Sexual Dimorphism of Brain and Behavior: The Role of Testosterone in Female Zebra Finch Behavior (*Taeniopygia gutatta*)

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As thesis advisor for Chelsea Gilpin, I have read this paper and find it satisfactory.

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Précis

Steroid hormones such as testosterone are produced by the sex organs as a result of stimulation of the hypothalamic-pituitary-gonadal (HPG) axis and travel through the bloodstream to tissues of the body (Goymann and Wingfield 2014). These signaling molecules regulate the transcription and expression of genes, leading to adjustments in physiology, morphology, and behavior, crucial for mediating responses to changes in the environment (Ketterson et al. 2005, Charlier 2009, Goymann and Wingfield 2014). The role of testosterone in the regulation of behavior has been extensively studied with respect to male aggression and paternal investment (Hegner and Wingfield 1987, Wingfield et al. 1987, Langmore et al. 2002, Cain and Ketterson 2013). However, the relationship between testosterone and behavior in females is still being investigated (Foecking et al. 2008).

I chose to undertake behavioral research, specifically behavioral endocrinology (the study of how hormones affect behavior), because I have always held an interest in animal behavior and its underlying causes. I observed the locomotor, aggressive and nesting behavior of female zebra finches (*Taeniopygia gutatta*) in mated pairs during the nesting period in two different social contexts: in the presence of another female of the same species, a socially unstable environment shown to increase testosterone production in other songbird species, (Langmore et al. 2002, Cain et al. 2011) and in the absence of a female conspecific. To determine the possible role of testosterone in the mediation female behavioral responses to an intruder, I measured the capacity of females to produce testosterone in response to GnRH (gonadotropin releasing hormone) injections. GnRH is a hormone of the HPG axis that stimulates the signaling cascade resulting in the production of testosterone (Cain and Ketterson 2012, Cain and Ketterson 2013, Rosvall et al. 2013). I hypothesized that if testosterone mediates the response

of female zebra finches to a same sex intruder (proposed as a less invasive mechanism to increase testosterone production), females would be expected to display an increase in locomotor and aggressive behaviors toward a female conspecific and a decrease in nesting behavior.

The results of this study showed no significant difference in plasma testosterone concentration between GnRH-treated and control females. Similarly, the presence of a female conspecific did not cause a significant change in the frequency of any behaviors observed in this study. The lack of a testosterone increase in response to GnRH suggests that testosterone most likely did not mediate the behaviors observed in this study and that age and stress may have skewed results.

Future research would benefit from exploring the effects of age and stress on female testosterone production as well as the effects of age on laying frequency. Comparing the behavioral responses to GnRH injections of free-living female zebra finches as well as individuals in captivity could also yield relevant results regarding the question of regulation of female reproductive behavior by gonadal steroid hormones.

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I. INTRODUCTION

The HPG Axis, Hormones and the Body

The hypothalamic-pituitary-gonadal (HPG) axis is a well-studied neuroendocrine system in vertebrates. Actions of the HPG axis include the production of sex steroid hormones, including the androgen testosterone, that regulate physiology, morphology and behavior in vertebrates (Charlier 2009, Rosvall et al. 2013, Goymann and Wingfield 2014). The activation of the HPG axis and the production of gonadal steroids begin with the perception of external or internal stimuli that regulate the HPG axis (Cain and Ketterson 2013, Rosvall et al. 2013, Goymann and Wingfield 2014). The hypothalamus is stimulated to release or inhibit the release of GnRH, (gonadotropin releasing hormone) which travels to the pituitary gland (Cain and Ketterson 2012, Cain and Ketterson 2013, Rosvall et al. 2013). The anterior portion of the pituitary releases the gonadotropins LH (luteinizing hormone) and FSH, (follicle stimulating hormone) which travel via the bloodstream to the gonads where they regulate testosterone production and release (Charlier 2009, Cain and Ketterson 2013, Rosvall et al. 2013, Goymann and Wingfield 2014). Testosterone molecules travel through the blood and affect target cells mostly through binding to nuclear androgen receptors (Ketterson et al. 2005, Foecking et al. 2008, Cain and Ketterson 2012, Cain and Ketterson 2013, Rosvall et al. 2013).

There are multiple courses of action once testosterone has entered a cell (Ketterson et al. 2005, Gorman and Wingfield 2014). It can bind directly to androgen receptors to regulate gene transcription, or be converted into estradiol by the enzyme aromatase (Foecking et al. 2008, Goymann and Wingfield 2014). Testosterone can also be converted into non-aromatizable forms such as dihydrotestosterone, which also binds to androgen receptors

(Goymann and Wingfield 2014). Through these courses of action, testosterone acts to regulate gene transcription, leading to changes in physiology, morphology and behavior (Ketterson et al. 2005, Charlier 2009, Goymann and Wingfield 2014).

Hormones traveling through the bloodstream regulate various physiological functions (Goymann and Wingfield 2014). An individual's response to the actions of the HPG axis (e.g., amount of sex steroids released) depends on many intricate variations such as hormone sensitivity at each stage of the HPG signaling cascade as well as hormone concentrations secreted (Rosvall et al. 2013, Goymann and Wingfield 2014). For example, variation in the number of pituitary GnRH or gonadal LH receptors could alter testosterone production (Rosvall et al. 2012, Rosvall et al. 2013). The HPG axis is also regulated by negative feedback loops, in which sex steroids inhibit GnRH or LH release at the levels of the hypothalamus or pituitary gland (Rosvall et al. 2013).

Across vertebrate taxa, the actions of neuroendocrine regulatory mechanisms are highly conserved (Goymann and Wingfield 2014). In general, sex steroids can exhibit either organizational or activational effects on the body (Arnold 2009). In the vertebrate brain, an organizational effect generally occurs early in development during a sensitive period to permanently alter organ function such as the brain's response to hormones (Arnold and Breedlove 1985, Arnold 2009). It has been suggested that organizational effects occur early in development when the brain is most sensitive to morphological changes such as axon dendrite length and cell synapse production (Arnold 2009). In contrast, activational effects are those that result in transient and reversible changes when hormones are secreted during adulthood (Arnold 2009).

Testosterone and Sexual Dimorphism

The most straightforward support of the organizational-activational hypothesis comes from the development of sexual dimorphism, or any other difference in characteristics between males and females of the same species (Wade and Arnold 2004). It has also been suggested, however, that sex-determination genes (XY in mammals, ZW in birds) are important in this process as well (Arnold 2009). During early development, androgens and estrogens act to differentiate primary sexual characteristics (e.g., gonadal development) as well as secondary sexual characteristics, including behavior (Foecking et al. 2008, Arnold 2009).

The relationship of testosterone and the HPG axis to physiology and behavior has historically been studied in males, while its role in females has not been at the forefront of behavioral endocrinology research (Viega et al. 2004, Ketterson et al. 2005, Foecking et al. 2008). However, experiments to determine the extent of the organizational effects of androgens during development and the activational effects in adults on primary and secondary sex characteristics have been performed to study the significance of testosterone in normally functioning females (Foecking et al. 2008). In mammalian systems, it has also been found that testosterone is responsible for the expression of male secondary sex characteristics, while the lack of testosterone is required to develop female sex organs (Arnold and Breedlove 1985). After this discovery, the "masculinizing" powers of testosterone in females has also been demonstrated in other vertebrates, including the development of male-like genitals after fetal treatment with testosterone in sheep (Roselli et al. 2011) and the development of hemipenes in adult female leopard geckos (Rhen et al. 1999).

A large body of research has been accumulated on the role of testosterone in male

sexual and aggressive behavior (Cain and Ketterson 2012, French et al. 2013). Aggression is well accepted by the scientific community as a testosterone-mediated behavioral trait of male vertebrates, and there are several studies that suggest that testosterone also mediates aggressive behavior in females (Langmore et al. 2002, Clotfelter et al. 2004). According to Cain et al. (2011), aggression can be defined as, "any overt fighting behavior or signal of imminent behavior with the capacity to harm" (Cain et al. 2011). A notable experiment performed by Phoenix et al. (1959) demonstrated that fetal treatment with testosterone led to the development of male-like sexual behavior in female guinea pigs (Arnold 2009). Activational effects of experimentally elevated testosterone in females have manifested in increased aggression in female leopard geckos (Rhen et al. 1999). Testosterone levels in the feces of female baboons have been shown to also correspond to levels of female social aggression (Beehner et al. 2005, Cain and Ketterson 2012).

Evidence has been presented that suggests testosterone mediates trade-offs in parental investment in current and possible future offspring in males and females (Fite et al. 2005). For example, in female marmosets, testosterone levels in urine were correlated to a decrease in maternal investment in young (Fite et al. 2005) and high circulating levels of androgens in female red squirrels yielded a similar result (Dantzer et al. 2011).

The Role of Testosterone in Female Songbird Behavior

The activational effects of increased testosterone on secondary sexual characteristics, such as aggression and parental behavior, have been well studied in male songbirds (Hegner and Wingfield 1987, Wingfield et al. 1987). While the activational role of testosterone in females is not as well understood, it has been suggested that testosterone may affect females

similarly to males, as both secrete testosterone and have androgen receptors in various tissues including the brain (Langmore et al. 2002, Cain and Ketterson 2013, Goymann and Wingfield 2014). However, not much is known about the exact mechanism through which testosterone operates to mediate the expression of "male-like" (or female-specific) characteristics in females (Goymann and Wingfield 2014).

In socially monogamous free-living dark-eyed juncos, a songbird species with biparental care, both males and females respond to experimental elevation of testosterone with increased intrasexual aggression, suggesting that testosterone may induce aggression in both sexes (Cain and Ketterson 2012). Junco females have been observed to be most aggressive toward simulated predators near the nest site, less aggressive toward intruders of the same sex, and least aggressive toward other males (Cain et al. 2011). Cain and Ketterson (2012) showed that female dark-eyed juncos that produced more testosterone in response to GnRH injections were more aggressive and had lower latency to show an aggressive response than females that produced less testosterone (Cain and Ketterson 2012).

These observations were evaluated in an evolutionary context. Those females with higher testosterone secretion were able to obtain higher quality mates, maintained paternal investment and defended mates and nesting sites from intruders, suggesting there may be fitness benefits for higher testosterone production in females (Cain et al. 2011, Cain and Ketterson 2012, Cain and Ketterson 2013). This hypothesis (known as the challenge hypothesis and proposed initially for males (Wingfield et al. 1990)) is supported by the observation that females are more aggressive in colonial breeding and socially monogamous species where competition for mates and paternal investment may be elevated (Ketterson et al. 2005,

Tomaszycki et al. 2006, Goymann and Wingfield 2014). However, when controlled for phylogeny this correlation was not found (Goymann and Wingfield 2014). Similar trends have also been observed in female spotted starlings (Veiga et al. 2004) and female tree swallows (Rosvall 2013). Intrasexual competition in female dunnocks has also been shown to elevate testosterone levels (Langmore et al. 2002, Cain et al. 2011).

Parental care has been evaluated in both sexes of songbirds for the activational effects of testosterone. According to Cain and Ketterson (2013), traits such as aggression are also associated with a decrease in maternal effort (Cain and Ketterson 2013). It was found that free-living dark-eyed junco females able to produce higher circulating testosterone in response to GnRH challenge spent less time brooding nestlings (Cain and Ketterson 2013). However, there was no correlation between increased testosterone and incubation time in juncos (Clotfelter et al. 2004). In contrast, tree swallow females showed a reduction in incubation time in response to experimentally elevated testosterone (Rosvall 2013).

There is debate as to the relationship between increased circulating testosterone and "male-like" behaviors such as aggression and parental investment in female songbirds. Goymann and Wingfield (2014) suggest that elevated testosterone likely does not interfere with maternal care because of the size discrepancy between male and female testosterone peaks (Goymann and Wingfield 2014). Moreover, hormone implants and GnRH challenges may elevate testosterone to levels higher than would be experienced in nature. Also, studies that combine data from free-living and captive birds may be confounded by the observation that plasma testosterone levels are significantly affected by captivity (Calisi and Bentley 2009).

Testosterone and the Female Zebra Finch

Native to Australia, the zebra finch (*Taeniopygia gutatta*), is a seasonal and communally breeding songbird that creates socially monogamous pair bonds and participates in biparental care (Case 1986, Adkins-Regan and Robinson 1993, Zann 1996, Tomaszycki et al. 2006). Males are known to participate in extra-pair copulations with other females in the breeding colony (Case 1986, Adkins-Regan and Robinson 1993, Zann 1996, Tomaszycki et al. 2006).

In this species, only males produce song due to various dimorphisms of the brain and vocal organ (Wade and Arnold 2004). The activational and organizational effects of sex steroids on the neurobiology of the zebra finch song and song system have been of specific interest to researchers (Gurney and Konishi 1980, Nespor et al. 1996, Wade et al. 1996). However, the role of hormones in controlling sex-specific differences in aggression and parental behavior has not been extensively investigated.

The existing research on the effects of experimentally elevated testosterone levels in zebra finches at times contradict similar studies performed on other species of songbird. In domesticated zebra finches, defense of mate and nest sites as well as a propensity toward intrasexual aggression toward “rival” females have been documented (Case 1986, Adkins-Regan and Robinson 1993, Tomaszycki et al. 2006). Aggressive behaviors have been documented more frequently during nesting periods in which eggs and young were present (Case 1986). However, males still displayed more aggressive behavior and a greater decrease in paternal investment compared to females (Case 1986). Female zebra finches have also been shown to respond to experimentally increased testosterone with increased aggression (Ketterson et al. 2005).

II. THESIS ACTIVITY

The purpose of my study is to contribute to a growing body of knowledge concerning testosterone and the behavior of female songbirds. The objectives of my study were to measure changes in the frequency of locomotor behaviors, intrasexual aggression and levels of maternal investment in female zebra finches in response to the presence of an "intruding female" as well as to measure the ability of female zebra finches to respond to a standard GnRH challenge. There is not a large volume of current literature examining activity (locomotor) levels in birds, however this behavior was chosen for observation because testosterone has been shown to increase activity levels in female dark-eyed juncos (Clotfelter et al. 2004).

My study had two objectives. First, I hypothesized that females would respond to the intrusion of a female conspecific (shown to increase testosterone production in other songbird species) with increased activity and aggression and reduced nesting behavior (incubation). Second, I hypothesized that GnRH challenges elevate testosterone levels in the female zebra finch.

III. MATERIALS AND METHODS

This study took place in Pullman, WA on the Washington State University campus in the Schwabl Laboratory between December 2013 and March 2014.

Study Species

Subjects were captive domesticated zebra finches (*Taeniopygia gutatta*). The study birds were born and raised in the lab and were between 3 and 5 years of age. All males and females were wild-type grey plumage morphs with the exception of one female, which was a fawn morph. Individuals were identified by numbered metal or colored plastic leg bands.

Housing

Pairs of males and females were kept in separate wire cages (n=34) in a colony room, each labeled with cage number, birds' identification numbers and the parents of each individual. 46 cm x 22 cm x 26.5 cm wire cages were kept on two metal shelves 106 cm and 152 cm off the ground approximately 8 cm apart. Each cage was furnished with a woven wooden nesting basket, 2 horizontal wooden perches on either side of the cage, a wooden and wire swing as well as a piece of cuttlebone. Food and water were distributed daily to each cage in plastic cups hung from the sides of the cage. Nesting material in the form of newspaper was provided on the floor of each cage and was replaced weekly or as needed. The room housing breeding pairs was kept at an average 21 degrees Celsius and an average humidity of 20% with a 14:10 light/dark cycle.

Behavioral Video Recording

All video recording took place in the early afternoon between the months of January and March 2014. The GoPro HERO3+ camera was chosen for this experiment for its small size, high resolution, quiet operation and ease of video file storage. All behavioral videos were recorded in a room adjacent to the colony room, which provided visual and partial auditory isolation from the colony. Preliminary observations in December 2013 showed that the repertoire of behaviors exhibited by the breeding pairs in isolation did not differ from that exhibited while in the colony room, therefore experimental videos were recorded in isolation to control for visual interactions with other individuals. Each video was recorded on the day the first egg in a clutch was laid and lasted between 40 to 50 minutes in length. This timing was also chosen because it has been shown that zebra finches are most aggressive during the nesting

period (Case 1986).

Two types of videos were recorded for this experiment: breeding pairs alone and breeding pairs with the introduction of an "intruding" female (referred to as lure female from this point on). Each breeding pair was recorded alone for observation of basal aggressive, nesting and locomotor activity as well as with a lure female to determine an increase or decrease in the frequency of these behaviors.

In both types of videos, cages were placed between two pieces of green tape on a metal shelf in the isolation room similar to the colony room 106 cm off of the ground in front of the camera mounted 23 cm from the cage. Eggs were removed, catalogued and replaced with plastic dummy eggs prior to filming. In order to differentiate video files, each cage was labeled with the date, time of recording, cage number and identification of the female(s) being observed as well as any morphological characteristics allowing distinction between them.

When recording a video of a breeding pair with a lure female, the breeding pair cage was placed in isolation before the introduction of the lure female. Lure females were removed from their cage and introduced into the cage of the breeding pair in question. Lure females were chosen randomly from the breeding colony based on morphological characteristics that would allow for distinction from the breeding pair female.

Behavioral Analysis

After recording, each video file was burned to a DVD and labeled with the date the video was taken, the cage and ID number of the breeding pair female as well as the lure female. Behavioral analysis for each video began at the time the experimenter left the isolation room or directly when the video started (activated by experimenter outside of isolation room via

remote control). Each video was scored with an ethogram of behaviors derived from Cain and Ketterson (2011) and Zann (1996) (Table 1).

All behaviors recorded were those initiated by the female of the mate pair. When the breeding pair was recorded alone, the duration and number of incidences of behaviors included pecking and chasing as well as duration of nesting behavior (egg incubation) and frequency of short and long range locomotion were recorded. When the lure female was introduced, all previously mentioned behaviors were recorded as well as pecking and chasing the lure female. A peck was defined as the female attacking the conspecific's bill or head with a closed bill. Chasing was defined as the female following the conspecific for multiple bouts of short or long-range locomotion. Short-range locomotion was defined as a hop or flight spanning less than half of the length of the cage. Long-range locomotion was defined as a flight spanning a distance of half or more than half the length of the cage. Nesting was defined as entering the nest cup with entire body.

Steroid Hormone Assays

GnRH challenges were performed on (n=12) females in the breeding colony in March and April of 2013 and were analyzed to determine the ability of each female to produce testosterone. Challenges were administered on the day of the first egg laid in each clutch between the hours of 1200 and 1600. Females were captured and blood (approximately 100 µL) was collected from the wing vein. Immediately after bleeding, the female was injected with either 50 µL phosphate-buffered saline (PBS) alone or 1.25 µL GnRH in 50 µL of PBS in the pectoral muscle. The female was then placed in a cloth bag in a quiet room for the duration of the experiment. Approximately 30 (median = 32 min., range 31-40 min.) and 60 minutes after

injection, second and third blood samples were collected. Plasma was separated by centrifugation at 6000g for 10 minutes and stored -20 degrees Celsius until analysis.

Testosterone assays were performed in February and March 2014 according to the protocol stated in Schwabl (2003), with some slight variations. Testosterone was extracted from plasma samples with diethyl-ether. Diatomaceous earth long-column chromatography was performed to separate testosterone, which was measured using radioimmunoassay (Schwabl 2003).

Statistical Analysis

Differences in behavior frequency between control and lure female videos were analyzed using paired 1-tail t-test. All t-tests were performed using XLSTAT. Hormone concentrations were transformed using a natural log transformation. Differences between bleed times and treatment groups of hormonal plasma concentrations were analyzed using a two-factor nested ANOVA. Within an individual, the testosterone plasma concentration from before the GnRH challenge was subtracted from both the concentrations at both 30 and 60 minutes, to ensure that it was the difference in plasma concentration that was evaluated. These values were then analyzed using a two-factor ANOVA. All ANOVA analyses were performed using JMP 8.0 SAS (Cary, North Carolina).

IV. RESULTS

Behavioral Observations

During the period of experimentation, 5 breeding pairs (Cages 2, 8, 10, 13, 16) laid two clutches allowing for the video recording of pairs alone and with a lure female. Three different responses to the introduction of an intruding female were observed with respect to locomotor

activity: a decrease in frequency of both long and short range locomotion, an increase in the frequency of short range flights and a decrease in frequency of long range flights, and an increase in the frequency of both long and short range locomotor activity. The female that exhibited the third response remained completely stationary prior to lure female introduction. Although variation in behavioral response was observed, there was no significant difference in the frequency of long and short range flights with the addition of the lure female (p=0.255 and p=0.176 respectively) (Figure 1).

Three females exhibited pecking behavior, two of which were directed toward the mate. There was one observed instance of intrasexual aggression in which the female (8) pecked the lure female while on top of the nest cup. There was no significant difference in frequency of pecking behaviors found (Peck Male p=0.187, Peck Lure p=0.187) (Figure 2).

There were no observed instances of nesting or chasing behavior. In every pair, the male demonstrated bouts of aggressive behavior toward the lure female, which subsided as time passed. Every male also attempted to mount the lure female, however the lure female was always uncooperative.

Hormone Analysis

There was no significant difference among testosterone concentrations at any bleed time or between the testosterone concentrations of the two treatment groups ($F_{5,31}$=1.19, p=0.3349) (Figure 3). The difference in testosterone concentrations between the second and first bleed and the third and first bleed were calculated within individual females. When using the difference in testosterone concentrations, there was no significant difference between control and treatment injected birds or among bleed times. ($F_{3,19}$=0.598, p=0.62) (Figure 4).

There was a trend toward decreasing testosterone concentrations in the control group 30 and 60 minutes post-bleed. This did not occur in the treatment group (Figure 4).

V. DISCUSSION

Based on previous zebra finch and other songbird literature, I hypothesized that females would respond with increased aggression and activity and reduced nesting behavior to the introduction of a female conspecific into the home cage. I also hypothesized that this response would be mediated by a transient increase in plasma testosterone concentrations (not measured in my study). I predicted that GnRH injections would result in a transient increase in plasma testosterone concentrations. I found that the introduction of a lure female into the mating pair cage did not cause a significant change in the frequency of any behaviors observed in any simple, straightforward way. GnRH challenges did not result in a significant increase in plasma testosterone concentrations compared to control injected females. Although not the direct focus of this study, the observation of attempted extra pair copulations and male aggression toward the lure female were consistent with current literature on zebra finches (Case 1986, Adkins-Regan and Robinson 1993, Zann 1996, Tomaszycki et al. 2006).

Aggression

There are several facts that could explain the lack of aggressive response toward the lure female and rejection of my hypothesis. First, the dimensions of the test cages may have influenced behavioral responses (Case 1986). Second, moving birds to the novel surroundings of the isolation room for behavioral observations may have affected results, possibly leading to decreased aggressive behavior. There is evidence that acute stress, characterized by increased levels of the steroid hormone corticosterone, in male rufous-winged sparrows was associated

with a decrease in plasma testosterone in response to capture and restraint (Deviche et al. 2012). A study of male zebra finches showed that even when isolated with the paired female, isolation from the flock in a novel environment is enough to cause elevated concentrations of corticosterone (Banerjee and Adkins-Regan 2011). If an acute stress response and associated increases in corticosterone levels influence testosterone production similarly in females and males, stress induced by my experimental design could have resulted in low levels of testosterone levels and lack of intrasexual aggression toward the lure female. However, the effect of acute stress and corticosterone on testosterone levels and behavior has not been studied in either sex of the zebra finch.

It is possible that testosterone may not play a role in female aggressive behavior (Rosvall et al. 2012) or that circulating androgen levels are not a good indicator of its importance. For example, a study by Rosvall et al. (2012) found that while levels of expression of androgen receptors and aromatase in the brain were predictors of aggression in both sexes in dark-eyed juncos, circulating testosterone levels did not predict aggressiveness in females (Rosvall et al. 2012). Although traits such as aggression could be beneficial to monogamously mated females in the context of acquiring and defending breeding resources, increased aggressive encounters could mediate tradeoffs such as increased stress and decreased immune function which decrease fitness (Rosvall 2008). As high testosterone levels are suggested to be "costly" to females, it may be evolutionarily favorable for females to adapt ways to express aggressive behavior other than those mediated by testosterone (Rosvall et al. 2012).

Locomotor and Nesting Behavior

I had predicted that the resident female would respond with increased activity to a

female intruder. However, my results do not show an increase in activity levels in response to the intrusion. Although instances in which overall locomotion increased in response to the addition of the lure female were observed, there was no significant difference between the frequency of long and short-range flights with the addition of the lure female and my hypothesis that the lure female would increase activity levels was not supported. The specific cases in which locomotion increased with the addition of a the lure female may be alternatively explained by a response to stress, as intermediate corticosterone levels were shown to increase perch hopping in white-crowned sparrows (Breuner et al. 1998).

Surprisingly, there were no observed instances of nesting behavior and the hypothesis was neither supported nor rejected. It is possible that daily removal of eggs from the nest cups in accordance with lab protocols have conditioned the birds in the flock to avoid wasting energy incubating young. As these birds have lived in captivity in separate mating pair cages, it is unlikely that rejection of dummy eggs is the result of coevolution to avoid intra-specific brood parasitism as seen in other species of free-living bird (Marchetti 2000).

Hormone Analysis

I hypothesized that GnRH injections would raise circulating plasma testosterone in female zebra finches, however there was no significant difference found between treatment groups. Interestingly, there was a trend toward decreasing testosterone concentrations in the control group 30 and 60 minutes post bleed, which did not occur in the treatment group. This shows that GnRH treatment did raise circulating testosterone levels, but not significantly when compared to the control. While these results failed to support my hypothesis, testosterone

concentrations observed were not trivially low when compared to other bird species showing that the females did have the capacity to produce testosterone. In a study by Egbert et al. (2013) with house sparrows, plasma testosterone concentrations were evaluated pre and 30 minutes post GnRH injection and there was a significant increase in concentration (Egbert et al. 2013). While the Egbert et al. (2013) concentrations are higher than those observed in my study, the trend toward an increase in plasma testosterone is similar (Egbert et al. 2013).

These GnRH trials were completed a year prior to behavioral observations. If age-related decrease in HPG responsiveness (discussed in the next section) occurred, this could explain a possible decrease in testosterone production and therefore behaviors in response to lure female introduction. Acute stress may have also suppressed testosterone production during the GnRH trials, explaining the lack of significant difference between treatment groups, as females were subject to similar conditions as the Deviche et al. (2012) study, which showed a stress-induced reduction in testosterone levels.

Improvements to Experimental Design

Multiple improvements could be made to the design of this experiment. A larger sample size of mating pairs would have provided a much larger data set to identify trends in aggression, maternal behavior and locomotor patterns. Although it was previously determined that isolation had little effect on the behaviors of the mating pairs, perhaps it would have been beneficial to allow pairs to better acclimate to isolation from the flock before the introduction of the lure female to control for novel surroundings. Because all breeding pairs were housed constantly in the same room before this study, they may have become accustomed visually or acoustically to each other's presence, decreasing inclinations toward aggression.

Age may have played a role in the results observed. In the wild, zebra finches can live up to 5 years of age, but have been shown to live longer in captivity (up to 9 years maximum; F. Nottebohm, D. Holmes personal communication) (Zann 1996). Aging is defined as, "a decline in physiological functioning with age accompanied by a decrease in reproductive performance and an increase in mortality" (Rose 1991). In a study relating BMR (basal metabolic Sinerate) and ageing in zebra finches, Moe et al. (2009) suggested that the ageing process is faster in zebra finches due to their short life span (Moe et al. 2009). Physiological changes in reproductive success resulting from aging are limited to studies of species of chickens and quail (Holmes et al. 2003). However, research in zebra finches has shown that egg productivity decreases as much as 80% after 4-5 years of age (Holmes, unpublished data). In quail, age brings less predictable ovulation and decreased egg production due to decreased hypothalamic sensitivity to steroids (Ottinger 2007) as well as decreased responsiveness to LH and reduced production of FSH (Holmes et al. 2003). It has also been shown that older birds display shorter laying intervals than younger birds (Williams and Christians 2003). These findings suggest that if younger zebra finch females were observed, it is possible that more females would have laid multiple clutches over the duration of the experiment which would have increased the sample size of the behavioral study.

Age-related defects in LH and FSH secretion, two hormones that control sex steroid production (Charlier 2009, Cain and Ketterson 2013, Rosvall et al., 2013, Goymann and Wingfield 2014), may have impacted my results. Such age effects could explain the absence of a GnRH-induced testosterone response and, in turn, low testosterone levels may have resulted in the absence of a behavioral response to an intruding female. If the zebra finches in my study

experienced similar aging related reproductive changes as quail (Holmes et al. 2003), it is possible that they may not have been able to respond to GnRH or social stimulation of the HPG axis with increased testosterone levels and hence aggressive behaviors.

Ideas for Future Research

Multiple avenues could be explored to further understand the role of testosterone in mediating behaviors in female zebra finches. To determine if an intruding female to a mating pair's environment raises testosterone in females, blood samples could be taken before and after mate pair isolation and lure female introduction and analyzed for changes in testosterone levels. Studies should be conducted to evaluate dose response relationships between GnRH and testosterone production. Another idea for future research is to compare testosterone production and behavioral observations of female zebra finch behavior in the wild compared to domesticated zebra finches in captivity to determine if studies of wild finches are applicable to laboratory settings and vice versa. To evaluate the proposed explanations for the behavior observed in this study, the effects of age and stress on female zebra finches should be researched further, specifically the mechanistic actions of corticosterone on female testosterone production and the effects of age on reproductive capability.

VI. CONCLUSIONS

The results of this study add to a growing body of knowledge examining the roles of testosterone and female behavior, specifically that the introduction of an intruding female elicited behavioral responses (though not statistically significant) in captive female zebra finches and that GnRH did not cause a significant elevation in circulating testosterone. My observations failed to support my hypothesis most likely due to small sample size, the age of

the females in the study, and my experimental design, which may have induced a stress response with effects on GnRH-induced testosterone elevation and response to an intruder. There is a gap in the knowledge of the mechanistic actions of testosterone in females regarding behavior. This study, as well as ideas for future research, lay a foundation for experimenters to further improve on to investigate testosterone and female behavior.

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VIII. APPENDIX

Zebra Finch Ethogram				
Date Recorded: **Date Analyzed:**		**Female ID:** **Female Cage:**	**Lure Female ID:** **Lure Female Cage:**	
Behavior	Time Start (Min)	Time Stop (Min)	Total Time (Min)	Total Number of Behavior Occurrence
Peck Male				
Chase Male				
Peck Lure				
Chase Lure				
Short Range Locomotion				
Long Range Locomotion				
Nesting				

Table 1. Sample ethogram used to analyze behavior videos. Time Start and Stop columns correspond to time during video.

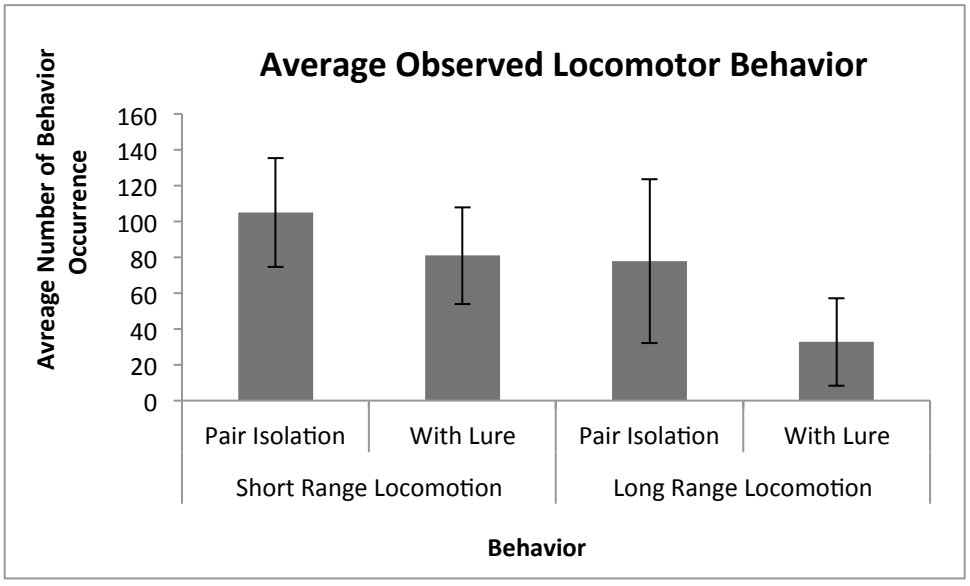

Figure 1. Average number of short and long-range locomotor behaviors of females in isolation with mate and after the introduction of the lure female (n=5). Error bars represent +/- SEM.

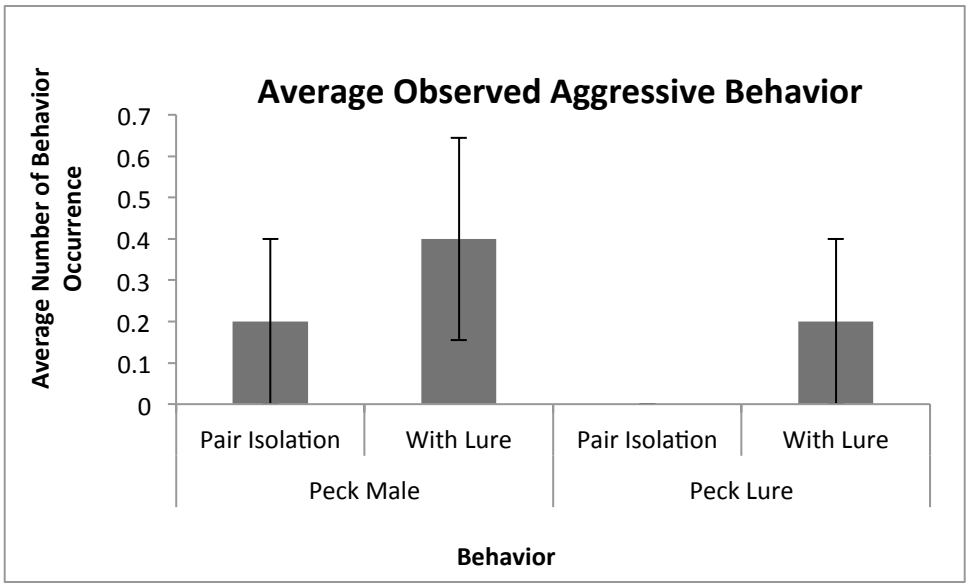

Figure 2. Average number of aggressive (pecking) behavior of female in isolation with mate and after the introduction of the lure female (n=5). Error bars represent +/- SEM.

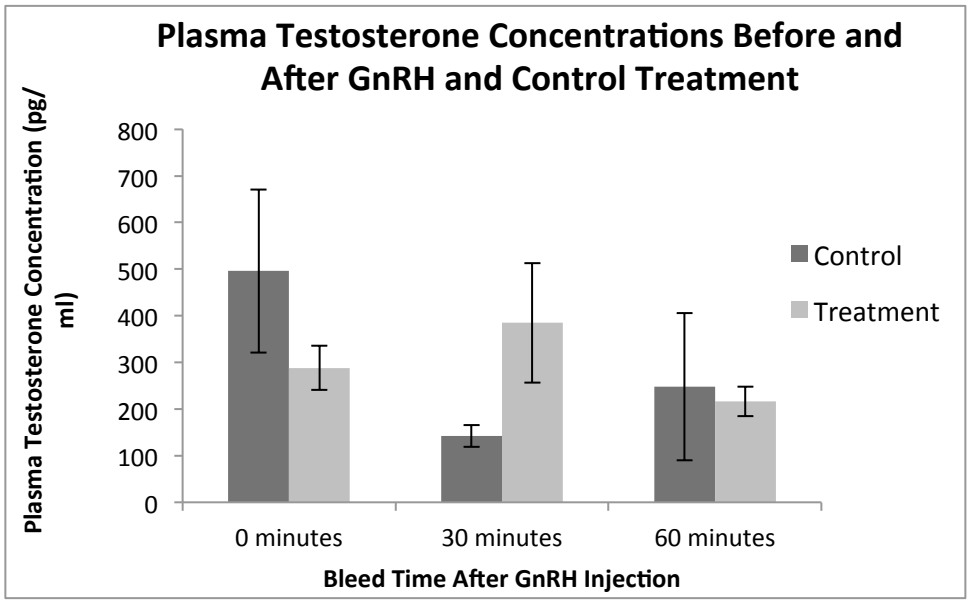

Figure 3. Female circulating plasma testosterone concentrations before a saline or GnRH injection, 30 minutes after injection and 60 minutes after injection (n=12). Error bars represent +/- SEM.

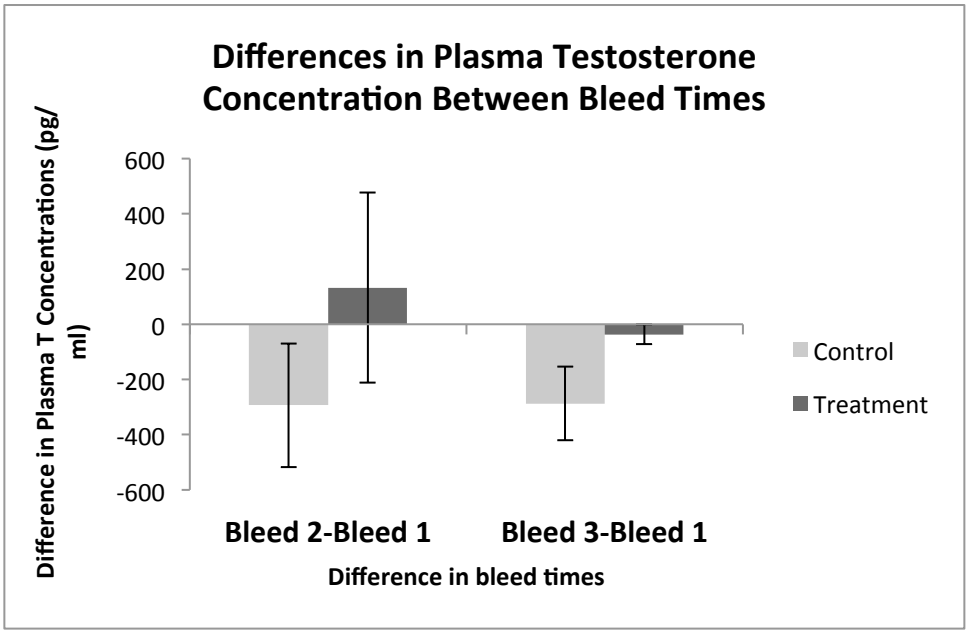

Figure 4. Difference in circulating plasma testosterone concentrations between 30 minutes post-injection (bleed 2) and the pre-bleed (bleed 1) and 60 minutes (bleed 3) post injection and the pre-bleed in GnRH and control injected females (n=12). Error bars represent +/- SEM.