined affiliative and reproductive behavior for females were not normally distributed despite several transformations so a Friedman's non-parametric ANOVA by ranks test was used. The test revealed a significant difference across the phases of social pair status (χ^2 = 27.08, df = 3, p < 0.001, Fig. 13). A Bonferroni post-hoc test revealed that much of the difference in bouts affiliative + reproductive behavior were found on one day prior to pair day (Fig. 13). Bouts of affiliative + reproductive one day prior to pair day were higher than introduction day ($\bar{X}$ = 3.92 *vs.* $\bar{X}$ = 1.75, p < 0.001, Fig. 13). Bouts one day prior to pair day were also higher than those on reintroduction day ($\bar{X}$ = 3.92 *vs.* $\bar{X}$ = 1.54, p < 0.001, Fig. 13) but were not significantly different from those on pair day ($\bar{X}$ = 3.92 *vs.* $\bar{X}$ = 2.79, p = 0.197). Bouts on reintroduction day were not significantly

different than those on introduction day ($\bar{X}$ = 1.54 *vs.* $\bar{X}$ = 1.75, p= 1.000) and those on pair day ($\bar{X}$= 1.54 *vs.* $\bar{X}$ = 2.79, p = 0.106). Bouts on introduction day were not significantly different than those on pair day ($\bar{X}$ = 1.75 *vs.* $\bar{X}$ = 2.79, p = 0.289).

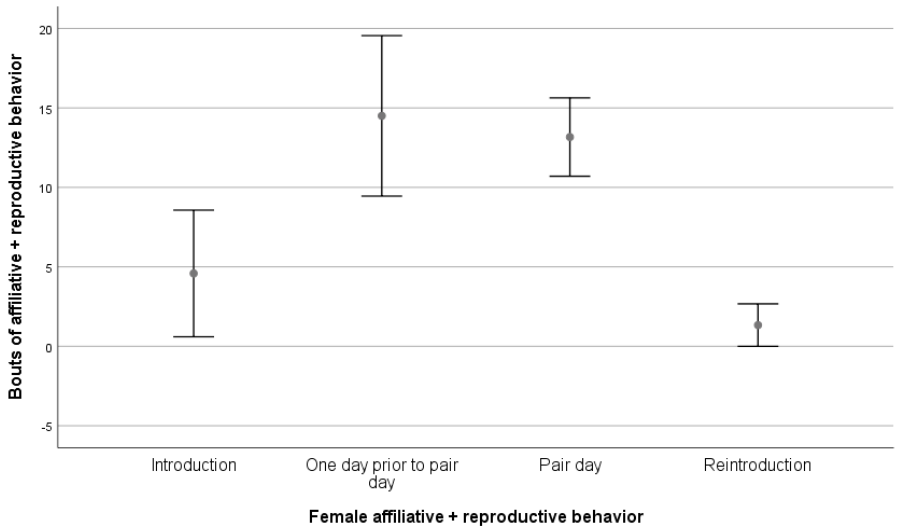

Figure 13. Means and 95% confidence intervals showing variation in mean bouts of female affiliative + reproductive behavior across the phases of social pair status. Bouts of affiliative + reproductive behavior one day prior to pair day and pair day were significantly higher those those on introduction day and reintroduction day. Bouts on introduction day were not significantly different than those on reintroduction day. Bouts one day prior to pair day were not significantly different than those on pair day.

Correlation tests

Correlation tests could only be performed between phases that included both hormonal analysis and behavioral analysis, which in this case was only introduction day phase and pair day phase. For male affiliative behavior, Spearman's correlation test showed no significant correlation between the circulating OT levels and the bouts of affiliative behavior observed in males (rho = -0.282, p = 0.091). There was no significant correlation between the circulating OT levels and the bouts of reproductive behavior observed in males (rho = 0.038, p = 0.431). There was no correlation between the

circulating OT levels and the bouts of aggressive behavior observed in males (rho = -0.140, p = 0.257).

Spearman's correlation test showed no correlation between the circulating OT levels and the bouts of aggressive behavior observed in females (rho = -0.338, p = 0.053) across the phases analyzed for hormones. The same test revealed no correlation between the circulating OT levels and the bouts of reproductive behavior observed in females (rho = 0.315, p = 0.067) during the phases analyzed for hormones. Female affiliative behavior data were normally distributed so a Pearson's correlation test was used instead, as this allowed testing for partial correlation based on "phase". A one-tailed Pearson's correlation test showed no correlation between circulating OT levels and the rate of affiliative behavior (r = 0.215, p = 0.157). When controlling for phase, Pearson's partial correlation test still showed that the circulating OT levels did not correlate with the affiliative behaviors observed in females (r = 0.191, p = 0.191) during the phases analyzed.

Combined affiliative and reproductive data for males were normally distributed for introduction and pair day data after a log transformation. A Pearson's correlation test was used to compare OT levels and the bouts of affiliative + reproductive behavior observed in males, both while controlling for day (2 phases) and without any control. If phase was not controlled for, there was no significant correlation between OT and combined affiliative and reproductive behavior (r = 0.-286, df = 22, p = 0.088, Table 1). However, when phase was controlled, there was a significant but weak negative correlation between OT level and combined rate of affiliative and reproductive behaviors during introduction and pair day (r = -0.367, df = 21, p = 0.042, Table 1). This technically

means that if OT increased from baseline to pair day, affiliative + reproducive behavior slightly decreased, though the r^2 value in this case is 0.1346, which means that only about 13.5% of the variation in the bouts of affiliative and reproductive behavior are explained by the variation in OT levels. In this case OT alone cannot explain most of the variation in the bouts of affiliative and reproductive behavior observed.

Control Variables			M_OT phase 1&2	Affiliation + rep	Day
-none-[a]	M_OT phase 1&2	Correlation	1.000	-.286	.180
		Significance (1-tailed)	.	.088	.199
		Df	0	22	22
	Affiliation + rep	Correlation	-.286	1.000	.315
		Significance (1-tailed)	.088	.	.067
		Df	22	0	22
	day	Correlation	.180	.315	1.000
		Significance (1-tailed)	.199	.067	.
		Df	22	22	0
day	M_OT phase 1&2	Correlation	1.000	-.367	
		Significance (1-tailed)	.	.042	
		Df	0	21	
	Affiliation + rep	Correlation	-.367	1.000	
		Significance (1-tailed)	.042	.	
		Df	21	0	

Table 1. There was a weak correlation between circulating OT levels and combined affiliative and reproductive behavior in males only when the day or phase was controlled for. If Phase was not accounted for, there was no correlation.

Combined affiliative and reproductive behavioral data for females were normally distributed for introduction day and pair day, which was a proxy for pair phase. A Pearson's partial correlation test was used to compare between the OT levels and the bouts of affiliative + reproductive behavior observed with and without accounting for day

(phase). There was a significant positive correlation between the OT levels and the combined bouts of affiliative and reproductive behavior both when phase is not controlled for ($r = 0.408$, $df = 22$, $p = 0.024$) and when phase is controlled for ($r = 0.402$, $df = 21$, $p = 0.029$) (Table 2). If phase is not controlled for the r^2 value is 0.166, which means that only about 16.6% of the variation observed in the bouts of affiliative and reproductive behavior is explained by the OT levels. If phase was controlled for, the r^2 value was 0.162, which means that, in females, only about 16.2% of the variation observed in the bouts of affiliative and reproductive behavior was explained by the variation in OT levels (Table 2). Like in males, most of the variation in the bouts of affiliative + reproductive behavior in females is not explained by OT alone (Table 2 and Fig. 13).

Control Variables			F_OT phase 1 & 2	F Affiliation + Rep	Day
-none-[a]	F_OT phase 1 & 2	Correlation	1.000	.408	.165
		Significance (1-tailed)	.	.024	.220
		Df	0	22	22
	F Affiliation + Rep	Correlation	.408	1.000	.652
		Significance (1-tailed)	.024	.	.000
		Df	22	0	22
	day	Correlation	.165	.652	1.000
		Significance (1-tailed)	.220	.000	.
		Df	22	22	0
day	F_OT phase 1 & 2	Correlation	1.000	.402	
		Significance (1-tailed)	.	.029	
		Df	0	21	
	F Affiliation + Rep	Correlation	.402	1.000	
		Significance (1-tailed)	.029	.	
		Df	21	0	

Table 2. Unlike males, females had a moderate positive correlation between circulating OT levels and combined affiliative and reproductive behaviors with or without controling the phase.

Experiment 2: Male Behaviors

During the second pair formation, certain behaviors in both males and females were similar to the first pair formation. Friedman's non-parametric ANOVA by ranks showed that male aggression was significantly different across the phases of social pair status ($\chi^2 = 11.294$, df= 2, $p = 0.004$). A Bonferroni post-hoc test revealed that male bouts of aggression significantly decreased from introduction day to one day prior to pair day ($\bar{X} = 2.73$ *vs*. $\bar{X} = 1.64$, $p = 0.032$) and pair day ($\bar{X} = 2.66$ *vs*. $\bar{X} = 1.64$, $p = 0.032$) (Fig. 14). Friedman's Non-parametric ANOVA by ranks test showed that bouts of reproductive behavior in males were not significantly different across the phases of social pair status ($\chi^2 = 4.105$, df = 2, $p = 0.128$, Fig. 15). Interestingly, unlike in the first pair formation, according to a Friedman's test, bouts of affiliative behavior by males during the second pair formation were significantly different across the phases of social pair status ($\chi^2 = 10.186$, df = 2, $p = 0.006$, Fig. 16). A Bonferroni post-hoc test revealed that male bouts of affiliative behavior, during this experiment, significantly increased from introduction day to pair day ($\bar{X} = 1.23$ *vs*. $\bar{X} = 2.32$, $p = 0.032$) and from introduction day to one day prior to pair day ($\bar{X} = 1.23$ *vs*. $\bar{X} = 2.45$, $p = 0.012$) (Fig. 16).

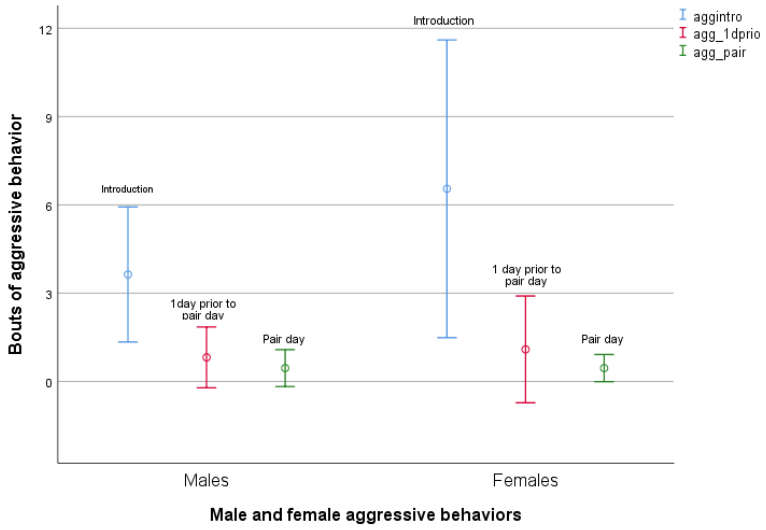

Figure 14. Mean and 95% intervals showing the variation in mean bouts of aggressive behavior between male and female cichlids across the phases of social pair status. In males, bouts of aggressive behavior one day prior to pair day and on pair day were significantly lower than introduction day. Male bouts of aggressive behavior one day prior to pair day was not significantly different from pair day. In females, there was a huge variation in bouts of aggressive behavior during introduction. Aggressive behavior was also much higher in females than in males. There was no significant difference in aggressive behavior in females across the phases of social pair status.

Experiment 2: Female Behaviors

Friedman's non-parametric ANOVA by ranks showed that female aggression differed across phases of social pair status ($\chi^2 = 8.063$, df = 2, p = 0.018). A Bonferroni post-hoc test, however, showed no significant difference between the phases in pairwise comparisons, although there was a large variation in mean bouts of aggressive behavior during introduction (Fig. 14). Female aggression during introduction was not significantly different than aggression during pair day ($\bar{X} = 2.59$ *vs*. $\bar{X} = 1.77$, p = 0.165) and aggression one day prior to pair day ($\bar{X} = 2.59$ *vs*. $\bar{X} = 1.64$, p = 0.076) (Fig. 14). Friedman's ANOVA by ranks test showed that female reproductive behavior in females was significantly different across the phases of social pair status ($\chi^2 = 7.161$, df = 2, p = 0.028, Fig. 15). However, a Bonferroni pairwise comparison test showed that no phase

was significantly different from any other. Reproductive behavior on introduction day was not significantly different from pair day ($\bar{X}$ = 1.55 *vs*. $\bar{X}$ = 2.50, p = 0.076) and one day prior to pair day ($\bar{X}$ = 1.55 *vs*. $\bar{X}$ = 1.95, p = 0.337) (Fig. 15). Reproductive behavior on pair day and one one day prior to pair day were not significantly different from each other either ($\bar{X}$ = 2.50 *vs*. $\bar{X}$ = 1.95, p = 0.602, Fig. 15).

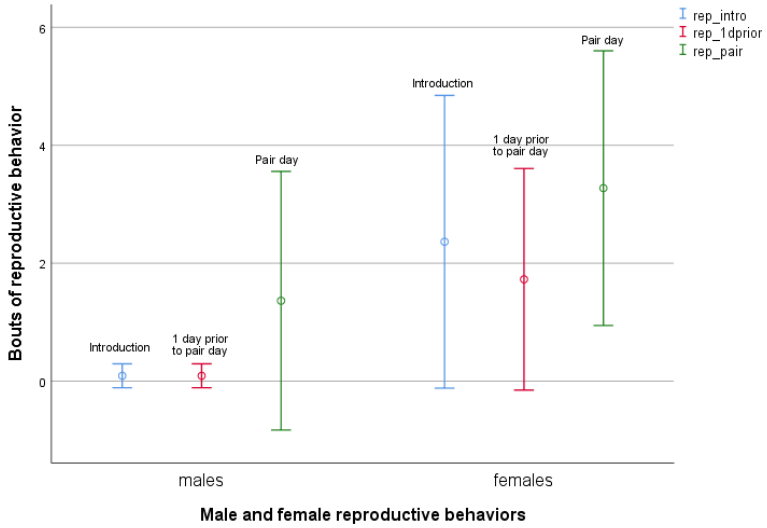

Figure 15. Means and 95% confidence intervals showing the variation in mean bouts of reproductive behavior between male and female cichlids across the phases of social pair status. There was no significant difference in mean bouts of reproductive behavior for both male and females across the phases of social pair-bond.

Female bouts of affiliative behavior were significantly different across the phases of social pair status (χ^2 = 8.727, df = 2, p = 0.013, Fig. 16) similar to the first paired formation. A Bonferroni post-hoc test revealed that female affiliation significantly increased from introduction day to pair day ($\bar{X}$ = 1.27 *vs*. $\bar{X}$ = 2.36, p = 0.032) and from introduction day to one day prior to pair day ($\bar{X}$ = 1.27 *vs*. $\bar{X}$ = 2.36, p = 0.032) (Fig. 16).

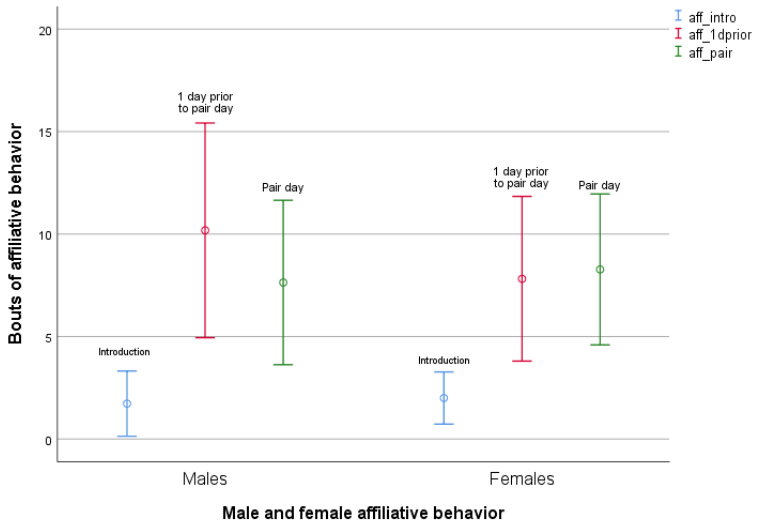

Figure 16. Means and 95% confidence intervals showing the variation in mean bouts of affiliative behavior between males and females across the phases of social pair status.

Combined Male Behaviors

When affiliative and reproductive behaviors were combined, the data were normally distributed in males after a square root transformation. A Repeated Measures ANOVA was used since male combined bouts of affiliative + reproductive behavior did not violate sphericity ($\chi^2 = 1.061$, df = 2, p = 0.588). The ANOVA revealed that there was a significant difference across the phases of social pair status (F = 12.078, df = 2, p < 0.0001, Fig. 17). A Bonferroni post-hoc test revealed that introduction day bouts of affiliative + reproductive behavior were significantly lower than both pair day behavior ($\bar{X} = 1.55 \pm 0.202$ *vs.* $\bar{X} = 2.96 \pm 0.367$, p = 0.002) and one day prior to pair day behavior ($\bar{X} = 1.55 \pm 0.202$ *vs.* $\bar{X} = 3.52 \pm 0.337$, p = 0.040) (Fig. 17).

Combined Female behaviors

Combined bouts of affiliative + reproductive behavior data for females were normally distributed without any transformation; moreover they did not violate sphericity (χ^2 = 1.940, df = 2, p = 0.379) so a Repeated Measures ANOVA was used. The ANOVA revealed a significant difference across the phases of social pair status (F = 10.401, df = 2, p = 0.001, Fig. 18). A Bonferroni post-hoc test revealed that introduction day behavior was significantly lower from both pair day behavior ($\bar{X}$ = 2.09 ± 0.595 *vs.* $\bar{X}$ = 11.82 ± 2.114, p = 0.003) and one day prior to pair day behavior ($\bar{X}$ = 2.09 ± 0.595 *vs.* $\bar{X}$ = 9.455 ± 2.024, p = 0.007) (Fig. 18).

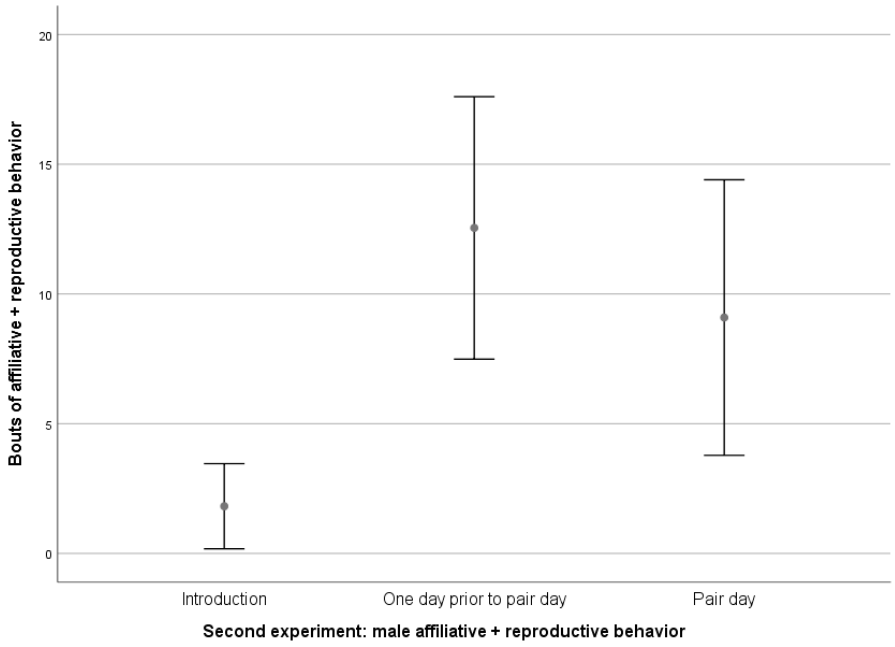

Figure 17. Means and 95% confidence interval showing the variation in mean bouts of male affiliative + reproductive behavior across the phases of social pair status. Introductin day bouts of affiliative + reproductive behavior were significantly lower than those of one day prior to pair day and pair day. One day prior to pair day and pair day were not significantly different than each other.

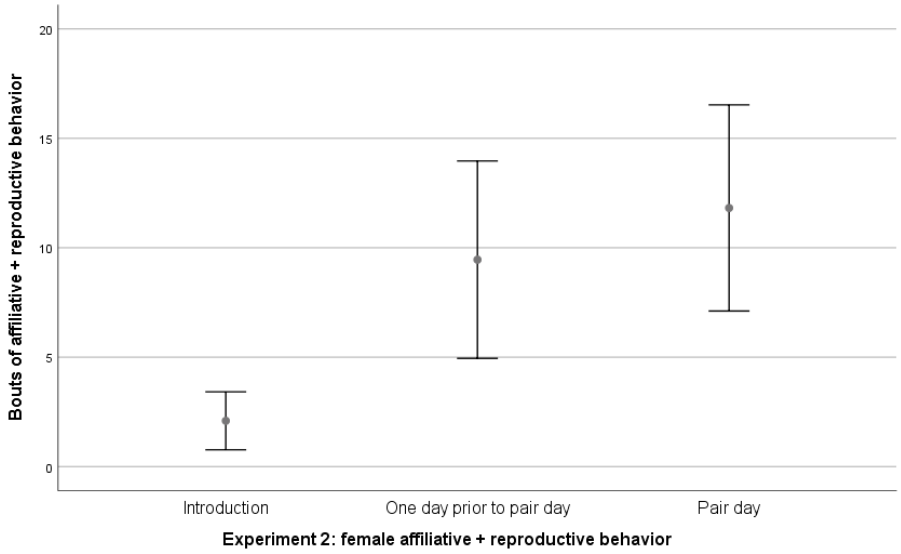

Figure 18. Means and 95% confidence intervals showing the variation in mean bouts of female affiliative + reproductive behavior. Introduction day bouts of affiliative + reproductive behavior was significantly lower than those of one day prior to pair day and pair day. One day prior to pair day and pair day were not significantly different from each other.

Discussion

Oxytocin levels

Circulating OT increased significantly for both male and female convict cichlids but only after the pair members were isolated from each other. This increase in OT was parallel in both males and females, with no significant difference in OT levels due to gender. There was no significant difference between baseline plasma OT concentrations and pair day plasma OT concentrations. This was contrary to my original hypothesis that the OT concentrations in the plasma would increase after the cichlids formed a pair-bond. Mean OT levels did increase slightly during the paired phase stage as seen in Figure 2, but the variation in OT during introduction, as observed in Figure 3, was so large that it overlapped with the OT levels measured during the paired phase so they were not significantly different from each other. Baseline variation in OT is actually not uncommon. Basal urinary OT in Cotton-top tamarins was found to have a 10-fold variation across individuals, with paired mates displaying similar levels of OT between each other (Snowdon *et al.*, 2010).

Although the OT levels during the paired phase of the experiment were not significantly higher than baseline, OT levels did increase from baseline (Figs. 2 and 3). The significant increase in OT levels during the separation phase of the experiment suggests that the OT levels observed may actually reflect other factors such as the presence or absence of the offspring. This is evident in the fact that one day after being separated from each other, male and female OT levels changed differently from each other. Females, which remained with the offspring, continued to have high OT levels,

one, two, or three days after separation with no significant change (Fig. 5). Males that were separated from both the female and the offspring, on the other hand, had a gradual reduction in mean OT levels one, two, or three days after separation (Fig. 4). Moreover, males that were separated for three days had significantly lower mean OT levels than did males separated for only 1 day (Fig. 4) although there was a large variation in mean OT levels for males separated for 3 days.

Besides social pair-bonding, OT is involved in other forms of attachment such as parent-offspring attachments, in many species, (Tabbaa *et al.,* 2016). For many mammals, including humans, OT is released in the peripheral system after parturition and after certain sensory stimulations such as suckling (Ross *et al.,* 2009). In rodents, even polygynous ones such as montane voles, OT receptor distribution in the brain changes drastically 24 hours after parturition. OT receptor density in the amygdala and the nucleus accumbens of the montane vole increases after parturition to densities resembling those of the monogamous prairie vole, which normally have high OT receptor densities in these regions outside of parturition (Insel and Shapiro, 1992). In convict cichlids OT promotes paternal care. Male convict cichlids that were administered an OT antagonist intraperitoneally one day after offspring hatched, did not have the significant increase in paternal care observed in males without the antagonist administration (O'Connell *et al.,* 2012).

Due to the close proximity in days between being pair-bonded and separated, it is possible that the OT levels observed during the separation phase actually reflect pair-bond OT levels, however OT usually has a half-life of about 3 to 9 minutes as a neurotransmitter (Witt *et al.,* 1990). This is confounded even further due to the similarity

in factors affecting both social pair-bonding and parental attachment, not to mention the fact, that OT release is stimulated by sensory imput during caregiving. As such, the timing of the blood sample collection is very important and may have had an effect on the results of this experiment.

It is also possible the variation observed could be due to errors during the analysis phase. ELISAs are usually run in duplicates or even triplicates to reduce intra-assay variation and to increase accuracy. Due to the small size of each cichlid and the fact that blood had to be extracted 3 separate times, blood samples collected tended to be small (about 25-40µL). Out of those blood samples, the amount of plasma obtained was usually about 10-25µL. To reduce intra-assay variation and to standardize the tests, only 10µL out of every plasma sample was used for the ELISA. The ELISA kit used was originally meant for 50µl of a sample, although 25 or even 20µL were no problem, according to manufacturer (personal communication, MyBiosource). Additionally, the kit specifically asked for undiluted samples only, therefore, samples could not be divided in any way and the analysis could not be run in duplicates. In this case, the ELISA plate containing the samples was read using the plate reader two times and the average of the two readings was used for the statistics analysis.

Behavior

Male and female aggressive behavior significantly decreased as a pair-bond developed, as was expected (Fig. 6 and 7). This aggression resurged for both male and female after the former pair were re-introduced. Since the female, at this point, had formed a social pair-bond with another male, this surge in aggression was expected, as

females in the wild normally attack non-mate conspecifics if they are able to, provided the opponent is not significantly larger (Fig. 6) (Lamprect and Rebhan, 1996; Oldfield and Hoffman, 2011; Leese, 2012). Males did not pair with any new females but were just as aggressive during re-introduction as they were when first introduced (fig. 7), suggesting no pair-bond between them.

Reproductive behavior was a bit more variable. It was expected that reproductive behavior would increase as a social pair-bond developed and a pair laid eggs. Reproductive behavior in males one day prior to pair day had significantly more bouts of behavior than introduction day, re-introduction day, and even pair day. This could be due to the fact that this species has biparental care of its offspring and will often divide parental roles (Itzkowitz *et al.,* 2001). Males will typically spend more time on brood and mate defense than the female (Itzkowitz *et al.,* 2003). In fact, the ability to defend territories is partly a reason female convict cichlids prefer larger males (Gagliardi-Seeley *et al.,* 2009; Wisenden, 1994; Wisenden, 1995). Therefore, it is possible that males spent more time defending brood after spawning, which would not directly count as a reproductive behavior under the ethogram used. Female reproductive behavior, on the other hand, increased significantly from introduction day to one day prior to pair day. It also increased significantly between introduction day and pair day, as was expected. Female convict cichlids tend to spend a greater amount of time around offspring than do the males unless an intruder is more easily fought off in unison with the male partner (Itzkowitz *et al.,* 2003; Oldfield and Hoffman, 2011).

Male affiliative behavior did not differ significantly between the phases of social pair status (Fig. 10). In fact, there was not much activity at all throughout the days of

observation, perhaps due to the cichlids' wariness of my presence. This result was not in accordance with my hypothesis, which stated that affiliative behavior would increase as a social pair-bond developed. This was partially clarified when affiliative behaviors were combined with reproductive behaviors to form a larger reproductive cycle data set. These could be combined because they both formed parts of a full reproductive cycle. When combined, male affiliative + reproductive behavior one day prior to pair day was significantly higher than introduction day. All other days were not significantly different from each other (Fig. 10).

Female affiliative behavior did follow my hypothesis. Female affiliative behavior significantly increased as a social pair-bond developed (Figs. 11). Bouts of affiliative behavior one day prior to pair day was significantly higher than introduction day but about the same as the affiliative behavior observed on pair day. Affiliative behavior on the day the female was re-introduced to the male was significantly lower than one day prior to pair day and pair day (Figs. 11). In fact, for some females, there was virtually no affiliative behavior observed during the re-introduction phase (Fig. 11).

When female affiliative behavior was combined with reproductive behavior, bouts of affiliative + reproductive behavior were also consistent with my hypothesis. Affiliative + reproductive behavior one day prior to pair day increased significantly from introduction day and were significantly higher than all the other phases of social pair status, including pair day (Fig. 13). These patterns of behavior are more consistent with the literature. In one study, there was an overall increase in affiliative + reproductive behavior as a pair formed but when the pair spawned affiliative behaviors were partly reduced and replaced with an increase in reproductive behaviors (Oldfield and Hoffman,

2011). Affiliative + reproductive behavior during the re-introduction phase was no different than that observed during the introduction phase, therefore it can be assumed that there was no bondage or attachment between the former cichlid pairs (Fig. 13).

It is very likely that the behaviors observed were affected by my presence. This effect was likely enhanced by the collection of blood samples in the three different phases. After the first blood collection, fish seemed wary of my presence and would hide for much of the observational period. This effect could have been avoided through the use of video cameras. Cichlid behaviors could have been calculated in a per minute proximate with one another, expressed as time spent in proximity to one another per minute of observation. Additoinally, behaviors could have been measured in time intervals, whereby any activity or inactivity is scored as a ratio of the total observation period. Alternatively, observation times could have started as soon as the cichlids expressed any of the behaviors in the established ethogram.

OT and behavior

There was no correlation between the circulating OT levels of male convict cichlids during the baseline phase and the pair phase nor most of the behaviors observed including aggressive bouts, affiliative bouts, and reproductive bouts. This contradicts my hypothesis that male affiliative and aggressive behavior would correlate with OT levels. There was, however, a partial correlation between circulating OT levels and affiliative + reproductive behaviors combined, when the phase of the experiment was controlled for (Table 1). This correlation was negative but very weak, accounting for only 13.5% of the variation in affiliative + reproductive behavior. This suggests that circulating OT levels in

the periphery of the male cichlid fish did not account for most of the variation in affiliative + reproductive behavior observed and there were likely other factors at play.

Female convict cichlids had similar results with no significant correlation between OT levels in the baseline and pair phase and affiliative bouts, aggressive bouts, and reproductive bouts. This also contradicted my hypothesis that an increase in circulating OT levels would correlate with an increase in affiliative behaviors and a decrease in aggressive behaviors. There was, however, a significant positive correlation and a positive partial correlation between the OT levels observed and the bouts of affiliative + reproductive behavior. Much like the results for the male, these correlations were very weak. If phase of the experiment was controlled for, OT explained only 16.2% of the variation in bouts of affiliative + reproductive behavior. If phase of the experiment was not controlled for, OT explained 16.6% of the variation in bouts of affiliative + reproductive behavior. As with the males, most of the variation in bouts of affiliative + reproductive behavior and all the other behaviors recorded, for that matter, were likely affected by other factors outside of circulating OT levels or at the very least, that circulating in the peripheral system.

The second part of the experiment was meant to have the female convict cichlid form a new social pair-bond with a different male, in order to induce a social pair loss with the first male. It also provided a chance to have a second round of behavioral observations without the males having gone through the blood collection phase of the experiment. These males were likely much less wary of my presence and thus may have acted much more naturally than the males from the first experiment. In prairie voles, environmental factors can affect pair-bonding well into adulthood (Johnson and Young,

2015). As expected for this experiment, the female formed a new social pair-bond and subsequently acted aggressively toward her former mate, thereby confirming social pair loss between the two. The results for this experiment were similar to the first experiment in that aggressive behavior, of both the male and female significantly decreased as a social pair developed. Reproductive behavior in females significantly increased as a social pair-bond developed but remained the same for the male. As was the case for the first experiment, this could be due to the fact that the male was mostly on patrol, defending the nest. Much like the first round, affiliative bouts of behavior for females increased significantly as a social pair-bond developed. Unlike the first experiment however, the male also expressed a significant increase in affiliative behaviors as the social pair-bond formed. This is what was expected based on my hypothesis and the literature, and it suggests that the affiliative behavior observed in the first experiment may well have been affected by outside factors, specifically, fear induced by my presence.

The behaviors observed in these experiment are likely the result of several factors, including the interplay between OT and other hormones. For this species, as with many, OT tends to work in concert with AVP when it comes to complex social behaviors (Johnson and Young, 2015; Carter, 2017). In the prairie vole, AVP tends to regulate male social behaviors, while female social behaviors are usually regulated by OT (Cho *et al.*, 1999). In the convict cichlid, the nonapeptide receptor antagonist for both OT and AVP ([β-Mercapto-β,β-cyclopentamethylenepropionyl1, O-Et-Tyr2, Val4, Arg8]-Vasopressin) prevented an increase in affiliative behaviors between potential mates during pair formation and reduced aggression toward conspecifics. Interestingly enough, in this same

experiment, this nonapeptide receptor antagonist did not affect behaviors within already established pairs. OT was found to be only involved in pair-bond formation but not pair maintenance or general affiliative behavior (Oldfield and Hoffman, 2011). Similar results were found in zebra finches, where OT/AVP antagonists delayed social pair-bond formations by reducing courtship behaviors, but the same antagonists did not affect behavior in already established pairs (Pedersen and Tomaszycki, 2012). Unfortunately for this study, I was unable to analyze circulating AVP levels due to the size of the fish and the low amounts of resulting plasma samples that were to already be used for OT analysis.

OT could have also interacted with steroid hormones, such as 11-ketotestosterone (11-KT) or estrogen. In rats, the behavioral effects of OT are tied to estrogen, which tends to up-regulate OT receptors . This link between OT and estrogen is not the same in prairie voles, where it is OT that facilitates the effects of estrogen (Cushing and Carter, 1999).11-KT has been found to be important for pair-bonding in male convict cichlids. In the wild, the highest concentrations of 11-KT in males occurs right before spawning during the courtship phase, with a significant decrease in 11-KT after spawning during the parental phase, although non-breeding male convict cichlids had the lowest concentrations of 11-KT (Van Breukelen, 2013). The antagonist to the steroid hormone, flutamide, was found to impair courtship behaviors in male convict cichlids, but it did not affect aggressive behaviors. Interestingly enough, in this same experiment, 11-KT concentrations in non-breeding laboratory convict cichlids resembled that of courting males, unlike those found in the wild (Van Breukelen, 2013). This brings the point that it is also possible that the laboratory setting, in general, may have contributed to the large

variation in baseline OT levels observed in my experiment. The baseline OT profile in wild convict cichlids may not necessarily be the same as that encountered in laboratory settings. In other species, such as prairie voles, OT can have a peripheral feedback mechanism with steroid hormones. When a female is sexually receptive or the cervix or uterus is stimulated through mating or parturition, estradiol increases which, in turn, upregulates OT receptors in the periphery, such as in the uterus. This then feeds back to the central nervous system through the systemic effects of OT, thereby affecting social behaviors and increasing affiliative behaviors (Cushing and Carter, 1999).

As mentioned in the introduction, OT can work with the dopamine system in the nucleus accumbens to regulate pair-bond formation. Mating causes the release of dopamine which then binds to D2R receptors in the nucleus accumbens, thereby promoting partner preference formations (Johnson and Young, 2015). The blockade of this receptor prevents pair-bond formations in prairie voles (Liu and Wang, 2003). Another dopamine receptor in the nucleus accumbens, D1R, is important for pair maintenance but not pair formation (Johnson and Young, 2015; Liu and Wang, 2003).

There is a theme with many of these social pair-bond experiments, including the present one. Like many of other hormones, OT affects behavior in a context-dependent manner. As seen in previous experiments, OT did not have an effect on social behavior in established pairs (Oldfield and Hoffman, 2011; Klatt and Goodson, 2013). In prairie voles, the presence of a partner can buffer the effects of a stressor (Smith and Wang, 2014). This social buffering can be induced by the administration of OT (Grippo *et al.*, 2012). Depending on the perceived context of safety or fear, the effect of OT and AVP can be one of social engagement, bonding, or reward, or one of avoidance and anxiety.

For instance, if there is fear, AVP (the older hormone) will override the effects of OT and promote avoidance, defense, aggression, and anxiety. If there is no fear, OT will interact with AVP and promote social interactions, bonding, sexual behavior, and parental behaviors (Carter, 2017). Social experience can also great affect social pair-bonding. Environmental and social experiences can cause neuroplastic changes in the parts of the brain responsible for pair-bonding (Johnson and Young, 2015). Therefore, the effect OT has on behavior is affected by social contexts and social experiences (Carter, 2017).

One other method through which OT can regulate social pair formations is through its effect on social recognition. Depending on the species, OT receptors tend to be distributed near areas of sensory processing, such as olfactory processing in rodents or visual and auditory processing in primates (Johnson and Young, 2015).

It is also possible that the circulating OT observed in this experiment may not have been the most accurate index for the central nervous system OT, and therefore the social behavior observed. Based on a meta-analysis of peripheral and central OT concentrations correlations, it seems that, without context, baseline plasma OT levels are not a good index for central nervous system OT levels since OT release in the peripheral and central nervous system seemed uncoordinated under basal conditions, with no stressors, in most species (Valstad *et al.,* 2017). Peripheral and central OT levels do correlate, however, for both humans and other species under specific situations such as after intranasal OT administration or induced stress. However, as mentioned earlier, OT may not even need to reflect the OT levels in the brain. It can affect the central nervous sytem through indirect paths, such as the stimulation of gonads and systemic organs that then stimulate OT in the central nervous system. There is ample evidence that shows that

agonists and antagonists administered in the peripheral system affect social behaviors as, for instance, the reduction in affiliative behavior in convict cichlid fish (Oldfield and Hoffman, 2011) or the reduction in courtship behaviors in zebra finches (Klatt and Goodman, 2013; Pedersen and Tomaszycki, 2012) after the peripheral administration of OT antagonists.

Lastly, in various voles, it is the mating system of the species, whether that be monogamous, promiscuous, or polygynous, that determines the OT receptor distribution in the brain, particularly in the nucleus accumbens (Insel and Shapiro, 1992). Therefore, it is possible that species across various taxa, such as convict cichlids, have similar patterns of OT receptor distribution in the brain, with monogamous species having a more dense distribution of OT receptors. Based on this hypothesis, OT release would be very localized to certain regions in the brain and circulating OT levels would not necessarily correlate with OT concentration in the brain.

Based on the literature and the results from the second pair formation, one cannot entirely dismiss an effect of OT on affiliation. The results from this experiment may not entirely reflect natural behaviors as behaviors were likely affected by human presence and future observations may benefit from the use of video cameras. On the other hand, it is possible to form social pair-bonds without necessarily increasing affiliative behavior. Pair-bond formations in convict cichlids does not just rely on increased affiliative behaviors but also on selective targets of aggression. In fact, often in experiments involving the use of pair-bonded convict cichlids, it is easier to note when a pair-bond forms when a male stops courting different females and both the male and female pair attack all conspecifics but themselves (Mackereth and Keenleyside, 1993). Additionally,

pair-bonds can develop faster in compartmentalized settings in which intruders are visible but physically separated to prevent attacks and co-housed pairs can identify each other rapidly (Oldfield and Hoffman, 2011), provided they have breeding sites available (Gumm and Itzkowitz, 2007). Unfortunately, in this experiment pairs were prevented from seeing neighboring cichlids with the intention of allowing pairs to solely focus on each other. That was also done for cases where larger males were unavailable but smaller males could still potentially form pair-bonds with larger females provided the female could not compare the co-housed male with neighboring males (Gagliardi-Seeley *et al.,* 2009). Thus, aggressive behaviors toward intruders was highly limited to charges directed at me on rare occasions.

In this experiment, circulating OT levels did not increase significantly from baseline to pair phase, but rather increased significantly during the separation phase. Since OT and behavioral correlations were only performed between baseline and introduction day, and pair phase and pair day, it is difficult to even get a significant positive correlation within the current framework as the separation phase was never included. If it were possible to analyze OT levels on a daily or at the very least a more frequent basis without actually harming the cichlid, there could very well have been a different result such as an increase in OT levels one day before spawning or perhaps there would have been a day to day significant fluctuation in OT levels. The use of less invasive methods of collecting and analyzing OT levels could prove very useful. This is already somewhat common in land mammals, for instance in cotton-top tamarins, OT levels can be analyzed from urinary samples (Snowdon *et al.,* 2010). There are methods to analyze hormones from waste in water samples as well, albeit they are a bit more

tedious than with land animals (Wong *et al.*, 2008). Indeed, it seems that several steroid hormones can be analyzed just from fish holding-water using enzyme immunoassays (Kid *et al.*, 2009). These methods unfortunately may not be the best for analyzing OT as it is released in very minute concentrations, especially in small fish, and the half-life of OT as a neurotransmitter is about 3 to 9 minutes and up to 20 as a hormone (Witt *et al.*, 1990). Nevertheless, there were some promising results and the influence circulating peripheral OT has on social behaviors in convict cichlids, particularly courting and reproductive behaviors, is still not out of the question.

Conclusion

OT plays an important role in social behavior across various vertebrae taxa. This role extends to social pair-bond formations in many species, including convict cichlids. In this species, an OT/AVP antagonist administered intraperitonealy during the pair-bond formation stage reduced affiliation toward the mate and reduced aggression toward intruders. This effect however, was no longer present during established pairs. In my experiment, since the spawning of a brood was one way to determine an established bond, following the above experiment, OT levels would not necessarily increase during the already established pair-bond. Rather, there may have been an increase in OT during the pair-bond formation stage, days prior to spawning, but the circumstances and the size of the cichlid fish did not allow for that analysis to happen for this thesis. OT levels did, however, increase after separation, which suggests that offspring and not necessarily the mate, was the larger determining factor of circulating OT levels. The mechanism by which OT regulates pair-bond formation in this species, requires the interplay of various factors including affiliative + reproductive behaviors, although this only accounts for 12-16.6% of the variation. More work is required to determine the extent that OT, and AVP for that matter, has on social pair-bonds. The use of different species across other vertebrae species is especially important, since at the moment there is no information available for the effect OT has on social behavior in most reptiles and amphibians.

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