The effects of prior social experience on reproductive behavior, spawning, and physiology in a monogamous fish, Amatitlania nigrofasciata

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The effects of prior social experience on reproductive behavior, spawning, and physiology in a monogamous fish, *Amatitlania nigrofasciata*

by

Kimberly P. Little

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Kimberly P Little
Prior social experience and reproductive behavior in a monogamous fish

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Dedication

For R.H.E., Jr.

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General Abstract

The overall goal of this research was to provide further understanding of the mechanisms involved in pair bonding in the monogamous convict cichlid. This project tested the influences of different aspects of social environment on subsequent reproductive behaviors. The effects of social experience, dominance and the role of visual and water-borne cues on pair bond formation as well as hormonal correlates of social experience were evaluated. The hypothesis tested in the first experiment was that prior experience in a mixed-sex group or dominance status affects subsequent pair formation or spawning. Individuals from mixed-sex groups paired with a novel fish more frequently than fish from single-sex groups, although dominance did not influence pair formation and spawning occurred infrequently. Behaviors in groups or pairs did not predict reproductive outcomes. To test the idea that hormones better explain the reproductive outcome of single-versus mixed-sex groups, 11-ketotestosterone (11-KT), 13,14-dihydro-15-keto prostaglandin F2α (PGF2α metabolite), and follicle-stimulating hormone (FSH) were measured. The hypothesis was that reproductive hormone levels would differ according to whether subjects had prior social experience with the opposite sex. 11-KT varied widely across males and was correlated with chasing behavior, but did not differ by social experience or dominance status. PGF2α metabolite did not differ among females as a function of prior social experience. FSH was elevated in subordinate males in single-sex groups only, and a similar trend in females was not statistically significant. The third experiment tested the hypothesis that visual and chemical cues influence pair bonding. Visual cues from the opposite sex did not influence pair bonding; pairs formed frequently among all the groups. Males exposed to female chemosensory
cues paired significantly less frequently than males exposed to male chemosensory cues, but there was no difference between the female groups. The difference in males might be related to group aggression as opposed to negative effects of female chemical cues on male behavior. Overall, results from these experiments indicate that being in mixed-sex groups facilitates pair formation, although the mechanism remains unclear. There was no relationship between dominance and pair formation. However, effects of dominance might be masked by the artificial laboratory environment in which pairs were assessed; individuals were removed from groups to evaluate pair formation. These results suggest that social environment influences pair formation in convict cichlids in a context-dependent way although how hormones contribute remains unclear.
I. General Introduction

Conspecific interactions can involve active participation in agonistic or affiliative behaviors or might be limited to passive observation. In a social environment, both types of interactions could influence mating behaviors and decisions. The effects of prior social experience on mate choice have been studied primarily in the context of mate choice copying (reviewed in Westneat et al. 2000), eavesdropping (see examples in McGregor and Peake 2000), and the effects of early-life experiences (e.g. Hebets 2003). A vast literature also exists on the relationship between stressful social encounters and reproduction (reviewed in Tilbrook et al. 2000). While the effects of social interactions on subsequent behavior are often evaluated experimentally using dyadic arrangements, a natural social environment typically consists of multiple individuals. Allowing for signals to occur in a network provides a more accurate representation of what information animals use to make decisions (McGregor and Peake 2000). Thus, various aspects of conspecific social interactions could potentially influence mating behavior and should be studied in a group context when applicable.

Information used for mate selection can be intentionally communicated (e.g. Warner et al. 1995) or provided inherently through group qualities such as operational sex ratio (Jirotkul 1999), the frequencies of different phenotypes present (van Gossum et al. 1999; 2001) or population density (Welch 2003). Regardless of whether information is provided though intentional or inherent signals, mate choice may rely on the ability to make comparisons in a social environment. For example, exposure to male phenotypes induces female mating preferences but only if the different males are presented simultaneously (Rosenqvist and Houde 1996). Understanding the natural history of
species during mating is paramount for experimental studies of mate choice. Laboratory experiments evaluating mate preferences and choice should consider the possible unnatural effects of limited choices and social experiences often present in laboratory settings before casting results in an evolutionary light.

Specific information from individuals that are part of a group can directly affect reproduction. Conspecific signals and cues can indicate social or reproductive status, and, for species that congregate in groups prior to breeding, social status may play a particularly important role. For example, resources and mates may be disproportionately acquired according to an individual’s dominance rank, as is typically the case in lekking and other strongly polygynous species (Krebs and Davies 1993). Monogamous species, including those that form long-term pair-bonds, also often congregate during or before the breeding season and choose partners after evaluating multiple potential mates, although their reproductive success will not be as skewed, presumably because of assortative mating (e.g. Bradley et al. 1995).

With monogamy the potential also exists for mutual mate choice instead of the unidirectional choice system typically found in polygynous or polyandrous species (Krebs and Davies 1993). However, in monogamous systems, unique selection pressures are often associated with each sex (e.g. Chippindale et al. 2001), causing males and females to use different criteria to evaluate mates. Additionally, as opposed to one sex being the “chooser” and the other being driven into intrasexual competition, competition may be part of the reproductive process within both sexes in monogamous systems. Furthermore, while having traits that enhance both competitive ability and attractiveness could be advantageous for males and females regardless of mating system, in systems in
which there are dichotomous sexual roles, selection pressures could be quite different between the sexes. However, in species having mutual mate choice, selection pressures should be more similar than in other mating systems. This sexual equality should be particularly true for monogamous animals that are also biparental where traits associated with competition, attractiveness, and parental care are more similar for each sex. It is also parsimonious that traits involved in multiple aspects of sociality are co-opted. For example, aggressive behavior is critical for success during both competition and defense of offspring. It might make sense, then, for aggressive behavior to be evaluated during monogamous mate assessment in place of more conventional signals of attractiveness. For monogamous species, a social environment consisting of both males and females would better allow observation of dominance relationships that could influence mate choice.

Mate assessment is not the only possible reproductive function of social experience. Animals congregating in groups are also exposed to sensory cues that could influence reproductive physiology and thus indirectly influence behavior. This indirect effect has been demonstrated in species without an extended period of pair bonding (i.e. Carassius auratus, reviewed in Munakata and Kobayashi 2010) as well as in monogamous animals. For example, prairie voles (Microtus ochrogaster) develop a partner preference and selective aggression toward strangers after being housed with a member of the opposite sex for 3 days, a change which is regulated by neuropeptides and can be induced experimentally (Insel et al. 1998; Young et al. 1998; Young and Wang 2004). Ring dove (Streptopelia roseogrisea) courtship causes physiological changes in both sexes that permit the onset of mating and incubation (Lehrman 1965). While these
examples highlight the importance of intersexual signals during pair bonding, the possibility that intersexual interactions prior to breeding physiologically prime individuals for pair formation has received little attention. Priming prior to pair formation would be particularly advantageous for monogamous seasonal breeders having extended biparental care, as pair formation and offspring care involve a considerable time constraint. Such priming mechanisms would allow seasonal breeders to maximize their limited time frame for selecting a mate and raising young to independence. Therefore, aspects of social experience prior to pair bond formation might physiologically prime individuals for mating or otherwise coordinate reproduction between partners in addition to environmental cues required for seasonal breeding to occur.

*Animal Model*

The convict cichlid (*Amatitlania nigrofasciata* Günther 1867) is a teleost fish species suitable for studies of monogamous social and reproductive behavior, including aggression, mating preferences, and parental care of individuals and pairs. Convicts are endemic to freshwater lakes and streams in Central America (McKaye 1977; Bussing 1987) and are easily kept in the laboratory. They form pair bonds during reproductive bouts and also exhibit biparental care of young until the fry stage. In the wild, both males and females may breed more than once per season, although this occurs more often in males than females (Wisenden 1995). Convict cichlids naturally congregate in mixed-sex groups having a slightly female-biased sex ratio (Cleveland-Roberts and Itzkowitz 2009). Typically, males and females select partners from within their own groups, and the pair then leaves the group to seek breeding habitat. Pairs usually remain together until fry are free swimming, approximately 4-6 weeks.
It is generally accepted that pair bond formation in convict cichlids occurs after both sexes assess potential partners behaviorally, primarily through courtship. Courtship behaviors may also serve a pair maintenance function (Leese 2012). However, there are also other, non-behavioral cues that could facilitate pair bond formation that have been largely overlooked by previous studies. For example, chemosensory cues act as pheromones to induce reproductive behaviors and spawning in other fishes (reviewed in Burnard et al. 2008). Visual cues, some as simple as tank background color, can also affect fish physiology (Hoglund et al. 2002). Fishes also produce courtship sounds (e.g. Rohmann and Bass 2011; Maruska et al. 2012), and such signals in addition to other auditory cues, such as the sound of nest building (Moore and Waring 1999), can influence physiology during the breeding season. Thus, olfactory, visual, and auditory cues could be potent modulators of pair bond formation in convict cichlids, and elucidating their roles may help explain the variation encountered when pairing fish in a laboratory setting.

An easily observable lab phenomenon is the rapidity with which pairs form following placement in small mixed-sex groups versus when a male is placed with a female after being housed with only their own sex. Pairs virtually always form in a mixed-sex group within 24-48 hours, whereas pairings may never occur between fish from single-sex conditions even after weeks of non-aggressive cohabitation (pers. obs., Leese pers. comm.). Exposure to cues from the other sex as well as having the ability to choose a mate could stimulate pair formation in mixed-sex groups by a currently unknown mechanism. The virtually universal laboratory practice of isolating the sexes
into single-sex stock populations may actually impair subsequent reproductive motivation compared to fish in a more natural setting.

Because convict cichlids are an aggressive species and establish dominance in groups, social status may have fitness consequences. If true, an individual’s propensity to pair might be predicted by behavioral or physiological correlates of individual dominance. Furthermore, pair formation in convict cichlids seems to require that a male and female have compatible aggressive dynamics, so it seems likely that dominance plays some role in pair formation.

As noted earlier, mechanisms that facilitate rapid pair formation in mixed-sex groups are currently unknown. The experiments in this dissertation were designed to examine whether behavioral and/or non-behavioral cues play a role in this phenomenon. Such cues could be actively used during mate assessment, but they may also serve a reproductive priming function. Therefore, an integrative approach combining a comprehensive behavioral analysis with an exploration of possible hormonal correlates was used. Because the reproductive behavior of convict cichlids has already been extensively studied, this species is particularly well suited for experiments elucidating physiological mechanisms of reproductive behavior.

*General Methods*

Prior to placement into experimental groups, all fish were housed in single-sex groups of approximately 30 individuals in 473.5 L stock aquaria containing gravel substrate and having relatively neutral pH (6.7-7.4). Fish were obtained from distributors in both Florida and Georgia through local pet stores; prior breeding history was not known. Stock and experimental fish were fed a commercial protein-rich pellet food.
(Zeigler Finfish Starter with Vpak mini-pellets) once daily. Rooms containing stock and experimental aquaria were maintained at 25±3° C on a 14L:10D hour cycle. The first experiment (Chapter II) was performed in two locations: Eastern Kentucky University (Richmond, KY) and Lehigh University (Bethlehem, PA), with approximately half of the replicates performed in each location. In both locations the fish were housed under the same water conditions, temperature, and light cycle. However, because of size differences in experimental aquaria, opaque plastic partitions were used to decrease tank volume at Lehigh to match tank dimensions at Eastern Kentucky. Experiments 2 (Chapter III), 3 (Chapter IV), and 4 (Chapter IV) were conducted exclusively at Lehigh University. Experimental fish were kept separate from primary stock after each replicate to avoid pseudoreplication. All experimental aquaria were drained and filled with fresh water in between each replicate to eliminate potentially confounding chemical cues from prior inhabitants.

Fish were individually marked for identification in 3 of the 4 experiments in this study. Tags were made in advance so that the procedure required less than 1 min per fish to perform. The tagging procedure required no anesthesia. All procedures were approved by the Lehigh IACUC (protocol A3877-01).

Colored sequins were used to tag individuals because of their visibility on high-definition video and light weight. To construct a tag, one clear glass 11/0 seed bead was secured to the end of approximately 13 cm of monofilament fishing line (5-8 lb test) by melting approximately 3.5 cm of the line onto the seed bead after threading it onto the line. A sequin was then threaded on top of the fastened bead with the concave side facing away from the seed bead.
In convict cichlids, a small area of muscle between the dorsal aorta terminus and the hypural plates was used as the attachment site to avoid bleeding and to minimize possible obstruction of swimming or other behaviors (see Figure 1 for exterior view). To insert the tag through the muscle, the free end of the monofilament was threaded into a 22 G hollow needle tip (previously removed from a syringe), which was then pierced through the dorsal caudal musculature and pulled out through the other side, leaving the monofilament running through the muscle to the other side of the fish. A second sequin of the same color was threaded onto the line and a second clear seed bead was threaded on top of the sequin. The monofilament was then trimmed to approximately 3.5 cm and melted onto the second seed bead. Fish were completely covered with wet paper towels during the entire procedure and had no direct contact with the heat source.

After the experiment, tags were removed before releasing the fish into designated stock aquaria. The small puncture healed without infection after one week. Treatment with antibiotics or use of a hospital tank was not required for proper healing. The tags did not interfere with swimming or other behaviors, and tag color was not correlated with any behaviors or social status.

Fish were placed with prospective partners of the opposite sex in 3 of the 4 experiments in this study. In these “forced pairings,” males and females were selected from single-sex stock aquaria so that males were 10 mm longer (standard length; length from snout to caudal peduncle) than their female partner (to reflect natural populations and female preference, see Santangelo and Itzkowitz 2004). All forced pairs were given a clay flower pot as breeding substrate.
The following ethogram was created for recording behavioral data:

1. Bite – an individual makes buccal contact with another individual on the body surface or fins of another individual. The intended target may or may not flee after being bitten.

2. Chase – rapid swimming in the direction of an individual (or group of individuals) at which point the individual(s) being chased flees and may be pursued by the chaser for up to several seconds. The quantification of a chase requires the intended target(s) to flee but does not require subsequent pursuit of the chaser.

3. Lateral Display – extension of paired and unpaired fins. Lateral displays may be brief or held for extended periods of time. For sustained displays, retraction of fins and subsequent re-extension are required for quantification.

4. Frontal Display – extension of gill opercula toward another individual. Often includes fin extension but not always.
Figure 1.1. Approximate location and relative size of sequin tag (indicated by circle). Line indicates where tag is threaded through muscle of fish. Image from http://www.daff.qld.gov.au/fisheries/pest-fish/noxious-fish/tilapia
II. The influences of prior social experiences on reproductive behavior

INTRODUCTION

Various abiotic ecological factors affect reproduction, most obviously among seasonal breeders, (e.g. food availability, O'Brien and Dawson 2011; rainfall, Chase et al. 2005; temperature, Wiebe and Gerstmar 2010), but social interactions can also shape reproductive success. Conspecific signals and cues may indicate qualities important for mate choice, such as social status (Bakker 1985; Bakker and Sevenster 1989; Rowland 1994; Drickamer 1995; Berglund 1997; Fox et al. 1997; Gilmour et al. 2005; Earley et al. 2006) and reproductive status (Drickamer 1995; Peeters et al 1999; Roberts and Uetz 2005) of individuals. Additionally, animals in groups are exposed to various behavioral and other sensory cues that could influence their reproductive physiology (see Wyatt 2003). Thus, conspecific intersexual interactions prior to breeding may not only be part of mate assessment but also may act to prime individuals for subsequent reproduction.

Dominance is often associated with enhanced reproduction due to greater resource control (Huntingford and Turner 1987; Alcock 2005), and social status can directly regulate factors that affect competitive and/or reproductive ability (e.g. growth rate in cichlid fish: Hofmann et al. 1999; Hofmann and Fernald 2000). Dominance may also directly affect factors involved in mate choice. For example, dominant males of some species court females more after winning a contest than subordinate males (Amorim and Almada 2005; LaManna and Eason 2010; Greenberg and Crews 1990) or exhibit other forms of increased mating effort, such as more frequent ejaculations (de Catanzaro and Ngan 1983). Male blockhead cichlids (*Steatocranus casuarius*) that win fights form pairs...
and spawn more often than males that lose contests or do not have a prior contest experience (LaManna and Eason 2010). Aggressive, dominant individuals may also behaviorally or chemically suppress reproduction of subordinates (Savage et al. 1988; Barrett et al. 1990; Cameron 1997; Gerlach 2006; Young et al. 2006), or the physiological stress response associated with being subordinate may cause reproductive impairment or decreased sexual behavior (Cameron 1997; Fox et al. 1997; Creel 2001; Hardy et al. 2002; Moore et al. 2005; Edeline et al. 2010). Reproductive advantages of being dominant may therefore result not only from increased attractiveness but also from directly or indirectly limiting the reproductive success of subordinates.

Alternatively, maintaining dominance can be stressful and, in some cases, could cause negative reproductive consequences, particularly when social ranks are unstable (Creel 2001; Sapolsky 2005; but see Goymann and Wingfield 2004). Highly aggressive dominant individuals may also make poor social partners or parents (Ward and FitzGerald 1987; Ros et al. 2004), and excessive aggression has been reported to interfere with pair formation in monogamous cichlids (*Amphilopus citrinelium*, Barlow et al. 1998; *Herichthys cyanoguttatum*, Itzkowitz and Draud 1992). Thus, contrasting predictions can be made regarding the relationship between dominance and reproduction among monogamous species.

Most studies examining the effects of dominance on reproduction involve dyadic contests to determine winner-loser effects (Chase et al. 2003). However, isolated, dyadic interactions would be a rarity for the many teleost fishes, birds, insects and some mammals that live in groups (Krebs and Davies 1993). Dominance relationships are often formed within a network of individuals and determined not only by fighting but
also by watching other individuals fight (Oliveira et al. 1998). Furthermore, dominance status can be reversed according to whether an individual is placed in a dyad or group (Verbeek et al. 1999; see also Chase et al. 2003). Individuals may also alter their sexual behavior in response to changes in context (Bierbach et al. 2011; Ung et al. 2011). Thus, in species that live in groups, the effect of dominance on reproduction should be studied in a group context rather than in isolated pairs to emulate interactions encountered in natural settings.

The relationship between reproduction and group social dynamics has been well studied among mammals, birds, and territorial fishes (reviewed in Rubenstein and Kealey 2012). However, fewer studies have considered how group interactions might affect reproductive behaviors in pair-bonding species, especially among non-avian taxa. Monogamous species are unique in that dominant individuals do not necessarily monopolize mates as in most polygamous systems (see Gratson et al. 1991). For example, socially subordinate monogamous males and females could form mating pairs, although there may be constraints associated with subordination that could decrease reproductive success, such as the consequences of stress. Intrasexual dominance hierarchies in both sexes could be important in monogamous species for mate assessment and choice in the form of behavioral assortative mating (see also Fawcett and Johnstone 2003).

Convict cichlids (*Amatitlania nigrofasciata*) are a monogamous, pair bonding, Central American cichlid species that naturally congregate in mixed-sex schools (Mackereth and Keenleyside 1993; Wisenden 1995; Cleveland-Roberts and Itzkowitz 2009). In typical habitat, males and females interact with multiple individuals of both
sexes prior to and as part of the mate selection process before establishing a breeding territory. However, in areas of high population density and extreme breeding site limitation, males establish territories first and females choose among these males (Alonzo et al. 2001). This contextual switch in pairing behavior suggests that dominance and aggression conveys a reproductive advantage, but it is uncertain if dominance is important when breeding sites are not limited. However, some degree of aggression is likely an advantage in any breeding environment because defense of young is a primary component of biparental care in this species.

A large proportion of research devoted to convict cichlid mate preference and choice emphasize “courtship behaviors” during the pre-spawning period. However, the frequencies of such behaviors are often quite low (e.g. Mackereth and Keenleyside 1993; Leese 2012) and might serve other functions (e.g. Santangelo 2005; Wisenden et al. 2008). The lack of solid evidence validating the significance of courtship behaviors in pair formation could be because other cues are more important. Because convict cichlids are seasonal breeders in the wild, their reproduction undoubtedly depends on a combination of environmental and social cues, with behavior representing only one of many multimodal signals potentially affecting reproduction. Courtship behaviors *sensu strictu* may instead be caused by reproductive pathways that have already been stimulated in a pre-spawning context. Thus, despite a rich behavioral groundwork, mechanisms causally related to pair formation, behavioral or otherwise, remain a mystery in this well-studied species.

Individual variation in aggression is well-documented for convict cichlids (e.g. Noel et al. 2005; Gumm and Itzkowitz 2007; Arnott and Elwood 2009a, 2009b; Barley et
al. 2010; van Breukelen 2013), although it is unknown if dominance status is transient and contextual or if individual differences are maintained throughout various life history events. Appropriate levels of aggression likely provide natural and sexual selective advantages in terms of convict cichlid competition and parental care, but aggressive dynamics have not been thoroughly evaluated as a factor in pair formation or mate choice. While female convicts mate with dominant males in an experimental group context (Weber and Weber 1976), it is not known if subordination impairs reproduction when breeding sites are more readily available (as they often are in natural environments). Although a theoretical link between mating and aggression in convicts has been made previously (see Cole et al. 1980; Haley 1983; Santangelo 2005), courtship is instead often used as the behavioral determinate or indication of pair formation.

Evaluating how individual differences in aggression might affect pair formation among male and female convict cichlids in a more natural context could provide key information about reproduction in this species. Introsexual dominance and intersexual behaviors and cues may stimulate fish in multiple ways. For example, regardless of a female’s dominance status, exposure to males might prime her for reproduction. These ideas are also important from a practical perspective because of their implications for laboratory-based studies. Studies of reproductive behaviors of captive animals maintained for long periods in single-sex groups (a nearly universal husbandry practice among research labs to prevent breeding and excessive aggression in stock populations) may not accurately reflect natural behavior. Levels of aggression are generally expected to be higher in mixed-sex groups compared to single-sex groups because of increased competition due to the presence of potential mates, but this has not been tested.
The following experiment was designed to compare whether prior experience in a mixed-sex group versus prior experience in a single-sex group affected an individual’s likelihood to form a pair bond and spawn. The relationship between dominance status and reproductive behavior was also evaluated in this context. I hypothesized that prior experience in a mixed-sex group would facilitate pair formation and spawning. I also hypothesized that the dominance status (aggressiveness) of individuals established in a group setting would be related to subsequent reproductive behavior.
METHODS

Treatment groups

Adults were placed into 3 different treatment groups of 6 individuals each in 189 L aquaria for the first part of this experiment. Prior to use, individuals were assessed for visible signs of ill health and lack of ovipositor emergence in females. Only animals perceived to be healthy were used, and females were not used in any part of the experiment if their ovipositor was visible. Fish were measured for standard length (SL; length from tip of snout to caudal peduncle) and tagged with colored sequins (described in general methods). Treatment groups consisted of either 6 males, 6 females, or 3 males and 3 females (Figure 2.1). Individuals in each single-sex group were chosen so that all individuals were size matched within 2 mm SL. Each mixed-sex group consisted of females size-matched to each other and males that were all 10 mm SL larger than the females and also within 2 mm of each other. Average standard length and standard error of individuals across groups was 57.0 ± 0.6 mm for females (N = 90) and 68.6 ± 1.3 mm for males (N = 90). After placement into groups, fish acclimated to their new tank for 1 hr before being video recorded for 1 hr (between 1000-1100 hrs). On each of the following 2 days, groups were also taped for 1 hr between 1000-1100 hrs.

Videos were watched to assess dominance prior to pairing. Dominance was defined by the total daily frequency of overt aggressive behaviors (bites and chases) as well as demonstration of dominance (not fleeing from attack) during dyadic interactions within groups. The least dominant individual was characterized by retreat from all approaching individuals and/or continual exhibition of subordinate display behavior.
Individual aggression was consistent between days 2 and 3 across groups, allowing unambiguous determination of dominance within the 48-hr time period.

Forced pairings

The second part of the experiment (“forced pairing”) began on the third day. The most and least aggressive individuals were removed from the group after the third video and placed with a novel stock fish of the opposite sex in a new 189 L aquarium between 1300-1400 hrs. Novel fish were taken from unisexual stock populations and also screened for health and ovipositor emergence before being size-matched to their potential partner. All stock fish were kept in unisexual tanks, as long-term housing in mixed-sex tanks results in pair formation and spawning within these groups (pers. obs.). Males in the “forced pairs” were approximately 10 mm SL longer than the female. Fish were placed into the new aquarium within 5 min. of each other, and which fish was placed into the aquarium first was randomized. The aquaria into which pairs were placed contained gravel, a clay flower pot for a breeding substrate, and a transparent divider used to create an intruder compartment on one end of the tank. After 48 hrs, a “juvenile” convict cichlid (approximately 30 mm SL and lacking obvious female coloration) was placed in the intruder compartment to assess pair bond formation. Small intruders that were not obviously female were used to prevent males from viewing the intruder as a potential mate. The observer was not blind to treatment when recording behaviors, as tanks were labeled for each replicate and visible on the video recording to ensure accuracy when reporting data by treatment and social status.

After placement into forced pairs, fish were allowed 20 min to acclimate before being video recorded for 1 hr. Forced pairs were videoed again on the second day.
between 1000-1100 hrs for 1 hr. On the third day (48 hr after being paired) an intruder was introduced into the intruder compartment of the tank, and the response of the putative pair was recorded for 1 hr.

Several criteria were used to judge if the adult male and female had formed a pair bond within 48 hrs. Low to moderate aggression toward each other, sharing time in the flower pot together, and coordinated, mutual overt aggression toward the intruder were used as indicators that a pair bond had formed. Aggression toward the small intruder by both the male and the female while maintaining no to low aggression toward each other was the minimum requirement to be considered paired. After the intruder test, each putative pair was assigned a status as “paired” or “not paired” and allowed an additional 48 hr to spawn. Individuals were removed from experimental aquaria after this final time period, and tags were removed before releasing them into designated stock aquaria for recovery. Any fish showing signs of injury (i.e. body damage, substantial fin damage, missing patches of scales) as a result of excessive aggression were removed from experimental aquarium, and that replicate was terminated. Premature termination was required for 7 of the 80 pairings in this experiment. All experimental aquaria were completely drained and refilled with fresh water after each replicate to prevent cues from previous groups or pairs from affecting results.

Statistical Analyses

The overall behavioral dynamics of individuals in each of the 3 treatment groups (females only, males only, and mixed-sex) were measured within and across days and among treatment groups within days. Behavior on the third day was used to compare social environment to subsequent behavior during forced pairing. The first and second
days allowed assessment of the stability of dominance relationships. Behaviors of males and females were analyzed separately for sex differences between the two single-sex groups and within mixed-sex groups. Differences in behavior between dominant and subordinate individuals were compared to establish legitimacy of dominance rank assignment. The immediate responses of experimental fish to novel fish and the responses of novel fish to the experimental fish were also analyzed. Only consistently observed behaviors are reported; behaviors that occurred too infrequently for statistical analysis were excluded.

The most frequent overt aggressive behaviors were chases and bites, and the most frequent display behaviors were frontal displays (flaring of opercula towards an individual in a head-on fashion, typically an aggressive act) and lateral displays (full extension of dorsal and ventral fins), which can be aggressive or submissive. Additionally, all these behaviors are performed by males and females and might also be considered courtship. The classification of the motivation underlying these behaviors depends both on the context in which it is observed and also its intensity. Because distinguishing motivation is often highly subjective, the results reported here for intersexual interactions in mixed-sex groups are reported as specific behaviors only, and no attempt was made to classify intersexual behaviors as “aggression” or “courtship” to avoid incorrect assignment of motivation.

Not all data were normally distributed, and Friedman’s tests were used when data violated normality assumptions of repeated measures ANOVA, and Greenhouse-Geisser corrected values are reported in cases where sphericity was violated. The Šidák correction was applied to post-hoc pairwise comparisons for significant repeated
measures ANOVA results, otherwise related-samples Wilcoxon Signed rank tests were used for post-hoc tests. All statistics were conducted using SPSS statistical software (SPSS Statistics for Window, Version 17.0).
Figure 2.1. Experimental design used in Aim 1 and Aim 2 to create the three treatment social groups. Arrows indicate which individuals were removed after 48 hrs of group treatment. In Aim 1, individuals were removed and paired. In Aim 2, individuals were removed and sacrificed for blood and brain collection.
RESULTS

Group Behavioral Data

Single-Sex Groups

Behaviors were recorded from the final 40 min of each 1 hr videotape so that fish were habituated to the camera before data recording. The following behaviors in all-male groups did not significantly differ across the 3 days: number of chases (F(2,18) = 0.73, P = 0.50), number of bites (Χ²(2) = 0.051, P = 0.98), number of lateral displays (F(2,18) = 0.45, P = 0.65), and number of frontal displays (Χ²(2) = 1.90, P = 0.38).

However, all-female groups were more variable across days (data not shown). The frequency of chases differed across days (Χ²(2) = 12.2, P = 0.002), with fewer chases on day 1 compared to day (Wilcoxon signed-ranks test Z = 2.78, P < 0.01) and day 3 (Wilcoxon signed-ranks test Z = 0.13, P < 0.01). Chases on days 2 and 3 were the same (Wilcoxon signed-ranks test Z = 2.78, P = 0.90). Bites also differed significantly across the 3 days (Χ²(2) = 7.13, P = 0.03). Days 1 and 3 were not different (Wilcoxon signed-ranks test Z = 0.74, P = 0.46), but more bites occurred on each of these days compared to day 2 (day 1: Wilcoxon signed-ranks test Z = 1.96, P = 0.05; day 3: Wilcoxon signed-ranks test Z = 2.37, P = 0.03). The following behaviors were not significantly different across the 3 days: lateral displays (F(1.28,11.55) = 2.07, P = 0.18) and frontal displays (Χ²(2) = 3.37, P = 0.19). Overall, the social or behavioral environment appeared stable within the 48 hr observation period in all-male groups but more variable in female groups.

Mixed-Sex Groups
The average frequency of chases in mixed-sex groups differed across days $(F(2,18) = 23.06, P < 0.01)$. Chases were significantly different between days 1 and 2 (paired $t(9) = 5.89, P < 0.01$), with more chases on day 2 than on day 1. Chases were also different on days 1 and 3 (paired $t(9) = 8.07, P < 0.01$), with more chases on day 3. Days 2 and 3 did not differ (paired $t(9) = 0.93, P = 0.38$). Bites did not differ across days $(F(2,18) = 2.13, P = 0.15)$.

The average frequency of frontal displays did not differ among days $(\chi^2(2) = 2.58, P = 0.28)$. Lateral displays were significantly different among days $(F(2,18) = 4.09, P = 0.03)$. As with chases, the frequencies of lateral displays were the same on days 2 and 3 (paired $t(9) = 0.9, P = 0.39$). Day 1 was significantly different from day 2 (paired $t(9) = 2.85, P = 0.02$) and day 3 (paired $t(9) = 2.24, P = 0.05$), with more lateral displays on days 2 and 3 than on day 1. Overall, the average frequencies of behaviors in mixed-sex groups seemed to stabilize by day 3 similar to single-sex groups.

Behaviors on Day 3 (approximately 1-2 hrs prior to forced pairing) were compared across the 3 treatment groups. As seen in Figure 2.2, neither overt aggressive behavior nor display behaviors differed (chases: $\chi^2(2) = 4.71, P = 0.10$; bites: $\chi^2(2) = 0.260, P = 0.88$; lateral displays: $\chi^2(2) = 5.66, P = 0.06$; frontal displays: $\chi^2(2) = 1.54, P = 0.46$).

The intra- and intersexual dynamics within mixed-sex groups were also examined. There was a significant difference between intra- and intersexual chases in males $(t(11.9) = 2.6, P = .023)$ and females $(t(18) = 4.2, P < 0.01)$, with both sexes directing more chases toward their own sex (Figure 2.3). The same was true for bites by males $(t(9.9) = 2.2, P = .050)$ and females $(t(18) = 2.2, P = .04$; Figure 2.4). There was no
difference in intra- versus intersexual lateral displays performed by males and females in mixed-sex groups.

**Dominance Status**

Dominance was assessed by measuring the amount of aggressive behavior displayed by an individual. Individuals with the highest levels of aggression were designated “dominant” and those with the lowest levels “subordinate.” Bites, chases, and intrasexual frontal displays were combined to create a measure of overt aggression, as these behaviors are most likely to be exclusively aggressive acts. Overall aggressive behavior of dominants and subordinates on day 3 was analyzed by treatment group and sex (Figure 2.5) and as an overall measure within each group (Figure 2.6). The average behaviors of individuals classified as dominant were not different across treatment groups (F(2) = 0.82, P = 0.45), and the same was true for subordinates (F(2) = 0.59, P = 0.56).

Aggression between dominants and subordinates was significantly different within all-male groups (t(9.2) = 6.2, P < 0.001), all-female groups (t(9.1) = 5.0, P < 0.001), and mixed-sex groups (t(18.1) = 6.7, P < 0.001). When separated by sex, mixed-sex dominant and subordinate females differed in aggression (t(8.3) = 5.5, P < 0.001), as did mixed-sex dominant and subordinate males (t(9.0) = 5.7, P < 0.001).

**Reproductive Data**

**Pair Bonding**

A total of 56 pair bonds occurred out of 80 forced pairings. Individuals from mixed-sex groups successfully formed pair bonds more frequently those from single-sex treatment groups (Table 2.1; Fisher’s exact test P = 0.03). Mixed-sex females paired
more often than single-sex females, and mixed-sex males paired more often than single-sex males, but these within-sex trends were not statistically significant (Table 2.1).

**Spawning**

To determine if prior experience might facilitate spawning, each pair was given 5 days in which to spawn. After 5 days, only 17 of the 80 forced pairings resulted in spawning. Spawning percentage was not different between social categories or by sex. Single-sex and mixed-sex females spawned with the same frequency (N=4).

**Dominance**

Dominance did not influence pair bonding or spawning. An equal number of dominant and subordinate individuals formed a pair bond within the 48-hr time period (N=28). An almost equal number of dominant (N=8) and subordinate (N=9) individuals spawned with their novel partner. There were no sex-specific effects of dominance.

**Behavior within forced pairs**

Overt aggression (combined chases, bites, and frontal displays) by experimental fish toward the novel partner was not related to treatment group, dominance rank, or sex of the group-housed fish (data not shown). There was also no combination of these categories that revealed any significant differences in aggressive response to a partner across groups.

Aggression by the novel fish toward its partner was also evaluated. Novel males and females did not behave significantly differently toward experimental fish based on their prior treatment group, dominance rank, or the combination of treatment group and dominance rank. The behaviors of experimental fish toward novel fish as well as behaviors of novel fish immediately after forced pairing are shown graphically in the
Appendix (Figures A1-A8). Only behaviors which occurred with relatively high frequency are shown; behaviors observed in less than half of the individuals within a comparison group are not included. In summary, pair bond formation but not within-pair behavior was related to prior social experience. In addition, whether or not a pair bond formed was not related to behavioral dynamics within pairs.
Figure 2.2. Median number of behaviors occurring in a 40 min period on Day 3 across the treatment groups. There were no significant differences among the groups for any behavior. N = 10 for each treatment group.
Figure 2.3. Mean ± SE intrasexual versus intersexual chases by males and females in 40 min in mixed-sex groups on Day 3. Significant differences within males (P = 0.02) and females (P < 0.01) are indicated by asterisks. N = 10 per sex.
**Figure 2.4.** Mean ± SE intrasexual versus intersexual bites by males and females in 40 minin mixed-sex groups on Day 3. Significant differences within males (P = 0.05) and females (P = 0.04) are indicated by asterisks. N = 10 per sex.
Figure 2.5. Average ± SE overt aggression (combined bites, chases, and intrasexual frontal displays) of dominant and subordinate individuals in 40 min among the three treatment groups. Differences indicated by letters are significant (p < 0.01). N = 10 per social group.
Figure 2.6. Average ± SE overt aggression by sex and treatment group. “SS Males” are males in single-sex groups. “MS Males” are males in mixed-sex groups, and so on. N=10 for each category. Significant differences (P < 0.02) are indicated by letters.
<table>
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<th>P-value for Fisher’s exact test</th>
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<td>12</td>
<td>8</td>
<td>0.16</td>
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<td>17</td>
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<td>11</td>
<td>9</td>
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**Table 2.1.** Pair bond formation within 48 hours (yes/no) by prior social treatment group with sexes separate (first four rows) and with sexes combined (“All”; bottom two rows). SS = single-sex group and MS = mixed-sex group. Fisher’s exact test P-values for each comparison given on right. Significant result in bold. N = 20 pairs for single-sex groups and N = 40 pairs for mixed-sex groups.
DISCUSSION

Behaviors or conspecific sensory cues experienced in a pre-reproductive context could affect subsequent reproductive behavior. Individuals from single- and mixed-sex groups were placed in a potential reproductive scenario to evaluate potential effects of opposite-sex cues on reproduction. Individuals previously kept in mixed-sex groups paired more frequently than those kept in single-sex groups. Consequently, behaviors in groups were examined to determine if they were related to subsequent pair formation. When compared to the single-sex groups, mixed-sex groups did not have a unique behavioral profile. Frequencies of behaviors (bites, chases, frontal displays, and lateral displays) were not different for individuals from the 3 treatment groups, and the idea that the presence of potential mates would significantly increase aggression was not supported. Furthermore, individuals in mixed-sex groups typically interacted more with members of their own sex than toward individuals of the other sex, mirroring a previous study in convicts (Weber and Weber 1976). These results are opposite to the prediction based on Lorenz’s (1950) psychohydraulic model (an earlier iteration described by McDougall in 1923) that following isolation from the other sex, individuals should have an increased motivation to mate.

Because there were no significant behavioral differences among individuals in the three treatment groups, prior mixed-sex experience may prime females and males physiologically so that they form pair bonds more quickly. Instead of pre-spawning behavioral suites (i.e. courtship, aggression, nesting), female reproductive status may instead be paramount for predicting pair bond formation (males significantly prefer gravid females, Nuttall and Keenleyside 1993; territory establishment occurs only when
the female is ready to spawn, Mackereth and Keenleyside 1993). An assumption of this hypothesis is that female physiology is affected by the presence of males. This hypothesis does not account for novel females pairing more frequently with males from mixed-sex groups, however. Exposure to water-borne cues certainly impacts reproductive behaviors in other fishes of both sexes, acting as potent sex pheromones in many cases (reviewed in Liley 1982, Munakata and Kobayashi 2010; Liley 1972; Stacey 1976; Stacey 1981; Liley and Stacey 1983; Kobayashi and Stacey 1993; Moore et al. 2002), and could be a potential mechanism by which male priming occurs. Chemosensory cues may act in concert with other cues, and the natural history of organisms should provide clues as to which sensory modalities are of primary importance. In fishes, chemosensation, vision, and audition have been exploited for reproductive purposes to varying degrees across species.

Frequent display behaviors, rapidly changing melanin-based coloration, and in females, the occurrence of bright ventral pigmentation (coloration described in Wisenden 1995; pers. obs.) suggest that visual signals are a predominant means of communication in convict cichlids. Visual signals or cues from the opposite sex may stimulate subsequent reproductive behavior or physiology. Visual cues significantly affect social and physiological status in other species (e.g. Anolis carolinensis, Korzan et al 2000; Salvelinus alpinus, Hoglund et al. 2002; Astatotilapia burtoni, Chen and Fernald 2011). Of course, chemosensory, visual, behavioral, and auditory cues (e.g. Maruska et al. 2012; see also Rollo et al. 2007) may all act in concert to prepare males and female convicts for pair bonding and spawning.
Another component of social experience that might influence pair formation is dominance status. Dominance was clearly established within 24 hrs and remained stable thereafter, as occurs in other fishes (Oliveira et al 1996; McGhee and Travis 2010), with dominant fish being significantly more aggressive than subordinates. Despite the obvious difference in aggression, dominance status was not related to pair formation or spawning with a novel partner, as dominant and subordinate fish were equally likely to form pair bonds. In contrast, in an African cichlid (Astatotilapia burtoni), social status and reproductive physiology are tightly linked (Fernald and Hirata 1977; Hofmann and Fernald 2001; Maruska et al. 2011; Fernald 2012; Fernald and Maruska 2012; Maruska and Fernald 2013). A biparental South American cichlid, Cichlasoma dimerus, also has a dominance-based breeding system and exhibits the same relationship between social status and reproduction (Alonso et al. 2011). However, in both species territorial and non-territorial males exhibit distinct morphological and behavioral traits associated with dominance, whereas convict cichlids do not. Thus, dominance may not impact reproduction as much in convicts because of the less rigid nature of their social hierarchies.

It is possible that when choice is removed from a reproductive scenario as it was in this experiment, convicts are more willing to accept a partner in spite of having behavioral preferences, assuming the partners are in a permissive physiological state. An alternative explanation is that dominance is transient in convicts, as opposed to other species in which strict hierarchies are accompanied by clear reproductive consequences, including morphological and physiological changes. Convicts in large, single-sex groups (for example, a 473 L stock tank containing 20-40 fish) are presumably mostly of
intermediate dominance, as only a few individuals display frequent aggressive behaviors or are clearly submissive in appearance (pers. obs.). I assumed that sampling from a typical stock group resulted in fish having approximately equal inherent aggressiveness when placed in experimental groups. While new dynamics are quickly established and maintained in the smaller groups, the effects of being newly dominant or submissive may not affect subsequent reproductive behavior simply because they are new. In other words, long-term experience may have an impact, but newly formed hierarchies may not affect behavior or reproduction.

The results from this study are in contrast to a previous study that examined the relationship between dominance and spawning in convicts (Weber and Weber 1976). However, different methodologies might account for the difference. In the Weber and Weber (1976) experiment, females and males were placed together in an aquarium with breeding substrate and allowed to interact freely. Dominant females consistently chose dominant males with which to spawn when given the opportunity to choose among several males. This finding indicated that dominance status was important for mate choice, but possibly only when individuals can be compared. My methodology may have prevented a dominance effect from being observed, as individuals placed into forced pairs could not observe their partner’s social status in a group setting. Social status could therefore be important for mate selection in convict cichlids even though it was not demonstrated in my experiment.

Behaviors in forced pairs were also evaluated to assess effects of prior experience on behavior in a reproductive context. While pair bond formation was facilitated by prior experience with the opposite sex, behavior within the forced pairs did not differ as a
function of prior treatment group or dominance status of the experimental fish. Perhaps, again, short-term experience did not significantly affect subsequent behaviors exhibited by individuals in a new context.

If the physiological state of the individual is what regulates behavior and/or pair formation, pre-spawning behaviors may not play a primary role in successful pair bonding for males or females. Not only were the immediate responses (typically “aggressive” behaviors) of experimental and novel fish similar among forced pairs based on prior experience or social status of the experimental fish, but behaviors in forced pairs that formed a pair bond were not different from those that did not. Courtship sensu strictu (behaviors such as brushes, quivering, and other non-aggressive interactions) were very infrequent both in the groups and in forced pairs and did not influence pair formation or spawning. These observations further support the idea that performance of “courtship” behaviors is not a driving force of pair formation in this species.

Mate preference and choice studies in convict cichlids have revealed preferences based on body size and female reproductive state (Noonan 1983; Nuttall and Keenleyside 1993; but see Keenleyside 1985 and Santangelo 2005), but the notion that there is an obvious, behavioral courtship component associated with mate choice is not well supported by previous research (see Santangelo and Itzkowitz 2004; Santangelo 2005; Leese 2012; current study). While convicts do exhibit particular behaviors only observed in a mating context, these “courtship” behaviors do not seem to causally affect pair formation but instead may only be a correlate of motivation to pair. In field studies, female convicts initiate courtship-type behaviors in a pre-mating context while male-initiated courtship does not often occur outside captivity (Meral 1973). A small
percentage of males have been observed to court in the wild after catastrophic brood loss (Wisenden et al. 2008), and such behavior likely signals motivation to pair again, as male convicts have a higher reproductive potential than females. Additionally, male courtship was directed toward females that were relatively close to spawning (Wisenden et al. 2008). These observations support the idea that female reproductive state is central to pair formation and that courtship behaviors indicate motivation but are not required for pair bonding to occur.

Courtship, if redefined to include all pre-spawning behaviors, would then be primarily characterized by low-level intersexual aggression by both individuals. Excessive levels of baseline aggression by either partner prevent pair formation (Barlow 1998; Itzkowitz and Draud 1992) and pair maintenance (pers. obs.). It is plausible that individuals gauge the aggressiveness of potential partner as a form of mate assessment. A previous study (Hirschenhauser et al. 2004) indicated that low to moderate aggression concomitant with high physiological responsiveness (rapid response to a challenge and rapid return to baseline) may be an ideal combination in a mate. This relationship might also explain why males and females often do not exhibit distinct courtship behaviors and instead aggress toward each other in a pre-spawning context, and why mild but not excessive levels of intersexual aggression result in more obvious pair bonding among convicts. This “aggressive compatibility hypothesis” for mate choice is further supported by dominant females selectively interacting with and spawning with dominant males in the absence of “courtship” behaviors (Weber and Weber 1976; personal obs.). Additionally, because convicts exhibit biparental care, I would expect a dynamic between aggression and mate choice, as parental defensive behaviors are critical for offspring
survival in the wild. Even within a 48-hr period, males and females in mixed-sex groups tended to selectively interact according to social rank, and on several occasions the dominant male and female would begin to form a territory (pers. obs.). It is noteworthy that these group interactions did not significantly hinder pair formation in individuals coming from mixed-sex groups. If given more time in a group, experimental individuals would likely identify any novel fish as an intruder instead of a potential mate, although assessment of other potential mates occurs throughout the pair bonding period (Santangelo and Itzkowitz 2006). Excessive aggression toward a novel fish was occasionally observed in experimental individuals from mixed-sex groups, and prior affiliation with an individual from the group may explain such occurrences.

There was a slight trend for display behaviors to be directed more often toward members of the other sex. As pointed out earlier, classification of such displays as “courtship” and not aggression is quite subjective, especially since these displays occurred with the same overall frequencies in all treatment groups. Furthermore, lateral displays are also often used to signal submission. It is therefore inappropriate to classify a lateral display as courtship simply because they are performed during encounters with the other sex. Other, less ambiguous behaviors exhibited by males and females toward the other sex may also have either agonistic or affiliative motivation. Although a “novel” ethogram was proposed as a solution to the problem of assigning motivation to behavior in convicts (Oldfield and Hofmann 2011), it bears remarkable similarity to numerous previously published descriptions of convict cichlid behavior, beginning decades ago (e.g. Baerends and Baerends-van Roon 1950; Cole et al. 1980). In addition, the assignment of affiliation or aggression in the Oldfield and Hofmann (2011) ethogram was
dependent on the action of the receiver, which may still be inaccurate. For example, males may not flee after being bitten by another male (pers. obs.), but this certainly does not imply the bite was affiliative. Thus, resolution of this rather confounding aspect of convict cichlid behavioral research remains dubious. This problem strengthens the argument that further research exploring the non-behavioral mechanisms involved in pair formation in this species is warranted and would be more revealing than studies of behavior alone.
III. Potential physiological correlates of social experience and dominance status

INTRODUCTION

The effects of social experience on reproductive physiology are well documented across taxa. An array of hormones are tightly linked to mating and dominance in species having various mating and parental care systems. For example, the two principal vertebrate gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), play obvious roles in reproduction as they modulate the synthesis of gonadal steroids, which affect reproductive and aggressive behavior. FSH and LH also affect breeding coloration and aggression in a cichlid fish (Blum and Fielder 1965), and conversely, intermale aggression affects FSH and LH levels in mice (Bronson et al. 1973). LH and FSH also may have sex-specific relationships with behavior. Dominance-related reproductive status is associated with FSH levels in the pituitary in male but not female cichlids (Cichlasoma dimerus, Alonso et al. 2011; Alonso et al. 2012), and stress-induced suppression of LH leads to interruption of the spawning cycle in female tilapia (Chabbi and Ganesh 2012).

FSH and LH synthesis and secretion are regulated by gonadotropin-releasing hormone (GnRH) from the hypothalamus, while increasing levels of gonadal steroids and certain peptides typically feedback negatively onto the hypothalamus to regulate GnRH (Nelson 2011). Evidence supports similar function of this hormonal axis in fishes as well, although the feedback relationships can depend on time of year and sexual maturation (i.e. negative feedback of sex steroids on gonadotropins occurs only during the breeding season in mature salmon, Dickey and Swanson 1998). LH and FSH are also
regulated differently, as administration of testosterone and estradiol results in positive feedback on LH but negative feedback on FSH in some instances (Dickey and Swanson; Zohar et al. 2010). The complexity of relationships between physiology and behavior cannot be underestimated, and such relationships likely involve multiple pathways, just as gonadotropins are influenced not only by sex steroid feedback but also by glucocorticoids.

Steroid hormones such as corticosteroids, androgens and estrogens are implicated in the regulation of a wide variety of behaviors across vertebrates. For example, maintaining dominance may either be more or less stressful than being subordinate, which in turn influences corticosteroid levels as well as gonadal steroids and various peptide hormones. Chronic elevated corticosterone is typically associated with submissive behavior and decreased aggression (Politch and Leshner 1977; Leshner et al. 1980; Øverli et al. 2002; Summers et al. 2005; but see Creel et al. 1996; Abbott et al. 2003), but acute cortisol increases are associated with high aggression (Munro and Pitcher 1985; Kruk et al. 2004; Summers et al. 2005). Changes in cortisol induced by acute stressors, including dominance interactions, also coincide with changes in circulating sex steroids (male and female reptiles: Grassman and Hess 1992; Jessop et al. 2002), and gonadal steroids acting on nuclear receptors in the brain influence an array of behaviors, including communication, reproduction, aggression, and affiliative behaviors (Insel 2010).

The role androgens (especially testosterone) play in reproduction and aggression is well studied among vertebrates (reviewed in Nelson 2011), and the effects on aggressive behavior are not limited to males (reviewed in Bouissou 1983; Schoech et al. 2003).
1991; Staub and De Beer 1997; Desjardins et al. 2006). In fishes, there are two behaviorally relevant androgens, testosterone and its 11-oxygenated form, 11-ketotestosterone (11-KT). Circulating 11-KT is directly related to changes in aggression and dominance status in several teleosts (e.g. Fostier et al. 1983; Liley and Stacey 1983; Oliveira et al 1996; Hay and Pankhurst 2005; Dzieweczyński et al. 2006; Parikh et al. 2006; Taves et al. 2009). Depending on the species and type of care, 11-KT can be either elevated or depressed during parental care (see Rodgers et al. 2006). Testosterone appears to be more relevant to female aggression in females, however, as females exhibit changes in testosterone but not 11-KT after contests (Oliveira 2004; Desjardins et al. 2008; Taves et al 2009). The aromatization of testosterone to estradiol also plays a regulatory role in aggressive behavior (Black et al. 2005; Huffman et al. 2013), and 11-KT is not aromatizable (Adkins-Regan 2005). Mirroring numerous mammalian and avian studies comparing testosterone (an aromatizable androgen) and dihydrotestosterone (DHT, a nonaromatizable androgen), 11-KT in a number of male telesosts, like DHT in mammals, has been shown to be directly related to expression of reproductive behaviors and secondary sex characteristics (Liley and Stacey 1983; Villars 1983; Kindler et al. 1989; Borg 1994). In male convict cichlids (Amatitlania nigrofasciata), 11-KT is elevated during pre-spawning behaviors, and its decrease after spawning coincides with the onset of parental care (van Breukelen 2008).

11-KT responsiveness to a territorial challenge is higher in species that are monogamous or biparental or that show at least some degree of pair bonding (Oliveira et al. 2001; Oliveira 2002; Hirschenhauser et al. 2004). Although male convict cichlids treated with the androgen receptor antagonist flutamide did not show decreased levels of
aggression (van Breukelen 2013), males treated with the antiandrogen cyproterone acetate (CPA) did (Sessa et al. 2013). The opposing results could be due to differences in action of these two antiandrogens. Flutamide is classified as a general androgen receptor blocker, while CPA also inhibits enzymes in the androgen biosynthesis pathway and has potent antigonadotropic effects (Moltz et al. 1980). Plasma levels of 11-KT might be correlated with differences in dominance or the propensity to form pair bonds in male convict cichlids because of its correlations with aggression, pre-spawning behaviors and parental care in convicts and other fishes.

In female fishes, reproductive physiology and sex behavior are largely regulated by sex steroids and prostaglandins. Female sex behavior and receptivity in fishes can be induced by pre-ovulatory $17α,20β$-dihydroxy-4-pregnene-3-one and $17,20β$-P-20β-sulfate as well as post-ovulatory prostaglandin F$_{2α}$ (PGF$_{2α}$). Surprisingly, ovarian sex steroids are not required for sex behavior in some female fishes (Kobayashi and Stacey 1993). In other vertebrate taxa, prostaglandins function in ovary maturation, ovulation, breakdown of the corpus luteum and female proceptivity and receptivity (Villars et al. 1985). Prostaglandins are also associated with vitellogenesis and even parental care in teleosts (reviewed in Ruggeri and Thoroughgood 1985).

PGF$_{2α}$ in particular induces spawning behavior in both male and female goldfish (Carassius auratus, Stacey 1976; 1981). PGF$_{2α}$ also successfully induced behavior and morphology associated with oviposition in females of a monogamous cichlid, which suggests that these hormonal mechanisms are generalizable across breeding systems (Cichlasoma bimaculatum, Cole and Stacey 1984) and should be explored as possible mechanisms of pair bonding. Additionally, PGF$_{2α}$ can be rapidly metabolized in vivo
(Samuelsson et al. 1975; Basu and Stjernschantz 1997), and difficulty in detection of the parent hormone can be resolved by assaying for its metabolites instead.

How PGF\textsubscript{2\alpha} (or 13,14-dihydro-15-keto PGF\textsubscript{2\alpha}) varies across the ovulatory cycle in convict cichlids or if it influences male reproductive behavior is unknown. Whether social experience affects PGF\textsubscript{2\alpha} levels in females should be investigated, as physiological priming by social cues and intersexual interactions has been demonstrated across taxa (e.g. Crews and Silver 1985; Dufty and Wingfield 1986; Ball and Bentley 2000; Sakata et al. 2002; Ros et al. 2009). Dominance or heightened aggression among female convicts is indicative of reproductive readiness (Weber and Weber 1976), so differences in aggression and social status are presumably correlated with changing levels of reproductive hormones. However, socially subordinate females do engage in pair bonding and spawning under certain circumstances and often appear as gravid as dominants upon dissection (pers. obs.). Overall, the relationships between aggressive or reproductive behaviors and hormone levels in the blood and brain are highly context-dependent and involve multiple hormonal axes.

The types of social interactions experienced by individuals when selecting a mate may be agonistic or affiliative in nature and are affected by various hormonal mechanisms regulating both behaviors and reproductive capabilities. Such interactions can also function beyond mate assessment, synchronizing reproductive physiologies (Lehrman 1965; Bruder and Lehrman 1967; Friedman and Lehrman 1968; Lehrman and Friedman 1969; Perrigo and Bronson 1982; Hurst and Nevison 1994; Stacey et al. 1994; Moore and Waring 1999; Moore et al. 2002) or future parental behaviors (Griggio and Hoi 2011), for example. The same type of cue or experience encountered in a pre-
reproductive setting may also differentially affect individuals; hormonal changes associated with aggressive encounters may facilitate pairing in dominant individuals and suppress reproduction in subordinates (Fichtel et al. 2007; Komers et al. 1997; Markiewicz and O'Donnell 2001; Mommsen et al. 1999; Moore and Waring 1999; Saltzman et al. 2009). Behavior-induced hormonal changes occur in multiple contexts across taxa, but how intersexual cues and dominance status might affect pair bonding in teleosts is poorly understood. Social interactions could be especially relevant for seasonally breeding, monogamous, biparental fishes to synchronize their reproductive biology and to judge the future parental abilities of prospective mates.

Elucidating mechanisms of behavior in convict cichlids would be helped by studies in other fishes having a similar social system, but monogamy with mutual mate choice and biparental care separates *A. nigrofasciata* from most teleost species (including other cichlids). In addition, even closely related species differ in physiological regulation of their behavior if they exhibit different mating and parental care systems, as in voles. Unfortunately, there are few studies of either close relatives of *A. nigrofasciata* or of more distantly related monogamous, biparental fishes. Presently, the hormonal mechanisms underlying mating behavior and pair bonding in monogamous fishes are largely unknown. Given the highly aggressive social dynamics of convicts in both reproductive and non-reproductive contexts, the potential relationship between individual aggression and pair formation could possibly be elucidated by tapping into physiological correlates of dominance and social experience.

The physiological mechanisms regulating reproduction have not been well described for convict cichlids for either sex, a surprising fact given the myriad studies of
their reproductive and parental behavior. Consequently, I examined plasma levels of 11-ketotestosterone in males and 13,14-dihydro-15-keto PGF$_2\alpha$ in females as well as whole brain content of follicle stimulating hormone in both sexes in different social contexts and between dominant and subordinate individuals. Because of the aggressive nature of convicts, dominant males were predicted to exhibit elevated 11-KT compared to subordinates. I also predicted that female experience with males would affect circulating PGF$_2\alpha$ levels. Lastly, I expected that follicle-stimulating hormone levels would be elevated in individuals in mixed-sex groups. Finding these correlations would support the idea that social stimulation of the reproductive axis is related to pair formation.
METHODS

Part I: Treatment Groups

The protocol for setting up the social groups were the same as in “Treatment Groups” in Chapter II. Single-sex male or female groups consisted of 6 size-matched individuals, and mixed-sex groups consisted of 3 males and 3 females, all of whom were 10 mm smaller (standard length) than the size-matched males in the group. Individuals were tagged and groups were video recorded for 1 hr approximately 48 hrs after initial setup to determine dominance relationships. Group and pair behaviors were recorded from 20 min. of the 1 hr video after at least 3 min. acclimation to cameras.

Part II: Blood and Brain Collection

After assigning dominance, the most and least aggressive individuals in a single-sex group and the most and least aggressive male and female in mixed-sex groups were euthanized with tricaine methanesulfonate (MS-222). Upon complete cessation of visible respiratory movements, fish were cut on a diagonal transect just anterior to the genital papilla to expose the dorsal aorta. Blood (between 20-40 µL) was collected using heparinized capillary tubes, placed in 0.5 mL snap cap tubes and centrifuged for 10 min. at 3,000 g. Plasma was removed and kept on ice until being frozen at -80 °C for storage. Whole brains were removed immediately after blood collection and also kept on ice until frozen at -80 °C.

Part III: Hormone Assays

Plasma samples were evaluated for 11-KT and PGF$_2$a metabolite at Washington State University’s Center for Reproductive Biology assay core facility by David de Avila. Plasma samples were extracted following the standard protocol provided by Cayman
Chemical for their enzyme-linked immunosorbant assay (ELISA) kits. Final values for hormone concentrations were adjusted for dilution factor and extraction efficiency. The 96 well plate 11-keto Testosterone EIA kit (Cayman Chemical; Michigan, USA) has a sensitivity of 100% for 11-KT, 2.9% for adrenosterone, 0.01% for 4-Androsten-11β,17β-diol-3-one, and less than 0.01% for other adrenosterone metabolites and testosterone. Coefficients of variation were calculated from the dose response; inter-assay variation for 11-KT was 9.48% and intra-assay variation was 5.59%. The 96 well plate 13,14-dihydro-15-keto prostaglandin F$_{2\alpha}$ kit (Cayman Chemical; Michigan, USA) has a sensitivity of 100% for the hormone of interest, 2.7% for 13,14-dihydro-15-keto Prostaglandin E$_2$, 1.8% for 15-keto Prostaglandin F$_{2\alpha}$, and less than 0.01% for thromboxane B$_2$, other prostaglandins (D,E, and F series) and their metabolites. Inter-assay variation for the PGF$_{2\alpha}$ metabolite was 5.77% and intra-assay variation was 6.48%.

**Part IV: Western Blots for Follicle Stimulating Hormone**

Whole brain extracts were prepared at Lehigh University for SDS-PAGE by mechanical homogenization of each brain in lysis buffer (PEM buffer supplemented with 0.05% NP-40 and 0.5 mM PMSF). Whole brains were used to account for FSH produced by gonadotropic cells present in brain tissue outside the pituitary (Parhar et al. 2003; Pandolfi et al. 2009). The brain and 150 µL lysis buffer were drawn into a 1 mL tuberculin syringe fitted with a 18 G needle, mixed by drawing up and expelling the brain/buffer mixture a few times and then was pushed through a 21 G needle, mixed again, and allowed to sit for 30 minutes on ice. The lysate was pelleted for 10 min. at 12,000 rpm (4°C) and the protein concentration of the soluble fraction was determined using the Bio-Rad Protein Assay reagent and IgG as a standard (Bradford Assay,
Bradford 1976). 80 µg of diluted protein (1:4) with loading buffer (120 nM Tris-HCl pH 6.8, 3% dodecylsulfate, 10% glycerol, 1% β-mercaptoethanol) was heated at 30°C for 5 min before being loaded into the gel. Proteins were separated on a 3-12.5 % SDS-PAGE, transferred to a PVDF membrane at 200 mA for 1 hour and blocked for 1 hour in 5% nonfat milk/TBS-Tween (0.9% NaCl, 20mM Tris, 2% Tween-20). The membrane was incubated overnight with rabbit anti-βFSH (Fundulus heteroclitus, 19 & 15 kDa ir-bands, provided by Akio Shimizo; 1:500 dilution). Membranes were rinsed in TBS-Tween (3 x 5 min washes) and incubated with anti-rabbit peroxidase conjugated antibody for 1-2 hours. Membranes were washed again in TBS-Tween and developed with ECL (Amersham). After being developed for βFSH, membranes were rinsed and re-probed with mouse anti-α tubulin as a loading control. The protocol above was repeated with anti-mouse peroxidase for the secondary incubation. βFSH content in the brain was semi-quantified using image analysis software (MetaMorph®, Molecular Devices, LLC) to generate a percentage scores for each sample obtained by comparing the intensity of each individual’s 15 kDa βFSH band to its tubulin control band.

Statistical Analyses

The behavioral dynamics of each of the 3 treatment groups were analyzed on the third day to compare their behavioral profiles with hormone levels of individuals in the groups. Differences in overt aggressive behaviors (bites, chases, and intrasexual frontal displays) by dominant and subordinate fish were analyzed according to treatment group and sex. When data did not violate assumptions of normality and sphericity, ANOVA was used for behavioral comparisons across groups and unpaired t-tests for comparisons between dominant and subordinate individuals. Non-parametric tests were used for non-
normal data (using the Shapiro-Wilk test). All statistics were conducted using SPSS statistical software (SPSS Statistics for Window, Version 17.0).
RESULTS

Group Behavioral Data

The average frequencies of each behavior in the 3 social treatment groups (chase, bite, lateral display, frontal display) can be seen in Figure 3.1. Chases and bites did not differ across groups (chases: $F(2,27) = 2.20, P = 0.13$, bites: $F(2,27) = 0.04, P = 0.96$). A trend existed for lateral displays to be higher in all-female groups, but the groups were not significantly different ($F(2,27) = 3.297, P = 0.052$). The average frequency of frontal displays also did not differ across groups ($F(2,27) = 0.27, P = 0.77$). Thus, the aggressive behaviors were similar among treatment groups prior to sampling.

Mixed-Sex Group Dynamics

The behaviors performed by either males or females in single- and mixed-sex groups were compared to determine if social group affected aggression by males or females differently. First, total mixed-sex group behavior averages were compared to single-sex group averages. Then, intra- and inter-sexual behaviors were separated to determine if sex-specific aggressive behavior was different based on social environment.

As seen in Figure A9, single-sex group females and mixed-sex group-housed females differed in the average number of bites ($U(18) = 2.0, Z = -3.63, P < 0.01$), chases ($U(18) = 4.0, Z = -3.48, P < 0.01$), lateral displays ($U(18) = 2.0, Z = -3.63, P < 0.01$) but not frontal displays. Females in single-sex groups performed more bites, chases, and lateral displays than females in mixed-sex groups (Figure A9). When I controlled for density (by including only intrasexual behaviors for mixed-sex females and dividing each treatment group by the number of females in the group) these differences were maintained (Figure 3.2), indicating that the all-female social experience results in
heightened female aggression. The number of frontal displays by females in single- and mixed-sex groups was again not significantly different.

As seen in figure A10, males in single-sex groups differed significantly from mixed-sex group males in the average number of total chases (t(18) = 3.72, P < 0.01) and lateral displays (U(18) = 16.5, Z = -2.54, P = 0.01) performed per group but did not differ for bites or frontal displays. Males in all-male groups performed more chases and lateral displays than males in mixed-sex groups (Figure A10). After controlling for density (by considering only intrasexual behaviors and dividing by the number of males in each treatment group) these differences were maintained (Figure 3.3), indicating that an all-male social experience affected some male behavioral parameters compared to being in a mixed-sex group.

Sex difference in behavior were also examined in mixed-sex groups. There was a significant difference in the number of bites (U(18) = 18.5, Z = -2.39, P = 0.02) lateral displays (t(18) = 2.23, P = 0.04) performed by males and females, with females biting more frequently and males performing more displays (Figure 3.4). Females, but not males, differed in the number of behaviors directed toward their own sex versus toward the other sex within mixed-sex groups (males: figure 3.5). Intrasexual and intersexual chases (U(18) = 2.0, Z = -3.65, P < 0.01), bites (U(18) = 1.5, Z = -3.71, P < 0.01) and lateral displays (U(18) = 21.0, Z = -2.20, P = 0.03) but not frontal displays differed among females, with intrasexual behaviors occurring more frequently in all cases (Figure 3.6).

*Dominance Status Across Groups*
Overall, aggressiveness did not differ across groups within a social status (dominants: F(2) = 2.8, P = 0.8; subordinates: X²(2) = 4.17, P = 0.13; Figure 3.7). Within a social group, the total aggressive behaviors performed by dominant and subordinate individuals of each sex were significantly different, with dominant fish performing more aggressive acts than subordinates (all female: U(20) = 2.0, Z = -3.98, P < 0.01; all male: U(18) = 1.0, Z = -3.73, P < 0.01; mixed-sex females: U(18) = 6.5, Z = -3.40, P < 0.01; mixed-sex males: (U(18) = 2.0, Z = -3.65, P < 0.01). When comparing dominant individuals by sex and social treatment, single-sex and mixed-sex males did not differ in overt aggression (t(18) = 1.59, P = 0.13). Dominant females, however, did differ (t(19) = 3.04, P = 0.01), with females in single-sex groups exhibiting more aggression overall (Figure 3.8).

**Hormone Assays**

Circulating 11-KT concentrations were extremely variable within treatment groups. The treatment subgroups (dominant single-sex males, dominant mixed-sex males, subordinate single-sex males and subordinate mixed-sex males) did not have significantly different 11-KT levels (X²(3) = 5.08, P = 0.17; Figure 3.9). Nor did dominant and subordinate males pooled across treatment groups differ in 11-KT (Figure 3.10). Because subordinate fish in all treatment groups performed few behaviors of interest, a correlation was computed between 11-KT and the various behaviors for dominant animals only. 11-KT correlated positively with chases in single-sex groups (rs(9) = 0.91, P < 0.01) but not in mixed-sex groups (rs(8) = 0.17, P = 0.69). 11-KT was not correlated with any of the other behaviors.
Female plasma 13,14-dihydro-15-keto prostaglandin $F_{2\alpha}$ was less variable but not different between single- and mixed-sex groups ($t(37) = 1.63, P = 0.39$) or between dominants and subordinates pooled across treatment groups ($t(37) = -0.10, P = 0.99$). There was also no difference when comparing the experimental social groups according to dominance status ($F(3) = 0.64, P = 0.56$). There was a significant positive correlation of PGF$_{2\alpha}$ metabolite with lateral displays in dominant females pooled for treatment group ($r_s(18) = 0.49, P = 0.04$). Data across subgroups are shown in Figure 3.11.

**FSH in Brain Tissue**

FSH did not differ between dominant and subordinate males ($t(14) = -1.32, P = 0.61$; Figure 3.12). There was a significant difference in FSH among treatment groups when not pooled by dominance, however ($X^2(3) = 11.7, P = 0.01$; Figure 3.13). Post hoc comparisons revealed a difference between dominant and subordinate males within single-sex group individuals (Sidak $P = 0.03$) and between mixed-sex dominant males and single-sex subordinate males (Sidak $P = 0.01$), with single-sex subordinate males exhibiting the highest level of FSH. Neither aggressive behaviors nor 11-KT levels were correlated with FSH values in males.

There was no difference in FSH levels between dominant and subordinate females pooled for social groups ($t(14) = -0.85, P = 0.07$). When compared across subgroups of dominance status within social environment, female FSH values displayed a similar pattern as male values, although the elevated FSH in single-sex subordinate females was not significantly different from the other subgroups ($X^2(3) = 6.4, P = 0.09$; Figure 3.14). Aggression was not correlated with FSH values in females. There was an overall sex difference in whole brain FSH content ($t(30) = -4.68, P <0.01$), with females exhibiting
higher levels overall compared to males (Figure 3.15). Combined bites and chases performed by each sampled individual in a 20 minute period on the day of sacrifice are shown in Figure A9 across the sexes, social groups, and dominance status.
Figure 3.1. Average ± SE number of behaviors per group in 20 min across treatments (all-female groups: AFG, all-male groups: AMG, and mixed-sex groups: MSG) on Day 3 prior to blood and brain sampling. There were no significant differences among groups for each behavior. N = 10 for AMG and MSG, N = 11 for AFG.
Figure 3.2. Differences between the two social conditions within females while controlling for density. Mixed-sex groups (MSG) versus all-female groups (AFG). Average ± SE frequency of intrasexual behaviors within a group performed by females only in 20 min. Asterisks indicate significant differences between treatment groups (P < 0.03). N = 10.
Figure 3.3. Differences between the two social conditions within males while controlling for density. Mixed-sex groups (MSG) versus all-male groups (AMG). Average ± SE frequency of intrasexual behaviors within a group performed by males only in 20 min. Asterisks indicate significant differences (P ≤ 0.01). N = 10.
Figure 3.4. Average ± SE behaviors performed by either males or females in mixed-sex groups. Intra- and intersexual behaviors are included. N = 10 per sex. Asterisk indicates significant difference between males and females (P < 0.05).
Figure 3.5. Male behavior toward females (light) and males (dark) in mixed-sex groups. Males did not show a significant aggressive preference for one sex. N = 10.
Figure 3.6. Female behavior toward females (dark) and males (light) in mixed-sex groups. Significant differences between categories indicated by asterisks (P ≤ 0.03). N = 10.
Figure 3.7. Differences in average overt aggression (combined chases, bites, and intrasexual frontal displays) between dominant and subordinate individuals in 20 min across treatment groups on day of blood and brain sampling. Letters denote significant differences ($P < 0.01$).
Figure 3.8. Differences in average overt aggression between dominant and subordinate individuals in 20 min across subgroups on day of blood and brain sampling. Letters denote significant differences (P ≤ 0.01), dashed line indicates that only within-sex statistical comparisons were performed.
Figure 3.9. Circulating levels of 11-KT in males by social group and dominance status. Concentrations are presented in picograms/mL. Open circles indicate statistical outliers.
Figure 3.10. Circulating levels of 11-KT in males by dominance status with treatment groups pooled. Concentrations are presented in picograms/mL. Open circles indicate statistical outliers.
Figure 3.11. Circulating levels of PGF$_{2\alpha}$ metabolite in females by treatment group and dominance status. Concentrations are presented in picograms/mL. Horizontal line segments denote the mean for each group.
Figure 3.12. Relative FSH in the brains of males and females. There was not a within-sex difference between dominant and subordinate individuals. $N = 8$. 
Figure 3.13. Relative FSH in the brains of males across subgroups. Asterisks denote significant differences (P < 0.04) between dominant and subordinate individuals. N = 4.
Figure 3.14. Relative FSH in the brains of females. There were no significant differences among the subgroups. N = 4.
Figure 3.15. Overall comparison of FSH in brain tissue between males and females. A greater concentration of FSH was found in females ($P < 0.01$).
DISCUSSION

The three social groups did not differ behaviorally, and the overall levels of behavior were similar to the previous study, indicating that behavioral profiles in these treatment groups are repeatable. Females in single-sex groups exhibited higher aggression than those in mixed-sex groups. Although a similar trend was seen in males, the pattern was less robust. In accord with these results, mixed-sex groups exhibited the smallest aggressive behavioral disparity between dominant and subordinate males and females. This was not because of elevated aggression of subordinates but because there were fewer behaviors performed by dominant individuals in mixed-sex groups. These results support the conclusion from Chapter II that single-sex environments do not reduce aggression.

Unexpectedly, males in mixed-sex groups aggressed toward males and females similarly. It is possible rapidly formed partner preferences of males in mixed-sex groups caused them to respond to females as intruders instead of potential mates. The higher intrasexual aggression in females observed in this study is consistent with other studies of female intrasexual competition in convicts (e.g. Arnott and Elwood 2009a; Cleveland-Roberts and Itzkowitz 2009). However, females may be less likely to exhibit equal aggression to males and females simply because of the size disparity between the sexes in the groups. Males would not face this obstacle because they were matched for size and experimental females were smaller, so it is possible that size alone could explain the lack of intrasexual aggressive preference in males but still allow for elevated intrasexual aggression among females.
Hormone assays were also performed to investigate possible physiological correlates of social experience. 11-Ketotestosterone levels were not different based on treatment group. These data were also extremely variable, making any patterns that may be present difficult to detect. There are several possible explanations for the variation and statistical similarity among groups, although it is not unusual for plasma 11-KT concentrations to be highly variable (de Avila pers. comm.). First, a primary difference in this study versus others that measure 11-KT levels was the timing of sampling. Other studies typically present males with a novel challenge and then measure steroid levels within 30 min after the challenge. In the present experiment, however, the aim was to determine if social context affected overall circulating levels of this steroid. Because 1-2 hrs elapsed during completion of recording behaviors in groups, assigning dominance status and obtaining blood samples, variation (especially among dominant individuals) could have resulted from differences in the types of interactions that may have occurred immediately prior to sampling.

Second, 11-KT may simply not play a primary role in or be indicative of physiological mechanisms involved in pair bond formation. It is possible that other hormones or even receptor distributions are more strongly affected by the types of social experiences in this experiment and that something among these might also act to facilitate pair formation. Nonapeptide systems are certainly important modulators of social behavior in other taxa, including monogamous, pair bonding species (see below). It is possible that 11-KT is thus relatively restricted in its behavioral roles, with primary relevance given to intrasexual aggressive interactions or paternal care but perhaps not direct involvement in pair formation. This idea is supported by the concurrent lack of
differences for behavioral and hormone data among groups and the significant correlation between 11-KT and chasing in all-male groups. Alternatively, it is the response itself and not circulating levels that is relevant for behavior in a reproductive or social context. Support for the hypothesis that 11-KT responsiveness is related to pair formation in this species (sensu Hirschenhauser et al. 2004) would require sampling individuals before and after a challenge in additional to providing a subsequent reproductive scenario.

Male mice reflexively release testosterone in response to females in a pre-mating context (Nyby 2008). Perhaps male convicts in mixed-sex groups exhibit a similar response. However, increased variation in circulating 11-KT occurred in dominant but not subordinate males in mixed-sex groups. In rodents, spontaneous androgen release is also caused by the pulsatile release of gonadotropins (Nyby 2008), and circulating levels of 11-KT in fishes might also vary because of a similar mechanism.

Lastly, another aspect of timing could have prevented detection of 11-KT correlations with social group or status in my experiment. If either too little or too much time had elapsed after placement into treatment groups, changes in hormone levels from the pre-treatment conditions would not be evident. Because social status and behaviors became stable before the third day, hormonal changes that potentially do occur might be transient and return to baseline as behavioral dynamics are established. Such a phenomenon would further indicate that baseline levels of 11-KT do not affect pair bonding in males.

Although a clear difference in resting 11-KT levels between dominant and subordinate males has been shown in an African cichlid species (Parikh et al. 2006), plasma 11-KT levels were not different based on social status in my study. This result
was surprising given the significant aggression disparity between dominant and subordinate males. The lack of difference between dominant and subordinate individuals could again be explained by excessive variation in concentrations possibly due to social challenges occurring just prior to sampling. The absence of significant 11-KT suppression in subordinates concomitant with the lack of relationship between dominance status and pair bond formation in the first experiment further supports the idea that the aggressiveness of dominant individuals (within an acceptable range) does not substantially negatively impact the reproductive potential of subordinate male convicts. However, aromatization of testosterone to estradiol is directly related to aggression in an African cichlid (Huffman et al. 2013), which may indicate that testosterone or estradiol but not 11-KT may be correlated with dominance status.

Relative levels of follicle-stimulating hormone in whole brain tissue were also measured as a potential correlate of social experience. This aspect of reproductive physiology has never been examined in convict cichlids, but studies in another cichlid species indicated that dominance and FSH levels in the pituitary are related (Alonso et al. 2011). One caveat to this component is that it is uncertain what the presence of gonadotropin in brain tissue means, as higher levels could indicate either increased storage of FSH in brain tissue without being released or increased synthesis and release into circulation (Santangelo pers. comm.). Prior studies comparing plasma and pituitary levels of gonadotropins in fishes have found that pituitary levels of FSH are positively correlated with circulating levels, although the same might not be true for LH (Swanson 1991; Oppen-Berntsen et al. 1994; Borg et al. 1998; Dickey and Swanson 1998; Maruska et al. 2010). While areas outside the pituitary also contain gonadotropic cells in fishes
(Parhar et al. 2003; Pandolfini et al. 2009), FSH is produced primarily in the pituitary, so results from whole brains likely do not differ significantly from studies using only the pituitary. Thus, increased FSH in the brain could reflect increased circulating levels, but this is uncertain in my experiment without measuring plasma FSH. While physiology does not always directly affect behavior or vice versa, a correlation between hormones and dominance would provide new evidence for a relationship between social status and reproduction in convict cichlids.

In males, LH and FSH regulate spermatogenesis and steroid synthesis via receptors in gonadal tissues, and LH activation of receptors in Leydig cells causes production of testosterone, from which 11-KT is derived (Nelson 2011; Dickey and Swanson 1998; Swanson et al 2003). Thus, growth and development of gonadal tissue in males caused by the release of gonadotropins from the pituitary ultimately results in increased plasma 11-KT (up to a putative negative feedback point from increased testosterone or estradiol; Dickey and Swanson 1998; Cavaco et al. 2001). Because 11-KT levels in this study were highly variable and not significantly associated with social group or status, it is expected that FSH might also be similar across categories of male social groupings, but this was only partially true.

Subordinate males in single-sex groups had higher relative FSH levels than dominant males, but the same trend in males in mixed-sex groups was not significant. The only notable behavioral difference among the subgroups was elevated aggression in single-sex male groups. One possible explanation for the difference in FSH levels would therefore be that increased exposure to aggression causes increased production of gonadotropins in subordinates, presumably through the stress axis. Elevated FSH could
be caused by a lack of negative feedback (or perhaps even positive feedback) in subordinate individuals via inhibition of androgen synthesis caused by increased circulating cortisol, although the 11-KT data do not support this conclusion. However, the influence of 11-KT on FSH production in the pituitary varies among species studied so far, as 11-KT has either a stimulatory or inhibitory role or no effect at all on FSH synthesis and release, depending on the species (Schulz and Miura 2002). Therefore, it is perhaps not unusual that 11-KT and FSH are not significantly correlated in this experiment, as feedback mechanisms might be driven by testosterone or estradiol and not 11-KT. What might cause disinhibition of FSH synthesis in subordinate males from only single-sex groups is unclear.

Another possible effect of cortisol on gonadotropin release is through action on the pituitary. In mammals, cortisol can decrease the sensitivity of the pituitary to signals from GnRH neurons that cause gonadotropin release (Oakley et al. 2009). Gonadotropin release from the pituitary would consequently be impaired, causing an accumulation of gonadotropins in the pituitary as FSH and LH continue to be synthesized. Social stress increases cortisol levels in other fishes (e.g. Ejike and Schreck 1980; Fox et al. 1997; Sloman et al. 2001), so it is likely that increased aggression experienced by subordinates in all-male groups results in increased circulating cortisol. Cortisol could therefore be acting at the gonadal or brain level to cause increased FSH in the brain, but whether it is due to synthesis or storage requires additional testing.

Alternatively, dominants individuals might have relatively less FSH because of negative feedback. If elevated FSH in subordinates reflects a baseline instead of an effect, then negative feedback caused by increased androgens (in conjunction with
increased aggression) among dominants may explain the trend. The significant
correlation between 11-KT and chases by dominant males from single-sex groups
supports this conclusion. It is therefore possible that the difference in FSH levels seen in
males is attributed to elevated androgens concomitant with higher aggression in dominant
fish in single-sex groups, with testosterone (and possibly estradiol) acting on the
hypothalamus to decrease FSH synthesis and release. If true, this supports a link between
dominance or aggression and reproduction, although previous behavioral tests do not
indicate that such a relationship impacts pair formation in a short-term scenario. The
possible relationship between 11-KT and pair bonding could be tested directly by
blocking the action of 11-KT with an androgen receptor antagonist, such as flutamide or
CPA, although preliminary data indicate flutamide treatment does not prevent pair
formation or spawning in males (Itzkowitz unpub. data).

Females were also sampled for FSH as well as the prostaglandin metabolite
13,14-dihydro-15-keto prostaglandin F\(_2\alpha\). In other female fishes, FSH is involved with
early stages of the ovulatory cycle (vitellogenesis), whereas LH seems to regulate final
maturation and ovulation of oocytes (Levavi-Sivan et al. 2010). LH stimulates the
ovaries to produce prostaglandins via maturation-inducing steroids (Munakata and
Kobayashi 2010). Prostaglandins are synthesized at multiple sites within the fish ovary,
and PGF is synthesized in the follicle walls of mature oocytes, although PGF\(_{2\alpha}\) production
can be induced in immature oocytes in vitro (Goetz 1991). PGF\(_{2\alpha}\) induces the ovaries to
contract (ovulation) and also facilitate spawning behavior in males and females via
pheromonal action in some species (Munakata and Kobayashi 2010). Thus, elevated
plasma levels of either FSH or PGF\(_{2\alpha}\) should provide some indication of what stage of the
ovulatory cycle is occurring. There were no differences in either FSH or PGF$_{2\alpha}$ metabolite among females, although for FSH there appears to be a trend across subgroups similar to that in males. In addition, plasma PGF$_{2\alpha}$ metabolite concentrations were not highly variable among individuals. These results could indicate that experimental females were all of similar reproductive state. Casual observations of internal anatomy upon sacrifice indicated that dominant and subordinate females often demonstrated similar gravidity and egg maturation, supporting this conclusion. It is therefore unlikely that different short-term social conditions significantly impact reproductive physiology, and instead females respond internally primarily to ecological cues unless conditions are extreme (malnutrition, stress, poor health, etc.).

If immediate reproductive capacity is generally similar across social contexts, then other mechanisms may modulate social bonding in this species or contribute to the variation seen in successful pair formation in captivity. Neuropeptides, for example, could act independently of the hormones tested here to facilitate pair formation, as behaviorally relevant vasopressin or vasotocin pathways are not always androgen dependent (Semsar and Godwin 2003). Furthermore, vasotocin and isotocin might be modulated by GnRH (Saito et al. 2003), and the increased presence of FSH in the brain in subordinates might reflect a compensatory response to subordination that facilitates pair formation via neuropeptide mechanisms. Vasopressin and oxytocin are well known modulators of social recognition (reviewed in Bielsky and Young 2004; Winslow and Insel 2004), pair bonding (Winslow et al. 1993; Williams et al. 1992, 1994; Insel and Hulihan 1995; Young et al. 1999; Pitkow et al. 2001; Lim et al. 2004; Lim and Young 2004; Donaldson and Young 2008), and parental care (Francis et al. 2002; reviewed in
In mammals and aggression and courtship (vasotocin: Goodson 1998a; Goodson 1998b; Goodson and Adkins-Regan 1999; see review by Goodson 2013) in birds. Although the majority of nonapeptide studies examine mammalian and avian taxa, there is growing evidence that these mechanisms serve similar functions in fishes. For example in fishes, vasotocin was linked to aggression and dominance status (Semsar et al. 2001; Lema and Nevitt 2004; Greenwood et al. 2008; Backstrom and Windberg 2009; Santangelo and Bass 2006; Almeida et al. 2012), courtship (Semsar et al. 2001; Salek et al. 2002; Grober et al. 2002; Carneiro et al. 2003) and parental care (Ripley and Foran 2010) (reviewed in Godwin and Thompson 2012). Although less studied, isotocin has also been implicated in fishes to modulate aggression (Kulczykowska et al. 2012) and parental care (O’Connell et al. 2012). The mechanisms that regulate pair formation could lie in peptide pathways, which may be complemented by other hormones involved in reproduction, such as sex steroids.

In summary, 11-KT and prostaglandin metabolite levels were not related to social experience or dominance status in my experiment. However, elevation of FSH in the brain coincided with subordination in single-sex groups. While the elevation occurred in both males and females, it was only statistically significant in males. Higher stress caused by being subordinate in single-sex groups could explain this pattern, as cortisol can act on the gonads to decrease steroid production (Consten et al. 2002). The decrease in steroids might cause increased FSH synthesis through reduction of negative feedback by steroids on gonadotropin synthesis or increased storage via cortisol-induced insensitivity to GnRH signals. Therefore, it remains unclear what might cause increased pair bond formation among individuals having mixed-sex social experience (Chapter II),
but evidence presented here suggests there might be a physiological link between dominance status and reproduction in convict cichlids. More direct tests would be more informative, but given the lack of groundwork in this area were not practical at these initial stages.
IV. The influence of visual and chemical cues on subsequent reproductive behavior

INTRODUCTION

When organisms interact socially, they perceive various sensory cues that could potentially influence their behavior or physiology. Additionally, different sensory components of a signal convey different types of information (Partan and Marler 1999; Taylor et al. 2011). Courtship involves multimodal signals that often have a strong visual component but may also include vocalizations, mechanosensory or chemosensory signals. A courtship performance lacking one or more of its components may be less effective or completely ineffective as a signal (sensu Page and Jaeger 2004). Also, if the signal receiver cannot interpret all of the modalities, the expected response may not occur (Page and Jaeger 2004; Campbell and Hauber 2009). Thus, having all modalities present in a particular behavioral suite may be required for effective communication.

Visual cues in particular are important for social dominance and reproduction across taxa. For example, “badges” or other markings correlated with variations in aggression or dominance are quite common among birds (Rohwer 1975; Rohwer 1982; Jarvi and Bakken 1984; Studd and Robertson 1985; Eckert and Weatherhead 1987; Whitfield 1987; Maynard Smith and Harper 1988; Jones 1990; Evans and Hatchwell 1992; Part and Qvarnstrom 1997; Pryke and Andersson 2003) but also occur in other taxa (Whiting et al. 2003; Tibbets and Dale 2004). In some of these studies, badges not only correlate with status but are actively assessed by conspecific rivals prior to conflict (Jarvi and Bakken 1984; Rohwer 1985; Jones 1990; Senar and Camerino 1998; Korzan et al. 2000; Tibbetts and Lindsay 2008; see also Earley et al. 2003). Similar to badges, other
morphological visual cues such as colorful patterns and skin darkening can also communicate information about both social status (Keenleyside and Yamamoto 1962; O’Connor et al. 1999; Hoglund et al. 2000; Grosenick et al. 2007; Chen and Fernald 2011) and reproduction (Pagel 1994; Watkins 1997; Torres and Velando 2005).

Teleosts are exceptionally diverse morphologically, often having striking colors and patterns along with behaviors that accentuate these traits. Teleosts use coloration to communicate in a variety of contexts (Kodric-Brown 1998), and even non-biological changes in visual environment can elicit changes in behavior and physiology in fish (aquarium background color; Hoglund et al. 2002). Skin darkening in teleosts can be induced by chronic activation of the hypothalamic-pituitary-interrenal axis, via alpha-melanocyte stimulating hormone and adrenocorticotropic hormone (Fuji and Oshima 1986). Changes in pigmentation in fishes may therefore provide information about social status or reproduction via the stress axis.

Convict cichlids (Amatitlania nigrofasciata) have a distinct barred pattern which quickly darkens in individuals of higher dominance status during aggressive encounters or when members of a pair repel intruders from a breeding site (pers. obs.). Subordinate or stressed convicts often display an overall dark coloration in which both bars and the spaces in between become darkened (pers. obs.). Differences in skin darkening might be related to acute versus chronic activation of the stress axis, and could therefore provide relatively specific information about the condition or current social status of an individual. Conspecifics, including potential mates, might be able to interpret these visual cues and use them in mate choice decisions in addition to behaviors or other signals.
In many taxa, olfaction is also exploited in regulating various aspects of social and reproductive behavior. Chemical cues are often used by males to evaluate female reproductive status or as sex pheromones that stimulate the reproductive axis in both sexes (reviewed in Rekwot et al. 2001; Stacey et al. 2003; Lemmen and Evenden 2009; Munakata and Kobayashi 2010; Enault et al. 2012). Prostaglandins acting as pheromones can activate pathways relevant for reproduction in males (Stacey 2003; Munakata and Kobayashi 2010) and induce male sex behavior (Sorensen et al. 1989; Stacey at al. 1989; Kitamura et al. 1994; Ogata et al. 1994). PGF$_{2\alpha}$ is rapidly metabolized in vivo in other species, so its metabolite may be more active than the parent compound as a pheromone, although both affect male behavior in goldfish (Munakata and Kobayashi 2010). Pheromonal action of PGF$_{2\alpha}$ on males also occurs in other fishes (*Misgurnus anguillicausatus*, Kitamura et al. 1994; Ogata et al. 1994; *Salmo salar*, Waring and Moore 1995; Moore and Waring 1996; *Salvelinus alpinus*, Sveinsson and Hara 2000; *Salmo trutta*, Moore et al. 2002). Pheromonal mechanisms in fishes are bidirectional, as compounds released from males also affect female sex behavior (*Danio rerio*, Chen and Martinich 1975; *Clarias gariepinus*, Resink et al. 1989; *Petromyzon marinus*, Weiming at al. 2002). While these examples indicate that chemical signaling in certain contexts can have profound effects on reproductive behavior and physiology in non-monogamous fishes, the importance of chemosensory mechanisms in regulating aspects of social monogamy (such as pair bonding) are unknown in fishes. Given the significance of olfactory cues in other teleosts, the reproductive relevance of this sensory modality is possibly conserved across mating systems. Water-borne pheromones that increase
readiness to pair would be particularly advantageous in seasonally breeding, pair bonding species, allowing them to maximize limited time for reproduction.

Chemical communication can also signal dominance in fishes, although this form of communication is less well understood. Steroids and aromatic compounds are excreted in urine, and some species urinate more frequently in the presence of competitors or potential mates (Oreochromis mossambicus, Almeida et al. 2005; Barata et al. 2007; Barata et al. 2008; Pimephales promelas, Martinovic-Weigelt et al. 2012; Astatotilapia burtoni, Maruska and Fernald 2012). Female goldfish (Carassius auratus) selectively urinate to indicate readiness to spawn (Appelt and Sorensen 2007). Such patterns (if not purely reflexive) suggest that urine signaling may actively communicate social or reproductive status in fishes.

Prior experiments (Chapter II) indicate pair formation in convict cichlids is facilitated by exposure to members of the opposite sex and that this facilitation is not primarily driven by behavioral interactions. Visual or chemosensory cues might be sufficient to induce such an effect, and these two modalities are well suited for experimental testing. How multimodal signals impact reproductive decisions, especially pair bonding, is not well understood, as the most conspicuous signal naturally receives the most attention from researchers. In convict cichlids, behavior may not be a prominent component of pair bond formation if other cues are more important. Fortunately, the role of visual and chemosensory cues can be explored relatively easily in this species.

Experience in mixed-sex groups facilitates pair formation in convict cichlids, and it is possible that visual and chemosensory cues underlie this effect. To test this idea, I manipulated individuals’ exposure to olfactory and chemical cues to reflect what is
experienced by individuals in intact single- or mixed-sex groups. The hypothesis that visual cues from the opposite sex are sufficient to induce reproductive priming will be supported if fish receiving visual signals typical of mixed-sex groups form pair bonds more frequently than fish having visual contact with only members of their own sex. The hypothesis that water-borne chemical cues from the opposite sex are sufficient to induce reproductive priming will be supported if fish receiving water containing chemical cues produced by the opposite sex form pair bonds more frequently than fish receiving water from only members of their own sex.
METHODS

Part 1: Visual Cues

All fish used had been in single-sex stock aquaria for at least 3 weeks, and I presumed this was sufficient isolation for the comparison of single- and mixed-sex treatments. Single-sex or mixed-sex groups were created by placing two 75.7 L aquaria side by side lengthwise, so that fish in each tank could see fish in the neighboring tank (Figure 4.1). Three tagged fish were placed in each aquarium to simulate a total group size of 6, which yielded a treatment effect in previous experiments. Groups were video recorded 24 hrs after setup and again 48 hrs after setup. Because dominance status did not impact subsequent pairing behavior in prior experiments, the individual exhibiting intermediate aggression in the group of 3 fish was removed and given a novel partner, so that each replicate yielded two forced pairings. The protocol for matching experimental fish with novel individuals was identical to the methods in “Part II: Forced pairings” in the first experiment (Chapter II). Forced pairs were again tested for pair bond formation 48 hrs after setup in the same manner as described in Chapter II. Group and pair behaviors were recorded from 20 min. of video after at least 3 min. acclimation to cameras.

Part II: Water-Borne Cues

Groups were set up similar to the methods for visual cues experiment, so that three fish of one sex were placed in a single 75.7 L aquarium. Three refugium boxes (Finnex External Hang On Box with Shin Mao Aqua Pump; flow rate approx 40 gallons per hour) were hung on the sides of the main tank and covered with cardboard to visually isolate fish in the refugia from the main tank (Figure 4.2). Water was pumped from the
main tank into each refugium, which then flowed back into the main tank. For single-sex groups, one fish of the same sex as individuals in the main tank was placed in each of the refugium boxes. For mixed sex groups, there were two possible arrangements. For “mixed-sex males,” three males were placed in the main aquarium and one female was placed in each of the three refugia, so that the three males were exposed to any water-borne cues from three females. For “mixed-sex females,” 3 females were placed in the main aquarium and 1 male was in each of the three refugia. Groups were video recorded for 1 hr 48 hrs after initial setup. For this experiment, fish were not tagged, and all individuals in the main tank were placed in new 75.7 L aquaria and given a novel individual with which to pair. Thus, each replicate generated three forced pairings. Pair bond testing was performed 48 hrs after forced pairing as described in Chapter II. Group and pair behaviors were recorded from 20 min. of video after at least 3 min. acclimation to cameras.
Figure 4.1. Experimental setup for testing the effect of visual cues from the opposite sex on subsequent pair bond formation. To create a “mixed-sex group,” males and females will be housed side-by-side, as shown above. For single-sex groups, females will be in both tanks or males will be in both tanks.
Figure 4.2. Experimental setup for testing chemical cues. Single-sex groups were created by placing same-sex individuals in the 3 refugia as the main tank. Mixed-sex groups consisted of either 3 males placed in the main tank with females in refugia or vice versa. Only 1 of the 3 refugia is illustrated in the mixed-sex group above.
RESULTS

Visual Cues Experiment

Small sample sizes and significant departures from normality required non-parametric analyses. Kruskal-Wallis one-way analysis of variance was used for comparing behaviors across groups with Mann-Whitney U for post-hoc tests. Because tanks were side-by-side in this design, each pair of tanks was counted as one replicate (two groups of three fish) when comparing behavior across the three different social group types. When testing social group effects within a sex, each group of three was considered an independent replicate to control for density effects when comparing single-sex versus mixed-sex groups. All statistics were conducted using SPSS statistical software (SPSS Statistics for Window, Version 17.0).

Group Behavioral Characteristics

The only treatment group behavioral difference was for lateral displays ($X^2(2) = 6.69, P = 0.04$; Figure 4.3). All-female groups and mixed-sex groups were different ($U(17) = 81, Z = -2.56, P = 0.01$), with more lateral displays occurring in groups that were part of the mixed-sex treatment (sexes combined). When single-sex group females were compared to mixed-sex group females, there was again a difference in lateral displays ($U(17) = 111.5, Z = 1.99, P = 0.045$), with females in mixed-sex visual groups performing more lateral displays. There were no differences in behaviors when males in mixed-sex visual groups were compared with males from single-sex visual groups, indicating that females in mixed-sex groups are driving the difference among groups.
Pair Bonding

Individuals having intermediate dominance were removed from each group of three and given a novel putative partner. Almost all the forced pairings across the treatment groups resulted in an obvious pair bond within 48 hrs. There were no significant differences between percents of pair bonds formed by single-sex and mixed-sex individuals of the same sex when compared using Fisher’s exact test (Table 4.1).

Water-borne Cues Experiment

Group Behavioral Characteristics

There were 5 replicates in each of 4 treatments: Males exposed to male water (single-sex males), females exposed to female water (single-sex females), males exposed to female water (mixed-sex males), and females exposed to male water (mixed-sex females). There were no significant differences among treatment groups for any behavior (Figure 4.4).

Pair Bonding

All individuals from groups were placed with a novel putative partner to assess pair bonding (60 forced pairings). Most of the forced pairings resulted in an obvious pair bond within 48 hrs of setup (Table 4.2). However, males in groups exposed to water from other males formed pair bonds less frequently than males exposed to water from females (Fisher’s exact test p=0.019). There was no difference between the two female treatments.
Figure 4.3. Behaviors across treatment groups in visual cues experiment. All-female groups interacted with and saw only females. All male groups interacted with and saw only males. Mixed sex groups consisted of females and males that had intrasexual interactions but visual contact only with the other sex. Difference in displays indicated by asterisk (P = 0.01). N = 10. Open circles are statistical outliers.
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<td>0</td>
<td>19</td>
<td>100</td>
</tr>
<tr>
<td>MS Females</td>
<td>20</td>
<td>3</td>
<td>17</td>
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<tr>
<td>MS Males</td>
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<td>89.5</td>
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</tbody>
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**Table 4.1.** Percent of forced pairings in visual cues experiment that resulted in pair bonds among females from single-sex groups (SS Females), females from mixed-sex groups (MS Females), males from single-sex groups (SS Males), and males from mixed-sex groups (MS Males).
Figure 4.4. Behaviors in water-borne cues experiment across treatment groups: males exposed to male water (M-M), males exposed to female water (M-F), females exposed to female water (F-F) and females exposed to male water (M-F). Chases in M-M groups appears elevated but is not significant when compared across groups. Open circles are statistical outliers.
## Pair Bond Formation

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<tbody>
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<td>3</td>
<td>12</td>
<td>80</td>
</tr>
<tr>
<td>MS Females</td>
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<td>14</td>
<td>93.3</td>
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<tr>
<td>MS Males</td>
<td>17</td>
<td>6</td>
<td>11</td>
<td>64.7</td>
</tr>
</tbody>
</table>

**Table 4.2.** Percent of forced pairings in water-borne cues experiment that resulted in pair bonds among females from single-sex groups (SS Females), females from mixed-sex groups (MS Females), males from single-sex groups (SS Males), and males from mixed-sex groups (MS Males).
DISCUSSION

Individuals were exposed to visual or chemical cues from fish of the same or opposite sex to examine possible effects on pair formation. The three types of visual experiences did not differentially affect subsequent behaviors, with the exception of more display behavior occurring when males and females had visual contact. Interestingly, males and females in same-sex visual groups formed pair bonds just as frequently as males and females having visual contact with the opposite sex. The overall high success rate in pair bonding was unexpected and difficult to interpret. A primary difference in the design of the visual and chemical cues experiments was the smaller group and tank size. Some might argue that keeping fish in closer contact could decrease stress (Itzkowitz pers. comm.), but my observations indicate that this was not the case in this study as several replicates had to be terminated due to extreme aggression in these small groups. The area provided by a smaller aquarium may therefore be more stressful, as escape from aggression is more limited.

Another design element of the visual cues experiment that differed from previous experiments was the selection of aggressive intermediates for the pairing component. Although prior dominance did not affect pair bond formation in my experiments, the added stress from the smaller tanks might have revealed more variation in pair formation among dominants and subordinates compared to intermediates. Individual differences in responsiveness to a stressor occurs in other fish species (Pottinger and Carrick 2001; Hirschenhauser et al. 2004), and by selecting intermediates, it is possible such differences were not observable.
Contrary to the well-established negative effect of stress on reproduction, increased stress can sometimes stimulate the reproductive axis. Cortisol synthesis increases as part of the stress response, and cortisol can suppress androgen synthesis (Consten et al. 2002). Gonadotropin synthesis could then increase because negative feedback by androgens has been removed (but see Zohar et al. 2010 for exceptions). In line with this reasoning, both acute and chronic stress suppressed both testosterone and 11-KT in brown trout, and plasma gonadotropins were elevated (Salmo trutta; Pickering et al. 1987). Convicts in these smaller-scale groups possibly experienced increased stress from intense aggression which caused increased gonadotropin synthesis and release, and the similarity in response across the groups in this experiment might be due to the selection of dominance intermediates. This idea is also supported by previous experiments in which subordinate individuals (who were likely experiencing an elevated stress response) exhibited higher levels of follicle-stimulating hormone in whole brain tissue (Chapter III).

Another possibility that explains the similar pairing success among groups is that a specific prior history is required for observing subsequent short-term effects of being in a single- versus mixed-sex social environment. Although the fish used may have previously bred at some point, the individuals used in Chapters II and III had been isolated in single-sex groups for at least several months. The individuals used in the experiments in this chapter may not have been isolated in same-sex stock aquaria for a comparable amount of time. Lastly, it is possible that experimental individuals might have responded to other visual cues in the laboratory, as tanks were not completely
visually isolated from the room. Proper husbandry did not allow for complete isolation of tanks, especially since the risk of injury to fish is heightened in these smaller groups.

Among fish exposed to water-borne cues, males but not females showed a subsequent difference in pair bond formation. Males exposed to putative female chemosensory cues formed pair bonds significantly less often when given a novel female than males exposed to male chemical cues. Although the overall group differences were not significant, males in same-sex chemosensory groups exhibited noticeably higher aggression than males in mixed-sex groups. All the males from same-sex groups also formed pair bonds with novel females. Following the reasoning presented for the visual cues experiment, the higher levels of aggression experienced by males in same-sex groups might have produced a more intense stress response that caused them to pair more frequently via stimulatory effects on the reproductive axis. The fact that females exposed to either male or female water-borne cues did not differ in pair formation may indicate similarity of reproductive state or simply be in agreement with the lack of behavioral differences that would indicate the potential for this type of stress response in one group but not the other.

Alternatively, males may respond either positively to male water-born cues or negatively to female cues in subsequent pair formation, although why this would be true is unclear in light of previous experiments. It is also possible that the decoupling of visual and olfactory cues had an unexpected effect, and that exposure to olfactory cues without the proper visual input somehow affects male reproductive behavior.

In summary, it remains unclear if either visual or chemical cues alone play a significant role in reproductive stimulation, as pair formation was generally high
regardless of visual or chemical exposure. These results in conjunction with prior experiments evaluating gonadotropin in the brain suggest that a short-term physiological response to aggression could positively influence subsequent reproductive behavior of males but not females. Evaluating responses of individuals to a mixed-sex social experience likely depends on sufficient prior long-term isolation in single-sex environments.
V. General Discussion

The experiments presented in this dissertation were performed to evaluate mechanisms involved in pair formation and spawning in the monogamous convict cichlid. My results are not consistent with the traditionally supported view that courtship *sensu strictu* is important for mutual mate choice and pair bonding in this species. On the contrary, laboratory studies performed in semi-natural conditions indicate pair bonding in convict cichlids may be driven by female choice and that dominant female convicts choose dominant males as their spawning partner (Weber and Weber 1976; pers. obs.). Such a conclusion is supported by the results presented here, as courtship behaviors were extremely infrequent and did not appear to influence pair bonding. I therefore hypothesize that females in mixed-sex groups who are relatively close to spawning will seek and choose a male partner based on his social status. The theoretical importance of female reproductive readiness for pair bond formation is supported experimentally by studies of male preferences for female gravidity over female size (Nuttall and Keenleyside 1993).

While I did not find support for dominance affecting pair bond formation, my experimental design may have masked such effects. Allowing fish to remain in groups through pair bond formation could reveal a relationship between social status on pair bonding as indicated by the Weber and Weber study (1976). Because subordinates were not reproductively suppressed in my study, I hypothesize that social status is a substrate for assortative mating in convicts and not a means of reproductive suppression as in other fishes. Furthermore, the hypothesis that males and females choose mates based on relative aggression as opposed to courtship behaviors is supported by my observations.
that excessive aggression by either sex prohibits pair bond formation as does excessive “timidity.” Differences in brain gonadotropin levels between dominant and subordinate fish in my study also supports a link between aggression and reproduction.

If we assume pair bond formation is driven only by female physiological state, then prior experiences of males are not likely to influence pair formation, but that was not the case in my study. In the experiment presented in Chapter II, mixed-sex social experience affected subsequent reproductive behavior in both male and female convict cichlids. Individuals exposed to members of the opposite sex formed pair bonds with novel fish more frequently than fish from single-sex groups. Additionally, the mechanism for this effect was not behavioral, as groups were behaviorally similar and individuals in pairs showed no behavioral differences according to the social experience or dominance status of the experimental fish. These results suggest that some sort of priming occurs in females and in males, a phenomenon which occurs in other teleosts (Stacey 2003; Munakata and Kobayashi 2010).

The mixed-sex group effect occurred in the absence of partner choice and with all novel individuals having only single-sex experience. This suggests that the effects of group social experience on individuals carry over into a reproductive context and might also influence novel partners, presumably though non-behavioral cues. Perhaps there is some communication of motivation that permits pair bonding to occur even though spawning is not imminent, as spawning was infrequent in general and did not occur more frequently among females having mixed-sex group experiences in this experiment. I expect that individuals from different mixed-sex groups that are force-paired would form pair bonds with each other at a still higher frequency, but this remains to be tested.
In Chapter III, I presented results for experiments evaluating potential hormonal correlates of single- and mixed-sex social environments. I was unable to detect changes in baseline 11-KT levels in males caused by the different social environments. 11-KT concentrations were extremely variable, although variation was largest in dominant males of mixed-sex groups. In male mice, exposure to females in a pre-mating context causes reflexive release of testosterone. It is possible that dominant convict cichlids also experience a reflexive release of testosterone, subsequently causing more variation in 11-KT in mixed-sex groups. It is unclear why this would not also cause increased variation in subordinate males exposed to females, however. Females did not exhibit differences in circulating levels of the prostaglandin metabolite 13,14-dihydro-15-keto PGF2α. Females in this experiment might have been of similar reproductive states, or PGF2α is simply not a sensitive measure of reproductive state or other factors influenced by social experience in this species.

Lastly, dominance status within single-sex groups was related to brain gonadotropin levels in males. The same trend was seen for females but was not statistically significant. Aggression was elevated in male groups, suggesting a potential interaction between single-sex aggressive environments and subordination that affects gonadotropin synthesis or release. This result was surprising given social status did not affect pair formation in my first experiment (Chapter II), although timing might play a substantial role; long-term differences in behavior may allow physiological effects to take hold in a way not discernable in short-term studies. Overall, my experiments exploring the link between physiology, behavior, and social experience in convict cichlids were inconclusive. A critical limitation of this experiment was the inability to pair individuals
after their group experience. While group data appeared repeatable, it is impossible to
know if the same mixed-sex group effect on pair bonding would have been seen if
individuals in the second experiment were allowed to pair.

Experiments in Chapter IV tested the roles of prior visual and water-borne cues in
subsequent pair formation. The visual experience of being in a mixed-sex group was
created by allowing males and females in small groups to interact freely with members of
their sex but to have unrestricted visual contact with members of the other sex.
Unexpectedly, almost all the fish removed from groups and given a novel putative partner
formed pair bonds, so there was no group effect on subsequent pair formation. It is
unclear why individuals in small single-sex groups would pair so readily given prior
results to the contrary (Chapter II). I suggest two of several possibilities to explain this
result. First, increased stress across groups caused by increased aggression in the smaller
aquaria used in latter experiments could stimulate the reproductive axis instead of
inhibiting it. This unexpected effect occurs in brown trout (Pickering et al. 1987).
Alternatively, increased stress could suppress the reproductive axis, possibly through
increased storage of gonadotropins in the pituitary and brain or through decreased
negative feedback by androgens.

In the chemical cues experiment, males exposed to water-borne cues of other
males demonstrated elevated aggression in groups and the highest frequency of pair bond
formation (100%) when given a novel female. The same stress-induced gonadotropic
effect proposed above could also explain this result; however several assumptions would
have to be met. Increased stress would have to cause increased gonadotropin synthesis
and release, and in turn reproductive behavior (pair bonding) would have to be affected
by increased gonadotropins in circulation. Interestingly, the other three groups exhibited
similar, lower aggression in groups and no statistical difference in pair bond formation,
which again suggests there is a link between aggression (and/or stress) and pair bond
formation. Elevated aggression among single-sex males but not mixed-sex males could
have been caused by water-borne cues, but the mechanism is unclear. Although I did not
find evidence for visual or pheromonal priming occurring in mixed-sex groups, the
potential link between aggression, stress and reproduction suggested by results from
experiments in Chapters III and IV open the door for more direct tests of these
mechanisms using monogamous convict cichlids.

In a natural setting, mate choice and pair bond formation in convict cichlids may
not occur according to our typical understanding of courtship, monogamy, and pair
formation established in other taxa. Pair bonding and mate choice in this species has
been a source of consternation for researchers perhaps because ideas developed using
other species are not appropriate. For example, it is possible that differences in the time
it takes to form pair bonds do not depend upon prolonged mate assessment (Leese 2012)
but instead simply reflect how close the female is to spawning relative to other females.
This would also explain why male convicts interact with other potential mates as opposed
to attacking them, in spite of having a prior affiliation with a female (e.g. Trifenbach and
Itzkowitz 1998; pers. obs.). Furthermore, given the behavior of females in mixed-sex
groups and in forced pairings, females seem to “control” pair bond formation. While
males do exhibit preferences when given an experimental choice, this does not
necessarily mean that male preferences would be important in a natural environment,
especially since the primary impetus of male preference seems to be female gravidity (Nuttall and Keenleyside 1993).

In an experimental setting, failure to form pair bonds can be caused by either female or male rejection of the partner. Thus in laboratory settings, convicts exhibit mutual mate choice. However, mate rejection by females as well as males might be based primarily on female reproductive state, thus eliminating the need to search for complex functions or mechanisms of assessment rooted in courtship behaviors. While convict cichlids are an excellent model for parental care in both the lab and field, they do not seem to be ideal for analyses of monogamous courtship dynamics unrelated to offspring defense. Courtship appears to serve functions other than mate assessment, such as signaling motivation (i.e. Wisenden et al. 2008; Santangelo 2005). Courtship might also be used as a means of persuasion or compensation when at a disadvantage. For example, courtship rates of rejected males increase when females form a preference for another male (Santangelo and Itzkowitz 2004; Santangelo 2005). Because females do not change their preference in response to increased courtship by a non-preferred male, it is unlikely that courtship serves a primary role in mate selection or pair bonding. Furthermore, courtship rates by preferred males were relatively low (Santangelo 2005). I hypothesize that courtship behaviors are a redundant signal to potential mates that an individual is in the correct motivational and physiological state for reproduction, and aggressive behavior or social status is instead the primary substrate for mate assessment in convict cichlids.

The ambiguity of the role of courtship in pair bonding does not preclude the importance of convict cichlids as a model for physiological mechanisms of pair bonding.
and monogamy, however, as they clearly form pair bonds and exhibit the hallmarks of selective aggression and partner preference after bonding. It is unclear if mechanisms involved in pair bonding in other taxa are generalizable to convicts, but convict cichlids seem to be an excellent teleost model for testing this idea. Work in this area is in its infancy, but could provide a novel, alternative perspective on the evolution of monogamy. Overall, these experiments indicate a need for research examining reproductive physiology and its relationship to behavior in convict cichlids and other monogamous fishes.


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Figure A1. Within-pair behavior immediately after forced pairing of experimental and novel fish in Aim 1 (Chapter II). Single-sex female lateral displays to novel males as well as bites and lateral displays by novel males to experimental females are shown. Females are grouped by their dominance status. Other behaviors were extremely infrequent and not included.
Figure A2. Lateral displays performed by single-sex females to novel males in a 20 min. period immediately after forced pairing (Chapter II). Females are grouped according to whether a subsequent pair bond formed within 48 hrs. Other behaviors were extremely infrequent and not included.
Figure A3. Behaviors of novel males toward experimental single-sex females in a 20 min. period immediately after forced pairing (Chapter II). Females are grouped according to whether a subsequent pair bond formed within 48 hrs.
Figure A4. Within-pair behavior in a 20 min period immediately after forced pairing of experimental and novel fish in Aim 1 (Chapter II). Single-sex male bites and lateral displays to novel females as well as lateral displays by novel females to the experimental males are shown. Males are grouped by their dominance status. Other behaviors were extremely infrequent and not included.
Figure A5. Chases and lateral displays performed by single-sex males to novel females after forced pairing (Chapter II). Males are grouped according to whether a subsequent pair bond formed within 48 hrs. Other behaviors were extremely infrequent and not included.
Figure A6. Lateral displays by novel females toward experimental single-sex males in a 20 min. period immediately after forced pairing (Chapter II). Males are grouped according to whether a subsequent pair bond formed within 48 hrs. Other behaviors were extremely infrequent and not included.
Figure A7. Lateral displays by mixed-sex males and females toward novel fish in a 20 min. period immediately after forced pairing (Chapter II). Experimental individuals are grouped according to sex and dominant status. Other behaviors were extremely infrequent and not included.
Figure A8. Lateral displays by novel males and females toward mixed-sex experimental fish in a 20 min. period immediately after forced pairing (Chapter II). Responses of novel fish are grouped according to the sex and dominant status of the experimental fish. Other behaviors were extremely infrequent and not included.
Figure A9. Differences in behavior between the two social conditions within females (Chapter III). Mixed-sex groups (MSG) versus all-female groups (AFG). Average ± SE frequency of behaviors within a group performed by females only in 20 min. Asterisks indicate significant differences (P < 0.01). Intra- and intersexual behaviors are included for mixed-sex group females. N = 10.
Figure A10. Differences in behavior between the two social conditions within males (Chapter III). Mixed-sex groups (MSG) versus all-male groups (AMG). Average ± SE frequency of behaviors within a group performed by males only in 20 min. Asterisks indicate significant differences (P ≤ 0.01). Intra- and intersexual behaviors are included for mixed-sex group males. N = 10.
Figure A11. Total combined bites and chases performed by dominant and subordinate males and females in 20 min. in both treatment types represented in Western blot FSH analysis on the day of sacrifice (Chapter IV).
Vita

EDUCATION

Auburn University, May 2006
Bachelor of Science, Zoology

Eastern Kentucky University, May 2010
Master of Science, Biology

Lehigh University, May 2014
Doctor of Philosophy, Integrative Biology

RESEARCH EXPERIENCE OUTSIDE DISSERTATION

• Reproductive Behavior of *Cyprinodon bovinus*, July 2009, July 2010
  - P. I. Murray Itzkowitz, Lehigh University
  - Funded by the Texas Nature Conservancy
  Population monitoring of the endangered Leon Springs pupfish. Recorded reproductive behavior of males and females and specific mating tactics of female pupfish after habitat restoration efforts. Additionally, gathered data on their interactions with another endangered sympatric species, *Gambusia affinis*, a predator of eggs and fry of the pupfish.

• Interspecific Aggression of Caribbean Damselfish Species, June 2009
  - P.I. Murray Itzkowitz, Lehigh University
  - Collaborator: Matthew Draud, Long Island University, C. W. Post Campus
  Presented two highly similar damselfish species with conspecific and congeneric damselfish or unrelated egg predators and recorded their aggressive responses to these intruders. Aggression was context-dependent, with conspecifics receiving the most aggression but only when presented simultaneously with another intruder. Research performed at Bellairs Research Institute in Barbados.

• Hybridization of Caribbean Damselfish Species, June 2009
  - P.I. Murray Itzkowitz, Lehigh University
  Collected fin clips from longfin (*Stegastes diencaeus*) and dusky (*S. adustus*) damselfish from several locations in Barbados. These were later combined with fin clips from fish in Jamaica for analysis of the effect of habitat degradation on hybridization. DNA extractions performed and sequences analyzed under the direction of Dr. Sean Mullen.

• Provisioning Behavior of Indigo Buntings (*Passerina cyanea*), January – June 2008
  - P.I. Gary Ritchison, Eastern Kentucky University
  Indigo Bunting nests were videotaped in 1996, and video analyses were performed and completed in 2008 to investigate the role of males in nestling parental care in this species.

  - P.I. Gary Ritchison, Eastern Kentucky University
  Collected blood samples from adult and nestling Eastern Phoebes from nests on the Blue Grass Army Depot in Richmond, KY. Microsatellite paternity analysis
performed using the lab facilities of David Westneat with the supervision of Ian
Stuart at the University of Kentucky.

**ACADEMIC WORK EXPERIENCE**

- **Thorne Graduate Research Fellow**, September 2012 – January 2013
- **Graduate Assistant to the Chair**, August 2011- August 2012, June 2013-May 2014
  Department of Biological Sciences, P.I. Murray Itzkowitz, Lehigh University
  Duties include animal husbandry for fresh- and saltwater fishes as well as
  management of graduate and undergraduate lab personnel
- **Graduate Teaching Assistant**, August 2008 – May 2011, January 2013
  Department of Biological Sciences, Lehigh University. Lab instructor for
  Genetics, Comparative and Integrative Biology, Animal Behavior and
  Experimental Neuroscience
- **Graduate Teaching Assistant**, August 2006 – May 2008
  Department of Biology, Eastern Kentucky University. Lab instructor for
  Principles of Biology for biology majors, General Zoology
  Fort Benning Restoration Assemblage. Diatom processing and analysis of
genera, chlorophyll fluorescence of algae, basic identification of freshwater
macroinvertebrates and data entry for stream ecology graduate students of Dr.
Jack Feminella.
- **Lab and Field Technician**, May 2004 - January 2006
  Department of Biological Sciences, Auburn University. Diatom processing and
analysis of genera, chlorophyll fluorescence of algae, wood identification,
 ergosterol condensation, basic identification of freshwater macroinvertebrates
and data entry for stream ecology graduate students of Dr. Jack Feminella.

**PUBLICATIONS**

**Little, K.P.,** Draud, M.J., and Itzkowitz, M.  Interspecific aggression in two highly similar
Mullen, S.P, **Little, K.**, Draud, M., Brozek, J., and Itzkowitz, M.  2012. Hybridization among
Caribbean damselfish species correlates with habitat degradation. *Journal of
Leiser, J.K., **Little, K.P.,** and Itzkowitz, M.  2011. Mate sampling in a natural population of
and Itzkowitz, M.  2012. Management of interactions between endangered species

**AWARDS AND FUNDING**

Lehigh University Gordon C. Thorne graduate fellowship, September 2012.
ACA Guy Jordan Research Fund Award, July 2012.
Lehigh University College of Arts and Sciences Research Grant, October 2011.
Lehigh University College of Arts and Sciences Summer Fellowship, June 2011.
Lehigh University Forum Grant, November 2009.