Neuroendocrine regulation of long-term pair maintenance in the monogamous zebra finch

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Abstract

Understanding affiliative behavior is critical to understanding social organisms. While affiliative behaviors are present across a wide range of taxa and contexts, much of what is known about the neuroendocrine regulation of affiliation comes from studies of pair-bond formation in prairie voles. This leaves at least three gaps in our current knowledge. First, little is known about long-term pair-bond maintenance. Second, few studies have examined non-mammalian systems, even though monogamy is much more common in birds than in mammals. Third, the influence of breeding condition on affiliation is largely unknown. The zebra finch (*Taeniopygia guttata*) is an excellent model system for examining the neuroendocrine regulation of affiliative behaviors, including the formation and maintenance of a long-term pair bond. Zebra finches form genetically monogamous pair bonds, which they actively maintain throughout the year. The genomic and neuroanatomical resources, combined with the wealth of knowledge on the ecology and ethology of wild zebra finches, give this model system unique advantages to study the neuroendocrine regulation of pair bonding. Here, we review the endocrinology of opportunistic breeding in zebra finches, the sex steroid profiles of breeding and non-breeding zebra finches (domesticated and wild), and the roles of sex steroids and other signaling molecules in pair-maintenance behaviors in the zebra finch and other monogamous species. Studies of zebra finches and other songbirds will be useful for broadly understanding the neuroendocrine regulation of affiliative behaviors, including pair bonding and monogamy.

Keywords: estradiol, pair bond, social behavior network, testosterone, water restriction, corticosterone

Introduction

Studies of the neuroendocrine regulation of pair bonding in monogamous species deepen our understanding of the neuroscience of sociality and social bonding, an important issue for both basic science and human health (Insel, 1997; McGraw and Young, 2010). Research on the neuroendocrine bases of prosocial and affiliative behavior, including pair bonding, has largely investigated pair bonding in a monogamous mammal, specifically within the first two weeks of pair-bond formation. Thus, little is known about the neuroendocrine regulation of pair-bond maintenance over the long term. Furthermore, less than 5% of mammals breed monogamously (Kleiman, 1977), while over 90% of birds breed monogamously (Lack, 1968; Black, 1996). Many avian species form life-long monogamous pair bonds and actively engage in pair-maintenance behaviors throughout the year. Some social behaviors, such as aggressive and reproductive behaviors, vary greatly across the seasons, but the extent to which pair-maintenance behaviors vary across seasons and breeding conditions is not clear.

Zebra finches (*Taeniopygia guttata*) are native to Australia, including the central deserts, where the environmental conditions are unpredictable and often severe (Morton, 2009; Zann, 1996). Zebra finches have many adaptations to these conditions, including the ability to breed opportunistically. The ability to form life-long monogamous pair bonds is also a likely adaptation (Adkins-Regan and Tomaszewski, 2007; Mariette and Griffith, 2012; Sánchez-Macouzet et al., 2014). Zebra finches are highly social and live in large groups; however, the extent to which a zebra finch depends on its partner is often underappreciated. The individuals within a large flock change greatly over time, and thus the most stable social relationship for a zebra finch is with its partner (Zann, 1996; Zann and Runciman, 1994). Zebra finches rarely engage in extra-pair courtship or copulations, and most pair bonds are socially as well as

65 genetically monogamous (Birkhead et al., 1988; Griffith et al., 2010). Maintaining life-long pair
66 bonds facilitates a rapid transition to breeding when environmental conditions are favorable for
67 breeding in unpredictable desert habitats (Adkins-Regan and Tomaszycki, 2007). For such
68 reasons, zebra finches are highly motivated to form and maintain a pair bond and are highly
69 dependent on it (Remage-Healey et al., 2003; Perez et al., 2012). These characteristics make the
70 zebra finch a [valuable](#) model system for examining the environmental and neuroendocrine
71 regulation of long-term pair maintenance.

72 Zebra finches can be studied in the wild and are also relatively easily maintained and
73 bred in captivity (Griffith and Buchanan, 2010). They have been used extensively to study the
74 neuroendocrine mechanisms of social behaviors (Griffith and Buchanan, 2010), such as male
75 song and courtship (Adkins-Regan, 1999; Reibel, 2009), female mate choice (Burley et al., 1996;
76 Reibel, 2009), and sociality (Goodson et al., 2005; Goodson et al., 2009a; Goodson and
77 Kingsbury, 2011). A few studies have examined pair bonding in this species, but primarily pair-
78 bond formation rather than pair-bond maintenance (Goodson et al., 2004; Svec and Wade, 2009).
79 Because pair bonds are life-long, individual zebra finches invest far more time in maintaining a
80 pair bond than in forming a new pair bond. Only a very small number of studies have focused on
81 the neuroendocrine regulation of long-term pair-maintenance behavior (Svec et al., 2009; Alger
82 et al., 2011; Smiley et al., 2012; Prior et al., 2014), arguably the most important social behavior
83 for a zebra finch.

84 Research exploring the neuroendocrine regulation of pair-maintenance behaviors in zebra
85 finches is in its early stages, and long-term pair-maintenance behaviors need to be situated within
86 social and reproductive contexts. Here, we will review the research on breeding-specific sex
87 steroid profiles in zebra finches and how sex steroids might regulate long-term pair maintenance.

We also highlight the value of using experimental water restriction as an efficient and effective way to manipulate the breeding condition of zebra finches in the laboratory.

Seasonal changes in sex steroid regulation of social behavior

For many vertebrate species, breeding is limited to specific and predictable times during the year, when environmental conditions are favorable for raising offspring (e.g., warmer temperature, greater food availability) (Wingfield and Kenagy, 1991). For species with short gestation or incubation durations, the breeding season occurs in spring, when photoperiod is increasing. Photoperiod is often the main environmental cue that initiates the physiological cascade in preparation for reproduction in seasonally-breeding vertebrates (Figure 1; Wingfield and Kenagy, 1991). Photostimulation causes the upregulation of hypothalamic-pituitary-gonadal (HPG) axis [activity](#), starting with the secretion of gonadotropin-releasing hormone (GnRH) from the hypothalamus to the pituitary, where it causes the secretion of luteinizing hormone (LH) and follicle stimulating hormone (FSH) into the systemic circulation (Figure 1). These hormones act on the gonads, stimulating the production of sex steroid hormones (e.g., testosterone (T) and estradiol (E2)) and the development of gametes, respectively.

Many social behaviors that are associated with reproduction (e.g., courtship, copulation, and mate guarding/defense) co-occur with the increase in circulating sex steroids during the breeding season. In these cases, sex steroids typically regulate relevant social behaviors through [activational](#) effects, priming appropriate neural circuitry (Adkins-Regan, 2005). Thus, sex steroids are often necessary and sufficient to initiate breeding-specific social behavior (Adkins-Regan, 2005). In this way, the classic endocrinology perspective provides a clear basis for the

relationship between social behavior specific to breeding and sex steroids; however, the nature of that relationship is less clear for social behaviors that occur throughout the year.

Seasonal expression of affiliative behaviors in monogamously-breeding species

In territorial species that are monogamous breeders, affiliative behaviors are often selectively expressed between pair-bonded mates and parents-offspring. Furthermore, in serially monogamous species, such affiliative behaviors are typically limited to the breeding season, when the HPG axis is upregulated. In avian species, for example, sex steroids are critical for singing and courtship behaviors (Adkins-Regan, 2005; Ball et al., 2002).

In species that form life-long monogamous pair-bonds, affiliative behaviors associated with pair maintenance occur both within and outside of the breeding periods. Despite the fact that life-long monogamy is widespread throughout the many avian families, extremely little is known about the neuroendocrine regulation of long-term pair maintenance (Black, 1996). Furthermore, only a few studies have examined the neuroendocrine mechanisms of pair maintenance in non-breeding condition birds (Kellam and Lucas, 2014; Prior et al., 2014).

Given the strong evidence that sex steroids regulate affiliative behaviors associated with courtship during the breeding season, we hypothesized that sex steroids also regulate affiliation associated with pair-bond maintenance during the non-breeding season. However, there are several alternative hypotheses. First, sex steroids may act via season-specific mechanisms, differentially regulating pair-maintenance behavior in the breeding versus non-breeding season. Second, sex steroids may only regulate pair-maintenance behaviors when they are expressed in the breeding season (Smiley et al., 2012), whereas other mechanisms support pair maintenance in the non-breeding season. Third, 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; Resendez and Aragona, 2012).

Breeding-specific mechanisms of steroid action

Relative to the breeding season, in the non-breeding season there can be shifts in: (1) the sites of sex steroid production (e.g., gonad vs. brain) (Schmidt et al., 2008; Heimovics et al., 2013), and (2) the mechanisms of sex steroid action (e.g., genomic vs. non-genomic) (Breuner & Orchinik, 2001; Trainor et al., 2007; 2008; Heimovics et al., 2012). First, while gonadal production of sex steroids is typically higher during the breeding season (when the HPG axis is upregulated), evidence from some species suggests that brain production of sex steroids is higher during the non-breeding season (when the HPG axis is downregulated) (Figure 2; Soma et al., 2000; Schmidt et al., 2008; Pradhan et al., 2010; Heimovics et al., 2012). These brain-synthesized ‘neurosteroids’ can be produced *de novo* from cholesterol or from circulating prohormones such as dehydroepiandrosterone (DHEA) (Figure 2; Balthazart and Ball, 2006; Schmidt et al., 2008).

Second, sex steroids have been traditionally thought to regulate behavior through genomic mechanisms, by binding to intracellular receptors that regulate gene expression. These genomic mechanisms typically have delayed and long-lasting effects on behavior (Figure 3; Schmidt et al., 2008; Soma et al., 2008). However, more recently, rapid and transient effects of sex steroids on social behaviors have been identified (Balthazart and Ball, 2006; Trainor et al., 2007; 2008; Soma et al., 2008; Heimovics et al., *in press*). These rapid effects are mediated by membrane-associated receptors that influence signal transduction pathways. These non-genomic mechanisms can affect behaviors within 5-30 min (Balthazart and Ball, 2006; Cornil et al., 2006;

Trainor et al., 2007; 2008). Rapid, non-genomic sex steroid signaling in the brain is likely to be coupled to changes in neurosteroid synthesis, rather than to changes in gonadal steroid synthesis. Whereas changes in neurosteroid synthesis can occur quickly and locally (including in pre-synaptic boutons), gonadally-synthesized sex steroids have to travel via the blood to the brain. It has been hypothesized that sex steroids are more likely to have rapid and transient effects on social behavior during non-breeding periods, when there is little gonadal steroid synthesis and increased neurosteroid synthesis (Figure 3; Schmidt et al., 2008; Heimovics et al., 2012; 2013; and 2015).

Evidence for season-specific mechanisms of steroid action comes from recent work examining aggression in male song sparrows (*Melospiza melodia*) (Heimovics et al., 2012; 2013; and 2015). Male song sparrows actively defend territories during both the breeding and non-breeding seasons. During the breeding season, systemic T levels are elevated. Furthermore, immediately following a stimulated territorial intrusion (STI) in the breeding season, systemic T levels increase even further (Wingfield, 1994; Wingfield and Hahn, 1994). In contrast, during the non-breeding season, systemic T levels are basal, but systemic and brain DHEA levels are elevated (Newman and Soma, 2009; Heimovics et al., 2013). E2 administration has rapid effects on signal transduction pathways in multiple brain regions, and these effects can vary by season (Heimovics et al., 2012). Furthermore, during the non-breeding season but not the breeding season, E2 rapidly increases aggression in males (Heimovics et al., 2015). Similar season-specific modes of sex steroid action in the regulation of aggression have been identified in California mice (*Peromyscus californicus*) (Trainor et al., 2007; 2008; Laredo et al., 2013; 2014). It is unclear to what extent this pattern of season-specific modes of sex steroid action is broadly

generalizable across species. Furthermore, it is unclear if the modes of sex steroid action vary with breeding condition in opportunistic breeders, such as zebra finches.

Reproductive physiology of zebra finches

Zebra finches are native to Australia and well-adapted to the extreme unpredictable environments in the central arid regions of the continent. One of these adaptations is the ability to breed opportunistically, which allows pairs to increase reproductive success by integrating several environmental cues when making decisions about breeding. These cues include photoperiod, water availability, presence of green grasses and seeds, and social information (Figure 1; Zann et al., 1995; Hahn et al., 2008; Perfito, 2010; Waas et al., 2005).

One of the most salient cues that brings zebra finches out of breeding condition is insufficient water availability (Zann, 1996). In the laboratory, water restriction has been used to bring 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 there are also zebra finch populations along the coast. The coastal habitat is more predictable in terms of seasonal variability, and here zebra finches do breed seasonally (Zann et al., 1995; Perfito et al., 2007). This variation in breeding across environments highlights the potential plasticity of zebra finch reproductive physiology.

Reproductive physiology of breeding and non-breeding zebra finches in the wild

Perfito and colleagues (2007) studied two populations of wild zebra finches and examined HPG axis function. First, in seasonally-breeding populations of zebra finches along the coast (“predictable habitat”), reproductive physiology was examined during the breeding season

(October/November) and non-breeding season (June/July). Both males and females had decreased gonad size during the non-breeding season (51% of females and 30% of males had completely regressed gonads). Similar patterns were seen with circulating LH levels. Second, zebra finches in the predictable habitat were compared to zebra finches in Alice Springs, in arid central Australia (“unpredictable habitat”). Note that the zebra finches in the unpredictable habitat were caught during a drought period and were not breeding. These non-breeding zebra finches in the unpredictable habitat were at an intermediate reproductive state between breeding and non-breeding zebra finches in the predictable habitat. This intermediate state was seen in both gonad size and circulating LH level (e.g., in Alice Springs, 42% of females and 8% of males had completely regressed gonads). Third, systemic T, dihydrotestosterone (DHT), and E2 levels were measured in zebra finches from both populations (non-breeding birds from the central habitat, and breeding birds from the coastal habitat) (Table 1). Systemic sex steroid levels were higher in breeding males and females from the coastal habitat than in non-breeding males and females from the central habitat (Perfito et al., 2007).

In a second study, Perfito and colleagues (2011) compared gonadotropin releasing hormone (GnRH) and gonadotropin inhibitory hormone (GnIH) in breeding zebra finches from a coastal population (predictable) and non-breeding zebra finches from Alice Springs in central Australia (unpredictable). These are the same predictable and unpredictable habitats that were previously studied (Perfito et al., 2007). Following a GnRH challenge, non-breeding males and females increased circulating LH levels. This effect was not seen in breeding males and females, suggesting a ceiling effect. Additionally, immunoreactive GnIH, GnRH-I, and GnRH-II neurons were quantified in males. Interestingly, numbers of GnRH+ and GnIH+ cells in the preoptic area

and median eminence were unaffected by breeding condition. Rather, breeding males had increased GnRH-II immunoreactive neurons in the midbrain (Perfito et al., 2011).

Experimental water restriction of zebra finches in the laboratory

Water restriction has been used to effectively manipulate the reproductive physiology of captive zebra finches since the mid-1900's (Table 2). Zebra finches are xerophilic and can be kept in the laboratory for months under water restriction (0.5-1 mL / individual / week) or even complete water deprivation (Table 2; Perfito et al., 2006; Prior et al., 2013). Importantly, other environmental cues such as humidity, photoperiod, and availability of green grasses have also been used to experimentally manipulate breeding in captive zebra finches (Vleck and Priedkalns, 1985; Hahn et al., 2008; Zann 1996; Perfito, 2010). Here we focus on water restriction for two reasons: (1) water restriction is a potent manipulation and sufficient to reduce breeding condition in zebra finches, and (2) water restriction is straightforward to implement in the laboratory setting. For such reasons, water restriction can be effectively used to manipulate breeding condition and associated changes in social behavior, including pair-maintenance behaviors.

While there is profound down-regulation of all levels of the HPG axis during the non-breeding season in seasonally-breeding birds, the extent of down-regulation during non-breeding periods is less straightforward in zebra finches. First, the numbers and sizes of GnRH-I and GnRH-II neurons in the preoptic area and median eminence are unaffected by water restriction in male zebra finches (Perfito et al., 2006). Furthermore, similar to the pattern seen in wild zebra finches (Perfito et al., 2011), GnRH-II neurons in the midbrain are reduced during non-breeding periods in male domesticated zebra finches (water restriction and isolation from females) (Perfito et al., 2006; Perfito, 2010). In contrast, water restriction has a clear inhibitory effect on

circulating LH levels in males (Perfito et al., 2006). Two weeks after water was returned (provided *ad libitum*), circulating LH levels were increased (Perfito et al., 2006).

The effects of water restriction on the gonads are more variable across studies (Perfito, 2010). For testes size, Perfito and colleagues (2006) reported a 46% reduction in testes volume and a 20% reduction in testis length. Similarly, Vleck and Priedkalns (1985) saw a decrease in testis length. However, other studies have found no effects of water restriction (including complete water deprivation) on testes size (Oksche et al., 1963; Sossinka, 1974; Prior et al., 2013). It is likely that the extent of reduction in testes size depends on other environmental factors, such as humidity and the presence of a female partner (Perfito, 2010). Importantly, while Perfito and colleagues (2006) reported a decrease in testes size, there was no concomitant change in measures of spermatogenesis. Relative to the testes, the effects of water restriction are greater on the ovary, although fewer studies have examined female zebra finches in this context. There is profound suppression of ovary size and largest follicle size, as well as oviduct size (Prior et al., 2013). In sum, experimental water restriction has profound effects on the reproductive physiology of captive zebra finches. These effects appear similar to the effects of natural droughts on wild zebra finches.

Sex steroid profiles in breeding and non-breeding zebra finches

Many studies have examined systemic sex steroid levels in zebra finches, primarily domesticated zebra finches in the laboratory. Furthermore, a few studies have also examined local steroid levels in the brain, which is important because systemic and local steroid levels can differ dramatically (Remage-Healey et al., 2008; 2009; Charlier et al., 2010; Schmidt et al., 2008). We used experimental water restriction to examine the effect of breeding condition on T

and E2 levels in the general circulation and in several brain regions in both male and female zebra finches (Prior et al., 2013). In males, water restriction had no effect on testes size but decreased systemic T levels. In females, water restriction did not decrease systemic E2 or T levels, but these were already quite low in control females with *ad libitum* water (Prior et al., 2013).

In the brain, water restriction affected T and E2 levels in a region-specific manner, sometimes in parallel with and sometimes independent of systemic steroid levels. In females, water restriction decreased T levels in the rostral telencephalon and E2 levels in central telencephalon, mid/hindbrain, and cerebellum. In males, water restriction had more effects, decreasing T levels in the rostral telencephalon, caudal telencephalon, and cerebellum, as well as E2 levels in the central telencephalon, caudal telencephalon, mid/hindbrain and cerebellum.

However, in the hypothalamus, water restriction did not affect T or E2 levels (Fig. 4A). The hypothalamus contains nuclei that are part of the “social behavior network” (e.g., preoptic area, ventromedial hypothalamus) and likely important in the regulation of pair-maintenance behaviors (Newman, 1999; Goodson, 2005). These data raised the hypothesis that local sex steroid synthesis in the hypothalamus is important for supporting pair-maintenance behavior in the water-restricted subjects.

Implications of breeding-specific endocrine states for laboratory studies of zebra finches

Domesticated zebra finches given *ad libitum* water and food are often considered to be uniformly in breeding condition. Furthermore, researchers often do not report breeding information in captive zebra finch studies. However, even within a single colony room, there can be considerable variation in reproductive readiness (Perfito, 2010). Furthermore, in all likelihood,

brain and circulating sex steroid profiles vary with reproductive readiness. Thus, this variation has implications for laboratory studies of zebra finch social behavior. The importance of considering breeding condition in captive zebra finches has been previously argued (Hahn et al., 2008; Perfito, 2010). There are many ways that researchers can control (e.g., water restriction) or assess (e.g., numbers of eggs and chicks, gonad size, oviduct size, LH levels, sex steroid levels) breeding condition in zebra finches.

Sex steroids and pair-bond formation and maintenance behaviors in zebra finches

Sex steroids in the regulation of courtship and pair-bond formation

In birds, many behaviors associated with courtship are regulated by sex steroids. In zebra finches, castration reduces courtship behavior and eliminates sexual activity (i.e., mounting) (Harding et al., 1983). These effects of castration can be rescued by both androgen and estrogen replacement (Harding et al., 1983). Note that castrating male zebra finches reduces, but does not eliminate, courtship behaviors, including male song (Arnold, 1975; Harding et al., 1983; Pröve, 1983).

The most widely studied courtship behavior in zebra finches is male song. Sex steroids are critical for the development of the song control circuit and the subsequent production of male song (Holloway and Clayton, 2001; Schlinger et al., 2001; Clayton, 2006; London et al., 2006). Male zebra finches produce two types of song, directed and undirected songs (Zann 1996). Following castration, estrogen replacement increased directed but not undirected singing beyond androgen replacement alone (Walters et al., 1991). In another study, androstenedione, a precursor to testosterone, rescued the inhibitory effects of exogenous arginine vasotocin on male song and courtship (Harding and Rowe, 2003).

Beyond initial courtship, there is mixed evidence for the role of sex steroids in regulating zebra finch pair-bond formation. The conflicting evidence may be partially due to the fact that sex steroids have specific effects depending on environmental/social contexts. For example, Hill and colleagues (2005) saw singing increase in T-treated males *only* during incubation. Tomaszewski and colleagues (2006), using the steroidal aromatase inhibitor 1,4,6-androstatriene-3,17-dione (ATD) in combination with the androgen receptor antagonist flutamide (FLUT), found that ATD+FLUT males were less likely to approach and greet females, but there were no other behavioral effects of ATD+FLUT treatment on courtship behavior, directed song, clumping, or pairing status of males or females. Because ATD is much less potent than non-steroidal aromatase inhibitors, such as fadrozole (Wade et al., 2004), future studies should examine the effects of fadrozole (or another potent aromatase inhibitor) on pair-bond formation in zebra finches.

Sex steroids in the regulation of pair-bond maintenance

Although courtship and pair-bond *formation* have been studied [to some extent](#), the neuroendocrine regulation of pair-bond *maintenance* has not been well-studied in zebra finches or other species. Furthermore, because sex steroids could differentially regulate such behavior depending on breeding and social contexts, their role in regulating pair-bond maintenance after initial pair-bond formation remains unclear. In birds, there are only a few studies that have examined the role of sex steroids in pair maintenance (Prior et al., 2014; Smiley et al., 2012; Kellam et al., 2006; Kellam and Lucas, 2014; Frigerio et al., 2004). For example, in non-breeding (winter) male downy woodpeckers (*Picoides pubescens*), T treatment increases the amount of time spent in close proximity with the partner (Kellam et al., 2006). Additionally, T treatment also decreases calling within a pair (Kellam and Lucas, 2014). In male greylag geese,

T treatment had no effect on pair-directed behavior but increased general “beak up” status signaling (indicating reproductive readiness) (Frigerio et al., 2004).

One of the only studies that specifically examined the neuroendocrine regulation of pair maintenance in zebra finches explored the role of progesterone (Smiley et al., 2012). Interestingly, progesterone administration to female zebra finches promotes courtship and nesting behaviors when administered prior to pairing. However, progesterone administration had no effects on pair-maintenance behavior (Smiley et al., 2012). This study did not examine the behavioral effects of reduced progesterone signaling or of estrogen manipulations.

Using experimental water restriction to study pair-bond maintenance in zebra finches

We used water restriction to manipulate the breeding condition of established zebra finch pairs in the laboratory (Prior et al., 2013) and examined the behavioral correlates, specifically pair-maintenance behaviors. Water restriction decreases circulating T levels in males and oviduct size (estrogen-sensitive) in females (see above). Consistent with previous studies of zebra finch behavior in the field (Zann, 1996), water restriction had no effect on pair-maintenance behavior, in 3 different behavioral paradigms (Prior et al., 2013). If sex steroids regulate pair-maintenance behavior during breeding and non-breeding periods, this raises the question whether sex steroids breeding-condition specific modes of action (Prior et al., 2013). We subsequently examined this hypothesis in a series of experiments. Specifically, we (1) correlated sex steroid levels and pair-maintenance behavior in both water-restricted pairs (WR) and control pairs (CON, *ad libitum* water) (Prior et al., 2013); (2) quantified acute and chronic effects of the aromatase inhibitor fadrozole on pair-maintenance behavior in WR pairs (Prior et al., 2014); and (3) examined the chronic effects of T treatment to males (Prior et al., unpublished results).

First, we have performed additional analyses of data from our study examining the effects of water restriction on pair-maintenance behavior and sex steroid levels (Prior et al., 2013), specifically [examining the relationships](#) between pair-maintenance behavior and sex steroid levels in plasma and the brain. We predicted that there would be positive [relationships](#) between hypothalamic sex steroid levels and proximity time during the Partner Reunion test. However, we found no significant [relationships](#) between sex steroid levels (T and E2) in the hypothalamus and pair-maintenance behavior (Table 3). Interestingly, in females, there was a positive correlation, independent of breeding condition (CON and WR groups were pooled), between E2 in central telencephalon and proximity time ($p=0.033$; Table 3). [While these results are only correlative and must be interpreted cautiously](#), these findings [are consistent with the hypothesis](#) that E2 [regulates](#) pair-maintenance behavior via estrogen receptors in the lateral septum (LS) or medial bed nucleus of the stria terminalis (BSTm), which are part of the social behavior network and regulate affiliation (Figure 5; Goodson, 2005; O'Connell and Hoffmann, 2011a; Goodson and Kingsbury, 2013).

Second, in non-breeding zebra finches, an increase in neurosteroid synthesis might be associated with an increase in non-genomic steroid signaling. In water-restricted zebra finch pairs, we non-invasively administered the aromatase inhibitor fadrozole, which decreases male E2 levels and increased male and female T levels both systemically and locally in the brain (Prior et al., 2014). We examined pair-maintenance behavior following both acute (~20 min) and chronic (1 week) fadrozole administration to either the male or female of an established pair (Prior et al., 2014). Interestingly, we only found effects of fadrozole with acute, but not chronic, administration. These data are consistent with the hypothesis that rapid estrogen signaling is important during non-breeding periods, when circulating sex steroids are low. Unexpectedly, we

found that acute fadrozole treatment *increased* pair-maintenance behavior (i.e., time spent in close proximity). The acute effect of fadrozole on pair-maintenance behavior could be due to either a decrease in E2 or an increase in T.

Third, we examined the effects of chronic T treatment to male zebra finches, in established pairs that received water *ad libitum*. Chronic T inhibited pair-maintenance behavior, specifically following a mild stressor (Prior et al., unpublished results).

Taken together, this series of studies suggests that sex steroids might have context-specific effects on pair-maintenance behavior. However, these steroid effects were rather limited in scope and magnitude, and it is possible that other neuroendocrine mechanisms play larger roles in regulating pair-bond maintenance. The prominence of other neuroendocrine mechanisms would be consistent with the results of Tomaszewski et al. (2006), who found no evidence for a role of androgens or estrogens in pair-bond formation in zebra finches.

Other potential regulatory mechanisms of pair-maintenance behavior in zebra finches

Several recent reviews have described other neuroendocrine mechanisms that regulate pair bonding and monogamy in vertebrates (Curtis et al., 2006; McGraw and Young, 2010; O'Connell and Hoffmann, 2011a,b). Extensive work on pair bonding in the monogamous prairie vole (*Microtus ochrogaster*) has elucidated the neural circuitry for pair-bond formation and the critical roles of nonapeptides (arginine vasopressin [AVP], oxytocin) and dopamine in the social behavior network and the reward system (Curtis et al., 2006; McGraw and Young, 2010). Studies in zebra finches have also examined the roles of nonapeptides (arginine vasotocin and mesotocin), dopamine, and vasoactive intestinal polypeptide (VIP) in pair-bond formation. Recent work has identified critical roles for mu and kappa opioid receptors in pair-bond

405 maintenance in prairie voles (Resendez and Aragona, 2012). Moreover, several studies have
406 examined glucocorticoids and pair bonding across taxa.

408 *Nonapeptides*

409 In prairie voles, the nonapeptides AVP and oxytocin are necessary and sufficient to
410 establish social bonds (Insel, 1997; McGraw and Young, 2010). In female prairie voles,
411 sociosexual interactions trigger a release of oxytocin in the nucleus accumbens (NAcc) and
412 prefrontal cortex (Young et al., 2008). In male prairie voles, AVP appears to be more important
413 than oxytocin in regulating pair bonding. Blocking AVP receptors in the ventral pallidum or
414 lateral septum (LS) using an AVP receptor antagonist prevents pair-bond formation in males
415 (Liu et al., 2001). More recently, adeno-associated viral vector gene transfer was used to increase
416 oxytocin receptor density in the NAcc, to compare the role of oxytocin in the monogamous
417 prairie vole versus the polygamous meadow vole (Ross et al., 2009). Interestingly, an elevation
418 of oxytocin receptors promoted pair-bond formation in female prairie voles, but had no effect in
419 the closely related but polygamous meadow vole (Ross et al., 2009). A similar pattern is seen in
420 males. Barret and colleagues (2012) decreased vasopressin receptor density in the ventral
421 pallidum of male prairie voles using RNA interference. This suppression of vasopressin
422 receptors disrupted pair-bond formation, and specifically decreased males' partner preference
423 (Barret et al., 2012).

424 The role of nonapeptides in zebra finch pair-bonding appears similar to what has been
425 described in prairie voles. First, peripheral administration of an oxytocin receptor antagonist
426 (OTA), which decreases mesotocin binding to its receptor, increased the latency to pair and
427 decreased pair formation in both sexes (Pedersen and Tomaszewski, 2012). Second, central (icv)

administration of an OTA to male and female zebra finches decreased the percentage of pair-bonded individuals that were stably paired, increased the latency to pair, decreased the duration of the pair bond, and decreased the percentage of individuals allopreening (Klatt and Goodson, 2013). These effects were more pronounced in females than males, similar to data from prairie voles (Young et al., 2008). Furthermore, experimentally reducing mesotocin levels in the paraventricular nucleus of the hypothalamus, using RNA interference, decreases pair bonding and clumping behavior in female, but not male, zebra finches (Kelly and Goodson 2014a). The wealth of evidence suggesting that nonapeptides are critical regulators of affiliation, and social behaviors more broadly, combined with the molecular techniques available to study VP-OT neurophysiology (such as RNA interference), makes future research on the role of nonapeptides in long-term pair maintenance promising (Kelly and Goodson, 2014b).

Dopamine

The majority of research on dopamine in prairie voles has been conducted during the first two weeks of pair bonding (Insel, 1997). A few studies suggest that dopamine signaling pathways are different during pair-bond formation and maintenance. After copulation, dopamine in the NAcc plays a critical role in bond formation for both males and females (Aragona et al., 2003; Aragona et al., 2006). Activation of D2-type dopamine receptors promotes pair-bond formation, while activation of D1-type dopamine receptors inhibits pair-bond formation (Aragona et al., 2006). After a pair bond has been established, D1-type receptors increase in the NAcc, likely inhibiting the formation of new pair bonds with other individuals (Aragona et al., 2006).

Dopamine also play a role in zebra finch pair bonding. During courtship and pair-bond formation in zebra finches, co-localization of tyrosine hydroxylase (TH, a critical enzyme for dopamine synthesis) and cFOS immunoreactivity in the ventral tegmental area and central gray is greater in males that court than males that do not court (Goodson et al., 2009b). When comparing newly-paired finches to non-paired finches, there is a positive correlation between dopamine signaling and pairing: (1) absolute levels of dopamine and its metabolite DOPAC are higher in several brain regions, including the ventral medial striatum, of newly-paired males and females than those that did not form a pair bond; and (2) TH-cFOS co-localization is higher in the ventral tegmental area of newly-paired birds (Banerjee et al., 2013). Interestingly, the one study that focused on the role of dopamine in pair-bond maintenance found the opposite relationship. Here, pair-maintenance behavior was negatively correlated with TH in the NAcc, preoptic area (POA), and ventral tegmental area (VTA) (Alger et al., 2011). Similar to the data in prairie voles, these results in zebra finches suggest that dopamine might be playing different roles during pair-bond formation and maintenance.

Vasoactive intestinal polypeptide (VIP)

One study in zebra finches found evidence for a role of VIP in zebra finch pair-bond formation. Kingsbury and Goodson (2014) found that a VIP receptor antagonist (VPAC) had two effects on pairing behavior. First, VPAC-treated males and females took longer to form a pair bond relative to controls. Second, VPAC-treated males and females appeared to form unstable bonds. Several of the bonds in the VPAC group lasted less than 1 day, whereas many of the bonds in the control group were stable for the duration of the study. Thus, VIP may be important in pair-maintenance behavior.

Opioid Receptors

Recent studies have examined opioids in prairie voles and their roles in pair-bond formation and maintenance (Resendez and Aragona, 2012). As with dopamine, different opioid receptor sub-types appear to be selectively involved in either pair-bond formation or maintenance. Opioid receptors are located throughout the motivational circuitry and have two sub-types (mu- and kappa-opioid receptors), which have opposing effects on reward processing. Prairie vole pair-bond formation is promoted by mu-opioid receptors (Burkett et al., 2011; Resendez and Aragona, 2012), whereas pair-bond defense associated with bond maintenance is promoted by kappa-opioid receptors within the NAcc shell (Resendez et al., 2012; Resendez and Aragona 2012).

While there are no studies directly examining the role of opioids in monogamy in birds, there is evidence that opioids regulate seasonally-appropriate vocal behaviors (Riters, 2012). In male dark-eyed juncos, opioid receptor densities in mPOA and VTA are related to gonad volume (Woods et al., 2010). Furthermore, administration of a mu-opioid receptor agonist reduces sexually-motivated singing behavior in male starlings (Riters, 2012). However, the opposite pattern has been observed in zebra finches (Khurshid et al., 2010). Taken together, there is evidence that opioid signaling changes seasonally and that it motivates sexual behavior and other social behaviors in birds.

Glucocorticoids

Depending on the circumstances, engagement in social behaviors can be a potent stressor or an effective buffer against a stress response. With regard to pair bonding, it is sensitive to

stress and glucocorticoids, but the presence of a pair bond can also ameliorate the negative effects of stress. In prairie voles, corticosterone administration interferes with pair-bond formation in females but facilitates pair-bond formation in males (De Vries et al., 1995; De Vries et al., 1996). Administration of corticotropin-releasing hormone to the NAcc promotes pair-bond formation in males (Lim et al., 2007). Finally, chronic stress causes bond loss in male prairie voles (Sun et al., 2013). There is evidence for a similar sex-difference in the role of corticosterone in zebra finch pair bond formation. LaPlante and colleagues (2014) found that acute corticosterone administration increased time males spent in close proximity to novel females, rather than novel males, in a 2-choice test. However, there was no effect of acute corticosterone on females (LaPlante et al., 2014).

As in many pair-bonding species, partner separation is a potent stressor and has long-term consequences for zebra finches (Ramage-Healey et al., 2003; Perez et al., 2012; Schweitzer et al., 2014). Partner separation increases circulating corticosterone levels more strongly in males than in females (Prior et al., 2013; Perez, 2013). Interestingly, there are two lines of evidence that suggest the ability of partners to synchronize their stress responses might be important in the expression of pair bonding. First, zebra finches produce modulated calls when alarmed, and these are “contagious” between partners (Perez, 2013). Hearing a partner’s stressed calls increases an individual’s circulating corticosterone levels (Perez, 2013). Second, in free-living monogamous great tits (*Parus major*), baseline corticosterone levels are correlated within a pair and become more similar over time in more successful pairs (Ouyang et al., 2014). Taken together, these studies raise the hypothesis that behavioral synchrony within a pair is modulated by glucocorticoids.

Based on these recent data, we re-analyzed some of our data on corticosterone and pair-bond maintenance (Prior et al., 2013) in order to examine correlations between circulating and brain corticosterone levels and time spent in close proximity (within 10 cm). The Partner Reunion test that we used constitutes an acute (~12 hr) stressor. There is a positive correlation in female zebra finches between corticosterone levels in the hypothalamus and pair-maintenance behavior ($p=0.019$; Table 3). Future studies should look at glucocorticoids in specific hypothalamic nuclei involved in social behavior (e.g., POA, AH and VMH, Figure 5).

Integrating sex steroids and other neuroendocrine mechanisms

Taken together, these lines of research suggest possible neuroendocrine mechanisms that might regulate zebra finch pair-maintenance behavior. They also highlight several brain nuclei in the social behavior network and the mesolimbic dopamine pathway that are likely to be involved (Figure 5). In future studies of these neuroendocrine mechanisms in zebra finches, it will be critical to consider the influence of breeding condition (Perfito, 2010). Nonapeptides, dopamine, etc. can vary with breeding condition seasons and might exert breeding-specific effects on social behavior (Riters et al., 2002; Heimovics and Riters, 2006, Heimovics et al., 2012; Goodson et al., 2012). Thus, sex steroids may still interact with these other signaling molecules (Kabelik, et al., 2011; Heimovics et al., 2012). To illustrate this possibility, in Figure 5, we also highlight brain nuclei that contain both estrogen receptors and aromatase in zebra finches (Saldanha and Coomaralingam, 2005).

Conclusions

The vast majority of research on affiliation has examined monogamy and parental behavior in mammalian model systems (Young et al., 2008). Importantly, affiliation in these systems is largely limited to the breeding season. The research to date then leaves at least three significant gaps in our current knowledge. First, little is known about the behaviors that are critical to the long-term maintenance of pair bonds. Second, little research has examined non-mammalian systems, despite the fact that monogamy is much more common in birds than in mammals (Adkins-Regan and Tomaszycki, 2007; Reichard and Boesch, 2003). Third, while breeding condition is well known to impact the regulation of aggressive behaviors (Soma et al., 2008; Heimovics et al., 2013), its influence on affiliation is largely unknown. Exploring the complexities of affiliation and its physiological regulation is not only fundamental to understanding sociality, but also relevant to our increasing recognition of how critical social bonding and prosocial behaviors are in humans.

Zebra finches are a [valuable](#) model system in which to study the regulation of long-term pair maintenance, and there is an extensive array of resources to aid in such investigations (Griffith and Buchanan, 2010). The combination of genomic and neuroanatomical resources together with the ethological relevance of pair bonding is unique and gives this model organism significant advantages for the study of pair bonding and monogamy. The effects of water restriction on captive zebra finches, as well as the effects of drought on wild zebra finches, show that non-breeding individuals have a distinct endocrine state from that of breeding individuals (Perfito, 2010; Prior et al., 2013). Our studies suggest that sex steroids modulate pair-maintenance behavior, depending on breeding condition. In addition, several lines of evidence suggest that other signaling molecules, such as nonapeptides and dopamine, are important for

long-term pair maintenance and can be steroid-sensitive. Future studies in the zebra finch and other monogamous songbirds will complement studies in monogamous mammals, such as the prairie vole, and elucidate conserved aspects of pair bonding in vertebrates.

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1022 **Table 1. Circulating androgen (ng/mL) and estrogen (pg/mL) levels in breeding and non-breeding zebra finches.**

	Reference	Breeding		Non-Breeding	
		Male	Female	Male	Female
<i>Non-Domesticated</i>	Perfito et al., 2007 ¹	T: 0.51 DHT: 0.28 E2: 390	T: 0.14 DHT: 0.21 E2: ND	T: 0.20 DHT: 0.27 E2: ND	T: 0.15 DHT: 0.22 E2: 370
	Priedkalns et al., 1984 ²	Androgen: <0.5	Androgen: <0.5	Androgen: <0.5	Androgen: <0.5
	Vleck and Priedkalns, 1985 ²	Androgen:<0.5 Estrogens: <300	Androgen:<0.5 Estrogens: <300	Androgen:<0.5 Estrogens: <300	Androgen:<0.5 Estrogens: <300
<i>Domesticated</i>	Prior et al., 2013 ²	T: 1.9 E2: 34	T: 0.3 E2: 40	T: 0.7 E2: 29	T: 0.2 E2: 31
	Prior et al., 2014 ²			T: 0.5 E2: 30	T: 0.1 E2: 28

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1025 ¹ Free-living zebra finches were compared during a breeding period in a predictable habitat (coast) and during a non-breeding period
1026 (drought) in an unpredictable habitat (arid central Australia).

1027 ² Experimental water restriction was used to bring subjects out of breeding condition.

1028 **Table 2. The use of experimental water restriction in captive zebra finches.**

Reference	Sex	Strain	Housing Conditions	Time to reduce water intake	Water per week (mL)	Total water restriction (days)	Primary Endpoints
Oksche et al., 1963	Both	Domesticated	Paired	NA	one 2 minute drinking bout/ week (0.5-1mL)	77-112	Gonad and neurosecretory system in brain
Calder, 1964	Male	Non-domesticated	?	?	~0.75	?	Water metabolism
Cade, 1965	?	Domesticated	groups	NA	0	80-112	Survival
Lee and Schmidt-Nielsen, 1971	?	Domesticated	individually	10 days	1, 0.5, & 0	10-110	Water metabolism
Sossinka, 1972	Both	Both	group	NA	0	513	Survival
Sossinka, 1974	Both	Non-domesticated	?	NA	0	60	HPG axis
Priedkalns et al., 1984	Both	Non-domesticated	Paired	NA	1	14	HPG axis
Vleck and Priedkalns, 1985	Both	Non-domesticated	Paired	12 days	1	14	HPG axis
Menon et al., 1989	?	?	?	NA	0	46	Water metabolism
Perfito et al., 2006	Male	Domesticated	same sex dyads	9 weeks	1	14	HPG axis
Prior et al., 2013	Both	Domesticated	Paired	5 weeks	1	40	Pair-maintenance behavior, HPG axis
Prior et al., 2014	Both	Domesticated	Paired	4 weeks	~ 0.5	70	Pair-maintenance behavior

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Table 3. Linear regressions (R-squared values) between brain steroid levels and pair-maintenance behavior.

		Testosterone	Estradiol	Corticosterone
Female	Plasma	0.009	1.92E-06	0.02
	Hypothalamus	0.003	0.03	0.26
	Rostral Telencephalon	0.05	0.16	0.0005
	Central Telencephalon	0.02	0.22	4.02E-05
	Caudal Telencephalon	0.04	0.007	0.0004
Male	Plasma	0.05	0.005	0.007
	Hypothalamus	3.20E-05	0.005	0.004
	Rostral Telencephalon	0.13	0.04	0.04
	Central Telencephalon	0.0007	0.07	0.001
	Caudal Telencephalon	0.006	0.181	0.04

Note. We performed additional analyses of data from our previous study (Prior et al., 2013). Our analyses presented here examine the relationships between pair-maintenance behavior and sex steroid levels in plasma and brain. Pair-maintenance behavior was measured as proximity time during a partner reunion, following an overnight separation. We were primarily interested in the relationships between sex steroids in the hypothalamus and telencephalon and pair-maintenance behavior. Evidence suggests a role for corticosterone in regulating pairing behavior, so we also examined the relationship between brain and plasma corticosterone levels and pair maintenance behavior. In females, there is a significant positive correlation between E2 in the central telencephalon and proximity time ($p = 0.033$) and also between corticosterone in the hypothalamus and proximity time ($p = 0.019$).

Figure Legends

Figure 1. HPG axis in a seasonally-breeding songbird (song sparrow) and an opportunistically-breeding songbird (zebra finch).

In many seasonally-breeding songbirds, such as the song sparrow, the HPG axis is up-regulated in response to increased photoperiod during the breeding (B) season in the spring. Conversely, the HPG axis is down-regulated during the non-breeding (NB) season, including fully regressed gonads and basal circulating sex steroid levels (Bentley et al., 1997; Bentley et al., 1998).

In contrast, in opportunistically-breeding songbirds, such as the zebra finch, there is not a strict dichotomy in reproductive state. Zebra finches integrate several environmental cues to make decisions about when to initiate and terminate breeding attempts (Waas et al., 2005; Perfito 2010). Following the cessation of breeding, zebra finches partially down-regulate the HPG axis. Hypothalamic GnRH and GnIH neurons are largely unaffected by breeding condition (Perfito et al., 2006; Perfito et al., 2011). Circulating LH levels are decreased during non-breeding periods (Perfito, 2010; Perfito et al., 2006; 2007; 2011). Male zebra finches may partial regress their testes during non-breeding periods, but gametogenesis in the testes appears to be largely maintained during non-breeding periods (Perfito, 2010). There is a greater suppression of female gametogenesis during non-breeding periods (Prior et al., 2013). In males, systemic T levels are reduced in non-breeding periods, but there is no change in systemic E2 levels in males or females (Prior et al., 2013).

Unless otherwise specified, the arrows in this figure represent general patterns for both males and females.

Figure 2. Sources of sex steroids vary with season or breeding condition. Testosterone (T) and estradiol (E2) regulate the brain and social behavior via binding to androgen receptors (AR) and estrogen receptors (ER). Traditionally, sex steroids are thought to be produced in the gonads and travel to the brain via the blood. However, some brain regions can also locally produce sex steroids from circulating prohormones (such as DHEA) or *de novo* from cholesterol. Stars (★) indicate possible starting points for sex steroid synthesis in the brain. In breeding (B) males and females, the gonads release sex steroids into the general circulation. In non-breeding (NB) males and females, the gonads release little, if any, sex steroids into the general circulation, and local synthesis of sex steroids in the brain can be upregulated.

Figure 3. Modes of sex steroid action vary with season or breeding condition. Traditionally, sex steroids, such as estradiol (E2), have been thought to affect gene expression in the brain through their intracellular receptors, such as estrogen receptors (ER), which can dimerize and act as transcription factors in the nucleus. However, sex steroid receptors can also be localized to the plasma membrane, and steroid binding can rapidly affect signal transduction pathways. While both genomic and non-genomic pathways are present throughout the year, the balance between genomic and non-genomic signaling is hypothesized to shift between breeding (B) and non-breeding (NB) periods (right-hand panel).

Figure 4. Effects of experimental water restriction on brain T and E2 levels. Brain sex steroid profiles in zebra finches that received water *ad libitum* (control) or were water restricted (1 mL per subject per week). (A) Hypothalamus, (B) Rostral Telencephalon, (C) Central Telencephalon, and (D) Caudal Telencephalon. Water restriction reduces T levels in the rostral

telencephalon of both sexes and in the caudal telencephalon of males only. Water restriction reduces E2 levels in the central telencephalon of the both sexes and in the caudal telencephalon of males only. Interestingly, the only region where there is no effect of water restriction for either males or females is the hypothalamus. Redrawn from data in Prior et al., 2013.

Figure 5. Potential brain regions that regulate pair bonding in birds and mammals.

This figure is synthesized from studies in mammals (McGraw and Young, 2010; O'Connell and Hoffmann, 2011a,b) and zebra finches (Goodson, 2005; Goodson and Kingsbury, 2013; Banerjee et al., 2013; Alger et al., 2011). Abbreviations: lateral septum (LS), nucleus accumbens (NAcc), medial bed nucleus of the stria terminalis (BSTm), anterior hypothalamus (AH), ventromedial hypothalamus (VMH), preoptic area (POA), ventral tegmental area (VTA), and [periaqueductal](#) grey (PAG). This neural circuitry is similar to the social decision-making network proposed in mammals. All nuclei from the social behavior network (yellow) are interconnected bi-directionally with each other. The primary connections between the mesolimbic dopamine system (purple) and the social behavior network are indicated. In addition, nuclei that contain both aromatase and estrogen receptors (ER α) are indicated ( ) (Balthazart et al., 1996; Saldanha et al., 2000; Saldanha and Coomaringam, 2005). The co-[expression](#) of aromatase and estrogen receptors in these nuclei raises the hypothesis that E2 modulates these brain regions that are involved in pair bonding.

Figure 1

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Figure 2

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Figure 4

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