The neuroendocrine regulation of pair-maintenance behavior in the opportunistically breeding zebra finch *(Taeniopygia guttata)*

by

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Abstract

Understanding affiliative behavior is critical to understanding social organisms. While affiliative behaviors are known to exist across taxa and a wide range of contexts, the bulk of what is known about the physiological regulation of affiliation comes from studies of mammals. The zebra finch (*Taeniopygia guttata*) is a good model to further our understanding of the neuroendocrine regulation of affiliative behaviors. Zebra finches form sexually monogamous pair bonds, which they actively maintain throughout the year. Thus, in this system we can examine the regulatory mechanisms of affiliation associated with long-term pair maintenance both within and outside of a breeding context. In this dissertation, I present a series of studies using the zebra finch to examine the hypothesis that sex steroids regulate pair-maintenance behavior differently depending on breeding condition. In brief, I report that, (a) zebra finches have distinct sex steroid profiles based on breeding condition, (b) levels of testosterone and estradiol levels are maintained in behaviorally-relevant regions of water-restricted (i.e. non-breeding) zebra finches, (c) aromatase inhibition rapidly increases pair-maintenance behavior (proximity time), (d) chronic male-testosterone treatment decreases pair-maintenance behavior (proximity time under stressed conditions), and (e) sex steroid profiles and pair-maintenance behavior are not correlated in wild-caught zebra finches. Taken together, this work suggests that sex steroids have breeding-specific and social-context-specific regulatory effects on pair-maintenance behavior. Finally, this research shows the importance of controlling for breeding condition in all behavioral neuroendocrinology research on zebra finches, and it highlights the role of seasonality in the expression and regulation of all affiliative behaviors.

Preface

A version of chapter two is published:

Nora H. Prior, Sarah A. Heimovics, Kiran K. Soma. (2013) Effects of water restriction on reproductive physiology and affiliative behavior in an opportunistically breeding and monogamous songbird, the zebra finch. *Hormones and Behavior,* 63(3): 462-74.

The protocol for this study was developed by KKS and NHP. Water restriction and behavioral tests were conducted by SAH and NHP. Behavior was scored by NHP. SAH and NHP did all tissue and blood collection. Brain dissection protocol was developed by NHP and SAH. All brain dissections, steroid extraction and assays were conducted by NHP. NHP analyzed data and wrote up the initial version of manuscript.

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The protocol for this study was developed by KKS and NHP. Water restriction and behavioral tests were conducted by KNY and NHP. Behavior was scored by KNY and NHP. Pilot study was conducted by NHP. Brain dissections, steroid extraction and assays were conducted by NHP and KNY. NHP analyzed data and wrote up the initial version of manuscript.

Chapter four is based on the work:

Nora H. Prior, Kang Nian Yap, Tian Qi D. Liu, Clementine Vignal, Kiran K. Soma. Chronic testosterone treatment in males inhibits pair-maintenance behavior in stressed zebra finches. *In preparation*

The protocol for this study was developed by KKS, NHP, and CV. NHP implanted birds. Behavioral tests were run by KNY. Behavior was scored by DL and NHP. CV provided expertise and training in vocal analyses. NHP scored vocal behavior. Brachial blood samples were collected by KNY and

NHP. Steroid extraction and assays were conducted by NHP and DL. NHP analyzed data and wrote up the initial version of manuscript.

Chapter five is based on the work:

Nora H. Prior, Kang Nian Yap, Hans Adomat, Mark C. Mainwaring, H. Bobby Fokidis, Emma S. Guns, Katherine L. Buchanan, Simon C. Griffith, Kiran K. Soma. Sex steroid profiles and pair-maintenance behavior in wild-caught zebra finches. *In preparation*

The protocol for this study was developed by SCG, KKS, KLB, and NHP. Behavioral data was collected by NHP. Brachial blood samples were collected by KNY, MCM, and NHP. Steroid extraction was conducted by HBF. Steroid levels were analyzed using the LC-MS/MS of ESG. ESG and HA provided expertise and trouble shooting for mass spec analyses. Samples were run on the mass spec by HA and NHP. NHP analyzed data and wrote up the initial version of manuscript.

All studies on captive zebra finches were conducted at University of British Columbia, Vancouver, BC, Canada. This work was conducted in accordance with CCAC guidelines, approved by CIHR projects number A09-0395. Work on wild pairs was conducted in collaboration with KL Buchanan at Deakin University, Geelong, Victoria, Australia (Animal Ethics Certificate: B24-2012 AECB) and SC Griffith at Macquarie University, North Ryde, NSW, Australia (License # ARA 2010/053).

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List of Abbreviations

μg – micrograms
μL – microliters
μm – micrometers
3β-HSD – 3β-hydroxysteroid dehydrogenase
17β-HSD – 17β-hydroxysteroid dehydrogenase
ACN – acetonitrile
ADIOL – androstanediol
AE – androstenedione
ANOVA – analysis of variance
ATD – 1,4,6-androstatriene-3,17-dione
AVT – arginine vasotocin
°C – degrees Celsius
C_{18} – carbon 18
caTEL – caudal telencephalon
CB – cerebellum
ceTEL – central telencephalon
$CHCl_2$ – dichloromethane
cm – centimeters
CON – control
CSS – charcoal stripped serum
d3T – deuterated testosterone,
d3DHT – deuterated 5α-DHT
d3Diol – deuterated ADIOL
d – days
D30– ~30-days post implant
D60 – ~60-days post implant
DHEA – dehydroepiandrosterone
DHT – Dihydrotestosterone
ERα – estrogen receptor alpha
F – flutamide
FAD – fadrozole
FMP – 2-fluoro-1-methylpyridinium *p*-toluene-4-sulfonate
g – gravity
HPG – hypothalamus pituitary gonadal axis
HPLC – high performance liquid chromantography
hr – hours
HYP – hypothalamus
i.p.– intraperitoneal
IS – internal standards
kV – kilovolts
LC-MS/MS – Liquid Chromatography-Tandem Mass Spectrometry

LOD – limit of detection
LOQ – limit of quantification
L/h – liters per hour
M/HB – mid/hindbrain
m/sec – meter per second
mbar – megabar
MeOH – methanol
mg – milligrams
mg/kg – milligrams per kilogram
min – minute
mL – milliliters
mm – millimeters
MRM – multiple reaction monitoring
NaOH – sodium hydroxide
NCM – caudomedial nidopallium
ng – nanograms
NSW – New South Wales
PBSG – phosphate-buffered saline containing 0.1% gelatin
pg – picograms
PRE – prior to implantion
RIAs – radioimmunoassays
rmANOVA – repeated measures analysis of variance
RPM – rotations per minute
rTEL – rostral telencephalon
s.c. injections – subcutaneous injection
sec – second
SEM – standard error of the mean
TEA – triethylamine
TEST – testosterone
VEH – vehicle
WR – water restricted

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Dedication

To my family, new and old

1 Introduction

1.1 Overview

Affiliation is fundamental to social bond formation and maintenance (Carter, 1998). However, our understanding of the neuroendocrine mechanisms regulating affiliation are largely based on research examining pair bond formation in breeding mammals (Insel and Young, 2001; Young and Alexander, 2012). These models leave at least three significant gaps in our current knowledge. First, as there are often distinct neuroendocrine mechanisms regulating the initiation and maintenance of behaviors (Ramenofsky, 1984; Nelson, 2005), little is known about the neuroendocrine mechanisms supporting the long-term maintenance of social bonding. Second, minimal research has examined non-mammalian systems, despite the fact that monogamy is dominant among birds and rare among mammals (Adkins-Regan and Tomaszycki, 2007). Finally, while seasonality is well-known to impact the regulation of aggressive behaviors (Trainor et al., 2007; Trainor et al., 2008; Soma et al., 2008; Heimovics et al., 2013; Laredo et al., 2013; Laredo et al., 2014), the influence of seasonality on affiliation is largely unknown. Elucidating the complexities of the ecological and physiological regulation of social bonding and affiliation is fundamental to understanding basic sociality. Moreover, it is critical in light of our increasing recognition of how importance social bonding and prosocial behavior are in human health.

My PhD research has investigated long-term pair-maintenance behavior of breeding and non-breeding zebra finches *(Taeniopygia guttata)* as a model to compare the neuroendocrine regulation of affiliative behaviors across breeding conditions. The zebra finch is highly gregarious and forms life-long pair bonds. These bonds are actively

maintained regardless of breeding condition, allowing us to study the neuroendocrine regulation of pair maintenance within and outside of breeding condition. As opportunistic breeders adapted to the arid conditions of Australia, zebra finches are highly sensitive to environmental cues, particularly water availability, to time breeding. Broadly speaking, behaviors that exclusively co-occur with breeding bouts may be triggered by the elevation of sex steroids during breeding; however, for behaviors that are present during breeding and non-breeding periods, sex steroids may have season-specific regulatory functions (Soma et al., 2008). My central hypothesis is that sex steroids regulate long-term pair-maintenance behavior differently depending on breeding condition. To explore this hypothesis, I have: (1) used experimental water restriction to describe the effects of breeding condition on reproductive physiology and pair-maintenance behavior; (2) quantified the effects of acute and chronic endogenous sex steroid manipulation on pair-maintenance behavior in water-restricted zebra finches; (3) described the effect of chronic exogenous testosterone administration on pair-maintenance behavior; and (4) correlated sex steroid profiles with pair-level variation in affiliative behavior in wild-caught zebra finches.

1.2 Affiliation

Affiliative behaviors are friendly/prosocial behaviors that occur across modalities and in a wide range of social contexts. Examples of affiliation include tactile behaviors (e.g., huddling in mammals and clumping in birds (Insel and Young, 2001), vocal communication (e.g. dueting in birds, Elie et al., 2010), and coordinated movement or activities. These affiliative behaviors mediate the formation of social bonds (such as parental or monogamous pair bonds) and help maintain social dominance hierarchies (Young et al., 1998). Affiliative

behaviors are expressed under a number of social contexts, including establishment of social bonds, courtship, reproduction, reconciliation, and coordination of group movements (Carter, 1998; Pellegrino, 2008). Evolutionarily, behaviors that stabilize groups and dyads are highly advantageous in many contexts and increases fitness (Carter, 1998; Sanchez-Macouzet et al., 2014).

For social species, maintaining positive and stable social relationships is a basic need. Disruption of social interactions is one of the most effective stressors in social animals (DeVries et al., 2003). Social isolation is a particularly profound stressor across a wide range of species (*Birds:* Perez et al., 2012; Remage-Healey et al., 2003; *Primates:* Levine and Wiener, 1988; Norcross and Newman, 1999; *Ungulates*: Ruis et al., 2001). In contrast, the mere presence of a conspecific can buffer the physiological effects of stress and pain in numerous species (*reviewed in:* Kikusui et al., 2006; Martin et al., 2014). Affiliative behaviors directly decrease stress and anxiety by dampening hypothalamic-pituitary-adrenal axis (HPA) activity. Broadly speaking, chronic activation of the HPA axis has long-term negative consequences on individual health (*reviewed in*: Sapolsky et al., 2000; McEwen and Wingfield, 2003). In primates, infants die without social contact (Harrison and Woods, 1991).

Affiliation is clearly important; however, it is significantly less studied than other social behaviors. For example, a Web of Science search for 'aggression*' gets over 40,000 hits (over 33,000 primary research articles, 3,500 from the field of zoology), whereas 'affiliation*' receives less than 15,000 (just under 12,000 primary research articles, less than 500 from the field of zoology). It is possible that research on affiliative behaviors is published without the keyword "affiliation", however note that the combined hits from 'affiliation*', 'social buffering*', and 'sociality*' still equals less than half of the hits from 'aggression*'. Even

'maternal behavior*' only receives 20,000 hits. Further research is needed from a range of disciplines to elucidate the behavioral and physiological complexities of affiliation.

1.3 Monogamy

Monogamous bonds are formed and maintained largely through affiliation within the pair. Bonds can be either socially or sexually monogamous: pairs that are only socially monogamous engage in extra-pair copulations. It is relatively easy to determine species with monogamous breeding systems and to identify paired individuals within those species, making the study of monogamous bonds a prime way to study affiliation (*reviewed in:* Carter, 1998; Insel and Young, 2001). Monogamous bonds can be transient (lasting only one breeding cycle) or life-long. Species that form sexually monogamous life-long bonds are particularly interesting because they engage in high levels of affiliation outside of the breeding season. During the non-breeding season, individuals almost exclusively engage in affiliative behaviors with their pair-bonded partner.

1.3.1 The monogamous prairie vole

Monogamy in prairie voles is one of the most well-studied systems of pair bonding (*review:* Young et al., 2008). In the wild, only around half of the individuals form monogamous pair bonds, although this percentage is higher in low population density areas (Getz and Hofmann, 1986). Of the monogamously bonded prairie voles, the average pair bond may last less than 50 days, due to the high mortality rate of voles (Getz and Hofmann, 1986). While short-lived, these bonds are usually maintained during both breeding and non-breeding seasons (Getz and Hofmann, 1986). In order for a pair bond to form in the territorial vole, first a male and female must encounter each other. An encounter requires a suppression

of territorial behavior and an increase in social curiosity and approach. Initial contact is usually followed by copulation, which will elicit the formation of a pair bond and social recognition/preference of the partner (Winslow et al., 1993). After bond formation, affiliative behaviors such as cohabitation and grooming help maintain partner preference and bonding (Winslow et al., 1993).

The vast majority of laboratory studies have taken advantage of two aspects of prairie vole behavior to explore the neuroendocrine regulation of bond formation (*review:* Carter, 1998; McGraw and Young, 2010). Pair-bond formation results in (1) selective preference for an individual's mate, and (2) active hostility against same-sex intruders (Insel, 1997). Importantly, these behaviors occur within 24 hours of copulation/ pair-bond formation (Insel 1997; McGraw and Young, 2010). Thus, through partner preference and resident-intruder paradigms, researchers have been able to identify critical components of the neurobiological basis of bonding.

In brief, this research has identified the nonapeptides oxytocin (OT) and vasopressin (AVP) as both necessary and sufficient to establish social bonds (Insel, 1997). In female prairie voles, sociosexual interactions trigger a release of OT in the nucleus accumbens and prefrontal cortex (*review:*Young et al., 2008). In males, AVP appears to be more important than OT in regulating pair bonding. Blocking vasopressin receptors (V1aR) in the ventral pallidum or lateral septum using a vasopressin antagonist (AVPA) prevents pair-bond formation (Liu et al., 2001).

Interestingly, after copulation, dopamine (DA) in the nucleus accumbens plays a critical role in bond formation for both males and females (Aragona et al., 2003; Aragona et al., 2006). Activation of D2-type DA receptors promotes pair-bond formation while activation of

D1-type receptors inhibits new bond formation (Aragona et al., 2006). After a pair bond has been established, D1-type receptors increase in the nucleus accumbens, likely prohibiting the formation of new bonds (Aragona et al., 2006).

1.3.2 Monogamy in birds

Monogamy is rare in mammals, fish and reptiles. Only around 5% of mammals are monogamous (Kleiman, 1977). In contrast over 90% of bird species are monogamous (Lack, 1968). The most commonly studied temperate songbirds are serially monogamous. However, for the many avian species that form and maintain life-long monogamous pair bonds (Black, 1996), it is as important to study the underlying mechanisms that support bond maintenance as it is to study initial bond formation (Aragona et al., 2003; Aragona et al., 2006). Importantly, pair-maintenance behaviors in life-long bonds occur during breeding and non-breeding periods, allowing researchers to ask questions about the role of seasonality in the expression and neuroendocrine regulation of affiliation. Thus, we can greatly expand our understanding of monogamy and affiliation through studies in birds.

1.4 Seasonality and the expression of social behavior

For many vertebrate species, breeding is limited to specific and predictable times during the year, during which environmental conditions (e.g., mild temperature, greater food) are favorable for raising offspring (Wingfield and Kenagy, 1991). For the many species with relatively short gestation or incubation durations, the breeding season occurs in spring, when daylength is increasing. Photoperiod is often the main environmental cue that initiates the physiological cascade in preparation for breeding in seasonally-breeding vertebrates (Wingfield and Kenagy, 1991). Photostimulation causes the upregulation of the

hypothalamic-pituitary-gonadal (HPG) axis, starting with the secretion of gonadotropin-releasing hormone (GnRH) from the hypothalamus to the pituitary, where it causes the secretion of lutenizing hormone (LH) and follicle stimulating hormone (FSH) into the systemic circulation. These hormones act on the gonads, stimulating the production of sex steroid hormones (e.g., testosterone and estradiol) and the development of gametes. Indeed, 'seasonality' is often used as an all-encompassing term, including both temporal changes in environmental condition and individual endocrinology, such that 'breeding season' implies elevated androgens in males and estrogen in females.

While this classic perspective provides a clear basis for the relationship between social behavior specific to breeding and sex steroids, the relationship between social behavior and sex steroids is less clear for behaviors that occur throughout the year. Both aggressive and affiliative behaviors occur in a wide range of contexts and do not have to be limited to the breeding season.

1.4.1 Seasonality and monogamy

Like monogamous prairie voles, many territorial species exclusively engage in affiliation with their pair-bonded mate and their offspring. For serially monogamous species, affiliative behaviors are typically limited to the breeding season when the HPG axis is upregulated. In avian species, sex steroids are thought to be responsible for many of the changes in the brain that are necessary for singing and courtship behaviors (Adkins-Regan, 2005; Ball et al., 2002). However, in life-long monogamous pairs, affiliation associated with pair maintenance occurs both within and outside of the breeding periods. Surprisingly little is known about pair-maintenance behavior during non-breeding periods, and the role of sex steroids in regulating pair maintenance is largely unknown. The evidence that sex steroids

regulate pair-affiliation associated with courtship during the breeding season may suggest they regulate pair-affiliation associated with bond maintenance in the same manner during the non-breeding season. However, there are three alternative hypotheses. Firstly, sex steroids may act via season-specific mechanisms, differentially regulating pair-maintenance behavior in the breeding versus non-breeding season. Secondly, sex steroids may only regulate pair-maintenance behaviors when they are expressed in the breeding season (Smiley et al., 2012), whereas other neuroendocrine mechanisms (not sex steroids) support pair maintenance in the non-breeding season. Finally, the neuroendocrine mechanisms that support pair maintenance may differ substantially from the mechanisms that support courtship and pair formation (Nelson, 2005; Aragona et al., 2003; Aragona et al., 2006). In this case, sex steroids may or may not be directly regulating pair-maintenance behavior across breeding and non-breeding seasons.

1.4.2 Seasonality of sex steroid effects on social behavior

While aggressive behaviors are often critical during breeding periods to defend territories or mates, they are not exclusive to breeding periods. Work on aggressive behavior in song sparrows has demonstrated that sex steroids can differentially regulate behavior in breeding and non-breeding seasons (Soma et al., 2008). Specifically, both the sources of sex steroids and their mechanisms of action are season-specific.

While gonadally-produced sex steroids are high during the breeding season, neural-production of sex steroids may be upregulated in the non-breeding season, when the HPG axis is downregulated (Soma et al., 2000; Pradhan et al., 2010; Heimovics et al., 2013). These neurosteroids can be synthesized *de novo* in the brain or from circulating prohormones, such as dehydroepiandrosterone (DHEA) (Balthazart and Ball, 2006). Classically, sex

steroids are thought to regulate behavior through genomic mechanisms, directly affecting gene expression, which typically has delayed and long-lasting effects on behavior. However, rapid and transient effects of sex steroids on social behaviors have more recently been identified (Balthazart and Ball, 2006; Soma et al., 2008). Rapid effects are mediated by membrane-bound receptors affecting signal transduction pathways, which can influence behaviors within 5-15 minutes. Local levels of steroids in specific brain nuclei are likely more rapidly and efficiently regulated through neurosteroid synthesis than systemic upregulation of the HPG axis. Taken together, these data raise the hypothesis that neurosteroid synthesis is upregulated during non-breeding periods when there is lower production of gonadal steroids. With this increase in neurosteroid synthesis, there may be a concomitant increase in rapid steroid signaling. Indeed, this effect is what is seen in the song sparrow model system (Soma et al., 2008; Heimovics et al., 2013).

1.5 The monogamous zebra finch

The zebra finch is an excellent model system to study the neuroendocrine regulation of affiliative behavior. Not only do they form sexually monogamous, life-long pair bonds, but they are also an extensively studied laboratory model organism for neuroscience and behavioral research (Griffith and Buchanan, 2010). While zebra finches are an extensively studied avian system, surprisingly little is known about the neuroendocrine mechanisms that promote pair-bond maintenance (Prior and Soma, *in prep*). Some of the most relevant background information is discussed below.

1.5.1 Zebra finch social behavior

Zebra finches form life-long monogamous pair bonds that are actively maintained in both breeding and non-breeding condition, and they engage in biparental care (Birkhead et al., 1988; Zann, 1996). Zebra finches have a large repertoire of affiliative behaviors including clumping (i.e., sitting, facing the same direction, touching each other), allopreening, following, cohabitation of the same nest, and several types of vocalizations (Birkhead et al., 1988; Zann, 1994; Elie et al., 2010). Zebra finches will engage in affiliative behaviors with other juveniles and their parents prior to sexual maturity. However, once mature, they will form a pair bond and engage in affiliative behaviors almost exclusively with their partner. The neuroendocrine mechanisms of zebra finch sociality (Goodson et al., 2005; Goodson et al., 2009; Goodson and Kingsbury, 2011) have been well studied. Furthermore, zebra finch courtship behavior has been well studied, although much of this work has focused on male song and mate competition, (Goodson et al., 1999; Goodson and Adkins-Regan, 1999; Harding and Rowe, 2003; Tomaszycki et al., 2006). Additional research has been done on pair-bond formation (Goodson et al., 2004; Svec et al., 2009; Svec and Wade, 2009; Kelly and Goodson, 2014; Banerjee et al., 2013). However, only a few studies (Svec et al., 2009; Smiley et al., 2010; Alger et al., 2011) have focused specifically on the neuroendocrine regulation of pair maintenance in zebra finches.

1.5.2 Opportunistic breeding in zebra finches

Zebra finches are common throughout most of Australia. A large portion of their range is desert, and they are well adapted to living in unpredictable habitats with minimal water. Thus, they are opportunistic breeders, integrating several environmental cues to make breeding decisions, including rainfall, humidity, green grasses, seeds and temperature (Zann, 1996; Perfito et al., 2007; Perfito et al., 2008). The most salient cue that brings zebra finches

out of breeding condition is insufficient water availability. In the laboratory, water restriction has been used to bring captive zebra finches out of breeding condition (Vleck and Priedkalns, 1985; Perfito et al., 2008). Although zebra finches are well adapted to unpredictable habitats, it is important to note that populations along the coast live in more predictable habitats and are seasonal breeders (Zann et al., 1995; Perfito et al., 2007). This variation in breeding system across environments highlights the potential plasticity of zebra finch reproductive physiology. However, it is unclear to what extent breeding condition affects zebra finch physiology.

1.5.3 Sex steroids and zebra finch pair-directed behavior

The neuroendocrine regulation of courtship and pair-bond formation has been widely studied in zebra finches (Arnold, 1975; Goodson and Adkins-Regan, 1999; Harding and Rowe, 2003; Tomaszycki et al., 2006; Smiley et al., 2012), and there is evidence that courtship behaviors (e.g., male song and sexual displays) may be regulated by sex steroids (Arnold, 1975; Adkins-Regan and Leung, 2006; Harding and Rowe, 2003; Hill et al., 2005; Remage-Healey et al., 2008: 2009). In contrast, administration of flutamide (an anti-androgen) and 1,4,6-androstatriene-3,17-dione (ATD, an aromatase inhibitor) to male zebra finches decreased male aggression and female approaches during pair-bond formation and courtship, but had no effect on any other courtship behaviors (Tomaszycki et al., 2006). Although courtship and pair-bond formation in zebra finches has been well studied, the neuroendocrine regulation of pair-*maintenance* behaviors has not. Furthermore, more broadly whether sex steroids have breeding-specific effects on zebra finch social behavior is unknown. Thus, research exploring breeding-condition specific regulation of pair-maintenance behavior in zebra finches involves several lines of novel inquiry.

1.6 Objectives

Here, I present a series of studies examining the neuroendocrine regulation of long-term pair-maintenance behavior in breeding versus non-breeding zebra finches. These studies used a combination of laboratory and field techniques. Experimental water restriction and pharmacological manipulations were used in the laboratory to examine the effects of (a) breeding condition on sex steroid profile and (b) sex steroid manipulations on pair-maintenance behavior. In the field, sex steroid profiles were correlated with pair-maintenance behavior in wild-caught zebra finches.

In Chapter 2, experimental water restriction was used to manipulate breeding condition in paired zebra finches. The effects of water restriction on male and female reproductive physiology, circulating sex steroid levels, and neurosteroid levels were quantified. In addition, the effect of water restriction on within-pair affiliative behavior was tested under three different behavioral paradigms: baseline conditions, a partner preference test, and a partner separation and reunion test. Interestingly, while water restriction produced distinct sex steroid profiles in both males and females, brain levels of testosterone and estradiol were maintained in behaviorally-relevant brain regions in both males and females. These results are consistent with the hypothesis that neurosteroid synthesis is upregulated in 'non-breeding' zebra finches.

Chapter 3 presents a direct test of the hypothesis that pair-maintenance behaviors are regulated by estradiol, acting via non-genomic or genomic mechanisms, in water-restricted zebra finches. In two experiments, subjects were treated with an aromatase inhibitor (fadrozole) either acutely or chronically, and a variety of pair-maintenance behaviors were

quantified. Additionally, we quantified the effect of acute fadrozole treatment on brain and circulating estradiol and testosterone levels. Acute fadrozole administration rapidly decreased estradiol levels in the circulation and brain of males and also rapidly increased testosterone levels in the circulation and brain of both males and females. However, neither the acute nor chronic fadrozole treatment decreased pair-maintenance behaviors. In one case, acute fadrozole treatment promoted affiliation. These data suggest that pair-maintenance behavior in non-breeding zebra finches is not promoted by estradiol acting via either non-genomic or genomic mechanisms.

The effect of fadrozole on pair-maintenance behavior presented in Chapter 3 could be due to the decrease in estradiol levels, or the concomitant increase in testosterone levels. The role of testosterone as a potential regulator of within-pair affiliation is further explored in Chapters 4 and 5.

Chapter 4 examines the effects of chronic testosterone treatment on long-term pair-maintenance behavior. Males were implanted with either an empty silastic implant or an implant packed with 8 mm crystalline testosterone. Physical and acoustic behavior was quantified in two behavior tests ('baseline' [unmanipulated] and 'stressed' [after a brief chase]) at three timepoints: prior to implanting, ~30-days post implant, and ~60-days post implant. Surprisingly, we found that pharmacologically elevated male testosterone reduced measures of affiliation *only* under a stressed behavioral paradigm. Interestingly, this significant effect was absent in the baseline behavior test. These results highlight the profound effect that social context has on the neuroendocrine regulation of behaviors.

Finally, while the first 3 data chapters present research conducted on domesticated zebra finches, we also investigated the role of sex steroids in regulating pair-maintenance

behavior in wild zebra finches. Chapter 5 reports a study that used liquid-chromatography tandem mass-spectrometry (LC-MS/MS) with wild-caught zebra finches to examine the relationship between sex steroid profiles and pair-maintenance behavior. Specifically, we quantified nine androgens and progestins (pregnenolone, progesterone, dehydroepiandrosterone (DHEA), androstenediol, pregnan-3,17-diol-20-one, androsterone, androstanediol (ADIOL), testosterone, and 5α-DHT). These LC-MS/MS generated sex steroid profiles allow us to look at sex steroid precursors as well as behaviorally relevant sex steroids, both of which have been correlated with social behavior (Soma et al., 2008; Fokidis et al., 2013). Overall, we found lower circulating sex steroid levels than have been reported for domesticated zebra finches. Only pregnenolone, progesterone, DHEA, and testosterone were quantifiable. A small number of samples had detectable, but non-quantifiable levels of androstenediol and androsterone. Sex steroid profiles were similar between males and females, with only circulating progesterone levels significantly higher in females. Additionally, we found no correlation between variation in pair-maintenance behavior and sex steroid profiles. However, these sex steroid profiles have interesting implications for behavioral neuroendocrinology of zebra finches. Firstly, the surprisingly high levels of circulating pregnenolone suggest it may be an important precursor for neurosteroid synthesis in zebra finches. Secondly, the low levels of sex steroids we found in wild zebra finches compared to domesticated raises the hypothesis that neurosteroid production may be higher in wild zebra finches.

In general, this series of studies furthers our understanding of the neuroendocrine regulation of affiliative behavior. Specifically, this work furthers our understanding of the role sex steroids play in regulating pair-maintenance behavior in zebra finches, and

highlights the importance of examining breeding condition in studies on long-term pair-bonding species. The overall findings and implications of this series of studies are discussed in Chapter 6.

2 Effects of water restriction on reproductive physiology and affiliative behavior in an opportunistically breeding and monogamous songbird, the zebra finch

2.1 Introduction

Zebra finches are opportunistic breeders and integrate many environmental cues to time breeding in arid habitats, including water availability, food availability, presence of green grasses, and photo period (Zann, 1994; Perfito et al., 2007; 2008). Water availability is one of the most important cues for wild zebra finches, and water restriction can bring captive male zebra finches out of breeding condition (Vleck and Priedkalns, 1985; Perfito et al., 2008; Morton, 2009). The hormonal profile of non-breeding zebra finches has not been fully characterized, but evidence to date suggests that circulating sex steroids are reduced in non-breeding zebra finches (Perfito et al., 2006; 2007).

Classically, sex steroids have been thought to be produced in the gonads and then travel to the brain to regulate behavior. However, there is now abundant evidence that sex steroids can also be produced locally in the brain, either de novo from cholesterol or from circulating prohormones such as dehydroepiandrosterone (DHEA) (Balthazart and Ball, 2006; Forlano et al., 2006; Schlinger and Remage-Healey, 2012). Behaviors that are regulated by gonadally-produced sex steroids during the breeding season can be regulated by neurally-produced sex steroids during the non-breeding season, when gonadal production of sex steroids is low or absent (Soma et al., 1999; 2000; Pradhan et al., 2010).

Taken together, these studies suggest that pair-maintenance behaviors in zebra finches might be regulated by gonadally-produced sex steroids while pairs are in breeding condition and regulated by neurally-produced sex steroids while pairs are in non-breeding condition.

As a first step towards testing this hypothesis, we examined the effects of water restriction on (1) male and female reproductive physiology, (2) pair-maintenance behaviors in a variety of behavioral paradigms, and (3) circulating and brain levels of estradiol, testosterone, and DHEA.

2.2 Materials and methods

2.2.1 Subjects

These experiments were carried out under a University of British Columbia Animal Care Committee protocol and followed the guidelines of the Canadian Council on Animal Care. Subjects were adult (> 120 d old) captive zebra finches housed in a colony maintained on a 14:10 hr light:dark cycle with an average temperature of 22°C and an average relative humidity of 31% (range: 21%-76%). All zebra finches had *ad libitum* access to seed (50/50, Panicum millet/white millet, Just For Birds, Langley BC), cuttlefish bone, and grit. Prior to experimental water restriction, all subjects had *ad libitum* access to water. Male-female dyads were housed together in cages (38 ½" x 19 ¾" x 19", Corner's Cages) that had a nestbox (5 ½ " x 5 ½ " x 7 ½ ") and a center groove into which a divider could be placed. Dyads were housed together for a minimum of 2 months prior to the start of the experimental manipulation. All pairs engaged in affiliative, courtship, and/or nesting behaviors, and were thus considered pair bonded.

Pairs were then assigned to one of two treatment groups: control (CON, n=10 pairs) or water restriction (WR, n=11 pairs). Treatment groups were counterbalanced with respect to the number of eggs laid and chicks hatched per pair during the previous 2 months. Water-restricted subjects were given decreasing amounts of water over the course of 5 weeks, to a

minimum of 1mL per subject per week, which they continued to receive for the duration of the experiment (Table 2.1). Water-restricted subjects always had access to empty water towers. When water was administered to the water-restricted pairs, a specific amount of water (Table 2.1) was added to the water tower for a limited period of time (30 to 120 min), and the amount of water consumed was estimated to the nearest 0.25 mL by measuring (and removing) the remaining water with a pipette (Table 2.1). Control pairs received water *ad libitum* from their water towers. After the start of the experimental manipulation, all eggs laid were removed from all pairs within 48 hours of laying, to prevent parental behavior from being a confound.

2.2.2 General timeline

A timeline for the experiment is shown in Figure 2.1. To assess the effects of water restriction on baseline behaviors and circulating steroid levels, Baseline Behavior Sessions were recorded and blood samples were collected before (Pre) and after (Post) water restriction (Figure 2.1). Next, we conducted two behavioral tests that elicited pair-maintenance behaviors under different conditions: the 'Partner Preference Test' and the 'Partner Reunion Test' (see below for details). Immediately following the Partner Reunion Test, blood and brain tissue were collected for quantification of circulating and brain steroid levels (Figure 2.1).

2.2.3 Baseline behavior and circulating steroid levels

Baseline behaviors were assessed during two 20-min sessions (40 min total) both before (Pre) and after (Post) water restriction. Pairs were recorded in their home cages in the colony room, between 09:00 and 13:00 hr, using a digital camcorder.

To measure the effects of water restriction on circulating steroid levels under normal conditions, blood samples were collected from the brachial vein before (Pre) and after (Post) water restriction (Figure 2.1). The male and female of each pair were caught simultaneously from their home cage, ~2 d after the second Baseline Behavior Session. Approximately 150 μL of blood was collected into heparinized capillary tubes within 10 min (6.8 ± 0.2 min) of entering the colony room and stored on wet ice. After centrifugation (10 min at 10,000g), plasma was collected and stored at -20ºC.

2.2.4 Partner preference test and partner reunion test

Both the Partner Preference Test and the Partner Reunion Test took place in a separate testing room (i.e., not the colony room). Between 11:00 and 13:00 hr on the day prior to testing, the pairs were moved to a testing room.

For the Partner Preference Test, the home cage was placed in between two smaller stimulus cages. Opaque partitions separated the stimulus cages and the home cage. The male or female in the pair was randomly assigned to be the focal animal, and the other individual was the partner stimulus. The pair was separated immediately prior to lights out (21:00 hr), the night before the test. The focal animal remained in the home cage, and the partner stimulus was placed in one of the stimulus cages. At the same time, a novel stimulus individual was placed in the other stimulus cage. Note that the novel stimulus was the same sex and in the same condition (control or water restricted) as the partner stimulus. The sides of the partner and novel stimuli were counterbalanced between the two treatment groups. The following morning, at the start of the test, the opaque partitions were removed, and behavior was recorded for 20 min using a digital camcorder. Note that during the overnight separation, the pair remained in acoustic contact, allowing them to maintain their pair bond (Zann, 1996).

Previous studies have used longer separation periods, without disruption of the pair bond (Remage-Healey et al., 2003).

For the Partner Reunion Test, the pairs were again separated immediately prior to lights out (21:00 hr), the night before the test. The male and female were placed on opposite sides of the home cage, and they were physically and visually isolated by a wire partition and an opaque partition, which were both inserted into a groove in the center of the cage. The following morning, at the start of the test, the opaque partition only was removed (the wire partition remained in place), so the pair was physically but not visually isolated for 10 min. Next, the wire partition was removed, so the pair was physically reunited for 10 min. Behavior was recorded using a digital camcorder.

2.2.5 Scoring behavior

All behaviors were scored by one researcher (NHP) who was blind to treatment condition. During the Baseline Behavior Sessions, behaviors that were quantified included general activity (feeding, drinking, and self-preening), nesting behaviors (time spent in the nestbox, and number of trips collecting nesting materials) and affiliative behaviors (clumping [i.e., sitting, facing the same direction, touching each other], allopreening, and proximity time [time spent within 10cm of each other]) (Goodson et al., 1999; Elie et al., 2011a). We did not quantify songs in the colony room because we were unable to reliably distinguish the focal male's vocalizations from other males' vocalizations.

In the Partner Preference Test, the cage was divided into three areas: adjacent to the partner, center/neutral area, or adjacent to the novel stimulus animal. Time spent adjacent to either the partner or novel stimulus was considered 'contact time.' The 'preference for

partner' was quantified as time spent adjacent to partner, as a percentage of contact time (Goodson et al. 2004).

During the Partner Reunion Test, behaviors were scored separately during the first and second halves of the test. Time spent on the center perch (close to partner) was scored in the first half of the test (visual reunion only), and proximity time within 10 cm was scored during the second half of the test (physical reunion). General activity and male song were also scored during both parts of the test.

2.2.6 Plasma and tissue collection

Systemic and/or local steroid levels might be rapidly and transiently increased after affiliative behavior is expressed. To measure circulating and brain steroid levels immediately after engaging in affiliative behavior, we captured both the male and female of a pair immediately following the Partner Reunion Test. Individuals were euthanized via rapid decapitation within 3 min (1.2 ± 0.07 min) of entering the testing room. Trunk blood, whole brain, gonads, and oviduct were collected. The length and width of the testes, diameter of the largest follicle of the ovary, and oviduct length were recorded by a researcher who was blind to treatment. Tissues were frozen on powdered dry ice and stored at -80°C. Masses of the testes, whole ovary, and oviduct were measured at a later date.

2.2.7 Brain dissection

Brains were sectioned in the coronal plane at 300μm on a cryostat at -12°C. Major neuroanatomical landmarks were used to divide the brain into several regions of interest (Figure 2.2). Specifically, a scalpel was used to dissect six regions: rostral telencephalon (rTEL), central telencephalon (ceTEL), caudal telencephalon (caTEL), hypothalamus (HYP), mid/hindbrain (M/HB), and cerebellum (CB) (Figure 2.2). Tissue of the same region (within

an individual) was pooled across multiple sections. The optic tectum was not collected. Brain samples remained frozen during this process and were stored at -80ºC.

2.2.8 Steroid extraction and measurement

Brachial plasma samples.

Plasma collected from the brachial vein was used to examine the effect of water restriction on circulating estradiol, testosterone, and corticosterone levels. Corticosterone was measured in unextracted plasma, as described previously (Newman et al., 2008b). For estradiol and testosterone measurements, steroids were extracted from plasma using solid phase (Newman et al., 2008a; Taves et al., 2010; 2011). Plasma (~40µL) was diluted in 10 mL water and loaded onto C18 columns (Agilent Bond-Elut OH, 500mg, cat # 12113045) that had been primed with 3mL HPLC-grade methanol and equilibrated with 10mL de-ionized water. Samples were then washed with 10 mL 40% HPLC-grade methanol, and steroids were eluted with 5 mL 90% HPLC-grade methanol. The eluted samples were dried at 40°C in a vacuum centrifuge (ThermoElectron SPD111V Speedvac) and stored at -20°C until assayed.

Extracted steroid samples were resuspended in PBSG (phosphate-buffered saline containing 0.1% gelatin) with absolute ethanol (0.8%) to aid resuspension (Newman et al., 2008a). These resuspended samples were used to measure estradiol and/or testosterone using sensitive and specific radioimmunoassays (RIAs) (Table 2.2). Plasma samples ≥30 µL were resuspended in 450 µL; 300 µL was used to quantify estradiol and 75 µL was used to quantify testosterone. Plasma samples between 20 and 30 µL were resuspended in 350 µL and used to quantify estradiol only. Plasma samples ≤20 µL were resuspended in 200 µL and used to quantify testosterone only. All samples were measured as singletons (Charlier et al.,

2011). All samples from an individual were run in the same assay. All values were corrected for recovery (Table 2.2). All brachial plasma samples had detectable levels of steroids.

Brain and trunk plasma samples.

Brain and trunk plasma samples were homogenized prior to extraction. Tissue was homogenized in 2mL polypropylene microcentrifuge tubes with 225µL ice-cold de-ionized water and 1200µL HPLC-grade methanol, using a bead homogenizer (Omni Bead Ruptor 24). Three small ceramic beads were added, and samples were homogenized for 1 min at a speed of 4 m/sec. Homogenates were left at 4°C overnight. Following centrifugation, supernatants were diluted in 10 mL of water, and samples were loaded onto primed and equilibrated C_{18} columns (~60µL of plasma and no more than 50 mg of brain tissue per C_{18} column). Samples were washed, eluted, and dried as described above. Steroids were resuspended using 400µL of PBSG with 1% absolute ethanol. From each sample, 150µL, 80µL, 100µL, and 20µL were taken as singletons for estradiol, testosterone, DHEA, and corticosterone RIAs, respectively. All samples from an individual were run in the same assay. All values were corrected for recovery (Table 2.2). Non-detectable samples were set to zero (estradiol, 97% detectable; testosterone, 100% detectable; DHEA, 93% detectable; corticosterone, 96% detectable).

2.2.9 Statistical analyses

To test for an effect of Treatment (control vs. water restriction) on reproductive physiology (size of gonads and oviduct, number of eggs laid), we used Welch's t-tests. As necessary, data were log-transformed to achieve homogeneity of variances.

Baseline behaviors and steroid levels in brachial plasma were analyzed using three-way repeated measures ANOVAs, with Treatment and Sex as between-subjects factors, and

Session (pre- vs. post-water restriction) as a within-subjects factor. When there was a significant interaction, we used a model reduction technique (simple main effects) and conducted follow-up ANOVAs within levels of one of the factors in the interaction. For baseline behaviors and brachial steroid levels, we conducted follow-up two-way ANOVAs separately by Session (pre- vs. post- water restriction). In cases where these two-way ANOVAs yielded a significant Treatment × Sex interaction, we then we conducted one-way ANOVAs to test for an effect of Treatment separately in males and females.

In Baseline Behavior Sessions, for infrequent behaviors (allopreening, clumping, copulation, and number of trips carrying nesting materials), Chi-squared tests were used on the frequency of occurrences in pairs.

For the Partner Preference Test, we measured partner preference (% contact time), total contact time, and total time spent adjacent to partner. The data from both sexes were pooled to increase sample sizes (as there were no sex differences) and then analyzed with Welch's t-tests.

For the Partner Reunion Test, time spent on the center perch during the first 10 min was analyzed by two-way ANOVA with Treatment and Sex as between-subjects factors. Time spent singing (by the male) during the first and second halves of the test and proximity time during the second half of the test were all analyzed using Welch's t-tests. The proportion of males singing was analyzed using Chi-squared tests.

Steroid levels in brain regions and trunk plasma were analyzed by two-way ANOVAs with Treatment and Sex as between-subjects factors. Steroid levels were analyzed by region because our primary interest was not to identify regional differences. When there was a

significant Treatment × Sex interaction, we conducted follow-up ANOVAs to examine the effect of water restriction within each sex.

All statistics were run in Cran R Statistics 2.14 (R Core Team, 2012). All data presented in tables and graphs represent means ± SEM.

2.3 Results

2.3.1 Reproductive physiology and steroid levels in brachial plasma

Water-restricted females and males consumed similar amounts of water (Table 2.1), but water restriction had very different effects on the two sexes. Female reproductive physiology was significantly affected by water restriction. The sizes of the largest ovarian follicle, ovary, and oviduct were significantly decreased in water-restricted females (Table 2.3 and Figure 2.3A). Further, the total number of eggs laid was significantly lower in water-restricted females (Figure 2.3C; $t = 5.67$, $p < 0.0001$). In males, however, there was no significant effect of water restriction on testes size (Table 2.3 and Figure 2.3B).

We also measured steroids in plasma collected from the brachial vein, both before and after water restriction (Figure 2.1). There was no effect of water restriction on circulating estradiol levels in either sex (Table 2.4). Water restriction significantly decreased circulating testosterone levels in males (Table 2.4, $F_{1,18} = 6.16$, $p = 0.022$) but not in females (Table 2.4). The decrease in plasma testosterone levels in males was not associated with an increase in plasma corticosterone levels (Table 2.4). Due to logistical limitations, the brachial plasma samples were not collected within 3 min (6.8 ± 0.2 min), so the corticosterone levels in Table 2.4 do not represent baseline levels.

2.3.2 General activity, nesting and affiliative behavior

Baseline Behavior Sessions

General activity was unaffected by water restriction. Feeding and self-preening levels were similar in control and water-restricted individuals (Table 2.5, all p values > 0.05). As expected, water-restricted individuals stopped visiting the water tower in their cage (which was empty during Baseline Behavior Sessions) (Table 2.5, p values < 0.001). Water restriction significantly decreased time spent in the nest box by both sexes (Figure 2.3D, $F_{1,38}$ = 13.23, p = 0.0008).

However, water restriction had no effect on baseline affiliative behaviors. Proximity time was similar in control and water-restricted pairs (Figure 2.4A, all p values > 0.05). Additionally, there was no significant effect of water restriction on allopreening (Table 2.6, X^2 (1) = 2.01, p = 0.157) or clumping (Table 2.6, X^2 (1) = 3.18, p = 0.074). There is a trend for more clumping in water-restricted pairs.

Partner Preference Test

Water restriction had no effect on partner preference (Figure 2.4B, t = 1.71, p = 0.106). There was also no effect of water restriction on contact time (CON: 797 ± 110 s, WR: 931 ± 41 s; t = -1.14, p = 0.276) or absolute time spent adjacent to the partner (CON: 516 ±105 s, WR: 425 ± 65 s; t = 0.73, p = 0.474).

Partner Reunion Test

During the first 10 min (visual reunion only), there was no effect of water restriction on time on the center perch in either males or females (Figure 2.4C, Treatment, $F_{1,36}$ = 1.29, p = 0.26; Sex, $F_{1,36}$ = 0.0043, p = 0.95; Treatment × Sex, $F_{1,36}$ = 1.004, p = 0.310). Almost all

males sang during the first half of the test: 9/10 control males and 8/10 water-restricted males. There was no effect of water restriction on time spent singing during the first half of the test (CON: 15.1 ± 4.7 s, WR: 11.6 ± 3.4 s; $t = 0.79$, $p = 0.44$).

During the second 10 min (physical reunion) there was no effect of water restriction on proximity time (Figure 2.4D, $t = 0.83$, $p = 0.42$). Relative to the first half of the test, fewer males sang during the second half of the test: 4/10 control males and 2/10 water-restricted males. There was no effect of water restriction on time spent singing during the second half of the test (CON: 13.7 ± 9.11 s, WR: 0.82 ± 0.55 s; $t = 1.41$, $p = 0.19$).

2.3.3 Steroid levels in trunk plasma and brain

We collected trunk plasma and the brain immediately after the Partner Reunion Test. All subjects were euthanized within 3 min of entering the testing room.

Estradiol

Water restriction had no effect on plasma levels of estradiol in males or females (Figure 2.5, Table 2.7). There was a significant main effect of Treatment on estradiol levels in ceTEL, M/HB, and CB (CON > WR, Figure 2.5, Table 2.7). There was a significant main effect of Sex in CB (female > male, Figure 2.5, Table 2.7). There was a significant Treatment × Sex interaction in caTEL; examining the effect of water restriction within each sex yielded a significant effect of Treatment in males only (Figure 2.5, Table 2.7, $F_{1,19} = 12.75$, $p = 0.002$). There was no effect of Treatment or Sex in any other region.

Testosterone

As expected, testosterone levels in plasma were higher in males than in females (Figure 2.6, Table 2.7). Water restriction significantly decreased plasma testosterone levels in males (Figure 2.6, $F_{1,17} = 13.61$, $p = 0.0018$) but not in females. There were significant main effects of Treatment and Sex on testosterone levels in the rTEL (Figure 2.6, Table 2.7). There was a significant Treatment × Sex interaction on testosterone levels in caTEL and CB. Examining the effect of water restriction within each sex yielded significant effects of Treatment in males only (Figure 2.6, Table 2.7, caTEL, $F_{1,19} = 4.94$, $p = 0.039$; CB, $F_{1,19} = 9.42$, $p = 0.006$). There was no effect of Treatment or Sex in any other region.

DHEA: Water restriction had no effect on plasma DHEA levels in males or females (Figure 2.7, Table 2.7). DHEA levels in plasma were higher in males than in females (Figure 2.7, Table 2.7). There were no significant main effects of Treatment or Sex, or a significant Treatment × Sex interaction in any brain region for DHEA (Figure 2.7, Table 2.7).

Corticosterone: Water restriction had no effects on plasma corticosterone levels (Figure 2.8, Table 2.7). Additionally, there were no significant main effects of Treatment or Sex, or a significant Treatment × Sex interaction in any brain region (Figure 2.8, Table 2.7).

2.4 Discussion

These data demonstrate for the first time in this important model species (1) the robust effects of experimental water restriction on female reproductive physiology, (2) the lack of an effect of water restriction on pair-maintenance behavior, and (3) the differential effects of water restriction on systemic and local sex steroid levels. Importantly, although water

restriction strongly affected the ovary and oviduct in females and plasma testosterone levels in males, water restriction did not significantly reduce estradiol levels in the hypothalamus or testosterone levels in the hypothalamus and central telencephalon. While these data are strictly correlational, they are consistent with the hypothesis that neurosteroids promote pair-maintenance behaviors, including male song, in non-breeding zebra finches, a hypothesis that will be tested further in future studies.

2.4.1 Effects of water restriction on reproductive physiology

Zebra finches are opportunistic breeders and can exist in a range or continuum of reproductive states (Perfito, 2010). We therefore did not expect to see dichotomous "breeding" and "non-breeding" conditions, as one would see in a temperate seasonally-breeding species. Nonetheless, control and water-restricted subjects did exhibit different physiological and endocrine profiles, consistent with field descriptions of breeding and non-breeding zebra finches, respectively (Perfito et al., 2007; Perfito, 2010).

The present water restriction protocol is intermediate between previous studies, which range from complete water deprivation (Sossinka, 1974) to slowly decreasing water over 11 weeks (Perfito et al., 2006). Most previous research has focused on the effects of water restriction on isolated males. In early studies, there were no effects of water restriction or deprivation on testis size (Oksche et al., 1963; Sossinka, 1974). However, in wild male zebra finches, testis volume is smaller in non-breeding males (15 to 40% reduction) (Perfito et al., 2007; 2011). Further, in captive male zebra finches, if a within-subjects design is used, then subtle effects of water restriction on testis size are detectable (Vleck and Priedkalns, 1985; Perfito et al., 2006). Here, water restriction had no significant effects on testis volume or mass. Possibly when male zebra finches are paired with females, a longer or more severe

water restriction is necessary, or perhaps we did not detect a subtle effect of water restriction because we used a between-subjects design.

In contrast, we saw a strong effect of water restriction on female reproductive physiology. Few previous studies have examined the effects of water restriction on female zebra finches. In wild female zebra finches, follicular volume is greatly reduced in non-breeding females (90% reduction) (Perfito et al., 2007), which is consistent with our results. Here, there was also a strong inhibitory effect of water restriction on oviduct size and egg laying. Overall, water restriction clearly reduced reproductive readiness in females.

We also report for the first time the effects of water restriction on circulating sex steroid levels. Circulating luteinizing hormone levels are decreased in wild non-breeding male and female zebra finches (Perfito et al., 2007). However, in captivity, water restricting males and females does not necessarily reduce luteinizing hormone levels (Vleck and Priedkalns, 1985). Additionally, Vleck and Priedkalns (1985) measured circulating androgens in males and estrogens in females, using pooled plasma samples. However, their steroid assays were not as sensitive, and most samples from control and water-restricted subjects had non-detectable values. Here, using ultra-sensitive assays, we saw that water restriction decreased circulating testosterone levels in males but not females. Interestingly, this effect on plasma testosterone is the opposite of what we observed with gonad size, where water restriction had a greater effect on females than males.

There were no effects of water restriction on circulating estradiol levels in males or females. The primary source of circulating estradiol in males is the brain, whereas the primary source in females is the ovary (Schlinger and Arnold, 1991). The lack of an effect of water restriction on female plasma estradiol levels is surprising, given the clear effects of

water restriction on the ovary and oviduct. The estradiol assay used here is highly accurate, precise, specific, and sensitive (can detect as little as 0.2 pg of estradiol) (Charlier et al., 2010, 2011; Taves et al., 2010, 2011). It is possible that plasma estradiol levels are generally low in control females, with only transient increases at specific times (e.g., ovulation). Alternatively, estrone, progesterone, prolactin, or arginine vasotocin (AVT) might be modulated by water restriction and affect female reproductive physiology (Liu and Bacon, 2005; Srivastava and Chaturvedi, 2010; Li et al., 2011), but these hormones were not measured here.

While stress is known to affect reproductive physiology (Roberts et al., 2007; Perfito, 2010; Lynn et al., 2010; Kelly et al., 2011), the effects of water restriction seen here do not appear to be the result of stress. Consistent with previous research (Perfito et al., 2007), there were no effects of water restriction on circulating or brain corticosterone levels, body mass (data not shown), or general activity.

2.4.2 Measures of pair bonding and pair-maintenance

Zebra finch social behavior has been extensively studied (Zann, 1996; Griffith and Buchanan, 2010; Healy et al., 2010; Goodson and Kingsbury, 2011); however, this is one of the few studies to focus on pair-maintenance behaviors in this species with long-term monogamy (Tomaszycki and Adkins-Regan, 2006; Dunn and Zann, 1996a; Elie et al., 2011a). Wild zebra finch pairs remain pair bonded regardless of breeding condition (Zann, 1994). However, how captive pairs would perform in traditional behavioral paradigms was unknown. Therefore, we examined a variety of affiliative behaviors under different paradigms. We also ensured that all of our pairs were bonded prior to the start of water restriction: pairs were housed for a minimum of 2 months, had laid eggs, and nearly all had chicks (Zann, 1994).

We used physical proximity as a primary measure of pair-maintenance behavior in all three behavioral testing paradigms. Physical proximity is a combination of coordination and following behaviors, both of which are very reliable measures of pair bonding and attachment (Zann, 1996). Water restriction had no effect on physical proximity in any testing paradigm. First, there was no effect of water restriction on physical proximity (time spent within 10 cm) during Baseline Behavior Sessions, in which we observed normal behavior in the home cage within the colony. Second, during the Partner Preference Test, there was no effect of water restriction on physical proximity (time spent adjacent to partner). Third, during the Partner Reunion Test, there was no effect of water restriction on physical proximity during the visual reunion phase (time spent on the center perch) or during the physical reunion phase (time spent within 10 cm). As expected, the amount of time spent within 10 cm was higher during the Partner Reunion Test than during Baseline Behavior Sessions. Taken together, these data indicate that water restriction does not reduce pair-maintenance behavior, consistent with observations of wild zebra finches (Zann, 1996).

Choice testing paradigms have been effectively used to determine zebra finch mate preferences and group size preference (Tomaszycki and Adkins-Regan, 2005; Adkins-Regan and Leung, 2006; Svec et al., 2009; Svec and Wade, 2009; Goodson et al., 2009), but partner preference was lower than what is typically seen in prairie voles (Goodson et al., 2004; Young et al., 2008). Zebra finches are highly gregarious and do not engage in high levels of extra pair copulations; thus one might expect the focal bird to interact with both the partner and the novel stimulus animal (Birkhead et al., 1988; 1990; Elie et al., 2011a; b).

In addition to physical proximity, we also measured other affiliative behaviors such as clumping, allopreening, and male song, all of which occurred infrequently here. While

clumping and allopreening are highly reliable measures of pair-bonding and attachment, levels vary considerably from study to study (Goodson et al., 2004; Tomaszycki et al., 2006; Elie et al., 2011b; Alger et al., 2011). In any case, water restriction did not reduce these behaviors in Baseline Behavior Sessions or the Partner Reunion Test, or reduce male song during the Partner Reunion Test. This is consistent with our data on physical proximity in these behavioral tests and previous work on wild zebra finches (Zann, 1996).

2.4.3 Effects of water restriction on brain steroid levels

Plasma and brain were collected immediately after the Partner Reunion Test. Thus, the steroid concentrations measured in the plasma and brain might not represent "baseline" levels but rather levels that are transiently elevated in response to reunion with the partner. Indeed, several studies have shown that social cues rapidly regulate neurosteroid synthesis (Remage-Healey et al., 2008; Pradhan et al., 2010; Dickens et al., 2012).

Plasma corticosterone levels increase following partner separation and return to baseline levels between 24 and 48 hr following reunion (Remage-Healey et al., 2003). Consistent with this, circulating levels of corticosterone in control subjects were elevated here, relative to previous work (Remage-Healey et al., 2003; Taves et al., 2010). Also, circulating DHEA and testosterone levels in control males were reduced compared to levels under normal conditions (see Table 2.4 here and Taves et al., 2010). However, circulating levels of estradiol were similar to levels under normal conditions (see Table 2.4 here and Taves et al., 2010).

DHEA is a prohormone and a precursor to testosterone. Here, circulating and brain levels of DHEA were not affected by water restriction in males and females. Given that circulating testosterone levels were low in water-restricted males and females, the

maintenance of DHEA levels could allow for local synthesis of testosterone and estradiol in brain regions that express the necessary steroidogenic enzymes (Schlinger et al. 2008; Pradhan et al. 2010). Similarly, in song sparrows, circulating and brain levels of DHEA are high in the breeding and non- breeding seasons (Soma and Wingfield, 2001; Newman et al., 2008b; Newman and Soma, 2009; 2011).

Water restriction affected brain testosterone and estradiol levels in a region-specific manner, sometimes in parallel with and sometimes independent of changes in plasma levels. Further, in some regions, such as the hypothalamus, brain testosterone and estradiol levels were far higher than plasma levels (especially in water-restricted subjects). Brain steroid levels are result of several factors, including circulating steroid levels, circulating steroid binding proteins, local steroid synthesis and catabolism, and tissue sequestration by steroid receptors or binding proteins (Schmidt et al., 2008; Taves et al., 2011).

Circulating testosterone levels in male zebra finches were strongly reduced by water restriction; plasma testosterone levels were extremely low in all females. Water restriction decreased testosterone levels in some brain regions: the female rTEL and the male rTEL, caTEL, and CB. In males, the above decreases in regional testosterone levels are likely the result of the decrease in circulating testosterone levels. One the other hand, the maintenance of testosterone levels in some regions of the male brain could be the result of increases in local testosterone production and/or sequestration. Testosterone levels in the hypothalamus are particularly intriguing. In females and males, testosterone levels were far higher in the hypothalamus than in the plasma. In addition, water restriction did not significantly reduce hypothalamic testosterone levels in males and even showed a trend to increase hypothalamic testosterone levels in females. This maintenance of testosterone levels in the male

hypothalamus (and central telencephalon) might be due to an up-regulation of neural steroidogenic enzymes such as 3β-hydroxysteroid dehydrogenase (3β-HSD), which plays a critical role in DHEA metabolism in the zebra finch brain (Soma et al., 2004; Schlinger et al., 2008). In male song sparrows, 3β-HSD activity in the forebrain is upregulated during the non-breeding season, when circulating testosterone levels are low (Pradhan et al., 2010; Pradhan and Soma, 2012). Future studies can measure 3β-HSD and other steroidogenic enzymes in brain regions from control and water-restricted zebra finches.

Water restriction had no effect on circulating estradiol levels in either males or females, but did reduce brain estradiol levels in some regions. Water restriction decreased estradiol levels in males in 4 regions (ceTEL, caTEL, M/HB and CB) and decreased estradiol levels in females in 3 regions (ceTEL, M/HB, and CB). Note that in some regions (e.g., central telencephalon), local estradiol levels, but not local testosterone levels, were reduced by water restriction. This pattern of results suggests that water restriction reduces aromatase activity or estrogen receptors in specific regions, and future studies can examine aromatase and estrogen receptors.

The hypothalamus contains nuclei that are part of the "social behavior network" (e.g., preoptic area, ventromedial hypothalamus) and likely important in pair-maintenance behavior (Newman, 1999; Goodson, 2005). Hypothalamic testosterone and estradiol levels were similar in males and females, and they were not significantly decreased by water restriction. Additionally, testosterone and estradiol levels were higher in the hypothalamus than in the plasma, which suggests local sex steroid synthesis (Taves et al., 2011). Previous studies have shown that the adult zebra finch hypothalamus expresses several steroidogenic enzymes, including CYP11A1, CYP17, 3β-HSD, 17β-HSD, and aromatase (London et al., 2006;

Schlinger and Remage-Healey, 2012) and thus has the capacity to synthesize sex steroids *de novo* from cholesterol. Future studies can focus on measuring sex steroids and steroidogenic enzymes within specific hypothalamic nuclei and portions of the social behavior network, as well as their roles in the expression of affiliative behavior.

In zebra finches, the roles of sex steroids in the regulation of pair-maintenance behaviors have not been studied. Numerous studies show that sex steroids promote courtship behavior and male song in zebra finches (Harding et al., 1983; Walters et al., 1991; Ball et al., 2002; Cynx et al., 2005). However, there is also evidence that sex steroids may not play a role in pair bond formation (Tomaszycki et al., 2006). This study used the steroidal aromatase inhibitor ATD (Tomaszycki et al., 2006), which is much less potent than non-steroidal aromatase inhibitors such as fadrozole (Wade et al., 1994). Future studies should examine the effects of fadrozole on pair-bond formation and maintenance. Moreover, overtly similar behaviors can be regulated differently across contexts (Wingfield et al., 2001), and therefore it is possible that pair-bond formation and pair-bond maintenance are regulated differently.

2.5 Conclusions

Water restriction affects the physiological and endocrine profiles of both male and female zebra finches, consistent with an induction of non-breeding condition, particularly in females. Nonetheless, pair-maintenance behavior and sex steroid levels in some brain regions, such as the hypothalamus, are not reduced by water restriction. While correlational, these data are nonetheless consistent with the hypothesis that local production of sex steroids in the brain promotes the expression of pair-maintenance behaviors in non-breeding zebra finches,

a hypothesis that will be directly tested in future experiments by pharmacologically inhibiting sex steroid synthesis or action in water-restricted individuals.

Table 2.1: Water consumption by water-restricted female (n=11) and male (n=11) zebra finches

WEEK	1	2	3	4	5	6	7	8
Water received	3 x 4mL	2 x 3mL	2 x 2mL	1 x 2mL	1 x 1mL	1 x 1mL	1 x 1mL	1 x 1mL
Water consumed, *females* (mL)	5.5 ± 0.41	2.6 ± 0.22	2.5 ± 0.2	1.2 ± 0.08	0.8 ± 0.02	0.8 ± 0.03	0.7 ± 0.05	0.7 ± 0.06
Water consumed, *males* (mL)	5.2 ± 0.36	2.9 ± 0.22	2.5 ± 0.14	1.2 ± 0.06	0.8 ± 0.05	0.8 ± 0.01	0.6 ± 0.04	0.7 ± 0.06

Note: Water received is given in number of times per week × volume at each "watering" (e.g., in Week 1, individuals received 4 mL of water on 3 separate days).

Table 2.2: Radioimmunoassay specifications

				% Recovery			
Steroid	RIA kit	Modification	Detection limit (pg/tube)	Plasma	Brain	Intra-assay variation (mean %CV)	Inter-assay variation (mean %CV)
17β-Estradiol	Beckman - Coulter, DSL-4800	Charlier et al. 2010	0.2	72.3	81.6	5.1	5.1
Testosterone	MP Biomedicals, cat. 07189102	Overk et al. 2013	0.3	90.2	78.9	12.0	11.0
DHEA	Beckman - Coulter, DSL-8900	Granger et al. 1999	2.0	85.0	90.0	12.2	12.5
Corticosterone	MP Biomedicals, cat. 07120103	Washburn et al. 2002	3.1	78.4	87.7	10.5	11.0

Table 2.3: Effects of water restriction (WR) on female and male reproductive physiology

females		Control	WR
Largest follicle	diameter (mm)	3.9 ± 0.7	**1.8 ± 0.4****
Ovary	mass (mg)	119.6 ± 10.6	**29.9 ± 1.2****
Oviduct	length (mm)	81.2 ± 5.6	**43.0 ± 5.6*****
	mass (mg)	230.7 ± 13.4	**65.5 ± 4.1****
males			
Left testis	length (mm)	4.0 ± 0.2	4.0 ± 0.2
	width (mm)	3.3 ± 0.2	3.1 ± 0.2
	volume (mm^3)	23.1 ± 0.3	21.5 ± 3.4
Right testis	length (mm)	3.4 ± 0.1	3.5 ± 0.2
	width (mm)	2.9 ± 0.2	2.7 ± 0.1
	volume (mm^3)	15.4 ± 1.8	13.3 ± 1.3
Total Testes	mass (mg)	41.32 ± 0.80	40.68 ± 1.02

Table 2.4: Steroid concentrations in plasma collected from the brachial vein

		Estradiol (pg/mL)		Testosterone (ng/mL)		Corticosterone (ng/mL)	
		Pre	Post	Pre	Post	Pre	Post
Female	CON	34.7 ± 3.1 (8)	40.3 ± 7.8 (10)	0.2 ± 0.01 (5)	0.3 ± 0.02 (10)	21.7 ± 3.4 (4)	13.5 ± 2.8 (10)
	WR	35.3 ± 4.8 (6)	31.4 ± 2.9 (11)	0.2 ± 0.02 (7)	0.2 ± 0.02 (10)	15.5 ± 3.4 (4)	17.2 ± 2.6 (10)
Male	CON	35.8 ± 1.4 (7)	34.0 ± 2.6 (9)	2.9 ± 0.2 (9)	1.9 ± 0.4 (9)	11.4 ± 3.6 (2)	9.4 ± 0.9 (7)
	WR	32.3 ± 2.0 (9)	28.9 ± 2.1 (11)	2.2 ± 0.6 (8)	**0.7 ± 0.2* (11)**	14.1 ± 4.3 (6)	17.2 ± 1.8 (11)

Note: Sample sizes are in parenthesis. Bolded value indicates significant difference between CON and WR in the Post-WR time period. * $p \leq 0.05$.

Table 2.5: Effect of water restriction on general activity during Baseline Behavior Sessions

		# Visits to the Water tower		% Time spent feeding		% Time spent self-preening	
		Pre	Post	Pre	Post	Pre	Post
Female	**CON**	1.75 ± 0.4	2.00 ± 0.5	4.39 ± 1.1	5.98 ± 1.8	8.53 ± 3.6	5.67 ± 2.3
	WR	2.09 ± 0.6	**0.00 ± 0.0*** **	5.65 ± 1.0	5.56 ± 0.8	6.63 ± 2.0	2.92 ± 1.5
Male	**CON**	1.45 ± 0.4	1.80 ± 0.5	1.96 ± 0.5	3.43 ± 1.2	6.80 ± 2.9	2.59 ± 0.6
	WR	1.64 ± 0.3	**0.05 ± 0.1*** **	2.34 ± 0.2	4.34 ± 1.0	7.37 ± 2.3	3.68 ± 1.6

Note: Bolded values indicate significant difference between CON and WR in the Post-WR time period. *** $p \leq 0.001$.

Table 2.6: Number of pairs that engaged in infrequent affiliative and reproductive behaviors during Baseline Behavior Sessions

	Allopreening		Clumping		Copulations		Carrying nesting	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post
CON	1	1	0	0	2	1	4	5
WR	6	4	0	3	2	0	6	**1***

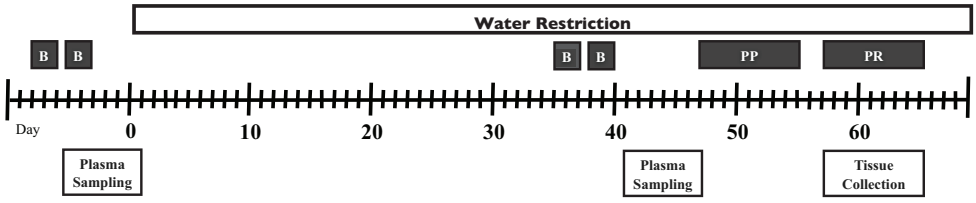
Note: Bolded value indicates significant difference between CON and WR in the Post-WR time period. * $p \leq 0.05$. n = 10 CON pairs and 11 WR pairs.

Table 2.7: Brain steroid levels

Region	Factor	Estradiol F	Estradiol P	Testosterone F	Testosterone P	DHEA F	DHEA P	Corticosterone F	Corticosterone P
Plasma	WR	1.07	0.309	**10.58**	**0.002**	1.15	0.290	0.07	0.796
	sex	1.08	0.306	**27.58**	**0.000**	**4.28**	**0.046**	1.79	0.189
	WR*sex	0.34	0.566	**16.08**	**0.000**	0.51	0.481	2.19	0.148
Hypothalamus	WR	2.80	0.103	0.73	0.398	1.59	0.215	0.02	0.869
	sex	1.82	0.185	0.20	0.660	1.19	0.282	0.20	0.651
	WR*sex	0.00	0.998	2.83	0.101	0.06	0.806	0.68	0.415
Rostral Telencephalon	WR	2.31	0.137	**4.77**	**0.035**	0.12	0.740	1.60	0.220
	sex	0.01	0.930	**4.33**	**0.044**	0.01	0.905	3.90	0.055
	WR*sex	0.44	0.509	0.14	0.708	0.53	0.820	1.20	0.288
Central Telencephalon	WR	**10.20**	**0.003**	1.69	0.202	0.06	0.801	0.72	0.400
	sex	0.22	0.639	7.75	**0.008**	1.59	0.215	3.82	0.058
	WR*sex	0.01	0.908	0.54	0.468	0.01	0.933	0.05	0.832
Caudal Telencephalon	WR	**16.52**	**0.000**	0.97	0.330	0.37	0.546	0.90	0.350
	sex	**10.50**	**0.002**	**6.14**	**0.018**	0.09	0.768	3.14	0.084
	WR*sex	**6.13**	**0.018**	**5.80**	**0.021**	0.00	0.973	0.19	0.669
Mid/Hindbrain	WR	**6.60**	**0.014**	3.46	0.071	0.28	0.599	0.77	0.385
	sex	0.01	0.924	1.24	0.271	0.09	0.762	0.00	0.995
	WR*sex	0.83	0.368	2.84	0.100	0.42	0.520	0.00	0.950
Cerebellum	WR	**19.57**	**0.000**	**9.94**	**0.003**	0.65	0.425	1.40	0.244
	sex	**7.32**	**0.010**	**12.12**	**0.001**	0.04	0.840	0.60	0.445
	WR*sex	0.14	0.708	**5.23**	**0.028**	0.83	0.369	0.00	0.960

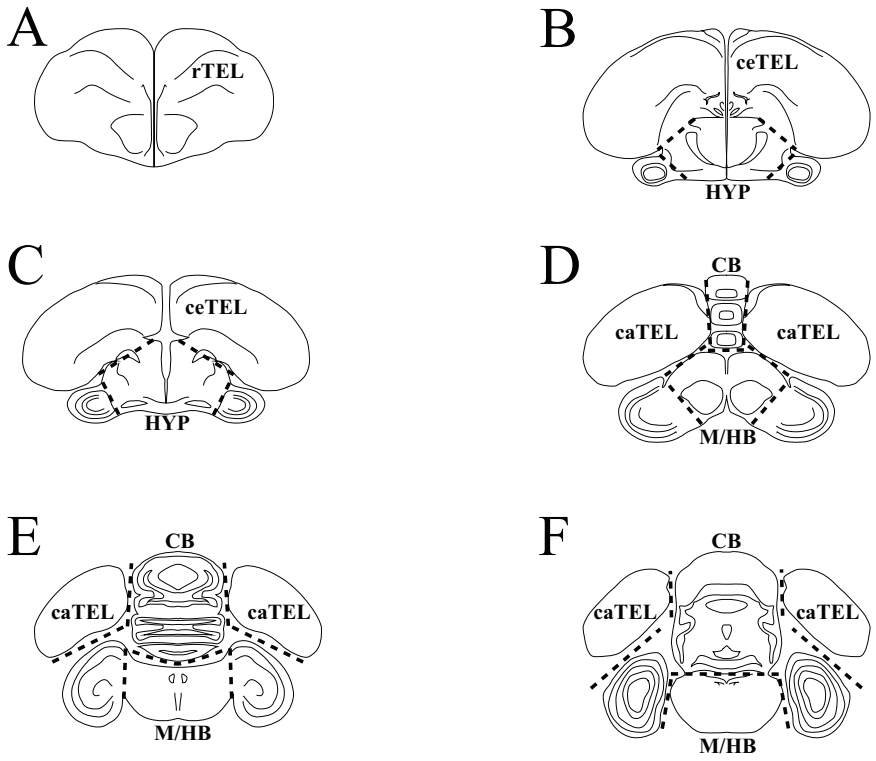
Note: Bolded values indicate significant effects of water restriction (WR) or sex or the interaction.
Significant interactions were followed up with simple or simple, simple main effects.

Figure 2.1 Experimental timeline 1



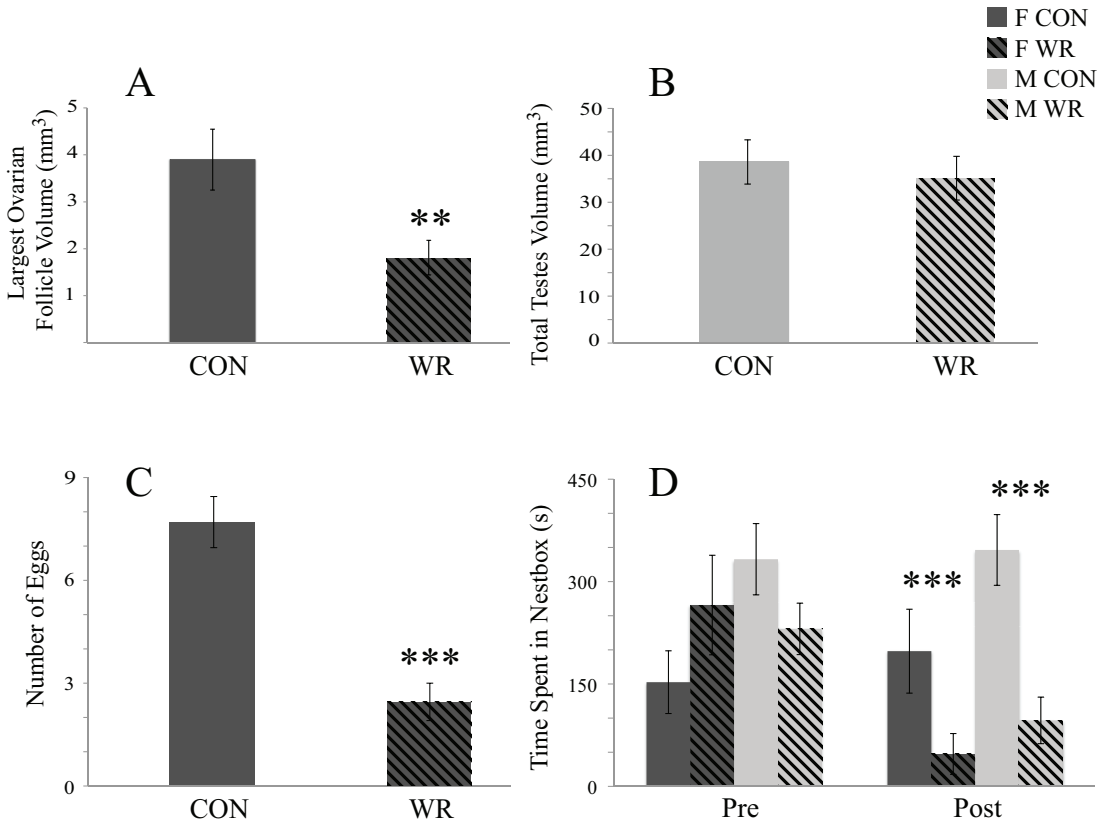
Experimental timeline. B = Baseline Behavior Sessions, in which subjects were recorded in the home cage in the colony room. PP = Partner Preference Test, and PR = Partner Reunion Test. Plasma samples were collected from the brachial vein from subjects in the home cage in the colony room. Brain tissue and trunk plasma were collected immediately after the Partner Reunion Test. Each tick mark indicates 1 d.

Figure 2.2 Diagram of brain dissections



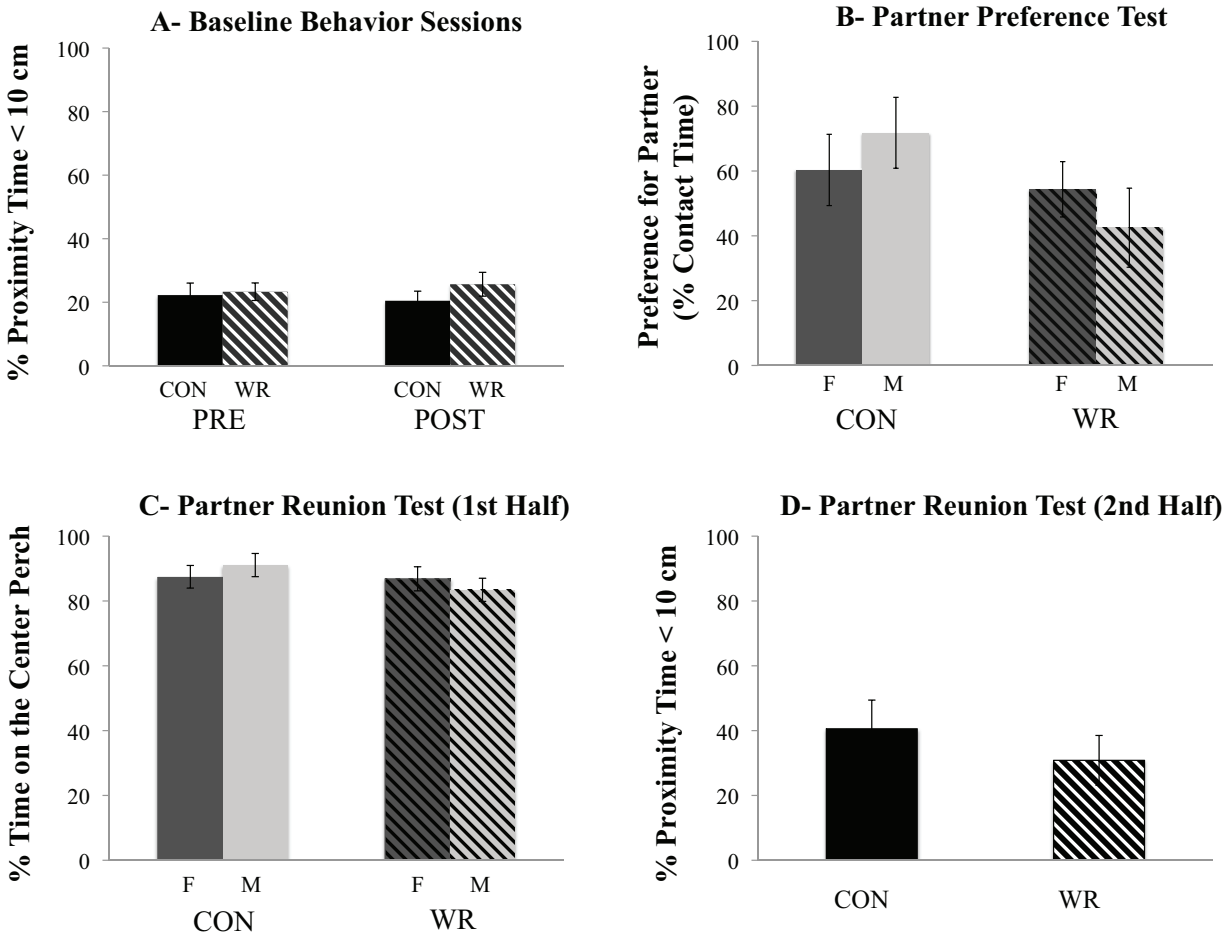
Coronal sections (300 μm) were made on a cryostat and a scalpel blade was used to dissect brain tissue. Prominent neuroanatomical landmarks were used to identify regions and make cuts with the scalpel blade (indicated by the dashed lines). The diagram in A is the most rostral section, and the diagram in F is the most caudal section. Tissue from the same region was pooled within an individual across sections. caTEL=caudal telencephalon, CB=cerebellum, ceTEL=central telencephalon, HYP=hypothalamus, M/HB=mid/hindbrain, rTEL=rostral telencephalon.

Figure 2.3 Effects of water restriction on reproductive physiology in females and males



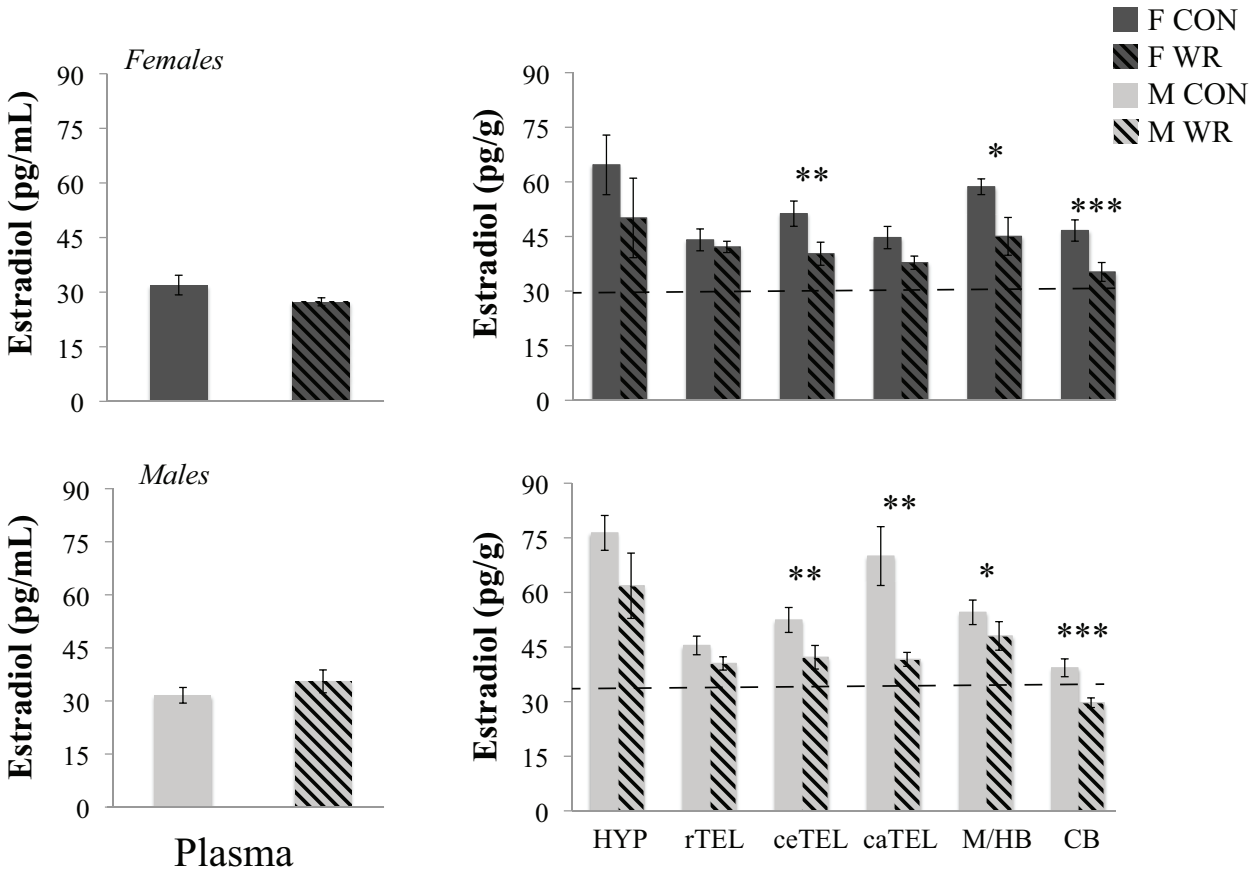
Effects of water restriction (WR) on reproductive physiology in females (F) and males (M). Effects of WR on (A) largest ovarian follicle volume, (B) total testes volume, (C) the number of eggs laid, and (D) the time spent in the nestbox. Relative to control subjects (CON) with *ad libitum* water, WR strongly reduced largest ovarian follicle volume ($t = 3.25$, $p = 0.004$) but had no effect on total testes volume ($t = 0.52$, $p = 0.61$). WR also decreased the number of eggs laid ($t=5.67$, $p < 0.0001$) and time spent in the nestbox in the post-WR period ($F_{1,38} = 13.23$, $p = 0.0008$). ** $p \leq 0.01$, *** $p \leq 0.001$

Figure 2.4 Effects of water restriction on pair-maintenance behaviors



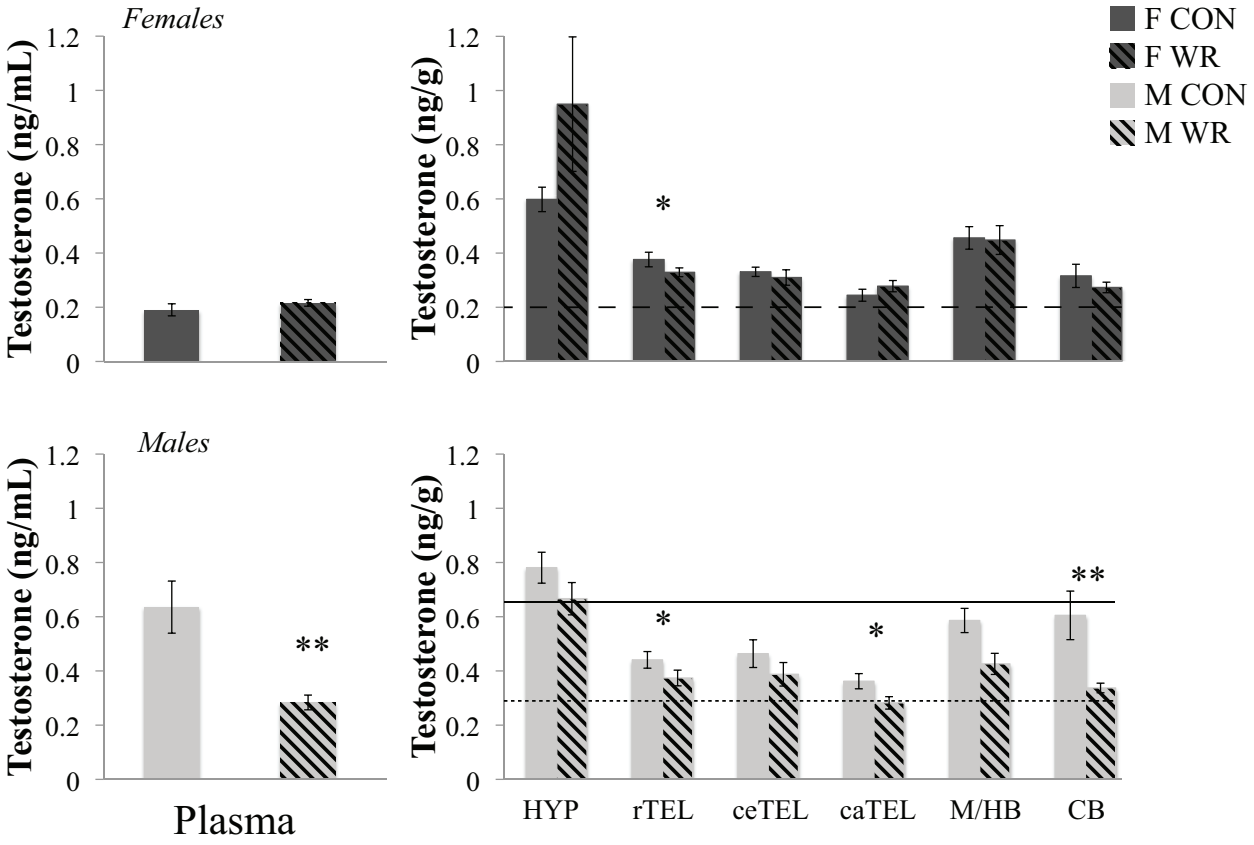
No effects of water restriction (WR) on pair-maintenance behaviors. WR had no effects on physical proximity, the primary measure of affiliation, in the 3 behavioral paradigms: (A) Baseline Behavior Sessions, (B) Partner Preference Test, (C) 1st half of the Partner Reunion Test (visual reunion only) and (D) 2nd half of the Partner Reunion Test (physical reunion).

Figure 2.5 Effects of water restriction on plasma and brain estradiol levels



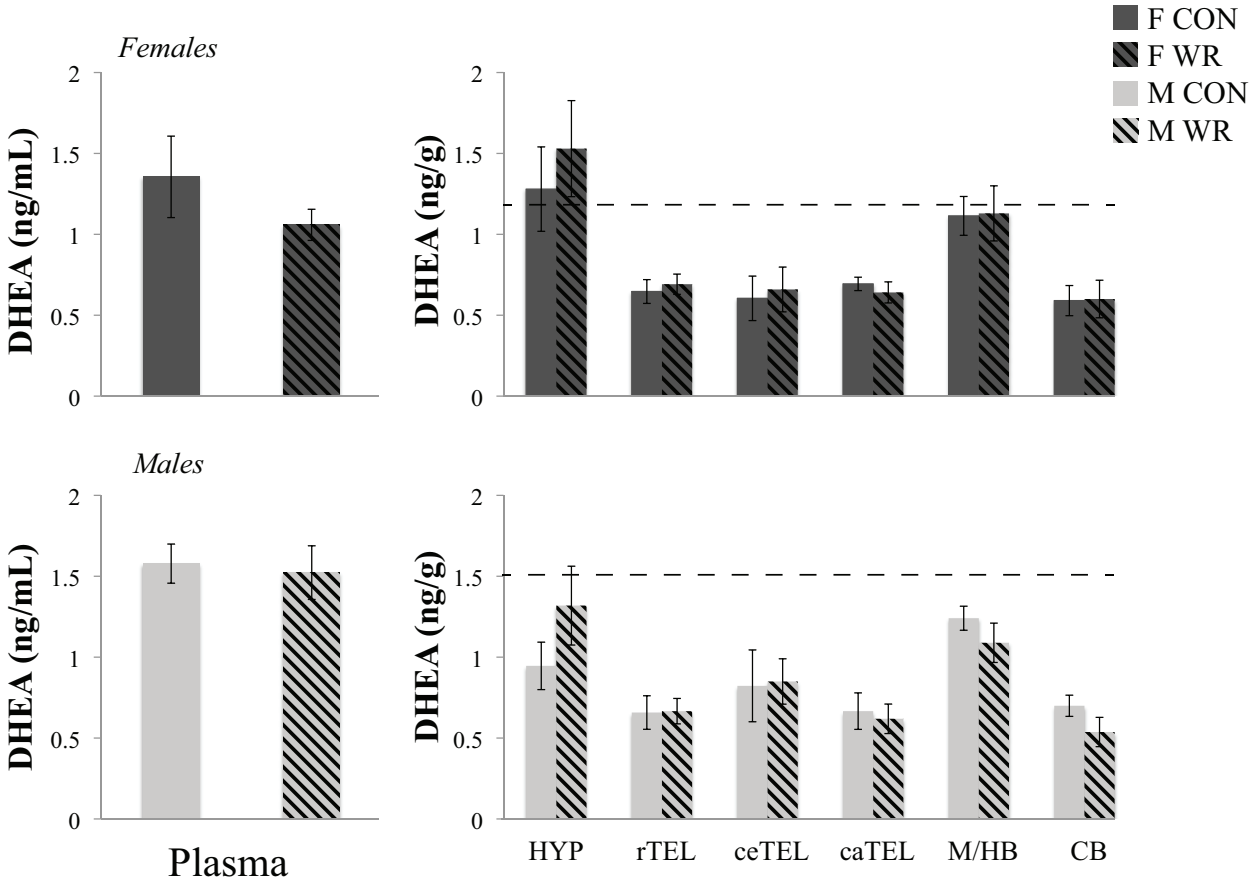
Effects of water restriction (WR) on plasma and brain estradiol levels. Samples were collected immediately after the Partner Reunion Test. WR had no effect on plasma estradiol levels in males or females, but decreased estradiol levels in some brain regions. The dashed lines on the brain graphs indicate the mean plasma estradiol level (CON and WR groups pooled) for females and for males. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

Figure 2.6 Effects of water restriction on plasma and brain testosterone levels



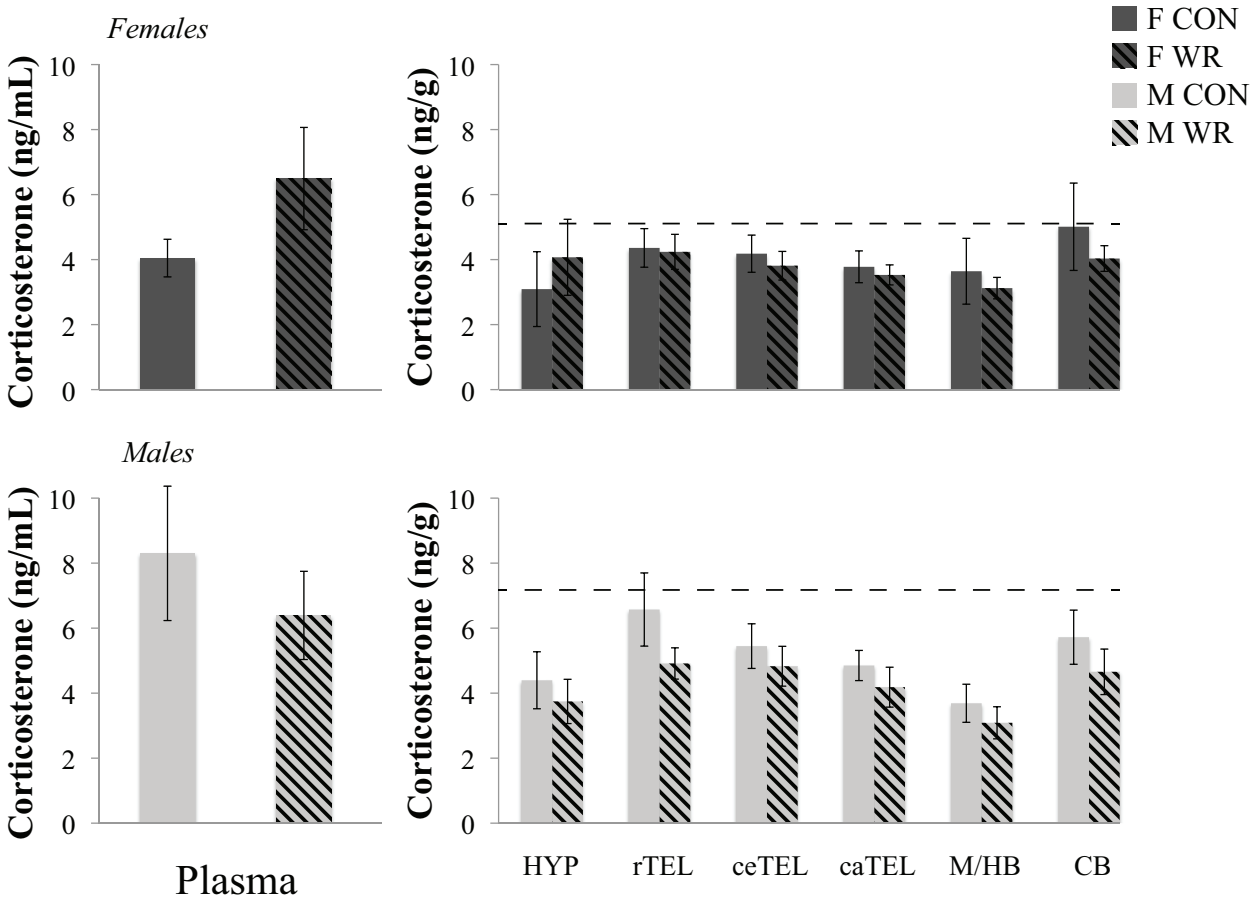
Effects of water restriction (WR) on plasma and brain testosterone levels. Samples were collected immediately after the Partner Reunion Test. WR had no effect on plasma testosterone levels females but significantly decreased plasma testosterone levels in males. Additionally, WR decreased testosterone levels in some brain regions. The dashed line on the female brain graph indicates the mean plasma testosterone level (CON and WR groups pooled) for females. The solid line on male brain graph indicates the mean plasma testosterone level in CON males, and the dotted line indicates the mean plasma testosterone level in WR males. * $p \leq 0.05$, ** $p \leq 0.01$

Figure 2.7 No effects of water restriction on plasma and brain DHEA levels



No effects of water restriction (WR) on plasma and brain dehydroepiandrosterone (DHEA) levels. WR had no effect on plasma or brain DHEA levels in males or females. The dashed lines on the brain graphs indicate the mean plasma DHEA level (CON and WR groups pooled) for females and for males.

Figure 2.8 No effects of water restriction on plasma and brain corticosterone levels



No effects of water restriction (WR) on plasma and brain corticosterone levels. WR had no effect on plasma or brain corticosterone levels in males or females. The dashed lines on the brain graphs indicate the mean plasma corticosterone level (CON and WR groups pooled) for females and for males.

3 Acute and chronic effects of fadrozole on pair-maintenance behavior in water-restricted zebra finch pairs

3.1 Introduction

Sex steroids such as estradiol can regulate behavior by modulating gene transcription (i.e., "genomic" mechanisms) or by modulating intracellular signal transduction pathways (i.e., "non-genomic" mechanisms) (Balthazart et al., 2006). Note, however, that intracellular signal transduction pathways can affect gene transcription (Björnström and Sjöberg, 2005). Genomic effects of estradiol on behavior generally take hours to days; non-genomic effects of estradiol on behavior can occur within 15 to 30 min. In general, brain-synthesized estradiol appears more likely than gonad-synthesized estradiol to act via non-genomic mechanisms (Balthazart et al., 2004; Schmidt et al., 2008; London et al., 2009).

Brain-synthesized estradiol can rapidly increase aggressive and sexual behaviors in several species (Cross and Roselli, 1999; Balthazart et al., 2004; Cornil et al., 2006). Brain synthesis of steroids (neurosteroids) can explain how steroids regulate behaviors even when gonadal secretion of sex steroids is low. For example, in song sparrows (*Melospiza melodia*), aggressive behaviors are regulated by brain-synthesized estradiol in the non-breeding season, whereas they are regulated by gonad-synthesized sex steroids in the breeding season (Soma et al., 2000; Schmidt et al., 2008). Breeding readiness is not dichotomous for the opportunistically-breeding zebra finch, but rather it is a complex continuum that appears to be largely regulated by water availability, both in the wild and laboratory (Vleck and Priedkalns, 1985; Zann et al., 1995; Perfito et al., 2007). However, distinct endocrine states (brain and circulating steroid levels) are seen in breeding and non-breeding zebra finches (Chapter 2: Prior et al., 2013). Furthermore, there may be an up-regulation of brain-

synthesized estradiol and testosterone in behaviorally-relevant brain regions of non-breeding male and female zebra finches, consistent with the pattern seen in song sparrows (Chapter 2: Prior et al., 2013). Taken together, these data raise the hypothesis that non-genomic steroid signaling mechanisms might be important in the regulation of behavior in water-restricted zebra finches than breeding zebra finches.

Here, we examine the effects of acute (Experiment 1) and chronic (Experiment 2) aromatase inhibitor (fadrozole) treatment on the pair-maintenance behaviors of water-restricted (i.e., non-breeding) zebra finch pairs. If estradiol promotes pair-maintenance behaviors via non-genomic mechanisms, then fadrozole treatment would decrease these behaviors in both experiments. If estradiol promotes pair-maintenance behaviors via genomic mechanisms only, then fadrozole treatment would decrease these behaviors in Experiment 2 only. Alternatively, if these behaviors are not promoted by estradiol, then neither acute nor chronic fadrozole treatment would decrease these behaviors.

3.2 Materials and methods

3.2.1 Subjects

Subjects were adult (> 120 d old) captive zebra finches housed in a colony maintained on a 14:10 h light:dark cycle. The colony typically has average temperature of 22ºC and average relative humidity of 31% (range: 21%-76%) (Chapter 2). All zebra finches had *ad libitum* access to seed (50/50, Panicum millet/white millet, Just For Birds, Langley BC), cuttlefish bone, and grit. Prior to experimental water restriction, all subjects had *ad libitum* access to water. Pairs were housed in cages (38 ½ x 19 ¾ x 19 in, Corners Cages), in which a solid divider had been placed down the middle. Each pair therefore occupied half of the cage.

Prior to the start of the study, pairs were provided a nestbox (5 ½ x 5 ½ x 7 ½ in) and nesting materials. Pairs were housed together for a minimum of 2 months prior to the start of the experimental manipulation, and all pairs engaged in affiliative, courtship, and/or nesting behaviors, and were thus considered pair bonded.

All subjects were water-restricted over the course of 4 wk, from 6 mL of water to a minimum of 1 mL per pair per week (i.e., 3 mL to 0.5 mL per subject per week). Here, the water for a pair was always split between two water towers, to prevent one individual from monopolizing all of the water. For half of the pairs, the female was designated the focal subject, and for the other half of the pairs, the male was designated the focal subject. This protocol for water restriction is intermediate between complete water deprivation (Sossinka, 1974) and more gradual water restriction over 11 weeks (Perfito et al., 2006). Additionally, this protocol is modified based on the amount of water consumed during the water restriction period of our previous study (1 mL per subject per week, Chapter 2: Prior et al., 2013). Zebra finches are opportunistic breeders, and water restriction is highly effective at reducing breeding readiness. In females, reproductive organs (oviduct and ovary) are profoundly reduced by water restriction (Chapter 2: Prior et al., 2013). Additionally, in males, circulating testosterone levels are significantly decreased (Chapter 2: Prior et al., 2013). Additionally, at the level of the pair, the number of eggs laid and the time spent engaging in breeding-related behaviors is decreased (Chapter 2: Prior et al., 2013). In the current study we saw very few eggs laid, and male plasma testosterone levels were similar to water-restricted males in our previous study (Chapter 2: Prior et al., 2013).

These experiments were carried out under a University of British Columbia Animal Care Committee protocol and followed the guidelines of the Canadian Council on Animal Care.

3.2.2 General timeline

A within-subjects design was used for the acute and chronic fadrozole (FAD) experiments (Experiment 1 and Experiment 2, respectively), and the same individual within a pair was the focal subject for both experiments. To minimize the stress of administration, fadrozole was delivered orally using a micropipette (Saldanha et al. 2004; Lee et al., 2007; Kabelik et al., 2011). The behavioral test was a Partner Separation and Reunion test (Figure 3.1, see below). The focal subject received both the vehicle and fadrozole treatments for both experiments, and the behavior of each pair was assessed a total of four times. The order of treatment was counterbalanced within each experiment. There were washout periods between treatments within an experiment and also between the two experiments (Figure 3.1A). In Experiment 1 (acute effects of fadrozole), the Partner Separation and Reunion test was administered immediately after the focal subject received fadrozole (Figure 3.1A). In Experiment 2 (chronic effects of fadrozole), the focal subject received fadrozole daily for 1 wk, and the following day (within 22 hr of the last dosing), the Partner Separation and Reunion test was administered (Figure 3.1A). In Experiment 2, immediately following the behavioral test, the focal subject was caught, and a blood sample was collected from the brachial vein and body mass was recorded. Blood samples were used for measurements of corticosterone, testosterone and estradiol.

We used a different dose of fadrozole for acute versus chronic administration. For Experiment 1, the focal subject received a single dose of 500 μg fadrozole in 20 μL of saline

(~36 mg/kg) or vehicle orally via micropipette. This dose was tested in a pilot study (see below). For Experiment 2, the focal subject received daily doses of 300 μg fadrozole in 20 μL of apple juice (~21 mg/kg) or vehicle orally, every day for 7 d (between 14:30 – 18:30 hr). In Experiment 2, apple juice was used as the vehicle to mask the taste of the fadrozole, which zebra finches appear to find unpalatable (our personal observations).

3.2.3 Pilot study: acute effects of fadrozole treatment

In a pilot study, we examined the rapid effects of orally administered fadrozole on estradiol and testosterone levels in plasma and brain. We used the same dose of fadrozole that was used in Experiment 1 (500 μg FAD in 20 μL of saline, ~36 mg/kg) or administered vehicle orally. This dose is slightly higher than what has been previously used because our treatment period was very short (Wade et al., 1994; Saldanha et al., 2004; Lee et al., 2007).

Water-restricted zebra finch pairs (n=20 pairs, same water restriction protocol as above) were used for this pilot study. Pairs were assigned to fadrozole treatment (n = 10) or vehicle (n = 10). In the pilot study only, both individuals in a pair were dosed at the same time with either FAD or vehicle, between 09:00 and 12:00 hr. Oral dosing was completed within 2 min (48.9 ± 7.4 sec) of capture. After 22 min, subjects were euthanized via rapid decapitation within 3 min of entering the testing room (63.3 ± 4.5 sec). This timepoint corresponds to the timeline of behavioral testing in Experiment 1 (see below and Figure 3.1B). Trunk blood and the brain were collected. The whole brain was flash frozen on powdered dry ice and stored at -80°C. Trunk blood was centrifuged for 10 min at 10,000 x g to obtain plasma, which was stored at -20°C. Brains were sectioned in the coronal plane at 300 μm on a cryostat at -12°C. Major neuroanatomical landmarks were used to divide the brain into several regions of interest (Chapter 2: Prior et al., 2013). Specifically, a scalpel

was used to dissect three regions: hypothalamus (HYP); a subsection of the caudal telencephalon containing predominantly caudomedial nidopallium (NCM), an extremely aromatase-rich region; and central telencephalon (ceTEL). Brain samples were stored at -80ºC.

3.2.4 Partner separation and reunion test

For both experiments, the behavioral endpoint we used was a partner separation and reunion test. We chose to use a Partner Separation and Reunion testing paradigm because pairs engage in higher levels of pair-maintenance behavior than they would at baseline (Chapter 2: Prior et al., 2013) (Figure 3.1B). At the start of the test, pairs were placed in a novel cage (set up similarly to the home cage) in testing rooms separate from the main colony. The testing cage was divided in half with both wire and opaque partitions. The male and the female within a pair were randomly placed on opposite sides of the partition, immediately prior to testing. Behavior was recorded with a camcorder during the full 20 min of the separation period (pairs are in acoustic contact during this period). Then the opaque partition was removed (leaving the wire partition in) for a 5 min visual reunion period, during which behavior was recorded. Finally, the wire partition was removed for a 5 min full reunion period, during which behavior was recorded. In Experiment 1, behavioral tests were conducted between 09:00 - 16:00 hr. In Experiment 2, behavioral tests were conducted between 09:00 - 14:00 hr.

3.2.5 Behavioral scoring

Behavior was then quantified by researchers (NHP and KNY) blind to treatment during three test periods: separation, visual reunion, and full reunion. Overall, the primary measures of pair-maintenance behavior were (1) time spent in close proximity (NHP), and

(2) total time spent vocalizing (primarily contact calls) (NHP). Calling was so rapid that we were unable to distinguish between female and male calls, thus this is a pair-level behavior. We also quantified locomotor activity [perch hops (KNY) and returns (NHP)] and other general behaviors (feeding, drinking, and self-preening) (NHP). These general behaviors were extremely rare and therefore are not presented here.

During the separation period (20 min), perch hops and time spent vocalizing were quantified separately for the first and second halves. We chose to do this because, in Experiment 1, it was possible that acute fadrozole administration might not have an effect on behavior within 10 min but might have an effect between 10 and 20 min. For Experiment 2, we were consistent to facilitate a comparison to Experiment 1. During the visual reunion period (5 min), time spent vocalizing, time spent on the center perch (immediately adjacent to the wire partition and thus an important measure of proximity), and returns (the number of times an individual left the center perch and returned to it within 4 sec) were scored. During the full reunion period (5 min), time spent vocalizing, proximity time (time within 10 cm of each other), and number of perch hops were scored. Throughout the entire test, other general behaviors (feeding, drinking, self-preening) were quantified. Perch hops and returns are expressed as a rate (number per min). Proximity time and time spent vocalizing are expressed as a percentage of time.

3.2.6 Tissue processing

Plasma and brain samples were processed as before (Chapter 2: Prior et al., 2013) and the procedures are summarized below.

Pilot Study: Acute Effects of Fadrozole Treatment

For estradiol and testosterone radioimmunoassays, brain and plasma samples were first homogenized and then were extracted from plasma using solid phase extraction with C_{18} columns (Agilent Bond-Elut OH, 500mg, cat # 12113045) (Newman et al., 2008a; Taves et al., 2011). Tissue was homogenized in 2 mL polypropylene microcentrifuge tubes with 225 µL ice-cold de-ionized water, 1200 µL HPLC-grade methanol and three small ceramic beads for 1 min at a speed of 4 m/sec (Omni Bead Ruptor 24). Homogenates were left at 4°C overnight. Following centrifugation, supernatants were diluted in 10 mL de-ionized water, and were loaded onto C_{18} columns, which had been primed with 3 mL of HPLC-grade methanol and equilibrated with 10 mL de-ionized water (no more than 50 mg brain tissue per C_{18} column). Columns were then washed with 10 mL 40% HPLC-grade methanol, and steroids were eluted with 5 mL 90% HPLC-grade methanol. The eluted samples were dried at 40°C in a vacuum centrifuge (ThermoElectron SPD111V Speedvac) and stored at -20°C until assayed.

Extracted steroid samples were resuspended in 450 µL PBSG (phosphate-buffered saline containing 0.1% gelatin), with absolute ethanol (0.8%) to aid with resuspension. These resuspended samples were used to measure estradiol and/or testosterone using radioimmunoassays. From each sample, 300 µL and 60 µL were taken in singleton for estradiol and testosterone, respectively. All samples from a given individual were run in the same assay. All values were corrected for recovery (estradiol in plasma, 72.3%; estradiol in brain, 81.6%; testosterone in plasma, 90.2%; testosterone in brain, 78.9%) (Chapter 2: Prior et al., 2013). Samples below the detection limit of the respective standard curve (estradiol, 0.2 pg/tube; testosterone, 0.3 pg/tube) were set to zero.

Experiment 2: Chronic Effects of Fadrozole Treatment

Corticosterone was measured in unextracted plasma. For estradiol and testosterone radioimmunoassays, steroids were extracted from plasma using solid phase extraction (as above). Plasma (~40 µL) was diluted in 10 mL water and loaded onto C_{18} columns (as above). Plasma samples ≥30 µL were resuspended in 450 µL; 300 µL was used to quantify estradiol and 75 µL was used to quantify testosterone. For a small number of samples (6 out of 50), there was insufficient plasma to measure both testosterone and estradiol; here, we resuspended in 350 µL to quantify estradiol only. All samples were measured as singletons. All samples from a given individual were run in the same assay. All values were corrected for recovery, and non-detectable samples were set to zero, as above. The detection limit for corticosterone was 3.1 pg/tube.

3.2.7 Statistical analyses

In the pilot study, to test for an effect of acute oral administration of fadrozole on circulating and brain estradiol and testosterone levels, a 3-way mixed-model ANOVA was used with Treatment (vehicle vs. fadrozole) and Sex (male vs. female) as between-subjects factors and Region (plasma, HYP, NCM, ceTEL) as a within-subjects factor. Significant Treatment × Sex interactions were followed up with a model reduction technique (simple main effects), where separate ANOVAs were conducted to examine the effect of fadrozole treatment within each sex.

To test for an effect of chronic fadrozole administration on circulating estradiol, testosterone, and corticosterone levels, we used a mixed-model ANOVA with Sex (male vs. female) as a between-subjects factor and Treatment (vehicle vs. fadrozole) as a within-

subjects factor. Significant Treatment × Sex interactions were followed up with a model reduction technique (simple main effects), where separate paired t-tests were conducted to examine the effect of fadrozole treatment within each sex. Body mass data were analyzed using a paired t-test with Treatment (control vs. fadrozole) as the within-subjects factor.

For each behavioral measure, the effects of acute and chronic fadrozole were analyzed separately. Additionally, each period of the partner separation and reunion test was analyzed separately (separation, visual reunion, and full reunion). To test for an effect of fadrozole on pair-level behaviors (i.e., time spent vocalizing and proximity time), we used two-way mixed-model ANOVAs with Sex (male vs. female) as the between –subjects factor and Treatment (vehicle vs. fadrozole) as the within-subjects factor. Individual-level behaviors (e.g., time spent on the center perch) were analyzed separately for the focal subject and the partner. These data were analyzed with mixed-model ANOVAs with Sex (male vs. female) as the between-subjects factor and Treatment (vehicle vs. fadrozole) as the within-subjects factor. All statistics were run in Cran R Statistics 2.14.

All behavioral data are presented using violin plots made in R Statistic 2.14 (R Core Team, 2012). Violin plots combine a box plot (white circles denote the median) with a kernel density plot (Hintze and Nelson, 1998). The width of the plot signifies the probability density at different y-values (i.e., the proportion of data at different y-values), similar to a histogram. These plots are smoothed to facilitate visual comparison of data distribution.

3.3 Results

3.3.1 Acute effects of fadrozole on circulating and brain steroid levels

Oral administration of fadrozole (500 μg) differentially affected males and females (Treatment × Sex interaction ($F_{1,59} = 17.47$, $P < 0.001$)). More specifically, FAD treatment significantly decreased estradiol levels in male plasma and brain tissue (Figure 3.2A, Treatment: $F_{1,27} = 14.35$, $P < 0.001$; Region: $F_{3,27} = 1.64$, $P = 0.20$; Treatment × Region: $F_{3,27} = 2.16$, $P = 0.12$). The effect of fadrozole on estradiol levels in the male NCM was particularly pronounced. Acute fadrozole treatment did not affect estradiol levels in females (Treatment: $F_{1,28} = 1.79$, $P = 0.19$; Region: $F_{3,28} = 18.29$, $P < 0.001$; Treatment × Region: $F_{3,28} = 0.81$, $P = 0.50$).

Oral fadrozole administration rapidly increased testosterone levels in plasma and brain tissue in both males and females (Figure 3.2B, Treatment: $F_{1,60} = 4.66$, $P = 0.035$; Sex: $F_{1,60} = 2.15$, $P = 0.15$; Region: $F_{3,60} = 4.47$, $P = 0.007$; all interactions, $P > 0.05$).

3.3.2 Chronic effects of fadrozole on circulating steroid levels

There was no significant effect of chronic fadrozole treatment on circulating estradiol levels in either male or female focal subjects (Figure 3.3A, Treatment: $F_{1,23} = 0.51$, $P = 0.48$; Sex: $F_{1,23} = 0.06$, $P = 0.80$; Treatment × Sex: $F_{1,23} = 0.006$, $P = 0.94$).

Chronic fadrozole treatment increased circulating testosterone levels differentially in males and females (Figure 3.3B, Treatment × Sex: $F_{1,32} = 5.92$, $P = 0.02$). More specifically, fadrozole significantly increased circulating testosterone levels in males only (females*:* $T_8 = 1.10$, $P = 0.30$; males*:* $T_9 = 3.18$, $P = 0.01$).

Chronic fadrozole treatment had no significant effect on circulating corticosterone levels (Treatment: $F_{1,32} = 0.03$, $P = 0.87$). However, males had higher circulating corticosterone levels than females (Figure 3.3C, Sex: $F_{1,32} = 8.31$, $P = 0.007$). There was no

significant Treatment × Sex interaction ($F_{1,32} = 0.18$, $P = 0.678$). Note also that chronic fadrozole treatment had no effect on body mass, another indicator of stress ($T_{23} = 0.59$, $P = 0.56$).

3.3.3 Effect of fadrozole on time spent in close proximity

In Experiment 1, which examined the acute effects of fadrozole treatment, there were no effects of Treatment or Sex on time spent on the center perch during the visual reunion period, for both the focal subject and partner (Figure 3.4A-B; Table 3.1). Furthermore, most individuals spent close to 100% of their time on the center perch during the visual reunion period (Figure 3.4A-B). During the full reunion period, acute fadrozole treatment significantly *increased* proximity time, regardless of whether males or females were treated with fadrozole (Figure 3.4C; Table 3.1).

In Experiment 2, which examined the chronic effects of fadrozole treatment, there were again no effects of Treatment or Sex on time spent on the center perch during the visual reunion period, for both the focal subject and partner (Figure 3.4D-E; Table 3.1). Again, most individuals spent close to 100% of their time on the center perch during the visual reunion period (Figure 3.4D-E). During the full reunion period, in contrast to Experiment 1, there was no effect of chronic fadrozole treatment on proximity time (Figure 3.4F; Table 3.1).

3.3.4 Effect of fadrozole on time spent vocalizing

In Experiment 1, there were no effects of Treatment or Sex on time the pair spent vocalizing during the partner separation period, for both the first half (Set 1, 0-10 min) and second half (Set 2, 10-20 min) (Figure 3.5A-B, Table 3.2). Similarly, there were no effects of Treatment or Sex on time spent vocalizing during the visual or full reunion periods (Figure

3.5C-D, Table 3.2). Most pairs spent the majority of the visual and full reunion periods vocalizing towards each other, and these vocalizations were primarily contact calls.

In Experiment 2, there were again no effects of Treatment or Sex on time spent vocalizing during any period of the behavioral paradigm (Figure 3.5E-H, Table 3.2). Again, most pairs spent the majority of the visual and full reunion periods vocalizing towards each other.

3.3.5 Effect of fadrozole on locomotor activity levels

In Experiment 1, locomotor activity was measured by perch hops during the partner separation period. There were no effects of Treatment or Sex on the number of perch hops by focal subjects (Figure 3.6A-B; Table 3.3). There was also no effect of fadrozole on perch hops by the partners (Table 3.4). During the visual reunion period, locomotor activity was measured by "returns," the number of times an individual left the center perch and returned within 4 sec. Fadrozole had no effect on returns made by focal subjects or by partners during the visual reunion period (Figure 3.6C; Tables 3.3-3.4). Finally, fadrozole had no effect on perch hops by focal subjects or by partners during the full reunion period (Figure 3.6D; Tables 3.3-3.4). There was also no effect of Sex of the focal subject on locomotor activity.

In Experiment 2, there was no effect of fadrozole on perch hops by male or female focal subjects during the partner separation period (Figure 3.6E-F; Table 3.3). Additionally, fadrozole had no effect on returns made by focal subject during the visual or perch hops during the full reunion period (Figure 3.6G-H; Table 3.3-3.4). Partner activity differed by treatment group and sex during the partner separation period and the visual reunion period (Table 3.4).

3.4 Discussion

Here we show that acute oral administration of fadrozole rapidly decreases estradiol levels in the circulation and brain of males and also rapidly increases testosterone levels in the circulation and brain of both males and females. We also show that acute and chronic administration of fadrozole to water-restricted zebra finches does not decrease pair-maintenance behaviors. In fact, acute fadrozole treatment increased time spent in close proximity during the full reunion period. Overall, these data provide no support for the hypothesis that pair-maintenance behaviors in water-restricted zebra finches are promoted by estradiol.

3.4.1 Oral administration of fadrozole

Fadrozole is a highly potent and specific competitive aromatase inhibitor in songbirds (Wade et al., 1994; Charlier et al., 2010). Fadrozole has been successfully used to alter songbird behavior (Soma et al., 2000; Belle et al., 2005) as well as neurochemistry (Lee et al., 2007; Kabelik et al., 2011). Furthermore, *in vitro* fadrozole significantly decreases aromatase activity in zebra finch telencephalic cultures within 15 min (Wade et al., 1994). Fadrozole administration via retrodialysis to the zebra finch NCM significantly decreases local estradiol levels and increases local testosterone levels within 30 min (Remage-Healey et al., 2008). We chose to administer fadrozole orally, as this is less stressful than s.c. or i.p. injections and therefore especially useful in studies that quantify behavior soon after administration. Chronic orally administered fadrozole has been used in several previous studies (Saldanha et al., 2000; Lee et al., 2007; Kabelik et al., 2010, 2011).

Here we show for the first time that orally administered fadrozole rapidly (within 25 min) decreases circulating and brain estradiol levels in male zebra finches. The percent decrease in estradiol levels differed depending on region, but was most pronounced in the

male NCM (~54% decrease), which has especially high levels of aromatase. Our overall percent decrease is consistent with results from previous studies (Wade et al., 1994; Charlier et al., 2010). Additionally, orally administered fadrozole rapidly increases circulating and brain testosterone levels in male and female zebra finches. The increase in testosterone levels is likely due, at least in part, to an accumulation of substrate for aromatase. Perhaps the effect of fadrozole on estradiol levels was greater in males than females because the dose we used was insufficient to inhibit ovarian aromatase, which is abundant (Schlinger, 1997). Alternatively, if we had examined microdissected brain regions (via Palkovits punch), we may have seen an effect of fadrozole on estradiol levels in specific nuclei in females and a more prominent effect in males. Overall, these data suggest that orally administered fadrozole rapidly inhibits brain conversion of testosterone to estradiol in males. While the increase in testosterone levels in females provides some evidence that fadrozole inhibited aromatase activity, there is no evidence that estradiol levels were affected in females.

In Experiment 2, we examined the effect of chronic fadrozole treatment on circulating estradiol and testosterone levels. Interestingly, we saw a significant increase in plasma testosterone levels without a significant decrease in plasma estradiol levels in males (there was a similar but non-significant pattern for testosterone in females). It is difficult to say whether measurement of steroids or aromatase activity is a better indicator of the *in vivo* effectiveness of fadrozole. While previous studies have used aromatase activity assays to examine the effects of fadrozole and vorozole (Wade et al., 1994; Cornil et al., 2006), most have not quantified effects on circulating estradiol and testosterone levels (Dittrich et al., 1999; Saldanha et al., 2000; Belle et al., 2005; Lee et al., 2007; Peterson et al., 2007; Spence et al., 2009; Rensel et al., 2013). Some of these studies also included a treatment group of

fadrozole plus estradiol, which successfully rescued the effect of fadrozole (Saldanha et al., 2000; Lee et al., 2007; Peterson et al., 2007; Spence et al., 2009). When circulating estradiol and testosterone levels are measured, there is often no effect on plasma estradiol levels but an increase in plasma testosterone levels (Soma et al., 2000; Kabelik et al., 2010; 2011). These effects of fadrozole can be rescued with estradiol (Soma et al., 2000; Kabelik et al., 2011).

Taken together, there seems to be a consistent disconnect between the effect of fadrozole on aromatase activity and the effect of fadrozole on systemic estradiol levels. Despite this disconnect, effects of fadrozole can be rescued by concomitant estradiol treatment. Therefore, it seems likely that depending on where aromatase is localized in the brain and in individual neurons (in pre-synaptic boutons or soma), the effect of fadrozole on the availability of estradiol may be variable throughout the brain. Furthermore, there are multiple mechanisms through which fadrozole could affect behavior: increases in systemic testosterone levels, possible decreases in systemic estradiol levels, and/or decreases in aromatase activity and estradiol levels in specific behaviorally-relevant neurons. It may be most effective to use gonadectomy or local fadrozole administration to specific brain regions to decrease brain estrogen levels.

3.4.2 Effects of fadrozole on pair-maintenance behavior

We measured ethologically relevant affiliative behaviors and saw no evidence that they were decreased by acute or chronic fadrozole treatment. In one case, acute fadrozole treatment increased an affiliative behavior (time spent in close proximity) during the full reunion period (see Figure 3.4C). Furthermore, we saw a similar non-significant trend for the time spent on the center perch during the visual reunion period (see Figure 3.4A). Thus, acute

fadrozole treatment increased an important metric of affiliation (time in close proximity) in both males and females.

This significant effect of acute fadrozole treatment on proximity time raises two questions: (1) how is fadrozole increasing proximity time, and (2) why is there a significant effect with acute but not chronic fadrozole treatment? With regard to the first question, fadrozole could be affecting affiliative behavior by (1) increasing testosterone, which may promote affiliation (Harding and Rowe, 2003; Hirschenhauser et al., 2008) or (2) decreasing a possibly inhibitory effect of estradiol on affiliation (Cushing and Wynne-Edwards, 2006; Lei et al., 2010). Testosterone promotes courtship in zebra finches (Harding and Rowe, 2003), and in other species, within-pair testosterone covariation may be a predictor of pair bond quality (Hirschenhauser et al., 2008). Conversely, in rodents ERα in the amygdala and hypothalamus is associated with reduced prosocial tendencies (Cushing and Wynne-Edwards, 2006; Wu et al., 2010).

With regard to the second question, chronic fadrozole treatment might affect brain steroid receptors. This phenomenon could result in the differential effect of acute versus chronic fadrozole treatment. More specifically, if testosterone is promoting affiliative behavior, then as testosterone levels increase in response to fadrozole, androgen receptor density may decrease over time; this would counteract the putative effects of testosterone in Experiment 2. Similarly, if fadrozole promotes affiliation via releasing the inhibitory effect of estradiol, then as estradiol levels decrease, estrogen receptor levels may increase over time to compensate. Alternatively, chronic oral administration (including the daily handling) may be more stressful to males than females, or may affect subsequent behavior more in male-treated pairs. There was a significant effect of Sex on proximity time during Experiment 2

(but not Experiment 1), such that male-treated pairs spent less time in close proximity compared to female-treated pairs. Only examining the female treated pairs, we see a similar trend in proximity time as the acute study. Interestingly, in Experiment 2, we also see that males have higher circulating corticosterone levels than females (regardless of pharmacological treatment). Males may be more affected by stress of daily dosing than females, and the decrease in proximity time could be a response to this stress.

3.4.3 Rapid, non-genomic versus genomic effects of estradiol on behavior

There are few studies of physiological mechanisms that regulate pair-maintenance behaviors (Elie et al., 2010; Alger et al., 2011; Smiley et al., 2012). While there has been mixed evidence regarding the role of sex steroids in regulating affiliation in zebra finch pairs (Harding and Rowe, 2003; Hill et al., 2005; Tomaszycki et al., 2006), this study was motivated by our previous results demonstrating there may be an up-regulation of brain-synthesized estradiol and testosterone in the hypothalamus of water restricted (i.e., non-breeding) males and females (Chapter 2: Prior et al., 2013). Those results suggested that non-genomic steroid signaling mechanisms might be particularly important for the behavior of non-breeding zebra finches, because neurosteroids have many rapid effects on behavior (Schmidt et al., 2008). While we saw no evidence that locally-synthesized estradiol promotes pair-maintenance behavior, neurosteroids may still be involved in other functions or behaviors of non-breeding zebra finches. Alternatively, locally-synthesized testosterone, rather than estradiol, may be more important in regulation of affiliation in the zebra finch.

3.5 Conclusions

Here we have used oral administration of fadrozole both acutely and chronically to examine the possible roles of estrogens in pair-maintenance behaviors of zebra finches. Our endocrine measures have raised questions about how to determine *in vivo* the effectiveness of fadrozole. We found no evidence that estrogens promote pair-maintenance behavior in males or females. On the contrary, in one case, acute fadrozole treatment rapidly stimulated an affiliative behavior, raising the possibility that estrogens inhibit affiliative behavior and/or that testosterone promotes affiliative behavior via non-genomic mechanisms.

Table 3.1: Effect of fadrozole on proximity time

		Visual Reunion *(Time spent on center perch)*					
		Focal Animal		Partner		Full Reunion *(Time spent within 10cm)*	
	FACTOR	F	P	F	P	F	P
Experiment 1 (Acute)	Treatment	1.36	0.250	0.21	0.646	**4.59**	**0.038**
	Sex	0.28	0.597	1.71	0.198	0.50	0.482
	Treatment × Sex	0.53	0.470	1.61	0.210	0.03	0.856
Experiment 2 (Chronic)	Treatment	0.06	0.806	0.93	0.339	0.05	0.824
	Sex	1.84	0.182	3.86	0.056	**8.61**	**0.005**
	Treatment × Sex	0.34	0.562	3.12	0.084	1.50	0.228

Note: Bolded values indicate significant main effects (Treatment in experiment 1 and Sex in experiment 2) on proximity measures.

Note: Bolded values indicate significant effect of factor (Treatment in Experiment 1 and Sex in Experiment 2).

Table 3.2: Effect of fadrozole on time spent vocalizing

		Partner Separation							
		0 - 10 min		10 - 20 min		Visual Reunion		Full Reunion	
	FACTOR	F	P	F	P	F	P	F	P
Experiment 1 (Acute)	Treatment	1.41	0.241	0.45	0.506	0.11	0.742	0.69	0.410
	Sex	0.25	0.621	1.63	0.208	2.35	0.132	0.13	0.716
	Treatment × Sex	1.63	0.209	0.02	0.900	**4.77**	**0.034**	0.05	0.818
Experiment 2 (Chronic)	Treatment	0.14	0.711	0.03	0.857	0.70	0.409	0.16	0.696
	Sex	0.43	0.519	1.18	0.283	0.06	0.806	0.30	0.583
	Treatment × Sex	2.65	0.111	1.71	0.198	0.16	0.695	0.0001	0.994

Note: Bolded value indicates significant Treatment by Sex interaction on time spent vocalizing. However, follow up analyses yielded no effect of Treatment in males (P=0.545) or females (P= 0.453).

Table 3.3: Effect of fadrozole on focal animal locomotor activity

		Partner Separation *(Perch Hops)*							
		0 - 10 min		10 - 20 min		Visual Reunion *(Returns)*		Full Reunion *(Perch Hops)*	
	FACTOR	F	P	F	P	F	P	F	P
Experiment 1 (Acute)	Treatment	0.55	0.462	0.02	0.883	0.15	0.706	0.98	0.328
	Sex	0.89	0.350	0.22	0.640	1.92	0.172	0.10	0.754
	Treatment × Sex	0.02	0.893	0.34	0.562	0.05	0.812	0.46	0.499
Experiment 2 (Chronic)	Treatment	0.19	0.664	1.25	0.270	0.001	0.991	0.27	0.601
	Sex	0.26	0.610	0.23	0.636	0.002	0.960	3.39	0.072
	Treatment × Sex	1.08	0.304	0.86	0.359	0.48	0.490	0.004	0.952

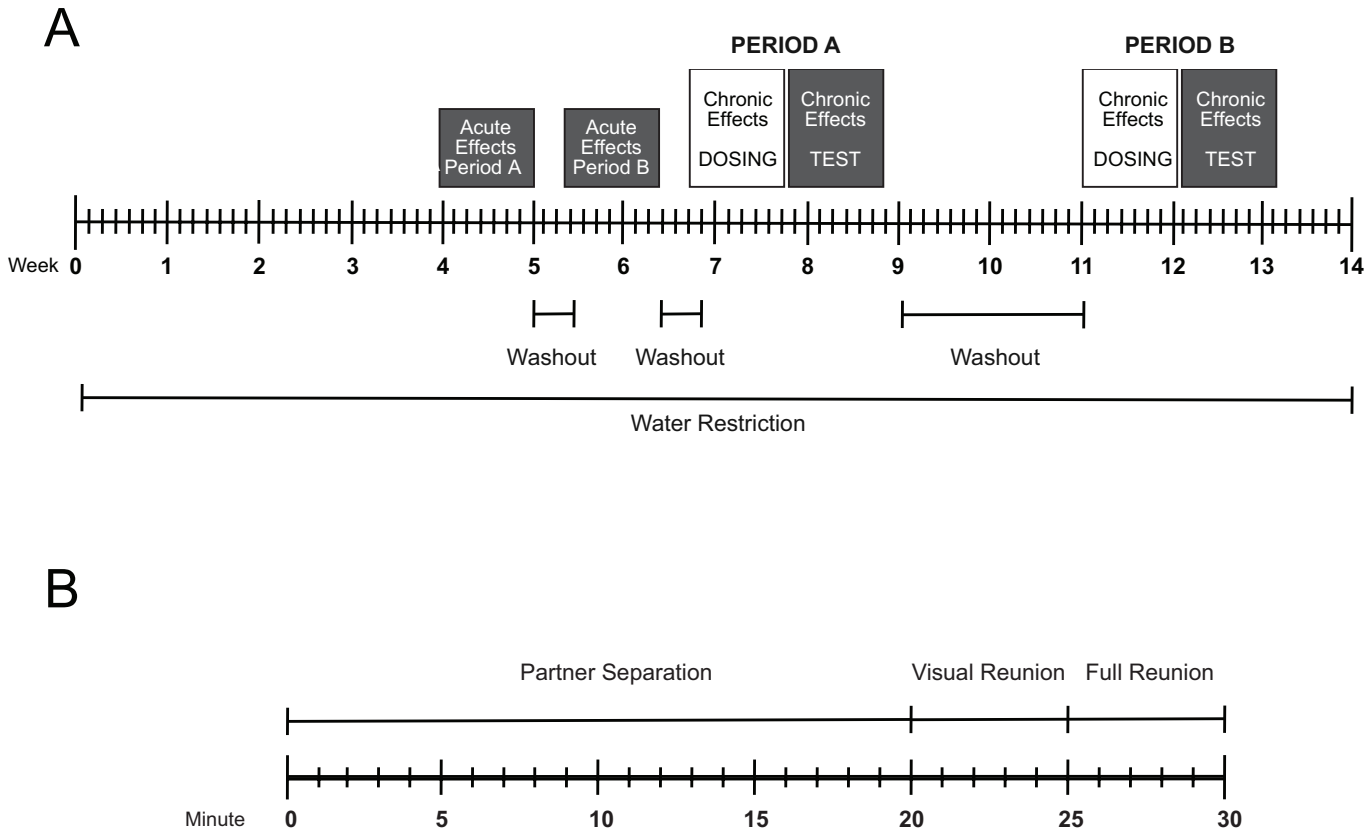
Note: There are no significant effects of either factor or interaction.

Table 3.4: Effect of fadrozole on partner locomotor activity

		Partner Separation *(Perch Hops)*							
		0 - 10 min		10 - 20 min		Visual Reunion *(Returns)*		Full Reunion *(Perch Hops)*	
	FACTOR	F	P	F	P	F	P	F	P
Experiment 1 (Acute)	Treatment	0.16	0.688	0.4	0.533	0.90	0.348	0.98	0.328
	Sex	0.85	0.361	0.07	0.788	3.08	0.086	0.10	0.754
	Treatment × Sex	0.03	0.855	0.005	0.946	0.40	0.528	0.46	0.500
Experiment 2 (Chronic)	Treatment	1.93	0.172	0.27	0.607	**4.64**	**0.037**	0.28	0.601
	Sex	**4.28**	**0.045**	2.85	0.099	**4.41**	**0.042**	3.39	0.073
	Treatment × Sex	1.37	0.249	3.82	0.057	0.10	0.746	0.004	0.952

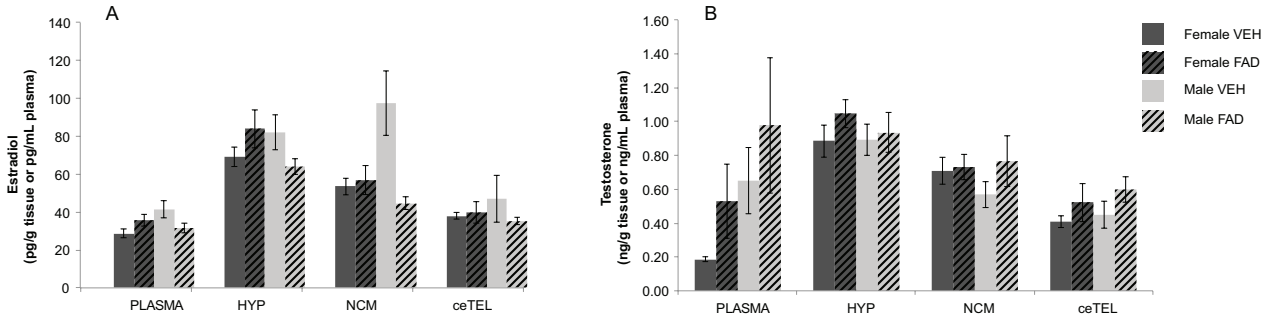
Note: Bolded values indicate significant effect of factor (Treatment or Sex).

Figure 3.1 Experimental timeline 2



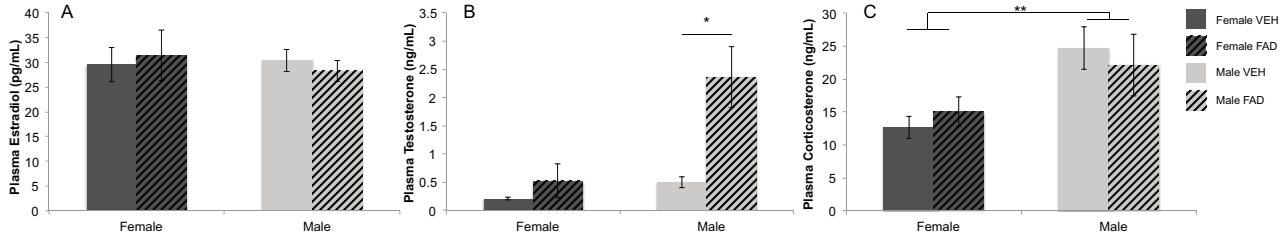
(A) A general timeline for this study. A within-subjects design was used so that the focal subject was the same for both the acute and chronic studies. Note the washout peroids between studies and rounds (period A and B). (B) Timeline of the partner separation and reunion behavioral test.

Figure 3.2 Effect of acute oral administration of fadrozole on steroid levels



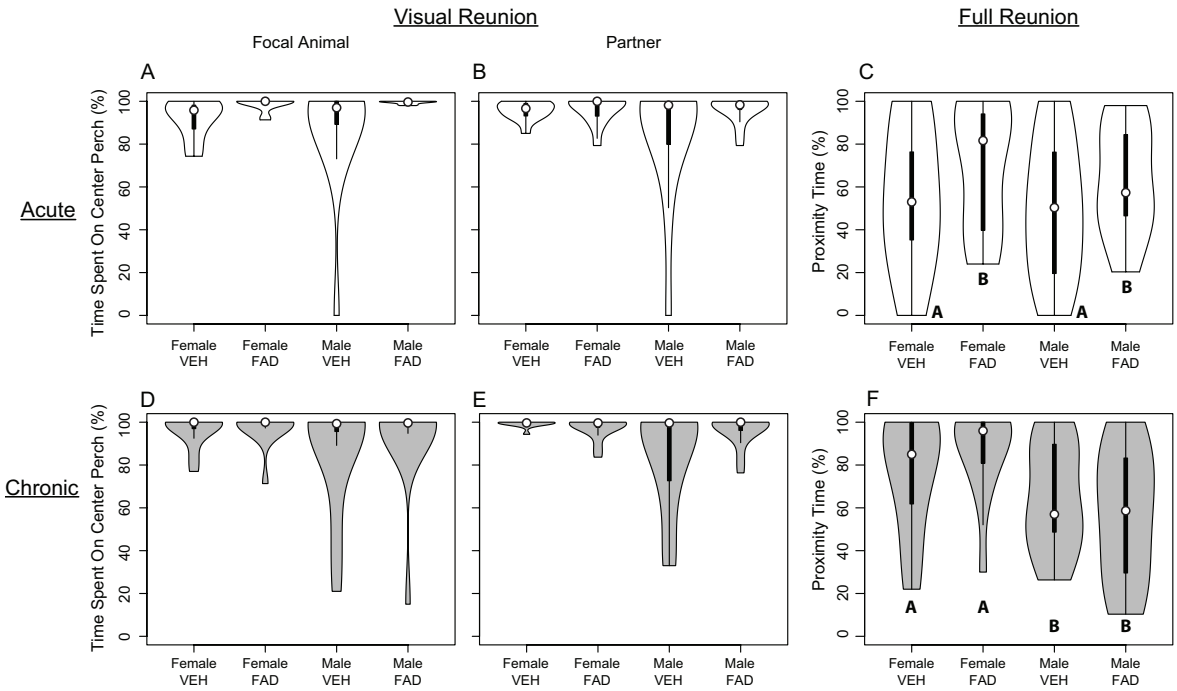
(A) Estradiol and (B) testosterone were measured in plasma, hypothalamus, NCM, and central telencephalon. Estradiol levels were decreased in plasma and brain by acute fadrozole administration in males only (simple main effects rmANOVA: $F_{1,27} = 14.35$, $P < 0.001$). Testosterone levels were increased in plasma and brain by acute fadrozole administration in both males and females (rmANOVA: $F_{1,60} = 4.66$, $P = 0.035$)

Figure 3.3 Validation of chronic fadrozole administration on circulating steroid levels



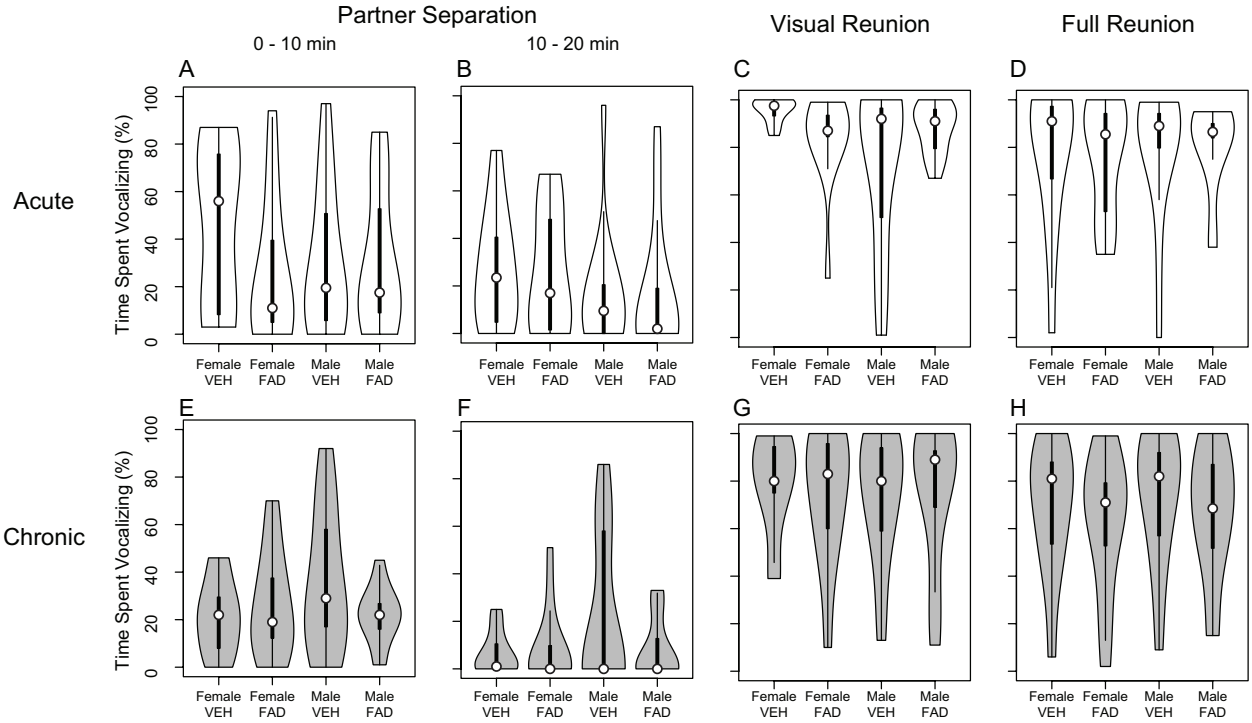
(A) Estradiol, (B) testosterone, and (C) corticosterone were measured in brachial plasma samples immediately after the partner separation and reunion test. There was no effect of fadrozole administration on circulating estradiol levels; however fadrozole increased circulating testosterone levels in males. Circulating corticosterone levels were higher in males. Significance is indicated ($P < 0.05$ = *; $P < 0.01$= **).

Figure 3.4 Effect of fadrozole on proximity behavior



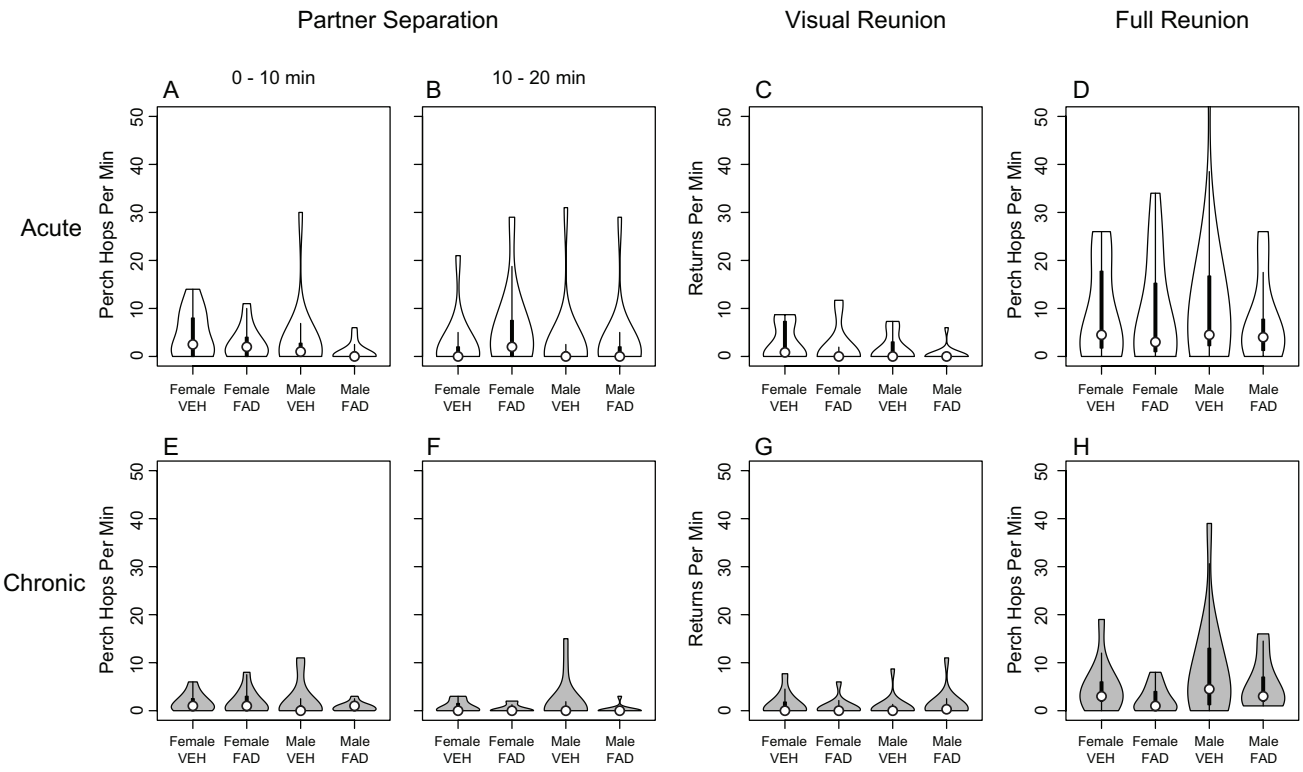
Violin plots combine a box plot (white circles denote the median) with a kernel density plot (Hintze and Nelson, 1998). The width of the plot signifies the probability density at different y-values (or the proportion of data at different y-values), similar to a histogram. Fadrozole administration increased proximity time during the full reunion of the acute fadrozole experiment in both male and female focal subject pairs (C: treatment, $F_{1,46} = 4.59$, $P = 0.038$). During the full reunion of the chronic fadrozole experiment female treated pairs had a higher proximity time than male treated pairs (F: sex, $F_{1,44} = 8.61$, $P = 0.005$). There was no effect on proximity during the chronic fadrozole administration (F) or on focal subject (A, D) or partner's (B, E) time spent on the center perch during the visual reunion period. These behavioral metrics are extremely high in control subjects, and thus a ceiling effect may have prevented us from seeing a significant stimulatory effect of fadrozole (especially time spent on the center perch).

Figure 3.5 Effect of fadrozole on vocalizations behavior



Panels A-D depict data from the acute fadrozole experiment and panels E-H represent the chronic fadrozole experiment. Fadrozole administration had no effect on the total time a pair spent vocalizing during any of the behavioral tests: (A&E) the first 10 min of the partner separation, (B&F) the second 10 min of the partner separation period, (C&G) the visual reunion, and (D&H) the full reunion.

Figure 3.6 Effect of fadrozole on locomotor activity



Panels A-D depict data from the acute fadrozole experiment and panels E-H represent the chronic fadrozole experiment. Fadrozole administration had no effect on the number of perch hops per min during any of the behavioral tests: (A&E) the first 10 min of the partner separation, (B&F) the second 10 min of the partner separation period, (C&G) returns per min during the visual reunion, and (D&H) perch hops per min during the full reunion. Perch hops were measured in both focal subject and partners (partner data is presented in Table 3.4).

4 Chronic testosterone treatment in males inhibits pair-maintenance behavior in stressed zebra finches

4.1 Introduction

Socially monogamous pair-bonding occurs across a wide range of species, from fish to mammals (Reichard and Boesch, 2003). Although monogamy is rare in mammals, fish and reptiles, it is seen in over 90% of bird species (Lack 1968; Kleiman, 1977). While many avian species are serially monogamous, forming new bonds with each breeding season, some species form life-long monogamous pair bonds (Black, 1996). However, the majority of what is known about the physiological mechanisms that supports monogamy comes from studies of mammals (Insel and Young, 2001; Adkins-Regan and Tomaszycki, 2007). Research focusing on the diversity of monogamy in birds will greatly further our current understanding of neuroendocrine regulation of pair-bonding.

The zebra finch *(Taeniopygia guttata*) is an excellent system to study the neuroendocrine regulation of pair-maintenance behavior associated with life-long monogamous bonds. Zebra finches form both socially and sexually monogamous pair bonds (Birkhead 1988; Griffith et al., 2010). Pair bonds are formed and maintained through affiliative behaviors (such as singing, clumping, allopreening) (Zann, 1996). It only takes 1-2 weeks for bonds to solidify, therefore for the majority of an individual's life affiliative behaviors primarily function as pair maintenance (Zann, 1996). While the neuroendocrine regulation of courtship and pair-bond formation has been well-studied in zebra finches (Arnold, 1975; Tomaszycki et al., 2006: Klatt and Goodson, 2013; Kelly and Goodson, 2014), the neuroendocrine mechanisms regulating pair-maintenance behaviors are largely unknown.

In seasonally breeding songbirds, sex steroids promote many of the changes in the brain that are necessary for singing and courtship behaviors associated with bond formation and maintenance (Ball et al., 2002; Adkins-Regan, 2005). For males, elevation of circulating androgens around breeding events coincides with courtship and bond formation. While zebra finches are opportunistic rather than seasonal breeders, circulating sex steroid levels are elevated in breeding males (Perfito et al 2010; Prior et al 2013). This suggests that androgens may regulate courtship and pair-bond formation in male zebra finches. Indeed, there are several lines of evidence that zebra finch courtship behavior is regulated by sex steroids, such as testosterone and estradiol (Harding et al., 1983; Adkins-Regan, 1999; Hill et al., 2005; Remage-Healey et al., 2008). However, it is still unclear whether sex steroids are also involved in pair-maintenance behavior.

Alternatively, there is also evidence that elevated male circulating testosterone beyond pair-bond formation has costs for pair bonds (Wingfield et al., 2001). Often, circulating testosterone is elevated for longer in polygynous male songbirds than in monogamous males (Wingfield et al., 2001). Furthermore, administration of testosterone implants to the serially monogamous male white-crowned and song sparrows causes them to court other females, effectively making them polygynous (Wingfield, 1984). This could suggest that circulating androgens inhibit pair-maintenance behavior in zebra finches. Pair-maintenance behavior has been largely unstudied in zebra finches, however circulating androgen levels are particularly low in wild zebra finches (Perfito et al., 2007; Prior et al., unpublished data), which may be consistent with this hypothesis.

Previously, we found evidence that sex steroids may regulate pair-maintenance behavior in zebra finches. Fadrozole (an aromatase inhibitor) administration to males or females, both

increased time spent in close proximity for zebra finch pairs (Prior et al., 2014). In addition to decreasing estradiol levels, fadrozole increases testosterone levels (Prior et al., 2014). Thus, we hypothesized that androgens promote affiliative behaviors associated with pair maintenance. Here we directly tested this hypothesis by quantifying the effect of a long-term chronic testosterone manipulation to males on pair-maintenance behavior.

4.2 Materials and methods

4.2.1 Subjects

Adult captive zebra finches (>120 days old) were paired in cages (38 ½ x 19 ¾ x 19 in, Corners Cages), in which a solid divider had been placed down the middle. The colony was maintained on a 14:10 h light:dark cycle. All individuals had *ad libitum* access to seed (50/50, Panicum millet/white millet, Just For Birds, Langley BC), water, cuttlefish bone, and grit. Pairs were housed together for a minimum of two months prior to the start of the study. Only pairs that showed interest in breeding (i.e. nest-building and egg laying) and engaged in affiliation were used in this study. Thus we were confident only successfully-bonded pairs were used.

These experiments were carried out under a University of British Columbia Animal Care Committee protocol and followed the guidelines of the Canadian Council on Animal Care.

4.2.2 Testosterone treatment

Pairs were divided into two treatment groups: Control (n=8) and Testosterone (n=7). Males were s.c. implanted with either an empty 10mm silastic implant (Dow Corning medical grade tubing: inner diameter 0.76mm, outer diameter 1.65mm) or with an implant packed with 8 mm crystalline testosterone (Steraloids A6950-000). All implants were sealed with liquid silastic glue. This manipulation has been shown to increase circulating testosterone levels within

a physiological relevant range in zebra finches (Vockel et al., 1990; Korsia, 1991). Implants were placed subcutaneously on the back. At the end of the study, the implants were removed. All of the testosterone implants contained some testosterone (< ½ full).

4.2.3 General timeline

Physical affiliative behavior, acoustic affiliative behavior, and blood samples were collected at three timepoints: prior to implantation (PRE), ~30 days post-implant (D30) (28.9 ± 0.6 days), and ~60 days post-implant (D60) (57.4 ± 0.4 days). Pairs were provided access to a nestbox and nesting material following the PRE timepoint, and had access for the remainder of the study. This was done to (1) ensure all pairs were at a similar point in the breeding cycle at the start of the study, and (2) allow us to examine the effect of testosterone treatment on any potential breeding attempts. All pairs laid at least one egg (minimum 1 egg, maximum 4 eggs) during the study. However, during the study, only 1 pair hatched a chick. Additionally, we only observed 3 pairs engaging in incubation behavior.

4.2.4 Behavioral tests

At each timepoint, two behavioral assays were conducted (between 08:00 and 14:00hr). First, we recorded unmanipulated baseline behavior for 30 min (Baseline Behavior Test). Second, in order to elicit higher levels of affiliation than would be seen at baseline, we recorded behavior following a mild stressor. Specifically, one researcher (KNY) consistently chased each pair for 15 sec in the cage; immediately following this we recorded behavior for 5 min (Stressed Behavior Test). The stressed behavior test was conducting immediately following Baseline Behavior Test.

Both behavioral assays were recorded with a camcorder for later quantification of physical affiliative behaviors. Vocal behavior was also recorded using 1-2 tie clip microphones

(AKG C417) and a Marantz PMD660 recorder. The first microphone was always placed on the outside of the cage (farthest from any neighboring cages), and the second microphone was placed inside the cardboard nestbox on D30 and D60. Acoustic and visual recordings were synchronized for quantification.

4.2.5 Blood samples

To confirm the efficacy of the testosterone treatment, blood samples were collected for steroid measurement. The morning after behavior was recorded, a blood sample was collected from the brachial vein in both the male and female of a pair (between 08:00 and 10:00hr). Two researchers (KNY and NHP) collected blood samples from both the male and female from a pair at the same time. First, approximately 20μL of blood was collected for corticosterone measurement within 3 min (1.7 ± 0.1 min). Next, an additional 50-100μL of blood was taken for testosterone measurement within 10 min (4.0 ± 0.1 min). Blood samples were centrifuged to obtain plasma (10 min at 10,000xg) which was stored at -20°C until further processing.

4.2.6 Quantifying physical affiliative behavior

All behavior was scored by researchers blind to treatment. The same behaviors were scored during both the 'Baseline Behavior Test (30 min) and the 'Stressed Behavior Test' (5 min). Three types of affiliative behaviors were scored: Proximity (% time within 15cm) (NHP), Clumping (NHP) and Allopreening (NHP and DL). Preening behavior was scored individually for the male and female. Additionally, we quantified the amount of time the male and female synchronized self preening bouts: 'coordinated preening' (NHP and DL). Time spent in the nestbox, copulations, nest-building, feeding and drinking were scored but were rare and are thus not presented here.

4.2.7 Quantifying acoustic affiliative behavior

Acoustic behavior was scored using Praat software (V 5.3.56) (Boersma, 2001). Zebra finches can produce several types of vocalizations including two prominent types of calls, distance calls and short (or "tet") calls (Zann, 1996). Distance calls are used when paired individuals are out of visual contact and also used with other individuals in a flock. Tet calls for communication over shorter distances (Zann, 1996). In a captive colony setting, distance calls are frequently used both within a pair and between members of different pairs. However, tet calls are used with cagemates, which in this instance would be exclusively within a pair. Thus, we only scored calls tet calls to give us a more conservative measure of pair-directed vocalization. This method of scoring does not allow us to discriminate between female and male tet calls.

During the Baseline Behavior Test (30 min) the total number of tet calls and male song bouts from three, two min sections (0-2, 14-16, and 28-30 min) were scored (NHP). Song bouts were defined as a single rendition of a song, typically including several introductory notes and several repetitions of a stereotyped sequence (motif). In addition to number of male song bouts, additional characteristics of male song were quantified: specifically, the number of motifs, introductory notes and distance calls inserted into male song were also quantified (NHP). During the Stressed Behavior Test (5 min), the latency to the first tet call and the first exchange of calls (a tet call with a rapid tet call response) was scored (NHP).

4.2.8 Steroid measurement

Corticosterone

Corticosterone was quantified with radioimmunoassay kit (MP Biomedicals-07120103) as previously described (Schmidt and Soma, 2008; Newman et al., 2008b). Briefly, 5 μL of unextracted plasma was diluted in 45 μL of steroid diluent. Next, 100 μL of tracer was added to standards and diluted samples, followed by immediately adding 100 μL of primary antibody and

incubating for 2 hr at room temperature. Finally, 250 µL of precipitating reagent was added to each sample. Following centrifugation at 4°C and decanting, samples were counted for 1 min on a gamma counter. Intra-assay variation was 8.51% and inter-assay variation was 9.68%.

Testosterone

Testosterone was extracted using solid-phase extraction (Newman et al., 2008a; Taves et al. 2010;2011). Approximately 20µL of plasma was added to 10 mL of de-ionized water and then loaded onto C_{18} columns (Agilent Bond-Elut OH, 500mg, cat # 12113045) that had been primed with 3 mL HPLC-grade methanol and equilibrated with 10 mL de-ionized water. Samples were then washed with 10 mL 40% HPLC-grade methanol, and steroids were eluted with 5 mL 90% HPLC-grade methanol. The eluted samples were dried at 40°C in a vacuum centrifuge (ThermoElectron SPD111V Speedvac) and stored at -20°C until assayed. Samples were resuspended in 400µL PBSG (phosphate-buffered saline containing 0.1% gelatin) with absolute ethanol (1.0%) to aid in resuspension (Newman et al., 2008a; Taves et al., 2011).

Testosterone was quatified using a radioimmunoassay kit (MP Biomedicals-07189102). Modifications to the kit instructions are described in (Overk et al. 2013). Briefly, 200 µL of primary antibody was added to 175 µL of standards and resuspended samples and incubated for 4 hr at room temperature. Next, 200 µL of tracer was added to each tube and incubated for 3 hrs at 37°C in a hot water bath. Finally, 50 µL of precipitating reagent was added to each sample and incubated for 60 min at 37°C and shaking at 90 rpm. Following centrifugation at 4°C and decanting, samples were counted for 2 min on a gamma counter. Final values were corrected for recovery (91.9%). Intra-assay variation was 10.76% and inter-assay variation was 12.75%.

4.2.9 Statistics

To control for individual/pair differences prior to manipulation, dependent variables are reported as change (Δ) at D30 and D60 relative to PRE (ex. D30-PRE= Δ D30), unless stated otherwise. Statistics were then conducted on these difference scores in R version 2.12.2 (R core team, 2012).

The effect of testosterone manipulation on circulating testosterone and corticosterone levels was assessed using repeated measures ANOVAs. Here, Timepoint (D30 vs D60) was the within-subjects factor, while Sex (Male vs Female, un-mainpulated) and Treatment (Control vs Testosterone) were between-subjects factors. When there were significant Sex × Treatment interactions, follow-up rmANOVAs were conducted on data separately for Male and Female. In these models, again, Timepoint was a within-subjects factor and Treatment was a between-subjects factor.

The majority of our behavioral metrics were quantified at the level of the pair. The effect of male testosterone administration on physical paired-level affiliative behaviors (Proximity, Clumping, Allopreening, and Coordinated Preening) was assessed with rmANOVAS separately for each behavior test (Baseline versus Stressed). Here, Timepoint was a within-subjects factor and Treatment was a between-subjects factor. Each behavioral parameter was analysed separately.

The effect of male testosterone manipulation on acoustic affiliative behaviors was assed using rmANOVAs. Timepoint was a within-subjects factor and Treatment was a between-subjects factor. From the Baseline Behavior Test, separate rmANOVAs were conduced for the # Tet calls and each of the parameters of Male Song (i.e. male song bouts, song motifs, #distance calls inserted into a song, and # introductory notes). From the Stressed Behavior Test, separate rmANOVAs were conducted for Latency to First Call and Latency to Exchange.

4.3 Results

4.3.1 Circulating testosterone and corticosterone levels

Absolute circulating testosterone and corticosterone levels are given in Table 4.1. Male testosterone administration increased male circulating testosterone levels at both D30 and D60 timepoints (Figure 4.1A: Treatment, $F_{1,24} = 33.93$, $P < 0.0001$; Timepoint, $F_{1,24} = 0.185$, $P = 0.671$; Treatment × Timepoint, $F_{1,24} = 1.89$, $P = 0.182$). There was no effect of male testosterone administration on female testosterone levels. At D30 and D60, female testosterone levels were unchanged (Figure 4.1B: Treatment, $F_{1,24} = 0.12$, $P = 0.736$; Timepoint, $F_{1,24} = 0.10$, $P = 0.761$; Treatment × Timepoint, $F_{1,24} = 0.18$, $P = 0.676$).

Male testosterone administration had no effect on Δ circulating corticosterone levels at D30 and D60 for either males or their un-manipulated partners (Figure 4.1C-D: Treatment, $F_{1,50} = 1.01$, $P = 0.319$; Time, $F_{1,50} = 0.80$, $P = 0.376$; Sex, $F_{1,50} = 0.001$, $P = 0.990$; all interactions $P > 0.05$).

4.3.2 Physical affiliative behaviors

The percent of time spent engaging in affiliative behaviors (close proximity, clumping, allopreening, and coordinated preening) is given in Table 4.2. Overall, percent of time in close proximity was higher during the Stressed Behavior Test than during the Baseline, as expected. Allopreening, clumping and coordinated preening were rare, and often less than half of the pairs engaged in these behaviors (Table 4.3).

Chronic testosterone treatment had no effect on physical affiliative behaviors during the Baseline Behavior Test (Table 4.4; Figure 4.2). However, during the Stressed Behavior Test, testosterone treatment in males decreased time in close proximity at both D30 and D60 (Table

4.4; Figure 4.3A). A similar trend was seen in percent of time spent coordinated preening, although this difference was not statistically significant.

4.3.3 Acoustic affiliative behaviors

The average of number of tet calls, male song bouts, and parameters is given in during the Baseline Behavior Test, and the average latency to first call and first exchange during the Stressed Behavior Test is provided in Table 4.5. All acoustic parameters measured were highly variable. Male testosterone treatment had no effect on Δ acoustic affiliation in the Baseline Behavior Test (Table 4.6A; Figure 4.4). Furthermore, there was no effect of testosterone on Δ latency to call or exchange at D30 and D60 (Table 4.6B; Figure 4.5) during the Stressed Behavior Test.

4.4 Discussion

Here we tested the hypothesis that increased testosterone in males promotes pair maintenance behavior in zebra finch pairs. We found that testosterone treatment in males reduced measures of affiliation following a mild stressor. Specifically, in the Stressed Behavior Test, testosterone treatment in males caused pairs to spend significantly less time in close proximity at both D30 and D60 timepoints. Interestingly, this significant effect was absent in the Baseline Behavior Test. This finding is consistent with several lines of evidence in other avian species showing that testosterone has costs for pair bonding (Wingfield 1984; Wingfield et al., 2001; Kellam and Lucas, 2014).

4.4.1 Behavioral metrics of pair maintenance

Interestingly, both here and in previous research, time spent in close proximity but not other affiliative behaviors were affected by endocrine manipulations (Chapter 3: Prior et al.,

2014). Time spent in physical proximity includes coordination of activities, affiliative behaviors (ie clumping and allopreening) and synchrony of movement (flights). This makes proximity a good overall metric of physical pair-maintenance behavior (in contrast with more specific behaviors such as clumping). Furthermore, proximity time is not sensitive to across-pair variation in the expression of affiliative (for example, one pair prefers clumping while another pair engages in more synchronized flights).

Here, we only saw the inhibiting effect of testosterone on proximity time under the stressed behavioral paradigm. Similarly, we previously reported effect of fadrozole on proximity time following partner separation (Chapter 3: Prior et al., 2014). Under stable conditions, pair-maintenance behaviors may reflect previously ingrained behavioral patterns, requiring less behavioral and physiological investment to maintain. In contrast, unstable conditions disrupt established patterns and necessitate both behavioral and physiological investments in pair maintenance. Furthermore, periods of instability may be associated with distinct endocrine profiles than during stable conditions (Sapolsky, 1983; Sapolsky, 2004). Thus, research exploring the neuroendocrine regulation of pair maintenance may need to look towards periods of natural or artificial disruption. Examples of "unstable" conditions include transitions into and out of breeding condition, and following a stressor.

Differences in the context (stable or unstable) may explain potentially contradictory results from previous studies: for example potentially conflicting results regarding the function of vocalizations in established zebra finch pairs. There is strong evidence that pair vocalizations are critically important in wild zebra finches. Male song appears to be important during transitions to breeding in wild populations of zebra finches (Dunn and Zann 1996a,b), and vocal duetting may synchronize wild pairs within a breeding cycle (Elie et al., 2010). In contrast, under

baseline conditions in the laboratory setting, Tomaskycki and collegues found that experimentally disrupted male vocalizations (either through tracheosyringeal nerve transection or puncturing the interclavicular air sac) had no effect on pair-maintenance behavior or pairing status of already established pairs (Tomaszycki and Adkins-Regan, 2005; 2006). It is possible that under naturally "unstable" conditions, individuals invest more in these behaviors making it easier for researchers to identify the role they play in pair maintenance.

4.4.2 Sex steroids and the regulation of pair-maintenance behavior

As already stated there are several lines of evidence suggesting sex steroids are involved in pairing and courtship behavior (Harding et al., 1983; Adkins-Regan, 1999; Hill et al., 2005; Remage-Healey et al., 2008). However, the neuroendocrine regulation of pair-maintenance behavior remains largely unknown. To our knowledge, this is the first report of inhibitory effects of testosterone on zebra finch pair-maintenance behavior. Although there are several lines of evidence that elevated testosterone negatively impacts pair bonds, potentially due to increased extra pair copulations (Wingfield, 1984). Furthermore, elevated testosterone can have negative impacts on fitness (O'Neal et al., 2008; Gerlach and Ketterson, 2013). Interestingly in the socially and sexually monogamous downy woodpecker, male testosterone administration appears to inhibit calling behavior associated with pair maintenance but not affect associations with non-mate females (Kellam et al., 2004; Kellam and Lucas, 2014).

Here, testosterone manipulation reduced proximity time, whereas previously it was increased with acute fadrozole treatment (an aromatase inhibitor) (Chapter 3: Prior et al., 2014). The effect of fadrozole could be due to the decrease in brain estradiol levels, or the concomitant increase in testosterone levels. Likewise, testosterone may act on androgen receptors, or once aromatized, on estrogen receptors. Thus both of these pharmacological manipulations could

impact both androgen and estrogen signaling. A consistent interpretation of our current results and Chapter 3 (Prior et al., 2014) is that estradiol inhibits pair affiliation: thus decreased estrogen levels from fadrozole administration promoted affiliative behavior, and the concomitant increase in estrogen from testosterone administration inhibited affiliative behavior. However, there are a number of differences between these two studies, which make it difficult to draw comprehensive conclusions. More specifically, the behavioral paradigm used previously was different than what we used here and our previous study pairs were water-restricted (experimental non-breeding condition). Therefore, it is also possible that the role steroids play in regulating pairing behavior is very specific to social and environmental contexts. However, an inhibitory effect of estradiol on affiliation is consistent with patterns seen in alloparenting in the highly social prairie vole (Lei et al., 2010).

Our inability to detect a robust effect of male testosterone on pair-maintenance may be affected by our inability to disentangle male and female contributions to pair level affiliative behaviors. Here, we only manipulated the male partner. When interpreting the results on pair-level behavior several possible scenarios must be considered: the pharmacological manipulation (1) affects only male behavior, (2) affects male behavior and causes a concomitant change in female her behavior, or (3) does not affect male behavior. Females may increase or decrease their affiliation to compensate for changes in male investment. For example, a recent study by Kellam and Lucas (2014) found that testosterone administration in free-living male downy woodpeckers decreased calling of both males and their female partners.

The role of female testosterone is largely unclear (Buchanan and Fanson, 2014). In other monogamous bird species circulating male and female testosterone may be tightly correlated over the year (Hirschenhauser et al., 2008; Kellman et al., 2004). Furthermore, the degree of

endocrine synchrony between males and females can correlate to increased fitness (Hirschenhauser et al., 2008). A possible intermediate mechanism between endocrine state and fitness is parental and pair-directed behavior. This raises the question of whether female testosterone could impact pair-maintenance behavior. Additionally, the effect of male testosterone implants on female behavior that Kellam and Lucas (2014) raises the question of whether changes in male testosterone could directly affect female testosterone levels. Here, we did not see any evidence that male testosterone levels influenced the endocrine state or behavior of the female partner. To determine the relationship between endocrine state and pair-maintenance behavior, future studies should look across the breeding cycle and at wild zebra finch pairs.

4.5 Conclusion

To our knowledge, this is the first report of an inhibitory effect of a sex steroid on zebra finch pairing behaviors, adding another dimension to the already unclear to picture of sex steroids in the regulation of zebra finch pairing behavior. A comparison of our results here to our previous study using fadrozole-administration suggests estradiol may have an inhibitory effect on pair maintenance (Prior et al., 2014). More importantly, it highlights the significant influence of environmental contexts (ie reproductive condition and social/environmental stressors). Future research should continue to take advantage of the strengths of the zebra finch as a model system for studying the neuroendocrine regulation of pair-bonding, while controlling for breeding condition (Prior et al., 2013). More broadly, our results here support the idea that research examining pair-maintenance behavior (or other established social behaviors) should focus on behavior during unstable environmental conditions.

Table 4.1: Circulating testosterone and corticosterone levels in males and females from Control and Testosterone implanted pairs

		Pre		D30		D60	
		Control	Testosterone	Control	Testosterone	Control	Testosterone
Testosterone	Male	0.73 ± 0.32	0.63 ± 0.15	0.32 ± 0.09	4.58 ± 0.26	0.76 ± 0.28	3.71 ± 1.10
	Female	0.05 ± 0.004	0.06 ± 0.01	0.04 ± 0.002	0.07 ± 0.01	0.07 ± 0.01	0.10 ± 0.02
Corticosterone	Male	5.06 ± 1.07	5.59 ± 1.94	5.79 ± 1.44	6.52 ± 1.08	5.03 ± 1.77	5.58 ± 1.34
	Female	5.85 ± 0.75	6.58 ± 1.24	3.51 ± 0.89	6.86 ± 1.70	6.51 ± 1.72	9.91 ± 3.08

Note: Concentrations are given in (mean ± SEM ng/mL). Note only male is implanted from each pair.

Table 4.2: Percent of time spent engaging in physical affiliative behaviors

		Pre		D30		D60	
		Control	Testosterone	Control	Testosterone	Control	Testosterone
Baseline Behavior Test	Proximity	54.51 ± 6.43	57.41 ± 7.59	53.09 ± 6.47	51.23± 8.87	57.96 ± 4.06	52.41 ± 6.46
	Clumping	3.65 ± 2.47	1.97 ± 1.49	0.52 ± 0.52	6.49 ± 6.49	1.82 ± 1.82	0.52 ± 0.46
	Allopreening	1.00 ± 0.58	1.05 ± 0.69	0.93 ± 0.71	0.23 ± 0.18	3.83 ± 2.73	1.87 ± 1.23
	Coordinated Preening	5.42 ± 2.10	3.27 ± 3.00	7.23 ± 4.38	0.77 ± 0.61	2.58 ± 2.17	1.77 ± 1.25
Stressed Behavior Test	Proximity	60.36 ± 5.75	86.39 ± 5.27	77.09 ± 11.01	71.51 ± 11.26	89.10 ± 4.93	65.04 ± 9.12
	Clumping	5.29 ± 5.29	14.62 ± 14.23	15.92 ± 11.04	6.24 ± 6.24	3.83 ± 2.70	3.24 ± 3.24
	Allopreening	2.04 ± 1.55	4.38 ± 4.38	0.67 ± 0.67	7.52 ± 6.09	1.25 ± 1.16	2.05 ± 1.20
	Coordinated Preening	0.08 ± 0.08	0.33± 0.33	11.79 ± 5.83	4.00 ± 2.08	6.54 ± 5.45	5.57 ± 5.04

Note: Out of 30 minutes for the baseline behavior test and out of 5 minutes for the stressed behavior test (mean ± SEM).

Table 4.3: The number of pairs from each treatment group that engaged in the rare affiliative behaviors

		Pre		D30		D60	
		Control	Testosterone	Control	Testosterone	Control	Testosterone
Baseline Behavior Test	Clumping	2	2	1	1	1	2
	Allopreening	4	3	3	2	2	3
	Coordinated Preening	5	2	3	2	2	3
Stressed Behavior Test	Clumping	1	2	2	1	2	1
	Allopreening	1	1	1	3	2	3
	Coordinated Preening	1	1	5	3	2	1

Table 4.4: Effect of male testosterone administration on percent time spent engaging in physical affiliative behavior

		Proximity		Clumping		Allopreening		Coordinated Preening	
FACTOR		$F_{(1,24)}$	P	$F_{(1,24)}$	P	$F_{(1,24)}$	P	$F_{(1,24)}$	P
Baseline Behavior Test	Treatment	0.72	0.406	1.68	0.207	0.15	0.701	0.003	0.960
	Timepoint	0.27	0.607	0.95	0.341	0.68	0.418	0.39	0.539
	Treatment × Timepoint	0.0009	0.977	1.67	0.208	0.09	0.773	0.68	0.420
Stressed Behavior Test	Treatment	**14.62**	**0.0008**	1.17	0.290	0.16	0.690	3.93	0.059
	Timepoint	0.06	0.812	1.08	0.308	0.36	0.557	1.24	0.277
	Treatment × Timepoint	1.18	0.288	0.04	0.844	1.77	0.200	0.23	0.637

Note: Data at D30 and D60 timepoints are corrected relative to PRE (ex. D30-PRE=DIFFD30). Timepoint is a within subjects factor and Treatment is a between subjects factor. Bolded value indicates significant main effect of treatment ($P<0.001$).

Table 4.5: Percent of time spent engaging in acoustic affiliative behaviors

		Pre		D30		D60	
		Control	Testosterone	Control	Testosterone	Control	Testosterone
Baseline Behavior Test	Tet Calls (#)	283 ± 29.31	248.57 ± 57.36	321.63 ± 27.87	270.57 ± 35.62	309.63 ± 72.29	234 ± 31.62
	Male Song (#)	2.50 ± 1.09	2.86 ± 1.37	1.25 ± 0.65	4.43 ± 2.74	3.38 ± 1.38	4.86 ± 2.27
	Song Motifs (#)	7.88 ± 4.51	7.71 ± 4.29	3.38 ± 1.68	15.14 ± 9.62	10.00 ± 3.72	16.43 ± 5.72
	Distnace Call (#)	9.50 ± 4.94	1.57 ± 1.57	0.63 ± 0.63	0.86 ± 0.86	0.75 ± 0.53	0.14 ± 0.14
	Introductory Notes (#)	2.63 ± 21.40	2.86 ± 1.39	2.13 ± 1.14	12.57 ± 8.03	6.88 ± 2.82	13.71 ± 7.82
Stressed Behavior Test	Latency to First Call (s)	7.63 ± 2.38	6.29 ± 4.65	4.00 ± 1.93	6.29 ± 3.54	5.50 ± 3.20	3.57 ± 1.62
	Latency to Exchange (s)	11.5 ± 4.72	21.29 ± 10.98	9.50 ± 4.53	10.14 ± 6.01	7.50 ± 2.97	7.14 ± 2.51

Note: Out of 30 minutes for the baseline behavior test and out of 5 minutes for the stressed behavior test (mean ± SEM).

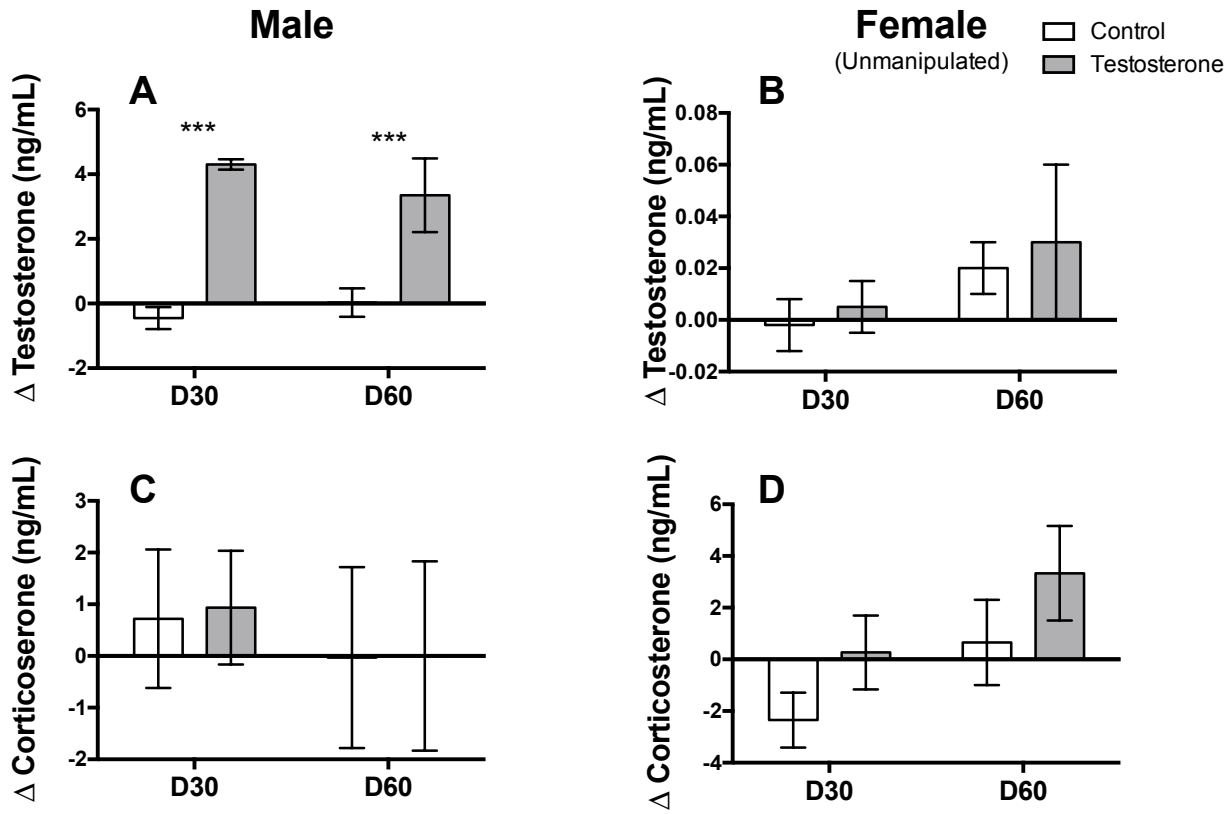
Table 4.6: Effect of male testosterone treatment on acoustic affiliative behavior

A		Tet Calls		Male Song Bouts		Song Motifs		Distance Calls		Introductory Notes	
FACTOR		$F_{(1,24)}$	P	$F_{(1,24)}$	P	$F_{(1,24)}$	P	$F_{(1,24)}$	P	$F_{(1,24)}$	P
Baseline Behavior Test	Treatment	1.70	0.205	0.92	0.348	1.13	0.299	2.88	0.103	1.67	0.210
	Timepoint	0.21	0.654	0.01	0.918	0.08	0.782	0.03	0.869	0.13	0.720
	Treatment × Timepoint	0.0003	0.987	0.22	0.643	0.15	0.700	0.03	0.868	0.07	0.791

B		Latency to First Call		Latency to Exchange	
		$F_{(1,24)}$	P	$F_{(1,24)}$	P
Stressed Behavior Test	Treatment	0.08	0.780	0.88	0.360
	Timepoint	0.0004	0.985	0.15	0.700
	Treatment × Timepoint	0.38	0.545	0.02	0.883

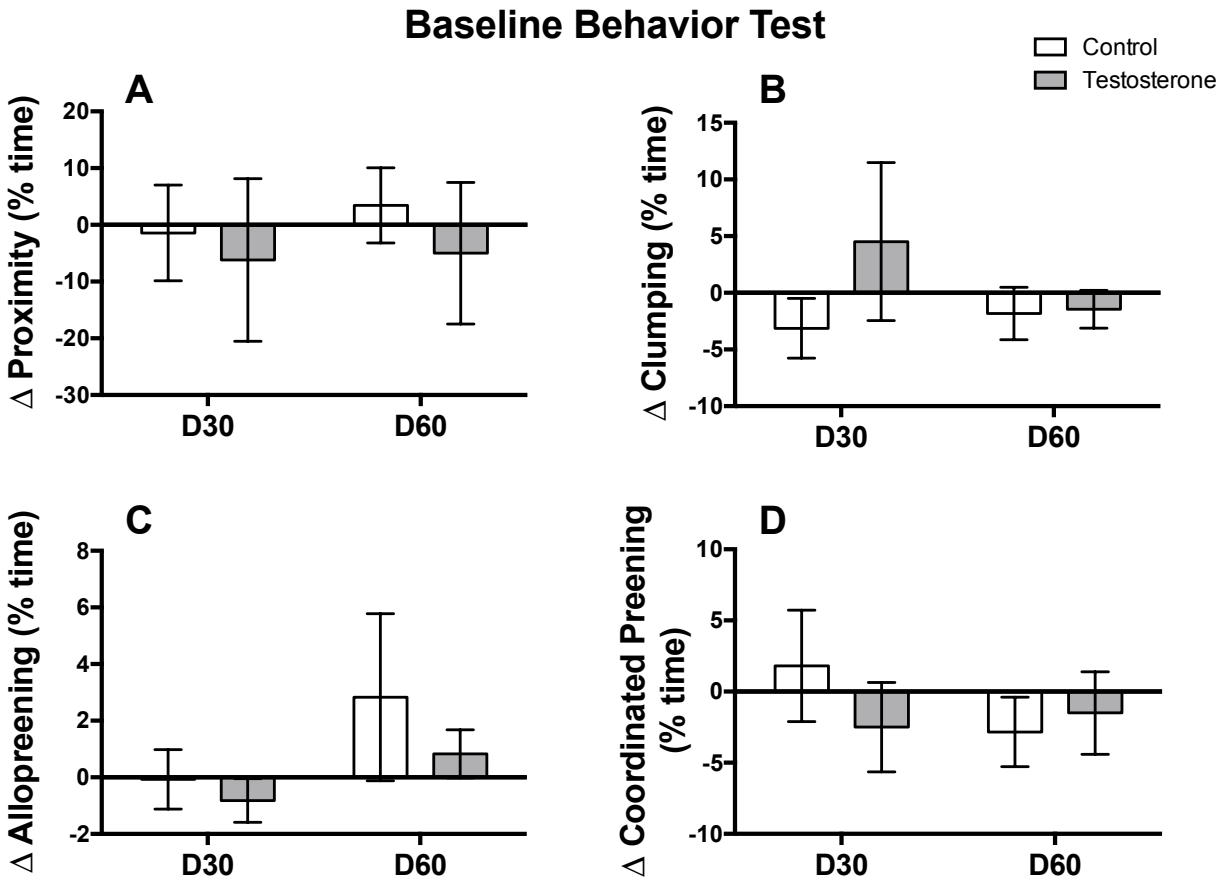
Note: Data at D30 and D60 timepoints are corrected relative to PRE (ex. D30-PRE=DIFFD30). Timepoint is a within subjects factor and Treatment is a between subjects factor. Bolded value indicates significant main effect of treatment ($P<0.001$). (A) Baseline Behavior Test, (B) Stressed Behavior Test.

Figure 4.1 Effect of male testosterone manipulation on circulating testosterone and corticosterone levels



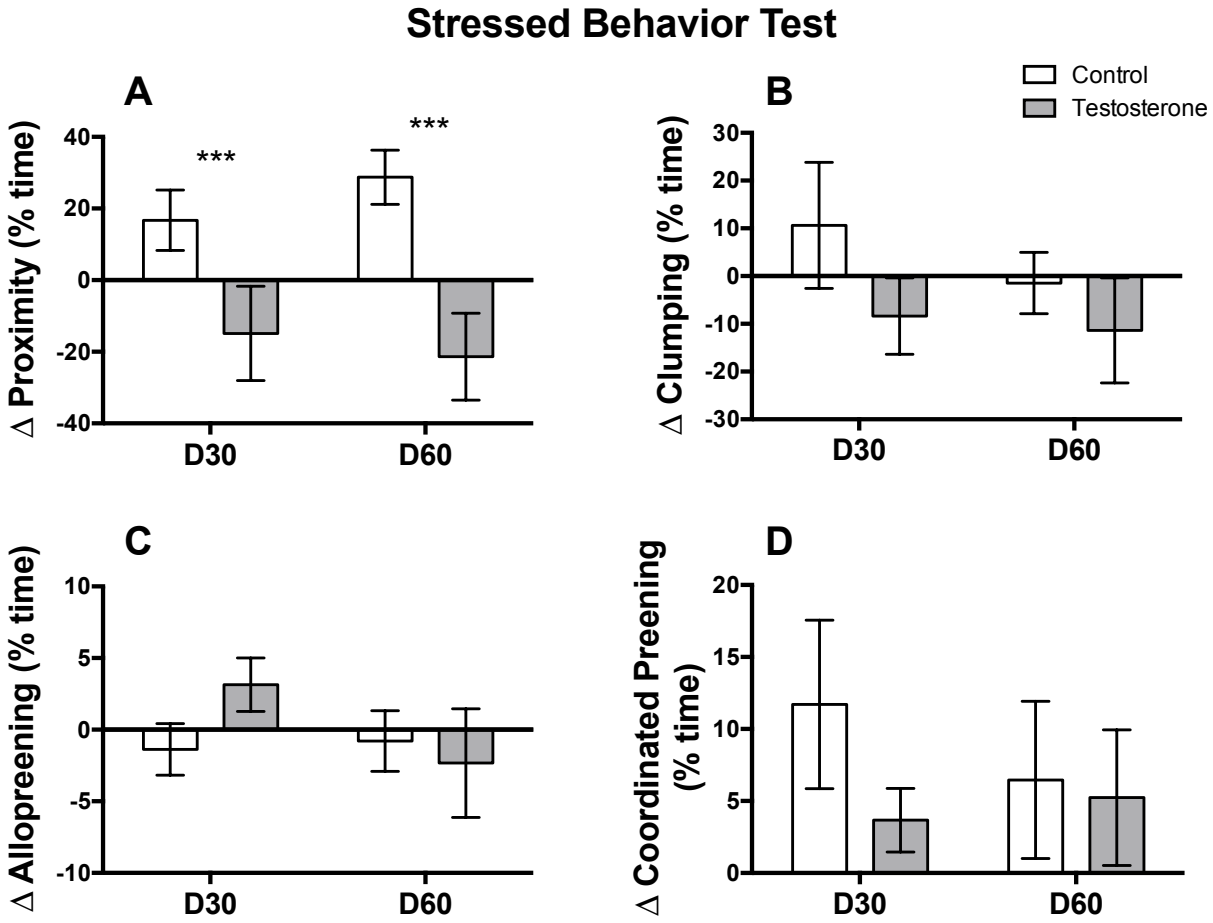
Change in circulating testosterone (A&B) and corticosterone (C&D) levels (ng/mL) at D30 and D60: Un-manipulated females (B&D) and manipulated males (A&C). Testosterone administration significantly increased male testosterone levels at both D30 and D60. Data at D30 and D60 timepoints are corrected relative to PRE (ex. D30-PRE=DIFFD30). *** $p \leq 0.001$

Figure 4.2 Change in percent of time engaging in physical affiliative behavior during the baseline behavior test



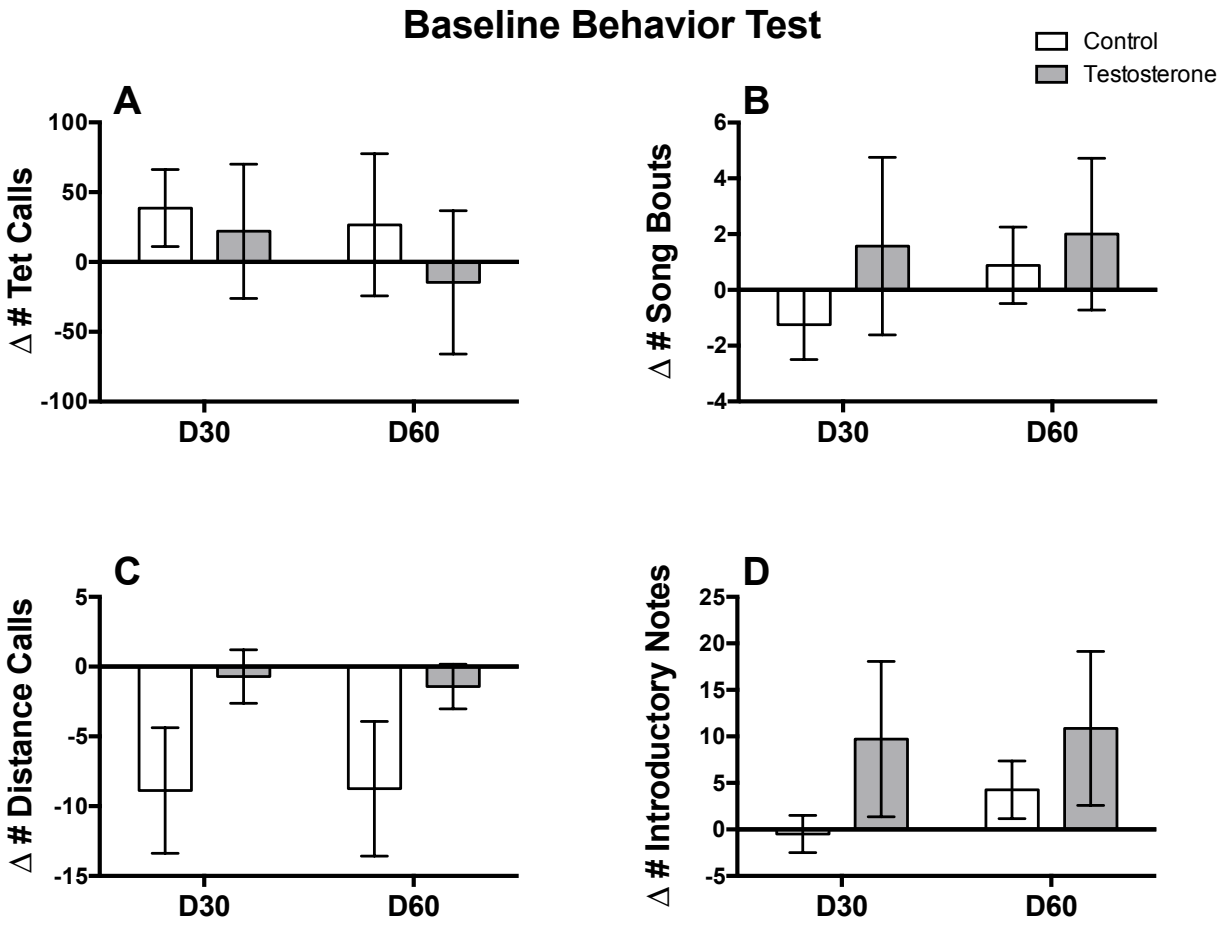
Change in percent time engaging in physical affiliative behavior during the baseline behavior test. (A) Proximity, (B) Clumping, (C) Allopreening, and (D) Coordinated Preening.

Figure 4.3 Change in percent of time engaging in physical affiliative behavior during the stressed behavior test



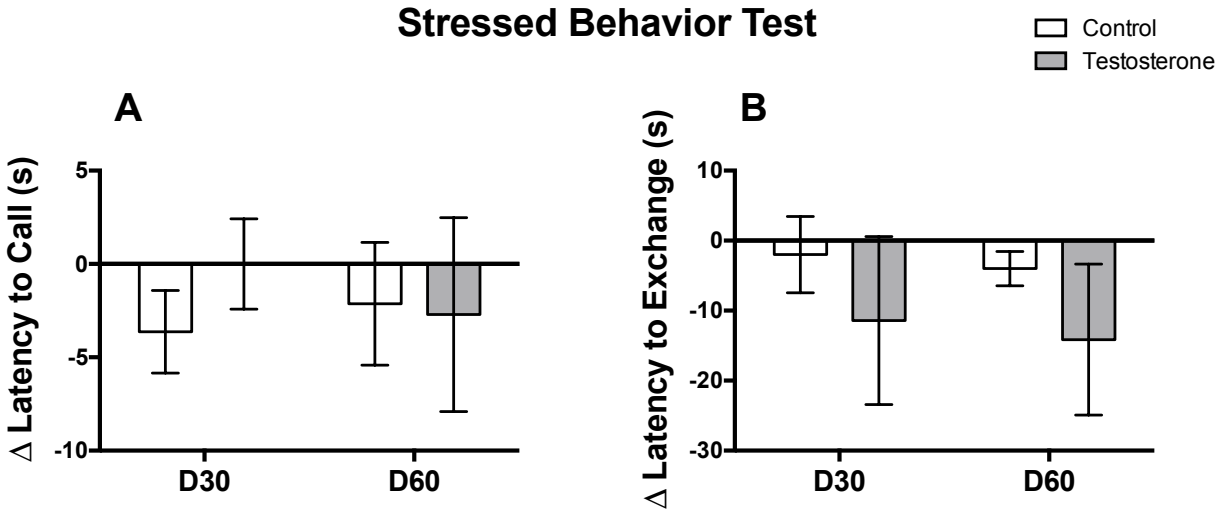
Change in percent time engaging in physical affiliative behavior during the stressed behavior test. (A) Proximity, (B) Clumping, (C) Allopreening, and (D) Coordinated Preening. *** $p \leq 0.001$

Figure 4.4 Change in acoustic affiliative behavior during the baseline behavior test



Change in acoustic affiliative behavior during the baseline behavior test. (A) # Tet Calls, (B) # Male Song Bouts, (C) # Distance Calls, and (D) # Introductory Notes.

Figure 4.5 Change in acoustic affiliative behavior during the stressed behavior test



Change in acoustic affiliative behavior during the stressed behavior test. (A) Latency to first tet call (s) (B) Latency to call exchange (s).

5 Sex steroid profiles and pair-maintenance behavior in wild-caught zebra finches

5.1 Introduction

Socially monogamous pair bonding occurs across a wide range of species, from fish to mammals (Reichard and Boesch, 2003). While monogamy is rare in mammals, fish and reptiles, it is the breeding strategy of over 90% of bird species (Lack, 1968; Kleiman, 1977). Pairs can be either socially or sexually monogamous, and bonds can last anywhere from one breeding cycle and to a lifetime (Zann, 1996; Griffith et al., 2010). While the ethological importance of pairs bonds has been widely documented, very little is understood about the suite of physiological mechanisms that support the maintenance of these bonds. Furthermore, the existing research has primarily focused on mammals (Insel and Young, 2001), where monogamy is clearly the exception.

Zebra finches (*Taeniopygia guttata*) form socially and sexually monogamous, life-long bonds (Birkhead et al., 1988; Griffith et al., 2010). Wild zebra finches are adapted to the unpredictability of life in Australian deserts. Thus it is not surprising that they are both nomadic and opportunistic breeders (Zann et al., 1995; Zann, 1996; Perfito et al., 2007). Maintaining year-round pair bonds may further facilitate flexibility, by improving the likelihood that pairs can rapidly begin breeding when environmental conditions are good (Adkins-Regan and Tomaszycki, 2007). This suggests that bond maintenance is fundamental to zebra finch pairs. Indeed, within a single breeding attempt, zebra finch pairs with higher behavioral synchrony have higher fitness (Mariette and Griffith, 2012).

The endocrine mechanisms that promote pair-maintenance behaviors have largely been unstudied. From pharmacological manipulations of laboratory zebra finches, there is evidence

that sex steroids may be important in promoting pair-directed behavior during bond formation (Harding and Rowe, 2003; Smiley et al., 2012). We recently found that fadrozole, an aromatase inhibitor, rapidly promoted time spent in close proximity in established pairs (Chapter 3: Prior et al., 2014). This effect of fadrozole may be due to decreases in estradiol levels, or due to the concomitant increase in testosterone levels. Taken together, this suggests that sex steroids may regulate pair-bond maintenance.

While pharmacological manipulations allow researchers to directly test the role of sex steroids in regulating pair maintenance, laboratory zebra finch pairs are fundamentally different from wild pairs (Griffith and Buchanan, 2010; Griffith et al., 2010). Therefore, it is critical that research is done examining the pair bonding in wild zebra finch populations. Typically, only one or two hormones can be measured from the blood samples collected from wild animals, greatly limiting investigations of behavioral endocrinology. The use of liquid chromatography – tandem mass spectrometry (LC-MS/MS) by wildlife biologists is in its early stages (Koren et al., 2012), and presents an opportunity to greatly increase the amount of information obtainable from limited number of small samples. LC-MS/MS allows us to produce more complete circulating sex steroid profiles and examine circulating precursors.

Precursors can themselves be correlated with behaviors (DHEA: Soma et al., 2004; Fokidis et al., 2013). Additionally, describing sex steroid profiles of precursors improves our ability to develop hypotheses about the upstream and downstream neuroendocrine mechanisms. For example, knowing what precursor is present in circulation allows us to make specific predictions about what steroidogenic enzymes will be active in target tissues. Recently, a novel steroidogenic pathway to 5α-DHT has benn identified ('backdoor pathway'; Figure 5.1) (Auchus, 2004). Importantly, the backdoor pathway of androgen synthesis allows DHT production to

occur independently of changes in testosterone levels (Auchus, 2004). Based on previous work in developing rats and tammar wallabies, we would predict that the 5α-reduced precursor, Androstanediol (ADIOL) would be present in circulation if the backdoor pathway was used (Shaw et al., 2000).

Here we used LC-MS/MS to produce sex steroid profiles of paired wild-caught zebra finches. Specifically, we looked at 9 androgens and progestins (pregnenolone, progesterone, dehydroepiandrosterone (DHEA), androstenediol, pregnan-3, 17-diol-20-one, androsterone, androstanediol (ADIOL), testosterone, 5α-dihydrotestosterone (5α-DHT)). This allowed us to (1) identify which steroids and precursors are present in wild zebra finches, and (2) examine the relationship between sex steroid profiles and pair-maintenance behavior. If the backdoor pathway is used in zebra finches, then we predicted to see elevated levels of circulating ADIOL. Additionally, if androgens promote pair maintenance we expected to see positive correlations between androgens and their precursors and pair-maintenance behavior.

5.2 Materials and methods

5.2.1 Subjects

Wild zebra finches (N = 22) were caught near Fowlers Gap Field Station, NSW, Australia in September 2012 (austral spring) and brought into outdoor aviaries (220 cm X 160cm X 200cm). Fowlers Gap Field Station is located in inland Australia where zebra finches breed opportunistically (Perito et al., 2007). All aviaries had a one-way viewing screen for behavioral observations. Subjects were given access to seed and water *ad libitum.* Subjects were set up in five aviaries housing 4 birds each (2 males and 2 females) with a sixth aviary housing 2 birds (1 male and 1 female). All birds were given a colored leg band for identification. We allowed the

birds to form new partnerships over a one month habituation phase after being placed in the aviaries. The duration of this study occurred during a period of favorable environmental conditions for breeding, and there was an abundance of free-roaming wild breeding pairs throughout the area. Thus the aviaries were outfitted with nest-boxes and nesting materials (natural grasses from the area), and we continued to supply nesting materials throughout the study. Even when not breeding, pairs build and maintain a roost nest that they use throughout the day and night (Kikkawa, 1980; Zann, 1996). Thus, we identified pairs as those clumping together (either in a nest or outside a nest). Pairs were stable for the duration of the study.

5.2.2 General timeline

Behavioral observations and blood samples were collected from each pair across 3 timepoints. Timepoint 1 began immediately following the initial 1-month habituation. Timepoint 2 and 3 began 31, and 38 days later respectively. One pair breed during this study. For the one pair that breed, the second and third behavioral sessions were taken at relevant life history points, incubation (Timepoint 2) and chick-rearing (Timepoint 3), 19 days and 34 days later respectively.

5.2.3 Behavioral observations

We collected 2 morning (08:00hr-12:00hr) and 2 evening (17:00-19:00hr), 10 minute behavioral observations at 3 timepoints (4 behavioral sessions at each timepoint: 12 session total/ pair). At timepoint 1 eight of the pairs had 1 evening observation. Thus we have a total of 11 or 12 behavioral observations for every pair. At each behavioral session, one observer (NHP) stood outside the aviary and scored behavior through the one-way viewing screen. Behavioral scoring was spoken into a microphone and later transcribed.

We scored several types of behavior, including foraging, affiliative behavior and aggression. Five behaviors were classified as affiliative: clumping (perching touching and facing

the same direction), allopreening, coordinated preening (male and female are self-preening at the same time), co-nesting, and overall time engaged in coordinated activities (coordinated foraging, preening, and nesting). Occasionally, we did observe chases associated with nest defense, however those behaviors were extremely rare and are not presented here.

5.2.4 Brachial blood samples

Blood samples were collected from the brachial vein in the morning (08:00-13:00hr). For Timepoints 2 and 3, the blood samples were collected 1-2 days after the last behavioral observation (1.5 ± 0.1 days). For Timepoint 1, blood samples were collected 2 days before the first behavioral observations for 7 pairs. Due to technical issues in the field, behavioral observations of the remaining 4 pairs were taken 1-2 weeks after first behavioral observations. All individuals in an aviary were caught and bled within 13 minutes (6.9 ± 0.40 min). All subjects in an aviary were sampled at the same time in order to reduce the stress. Plasma was obtained by centrifugation of heparinized capillary tubes. Samples were kept frozen until further processing. Of the 66 samples, 62 were successfully processed.

5.2.5 Steroid analysis: liquid chromatography-tandem mass spectrometry

Steroid extraction and LC-MS/MS protocols were adapted from Adomat et al., 2012.

Samples (~30 µl of plasma) were adjusted for pH using 20 µl of 1M NaOH and deuterated testosterone, 5α-DHT and ADIOL (d3T, d3DHT, d3Diol C/D/N Isotopes) were added as internal standards (IS). Steroids were then extracted using 2000 µl of 60:40 (v/v) hexane:ethyl for 30 minutes .Two additional 2000µl extractions were pooled with the initial extract to ensure high extraction efficiency. Pooled extracts were dried in a centrifugal vacuum evaporator (Labconco).

The resulting residues were derivatized using 2-fluoro-1-methylpyridinium *p*-toluene-4-sulfonate (FMP), which enhances sensitivity for hydroxylated steroids. More specifically, FMP (Sigma, Oakville, Ontario, Canada) was dissolved in dichloromethane ($CHCl_2$) to yield a 20 mM solution, and then 4 µl/ml of triethylamine (TEA) was added. This solution was prepared immediately prior to use. Individual samples were then treated with 400 µl of the FMP solution and allowed to react at room temperature for 1 hour. 50µl of methanol (MeOH) was added to quench any residual reagent for 15 minutes after which they were again dried in the Centrivap. The dried extracts were then reconstituted in 50 µl of 50% MeOH, centrifuged at 20000g for 5 minutes to sediment any remaining particulates and transferred into LC vials with low volume inserts for analysis by LC-MS/MS.

Analysis was carried out with a Waters Aquity UPLC Separations Module coupled to a Waters Quattro Premier XE Tandem Mass Spectrometer. A 2.1x100mm BEH 1.7µM C18 column was used for the steroid samples. The mobile phases were water and acetonitrile (ACN) both containing 0.1% formic acid using the following gradient: 0 min, 10%; 0.5 min, 10%; 1 min, 20%; 7 min, 30%; 13 min, 35% (% ACN). This was followed by a column flush of 95% ACN and re-equilibration for a total run length of 18 min. Column temperature was 35 °C and injection volume was 15 µl. The MS was set at unit resolution, capillary was 1.5 kV, source and desolvation temperatures were 120 °C and 300 °C respectively, desolvation and cone gas flows were 1000 L/h and 50 L/h and the collision cell pressure was held at 4.6×10^{-3} mbar. All data were collected in ES+ by multiple reaction monitoring (MRM) for steroids. Instrument parameters were optimized for the m/z's and corresponding fragments of the oxime-steroids monitored for each MRM.

Steroid quantification

Data processing was conducted using Quanlynx (Waters Corp.) using area under curve (AUC) of analyte/IS. Calibration samples consisted of neat standards and equivalently extracted spiked charcoal stripped serum (CSS) (6 standards ranging from 0.02 to 10ng/ml) and normalized to sample volume. The limit of detection (LOD) ranged from approximately 0.01-0.02 ng/mL (3x the background); limit of quantification (LOQ) were approximately 10x background (+/- 20% accuracy). Recoveries and conversions to derivatized steroid species were greater than 80% for each steroid. Unfortunately, our ability to detect 5**α-**DHT was affected by an internal artifact co-eluting with this steroid. This artifact appears to be unique to the zebra finch matrix, unfortunately making reliable quantification impossible for this analyte/derivatization protocol for these samples.

5.2.6 Statistics

All statistics were conducted in R (v 2.12.2) (R Core Team, 2012).

Given that only 1 pair had changes in breeding status throughout the testing and that initial statistical models showed no effect of Timepoint (within-subjects factor, rmANOVA), sex steroid levels were averaged across timepoints for each individual (*see results)*. To determine if there was a sex difference in steroid levels, welsch's t-tests were conducted on the average sex steroid levels for males versus females. Additionally, linear regressions were conducted to determine if female and male steroid levels were correlated within pairs.

Affiliative behavior (All clumping) was stable across timepoint (within-subjects factor, rmANOVA), and was thus averaged across timepoints for each pair. Linear regressions were conducted on the averaged female and male sex steroid levels and pair-level affiliative behavior, to determine if sex steroid profiles correlated with affiliation.

5.3 Results

5.3.1 Description of sex steroid profiles

Unfortunately, our ability to detect 5α-DHT was affected by an internal artifact co-eluting with this steroid. Of the remaining 8 steroids that we investigated, 4 were present in concentrations above our limit of quantification (LOQ): Pregnenolone, Progesterone, DHEA, and Testosterone (Table 5.1). Circulating steroid levels were stable over time (Pregnenolone: $F_{(1,58)}$=1.04, P=0.312; Progesterone: $F_{(1,58)}$=0.24, P=0.625; DHEA: $F_{(1,58)}$=0.01, P=0.917; Testosterone $F_{(1,58)}$=1.07, P=0.306). Thus, sex steroid levels were averaged across Timepoint for each individual.

Average circulating concentrations sex steroids were log transformed to aid in assumption of normality. Circulating pregnenolone, DHEA and testosterone were similar in males and females (Figure 5.2A,C&D: Pregnenolone: T = 0.15, P=0.879; DHEA: T=1.28, P=0.217; Testosterone: T=-1.47, P=0.171). Circulating progesterone levels were higher in females than in males (Figure 5.2B: Progesterone: T=2.17, P=0.046). There was no correlation of sex steroid levels within a pair (Pregnenolone: R^2=0.04, P=0.568; Progesterone: R^2=0.0005, P=0.947; DHEA: R^2=0.13, P=0.280; Testosterone: R^2=0.05, P=0.525). Interestingly, circulating pregnenolone concentrations are an order of magnitude higher than any other steroid measured (Table 5.1). Additionally, circulating testosterone levels were extremely low (Table 5.1).

The other precursors we examined were below our limit of quantification (LOQ): Androstenediol, pregnan-3, 17-diol-20-one, androsterone, and androstanediol (ADIOL). However, we were able to detect trace amounts of androstenediol and androsterone in some samples (Table 5.1). The 5α-reduced steroid precursors associated with the backdoor pathway

were largely non-detectable despite the low detection limit of the LC-MS/MS assay. Specifically, circulating ADIOL was non-detectable, which we had predicted would be elevated if the backdoor pathway was used.

5.3.2 Behavioral summary

A summary (mean ± SEM) of common behaviors, including affiliation, nesting, preening and foraging behavior is presented in Table 5.2. Overall, affiliative behaviors were higher in the evening. Co-nesting and clumping were the most common affiliative behaviors (Table 5.2). Thus we combined co-nesting and clumping for each observation as a metric of affiliation: All Clumping. Affiliative behavior (All Clumping) was stable across timepoint ($F_{(1,120)}$=1.29, P=0.258). Thus, affiliative behavior levels were averaged across timepoints (11-12, 10-min behavioral observations total) for each pair.

Average affiliation (% time all clumping) within pairs did not correlate to either averaged male or female sex steroid levels (Table 5.4, Figure 5.3).

5.4 Discussion

Maintaining a pair bond is critical to a zebra finch, and therefore understanding the proximate mechanisms that promote affiliative behavior is fundamental towards understanding long-term pair maintenance. By using LC-MS/MS, we were able to develop the most comprehensive sex steroid profile to date for zebra finches and correlate these sex steroid profiles with pair-maintenance behavior. Sex steroid profiles were similar in males and females. Circulating pregnenolone was present at extremely high levels, an order of magnitude greater than the other steroids. DHEA, a precursor for the traditional pathway (Figure 5.1), was the next most prominent steroid precursor, and there was no evidence of circulating precursors from the

backdoor pathway. Specifically, we did not detect any circulating ADIOL. Finally, while the pairs did vary in the amount of pair-maintenance behavior they engaged in, there was no correlation between this variation and sex steroid profiles of males and females.

5.4.1 Sex steroid profiles and neurosteroid synthesis

The circulating testosterone levels reported here are similar to what has been reported previously for wild zebra finches (Perfito et al., 2007; Vleck and Priedkalns, 1985), and are considerably lower than what has been reported in laboratory populations of zebra finches (Chapter 2: Prior et al., 2013). To our knowledge, none of the other steroids examined here have been quantified in wild zebra finches. Consistent with the pattern for testosterone, circulating DHEA and progesterone were also lower than what has been reported from laboratory populations of zebra finches (DHEA: Soma et al., 2004; Chapter 2: Prior et al., 2013; Fokidis et al., 2013; Progesterone: Taves et al., *in prep*). Furthermore, the amount of circulating DHEA reported here is also lower than what has been reported in wild song sparrows (Newman et al., 2011; Soma and Wingfield, 2001). These levels of circulating steroids are also low compared to previous reports of circulating testosterone and DHEA in stressed or non-breeding songbirds (Soma et al., 2004; Newman et al., 2011; Taves et al., 2010; Chapter 2: Prior et al., 2013). In contrast, we measured extremely high levels of circulating pregnenolone. While, pregnenolone has been largely unstudied in songbirds, the circulating concentrations of pregnenolone in Japanese quail are less than half of what we report here (Tsutusi and Yamazaki, 1995).

Neurosteroids production is especially high in songbirds (Schlinger and Arnold, 1991). It is possible pregnenolone is a primary precursor for neurosteroid synthesis. Furthermore, the surprisingly low levels of other sex steroids, specifically testosterone, may suggest that neurosteroid synthesis is higher in populations of wild zebra finches than in previously studied

laboratory populations. In song sparrows, neurosteroid synthesis of androgens and estrogens is higher during the non-breeding season (Pradhan et al., 2010; Schmidt et al., 2008). As opportunistic breeders, it may be adaptive for the zebra finch to have high levels of neurosteroid synthesis to allow for stable brain steroid levels despite rapid peripheral changes in reproductive status. Indeed, there is some evidence this may be the case for water restricted laboratory populations of zebra finches (Chapter 2: Prior et al., 2013). Future work should compare the role of pregnenolone as a precursor for neurosteroid synthesis in breeding and non-breeding (water restricted) laboratory zebra finches.

5.4.2 The backdoor pathway of androgen synthesis

Pregnenolone and progesterone are precursors in both the traditional and backdoor pathway to androgen synthesis (Figure 5.1). Here, none of the precursors specific to the backdoor pathway were detectable in circulation, including ADIOL, which we had predicted would be elevated. Despite the lack of systemic evidence for the backdoor pathway, we cannot rule out the possibility that it is used for local androgen synthesis in zebra finches.

In the tammar wallaby, ADIOL is released from the testes and travels to the skin and urogenital tract to promote male sexual differentiation during early development (Auchus, 2004). During this time, male plasma levels 5α-DHT and ADIOL are elevated compared to females (Shaw et al., 2000). Thus the backdoor pathway is particularly important and visible in circulation when circulating testosterone levels are low (Auchus, 2004). Here, we did see low plasma levels of testosterone in both males and females. While, this led us to think we were more likely to detect systemic evidence of the backdoor pathway, it is also possible that the backdoor pathway is acting locally. Future work should focus on local steroid metabolism in the brain of the traditional versus backdoor pathway of androgen synthesis.

5.4.3 The role of sex steroids in within-pair affiliation

Classically, progesterone is considered to be a reproductive steroid in females. However, there is also evidence that it may be involved in regulating pairing behavior in zebra finches. Smiley et al (2012) found that exogenous progesterone administration in female zebra finches promoted courtship and nesting behaviors when administered prior to pairing, however progesterone administration had no effect on pair-maintenance after initial bond formation. Our results are consistent with Smiley and colleagues (2012). We found no correlation between male or female progesterone levels and pair-maintenance behavior. We did not look during initial pair bond formation, and it is possible there was have been a relationship between circulating progesterone levels and pair affiliation during that time.

Research examining the regulatory role of testosterone in pair bonding has largely focused on male song. While, there is extensive work highlighting the importance of testosterone in the development and regulation of male song (Pröve, 1983; Williams et al., 2003; Remage-Healey et al., 2009), it is unclear what the significance of this is for pair affiliation associated with long-term bond maintenance. After initial bond formation (~2 weeks), directed and undirected male song functions as pair affiliation associated with bond maintenance and appears to synchronize pairs throughout the year (Zann, 1996; Dunn and Zann, 1996b). However, male song is not necessary for pair-bond maintenance (Tomaszycki and Adkins-Regan, 2006).
The role of androgens in regulating non-song pairing behavior is even more mixed. Administration flutamide (an anti-androgen) and 1,4,6-androstatriene-3,17-dione (ATD, an aromatase inhibitor) in male zebra finches, decreased male aggression and female approaches during pair-bond formation and courtship, but had no effect on any other courtship behaviors (Tomaszycki et al., 2006). There have only been a few studies examining the role of androgens in zebra finch pair maintenance. Recently, we even found that chronic testosterone

administration in males inhibits pair affiliation (Chapter 4). Our results here are difficult to interpret partly because circulating testosterone levels were so low in both males and females. We saw neither a positive nor negative relationship between circulating testosterone and pair affiliation.

5.5 Conclusions

The zebra finch is an extensively used model for neuroscience and behavioral studies, and yet surprisingly little is known about its physiology in the wild. To our knowledge, this is the first study to take advantage of the sensitivity of LC-MS/MS to investigate the neuroendocrine regulation of behaviors in wild birds. Our sex steroid profiles have highlighted the potential importance of circulating pregnenolone in both males and females. Furthermore, this research raises the question of whether neurosteroid synthesis may be higher in wild populations of zebra finches. Finally, we found no evidence that sex steroids are involved in regulating pair-maintenance behavior in wild zebra finches.

Table 5.1: LC/MS-MS results: Describing sex steroid profiles

	Pregnenolone	Progesterone	DHEA	Androstenediol	Pregnan-3,17-diol-20-one	Androsterone	Androstanediol (ADIOL)	Testosterone
% Total samples >LOD	100	74	100	19	0	5	0	39
% Total samples >LOQ	100	48	100	0	0	0	0	31
Min	0.23	0.04	0.03					0.04
Max	18.88	1.81	1.67					1.60
Mean ± SEM	5.54 ± 0.50	0.41 ± 0.09	0.46 ± 0.04					0.26 ± 0.08

Note: Summary of sample results of sex steroid profiles in zebra finch plasma (ng/mL). Sixty-two samples were run on LC/MS-MS (Female =29; Male =33). The number of samples that were greater than the limit of detection (LOD) and limit of quantification (LOQ) are given separately. For the samples that were greater than our LOQ, descriptive statistics are given (minimum, maximum, and mean± SEM). There were detectable levels of androstenediol and androsterone, however no samples were above the LOQ.

Table 5.2: Summary of behavior in the morning and evening

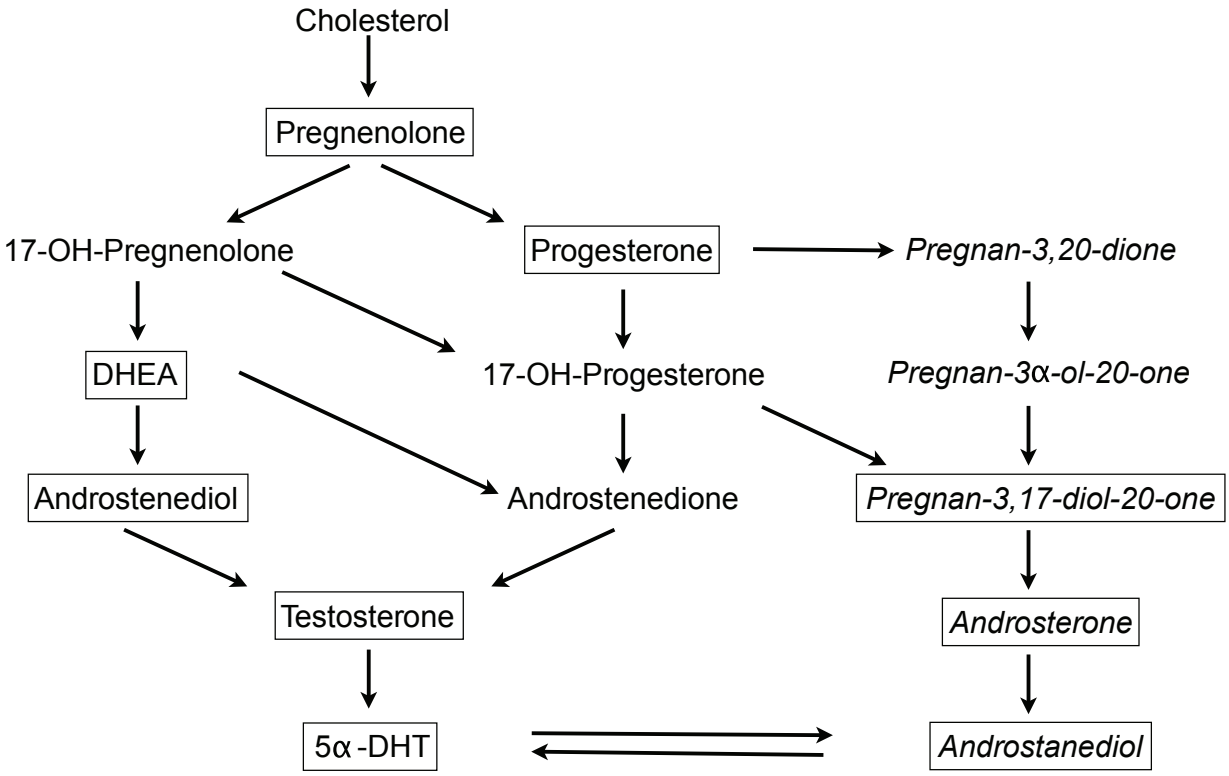
		Morning	Evening
Affiliation	**Clumping (outside nestbox)**	12 ± 5	51 ± 18
	Allopreening	2 ± 1	6 ± 3
Nesting	**Female**	35 ± 14	50 ±19
	Male	20 ± 8	36 ± 14
	Coordinated	36 ± 15	83 ± 24
Preening	**Female**	3 ± 1	10 ± 3
	Male	16 ± 5	14 ± 3
	Coordinated	4 ± 1	6 ± 3
Foraging	**Female**	1 ± 1	6 ± 5
	Male	2 ± 1	1 ± 1
	Coordinated	1 ± 1	1 ± 1

Note: Time (s) spent engaging in behaviors in the morning and evening (mean ± SEM). Here Clumping in and outside of the nestbox are presented separately.

Table 5.3: Correlation between sex steroid and pair-level affiliative behavior

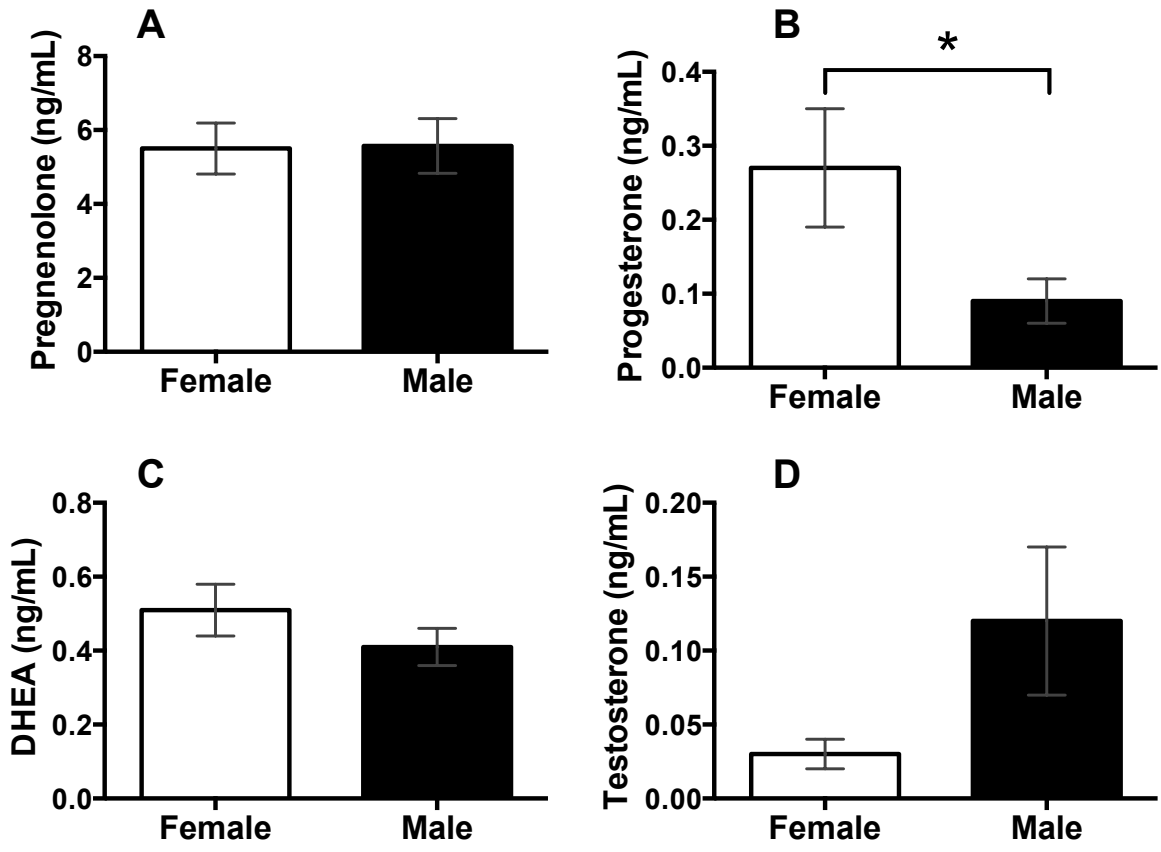
		R-squared	P value
Pregnenolone	**Female**	0.08	0.410
	Male	0.04	0.567
Progesterone	**Female**	0.31	0.078
	Male	0.10	0.332
DHEA	**Female**	0.0005	0.944
	Male	0.0002	0.962
Testosterone	**Female**	0.002	0.896
	Male	0.12	0.288

Figure 5.1 Steroidogenic pathway



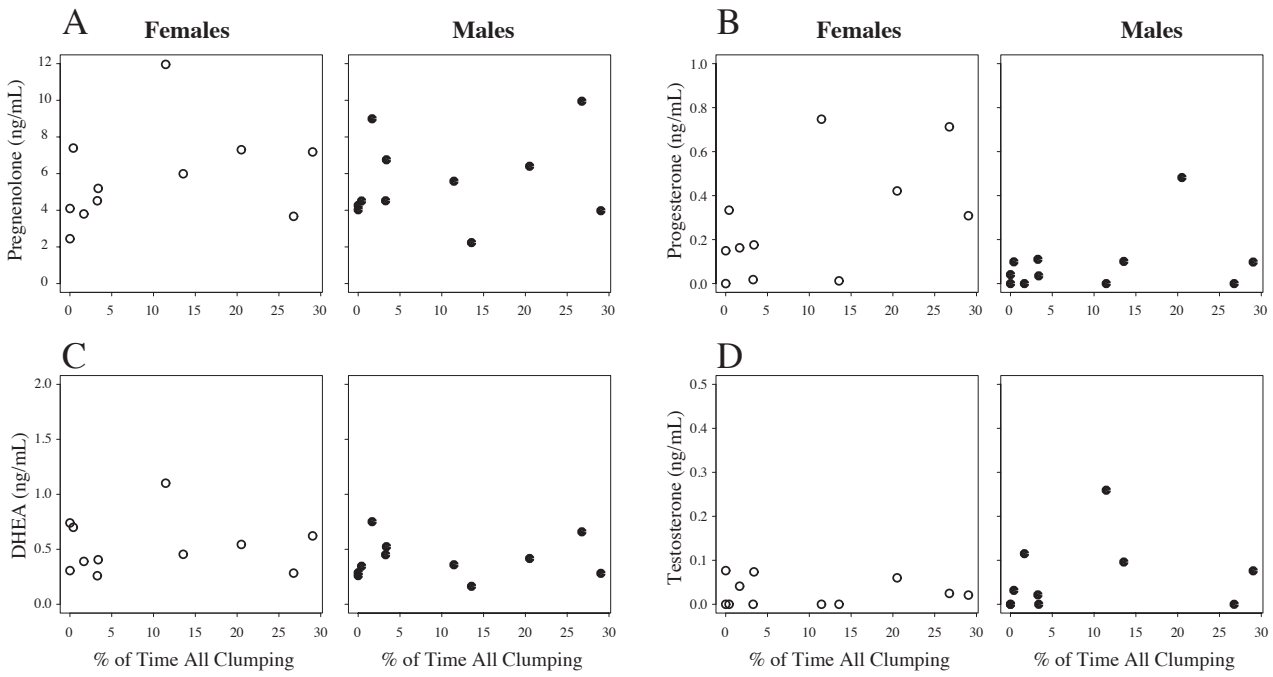
Steroidogenic pathway highlighting traditional pathway and backdoor pathway to 5α-DHT (italicized). Steroids that we examined with LC-MS/MS are boxed. Four steroids were present in levels above our limit of quantification (LOQ): pregnenolone, progesterone, DHEA, and testosterone. Trace levels (above the limit of detection (LOD) but not LOQ) were present for androstenediol and androsterone.

Figure 5.2 Sex differences in circulating sex steroid levels



Circulating sex steroid levels of (A) pregnenolone, (C) DHEA and (D) testosterone were similar in males and females. Circulating progesterone levels were higher in females than in males (T=2.17, P=0.047) (B). Overall steroid levels are very low, with the exception of circulating pregnenolone. Circulating pregnenolone levels are an order of magnitude larger than other steroid levels in both males and females. Statistical significance is indicated with *, $p < 0.05$.

Figure 5.3 Correlation between clumping and circulating sex steroids



Sex steroid levels were averaged across timepoints for each individual. Affiliative behavior (% of time all clumping) was averaged across timepoints (11-12, 10-min behavioral observations total) for each pair. There was no significant correlation between sex steroid levels in males or females and affiliative behavior within a pair: A) pregnenolone, B) progesterone, C) DHEA, and D) testosterone. Female data points are darkened circles and male data points are open circles.

6 General Discussion

Affiliation is a fundamental and relatively understudied component of social behavior. Moreover, the bulk of research on the neuroendocrinology of affiliation has focused on initial monogamous pair-bond formation in mammals (Insel and Young, 2001; Young and Alexander, 2012). Although valuable, that line of research cannot address the variation that exists across monogamously breeding species. Particularly for the many avian species that form life-long monogamous bonds (Black, 1996), it as important to study the underlying mechanisms that support bond maintenance as it is to study initial bond formation. Importantly, studies on life-long pair bonds allow researchers to explore breeding-specific neuroendocrine regulation of pair-maintenance behavior. Zebra finches are a great model system in which to study the regulation of long-term pair maintenance, and there is an extensive array of resources at our disposal to aid in such investigations (Griffith and Buchanan, 2010). The combination of laboratory resources (such as a sequenced genome) together with the ethological relevance of pairing behaviors is unique and gives this model organism significant advantages for the study of ethologically relevant pair maintenance. While they are an extensively used model for neuroscience and behavioral studies, surprisingly little is known about the neuroendocrine regulation of pair maintenance in this species.

In this dissertation, I have presented a series of studies using the zebra finch to examine the hypothesis that sex steroids regulate pair-maintenance behavior differently depending on breeding condition. In brief, I report that, (a) testosterone and estradiol levels are maintained in behaviorally-relevant regions of water-restricted (i.e. non-breeding) zebra finches (Chapter 2), (b) fadrozole rapidly increased pair-maintenance behavior (proximity time) (Chapter 3), (c)

chronic male-testosterone treatment decreased pair-maintenance behavior (proximity time under stressed conditions) (Chapter 4), and (d) sex steroid profiles and pair-maintenance behavior were not correlated in wild-caught zebra finches (Chapter 5). Taken together, this series of studies suggests that sex steroids have breeding-specific and social-context-specific regulatory effects on pair-maintenance behavior. However, the inconsistent effects of sex steroids may indicate that other neuroendocrine mechanisms are primarily regulating pair maintenance. This conclusion would be consistent with the results of Tomaszycki et al. (2006), who saw no evidence for the role of androgens and estrogens in the regulation of pair-bond formation in zebra finches, and with the body of evidence for other neuroendocrine mechanisms that regulate pair-bond formation in zebra finches. Regardless of the neuroendocrine mechanisms regulating pair-maintenance behavior, this series of studies emphasizes the importance of considering breeding condition in zebra finches and other monogamous species.

6.1 Major findings: Breeding condition matters in studying the behavioral endocrinology of zebra finches.

6.1.1 Zebra finches have distinct breeding and non-breeding states

It was previously unclear the extent to which zebra finches, as opportunistic breeders, have distinct endocrine states associated with breeding readiness (Perfito, 2010). Here we found that in females, water restriction profoundly reduced largest ovarian follicle size, ovary size, oviduct size, and egg laying. In males, water restriction had no effect on testes size but decreased systemic testosterone levels (Chapter 2). The effect of water restriction on levels of sex steroids in the brain were only sometimes mirrored in levels of circulating sex steroids. Furthermore, in the brain, water restriction affected testosterone and estradiol levels in a region-specific manner.

Specifically, water restriction decreased sex steroid levels in several brain regions [including: testosterone levels in the female rTEL and the male rTEL, caTEL, and CB, and estradiol levels in female ceTEL, M/HB, and CB and male ceTEL, caTEL, M/HB and CB (Chapter 2)]. Because pair-maintenance behaviors are similar across breeding context despite changes in sex steroid profiles, one hypothesis is that pair-maintenance behaviors are regulated by neuroendocrine mechanisms that are unaffected by breeding condition.

6.1.2 Sex steroids regulate pair-maintenance behavior

The direct effects of sex steroids on pair-maintenance behavior were assessed across two experiments. We found significant effects of both pharmacological manipulations conducted, specifically: (1) a rapid promoting effect of fadrozole on proximity behavior in water restricted (i.e. non-breeding) zebra finches (Chapter 3) and, (2) an inhibitory effect of exogenous male testosterone on proximity time in control (i.e. breeding condition) individuals (Chapter 4).

The rapid promoting effect of fadrozole (Chapter 3) could be due to either the decrease in brain estrogen levels or to the concomitant increase in brain testosterone. The inhibitory effect of testosterone (Chapter 4) suggests that the effect of fadrozole (Chapter 3) was due to a decrease in brain estrogen levels. However, these two experiments differed in several ways, making it difficult to compare the results. Firstly, the effect of fadrozole was seen in water-restricted (i.e. non-breeding) males and females, while the effect of testosterone was seen in control (i.e. breeding) males. Secondly, the effect of fadrozole was seen during a partner separation behavioral paradigm, while the effect of testosterone was seen in the stressed test. Together, these experiments may suggest that sex steroids regulate pair-maintenance behavior differently depending on breeding condition, sex, and social context.

6.1.3 Sex steroids act via non-genomic mechanisms in non-breeding zebra finches

Although water restriction produced distinct sex steroid profiles in both the circulation and the brain, we also found that hypothalamic levels of testosterone and estradiol were unaffected by water restriction in both sexes (Chapter 2). The hypothalamus contains nuclei that are part of the "social behavior network" (e.g., preoptic area, ventromedial hypothalamus) and are likely important in the regulation of pair-maintenance behaviors (Newman, 1999; Goodson, 2005; Banerjee et al., 2013). Thus, this maintenance of sex steroid levels in the hypothalamus is consistent with the hypothesis that sex steroids regulate pair-maintenance behavior in non-breeding zebra finches. When gonadal production is low during non-breeding periods, levels of sex steroids in the brain can be maintained through increased neurosteroid synthesis (*reviews:* Schmidt et al, 2008; Soma et al., 2008). In non-breeding song sparrows an increase in neurosteroid synthesis corresponds to an increase in non-genomic signaling (Soma et al., 2008; Heimovics et al., 2012), which raises the question of whether there is non-genomic signaling in non-breeding zebra finches. We directly tested this hypothesis using an acute and chronic fadrozole administration in non-breeding zebra finches. We found rapid effects of acute (but not chronic) fadrozole administration in water-restricted pairs (Chapter 3). However, to further test this hypothesis, a fadrozole administration study should also be conducted on breeding-condition zebra finches.

6.2 Additional contributions to novel lines of inquiry

A significant portion of this thesis has been devoted to developing zebra finches as a model system to examine breeding condition in relation to pair-maintenance behavior. This has involved several novel lines of inquiry

6.2.1 Water restriction and studies of zebra finch behavior

While water restriction has been used previously in zebra finches (Oksche, 1963; Sossinka, 1974; Vleck and Priedkalns, 1985; Perfito, 2010), the majority of research has examined the regulation of opportunistic breeding in male zebra finches (*review:* Perfito, 2010). Here we validated the use of water restriction for behavioral research in zebra finches. Critically, we described the effect of water restriction on female reproductive organs as well as both systemic and brain male and female steroid levels. Examining breeding condition in the regulation of zebra finch behavior is an important question, as we have highlighted above, and thus this water restriction paradigm will be useful in future work.

6.2.2 Behavioral testing paradigms

In addition to water restriction, we had to adapt behavioral paradigms to study within-pair affiliation in established pairs. The limited previous work on zebra finch pair-maintenance behavior used either extreme behavioral endpoints, such as divorce, or relied on baseline behavior (Tomaszycki et al., 2006; Smiley et al., 2012). Pair maintenance is difficult to study under baseline conditions, as it reflects highly stable behavioral patterns that are likely to require less behavioral and physiological investment to maintain. Therefore, we tested several new or adapted behavioral paradigms, with the goal of using unstable social conditions to elicit higher levels of affiliation. Specifically, we quantified behavior following two types of stressors: partner separation (Chapter 2 and 3) and chase (Chapter 4). While isolation and partner separation have been used previously to examine social stressors (e.g. Remage-Healey et al., 2003), we adapted these previous paradigms to elicit both vocal and physical affiliative behavior by using cages with both opaque and wire partitions. Following a separation we were able to first elicit vocal behavior using only visual contact (wire partition), and then allow full physical contact (no partition). A brief visual separation was sufficient to obtain elevated levels of affiliation in a pair.

We also relied heavily on proximity time in freely-moving individuals as a comprehensive metric of pair maintenance. While proximity has been used in a wide range of behavioral tests including partner preference or choice tests (e.g. Tomaszkycki and Adkins-Regan, 2005; Goodson et al., 2009), to our knowledge this is the first use of it as a measure of synchrony in freely-moving individuals. Interestingly, proximity time was the only behavioral metric where we saw effects of our sex steroid manipulations (Chapter 3 and 4). This sensitivity to manipulation suggests proximity is a useful behavioral metric to consider in future studies.

Using different behavioral paradigms, unfortunately, makes it more difficult to compare results across experiments. However, one of the major strengths of this work is developing behavioral paradigms that can be used for future studies.

6.2.3 Vocal behavior in breeding and non-breeding zebra finch pairs

As songbirds, zebra finches engage in a wide range of vocal behaviors. It has long been known that zebra finches produce short "tet" calls as a means to stay in contact (Zann, 1996); however, the vast majority of research has focused on male song (Williams, 2001; Riebel, 2009). Recently, it was discovered that wild zebra finch pairs engage in quiet and private duets during the breeding season (Elie et al., 2010). Furthermore, there is evidence of greater pair-level variation in zebra finch vocal duets than in physical behaviors (C. Vignal, *personal communication)*. Despite this rich range and variability in vocal behaviors, the majority of work on pairing in zebra finches has focused on physical metrics of affiliation (such as clumping) or male song (Tomaszycki and Adkins-Regan, 2005: 2006; Svec and Wade, 2009; Goodson et al., 2009). Clearly, it is important to consider a wider range of both male and female vocal behavior when studying pair maintenance. In these studies, we measured particular features of calling behavior in zebra finch pairs (Chapter 3 and 4); however, there was no effect of either sex steroid

manipulation on those vocal behaviors. It is possible that more sophisticated measures are needed than time spent calling (Chapter 3) or number of calls (Chapter 4). It was also our intention to measure duetting behavior in Chapter 4 as well; however, duets are only expressed during breeding periods. Because the pairs did not breed in the study reported in Chapter 4, we were unable to examine the effect of testosterone treatment on duetting. Further work needs to be done to develop behavioral paradigms that would allow for better analyses of neuroendocrine regulation of vocal behavior in captive pairs.

6.2.4 Wild versus domesticated zebra finches

Wild populations of zebra finches have lower rates of extra-pair copulations and perform differently on mate-choice tests than domesticated zebra finches (Rutstien et al., 2007; Griffith et al., 2010). This suggests that domestication has altered aspects of pairing behavior for the zebra finch (Griffith and Buchanan, 2010). However, the majority of zebra finch research has been conducted on domesticated zebra finches (Griffith and Buchanan, 2010). While the majority of my dissertation examined domesticated zebra finches, one study investigated the role of sex steroids in regulating pair-maintenance behavior in wild zebra finches.

However, there are still steep methodological challenges to doing longitudinal studies on wild zebra finches. Zebra finches are largely nomadic, and it is very difficult to follow them within a breeding season much less across breeding seasons. Because of these challenges, we brought wild individuals into captivity for the purpose of this study. We were thus limited by the outdoor aviary space available. In the 6 aviaries, we were only able to study 11 pairs. To maximize the information we gathered from these pairs, we used LC-MS/MS assays to quantify nine steroids. Examining these questions in free-roaming, wild pairs is critical to our understanding of pair-maintenance behavior and the neuroendocrine mechanisms supporting it.

Thus, further work and resources are needed to examine these questions in free-roaming, wild pairs.

6.2.5 LC-MS/MS-generated sex steroid profiles

Typically, only one or two hormones can be measured from the small blood samples collected from small wild animals, greatly limiting investigations of behavioral endocrinology. The use of liquid chromatography – tandem mass spectrometry (LC-MS/MS) presents an opportunity to measure multiple steroids simultaneously with the additional benefit of much higher specificity than is achieved with traditional use of immunoassays (Koren et al., 2012).

LC-MS/MS-generated sex steroid profiles can include sex steroid precursors as well as 'behaviorally relevant' sex steroids. The LC-MS/MS assay we used here allowed us to measure sex steroid precursors involved in both the 'traditional' and 'backdoor' steroidogenic pathway to 5α-DHT (Figure 5.1) (Auchus, 2004). Measuring sex steroid precursors has three significant benefits. Firstly, sex steroid precursors themselves can be correlated with behaviors (*DHEA*: Soma et al., 2004; Soma et al., 2008; Fokidis et al., 2013). Secondly, describing sex steroid profiles of precursors improves our ability to develop hypotheses about upstream and downstream neuroendocrine mechanisms. More specifically, 'behaviorally relevant' sex steroids can be produced in peripheral endocrine tissues or locally in the brain (neurosteroids) (Schmidt et al., 2008). Thus, primary circulating sex steroid precursors may be used for neurosteroid synthesis in the brain. Finally, because the backdoor pathway to 5α-DHT bypasses testosterone, utilizing both pathways allows for a tight regulation of DHT:testosterone ratios. This tight regulation may be particularly important in local tissues during times when systemic levels of testosterone are low (Auchus, 2004). Evidence for the backdoor pathway has not been previously investigated in songbirds. Overall, we found lower circulating sex steroid levels than has been

reported for domesticated zebra finches. Only pregnenolone, progesterone, DHEA, and testosterone were quantifiable (Chapter 5). While, the LC-MS/MS generated sex steroid profiles did not correlate with pair-maintenance behavior, these profiles themselves have interesting implications for behavioral neuroendocrinology of zebra finches. The low levels of sex steroids we found in wild zebra finches compared to domesticated raises the hypothesis that neurosteroid production may be higher in wild zebra finches. Furthermore, the surprisingly high levels of circulating pregnenolone suggest it may be an important precursor for neurosteroid synthesis in zebra finches.

However, there were several challenges to developing the LC-MS/MS assay (Chapter 5). While the LC-MS/MS assay we used had many benefits, it was originally validated for human and rodent models (Adomat et al., 2012). Future work is needed to refine the LC-MS/MS assay in songbirds. Not only could we make the assay more sensitive allowing us to quantify more steroids and precursors (if they are present), but we will be able to quantify DHT which is known to be present in circulation and tissue of zebra finches (*wild zebra finches:* Perfito et al., 2007).Here we discovered too late that songbirds have an internal artifact that co-elutes with 5α-DHT. Measuring 5α-DHT in wild zebra finches would have been beneficial for assessing DHT:testosterone ratios of wild zebra finches.

6.3 Future directions

These studies represent a first step towards examining the neuroendocrine regulation of zebra finch pair-maintenance behavior and the influence of breeding system on these mechanisms. Further research needs to be conducted in relation to all the questions studied here.

6.3.1 Do non-breeding zebra finches upregulate neurosteroid synthesis?

While we demonstrated breeding-specific differences in brain sex steroid levels, there are many ways these differences could be achieved. Brain steroid levels are a result of several factors, including circulating steroid binding proteins, local steroid synthesis, and tissue sequestration by steroid receptors or binding proteins (Taves et al., 2011). In order to determine whether the maintenance of sex steroid levels in non-breeding zebra finches is due to an upregulation of neurosteroid synthesis, future studies need to examine sex steroid receptors and the necessary steroidogenic enzymes. Steroidogenic enzyme levels can be examined using in-*situ* hybridization for mRNA or immunohistochemistry, and their activity can be assessed using activity assays. Given evidence in the song sparrow system where 3β-HSD activity in the forebrain is upregulated in non-breeding males (Pradhan et al., 2010; Pradhan and Soma, 2012), we might predict that 3β-HSD is also upregulated in water-restricted zebra finches, Finally, LC-MS/MS can be used to quantify sex steroid precursors. This ability to assay many precursors at once may help identify which circulating precursors are used for local neurosteroid synthesis, which will allow more specific upstream hypotheses to be developed.

6.3.2 Do sex steroids directly regulate pair-maintenance behavior?

The results of this work provide several lines of evidence that sex steroids may be regulating pair-maintenance behavior. Firstly, future studies should further elucidate the behavioral expression of pair maintenance. In particular, research should (1) further identify which behaviors are important under specific contexts or paradigms, and (2) examine the contribution of females versus males to these behaviors. More precise understandings of these behaviors will be critical for designing behavioral assays for future pharmacological manipulation experiments. Secondly, more precise pharmacological manipulations could be done centrally (rather than the systemic manipulations we did). More precise sex steroid

manipulations could clarify the role of specific nuclei in regulating pair-maintenance behavior. For example, for brain nuclei near the surface, microdialysis or cannulations can be used to manipulate steroids in freely behaving individuals.

6.3.3 Does estrogen act via membrane-bound receptors in non-breeding zebra finches?

Here we suggest that estrogen is acting via non-genomic signaling mechanisms in water-restricted zebra finches. In order to examine this hypothesis more closely, future research needs to compare membrane-bound estrogen receptor levels in water-restricted versus control zebra finches. Additionally, the effect of estrogen on signal transduction pathways can be examined using immunohistochemistry (Heimovics et al., 2012). The role of non-genomic signaling in regulating behavior could also be studied through the more precise brain-specific pharmacological manipulations as discussed in section 6.3.2.

6.3.4 Do non-steroidal neuroendocrine mechanisms regulate pair-maintenance in zebra finches?

Beyond the role of sex steroids, there are several other neuroendocrine mechanisms that should be examined in relaton to zebra finch pair-maintenance behavior: including, (1) the regulatory role of nonapeptides in pair-bond maintenance, (2) the differential effect of dopamine during pair-bond maintenance and formation, and (3) the interplay between the HPA axis and pair-bonding. Broadly, each of these avenues of research makes sense in light of what is known about the overall neural circuitry underlying stress physiology, motivation and affiliation. A comprehensive discussion of the neuroendocrine regulation of pair-bonding cannot be done here (*relevant reviews*: Curtis et al., 2006; McGraw and Young, 2011; O'Connell and Hoffmann, 2011a,b); however, I will note some of the grounds for these three avenues of study.

Firstly, the nonapeptides oxytocin and arginine vasopressin (AVP) have been identified as both necessary and sufficient to establish social bonds in prairie voles (Insel, 1997). Recently,

many similarities between the neuroendocrine regulation of pair-bond formation in zebra finches and prairie voles have been identified. For example, administration of an oxytocin receptor antagonist to male and female zebra finches increased the latency to form a pair bond, decreased the overall percentage of pair-bonded individuals and decreased engagement in affiliative behaviors (Klatt and Goodson, 2012). Furthermore, oxytocin knockdown, using RNA interference (antisense) in female zebra finches, decreased pair bonding and clumping behavior (Kelly and Goodson 2014).

Secondly, dopamine in the NAcc plays a critical role in bond formation for both males and females prairie voles (Aragona et al., 2003; Aragona et al., 2006). However, while activation of D2-type dopamine receptors promotes pair-bond formation, activation of D1-type receptors inhibits bond formation (Aragona et al., 2006). In zebra finches, during courtship and pair-bond formation there is a positive relationship between dopamine and pair bonding (Goodson et al., 2009; Banerjee et al., 2013). Interestingly, the one study that focused on the role of dopamine in pair maintenance in zebra finches found the opposite relationship. Here TH immunoreactivity was negatively correlated with pair-maintenance behavior in the NAcc, preoptic area (POA), and ventral tegmental area (VTA) (Alger et al., 2011). This potential converse relationship between dopamine and affiliation in zebra finches suggests that dopamine might differentially regulate pair-bond formation and maintenance.

Finally, corticosterone administration interferes with pairing in females but facilitates pairing in male prairie voles (De Vries et al., 1995; De Vries et al., 1996). Additionally, corticotrophin-releasing hormone administered centrally promotes pair bonding (Lim et al., 2007). In zebra finches, acute corticosterone administration increases male preference for females (in non-bonded individuals), but had no effect when administered to females, suggesting

corticosterone has sex-specific effects on pairing in zebra finches (LaPlante et al., 2014). Furthermore, the ability of partners to synchronize their stress response might be important in the expression of pair bonding (Perez, 2013;Ouyang et al., 2014).

These neuroendocrine mechanisms, which may be involved in regulating pair-bond maintenance, act in brain regions that also contain aromatase and estrogen receptors. Thus, it is possible that sex steroids may modulate these other neuroendocrine mechanisms in breeding-condition dependent ways (Prior and Soma, *in prep*).

6.3.5 Are the neuroendocrine mechanisms regulating zebra finch social behavior, more broadly, affected by breeding condition?

Previously, behavioral studies in zebra finches have focused on captive breeding-condition individuals (Griffith and Buchanan, 2010). Even when studies refer to "non-breeding" pairs, they typically are referring to pairs that are not actively breeding, leaving the endocrine state ambiguous (e.g. Kabelik et al., 2010). Here we have demonstrated the impact that breeding condition may have on the regulation of zebra finch behavior. The water restriction paradigm we used here can be applied to studies focusing on other behaviors (such as courtship, aggression, and sociality). Under water restriction, the neuroendocrine mechanisms regulating behaviors may be similar to or very different from what has been previously reported.

6.4 Conclusion

To our knowledge this is one of the first series of studies examining the effect of seasonality on the neuroendocrine regulation of affiliative behavior, including the first study looking at the relationship between sex steroid profiles and affiliation in wild pairs. Both pharmacological manipulations that we conducted affected pair-maintenance behavior.

Furthermore, we have demonstrated that breeding and non-breeding zebra finches correspond to distinct endocrine states in males and females and that sex steroid levels are maintained in the hypothalamus regardless of condition. These findings highlight the importance of considering breeding condition when examining the relationship between zebra finch pair-maintenance behavior (as well as other behaviors) and sex steroids. That previous work has not controlled for breeding condition may explain discrepancies in the results of studies looking at the role of sex steroids in regulating zebra finch behavior. Taken together, these studies have furthered our understanding of the neuroendocrine regulation of affiliative behavior, supported several new avenues of research, and demonstrated the value of the zebra finch for studies of long-term pair-maintenance.

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