

1-2-2013

Stress And Social Relationships: The Role Of Corticosterone In The Formation And Maintenance Of Pair Bonds In The Monogamous Zebra Finch (*taeniopygia Guttata*)

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**STRESS AND SOCIAL RELATIONSHIPS: THE ROLE OF CORTICOSTERONE IN
THE FORMATION AND MAINTENANCE OF PAIR BONDS IN THE MONOGAMOUS
ZEBRA FINCH (*Taeniopygia guttata*)**

by

KIMBERLY A. LAPLANTE

DISSERTATION

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

2013

MAJOR: PSYCHOLOGY (Behavioral and
Cognitive Neuroscience)

Approved by:

Advisor

Date

DEDICATION

This work is dedicated my parents, Mario and Jean LaPlante, for their love, encouragement and unwavering support. To my sister, Erin Brule, who always seems to have the answer. Finally, to Seth Arbogast, my partner in love and life. I can never express how much you mean to me.

ACKNOWLEDGMENTS

I would like to acknowledge those who have helped make this research possible. First, I would like to acknowledge my advisor on this work, Dr. Michelle Tomaszycski for her support, advice, and technical training. I would like to thank Drs. George Borszcz, Thomas Fischer, Shane Perrine, and Richard Slatcher for serving on my dissertation committee and providing advice whenever asked. I truly could not have done this without the technical and emotional support of fellow graduate students, Ana Daugherty, Erin Lowrey Ondercin, and Bayley Thompson. Finally, these studies could not have been completed without the dedicated work of undergraduate assistants, Bill Lacey, Enida Huremovic, Kelly Quednau, Brendon Zatirka, and Alyssa Pedersen.

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CHAPTER 1 - BACKGROUND LITERATURE

Stress and social relationships are strongly connected. Negative social relationships can be a source of stress in people's lives (Cranford, 2004; Fiore, Becker & Coppel, 1983), but positive relationships can also buffer against external sources of stress (Cohen & Wills, 1985; Kikusui, Winslow & Mori, 2006). It is important to understand the interaction between stress and social relationships, since both factors profoundly impact physical and mental health. Chronic high levels of stress are associated with a weakened immune response (reviewed in Chrousos, 1995; Segerstrom & Miller, 2004) that lead to slower wound healing (Ebrecht *et al.*, 2004), a greater propensity to become sick (Cohen *et al.*, 1998), and increased severity of illness (Cohen, Doyle & Skoner, 1999; Sephton & Spiegel, 2003). Additionally, chronic high-level stress is associated with a greater incidence of cardiovascular disease (Black & Garbutt, 2002), gastrointestinal ailments (Mayer, 2000) and affective disorders such as depression (Tafet & Bernardini, 2003) and anxiety (McEwen, 2006).

Social relationships can function to buffer against the effects of a stressor, thereby reducing the physiological and behavioral stress response. Social buffering is apparent in a range of species including humans, non-human primates, ungulates, canines, rodents, and birds (reviewed in Hennessy, Kaiser & Sachser, 2009). The social organization of a species seems to influence the types of social relationships that effectively buffer against the effect of a stressor on an organism (Hennessy *et al.*, 2009). For example, the presence of a bonded pair-mate reduces the elevated stress response to novelty in both sexes of the monogamous titi monkey (*Callicebus cupreus*), but presence of a familiar cagemate does not in the closely related, promiscuous, squirrel monkey (*Saimiri sciureus*) (Hennessy, Mendoza, Mason & Moberg, 1995). In monogamous, pair-bonded species, the presence of pair-mates tends to provide a greater social

buffering effect than the presence of an unfamiliar conspecific (reviewed in Hennessy *et al.*, 2009). This is likely because monogamous pair-bonds, such as married relationships, tend to involve more frequent, intimate and emotionally intense interactions compared to other types of relationships (Cutrona, 1996).

While it has been well established that pair-bonded relationships provide a buffer the stress response (Hennessy *et al.*, 1995; 2009), the converse (effects of stress on pair relationships) remains poorly understood. Thus, the present studies experimentally tested 1) the effect of acute CORT treatments on social affiliation preferences, 2) the effect of chronic corticosterone (CORT) administration on affiliative behavior and neural expression of corticotropin releasing hormone (CRH) mRNA and glucocorticoid receptor (GR) mRNA, and 3) the effect of pair formation and maintenance on circulating CORT and on CRH and GR mRNA.

I examined these effects using zebra finches (*Taeniopygia guttata*). Zebra finches are highly social, monogamous, form long term pair-bonds, and are selective in their choice of mates (Zann, 1996). The zebra finch has been used extensively to study the mechanisms of social and affiliative behavior in the laboratory (for reviews see: Adkins-Regan, 2009; 2011; Adkins-Regan & Tomaszycki, 2007; Goodson & Kingsbury, 2011). Previous work examining the effect of stress on pair-bond formation, however, has focused on the prairie vole (*Microtus ochrogaster*), a monogamous rodent. These studies focus exclusively on partner preference, the initial step in pair-bond formation, and are typically conducted using a two-choice paradigm. The zebra finch model allows for long-term pair-bonds to be examined in a naturalistic, colony setting. Prairie voles, like most rodents, but unlike humans, rely heavily on olfaction for mate selection and recognition. Similar to humans, zebra finches rely more heavily on visual and vocal communication. Finally, prairie voles have aberrant adrenal responses, characterized by

glucocorticoid hypersecretion up to 10 orders of magnitude higher than other rodents (Carter *et al.*, 1995). Monogamy is rare in the rodent family—less than 3% of rodents form monogamous pair-bonds (Kleiman, 1977). Some have suggested that the characteristically high adrenal activation in the prairie vole, particularly during development, uniquely primes the brain for formation of a monogamous pair-bond (Carter *et al.*, 1995; DeVries, DeVries, Taymans & Carter, 1996; Lim, Nair & Young, 2005). The abnormal adrenal response seen in prairie voles may limit the generalizability of stress and social affiliation findings across taxa.

Stress

The goal of the present studies was to test the relationship between isolated components of the stress response (circulating total CORT, CRH and GR mRNA) and social affiliative behaviors. In vertebrate animals, the hypothalamic-pituitary adrenal (HPA) axis mediates the physiological response to stressors. Following a stressor, direct inputs from the brain stem and indirect inputs from the basal forebrain and limbic system activate the paraventricular nucleus of the hypothalamus (PVN) to release CRH (Ziegler & Herman, 2002). CRH binds to receptors in the anterior pituitary to trigger the release of adrenocorticotrophic hormone (ACTH) into the bloodstream (Sapolsky, 1992). ACTH acts on the adrenal cortex to release glucocorticoids (such as CORT), steroid hormones acting systemically in concert with catecholamines, particularly epinephrine (E) and norepinephrine (NE), to produce the stress-response (Sapolsky, 1992).

Glucocorticoids facilitate the stress response. This response evolved as an adaptive reaction to short-term environmental challenges that have required increased energy availability, increased cardiovascular tone and alteration of immune function to suppress inflammation and analgesia (Sapolsky, 1992). To accommodate these energy-expensive processes, the body slows down systems that are not essential for responding to the stressor such as digestion, reproduction,

and normal growth (Sapolsky, 1992). Evolutionarily, the stress response has proven to be a beneficial reaction to short-term stressors such as fleeing a predator, chasing prey or physically responding to a threat. However, exposure to chronic stressors has a detrimental effect on physiological function and overall health (reviewed in McEwen, 2006; Sapolsky, 1992; Sephton & Spiegel; 2003; Segerstrom & Miller, 2004).

Inhibition of the HPA axis is accomplished by both glucocorticoid-mediated and glucocorticoid-independent pathways (Ziegler & Herman, 2002). Within the glucocorticoid-mediated pathway, dedicated receptors are expressed in the PVN and in a number of HPA-regulatory regions including the amygdala and NE/E subgroups of the brainstem (Ziegler & Herman, 2002). There are two types of glucocorticoid receptors; the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR). These receptors are both located in the PVN, across other regions of the hypothalamus and the limbic system; but these receptor subtypes bind glucocorticoids with different affinities and serve different functions to regulate the HPA response (de Kloet, 1991; 2000). MR has a high affinity for CORT and is often bound during basal CORT secretion. Activity at MR is implicated in the onset of the stress response. GR, as a complement, binds CORT with low affinity and is activated only by large amounts of CORT. Activation of GR provides the negative feedback necessary to terminate the stress response and facilitate recovery from stress. Both chronic and early life stress reduce expression of both GR and MR in the PVN (Banerjee, Arterbery, Fergus, & Adkins-Regan, 2012; Herman, Adams & Prewitt, 1995; Zhe, Fang, & Yuxiu, 2008). Chronic CORT treatment has also been shown to sensitize the stress response and up regulate HPA activity (Makino, Smith & Gold, 1995; Ulrich-Lai & Herman, 2009).

Reproduction

Stress suppresses reproduction in both males and females (Sapolsky, Romero & Munck, 2000). In addition to activating the HPA axis, stressors also trigger a reaction in the hypothalamic pituitary gonadal (HPG) axis (reviewed by Wingfield & Sapolsky, 2003), which inhibits the release of gonadotropin releasing hormone (GnRH) from the hypothalamus. This suppresses the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the anterior pituitary which then suppresses the release of sex steroids, such as estrogen and testosterone, from the gonads (reviewed by Wingfield & Sapolsky, 2003). Thus, there is an inverse relationship between circulating levels of glucocorticoids and sex steroids. In females, activation of the stress response inhibits reproduction on three levels; disruption of ovulation, reducing the likelihood that a fertilized egg will implant and affecting behavior by reducing behaviors that indicate willingness to copulate, thereby reducing copulations (Rivier & Vale, 1984). In males, stressors inhibit testosterone release, impair the ability to achieve and maintain an erection, and disrupt libido (Sapolsky *et al.*, 2000).

Behaviorally, central injections of CRH decrease lordosis, the female mating posture in rats (Sirinathsingji, Rees, Rivier & Vale, 1983; Sirinathsingji, 1986) and suppress copulation solicitation displays in the white-crowned sparrow (*Zonotrichia leucophrys*) (Maney & Wingfield, 1998). However, CRH does not block sexual behaviors in all species. A study in the female musk shrew (*Suncus murinus*) showed that intracerebroventricular (i.c.v.) injections of CRH actually increased sexual behaviors that include rump presenting and tail wagging (Schiml & Rissman, 2000).

Pair-Bonding

Short-term fasting (four to ten hours) increases CORT and decreases testosterone in zebra finches (Lynn, Stampelis, Barrington, Weida & Hudak, 2010). Behaviorally, fasted males engaged in fewer courtship behaviors including directed singing, approach, beak wiping and clumping (physical contact), as compared to control males (Lynn *et al.*, 2010). Females were not studied. It remains unclear whether the suppression of courtship displays in males was driven by the increased CORT, decreased testosterone, some other unknown variable altered by fasting or a combination of factors. A previous study in zebra finches found that blocking sex steroid actions (with flutamide, an androgen receptor antagonist, and an aromatase inhibitor) did not affect the likelihood to pair or frequency of pair bonding behaviors such as clumping and nest box cohabitation in males or females (Tomaszycki, Banerjee, & Adkins-Regan, 2006). Blocking sex steroids in females did increase same-sex pairing, which could reflect an increased motivation to pair. Males treated with an aromatase inhibitor and an androgen receptor antagonist did not differ from controls in amount of directed singing, but were less likely to approach females compared to control males (Tomaszycki *et al.*, 2006). This suggests that CORT, and not sex steroids, may be driving the effect of short-term fasting to suppress courtship in males. This hypothesis was directly tested in the present studies.

The monogamous prairie vole has been used extensively to examine the biological underpinnings of the formation of pair-bonds (DeVries, 2002; Young & Wang, 2004). CORT plays a crucial and sexually dimorphic role in the formation of partner preference –the initial step in forming a pair-bond in prairie voles (DeVries, DeVries, Taymans & Carter, 1995; 1996). In sexually naïve females, baseline CORT is reduced upon introduction to an unfamiliar male (DeVries *et al.*, 1995). The suppression of baseline CORT is a necessary component of pair-bond formation: a peripheral CORT injection upon introduction to a male hinders the formation

of a partner preference (DeVries *et al.*, 1995). Complete suppression of CORT, by removal of the adrenal glands, accelerates the formation of partner preferences in females (DeVries *et al.*, 1995). However, the opposite effect has been demonstrated in male prairie voles, in which CORT facilitates partner preference formation (DeVries *et al.*, 1996). Under normal laboratory conditions, six hours of cohabitation is sufficient for female prairie voles to form partner preferences, but this is an insufficient amount of time for males. Yet, when stress is induced, either by a forced-swim paradigm or injection of CORT, directly preceding the six hour period of cohabitation, males develop partner preferences while females do not (DeVries *et al.*, 1996).

Geoffroy's tufted-ear marmosets (*Callithrix geoffroyi*) form a pair-bonded relationship with the opposite sex individual with which they are housed. Marmosets co-housed following a period of isolation or co-housed directly from their natal group both had higher levels of CORT during the first 10 days of pairing with their new social partner compared to baseline (Smith, Birnie & French, 2011). However, only the isolated males and females maintained elevated CORT after this initial pair formation period. Isolated animals of both sexes also displayed a greater amount of social contact when paired. In females, urinary CORT was highly correlated with social-seeking, approach behaviors (Smith *et al.*, 2011). The findings of this study are limited in that isolation may affect a number of hormonal systems, not only the HPA axis and, as such, changes in behavior may not be directly attributable to changes in CORT. The correlation between changes in CORT and changes in behavior is not sufficient to infer causation in either direction. Also, all animals were equally likely to form a pair-bond. Furthermore, there was no attempt to test of bond fidelity. While this study does not specifically examine the effect of CORT on pair-bond formation, it does provide evidence that elevated CORT may increase social affiliation.

The relationship between HPA activity and pair formation may be bi-directional. CORT affects the propensity to form a partner preference; the first step in pair-bond formation (DeVries *et al.*, 1995; 1996; DeVries, Guptaa, Cardillo, Cho & Carter, 2002; Lim *et al.*, 2007). Conversely, the formation of a pair-bond alters HPA activity. Mere exposure to a member of the opposite sex increases CORT. In human males, exposure to a female rapidly increases salivary CORT (Roney, Lukaszewski & Simmons, 2007), but CORT is not affected if the female is appraised as unattractive (van der Meij, Buunk & Salvador, 2010). Female humans have elevated CORT after watching a video of an attractive male courting a female (Lopez, Hay & Conklin, 2009). The formation of a pair-bond is associated with higher levels of CORT across taxa (black-legged kittiwake (*Rissatridactyla*): Angelier, Moe, Clement-Chastel, Bech & Chastel, 2007; Geoffroy's tufted-ear marmosets: Smith *et al.*, 2011; humans: Marazziti & Canale, 2004).

There is a dearth of study exploring the effect of stress on pair-bond formation. The effect of CORT on partner preference has been studied only in prairie voles, a model with methodological limitations due to the unnatural paradigms for assessing partner preference, their primary reliance on olfactory cues for mate selection and recognition, and their atypical adrenocortical response. To investigate the generalizability of these findings to zebra finches, I directly tested the effect of acute CORT administration on social preference in a two-choice paradigm in Experiment 1. Here, I used two doses to assess the effect of CORT on preference for opposite sex birds using two different alternate choices: a single same sex bird and a group of same sex birds.

The effect of CORT on pair bond formation has only been explored using acute stress models. To understand the effect of chronic stress on pairing, I directly manipulated CORT by

subcutaneously implanting a 21-day release pellet and observed pairing behaviors in a colony setting in Experiment 2A. Presumably, the effect of stress on alterations of pair bonding behavior occurs due to changes at the neural level. Thus, in Experiment 2B I examined neural markers of stress to assure that my peripheral administration of CORT had an effect at the neural level, altering expression of CRH and GR mRNA.

There is evidence across taxa that an increase in circulating CORT is associated with pair bond formation (Angelier *et al.*, 2007; Smith *et al.*, 2011; Marazziti & Canale, 2004), but this has not been examined at the neural level. Here, I investigated the effect of pairing on the stress response at both a peripheral (Experiment 3A) and neural level (Experiment 3B). I tested the effect of pairing on total blood CORT in untreated, paired birds compared to unpaired birds and birds maintained in same-sex conditions. I also tested the effect of pair-bond formation (short- and long-term) on neural expression of CRH and GR mRNA.

Taken together, these studies provide evidence to support a relationship between stress physiology and social behavior in the zebra finch, as shown in Figure 1. This improved understanding of the biological underpinnings of pair-bond formation facilitates the study of social relationships in humans and pathological interruptions to the formation and maintenance of social relationships, such as those that occur in autistic spectrum disorders.

CHAPTER 2 – EFFECTS OF ACUTE CORTICOSTERONE ON PREFERENCE FOR SOCIAL AFFILIATION

There is evidence the physiological response to stress may alter motivation to form a pair-bond with a member of the opposite sex. For example, acute stress increases attraction for females in male humans (Dutton & Aron, 1974). In prairie voles, the effects of CORT on partner preferences are sexually dimorphic. Acute peripheral administration of CORT increases partner preference in males, but inhibits it in females (DeVries *et al.*, 1995; 1996). The effect of centrally administered CRH on partner preference in males is dose-dependent (DeVries, *et al.*, 2002). Moderate doses of CRH (0.1, 1 ng) facilitated the formation of partner preference, but extreme doses (10, 100 ng) did not (DeVries *et al.*, 2002). Injections of a CRH receptor antagonist (CRH Receptor 1 or CRH Receptor 2) administered along with CRH, blocked the effects of CRH on partner preferences in males (Lim *et al.*, 2007). Thus, activities at either CRH receptor support social bonding in males.

In zebra finches, short-term fasting (4 to 10 hours) increases CORT and decreases testosterone in zebra finches (Lynn *et al.*, 2010). Behaviorally, fasted males engaged in fewer courtship behaviors than control males, including directed singing, approach, beak wiping and clumping (physical contact) (Lynn *et al.*, 2010). It remains unclear whether the suppression of courtship displays in males was driven by the increased CORT, decreased testosterone, some other unknown variable altered by fasting, or a combination of factors. A previous study in zebra finches found that blocking sex steroid actions (with flutamide, an androgen receptor antagonist, and an aromatase inhibitor) did not affect the likelihood to pair or frequency of pair bonding behaviors in males (Tomaszycki *et al.*, 2006). This suggests that CORT may be driving

the effect of short-term fasting that suppresses courtship in males. This hypothesis was directly tested in the present experiment.

Females are commonly overlooked in the study of pair-bond formation. In prairie voles, CORT suppresses the formation of partner preference. Blocking sex steroids in female zebra finches increases same-sex pairing, which could reflect an increased motivation to pair (Tomaszycki *et al.*, 2006). To date, there are no studies in birds examining the effect of CORT on motivation to pair.

The two-choice paradigm has been used extensively with prairie voles to determine partner preference (DeVries, 2002; Young & Wang, 2004) and has been used in birds to assess both mate and social group preferences (Adkins-Regan & Leung, 2006; Goodson & Thompson, 2010; Kelly *et al.*, 2011). Untreated adult birds reared in a bi-parental environment show a preference for an opposite-sex bird over a same-sex bird (reviewed in Adkins-Regan, 2002). Furthermore, zebra finches are a highly social species (Zann, 1996) and when given the choice between 2 or 10 same-sex conspecifics, untreated zebra finches prefer the larger group (Goodson & Thompson, 2010; Kelly *et al.*, 2011). The present study used two separate paradigms to determine social preference; one in which birds are given the choice between a single opposite sex or single same sex bird. In a second paradigm, I assessed preference between two highly appealing options – an opposite sex-bird and a group of same-sex birds. The use of this novel testing paradigm allows for detection of an increase in pairing motivation that may not be revealed by using only the standard two-choice test between a single-male and a single-female, due to a ceiling effect from the high expected baseline opposite-sex preference.

Based on the previous findings that stress decreased pairing behaviors in zebra finches of both sexes, males and females treated with CORT are expected to spend less time in proximity to

opposite-sex birds as compared to vehicle-treated controls, due to a decreased interest in pairing. An alternative hypothesis, in line with the findings in prairie voles, is that acute CORT treatment will have a sexually dimorphic effect to increase pairing motivation in males and decrease pairing motivation in females.

Methods

Subjects

Socially reared adult male ($n = 8$) and female ($n = 8$) zebra finches were maintained on a 12:12 hour light/dark cycle (lights on 6:00) in a temperature and humidity controlled room under conditions ideal for mating (24°C, 50% humidity). Seed and water were provided *ad libitum* and their diet was supplemented with hard-boiled chicken egg and calcium-enriched grain (Simple System Breeder Crumb 5-Day Product, The Bird Care Company) twice per week. Prior to the experiment, subjects were housed in same-sex aviaries (152.4 × 76.2 × 198.1 cm) to prevent pair formation. Throughout the 4-week testing period, subjects were housed in same sex groups ($n = 8$) per cage (78.7 × 55.9 × 88.9 cm).

Testing Apparatus

During testing, subjects were singly housed in an observation cage (41.9 × 26.7 × 33.0 cm). The observation cage contained one perch running along the center of the cage. The perch had 2 pieces of tape wrapped around it at 11 cm from each end to mark zones proximal to each of the stimulus cages. Stimulus cages of the same size were placed to the right and left of the observation cages and contained food, water and 2 perches (See Figure 2 for a representation of the apparatus).

In the Single/Single condition, one stimulus cage contained 1 same-sex bird and the other stimulus cage contained 1 opposite-sex bird. In the Single/Group condition, one stimulus cage

contained 4 same-sex birds and the other contained 1 opposite-sex bird. Birds were exposed to both conditions for all doses. Condition order (Single/Single versus Single/Group) and stimulus sides (right vs. left) were randomized across doses for each bird.

Injections & Observations

Birds were injected intra-muscularly with either vehicle (peanut oil) or CORT (low dose 10 µg; high dose 20 µg) in a 50 µl volume. The high dose produces safe, supraphysiological levels of circulating CORT within 15 minutes of treatment (Gam, Mendonc, & Navara, 2011). All animals received both doses and the control. For the first test, all subjects received the vehicle injection to obtain baseline data on social preference and familiarize the animals with the testing procedure and apparatus. Dose order was randomized for the next 3 tests and doses were separated by 7 treatment-free days. All animals received the vehicle dose twice to allow for analysis between baseline and vehicle doses administered after CORT treatments.

Immediately following injection, animals were released into the first observation cage and allowed 15 minutes for the treatment to become effective and to habituate to the testing cage. During this time dividers were in place to prevent visual contact with the stimulus cages. Then, the dividers were removed and the amount of time the bird spent nearest to, and facing, each stimulus cage was recorded for 10 minutes. Duration of male singing behavior directed at the female stimulus bird was also recorded. After the 10 minute observation period, the test bird was moved into the observation cage for the second condition and allowed 15 minutes for habituation which was followed by a second 10 minute observation period.

Statistical Analysis

The duration of time that the subject spent nearest to the cage containing a single animal of the opposite sex was converted into a percentage of the total time spent next to either cage.

Baseline vehicle dose was compared to the second vehicle dose in a one-way repeated measure Analysis of Variance (ANOVA). To account for variance due to treatment day, only the second repeated vehicle dose was used in the analyses. To examine the effect of treatment on proximity to the opposite sex, separate one-way repeated measure ANOVAs were conducted on Treatment (2 doses and vehicle) for each sex in each condition. To test an interaction between Treatment and Sex, a 3 (Treatment) x 2 (Sex) mixed factorial ANOVA was conducted on the percentage of time spent in proximity to the opposite sex bird in each condition. To test the effect of CORT on singing behavior in males, two separate one-way repeated measure ANOVAs (Treatment; 2 doses and vehicle) were conducted: one for each condition.

Results

There was no significant difference in the time spent in proximity to opposite-sex animals between the 2 control administrations when males were treated with vehicle (Figure 3A, Single/Single: $F(1, 7) = 0.55, p = 0.48$; Single/Group: $F(1, 7) = 0.18, p = 0.68$). Similar results were obtained with females that were treated with vehicle (Figure 3B, Single/Single: $F(1, 7) = 0.89, p = 0.38$; Single/Group: $F(1, 7) = 0.56, p = 0.48$). This suggests that there was no carry-over effect from previous tests due to CORT administration or repeated exposure to the testing paradigm. At baseline, opposite sex preference was higher in the Single/Single condition compared to the Single/Group condition for females ($t(7) = 5.59, p < 0.01$), but this was not true for males ($t(7) = 1.30, p = 0.24$). In the Single/Single condition, females showed a stronger opposite-sex preference compared to males ($F(1, 14) = 8.74, p = 0.01$), but this sex difference was not apparent in the Single/Group condition ($F(1, 14) = 0.04, p = 0.84$, Figure 4).

In males, a repeated measure ANOVA revealed a significant effect of Treatment on the percentage of time spent in proximity to the female relative to the social group in the

Single/Group condition ($F(2, 14) = 5.46, p = 0.02$), but not relative to the same-sex individual in the Single/Single condition ($F(2, 14) = 2.17, p = 0.15$, Figure 5A). Post hoc paired-samples t -tests indicated that, when males were treated with 10 μg of CORT, they spent a greater percentage of time in proximity to the female than to the social group, compared to when they were treated with 20 μg of CORT ($t(7) = 3.13, p = 0.02$). When males were treated with the vehicle, their proximity to the female relative to the social group did not differ from either treatment group (10 μg : $t(7) = -1.92, p = 0.10$; 20 μg : $t(7) = 1.23, p = 0.26$).

As shown in Figure 5B, CORT treatments did not alter female preferences for males in either the Single/Single condition ($F(2, 14) = 0.35, p = 0.71$) or the Single/Group condition ($F(2, 14) = 0.93, p = 0.42$). A 3 (Treatment) \times 2 (Sex) mixed factorial ANOVA revealed no main effect of Treatment or interaction between Treatment and Sex for the Single/Single condition (Treatment: $F(2,28) = 0.30, p = 0.75$; Interaction: $F(2,28) = 1.41, p = 0.26$) or the Single/Group condition (Treatment: $F(2,28) = 3.08, p = 0.06$; Interaction: $F(2,28) = 1.12, p = 0.34$).

Male singing did not differ by Treatment in either the Single/Single condition ($F(2, 14) = 0.50, p = 0.62$) or the Single/Group condition ($F(2, 14) = 1.28, p = 0.31$, see Figure 6). The rate at which females were sung to by stimulus males also did not differ by Treatment in either condition (Single/Single: $F(2, 14) = 0.15, p = 0.86$; Single/Group: $F(2, 14) = 0.54, p = 0.60$).

Discussion

It was expected that acute CORT treatment would decrease opposite-sex preference and singing in males in a simple 2-choice paradigm between a single male and a single female, since fasting stress decreases courtship and pairing behaviors in zebra finches (Lynn *et al.*, 2010). However, there was no effect of acute CORT treatment on opposite-sex preference in the Single/Single paradigm or on singing in males. The previously shown effect of fasting-stress on

pairing behaviors was apparent after a prolonged stressor (4- and 10-hours of fasting) and was shown using a semi-naturalistic environment. Thus, methodological differences may account for differences in results. Females are understudied, but the present study suggests that acute CORT treatment does not alter preference for males in either condition studied here.

When I examined the effects of CORT on the choice of an opposite-sex bird vs. a social group, there was an effect of treatment on opposite-sex preference in males. Post hoc analysis revealed no difference between vehicle and acute CORT treatment, but showed that the low dose of CORT increased opposite-sex preference compared to the high dose of CORT (Figure 5A). This result is in agreement with findings in male prairie voles that effects are dose dependent (DeVries *et al.*, 2002).

Since females are understudied, sex differences in pairing are seldom discussed. Interestingly, there was a sex difference at baseline –opposite-sex preference was higher in females compared to males (Figure 4). Opposite-sex preference in untreated females is typically very high, but is reduced by altering the rearing environment or levels of sex-steroids during development (Adkins-Regan, 2002). Previous literature has shown that untreated males develop a preference for females over males or family members around day 90 (early adulthood) and initially spent approximately 80% of their time with females in a choice paradigm similar to that used here (Adkins-Regan & Leung, 2006). However, opposite-sex preference drops off to 40-50% in later adulthood in males (Adkins-Regan, 1999; Adkins-Regan & Krakauer, 2000). This is consistent with the sex difference shown here. One possible explanation for this effect is that same-sex housing immediately prior to the experiment, in contrast to the mixed-sex social groups that occur naturally, may alter opposite-sex preference (Adkins-Regan, 2002; Mansukhani, Adkins-Regan & Yang, 1996).

There is evidence in zebra finches that females prefer birds bred for low-stress reactivity over those bred for high-stress reactivity (Roberts, Buchanan, Bennett & Evans, 2007). However, this effect of CORT activity to influence mate preference is not consistent across taxa. For example, HPA reactivity in males does not reliably predict female preference in guinea pigs (Bauer, Dittami & Machatschke, 2008). This is the first study to show that there was no difference in the rate to which male stimulus birds sang to females based on treatment, suggesting that acute CORT treatment does not alter attractiveness to the opposite sex. Rate of directed male song in this situation, however, is a coarse operational definition of attractiveness, primarily because the stimulus males were not exposed to other females at the time and thus had limited choice. To better understand the effect of CORT on female attractiveness, future studies should employ a three-choice paradigm and allow males to select between high, low and control CORT females (as was tested in males: Roberts *et al.*, 2007).

Nonetheless, the present study suggests that the effects of CORT on partner preferences do not directly generalize across species. This study used novel, unfamiliar stimulus animals in the choice cages. While this allowed for testing the effect of CORT on preference based on factors of interest (sex and group size), it does not directly generalize to findings in prairie voles which used a familiar animal to test partner preference. Additionally, the two-choice paradigm used here allowed for a high level of experimental control, but it lacked the face validity of a semi-naturalistic colony environment. Finally, this study only examined the effect of acute CORT on opposite-sex preference and the effect of chronic high levels of CORT on pairing is still unclear. The effect of chronic CORT administration on pairing will be explored in the following chapter.

CHAPTER 3 – EFFECTS OF CHRONIC CORTICOSTERONE ON PAIR-BONDING

While it has been well established that pair-bonded relationships provide a buffer against the stress response (Hennessy *et al.*, 1995; 2009), the converse (effects of stress on pair relationships) remains poorly understood. Acute stress has been shown to increase attraction for members of the opposite sex and to facilitate pair-bonding in male humans (Dutton & Aron, 1974) and male prairie voles (DeVries *et al.*, 1996), a frequently used rodent model of monogamy. Inconsistent with this evidence, male zebra finches subjected to short-term fasting (4 to 10 hrs) have increased circulating CORT and decreased courtship behaviors, including directed singing, approach, beak wiping and clumping (physical contact) (Lynn *et al.*, 2010). However, the short-term fasting stress paradigm also suppresses free testosterone (Lynn *et al.*, 2010), and thus it is uncertain what direct effects CORT has on pair-bond formation that are independent of additional biological factors. Furthermore, the effect of CORT on pair-bond formation may be sexually differentiated. Although females are commonly overlooked across taxa, one study in prairie voles showed that elevated CORT suppresses the formation of partner preferences in females (DeVries *et al.*, 1995).

The limited extant literature describing the effect of CORT on pairing is further restricted by a focus on acute CORT administration and/or acute stress paradigms. Studies in humans suggest that the effect of acute stress on interpersonal relationships may be similarly acute and thereby have little influence on long-term outcomes (reviewed in Randall & Bodenmann, 2009). However, chronic stress, which is an increasingly natural phenomenon in humans, can affect relationship quality over extended periods of time (reviewed in Randall & Bodenmann, 2009). Indeed, prolonged stress has been shown to increase rates of divorce (Cohan & Cole, 2002). There is a wealth of information on interpersonal dynamics, attachment styles and coping

mechanisms that mediate the relationship between stress and the quality of pair-bonded relationships (see Randall & Bodenmann, 2009; Story & Bradbury, 2004 for reviews), but relatively little is known about the biological mechanisms that underlie this association. In other words, a stressor (either external or internal) may disrupt interpersonal relationships, but it is unknown whether this occurs through the traditional biological stress response as measured by sustained elevations in CORT.

Indeed, the accepted biological model of stress is complex, including multiple brain regions and neurotransmitter systems. In vertebrate animals, the HPA axis mediates the physiological response to stressors. Following a stressor, direct inputs from the brain stem and indirect inputs from the basal forebrain and limbic system activate the PVN to release CRH, which then binds to receptors in the anterior pituitary to trigger the release of ACTH into the bloodstream (Ziegler & Herman, 2002). ACTH acts on the adrenal cortex to release glucocorticoids (such as CORT), steroid hormones acting systemically in concert with catecholamines, particularly epinephrine (E) and norepinephrine (NE), to produce the stress-response (Sapolsky, 1992). Of these candidate neural chemical substrates, CORT expression is considered a main impetus in the onset and sustained stress response (glucocorticoid-mediated pathway) and is therefore a popular focus in experimental study.

The glucocorticoid-mediated pathway is also responsible for inhibition of the HPA axis, although there are secondary inhibitory glucocorticoid-independent pathways (Ziegler & Herman, 2002). Within the glucocorticoid-mediated pathway, dedicated receptors are expressed in the PVN and in a number of HPA-regulatory regions including the amygdala and NE/E subgroups of the brainstem (Ziegler & Herman, 2002). There are two types of glucocorticoid receptors; GR and MR. Both of these receptors are located in the PVN, across other regions of the hypothalamus,

and the limbic system; but these receptor subtypes bind glucocorticoids with different affinities and serve different functions to regulate the HPA response (de Kloet, 1991; 2000). MR has a high affinity for CORT and is often bound during basal CORT secretion. Activity at MR is implicated in the onset of the stress response. GR, as a compliment, binds CORT with low affinity and is activated only by large amounts of CORT. Activation of GR provides the negative feedback necessary to terminate the stress response and facilitates recovery from stress. Both chronic and early life stress reduce expression of both GR and MR in the PVN (Banerjee *et al.*, 2012; Herman *et al.*, 1995; Zhe *et al.*, 2008). Chronic CORT treatment has also been shown to sensitize the stress response and up-regulate HPA activity (Makino *et al.*, 1995; Ulrich-Lai & Herman, 2009).

Whereas the biological stress response is well understood, the neurochemical substrates of stress that specifically affect relationships are not, despite the abundant evidence of a critical role of stress in relationships. The present study was designed to address this dearth in the literature by inducing a state of increased CORT and then examining pair-bond formation and maintenance in a semi-naturalistic colony environment.

In addition to administering chronic CORT treatments, the unique effects of CORT on CRH and GR mRNA were measured in the brain. This thorough examination allowed for an inclusive experimental model of the relationship between circulating blood CORT, neural expression of glucocorticoids, and the behavioral outcome of pairing. Based on previous findings in male zebra finches (Lynn *et al.*, 2010), animals treated with CORT were expected to have a decreased propensity to pair as compared to controls. An alternative hypothesis, in line with the findings in prairie voles (DeVries *et al.*, 1995; 1996), is that acute CORT treatment would have a sexually dimorphic effect—increased pairing in males and decreased in pairing in

females. In addition, I expected that the chronic CORT treatment would decrease GR mRNA expression (Herman *et al.*, 1995) and increase CRH in the PVN, both of which are believed to underlie the common sensitization of the stress response under chronic conditions (i.e., up-regulation of HPA activity; Makino *et al.*, 1995; Ulrich-Lai & Herman, 2009).

Methods

Subjects

Socially reared adult male ($n = 20$) and female ($n = 16$) zebra finches (*Taeniopygia guttata*) were maintained on a 12:12 hour light/dark cycle (lights on 6:00) in a temperature and humidity controlled room under conditions ideal for mating (24°C, 50% humidity). Seed and water were provided *ad libitum* and their diet was supplemented with hard-boiled chicken egg and calcium-enriched grain (Simple System Breeder Crumb 5-Day Product, The Bird Care Company) twice per week. Prior to the experiment, subjects were housed in same-sex aviaries (152.4 × 76.2 × 198.1 cm) to prevent pair formation. During the experiment subjects (4 of each sex) were housed in observation cages (91.4 × 76.2 × 76.2 cm). Each cage contained water and food dishes, a grit box, perches and four empty nest boxes. Nest material was provided as needed.

Male ($n = 3$) and female ($n = 3$) birds were maintained in same-sex observation cages with equal numbers of animals as the experimental birds to control for physiological effects due to exposure to opposite-sex animals.

Implants

Birds were implanted subcutaneously in the flank with either a 0.1 mg corticosterone (CORT) or a sham pellet. All birds in the same-sex condition were implanted with a CORT pellet. Pellets (1.5 mm diameter) are commercially available biodegradable 21-day continuous

time-release pellets (Innovative Research of America, Sarasota, Florida). This dose has been shown to increase plasma CORT to 186% of baseline levels in similarly sized mountain chickadees, *Poecile gambeli* (Pravosudov, 2003). Acute stress in mountain chickadees has been shown to increase plasma CORT to 600% above baseline (Pravosudov, Mendoza & Clayton, 2003), so the artificially induced elevation of CORT in this study was anticipated to be modest.

Behavioral Observations

Methods are outlined in Figure 7. Specifically, on Day 1, animals were implanted with either a CORT (male, $n = 13$; female, $n = 11$) or sham (male, $n = 10$; female, $n = 8$) pellet. For the same-sex control group, CORT-implanted males ($n = 3$) and females ($n = 3$) were housed in same sex aviaries in groups of 8. For the mixed-sex group, birds and housed in an observation cage in a group of 4 same-sex birds (2 CORT-implanted and 2 sham-implanted). On day 2, novel, opposite-sex stimulus birds were introduced to birds in the mixed sex condition (4 per cage for an equal sex ratio, male, $n = 16$; female, $n = 20$ overall). Birds in the same sex condition remained in the same housing and their behaviors were not recorded. For those in the mixed-sex condition, pairing, courtship and aggressive behavior were recorded for 1 hour following introduction. See Table 1 for a list of behaviors that were scored. Animals remained in the mixed-sex cage throughout the experiment and were further observed for 1 hour on Days 3, 4, 5, 9, 16, 23 and 30. All observations were conducted between 9.00-11.00 hr. On Day 31, implanted animals were sacrificed by rapid decapitation, brains were removed and frozen in cold methyl-butane and stored at -80° C until processing. Trunk blood was collected at this time. Also, on Day 31 eggs were collected from the observation cage nest boxes and checked for viability by holding them to a bright light and looking for evidence of an embryo. Ownership of

eggs was determined as the pair that spent more than 50% of their nesting time in that box during the last two periods of observation.

Blood Collection

To test the extent to which circulating plasma CORT levels were elevated in the implanted birds, blood was taken from 2 CORT and 2 sham birds on Days 2, 4, 10, 16, and 23. Blood was taken from same-sex control birds only at Time 1 and Time 2, since subject numbers were low. Birds were randomly ordered for blood collection and blood was taken from each bird only once. Two capillary tubes of blood was collected from each animal by puncturing the brachial vein with a 26-gauge needle within 3 minutes of opening the cage between 10.00 -12.00 hr. Blood was emptied into 1.5 ml vials and kept on ice. All samples were centrifuged within 1 hour of blood collection and plasma samples were frozen at -80° C until assay. Total plasma CORT levels were estimated using a Corticosterone Enzyme Immunoassay Kit (Assay Designs, #901-09, Enzo Life Sciences, Ann Arbor, MI) that has been used previously in zebra finches (Wada *et al.*, 2008).

Histology

Brains were sectioned coronally (20 μ m) on a cryostat (Leica CM3050S, Buffalo Grove, IL) and mounted onto SuperFront Plus slides (Fisher Scientific, Hampton, NH). Six series of sections representing the whole brain were collected and stored at -80° C with desiccant.

Nissl Staining

One series from each brain was Nissl stained for neuroanatomical localization. Slides were fixed in potassium buffered formalin, dipped in Nissl stain for 5 minutes, and then rinsed in distilled water. After a second rinse in distilled water they were dehydrated in a series of

ethanols, the first of which contained glacial acetic acid to allow for better contrast and visualization of cells.

Fluorescence In Situ Hybridization

Genes coding for the following were used: CRH (GENBANK: NM_001245612), GR (GENBANK: XM_002192952). Primers were developed using the National Center for Biotechnology Information primer tool and polymerase chain reaction was conducted on reverse-transcribed isolated RNA to obtain DNA. Probes were prepared using Roche Applied Science DIG RNA Labeling kits and Biotin kits (Indianapolis, IN) according to manufacturer's instructions. Correct product size of probes and sequences were confirmed using the Wayne State Applied Genomics Technology Center. Specificity of probes to zebra finch CRH and GR was confirmed using the BLAST tool on the NCBI database. Dot blot assays were used to determine ideal probe concentrations.

Fluorescence *in situ* hybridization (FISH) followed protocol previously established in our lab (Thompson, Dzubur, Wade & Tomaszycski, 2011). All tissue was run at the same time to control for differences in technique. At room temperature, all slides were fixed in 3% paraformaldehyde and rinsed in PBS. Slides were incubated in 0.1M triethanolamine hydrochloride with 0.25% acetic anhydride for 10 minutes and then rinsed 3x in 0.2M sodium phosphate, sodium chloride and ethylenediaminetetraacetic acid (SSPE). Slides were then dehydrated in a series of ethanols and air dried for 10 minutes before being covered with parafilm and incubated overnight at 55°C in 200 µl of hybridization buffer containing the probes at ideal concentrations for each gene (GR 1:100; CRH 1:1,000) as determined by the dot blot assay.

The next day, parafilm coverslips were removed by rinsing in 2x SSPE. Slides were then washed in 2x SSPE for 30 minutes at room temperature, followed by a 1 hour wash in 2x SSPE and 50% formamide at 65°C and 2 x 30 minute washes in 0.1X SSPE at 65°C. Slides were then incubated in 0.3% hydrogen peroxide in Tris-NaCl-Tween (TNT) buffer for 10 minutes followed by 3 x 5 minute rinses in TNT buffer. Slides were then washed in TNB buffer (TNT buffer and 2 mg/ml BSA) for 30 minutes at 37°C and incubated for 2 hours in TNB buffer with Anti-DIG-POD antibody (1:100; 10 µg/ml, Roche Diagnostics, Indianapolis, IN). Slides were then washed in TNB buffer and incubated in tyramide-conjugated TRITC fluorophore in the manufacturer's buffer (1:100; Alexa 594, Molecular Probes, Carlsbad, CA) for 30 mins. Double-labeled slides were once again incubated in 0.3% hydrogen peroxide in TNT buffer for 10 minutes and washed for 5 minutes in TNT buffer. To detect biotin, slides were incubated for 1 hour in TNT buffer containing anti-biotin antibody (1:500, 10 µg/ml, Vector Labs, Burlingame, CA) and visualized with tyramide-conjugated FITC fluorophore in the manufacturer's buffer (Alexa 594, Molecular Probes, Carlsbad, CA). Finally, the slides were washed in TNT buffer, coverslipped with Slow Fade (Molecular Probes, Carlsbad, CA) and cured in a lightproof box overnight. Twenty-four hours later, the edges of the coverslips were sealed with clear nail polish. Slides were then stored at -20°C until quantification.

Quantification

Slides were analyzed using a Nikon (Eclipse 80i) microscope and Nikon Elements software (AR 3.0). The PVN was located using Nixdorf-Bergweiler and Bischof's zebra finch atlas in the Nissl stained series. One observer, blind to treatment group, quantified the number of cells for each mRNA in the PVN. A standard counting threshold was maintained across sections

to insure comparable fluorescence intensity among counted cells. Four brains from CORT treated animals (3 females, 1 male) were excluded from the analyses due to tissue damage.

Statistical Analysis

To assess the efficacy of the CORT treatment, total plasma CORT and neural expression of CRH and GR mRNA were compared across treatment groups. To account for low sample sizes, total CORT levels were collapsed into Time 1 (Days 2 & 4), Time 2 (Days 10 & 16), and Time 3 (Days 23 & 31) and compared across treatment groups using a one-way ANOVA for each time-point. For both CRH and GR mRNA, the slice with the maximum count for the PVN was averaged across hemispheres to achieve a total count per subject. This quantification was then analyzed separately for each mRNA using one-way ANOVAs to explore differences based on treatment.

As has been found in the past in our lab, the behavioral data was largely variable and non-normally distributed. Therefore, a parametric statistical test (i.e. ANOVA) on the raw data was not a viable option. To control for the large variability each behavior was calculated as the percentage of time (or bouts) the bird exhibited that behavior towards their final preferred partner. These percentages were then arc-sin transformed to normalize the distribution. Subjects were considered paired if they spend 75% or more of their total time clumping with one specific partner compared to other opposite sex individuals (Mabry, Streatfeild, Keane & Solomon, 2011). Motivation to pair was assessed using a latency to pair measure, which was the first day a bird spent 75% or more of its time clumping with a specific partner. Latencies were compared across treatment groups. Pair-bond stability and maintenance were also examined using the same pairing index described above.

To assess the effects of CORT treatment on courtship and pair bonding behavior, observation days were collapsed and analyzed as Time 1 (Days 2-5), Time 2 (Days 9 & 16), and Time 3 (Days 23 & 31). Pairs were expected to form during Time 1, and be maintained during Time 2. The 21-day release pellets were expected to become inactive at Time 3, so these observations showed whether or not pairing behaviors changed following cessation of treatment. Using the arc-sin transformed partner preference percentage data, all behaviors from Table 1, except aggression, were compared across treatment groups using a 2 x 3 (Treatment Group x Time) ANOVA. Frequency of aggressive attacks was log transformed to normalize the data and compared across treatment groups using a repeated measure ANOVA for each sex. Reproductive function in females was assessed by comparing number of viable eggs across treatment groups using a one-way ANOVA.

Results

There was no significant effect of CORT treatment on circulating total CORT levels at any time point, as shown in Figure 8 (Time 1, $F(1, 13) = 0.40, p = 0.54$; Time 2, $F(1, 17) = 1.25, p = 0.28$; Time 3, $F(1, 30) = 2.81, p = 0.10$). There was also no significant effect of CORT treatment on expression of CRH ($F(1, 30) = 0.05, p = 0.94$) or GR ($F(1, 30) = 0.25, p = 0.62$) in the PVN (Figure 9).

CORT treatments did not alter total blood CORT or neural substrates. One explanation for this is that the method used to elevate CORT levels was ineffective. However, an alternative possibility is that the levels of free CORT were affected by treatment, but I was unable to detect it. Due to the small body size of the zebra finches it was difficult to collect a large volume of blood and thus there was an insufficient sample to analyze levels of both total CORT and

corticosterone binding globulin. To explore the possibility that the treatment was, in fact, effective, the behavioral data was analyzed.

In males, there was no significant effect of treatment on pairing at any time point, as shown in Figure 10A (Time 1, $F(1, 18) = 0.78, p = 0.39$; Time 2, $F(1, 18) = 0.00, p = 1.00$; Time 3, $F(1, 18) = 0.24, p = 0.63$). In females, there was no significant effect of treatment on pairing at either Time 1 ($F(1, 14) = 0.26, p = 0.62$) or Time 2 ($F(1, 14) = 0.00, p = 1.00$), but CORT-treated females were significantly less likely to be paired at Time 3 than controls ($F(1, 14) = 5.09, p = 0.04$), as shown in Figure 10B.

As shown in Figure 11, of the animals who paired, there was no significant effect of treatment on the latency to pair in males ($F(1, 15) = 0.09, p = 0.77$), but the CORT treated females that paired did so more quickly than controls ($F(1, 12) = 8.88, p = 0.01$). There were no incidences of separation from a pair-bonded partner in males, but two females did separate, one from the treatment group and one control, thus there were no effects of treatment on pair bond stability.

Males increased the percentage of singing directed at their partner over time ($F(2, 36) = 11.41, p < 0.01$), but there was no significant effect of Treatment ($F(1, 18) = 0.38, p = 0.54$) or Treatment by Time interaction ($F(2, 36) = 1.99, p = 0.15$) (Figure 12).

As shown in Figure 13, the percentage of time spent clumping with the preferred partner increased over time in females ($F(2, 28) = 3.95, p = 0.03$), but this effect was not statistically significant for males ($F(2, 36) = 3.05, p = 0.06$). For either sex, there was no significant effect of Treatment (males: $F(1, 18) = 0.09, p = 0.77$; females: $F(1, 14) = 0.54, p = 0.48$) or interaction between Treatment and Time (males: $F(2, 36) = 0.54, p = 0.59$; females: $F(2, 28) = 0.03, p = 0.97$).

There was no significant effect of Time (males: $F(2, 36) = 1.59, p = 0.22$; females: $F(2, 28) = 1.42, p = 0.26$), Treatment (males: $F(1, 18) = 0.46, p = 0.51$; females: $F(1, 14) = 0.21, p = 0.66$) or an interaction between Time and Treatment (males: $F(2, 36) = 0.40, p = 0.67$; females: $F(2, 28) = 0.66, p = 0.52$) in either sex for the percentage of time spent allopreening with the preferred partner (Figure 14).

As shown in Figure 15, the percentage of time spent in the nestbox with the preferred partner increased over time for both sexes (males: $F(2, 36) = 4.66, p = 0.02$; females: $F(2, 28) = 4.52, p = 0.02$). For either sex, there was no significant effect of Treatment (males: $F(1, 18) = 0.50, p = 0.48$; females: $F(1, 14) = 0.52, p = 0.49$) or interaction between Treatment and Time (males: $F(2, 36) = 2.20, p = 0.13$; females: $F(2, 28) = 0.63, p = 0.54$). The frequency of tail quivers and copulations was too low to permit statistical analysis.

Aggressive behavior was suppressed by CORT treatment in males ($F(1, 18) = 4.81, p = 0.04$) (Figure 16A). Post hoc t -tests revealed a significant effect at Time 3 ($t(18) = 2.46, p = 0.02$), but not at Time 1 ($t(18) = 0.39, p = 0.71$) or Time 2 ($t(18) = 2.00, p = 0.61$). There was no significant effect of treatment on aggressive behavior in females ($F(1, 14) = 1.96, p = 0.18$) (Figure 16B). For either sex, there was no significant effect of Time (males: $F(2, 36) = 2.34, p = 0.11$; females: $F(2, 28) = 0.67, p = 0.52$) or interaction between Treatment and Time (males: $F(2, 36) = 1.04, p = 0.36$; females: $F(2, 28) = 1.15, p = 0.33$). Both sexes, regardless of Timepoint (males: $F(2, 36) = 2.81, p = 0.07$; females: $F(2, 28) = 0.68, p = 0.51$), Treatment (males: $F(1, 18) = 0.07, p = 0.80$; females: $F(1, 14) = 0.55, p = 0.47$), or the interaction between Treatment and Time (males: $F(2, 36) = 0.78, p = 0.47$; females: $F(2, 28) = 1.18, p = 0.32$), were equally likely to receive aggression.

Reproductive function, as measured by number of viable eggs did not differ by treatment group for females ($F(1, 15) = 0.75, p = 0.40$) and was not assessed in males. This suggests that reproductive function was not impaired by CORT treatment.

Discussion

This study was designed to be a thorough examination of the effect of chronic high level CORT on pair-formation and pair-maintenance. However, there was a lack of treatment effect in total CORT (Figure 8) or neural expression of CRH and GR (Figure 9). It is possible that levels of bound CORT were affected by treatment, but were not measured here. Behavioral data do support some effect of treatment indicated by a decreased likelihood to pair for females. This is consistent with the literature in prairie voles, such that administration of CORT blocks the formation of a partner preference (DeVries *et al.*, 1995). Interestingly, CORT-treated birds that did pair did so more rapidly than control animals. Furthermore, there was an effect of CORT treatment to suppress aggressive behavior in males, but this was only apparent at Time 3, a time after the stated effective period of the treatment (after the 21-day release).

The alternative explanation is that behavioral effects seen here were spurious findings due to chance and that the implant was ineffective. The formulation of the pellet may likely have been insufficient for use in the zebra finch. The manufacturer of the CORT pellet, Innovative Research of America, has a long history of creating continuous release drug and hormone delivery pellets. A search of the company's website indicated that their CORT pellets have been used in 574 published research studies of different taxa between 1981 and 2013. Innovative Research of America formulates the matrix of the drug-delivery pellet differently depending on the species of the research subject. Due to the high metabolic rate of avian species compared to rodents, for example, the matrix formulated for birds is developed to allow for a slower release

of the hormone. Use of the 21-day release CORT pellets formulated for rodents in the zebra finch led to death within 48 hours, presumably due to the rapid release of CORT (personal observation).

Further examination of the literature in which Innovative Research of America CORT pellets were used revealed that the vast majority of studies were conducted in rodents. I am aware of only four studies conducted using avian models. Doses for this study were selected based on a dose-response curve shown in a similarly sized bird (Pravosudov, 2003); however, further study from that lab did not verify the efficacy of the CORT pellets and found no neural effect of treatment (hippocampal volume and cell proliferation; Pravosudov & Omanska, 2005). In another study, there appeared to be a brief, highly variable effect of the CORT implant to increase levels of total CORT in white-crowned sparrows 7 days following implantation. But, control data from sham-implanted animals were not reported, so it cannot be determined if the elevation in CORT was due to the pellet or to the implantation procedure and handling of the subjects (Bonier, Martin, & Wingfield, 2007). Bonier and colleagues (2007) measured CORT at multiple time-points, but did not report any significant effect of treatment.

Unfortunately, many experiments that fail to find effects of treatment are often subject to the “file-drawer phenomenon” and do not get published. The dissemination of findings of only positive results presents a serious limitation within the literature and inaccurately reflects the efficacy of these drug-delivery models. Indeed, it is only through direct communication with other researchers in the field that I discovered many others have found these pellets to be ineffective using the model of delivery recommended by Innovated Research of America (subcutaneous implantation in the flank as used in this study). Furthermore, only a handful of published studies have used these pellets in avian models. This is unfortunate, as each failed

attempt to use these pellets results in a substantial loss of research funds, unnecessary loss of animal life, and wasted months of study.

While the present study appeared to be unsuccessful at increasing levels of CORT, the effect of chronic high levels of CORT on pair formation and pair maintenance remains an interesting question. There are a number of alternative methods that could be used in future study to address this research question. One alternative for pharmacological manipulation is to use a different method of sustained CORT delivery. There are a number of studies that use an implant created by filling a silastic tube with pharmaceutical-grade powdered hormone (Breuner & Hahn, 2003; Martin, Gilliam, Han, Lee & Wikelski, 2005; Tomaszycski *et al.*, 2006). The benefits of using this method are the allowance for chronic delivery of hormone without repeated injections and it is cost effective. However, the rate and duration of drug delivery is variable across subjects and it is not always effective at raising plasma CORT (Martin *et al.*, 2005). Osmotic pumps have been used for drug delivery in other avian studies (Horton & Holberton, 2009), but would be difficult to use in zebra finches due to their small body size. Another method is to provide repeated (daily) doses of CORT either orally (Spencer & Verhulst, 2008) or with subcutaneous injection across a period of time. This may be accomplished by supplementing the animal's food source, but due to variable rates of ingestion doses may differ across subjects. Direct administration of oral CORT and subcutaneous injection both require the use of restraint, which is a known stressor (McGraw, Lee & Lewin, 2011; Schmidt, Chin, Shah & Soma, 2009; Wada *et al.*, 2008) and thus also subject the vehicle-treated group to a chronic stress condition.

Beyond pharmacological manipulation, chronic stress conditions can be induced using a variety of other methods. A stress response may be elicited in the zebra finch by using

prolonged periods of isolation (Banerjee & Adkins-Regan, 2011), capture and restraint (McGraw *et al.*, 2011; Schmidt *et al.*, 2009; Wada *et al.*, 2008), and food restriction (Lynn *et al.*, 2010). While more ecologically valid than pharmacological manipulation, exposure to these conditions can more broadly affect hormone and neurotransmitter activity and, as such, would limit the ability to infer causal relationships between specific physiological markers and the behaviors of interest. These potential methods should be further explored in future studies.

In sum, the effect of chronic high-levels of CORT on the formation and maintenance of pair-bonds remains an interesting, but unanswered question. In humans, chronic stress can affect relationship quality over extended periods of time (reviewed in Randall & Bodenmann, 2009) and I believe further study is warranted to explain the neurochemical substrates of stress that specifically affect relationship formation and maintenance.

CHAPTER 4 – EFFECTS OF PAIRING ON STRESS RESPONSE PHYSIOLOGY

Complex biological mechanisms underlie pairing behaviors. One component, identifiable across taxa, is the physiological stress response. A meta-analysis of studies with humans suggests that social interactions can elicit a stress response, but the degree of the response is dependent on the nature of the stressor (Dickerson & Kemeny, 2004). Social interactions that include an overt evaluation by others elicits a strong HPA axis response ($d = 0.86$; Dickerson & Kemeny, 2004). The commonly observed increase in glucocorticoids (including cortisol) during pair-bond formation may be attributed to the overt social evaluation that occurs when selecting a romantic partner. Indeed, mere exposure to a member of the opposite sex will increase HPA activity (Roney *et al.*, 2007; Lopez *et al.*, 2009). However, at least in humans, this effect may be modified by the perceived attractiveness of the partner. In human males, cortisol levels may remain unaffected if the female is appraised as unattractive (van der Meij *et al.*, 2010), whereas normally exposure to a female rapidly increases salivary cortisol (Roney *et al.*, 2007). Similarly, female humans have elevated cortisol after watching a video of an attractive male courting a female (Lopez *et al.*, 2009), although the effect of attractiveness has not been explicitly tested. In animal models, however, the inverse relationship is found. Birds with access to unattractive potential mates have higher levels of CORT compared to those with ideal mates (Griffith, Pryke & Buttemer, 2011).

Human males and females are assumed to have a similar HPA response to pairing; however, effects are not consistent across taxa and can differ between the sexes (DeVries *et al.*, 1995; 1996). A sexually-dimorphic response to the presence of an opposite sex conspecific is shown in prairie voles—males have an increased CORT response whereas females have a decreased response (DeVries *et al.*, 1995; 1996). In Japanese quail, females show increased

CORT in response to the presence of a conspecific only when there is the possibility to mate (Rutkowska, Place, Vincent & Adkins-Regan, 2011). Female quail also show an elevated CORT response following interaction with same-sex conspecifics, and thus CORT elevation may be a generalized response to social interaction and is not specifically attributable to an opposite-sex interaction (Rutkowska *et al.*, 2011).

Acute increases of cortisol/CORT have been identified as a precursor to the formation of a pair-bond, either as a unique response to a conspecific or as a generalized response to the socialization that is necessary to form a bond. Yet, studies have identified sustained elevations in cortisol/CORT during the maintenance of the partnership over extended periods of time (days to months) in various species (black-legged kittiwake; Angelier, *et al.*, 2007; Geoffroy's tufted-ear marmosets: Smith *et al.*, 2011; humans: Marazziti & Canale, 2004). According to theories of pair-bond formation, it is the acute response that facilitates the initial pair-bond and thus, the sustained elevation in basal CORT after the formation of a pair-bond would not be expected, particularly in light of social buffering theory, in which the presence of a pair-bonded partner dampens the physiological response to stressors (for review across taxa see Hennessy *et al.*, 2009). However, basal CORT levels and stress reactivity are separate processes and often do not correspond in avian models of pairing (Angelier *et al.*, 2007). With one exception (Smith *et al.*, 2011), previous studies have only examined the acute effect of pairing on HPA axis activity at one time point. This study explored the differences at the neural level between pairing in either short-term (48-hours) or long-term (2-week) conditions and explored effects on long-term (2-week) pairing on blood levels of total CORT.

This study further sought to broaden the extant literature by examining and modeling the larger physiological stress system. Previous studies have only used peripheral markers of stress

(*e.g.* salivary, blood or urinary cortisol/CORT), which limits conclusions about the neural mechanisms that are hypothesized to drive pairing behavior. Thus, I sought to demonstrate that paired animals have elevated basal CORT measured peripherally (blood) compared with unpaired animals. In additional subjects, I expected to show that paired animals had increased neural expression of CRH and decreased GR mRNA in the PVN. Chronic stress decreases GR expression and increased CRH expression in the PVN (Herman *et al.*, 1995; Makino *et al.*, 1995; Ulrich-Lai & Herman, 2009). Thus, long-term pairing, which is expected to elevate CORT, is also expected to increase CRH and decrease GR mRNA expression. With the careful observation of multiple physiological markers of stress, this study also explored the relationship between specific pairing behaviors (*e.g.* clumping, nestbox cohabitation, and allopreening) and specific markers of HPA axis activity.

A further innovation of this study was in the contrast of animals that have paired with those who had an equal opportunity but did not pair—an ecologically valid comparison that translates well to the human experience. Previous literature has often used isolated conspecifics as control animals for the paired group. This is likely because species commonly used to study these phenomena pair almost immediately and with minimal selectivity. This is not the case for zebra finches, which are particularly selective in choosing a mate (Zann, 1996). Approximately 75% of birds will pair within 3 days when given access to a limited mate pool (Pedersen & Tomaszycki, 2012; Silcox & Evans, 1982). Thus, I created a unique opportunity to compare animals that paired with those who remained unpaired, despite an opportunity to do so. This contrast allows for the explicit testing of the effects due to social interaction alone, and the composite effects of pair-bond formation and social evaluation, which to date has been

impossible to differentiate. A third group, maintained in same-sex conditions, provided an experimental control for these multiple comparisons.

Methods

Experiment 1: Effects of Pairing on Blood Levels of CORT

Subjects & Blood Collection

This study utilized control subjects from the chronic CORT administration study (Chapter 3; male, $n = 10$; female, $n = 8$) and split these subjects into groups (paired or unpaired in mixed-sex conditions) at two time points (Time 1, within 4 days of cohabitation and Time 2, between days 5 and 14 following cohabitation). During the experiment subjects (4 of each sex) were housed in observation cages ($91.4 \times 76.2 \times 76.2$ cm) and pairing behaviors were recorded. Subjects were considered paired if they spend 75% or more of their total time clumping with one specific partner compared to other opposite-sex individuals (Mabry *et al.*, 2011). A third group (male, $n = 6$; female, $n = 5$) were used as same-sex controls and maintained in the similar, but same-sex conditions. Each cage contained a water dish, food dish, grit box, perches and four empty nest boxes.

To assess circulating plasma CORT levels, blood was taken from each animal a maximum of two times at Time 1 or Time 2. For blood collection, two capillary tubes of blood were collected from each animal by puncturing the brachial vein with a 26-gauge needle within 4 minutes of opening the cage between 10.00 and 12.00 hr. Only samples collected within the 4 minute window were analyzed. Blood was emptied into 1.5 ml vials and kept on ice. All samples were centrifuged within 1 hr of blood collection and plasma samples were frozen at -80° C until assay. Plasma CORT levels were estimated using Corticosterone Enzyme Immunoassay

Kit (Assay Designs, #901-09, Enzo Life Sciences, Ann Arbor, MI) that has been validated previously in zebra finches (Wada *et al.*, 2008).

Statistical Analysis

To assess the effect of pairing condition (mixed-sex paired, mixed-sex unpaired, and same-sex) on blood CORT at Time 1 and Time 2, a Kruskal–Wallis one-way analysis of variance was conducted for each sex. This statistic was chosen since it is robust to unequal sample sizes and non-normal distributions. Post hoc Mann-Whitney U tests were conducted as necessary.

Experiment 2: Effects of Pairing of GR and mRNA Expression

Subjects

A total of 42 adult zebra finches (male, $n = 21$; female, $n = 21$) were used in this study. There were 36 birds assigned to a pairing group in one of two conditions; those that were allowed to pair for either 48 hours or 2 weeks. Of these, 24 birds (12 of each sex) formed strong pairs within the first 48 hours. Twelve pairs (6 of each sex) were assigned to the 48-hour group and the other 12 pairs were allowed to maintain their bond for 2 weeks. To control for the effects of the testing environment, animals that exhibited no pairing behavior were included as unpaired controls (male, $n = 4$; female, $n = 4$). To control for the possibility that the animals that did not pair were deficient in some way and control for effects of the opportunity to pair additional subjects maintained in same-sex conditions were used as controls (male, $n = 5$; female, $n = 5$). All subjects were maintained on a 12:12 hour light/dark cycle (lights on 6:00) in a temperature and humidity controlled room under conditions ideal for mating (24°C, 50% humidity). Seed and water were provided *ad libitum* and their diet was supplemented with hard-boiled chicken egg and calcium-enriched grain (Simple System Breeder Crumb 5-Day Product, The Bird Care Company) twice per week. Prior to the experiment, subjects were housed in same-sex aviaries

(152.4 × 76.2 × 198.1 cm) to prevent pair formation. During the experiment subjects in the pairing condition were housed in observation cages (91.4 × 76.2 × 76.2 cm) in groups of 8 (4 of each sex). Each cage contained water and food dishes, a grit box, perches and 4 empty nest boxes. Nest material was provided as needed.

Pairing Behavior

Birds in the pairing condition were allowed to pair for either one of two time periods: 48 hours or 2 weeks. Most animals form a pair within 48 hours (Silcox & Evans, 1982). Two weeks is sufficient time to maintain a pair relationship, but not long enough to allow for offspring to be produced, which eliminates the confound effect of parenting (Tomaszycki & Adkins-Regan, 2006).

For the pairing group, observation to assess pairing behaviors (see Table 1a) occurred for 30 minutes on the first day: 15 minutes between 11:00 and 12:00 hr and 15 minutes between 13:00 and 15:00 hr. Pairing behaviors were again recorded for 15 minutes on the second and third days between 11:00 and 12:00 hr. Pairing status was assessed using an association index, in which a subject was considered paired if more than 75% of the subject's pairing behaviors were with one partner relative to other opposite-sex animals (as in Mabry *et al.*, 2011). To be included in the paired group, animals must have paired by Day 2 and maintained the pair on Day 3. Animals in six pairs of the pairs were sacrificed on Day 3, the remaining six pairs were observed 3 times per week until 2 weeks post-pairing when they were sacrificed. Only animals that maintained the same partner throughout the 2 weeks were included in the analyses.

Brain Collection

Directly following observation on the last day of testing for the pairing group (48-hour or 2-week), animals were sacrificed by rapid decapitation, brains were removed and frozen in cold

methyl-butane and stored at -80° C until processing. The brains of unpaired controls were also collected at these time points. The brains from same-sex control animals were collected using the same protocol. Brains were then sectioned coronally at $20\mu\text{m}$ on a Leica cryostat and mounted directly onto SuperFrost Plus slides (Fisher Scientific, Pittsburgh, PA).

Nissl Staining

One series from each brain was Nissl stained for neuroanatomical localization. Slides were fixed in potassium buffered formalin, dipped in Nissl stain for 5 minutes, and then rinsed in distilled water. After a second rinse in distilled water they were dehydrated in a series of ethanols, the first of which contained glacial acetic acid to allow for better contrast and visualization of cells.

Fluorescence In Situ Hybridization

Genes coding for the following were used: CRH (GENBANK: NM_001245612), GR (GENBANK: XM_002192952). Primers were developed using the National Center for Biotechnology Information primer tool and polymerase chain reaction was conducted on reverse-transcribed isolated RNA to obtain DNA. Probes were prepared using Roche Applied Science DIG RNA Labeling kits and Biotin kits (Indianapolis, IN) according to manufacturer's instructions. Correct product size of probes and sequences were confirmed using the Wayne State Applied Genomics Technology Center. Specificity of probes to zebra finch CRH and GR was confirmed using the BLAST tool on the NCBI database. Dot blot assays were used to determine ideal probe concentrations (1:100 for CRH; 1:1,000 for GR).

Fluorescence *in situ* hybridization (FISH) followed protocol previously established in our lab (Thompson *et al.*, 2011). All tissue was run at the same time to control for differences in technique. At room temperature, all slides were fixed in 3% paraformaldehyde and rinsed in

PBS. Slides were incubated in 0.1M triethanolamine hydrochloride with 0.25% acetic anhydride for 10 minutes and then rinsed 3x in 0.2M sodium phosphate, sodium chloride and ethylenediaminetetraacetic acid (SSPE). Slides were then dehydrated in a series of ethanols and air dried for 10 minutes before being covered with parafilm and incubated overnight at 55°C in 200 µl of hybridization buffer containing the probes at ideal concentrations for each gene (GR 1:100; CRH 1:1,000) as determined by a dot blot assay.

The next day, parafilm cover slips were removed by rinsing in 2x SSPE. Slides were then washed in 2x SSPE for 30 mins at room temperature, followed by a 1 hour wash in 2x SSPE and 50% formamide at 65°C and 2 x 30 minute washes in 0.1X SSPE at 65°C. Slides were then incubated in 0.3% hydrogen peroxide in Tris-NaCl-Tween (TNT) buffer for 10 minutes followed by 3 x 5 minute rinses in TNT buffer. Slides were then washed in TNB buffer (TNT buffer and 2 mg BSA) for 30 minutes at 37°C and incubated for 2 hours in TNB buffer with Anti-DIG-POD antibody (1:100; 10 µg/ml, Roche Diagnostics, Indianapolis, IN). Slides were then washed in TNB buffer and incubated in tyramide-conjugated TRITC fluorophore in the manufacturer's buffer (1:100; Alexa 594, Molecular Probes, Carlsbad, CA) for 30 minutes. Double-labeled slides were once again incubated in 0.3% hydrogen peroxide in TNT buffer for 10 minutes and washed for 5 minutes in TNT buffer. To detect biotin, slides were incubated for 1 hr in TNT buffer containing anti-biotin antibody (1:500, 10 µg/ml, Vector Labs, Burlingame, CA) and visualized with tyramide-conjugated FITC fluorophore (Alexa 594, Molecular Probes, Carlsbad, CA). Finally, the slides were washed in TNT buffer, coverslipped with Slow Fade (Molecular Probes, Carlsbad, CA) and cured in a lightproof box overnight. Twenty-four hrs later, the edges of the coverslips were sealed with clear nail polish. Slides were then stored at -20°C until quantification.

Quantification

Slides were analyzed using a Nikon (Eclipse 80i) microscope and Nikon Elements software (AR 3.0). The PVN was located using Nixdorf-Bergweiler and Bischof's zebra finch atlas in the Nissl stained series. Observers, blind to treatment group, quantified the number of cells in each brain area. A standard counting threshold was maintained across sections to insure comparable fluorescence intensity among counted cells. Six brains were excluded from the analyses due to tissue damage (3 of each sex). Final sample sizes are shown in Table 2.

Statistical Analysis

For both CRH and GR mRNA, the slice with the maximum count for the PVN was averaged across hemispheres to achieve a total count per subject. This mean quantification was log-transformed to normalize the data and analyzed separately for each sex and mRNA using one-way ANOVAs to explore differences based on group.

Due to low levels of expression of GR mRNA, only the relationship between CRH mRNA and behavior was analyzed. Data from pairing behaviors (Table 1a) were averaged across days and then log-transformed to normalize the data. CRH mRNA cell counts were similarly log-transformed. Separate multiple linear regression were conducted for each sex to assess the ability of pairing behaviors to predict expression of CRH mRNA in the PVN. In females, clumping, nest box cohabitation, and allopreening were used as predictors. The same predictors were used in males with the addition of directed singing. All predictors were entered simultaneously.

Results

Experiment 1: Effects of Pairing on Blood levels of CORT

There was a significant effect of pairing condition on median blood CORT in males at Time 2 ($\chi^2(2, n = 9) = 6.28, p = 0.04$), but not at Time 1 ($\chi^2(1, n = 8) = 1.00, p = 0.32$) (Figure 17A). A post-hoc Mann-Whitney test indicated higher levels of Blood CORT for the mixed-sex, paired group compared to the same-sex group ($U = 0, p = 0.01$). Note that the Mann-Whitney value of zero occurred because all values in the mixed-sex, paired group were higher than the same-sex group, as shown in Figure 17A. There were no significant differences between mixed-sex, paired and unpaired groups ($U = 2, p = 0.36$) or between the same-sex and mixed-sex, unpaired groups ($U = 3, p = 0.32$). There was no significant effect of pairing condition on median blood CORT in females at either Time 1 ($\chi^2(2, n = 8) = 2.43, p = 0.30$) or Time 2 ($\chi^2(1, n = 9) = 2.16, p = 0.14$) (Figure 17B).

Experiment 2: Effects of Pairing of GR and mRNA Expression

GR expression was very low across all subjects (see Figure 18), which indicates that staining for GR did not work. Studies in zebra finches clearly demonstrate GR expression in the PVN (Shahbazi, Schmidt & Carruth, 2011). A scan across all tissue revealed overall low levels of expression of GR, so the lack of staining was not isolated to the PVN. A main effect of pairing group on GR expression was not found in males (Figure 18A; $F(3, 17) = 0.35, p = 0.79$) or females (Figure 18B; $F(3, 16) = 0.53, p = 0.67$). It cannot be determined if effects of pairing group would be found with adequate staining of GR mRNA.

The level of expression of CRH mRNA was in the expected range, however there was also no significant effect of pairing group on CRH expression in males (Figure 18A; $F(3, 17) = 0.50, p = 0.69$) or females (Figure 18B; $F(3, 16) = 0.53, p = 0.67$).

In the regression models, pairing behaviors together did not significant predict expression of CRH mRNA in the PVN in males ($R^2 = 0.05, F(4, 14) = 0.13, p = 0.97$), females ($R^2 = 0.59, F$

(3, 11) = 3.91, $p = 0.06$), or both sexes together ($R^2 = 0.36$, $F(3, 26) = 1.10$, $p = 0.37$). Furthermore, there was no significant correlation between CRH mRNA expression and any pairing behavior in either sex ($p > 0.05$).

Discussion

The present study found sustained higher levels of CORT total in long-term pair bonding, but the effect was isolated in males. In males only, blood levels of CORT were higher in paired animals compared to controls housed in same-sex conditions. Other studies across species have found similar effects (Angelier *et al.*, 2007; Smith *et al.*, 2011; Marazziti & Canale, 2004). Unfortunately, most males did not pair by Time 1, so it is unclear the exact time course of CORT elevation throughout the duration of the pair bond. Nonetheless, elevated levels of total CORT in males was associated with the long-term maintenance of a partnership.

No effect was found in females. The null effect is likely due to small sample sizes that could not be controlled during the mixed-sex pairing condition. At Time 2, an effect was trending to suggest that paired females had lower levels of total CORT than same-sex controls; the opposite effect that was observed in males. Due to the statistical limitations, I am hesitant to interpret this effect. Future study should attempt to replicate the sustained levels of CORT in females that were observed in males, possibly identifying a reciprocating effect within the pair-bond.

Conclusions of the biological expression of stress reactivity by pairing are limited by the lack of evidence from GR mRNA. It is unclear why staining for GR mRNA was insufficient, which precluded analysis. The concentration used for *in situ* hybridization for GR (1:100) was higher than what was used for CRH (1:1,000). After reviewing the protocol, there is no obvious reason for the low level of expression. Further troubleshooting will be conducted in the

Tomaszycki lab. However, CRH was successfully measured and indicated no neural modulation that was associated with pairing. There were no significant effects of group on CRH expression in the PVN. Further, CRH expression did not account for differences in pairing behaviors. Therefore, pairing did not alter stress reactivity at the neural level.

There remains an intriguing question of the relevance of elevated CORT in pair bonding if the traditional neural mechanisms do not demonstrate a change. Potentially there are other related biological mechanisms that account for the behaviors that are associated with elevated CORT. Previous study in our lab has shown that the mRNA expression of nonapeptides that are important in the pairing process (mesotocin, the avian homologue of oxytocin, and vasotocin, the avian homologue of vasopressin) are elevated in paired animals as compared unpaired counterparts (Lowrey & Tomaszycki, in revision). Similarly, manipulation of nonapeptides has been shown to regulate partner preference in prairie voles (Cho, DeVries, Williams & Carter, 1999; Wang & Aragona, 2004). It is feasible that the role of stress in pair-bond formation is not direct, but rather an indirect impetus or consequence of other neurochemical systems.

The role of stress in the formation and maintenance of pair-bonds has been largely assumed. Most studies have observed or manipulated circulating peripheral cortisol/CORT, but few have observed the effects long-term or with additional measures of neural expression. In male prairie voles, increasing central CRH expression increases the propensity to form a pair bond (although this model does not translate well to the human condition). However, the relationship between CRH and long-term bonding behavior has not been examined in any species. The lack of relationship between CRH mRNA and behavior observed here suggests that the relationship may not be direct. An interesting alternative hypothesis is a broader biological mechanism in which elevated CORT may interact with but not wholly drive behavioral

outcomes. Future study should consider additional related factors, such as nonapeptides, that may be mediating the relationship between CORT and pair-bonding.

In conclusion, elevated total CORT was associated with long-term pair bonding, although this effect was only observed in males. Despite elevations in CORT, the neural expression of CRH was unchanged under pairing conditions, which suggests that there is no neural modulation of the stress physiology under chronic pairing conditions. While this was unexpected, it is plausible that CORT is an indirect factor in a broader biological mechanism. Future studies are encouraged to include additional factors that may act in concert with CORT to produce pair-bonding behaviors.

CHAPTER 5 – SUMMARY OF FINDINGS AND GENERAL DISCUSSION

The studies in this dissertation were the first to investigate the relationship between the physiological response to stress and pair-bonding behavior in the zebra finch. As summarized in Figure 19, this set of studies demonstrated that CORT may facilitate the formation of partner preference and has a potential role in the long term maintenance of pair-bonds.

Consistent with data from prairie voles (DeVries *et al.*, 1996), acute CORT treatment increased preference for the opposite sex in males. This, however, is inconsistent with previous findings in zebra finches (Lynn *et al.*, 2010), which found that stress induced by fasting decreased courtship and pairing behaviors. Methodological differences may account for discrepant results. My study was acute and conducted in a two-choice paradigm, whereas the previously shown effect of fasting-stress on pairing behaviors was apparent after a more prolonged stressor (4 and 10 hours of fasting) in a semi-naturalistic environment.

In the present study, pairing behaviors increased blood levels of total CORT in males, but did not significantly alter neural expression of CRH. Elevated CORT due to pairing in males was expected based on data reported across taxa (Angelier *et al.*, 2007; Smith *et al.*, 2011; Marazziti & Canale, 2004). The lack of neural modulation of CRH would suggest a lack of chronic stress exposure, as was hypothesized. To my knowledge, this was the first study to examine the effect of pairing on CRH expression in any species in a short-term pairing model. Additionally, this was the first to examine long-term effects of pairing on CRH or to assess the effect of chronic stress on pair formation and long-term maintenance.

Unexpectedly, there was a sexually dimorphic effect of CORT. Acute CORT treatment did not affect preference for the opposite sex in females. Based on findings in prairie voles (DeVries *et al.*, 1995), I expected a decreased preference for the opposite sex following CORT

treatment. However, my data did not support this hypothesis. Interestingly, I did find a sex difference in opposite sex preference –at baseline, females showed a stronger preference for males than males showed for females. However, there were no effects of pair bonding on blood levels of total CORT or neural expression of CRH in females. Studies across taxa have shown elevated CORT levels in both sexes as a response to pair-bond maintenance, but data from prairie voles support a sexually dimorphic effect in response to pair bond formation, consistent with the trends in my findings. In female prairie voles, a suppression of CORT is necessary to support pair-bonding (DeVries *et al.*, 1995), however this has not been studied in pair maintenance.

Due to several limitations, the effect of chronic CORT treatment on pairing remains unclear. I have no evidence that the CORT implant significantly altered circulating CORT. Personal communication with others in the field led us to understand that sustained-release pellets have frequently been found ineffective in avian models. However, these null results were not published. This underscores the need to publish null results along with positive findings to accurately depict behavioral phenomena and efficacy of drug-delivery methods.

An additional significant limitation of the present studies was the lack of adequate staining to accurately quantify and analyze expression of GR mRNA in the PVN. I used a relatively high concentration of the probe and I am confident in the methodology used for *in situ* hybridization. The sequencing for the probe was confirmed through the Wayne State University genomics center. Dot blot analysis was used to determine adequate concentrations and this tissue was double-labeled with CRH mRNA, which was successfully visualized. I remain uncertain as to why the expression of GR mRNA was undetectable.

One further limitation was the necessary restriction of scope. The physiological response to stress occurs through a range of hormonal and neural systems including the sympathetic adrenergic/noradrenergic system. We do know that depletion of the noradrenergic system decreases pairing in both sexes of zebra finches (Vahaba, Lacey, & Tomaszycski, 2013). The studies here focused only on CORT, an important component of the stress response that has been implicated in pair bond formation and maintenance. But CORT and CRH were not a significant correlates to observed pairing behaviors and we did not directly measure or manipulate other hormonal factors or neurotransmitters. Thus, findings here can only address the direct relationship between CORT and pairing behaviors. Based on the pattern of effects a CORT-related stress response is common to at least of the formation, if not long-term maintenance of a pair bond. However, it is unlikely that CORT fully explains pair-bonding behaviors and may act in concert with other known neurochemical systems.

Studies that employ more ecologically-valid stressors may be used to more effectively understand the relationship between stress as a construct and pairing. However, studies of this type limit the ability to discern which physiological factors are directly related to behaviors. Future studies should account for changes in these complimentary stress-response systems to better understand the role that stress as an overall construct plays on pair formation and maintenance.

In sum, these studies provide some evidence to support a relationship between stress physiology and social behavior in the zebra finch. While aspects of these associations remain inconclusive, this improved understanding of the biological underpinnings of pair-bond formation could provide insight to the social relationships in humans.

APPENDIX

Table 1

Behaviors to be Scored During Observation Periods

Adapted from Tomaszycski, Banerjee & Adkins-Regan (2006)

Behavior	Description	Scored as
Directed Singing	Male sings directly to specific female	Duration (sec)
Clumping	Direct physical contact with a conspecific	Duration (sec)
Allopreening	Preening between two individuals	Frequency (bouts)
Nest Box Together	In a nest box with another individual	Duration (sec)
Tail Quiver	Female-typical sexual solicitation	Frequency (bouts)
Copulation	Male mounts female	Frequency (bouts)
Aggression (actor)	Instigated physical attack	Frequency (bouts)
Aggression (recipient)	Received physical attack	Frequency (bouts)

Table 2.

Number of Subjects by Treatment Group and Sex Included in the Study of the Effects of Pairing on Corticotrophin Releasing Hormone and Glucocorticoid Receptor mRNA in Zebra Finches

	2 Week Paired	48 Hour Paired	Unpaired	Same-sex control
Male	6	5	4	3
Female	6	4	3	5

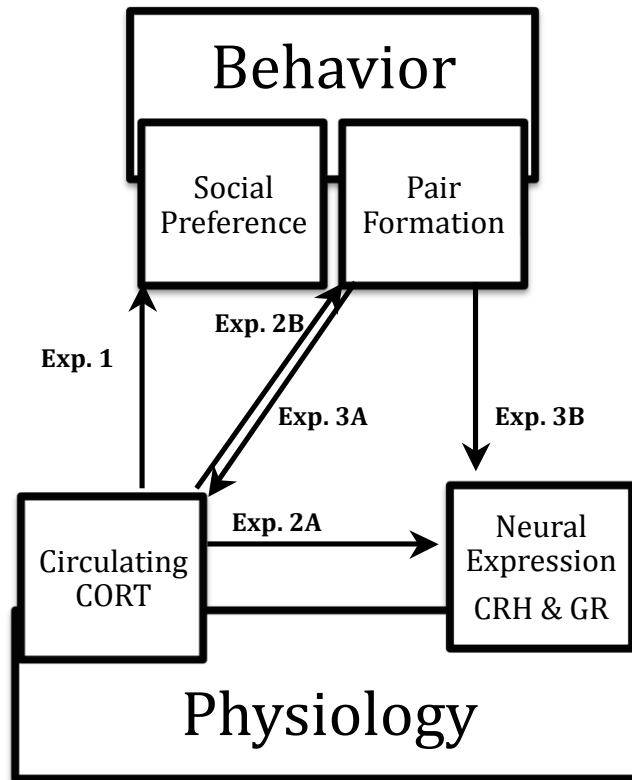


Figure 1. Dissertation Overview. The theoretical relationships between constructs of interest are depicted and labeled with each experiment for hypothesis testing. CORT = corticosterone, CRH = corticotrophin releasing hormone, GR = glucocorticoid receptor.

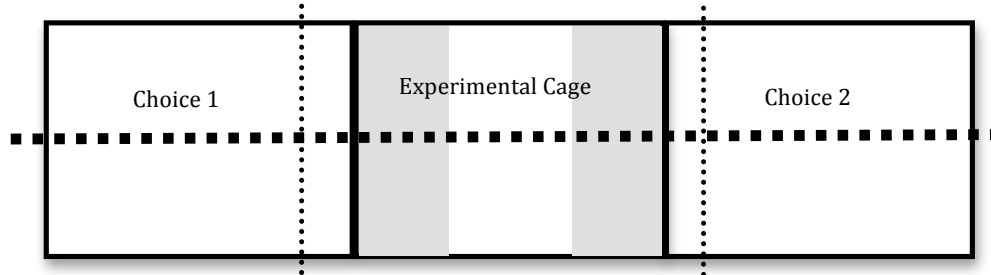


Figure 2. Schematic of Two-Choice Testing Apparatus in a Study of the Effects of Acute CORT Administration on Partner Preference. Dashed lines indicate the location of perches and grey areas indicate the zones proximal to choice cages. In the Single/Single condition, choice 1 was a single opposite-sex bird and choice 2 was a single same-sex bird. In the Single/Group condition, choice 1 was a single opposite-sex bird and choice 2 was a group of 4 same-sex birds. Condition order and side of stimulus presentation were counterbalanced. Percent of time in each shaded area of the total time in shaded areas was computed. CORT = corticosterone.

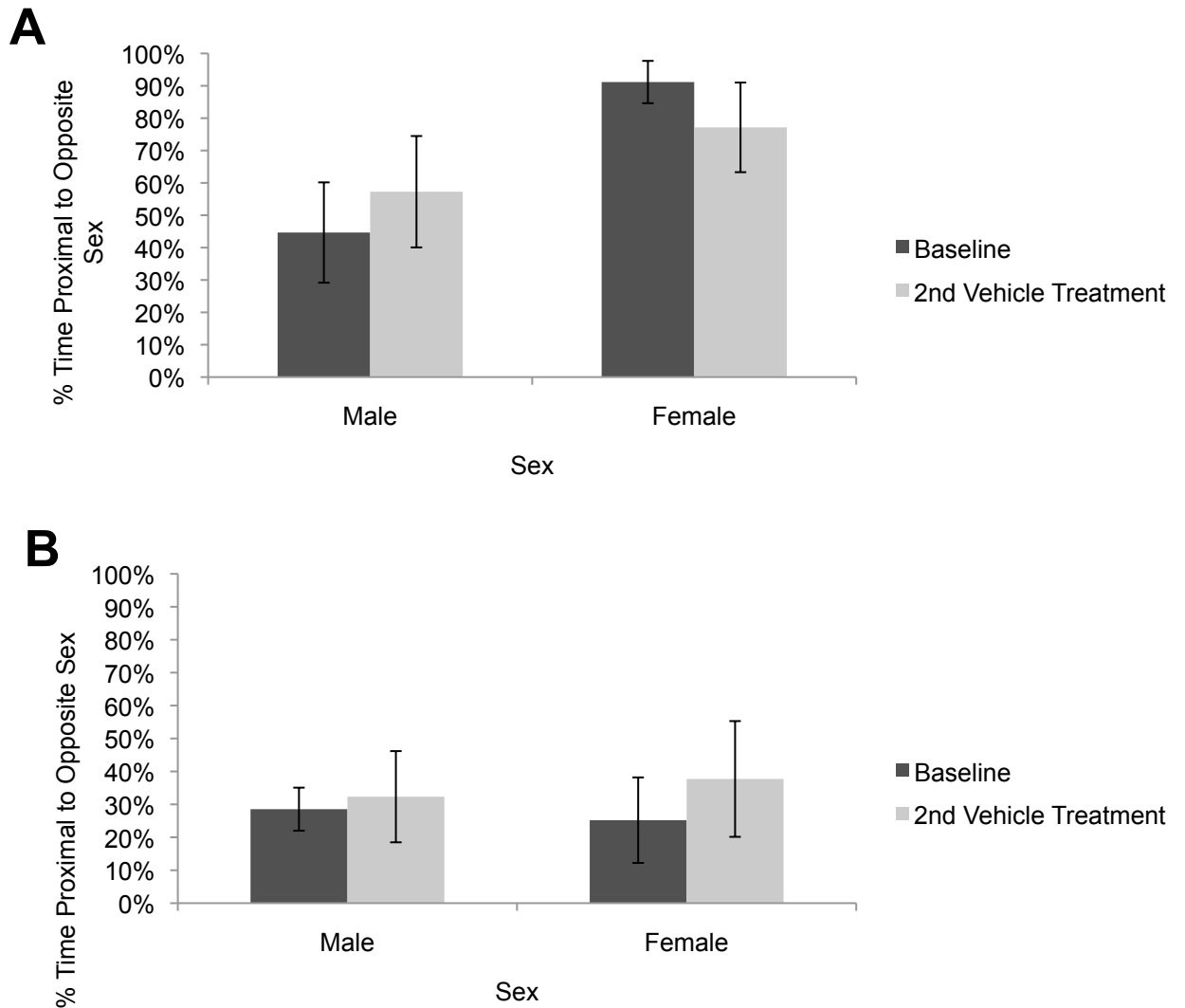


Figure 3. Comparison of Baseline and Second Administration of Vehicle across Testing Conditions in a Study of the Effects of Acute CORT Administration on Zebra Finch Partner Preferences. There was no significant difference in the percentage of time spent in proximity to the opposite-sex bird between the initial baseline control treatment and the control treatment given in the injection sequence for either sex in either the A) single opposite-sex, single-same sex or the B) single opposite-sex, 4 same-sex conditions. Error bars indicate SEM. CORT = corticosterone.

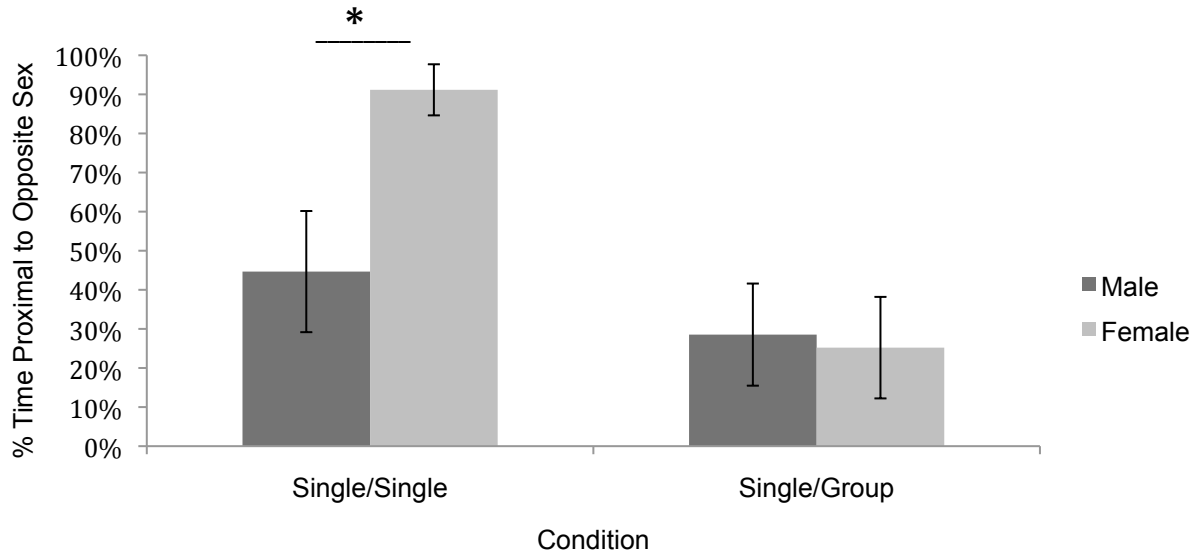


Figure 4. Percentage of Time Spent in Proximity to the Opposite-Sex Animal at Baseline in a Study of the Effects of Acute CORT Administration on Zebra Finch Partner Preferences. At baseline, males spent significantly less time in proximity to females in the Single/Single condition than females did to males, but there was no sex difference in the Single/Group condition. * Indicates ($p < 0.05$). Error bars indicate SEM. CORT = corticosterone.

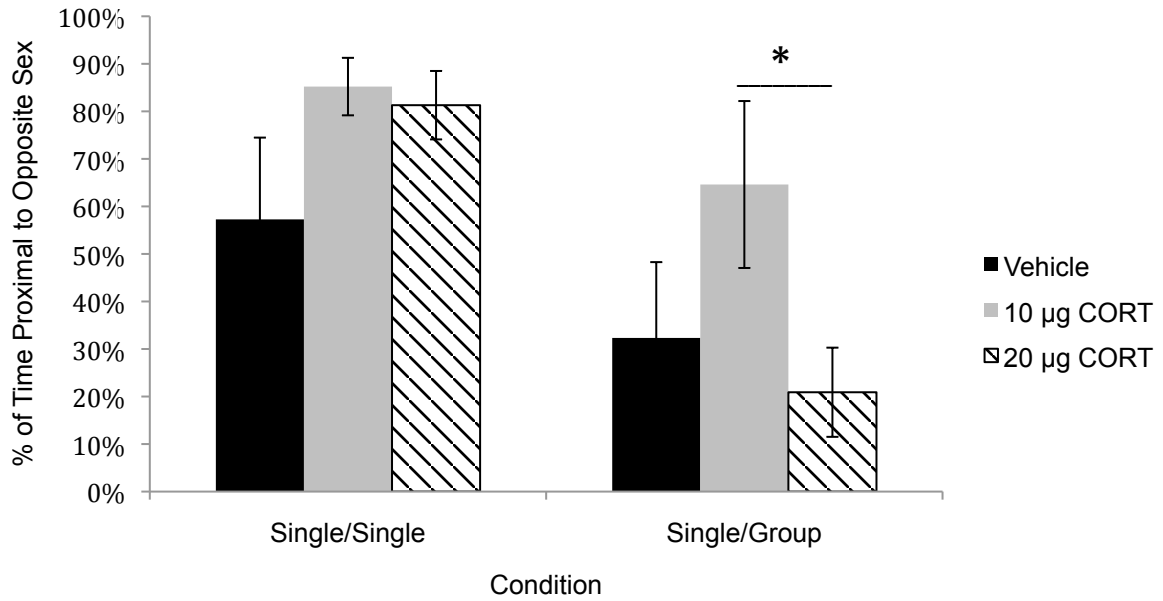
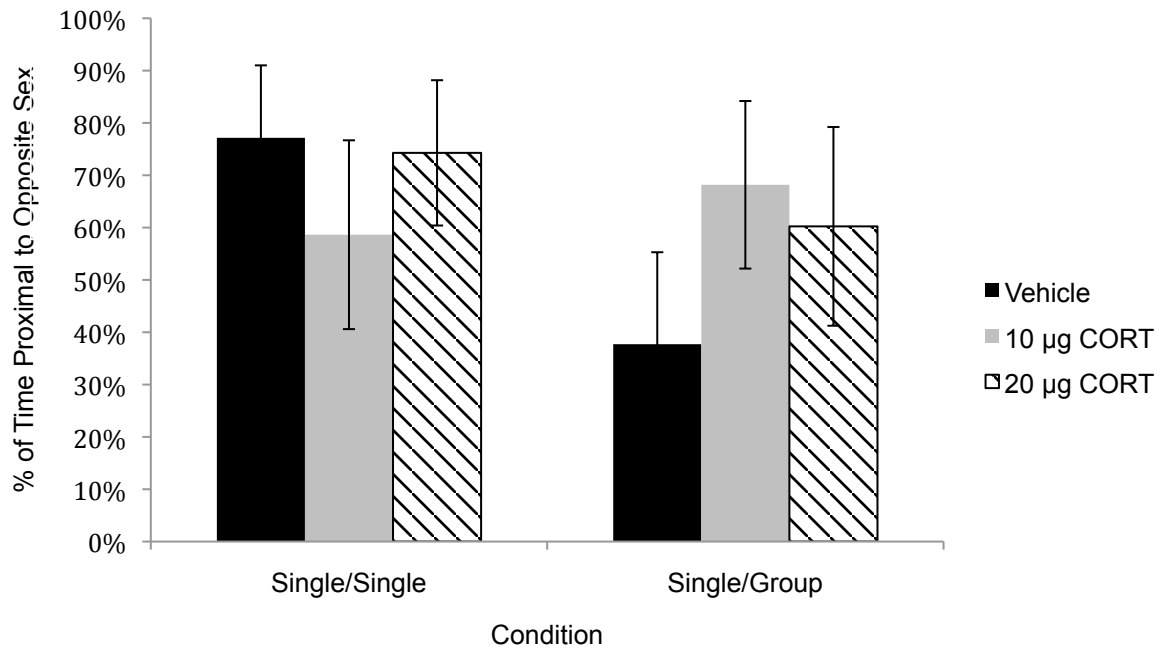
A**B**

Figure 5. Percentage of Time Spent in Proximity to Opposite-Sex Animal by Treatment and Condition in a Study of the Effects of Acute CORT Administration on Zebra Finch Partner Preferences. A) In males, there was no significant difference in the percentage of time spent in proximity to the opposite-sex bird by treatment in the Single/Single condition, but there was a significant overall effect of treatment in the Single/Group condition ($p < 0.05$). *Post hoc analysis indicated a significant difference between 10 μg and 20 μg doses of CORT ($p < 0.05$). B) In females there was no significant difference in the percentage of time spent in proximity to the opposite-sex bird by treatment in either condition. Error bars indicate SEM. CORT = corticosterone.

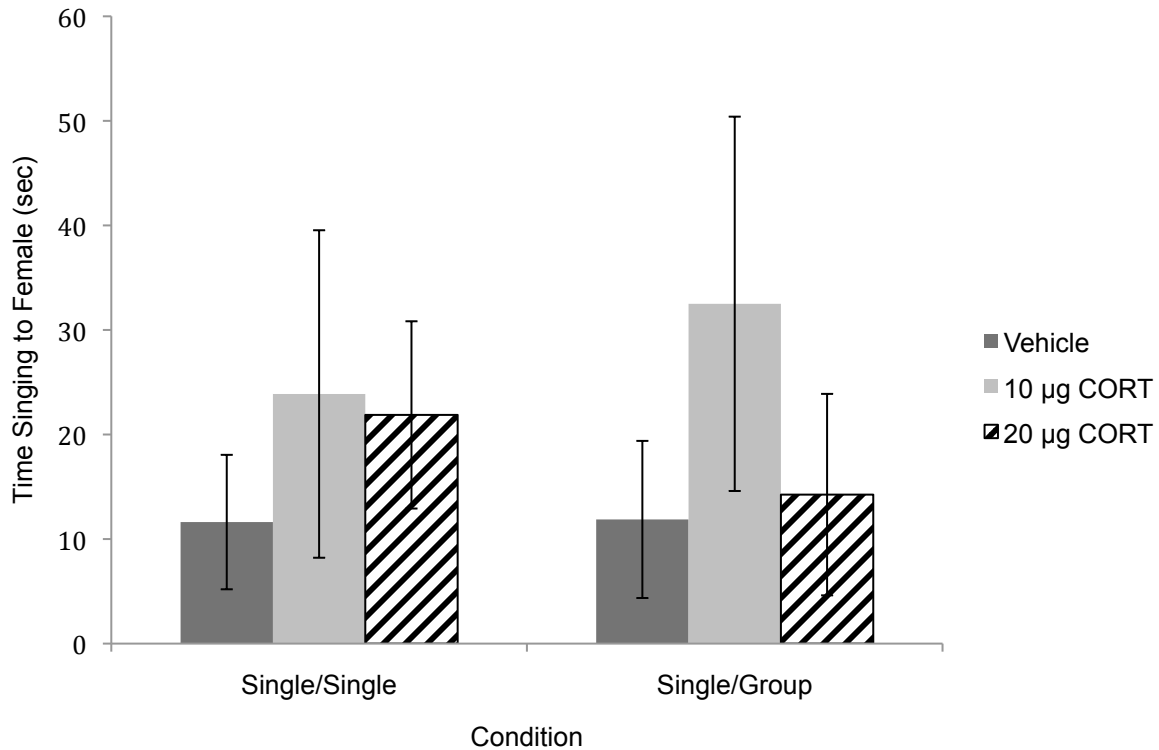


Figure 6. Time Spent Singing to Females by Treatment and Condition in a Study of the Effects of Acute CORT Administration on Zebra Finch Partner Preferences. There was no significant effect of treatment on the time males spent singing to females in either the Single/Single or Single/Group condition. Error bars indicate SEM. CORT = corticosterone.

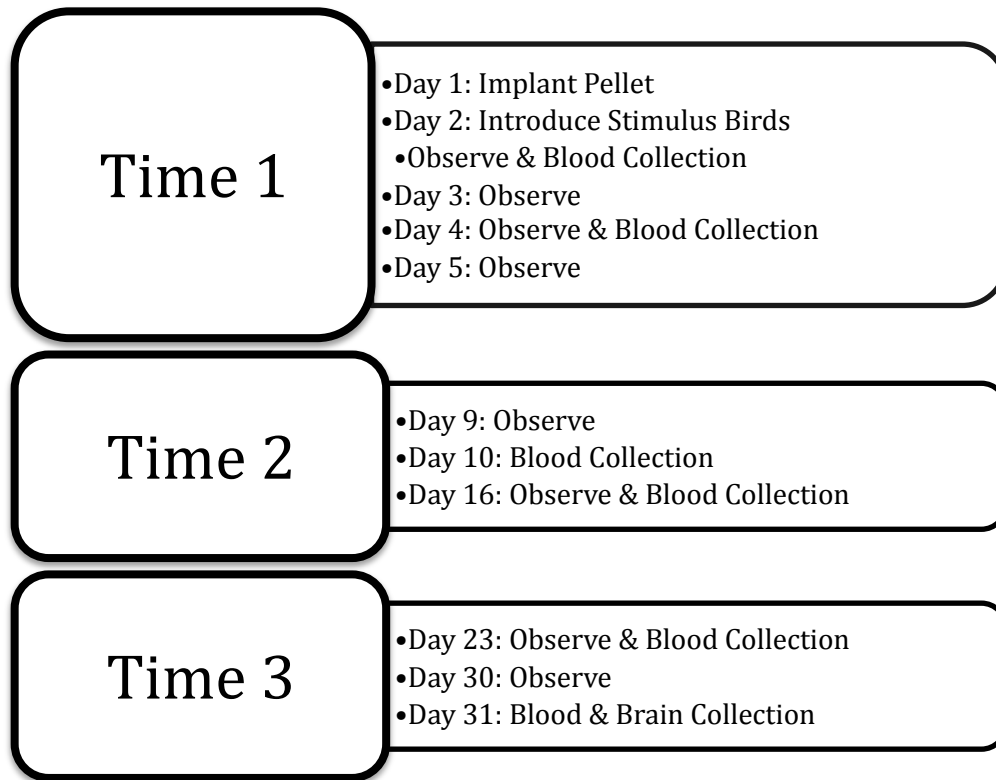


Figure 7. Schematic of Data Collection Timeline in a Study of the Effects of Chronic Corticosterone Administration on Pairing in Zebra Finches. Data were collected according to the detailed timeline and analyzed as time composites (Time 1, 2, & 3).

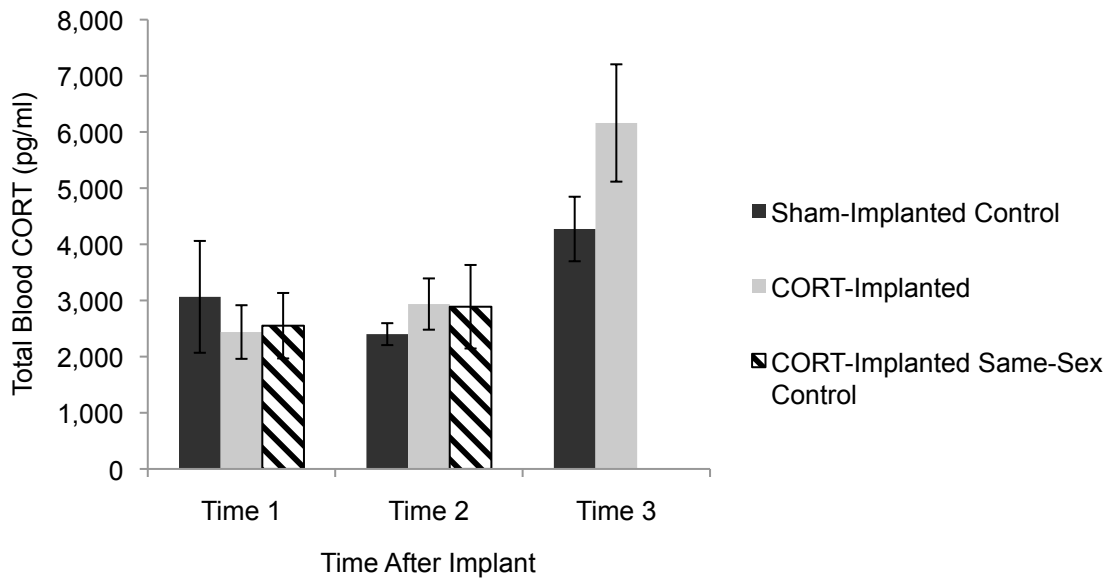


Figure 8. Effects of Chronic Corticosterone Treatment in Total Blood Corticosterone in a Study of the Effects of Chronic Corticosterone Administration on Pairing in Zebra Finches. Due to low sample sizes, blood collection was averaged into 3 Time Points. Blood collected on Day 2 and 4 following implant were considered Time 1, Time 2 blood was collected between Days 9 and 16 and Time 3 data was collected on Day 23 and 31. Blood was not collected from birds in the same-sex condition at Time 3. There was no significant effect of treatment at any time-point ($p > 0.05$). CORT = corticosterone.

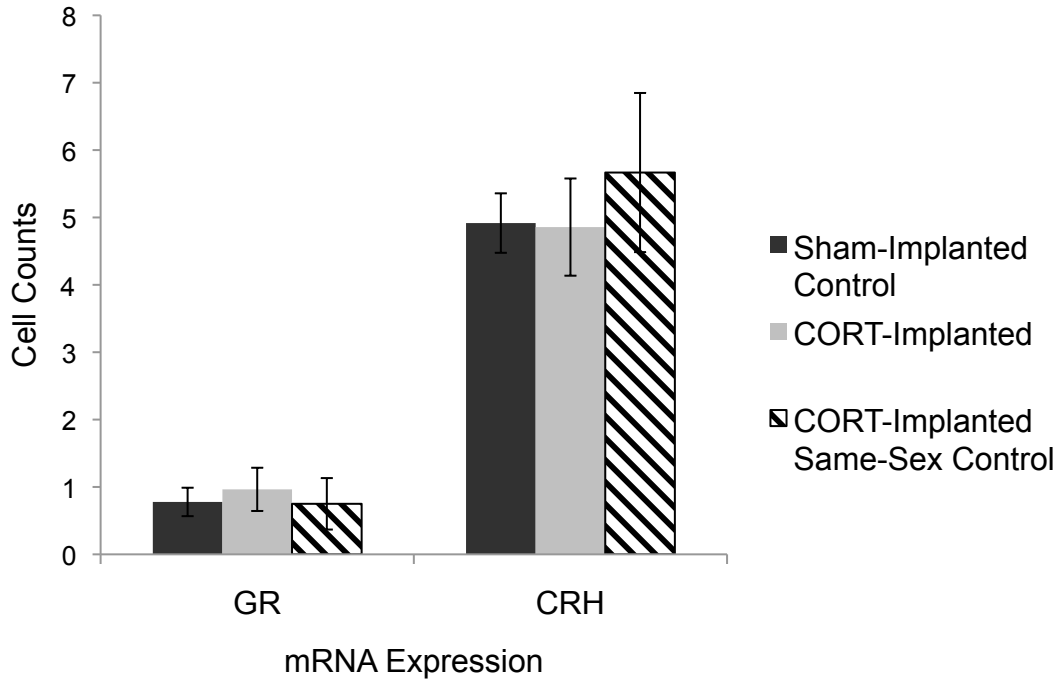


Figure 9. Effects of Chronic Corticosterone Treatment on Expression of Glucocorticoid Receptor and Corticotropin Releasing Hormone mRNA in the Paraventricular Nucleus of the Hypothalamus in a Study of the Effects of Chronic Corticosterone Administration on Pairing in Zebra Finches. There was no significant effect of treatment on expression of GR or CRH mRNA ($p > 0.05$). CORT = corticosterone, CRH = corticotropin releasing hormone, GR = glucocorticoid receptor.

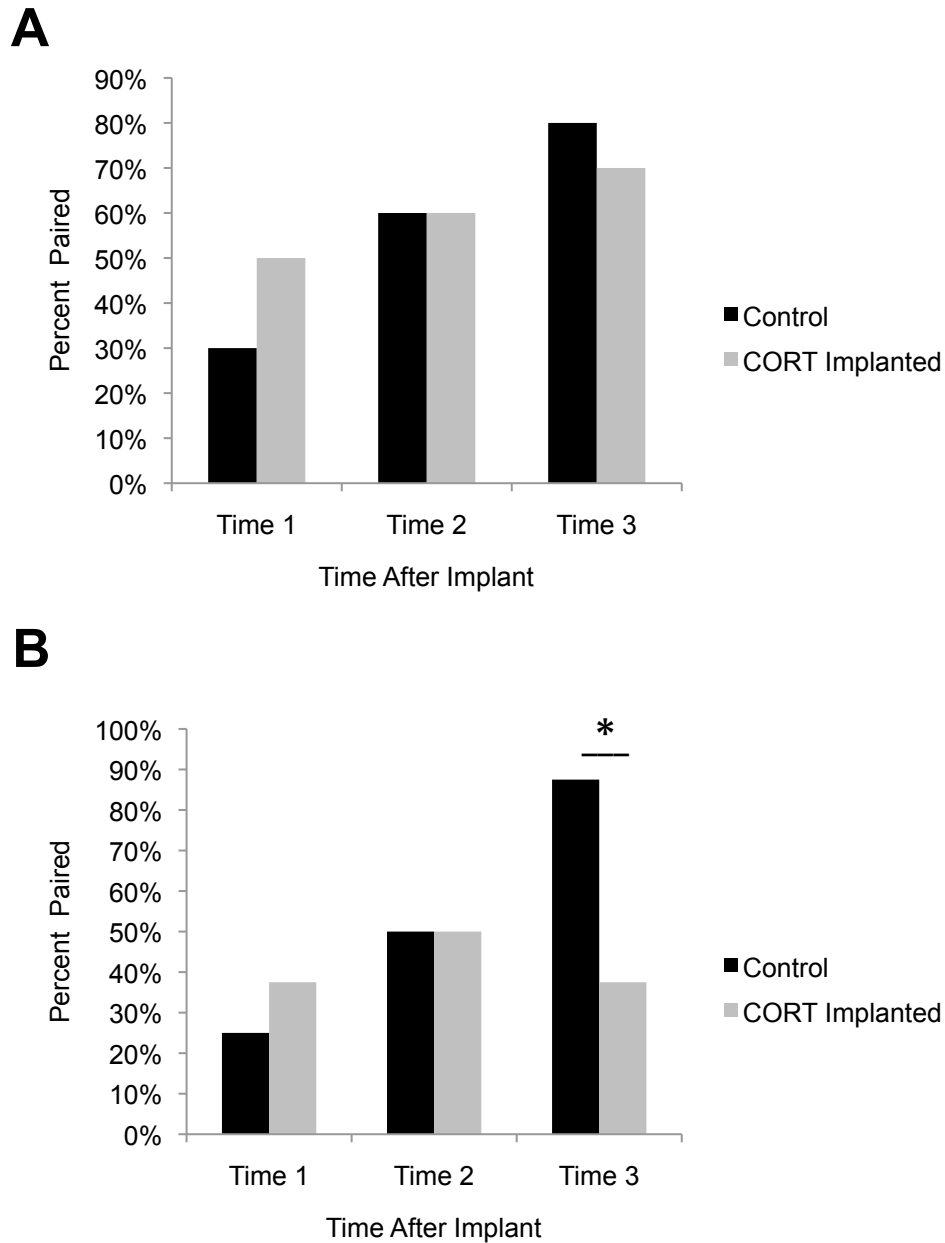


Figure 10. Effects of Chronic Corticosterone Treatment on the Percent of subjects that Paired in a Study of the Effects of Chronic Corticosterone Administration on Pairing in Zebra Finches. A) In males, there was no significant effect of treatment on pairing at any time point ($p > 0.05$). B) In females, CORT treatment suppressed pairing at Time 3 as indicated with * ($p < 0.05$), but not at Time 1 or Time 2 ($p > 0.05$). CORT = corticosterone.

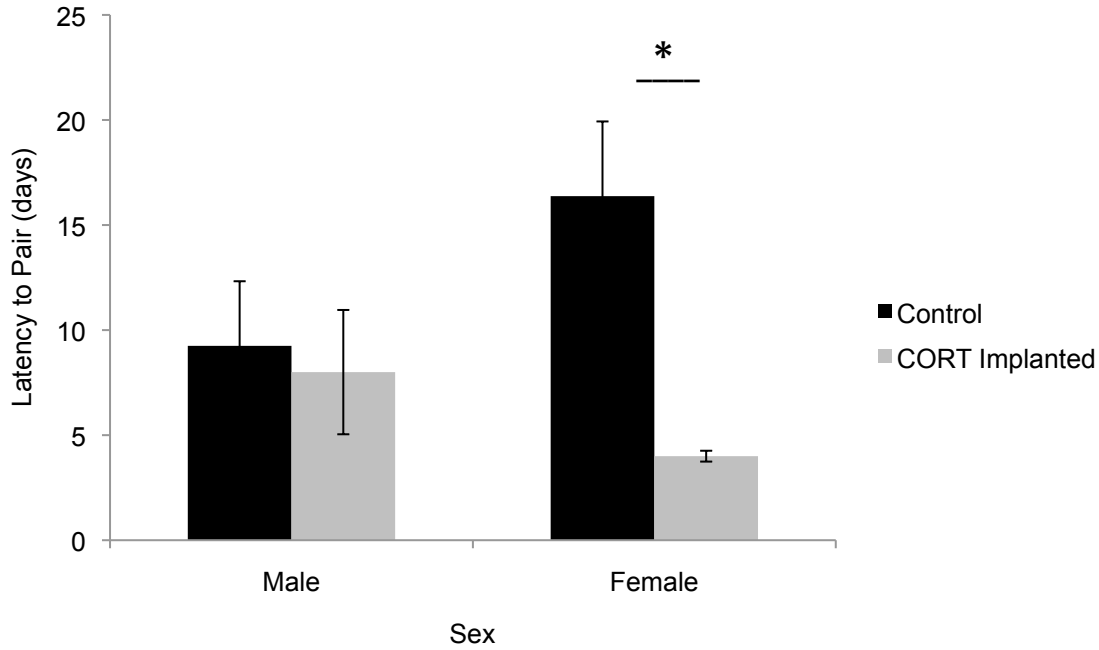


Figure 11. Effects of Chronic Corticosterone Treatment on the Latency to Pair in a Study of the Effects of Chronic Corticosterone Administration on Pairing in Zebra Finches. There was no significant effect of treatment on latency to pair in males ($p > 0.05$), but there was a significant effect of treatment to decrease latency to pair in females as indicated with * ($p < 0.05$). Error bars indicate SEM. CORT = corticosterone.

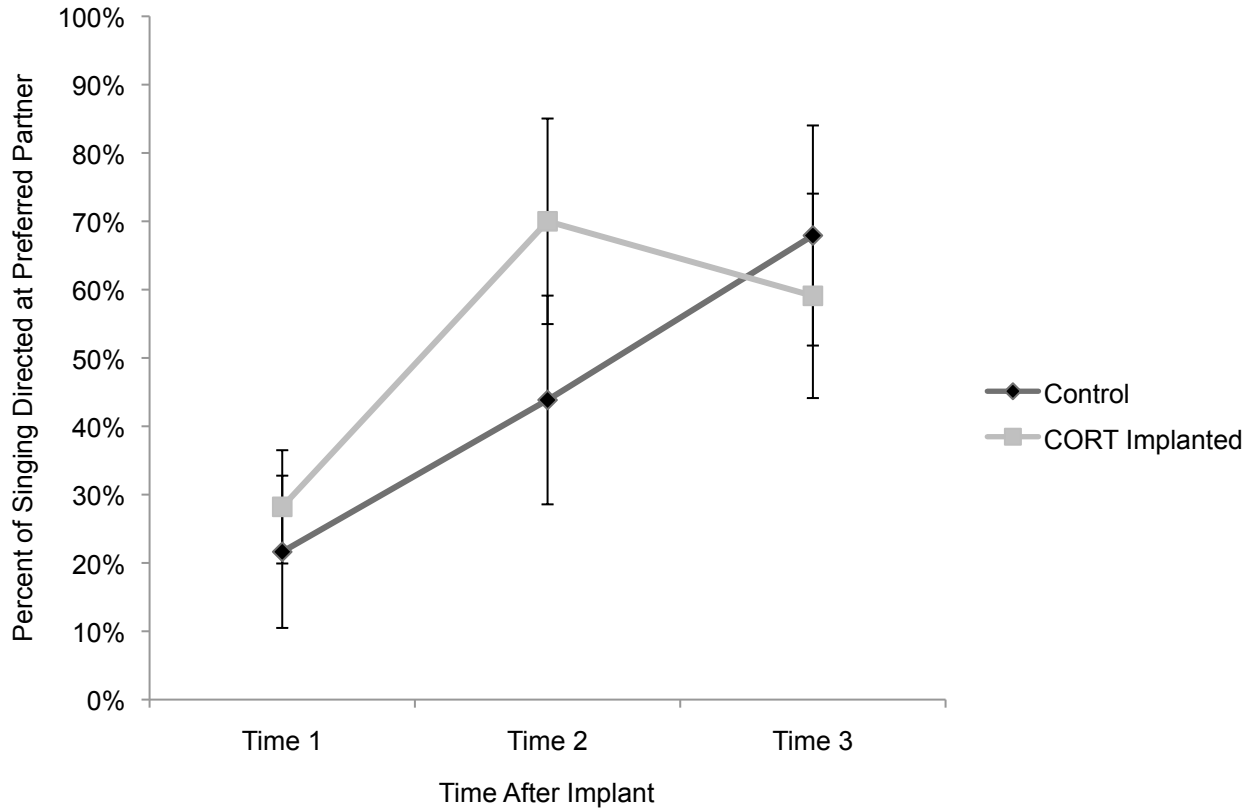


Figure 12. Effects of Chronic Corticosterone Treatment on Directed Singing in Males in a Study of the Effects of Chronic Corticosterone Administration on Pairing in Zebra Finches. Percentage of singing to the preferred partner increased across time, but there was no significant effect of treatment ($p > 0.05$) or interaction between treatment and time. Error bars indicate SEM. CORT = corticosterone.

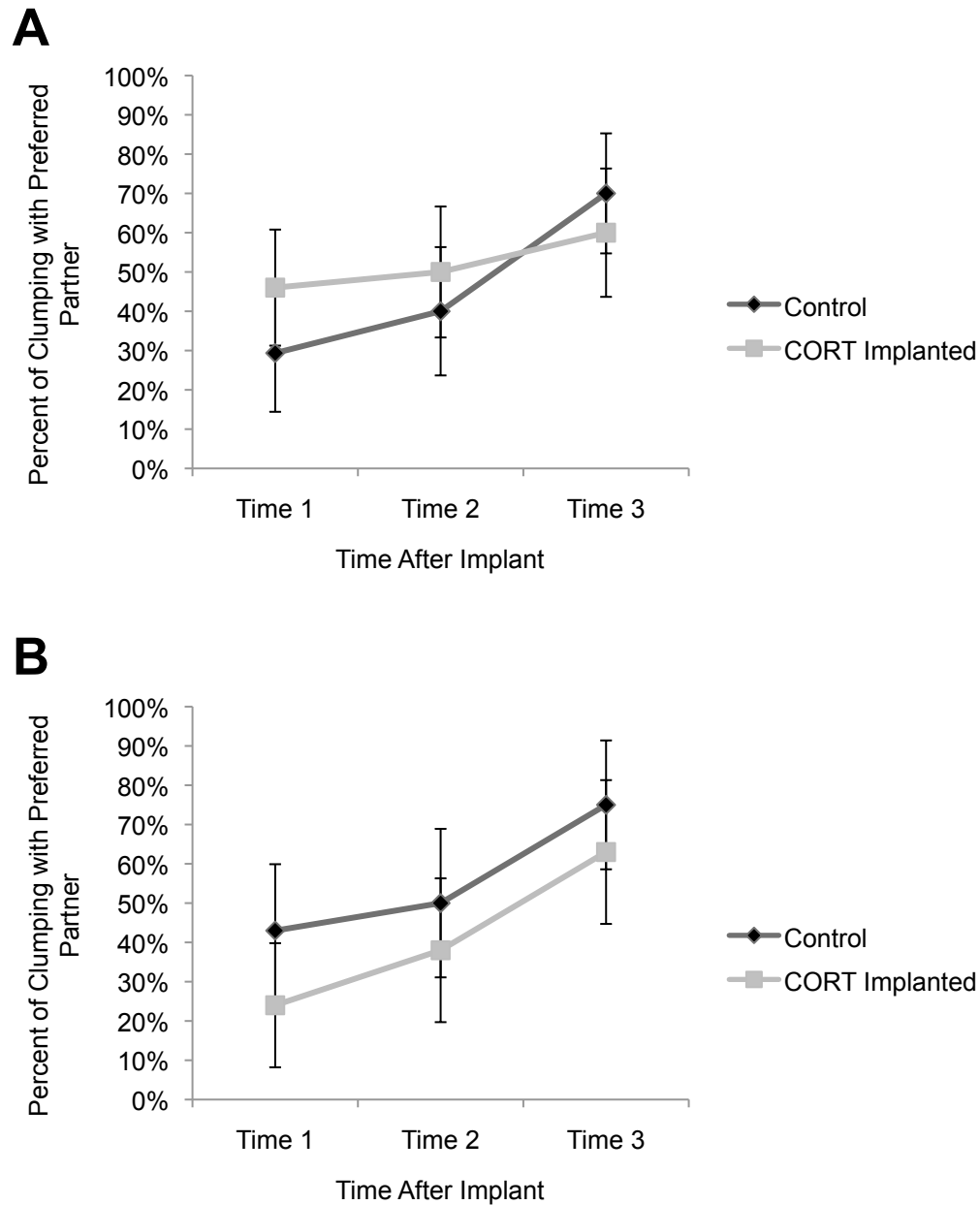


Figure 13. Effects of Chronic Corticosterone Treatment on Clumping in a Study of the Effects of Chronic Corticosterone Administration on Pairing in Zebra Finches. Percentage of time spent clumping with the preferred partner increased over time for B) females ($p < 0.05$), but not for A) males ($p > 0.05$). There was no significant effect of treatment or interaction between treatment and time for either sex ($p > 0.05$). Error bars indicate SEM. CORT = corticosterone.

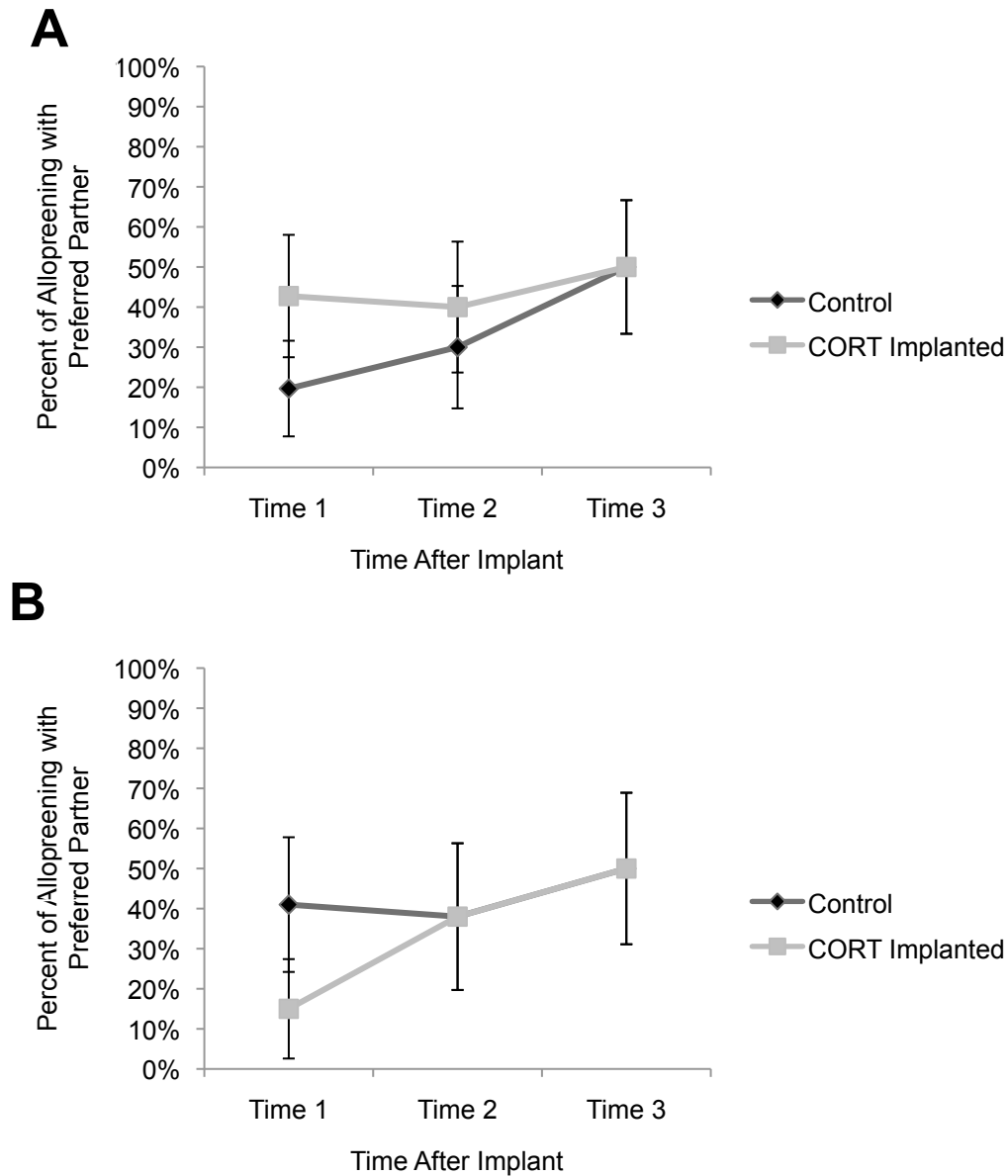


Figure 14. Effects of Chronic Corticosterone Treatment on Allopreening in a Study of the Effects of Chronic Corticosterone Administration on Pairing in Zebra Finches. In both A) males and B) females, there was no significant effect of time, treatment or interaction between time and treatment for the percentage of time spent allopreening with the preferred partner ($p > 0.05$).

Error bars indicate SEM. CORT = corticosterone.

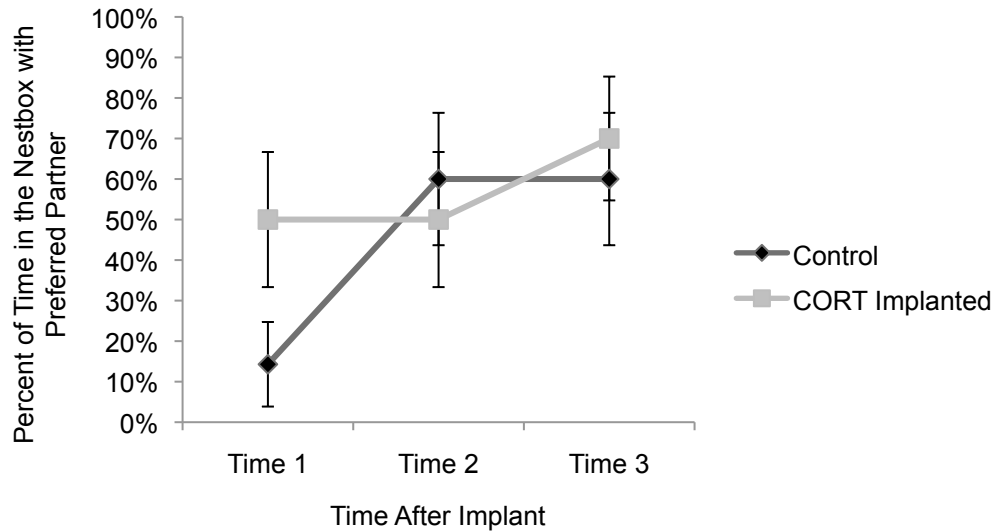
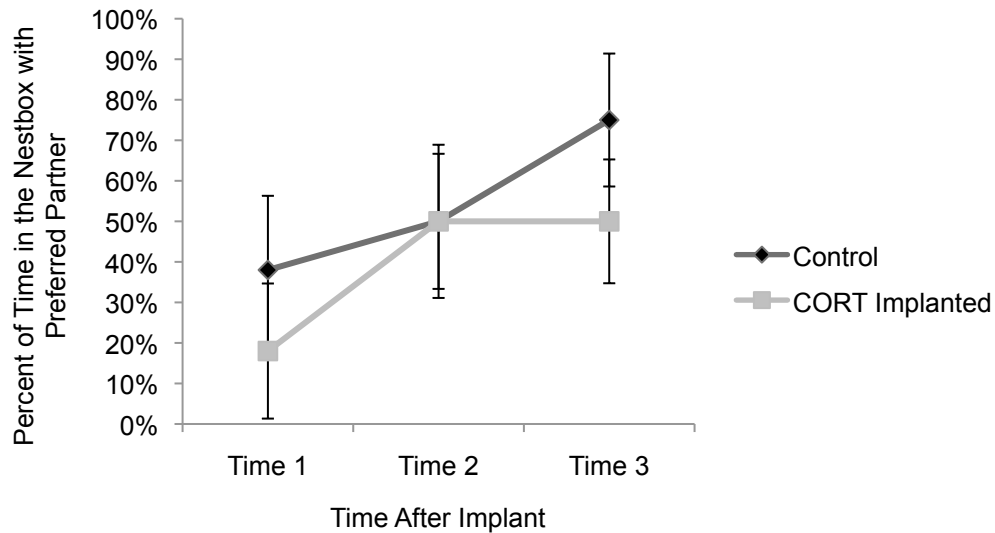
A**B**

Figure 15. Effects of Chronic Corticosterone Treatment on Nestbox Cohabitation in a Study of the Effects of Chronic Corticosterone Administration on Pairing in Zebra Finches. The percentage of time spent in the nestbox with the preferred partner increased significantly over time for A) Males and B) Females ($p < 0.05$), but there was no effect of treatment or time by treatment interaction ($p > 0.05$). Error bars indicate SEM. CORT = corticosterone.

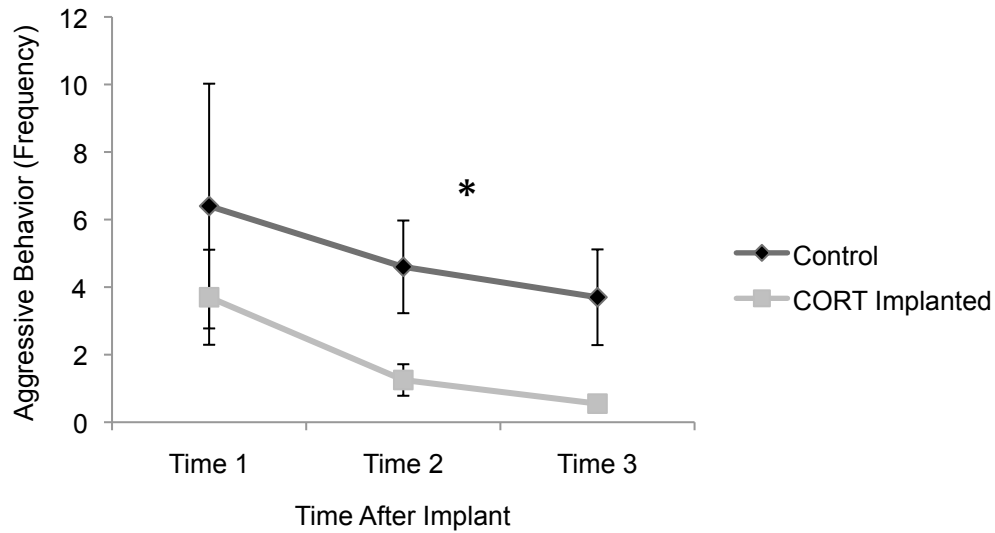
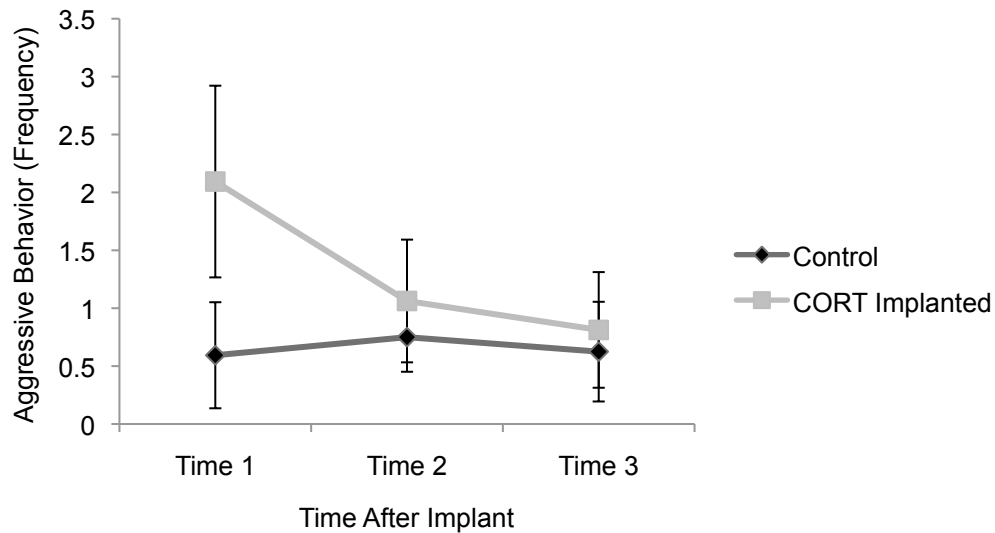
A**B**

Figure 16. Effects of Chronic Corticosterone Treatment on Aggressive Behavior in a Study of the Effects of Chronic Corticosterone Administration on Pairing in Zebra Finches. A) There was a significant effect of treatment on aggression in males ($p < 0.05$). Post-hoc analysis revealed a significant effect only at Time 3 ($p < 0.05$, indicated by *). B) There was no significant effect of treatment in females ($p > 0.05$). There was no effect of time or time by treatment interaction ($p > 0.05$) in either sex. Error bars indicate SEM. CORT = corticosterone.

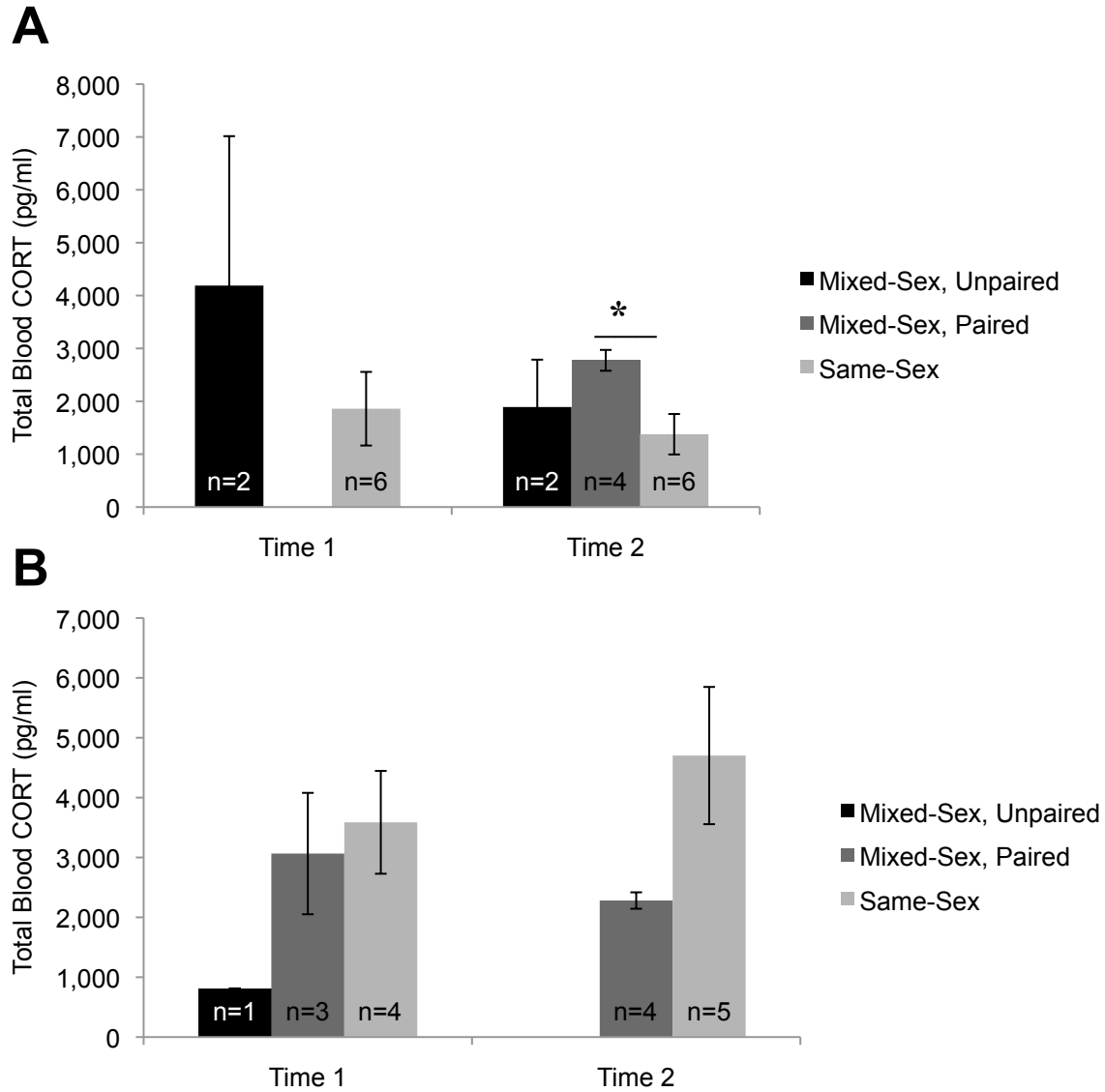


Figure 17. Effects of Pairing Condition on Total Blood Corticosterone in a Study of the Effects of Pairing on Stress Response Physiology in Zebra Finches. A) In males, there was no significant effect ($p > 0.05$) of Pairing Condition on Blood CORT at Time 1, but there was a significant difference between the Mixed-Sex, Paired Group and the Same-Sex Group at Time 2 ($p < 0.05$). B) In females, there was no significant effect of Pairing Condition on Blood CORT at Time 1 or Time 2 ($p > 0.05$). Error bars indicate SEM. CORT = corticosterone.

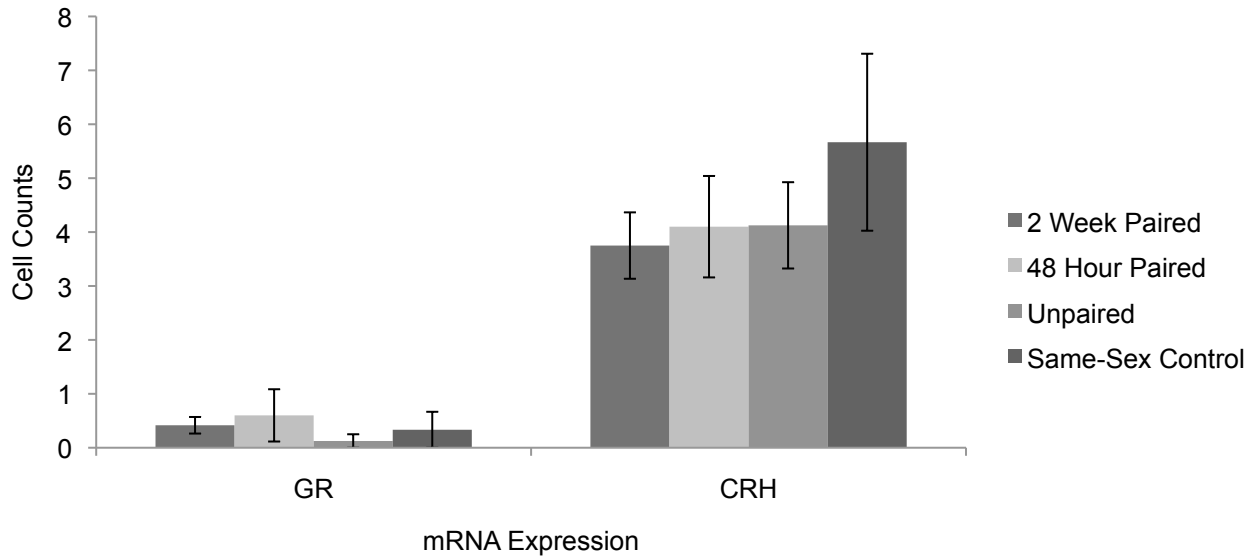
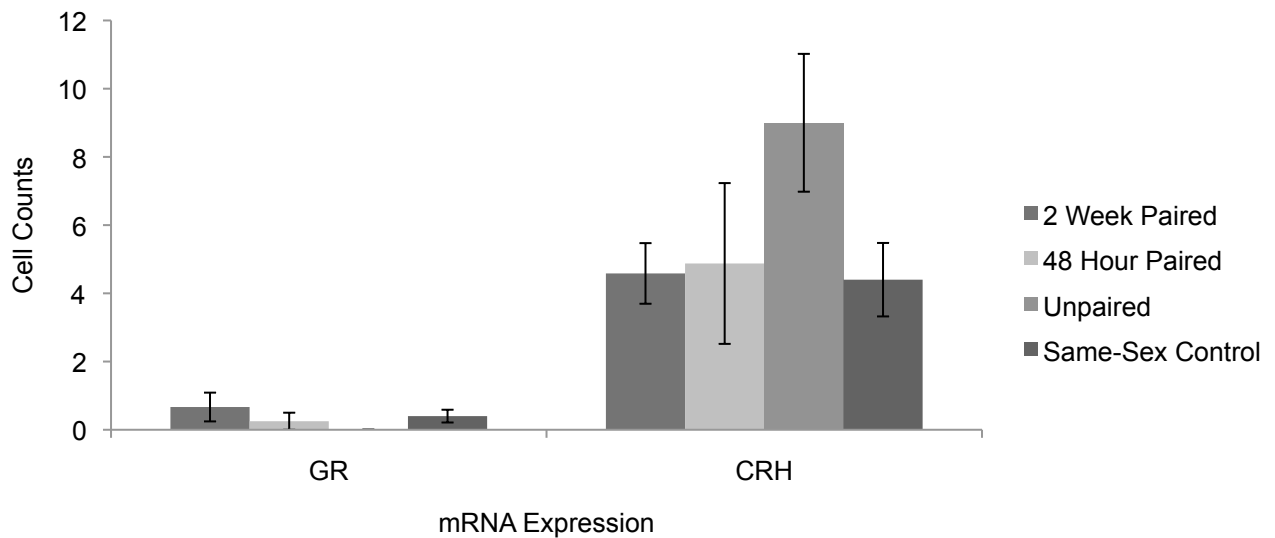
A**B**

Figure 18. Effects of Pairing Condition on Expression of Glucocorticoid Receptor (GR) and Corticotropin Releasing Hormone (CRH) mRNA in the Paraventricular Nucleus of the Hypothalamus in a Study of the Effects of Pairing on Stress Response Physiology in Zebra Finches. A) In males and B) females there was no significant effect of pairing condition on expression of GR or CRH ($p > 0.05$). Error bars indicate SEM.

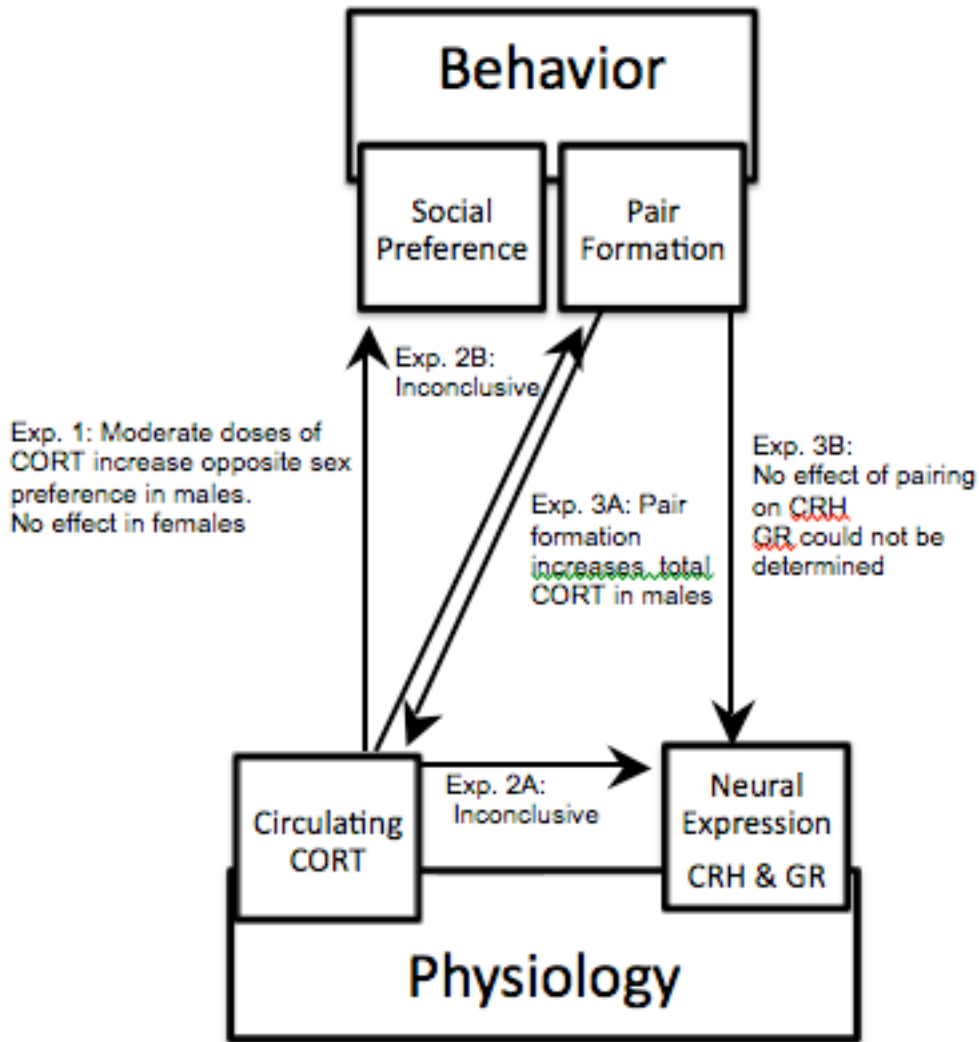


Figure 19. Summary of Findings. The theoretical relationships between constructs of interest are depicted and indicated as supported or not based on the experiments in this dissertation. In males, CORT treatment increases preference for the opposite sex and pair formation increases circulating total CORT. There is no effect of CORT on preference for the opposite sex and pair formation decreases circulating free CORT. The results from Experiment 2 were unclear, due to questionable treatment efficacy. Furthermore, the effects of pairing of GR mRNA could not be determined due to insufficient staining. CORT = corticosterone, CRH = corticotrophin releasing hormone, GR = glucocorticoid receptor.

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ABSTRACT**STRESS AND SOCIAL RELATIONSHIPS: THE ROLE OF CORTICOSTERONE IN THE FORMATION AND MAINTENANCE OF PAIR BONDS IN THE MONOGAMOUS ZEBRA FINCH (*Taeniopygia guttata*)**

by

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Stress and affiliative social relationships are bi-directionally related. It is well understood that social relationships can buffer the physiological response to a stressor. Conversely, there is some evidence to suggest that stress can affect the propensity to form a pair bond. Data from humans and prairie voles, monogamous rodents, suggest that activation of the hypothalamic-pituitary-adrenal axis (HPA) in males increases attraction for females. Across species, females are understudied, but evidence from the prairie vole suggests HPA activation impairs formation of partner preference, the first step in establishing a pair bond. Across taxa there is evidence that formation and maintenance of a pair bond may also increase HPA activity in both sexes. The studies presented here explored the relationships between circulating corticosterone, neural expression of corticotropin releasing hormone and glucocorticoid receptors, social preference and pair bonding using a model species uniquely suited to studying long-term, monogamous relationships.

AUTOBIOGRAPHICAL STATEMENT

While attending Crookston High School (Crookston, MN) I took four semesters of classes at the University of Minnesota – Crookston. Upon graduation in 2002, I enrolled at Northern Michigan University in Marquette, MI where I majored in Psychology double minored in Human Biology and Human Nutrition. While at Northern, I studied a mouse model of Tinnitus under the direction of Dr. Cynthia Prosen and graduated magna cum laude in 2005. I then studied Humanistic Psychology at the University of West Georgia (Carrollton, GA) for one year before enrolling in the Behavioral and Cognitive Neuroscience program at Wayne State University in Detroit, MI. I completed my Masters of Arts degree in 2010 with Dr. Donald Coscina. My master's research was an exhaustive study of the effect of agonists and antagonists specific to corticotropin releasing hormone receptors 1 and 2 in a rat model. Following completion of my masters, I began to work with Dr. Michelle Tomaszynski on understanding the effect of stress on social behavior in the monogamous zebra finch.