**Context-dependent effects of testosterone treatment to males
on pair-maintenance behavior in zebra finches**

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Running Head: context-dependent effects of testosterone on pair maintenance

Word Count: 8005

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Abstract

Monogamous pair bonds can be transient or long-lasting, which varies across species. The neuroendocrine mechanisms regulating pair-maintenance behaviors are largely unknown, yet fundamental to our understanding of monogamy. Furthermore, the expression and regulation of pair-maintenance behavior is likely to be greatly influenced by social and environmental contexts. Our previous research suggested that androgens might regulate long-term pair-maintenance behavior in the monogamous zebra finch. Here, we tested the hypothesis that testosterone treatment to males affects long-term pair-maintenance behavior in zebra finches. Established pairs were randomly assigned to one of two groups: Control (n=8) and Testosterone (n=7). Males were given either an empty or a testosterone-filled silastic implant. Physical and acoustic affiliative behaviors and plasma testosterone levels were examined at three timepoints: pre-implantation, 30 days post-implant, and 60 days post-implant. Importantly, we examined affiliative behaviors under two contexts: in the homecage (Baseline) and following a brief chase (Post Stressor). Male testosterone treatment had no effects on behavior during the Baseline period, but significantly affected behaviors during the Post Stressor period. Specifically, testosterone-treated males spent less time in close proximity to their partner and sang more. To our knowledge, this is the first report of a context-dependent effect in the neuroendocrine regulation of pair-maintenance behavior, as well as the first report of an inhibitory effect of testosterone on zebra finch pairing behaviors. These results raise interesting questions about the function of affiliative behaviors in established pair bonds.

Keywords: androgen, corticosterone, affiliation, song, songbird, monogamy, pair bonding, vocalizations, tet calls

Introduction

Socially monogamous pair bonding occurs across a wide range of species, from fish to mammals, and it is the dominant mating strategy amongst birds (Reichard and Boesch, 2003). However, there is considerable variation within monogamous mating systems. For serially monogamous species, pair bonds are only maintained for one breeding cycle, with new bonds being formed with each new breeding season. In contrast, for life-long monogamous species, pair bonds are formed when individuals reach adulthood and then maintained indefinitely. While understanding monogamy has been a key area of research in evolution and behavioral ecology (Reichard and Boesch, 2003), surprisingly little is known of the physiological regulation of pair-maintenance behavior (*review*: Prior and Soma, 2015).

Pair bonds are formed and maintained through affiliative behaviors (Black, 1996). Whereas it is relatively easy to identify behaviors that are relevant during pair-bond establishment (courtship and bond formation), it is less clear which behaviors are necessary for pair-bond maintenance (e.g., allopreening, clumping, synchronization of activities, intra-pair vocal exchanges). It is likely that multiple affiliative behaviors promote pair maintenance in established pairs. However, the relative importance of specific affiliative behaviors likely depends on the social and environmental context. Therefore, situating behaviors within their social and environmental contexts could offer a more nuanced understanding of pair maintenance. Examining pair affiliation under ethologically-relevant contexts might clarify the neuroendocrine regulation of pair maintenance (Prior and Soma, 2015; Tomaszewski et al., 2015).

After a stressor, pairs frequently engage in elevated levels of affiliative behavior (Remage-Healey et al., 2003; Prior et al., 2013). Affiliation can dampen the hypothalamic pituitary adrenal (HPA) axis, thus acting as a social buffer (Kikusui et al., 2006; Martin et al.,

2014). Furthermore, in the absence of a stressor, pair-maintenance behaviors can reflect established behavioral patterns, which have become habits and are easily maintained (Yin and Knowlton, 2006). A disruption to this established pattern, such as an external stressor, can make pair-directed behaviors more important. For example, motivation to engage in affiliation with a partner could increase. A pair's behavioral response after a stressor may be a valuable indicator of pair-maintenance behavior.

Zebra finches are a valuable model for examining the relationship between sex steroids, context, and pair maintenance (review: Prior and Soma, 2015). It takes 1-2 weeks for these life-long bonds to solidify (Zann, 1996). Once bonded, individuals engage in affiliative behavior predominately with their partner. For the majority of an individual's life, affiliative behaviors primarily function in pair maintenance (Zann, 1996). There are several lines of evidence that zebra finch behavior associated with courtship and pair-bond formation is partially regulated by sex steroids, such as testosterone and estradiol (Harding et al., 1983; Adkins-Regan, 1999; Hill et al., 2005; Ramage-Healey et al., 2008). However, it is still unclear how sex steroids are involved in pair-maintenance behavior. In a recent study (Prior et al., 2014), we found evidence that sex steroids may regulate pair-maintenance behavior in zebra finches. When fadrozole (an aromatase inhibitor) was administered to either the male or female partner, the time pairs spent in close proximity increased. This effect of fadrozole could be due to 1) the decrease in estradiol levels or 2) the concomitant increase in testosterone levels (Prior et al., 2014).

In this experiment, we tested the hypothesis that testosterone treatment promotes pair-maintenance behavior. Males from established pairs were implanted either with testosterone-filled or control (empty) silastic implants. We then quantified pair maintenance during two conditions: Baseline (unmanipulated in the homecage) and Post Stressor (following a 15 sec

chase). This very brief stressor was designed to elicit a similar behavioral response to predator avoidance in wild zebra finches (NH. Prior, *personal observation*). Such brief stressors resulting in increased activity are common in the wild and are often followed by periods of increased pair maintenance (NH. Prior, *personal observation*).

Materials and Methods

Ethics Statement

This work was conducted in accordance with the Canadian Council on Animal Care guidelines with a UBC Animal Care Committee protocol (A09-0395).

Subjects

Adult captive zebra finches (>120 days old) were paired in cages (49 x 50 x 48 cm, Corners Cages). 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 evidence of breeding (i.e., nest building and egg laying) and that engaged in affiliation were included. Thus, we were confident that only successfully-bonded pairs were used.

Testosterone treatment

Pairs were randomly assigned to one of two treatment groups: Control (n=8) and Testosterone (n=7). Males were subcutaneously implanted with either an empty 10mm silastic implant (Dow Corning medical grade tubing: inner diameter 0.76mm, outer diameter 1.65mm) or an implant

packed with 8mm crystalline testosterone (Steraloids A6950-000). All implants were sealed with liquid silastic glue (1mm at each end). Similar manipulations have been effectively used previously 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 still contained testosterone ($< \frac{1}{2}$ full).

General timeline and assessment of breeding condition

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).

The effect of sex steroids on behavior might depend on breeding condition (Adkins-Regan, 2005; Heimovics et al., 2015; Prior and Soma, 2015). Typically researchers do not report breeding condition in zebra finches; it is generally assumed that, because they are opportunistic breeders, they are in a constant state of breeding readiness (Perfito, 2010; Prior and Soma, 2015). However, that assumption may not be accurate. Zebra finches within a single colony can often be at different breeding states (Perfito, 2010). Thus, it is important to control or assess breeding condition of zebra finches.

Here we assessed breeding condition in two ways. Firstly, we controlled access to nesting materials. Specifically, to ensure that all pairs were at a similar life-history stage at the beginning of the experiment, nesting material was withheld from all pairs during the PRE timepoint. Following the PRE timepoint, all pairs were given *ad libitum* access to nestboxes and nesting materials for the remainder of the study. Only pairs that showed an interest in breeding (i.e. nest building and egg laying) were included. Thus, we can confidently say that no pairs were included

that were in non-breeding condition.

Secondly, we recorded information daily on egg laying, nest building, and incubation behavior to assess each pair's investment in breeding. Importantly, while all pairs laid at least one egg (range 1-4 eggs) during the study, most of these eggs were not viable. Laying eggs outside of coordinated breeding attempts (e.g. while housed in same-sex flocks) is common for female zebra finches. During our experiment, only one chick was hatched and three pairs were observed incubating eggs. Taken together, we classified most of these pairs as "pre-breeding," and importantly we were able to ensure that both of our treatment groups were comprised of pairs in a similar breeding state.

Behavioral testing conditions

Behavior was scored during two different conditions. For the first condition (Baseline), we recorded behavior in the home cage for 30 min. For the second condition (Post Stressor), we recorded behaviors immediately after a brief chase. Specifically, one researcher (KNY) reached into the cage and chased each pair for 15 sec with his hand. Both behavioral conditions were recorded on the same day between 08:00 and 14:00h. The Post Stressor condition always occurred immediately after the Baseline condition.

Our Post Stressor condition was designed to mimic periods of brief stress, and thus elicit an increase in activity similar to that seen during predator avoidance or nest defense (against conspecifics) in the wild (NH. Prior, *personal observations*). Such brief stressors resulting in increased activity are common in the wild and are often followed by periods of pair affiliation (NH. Prior, *personal observation*). These events are brief, and therefore we restricted the behavioral assay after the stressor to 5 min.

Behavior was recorded with a camcorder for quantification of physical affiliative behaviors. Vocal behavior was 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.

Blood samples

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

Quantifying physical affiliative behavior

All behavior was scored by researchers blind to treatment (Testosterone vs. Control). The same behaviors were scored for both behavioral conditions [Baseline (30 min) and Post Stressor Chase (5 min)]. Three types of pair-level affiliative behaviors were scored: Proximity (% time within 15cm) (NHP), % Time Clumping (NHP) and % Time Coordinated Preening (NHP and TQDL). While preening behavior was scored individually for the male and female, we also quantified “coordinated preening,” the amount of time the male and female synchronized self-

preening bouts (NHP and TQDL). Additionally, we quantified one type of individual-level affiliative behavior: % Time spent allopreening was divided into male allopreening the female partner (Male AP) and female allopreening the male partner (Female AP) (NHP and TQDL). Finally, several other behaviors (i.e. time spent in the nestbox, nest-building, feeding, drinking and the number of copulations) were scored; however, because they were rare, they are not presented here.

Quantifying acoustic affiliative behavior

Acoustic behavior was scored using Praat software (V 5.3.56) (Boersma, 2001).

Male song was scored from three, two min sections (0-2, 14-16, and 28-30 min) during the Baseline condition, and during the entire five minutes of the Post Stressor condition. Specifically, the number of song bouts, motifs and introductory notes were quantified (NHP). Song bouts were defined as single renditions of a song, separated from one another by at least 1sec, typically including several introductory notes (short calls) and several repetitions of a stereotyped sequence (a motif).

In addition to song, zebra finches produce several types of vocalizations including two prominent types of calls, distance calls and short calls (Zann, 1996). Short calls can be further classified into several subtypes (e.g. tet and stack) (Zann, 1996). Distance calls are used when paired individuals are out of visual contact and are also used with other individuals in a flock. Short calls are used for communication over shorter distances (Zann, 1996), and typically for individuals within visual contact. In our experimental set up, each cage contained one pair. Thus, we assumed that short calls were used predominately within a pair, whereas distance calls were used with other birds in the colony room. Here, we only quantified short calls.

Short calls were scored from three, two min sections (0-2, 14-16, and 28-30 min) of the Baseline condition, and during the entire five min of the Post Stressor condition (NHP). Additionally, we scored the latency for a pair to resume calling following the chase (Latency to first call) (NHP). Note that, while females and males have distinct distance calls, male and female short calls are non-distinguishable, and thus calls were quantified at the level of a pair.

Steroid measurement

Corticosterone

Corticosterone was quantified with a radioimmunoassay kit (MP Biomedicals-07120103) as previously described (Schmidt and Soma, 2008; Newman et al., 2008a). Briefly, 5 μ L of unextracted plasma was diluted in 45 μ L of steroid diluent. Next, 100 μ L of tracer was added to standards and diluted samples, followed by immediately adding 100 μ L of primary antibody and incubating for 2 h at room temperature. Finally, 250 μ L 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., 2008b; Taves et al., 2010; 2011). Approximately 20 μ L of plasma was added to 10 mL of de-ionized water and then loaded onto C₁₈ 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 quantified 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 hr at 37°C in a water bath. Finally, 50 µL of precipitating reagent was added to each sample, and samples were then incubated at 37°C and shaken at 90 rpm for 60 min. 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%.

Statistics

To control for the effect of individual/pair variation in the expression of affiliative behavior, each dependent variable is reported as change at D30 and D60 relative to PRE (ex. D30-PRE), unless stated otherwise. Statistics were then conducted on these difference scores in R version 3.0.2 (R core team, 2015). The assumption of sphericity was met for all models as there were only two levels for the within-subjects factor. Type II sum of squares calculated from the R package “car” was used for all ANOVAs. Data was transformed as necessary. For statistically significant effects of testosterone on behavior, Cohen’s D calculations were conducted based on the F statistic.

The effect of testosterone manipulation on circulating testosterone and corticosterone levels was assessed separately in males and females using rmANOVAs (function lm). Timepoint (D30 vs D60) was the within-subjects factor, and Treatment (Control vs Testosterone) was a between-subjects factor.

The majority of our behavioral metrics were quantified at the level of the pair. The effect of behavioral condition (Baseline versus Post Stressor) on overall level of affiliative behavior (Proximity, Clumping and Coordinated Preening) was assessed using Wilcoxon-signed-rank-tests. The effect of male testosterone treatment was then assessed separately for each behavioral condition and each affiliative behavior (rmANOVA). Again, Timepoint was a within-subjects factor and Treatment was a between-subjects factor.

The only physical behavior that was subdivided by sex was percent time allopreening. However, because allopreening was so rare we did not conduct rmANOVAs. Instead, we used Fisher's exact test of independence to determine if the Testosterone and Control pairs differed in the number of observations where male-allopreening (Male AP) or female-allopreening (Female AP) occurred (across both D30 and D60). This allowed us to examine if there is evidence that male testosterone treatment shifted which individual was initiating (or investing more) into pair maintenance. Separate Fisher's tests were conducted for the Baseline Behavior and the Post Stressor separately.

The effect of behavioral condition (Baseline versus Post Stressor) on overall level of acoustic behavior (Song Rate and Call Rate) was assessed using Wilcoxon-signed-rank-test. The effect of male testosterone treatment on parameters of male song and calling behavior was assessed using rmANOVAs separately for each behavioral condition (Baseline versus Post Stressor). Timepoint was a within-subjects factor and Treatment was a between-subjects factor.

Separate rmANOVAs were conducted for the Call Rate, Song Rate, Motif Rate, the number of Motifs per Song Bout, and the number of Introductory Notes per Song Bout. Additionally, from the Post Stressor, a rmANOVA was conducted for latency to first call.

Results

Efficacy of testosterone implants

Testosterone treatment increased males' circulating testosterone levels at both the D30 and D60 timepoints (Figure 1A: $R^2=0.625$; Treatment, $F_{1,26} = 49.23$, $P < 0.001$; Timepoint, $F_{1,26} = 0.13$, $P = 0.730$; Treatment $\times$ Timepoint, $F_{1,26} = 1.82$, $P = 0.189$). Plasma T levels in the testosterone-treated males were within the physiological range for this species (McGraw et al., 2004; Schmidt et al., 2014), and is consistent to previous manipulations using similar silastic implants (Vockel et al., 1990; Korsia, 1991). There was no effect of male testosterone treatment on female testosterone levels (Table 1: $R^2=0.009$; Treatment, $F_{1,26} = 0.36$, $P = 0.555$; Timepoint, $F_{1,26} = 2.91$, $P = 0.100$; Treatment $\times$ Timepoint, $F_{1,26} = 0.01$, $P = 0.926$).

Importantly, male testosterone treatment had no effect on circulating corticosterone levels at D30 and D60 (Figure 1C: $R^2=-0.103$, Treatment, $F_{1,26} < 0.01$, $P = 0.941$; Timepoint, $F_{1,26} = 0.295$, $P = 0.592$; Treatment $\times$ Timepoint, $F_{1,26} < 0.01$, $P = 0.953$). Additionally, male testosterone treatment had no effect on the circulating corticosterone levels of the un-manipulated female partner (Table 1: $R^2=0.058$, Treatment $F_{1,26} = 2.07$, $P = 0.161$; Timepoint, $F_{1,26} = 2.72$, $P = 0.111$; Treatment $\times$ Timepoint, $F_{1,26} < 0.01$, $P = 0.989$).

Physical affiliative behaviors

Qualitatively, pair affiliation was higher during the Post Stressor condition than during the Baseline condition; however, this difference was only statistically significant for time spent in close proximity (Table 2: Proximity Time, $W=485.5$, $P<0.001$; Clumping, $W=990$, $P=0.800$; Coordinated Preening, $W=1058.5$, $P=0.665$).

Male testosterone treatment had no effect on physical affiliative behaviors during the Baseline condition (Table 3; Figure 2). However, during the Post Stressor condition, time spent in close proximity was significantly lower in testosterone-treated pairs than in control pairs (Table 3; Figure 2A; Effect size, Cohen's $D = 2.01$).

Table 4 shows time spent allopreening (Male AP and Female AP) for each pair. Because allopreening was rare, we could only test whether male testosterone treatment influenced frequency of male versus female allopreening within pairs. There was no effect of male testosterone treatment on allopreening (Baseline: $P=0.138$; Post Stressor: $P=0.133$; Fisher's Exact Test).

Male Song

Overall, there was no effect of testing condition on the amount of song (Table 5: Song Rate, $W=1079$, $P=0.583$). During the Baseline condition, there was no effect of testosterone administration on any measure of song (Table 6; Figure 3). Interestingly, male testosterone treatment increased male song during the Post Stressor condition. More specifically, Song Rate, Motif Rate and Motifs/Bout were higher for testosterone-treated males (Table 6; Figure 3; Effect sizes: Cohen's D : Song Rate=1.43, Motif Rate=1.75, Motifs/Bout = 1.40). There was a statistical trend for the same effect for number of Introductory Notes/Bout (Table 6).

Intra-pair calling

We quantified call rate for each pair during six minutes of the Baseline condition and the entire five-minute Post Stressor condition (Table 5). Overall, there was less calling during the Post Stressor condition than during the Baseline condition (Table 5: Call Rate, $W=566$, $P<0.001$).

Male testosterone treatment had no effect on call rate during the Baseline condition ($R^2=-0.083$; Treatment, $F_{1,26}=0.27$, $P=0.607$; Timepoint, $F_{1,26}=0.41$, $P=0.528$, Treatment $\times$ Timepoint, $F_{1,26}=0.07$, $P=0.788$). Furthermore, there was no effect of male testosterone treatment on call rate during the Post Stressor condition ($R^2=-0.083$; Treatment, $F_{1,26}=0.03$, $P=0.859$; Timepoint, $F_{1,26}=0.34$, $P=0.563$, Treatment $\times$ Timepoint, $F_{1,26}=0.41$, $P=0.526$).

Finally, there was no effect of male testosterone treatment on latency to resume calling following the chase (Table 5: $R^2=-0.090$; Treatment, $F_{1,26}=0.20$, $P=0.660$; Timepoint, $F_{1,26}=0.02$, $P=0.892$, Treatment $\times$ Timepoint, $F_{1,26}=0.38$, $P=0.542$).

Discussion

Elucidating the neuroendocrine regulation of long-term pair-maintenance behavior will deepen our understanding of social bonding. Furthermore, research examining the neuroendocrine regulation of pair-bond maintenance needs to be situated within ethologically relevant social and environmental contexts. Here, we show that male testosterone treatment has both context- and behavior-specific effects on pair maintenance of established zebra finch pairs. While male testosterone treatment had no effect on physical or vocal behavior during the Baseline condition, following a brief chase (Post Stressor), testosterone-treated males spent less time in close proximity with their partner, but more time singing. Importantly, based on our operational definitions, singing and proximity time can co-occur, so the increase in singing

behavior does not directly explain the decrease in physical pair-maintenance behavior. Rather, it appears that testosterone administration to males shifts a male's response to this brief stressor.

Context-specific effects of testosterone on pair maintenance

While social and environmental contexts are well known to influence the physiological regulation of behaviors across taxa [mice (Bartolomucci et al., 2001; Haller et al., 2004), woodrat (Monaghan & Glickman, 1992), humans (Bartz et al., 2011), sunfish (Colemann and Wilson, 1998), zebra finch (Hessler and Doupe, 1999; Goodson and Thompson, 2010; Kelly and Goodson, 2014)], to our knowledge, this is the first report of a context-dependent regulation of pair maintenance.

Importantly, during the Post Stressor condition, individuals would 1) have a different internal endocrine state (e.g., elevated corticosterone and/or catecholamines), and 2) be primed to have context-specific responses (e.g., escape behavior). Our testosterone administration could have modulated behavior by impacting either of these factors. In mammals, testosterone administration inhibits the male's stress response (Hanada et al., 1994; McCormick et al., 2002). However, in wild birds, testosterone can increase circulating corticosterone levels post stressor (Ketterson et al., 2001). Here, we did not measure corticosterone levels after the brief chase, but we saw no effect of testosterone implants on baseline corticosterone levels in males. While it appears unlikely that our testosterone administration affected HPA axis activity, further experimentation would be needed to rule this out. The second and more likely scenario is that testosterone shifted an individual's the behavioral response to a single stressor (such as increased vigilance or antipredator behavior) (Fusani et al., 1997).

Behavior-specific effects of testosterone on pair maintenance: opposing behavioral effects?

While it seems likely that testosterone administration caused a behavioral shift during the Post Stressor condition, it is unclear whether this change could have negative consequences for the zebra finch pair. Our operational definition of proximity time simply reflects spatial coordination within a pair, regardless of whether the members of the pair are active or sedentary. Importantly, if testosterone-treated males increased their activity, well-coordinated pairs could have maintained their spatial proximity through a variety of mechanisms. For example, if males increased their number of flights, but consistently landed near their partner, we would have quantified this as time in close proximity. Alternatively, the female partner could have increased her activity to match the change in her partner's. The decrease in proximity time that we report may be caused by a change in either active or sedentary behavior of the males and/or the male's ability to coordinate this change with his female partner. Regardless of the mechanisms, because coordination is important for wild zebra finch pairs (Mariette and Griffith, 2012; 2015), we hypothesize that a decrease in proximity time is detrimental for pair maintenance.

In contrast to the decrease in proximity time, our testosterone administration increased male singing. Male song can be used for: 1) territorial defense (Nowicki and Searcy, 2004), 2) mate choice and courtship (Nowicki and Searcy, 2004), 3) pair-bond formation, and 4) pair-bond maintenance (Dunn and Zann, 1996). For established zebra finch pairs, song likely functions either to elicit extra-pair courtship or to support bond maintenance (Zann, 1996). Zebra finches do not hold or defend territories, and there is no evidence that song is used in any aggressive context (Zann, 1996). In our experimental set up, pairs were visually isolated from other colony members. We did this to decrease the likelihood of males singing in response to social stimuli other than their partner. Thus, here song was most likely associated with pair maintenance.

Importantly, both directed and undirected song could function towards pair-bond maintenance in zebra finches (Zann, 1996; Dunn and Zann, 1996).

Assuming that both proximity time and male song promote bond maintenance, it is difficult to interpret overall results of the opposing effect of testosterone on male song versus proximity time. It is possible that these opposing effects could cancel each other out, making the overall effect of testosterone administration neutral. Functionally, testosterone-treated male zebra finches may simply be shifting the modality of communication with their partner (from physical to acoustic). Alternatively, the overall effect of testosterone on pair maintenance could depend on whether male song or proximity time is more important for pair maintenance. In breeding wild zebra finches, pair synchrony correlates with breeding success (Mariette and Griffith, 2012; 2015). Additionally, the structure of male song can impact breeding success in wild zebra finches (Woodgate et al., 2012); however, Tomaszycski and Adkins-Regan (2006) found no evidence that song is necessary for pair-bond maintenance in zebra finches (Tomaszycski and Adkins-Regan, 2006). Therefore, it is difficult to say whether male song or proximity is more important for pair maintenance.

Taken together, testosterone administration appears to have inhibited pair-maintenance behavior via shifting male's behavior Post Stressor away from his partner. Such a behavioral shift could be due to testosterone promoting a different behavioral response or decreasing the saliency of his partner. Future studies should directly test the effect of testosterone administration on the saliency of sensory inputs (e.g., impact of testosterone of auditory processing) and what alternative behaviors testosterone may be promoting, such as aggression or extra-pair courtship (Garamszegi et al., 2005; Wingfield, 1984; Raouf et al., 1997). Finally, while it seems that

testosterone inhibited pair maintenance, future research needs to directly test to what extent this effect is detrimental to zebra finch pairs.

Testosterone and the neuroendocrine mechanisms affecting pair maintenance

Based on our previous result that fadrozole increased proximity time (Prior et al., 2014), we hypothesized that testosterone administration has promoting effects on pair maintenance. Our results here do not support this hypothesis. It is important to note key difference between our two studies: firstly, here pairs were breeding-ready (water *ad libitum*) whereas in Prior et al., 2014 pairs were water-restricted, and secondly, here behavior was quantified after a chase, whereas previously it was quantified during a partner separation and reunion behavioral paradigm. Despite these differences, both fadrozole and testosterone manipulations affected behavior allowing us to compare these results. Our previous effect of fadrozole could have been due to the decrease in brain estradiol levels, or the concomitant increase in testosterone levels (Prior et al., 2014). Here, testosterone can act on androgen receptors, or once aromatized in the brain, on estrogen receptors. Taken together, we can hypothesize that estradiol inhibits pair maintenance behavior. An inhibitory effect of estradiol on affiliation is consistent with patterns seen in alloparenting in the highly social prairie vole (Lei et al., 2010).

In contrast to the inhibitory effect of testosterone on proximity time, testosterone administration increased Song Rate, Motif Rate, and Motifs/Bout. A promoting role of testosterone on song is well described in zebra finches (Arnold, 1975, Adkins Regan, 1999), However, here testosterone only promoted song Post Stressor and not under Baseline conditions. Previous research in zebra finches has also suggested that the effect of testosterone on song in actively breeding birds depends on context. Cynx and colleagues (2005) found that testosterone

promoted song only during courtship, whereas Hill and colleagues (2005) reported that testosterone-treated males only increased singing behavior while actively incubating eggs. This study is the first investigation of the effect of testosterone on song in well-established zebra finch pairs.

We might have expected that any effect of testosterone administration would be seen for both male song and intra-pair calling. Previous research in downy woodpeckers found evidence that testosterone influences calling within established pairs (Kellam and Lucas, 2014). Additionally, Ter Maat and colleagues (2014) found evidence that the zebra finch “stack” call is produced through similar mechanisms as male song. However, there are several reasons this may not be the case. Firstly, zebra finches produce several subtypes of short calls (Zann, 1996). (Zann 1996). Unfortunately, we were not able to distinguish between subtypes of short calls on our recordings; however, if we had been able to do this, we may have seen an effect. Secondly, we were not able to distinguish between male and female short calls, and thus our measure of intra-pair calling was at the level of the pair, not the individual. It is possible that male testosterone treatment had subtle effects on male calling but not female calling. Alternatively, the female could have compensated for a change in male calling. Finally, it is possible that the motivation to sing and the motivation to produce short calls are different. Taken together, it is difficult to interpret our lack of an effect on intra-pair calling. Future studies should continue to examine the neuroendocrine regulation of intra-pair calling as it likely represents a critical expression of pair maintenance.

Conclusions

Taken together, our results clearly indicate behavior- and context-dependent effects of testosterone treatment on pair-maintenance behavior in zebra finches. More broadly, these results highly the importance of context-dependent mechanisms in the neuroendocrine regulation of pair maintenance. Additionally, it is the first report of a potential inhibitory effect of testosterone on zebra finch pairing behaviors. We propose that testosterone administration caused a shift in the male's physiological or behavioral response to a stressor, away from physical affiliative behavior and toward song. Further research is needed to disentangle the ultimate function of this effect. A comparison of our results here to our previous study using fadrozole-administration raises the hypothesis that estradiol may have an inhibitory effect on pair-maintenance (Prior et al., 2014).

Acknowledgements

We thank Alice Chan and Anne Cheng for help with animal husbandry and data collection; and Matthew D. Taves, Benjamin A. Sandkam, and 2 anonymous reviewers for feedback on the manuscript.

Funding

Funding sources included: Canadian Institutes of Health Research (CIHR) Operating Grant 133606 to KKS, a graduate research fellowship from the National Science Foundation (NSF GRFP) to NHP, a French Agence Nationale de la Recherche (project 'Acoustic Partnership') to CV, and an IUF grant (Institut Universitaire de France) to CV.

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669

670 Table 1: Circulating testosterone and corticosterone concentrations (ng/mL) in females from Control and Testosterone pairs (mean ±
671 SEM).

	Pre		D30		D60	
	Control	Testosterone	Control	Testosterone	Control	Testosterone
Testosterone	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	5.85 ± 0.75	6.58 ± 1.24	3.51 ± 0.89	6.86 ± 1.70	6.51 ± 1.72	9.91 ± 3.08

672

673 Note: Only males received the testosterone treatment.

Table 2: Percent of time spent engaging in physical affiliative behaviors (mean $\pm$ SEM).

		Pre		D30		D60	
		Control	Testosterone	Control	Testosterone	Control	Testosterone
Baseline	Proximity	54.51 $\pm$ 6.43	57.41 $\pm$ 7.59	53.09 $\pm$ 6.47	51.23 $\pm$ 8.87	57.96 $\pm$ 4.06	52.41 $\pm$ 6.46
	Clumping	3.65 $\pm$ 2.47	1.97 $\pm$ 1.49	0.52 $\pm$ 0.52	6.49 $\pm$ 6.49	1.82 $\pm$ 1.82	0.52 $\pm$ 0.46
	Coordinated Preening	5.42 $\pm$ 2.10	3.27 $\pm$ 3.00	7.23 $\pm$ 4.38	0.77 $\pm$ 0.61	2.58 $\pm$ 2.17	1.77 $\pm$ 1.25
Post Stressor	Proximity	60.36 $\pm$ 5.75	86.39 $\pm$ 5.27	77.09 $\pm$ 11.01	71.51 $\pm$ 11.26	89.10 $\pm$ 4.93	65.04 $\pm$ 9.12
	Clumping	5.29 $\pm$ 5.29	14.62 $\pm$ 14.23	15.92 $\pm$ 11.04	6.24 $\pm$ 6.24	3.83 $\pm$ 2.70	3.24 $\pm$ 3.24
	Coordinated Preening	0.08 $\pm$ 0.08	0.33 $\pm$ 0.33	11.79 $\pm$ 5.83	4.00 $\pm$ 2.08	6.54 $\pm$ 5.45	5.57 $\pm$ 5.04

Note: Behavior was quantified from the entire 30 min of the Baseline period and the entire 5 min of the Post Stressor period at each Timepoint (Pre, D30, and D60).

684

685 Table 3: Effect of testosterone treatment to males on percent of time spent engaging in physical affiliative behaviors.

CONDITION	FACTOR	Proximity			Clumping			Coordinated Preening		
		F _(1,26)	P	R ²	F _(1,26)	P	R ²	F _(1,26)	P	R ²
Baseline	Treatment	0.39	0.537	-0.094	1.05	0.316	-0.045	0.22	0.644	-0.057
	Timepoint	0.09	0.767		0.08	0.775		0.40	0.530	
	Treatment × Timepoint	0.03	0.863		0.63	0.436		0.79	0.381	
Post Stressor	Treatment	15.68	<0.001	0.319	2.01	0.168	-0.008	0.85	0.364	-0.033
	Timepoint	0.11	0.745		0.57	0.455		0.43	0.520	
	Treatment × Timepoint	0.80	0.378		0.17	0.684		0.79	0.381	

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687 Note: Statistics were conducted on the absolute difference at D30 and D60 timepoints. Bolded values indicate significant main effects
688 of Treatment.

689 Table 4: Alloprenening (AP) behavior by sex for Control and Testosterone pairs.

690

Treatment	Pair	Baseline				Post Stressor			
		Δ D30 (sec)		Δ D60 (sec)		Δ D30 (sec)		Δ D60 (sec)	
		Male AP	Female AP	Male AP	Female AP	Male AP	Female AP	Male AP	Female AP
CON	1	0	0	0	0	0	0	0	0
	2	92	12	-15	-12	0	0	0	0
	3	-16	-8	0	0	-9	0	0	0
	4	0	0	0	0	0	0	2	0
	5	-5	16	309	78	0	0	0	0
	6	-30	14	0	58	0	16	0	-16
	7	0	0	0	0	0	0	28	0
	8	0	-85	0	0	0	-12	0	0
	Mean	5	-6	37	16	-1	1	4	-2
TEST	9	0	-6	0	84	0	0	0	0
	10	0	0	0	0	19	0	2	0
	11	-86	0	147	0	23	0	-94	0
	12	23	0	-23	0	0	0	0	0
	13	6	0	-1	0	8	0	-5	0
	14	-40	0	0	0	0	0	0	0
	15	0	0	0	0	0	0	0	0
	Mean	-14	-1	18	12	7	0	-14	0

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694 Table 5: Measures of acoustic affiliative behaviors (mean $\pm$ SEM).

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		Pre		D30		D60	
		Control	Testosterone	Control	Testosterone	Control	Testosterone
Baseline	Call Rate (per min)	47.2 $\pm$ 4.9	41.4 $\pm$ 9.6	53.6 $\pm$ 4.6	45.1 $\pm$ 5.9	51.6 $\pm$ 12.0	39.0 $\pm$ 5.2
	Song Rate (per min)	0.4 $\pm$ 0.2	0.5 $\pm$ 0.2	0.2 $\pm$ 0.1	0.7 $\pm$ 0.5	0.6 $\pm$ 0.2	0.8 $\pm$ 0.4
	Motifs / Bout	2.5 $\pm$ 1.0	1.1 $\pm$ 0.6	1.1 $\pm$ 0.5	1.9 $\pm$ 0.7	3.0 $\pm$ 1.1	3.2 $\pm$ 1.0
	Introductory Notes / Bout	2.2 $\pm$ 1.4	0.5 $\pm$ 0.2	0.7 $\pm$ 0.4	1.3 $\pm$ 0.6	2.6 $\pm$ 1.0	1.9 $\pm$ 0.5
Post Stressor	Call Rate (per min)	29.0 $\pm$ 5.3	32.8 $\pm$ 5.8	30.2 $\pm$ 5.5	34.4 $\pm$ 7.8	36.0 $\pm$ 9.5	37.6 $\pm$ 6.5
	Song Rate (per min)	0.4 $\pm$ 0.1	0.1 $\pm$ 0.06	0.6 $\pm$ 0.2	1.1 $\pm$ 0.5	0.7 $\pm$ 0.3	1.3 $\pm$ 0.7
	Motifs / Bout	2.3 $\pm$ 0.9	1.6 $\pm$ 1.1	1.7 $\pm$ 0.5	4.6 $\pm$ 1.3	1.5 $\pm$ 0.6	2.9 $\pm$ 1.0
	Introductory Notes / Bout	2.0 $\pm$ 0.7	1.3 $\pm$ 0.8	2.0 $\pm$ 0.8	3.3 $\pm$ 1.0	1.9 $\pm$ 0.7	2.9 $\pm$ 1.0
	Latency to First Call (sec)	7.6 $\pm$ 2.4	6.3 $\pm$ 4.7	4.0 $\pm$ 1.9	6.3 $\pm$ 3.5	5.5 $\pm$ 3.2	3.6 $\pm$ 1.6

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698 Note: Behavior was quantified from a subset of 6 min of the Baseline period and the entire 5 min of the Post Stressor period at each

699 Timepoint (Pre, D30, and D60).

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701

702 Table 6: Effect of testosterone treatment to males on parameters of male song.

		Song Rate			Motif Rate			Motif/ Bout			Introductory Notes/ Bout		
		F _(1,26)	P	R ²	F _(1,26)	P	R ²	F _(1,26)	P	R ²	F _(1,26)	P	R ²
Baseline	Treatment	0.43	0.520	-0.057	1.75	0.197	-0.023	3.28	0.082	0.094	<0.01	0.956	-0.115
	Timepoint	0.77	0.390		0.48	0.493		2.66	0.115		0.30	0.592	
	Treatment × Timepoint	0.25	0.624		0.12	0.731		0.07	0.789		0.01	0.931	
Post Stressor	Treatment	6.66	0.016	0.115	9.63	0.004	0.187	6.38	0.018	0.133	3.41	0.076	0.020
	Timepoint	0.08	0.773		0.02	0.877		0.69	0.413		0.19	0.671	
	Treatment × Timepoint	<0.01	0.962		0.03	0.861		0.38	0.542		<0.01	0.977	

703

704 Note: Statistics were conducted on the absolute difference at D30 and D60 timepoints. Bolded values indicate significant main effects
705 of Treatment.

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Figure Legends

Figure 1: Circulating testosterone (A) and corticosterone (B) concentrations (ng/mL) in males from Control and Testosterone groups (mean $\pm$ SEM). Testosterone treatment significantly increased testosterone levels in males at both D30 and D60. Female steroid levels are shown in Table 1.

Figure 2: Data is presented as absolute difference at D30 and D60 relative to PRE (e.g. D30-PRE) for (A) Proximity, (B) Clumping, and (C) Coordinated Preening, during the Baseline and Post Stressor periods. During the Post Stressor period, testosterone treatment significantly decreased time spent in close proximity. These behaviors are operationally defined at the level of the pair. Each data point represents 1 pair. The central line-and-whiskers indicate the mean $\pm$ SEM. Note: the range of the Y-axis is different across panels.

Figure 3: Data is presented as absolute difference at D30 and D60 relative to PRE (e.g. D30-PRE) for (A) Song Rate, (B) Motif Rate, and (C) Motifs/bout, during the Baseline and Post Stressor periods. During the Post Stressor period, testosterone-treated males had significantly higher song rate, motif rate and number of motifs per song bout. Each data point represents 1 pair. The central line-and-whiskers indicate the mean $\pm$ SEM. Note: the range of the Y-axis is different across panels.

Figure1

A

B

Figure2

Baseline Post Stressor

○ Control ● Testosterone

Figure3

Baseline Post Stressor