BIRDS ON BIRTH CONTROL: THE EFFECTS OF 17α-ETHINYLESTRADIOL ON ZEBRA FINCH (*TAENIOPYGIA GUTTATA*) COURTSHIP BEHAVIOR, PARENTAL CARE, AND STRESS PHYSIOLOGY

By

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Abstract: Hormones are key regulators of behavioral expression and reproductive investment. Individual behavioral expression can also reflect environmental influences, such as contaminant exposure. Many environmental contaminants can specifically disrupt the endocrine system by binding to hormone receptors, thereby mimicking hormones or blocking hormone production. 17α-Ethinylestradiol (EE2), found in oral contraceptives, is considered an endocrine disrupter and is often detected in sewage effluent. Both aquatic and terrestrial animals can be exposed to EE2 in the environment with potential effects on behavior and physiology. I observed the effects of EE2 on courtship, parental care, offspring growth, reproductive success and stress response in captive zebra finches (*Taeniopygia guttata*). I used three levels of EE2 exposure, 0 ng (control); 4 ng, which is a level found in streams near wastewater effluent sites; and 100 ng, which serves as a higher level not recorded in nature. Birds were exposed to their respective treatments every other day for three weeks before behavioral and stress tests. Treatment was continued through nest building and incubation. In Chapter II of my dissertation, I conducted mate choice trials on both males and females choosing between control and EE2 treated birds of the opposite sex. I found that on average focal bird preference was not influenced by EE2 treatment of choice birds. In Chapter III, I observed the effect of EE2 on male courtship behaviors over 48 hours, male parental care via incubation and whether offspring and reproductive effort were influenced by paternal treatment. EE2 decreased the amount of male pair bonding behaviors directed towards females during incubation and significantly decreased brood size in pairs with 100 ng EE2 treated males but male EE2 treatment did not significantly affect courtship behaviors, offspring growth or additional reproductive success. Finally, in chapter IV, I tested the effects of EE2 on baseline and stress-induced corticosterone (CORT) levels, a hormone released in response to stress, in males and females and the indirect effects of EE2 on offspring of treated males. I induced the stress response by fasting the birds for 4 hours and I collected pre-and post-fasting blood samples to analyze baseline and stress-induced CORT. I found that baseline CORT responses were increased in 4 ng EE2 treated females in comparison to control females and 100 ng EE2 treated females. Males and offspring were not influenced by EE2 treatment. My dissertation research shows that EE2 can affect some aspects of behavior, reproductive outputs and CORT levels in zebra finches, thereby, providing novel information on the potential for EE2 to influence avian behavior and physiology.

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CHAPTER I

GENERAL INTRODUCTION

INTRODUCTION

Hormones are key regulators of behavioral expression and reproductive investment (Adkins-Regan 2005). For example, both testosterone and estradiol can influence courtship and reproductive behaviors, with low hormone levels decreasing the expression of these behaviors and increased hormone levels restoring behavioral expression (Buntin 1996; Eisner 1960; Murton et al. 1969; Tomaszycki et al. 2006).

Individual behavioral expression can also reflect environmental influences, such as contaminant exposure (Frye et al. 2011; Hirsch et al. 2003). Many environmental contaminants can specifically disrupt the endocrine system by binding to hormone receptors, thereby mimicking hormones or blocking hormone production (Salierno and Kane 2009). One such contaminant with endocrine disrupting effects is 17α-ethinylestradiol (EE2), which is a synthetic estrogen found in birth control pills (Berg et al. 1999). Women taking oral contraceptives excrete EE2 in their urine but because EE2 is a stable hydrophobic compound it does not degrade readily and can enter the environment via wastewater effluent (Bell 2004). Estrogen receptors are highly conserved across vertebrates, therefore, EE2 has the potential to affect many different species because it is structurally similar to the endogenous hormone 17β-estradiol and

can produce similar effects (Bell 2004; Berg et al. 1999; Kaspar and Witzel 1985; Welshons et al. 2003). Many studies of aquatic organisms have shown that exposure to EE2 can influence both behavior and physiology (Coleman et al. 2009; Kidd et al. 2007; Saaristo et al. 2010; Salierno and Kane 2009). Studies of terrestrial organisms have addressed potential effects of EE2 exposure on offspring development and physiology (Berg et al. 1999; Halldin et al. 1999), but research has not addressed the potential for EE2 exposure to influence adult behavior.

In addition to affecting behavior, EE2 has the potential to influence the production of other hormones, such as glucocorticoids. Corticosterone (CORT) can reflect environmental influences, as well, and both baseline and stress-induced CORT concentrations can be affected due to contaminant exposure (Lattin et al. 2014; Tartu et al. 2015). Although there is hormonal cross-talk between the hypothalamo-pituitary-gonadal (HPG) axis and the hypothalamo-pituitary-adrenal (HPA) axis (Lynn et al. 2010; Wingfield 1985; Zuloaga et al. 2011), very little research has addressed the potential for EE2 to influence CORT production.

EE2 is likely to remain an ecologically relevant environmental contaminant due to its importance as a synthetic estrogen in oral contraceptives. Studying the effects of EE2 on behavior and physiology in terrestrial species can elucidate whether this contaminant affects other organisms besides those which are primarily aquatic.

OBJECTIVES

Chapter II) a. Assess whether EE2 exposure influences male and/or female mate choice.

b. Evaluate the knowledge gained by quantifying behavior of the focal individual in addition to time spent with each choice individual during mate choice trials.

Chapter III) a. Determine if EE2 exposure influences male courtship and pair bonding behavior.

b. Determine if EE2 exposure directly affects male incubation behavior and/or indirectly affects female incubation behavior.

c. Determine if male EE2 exposure affects nesting success in captivity as quantified by clutch size, hatching success, brood size, offspring growth and fledging success.

Chapter IV) a. Assess if EE2 exposure influences male and female baseline and stress-induced CORT responses to a natural stressor.

b. Determine if paternal EE2 exposure indirectly affects the CORT responses of offspring to a natural stressor.

METHODOLOGICAL OVERVIEW

For my research, I used a captive population of zebra finches (*Taeniopygia guttata*). Birds were housed in cages of 4 (45Wx45Dx45H cm) to 8 (90Wx45Dx40H cm) same sex individuals and provided with seed and water *ad libitum*. The aviary was maintained at a temperature of approximately 22°C, a humidity of 20-50% and a light:dark cycle of 14:10h. Zebra finches were randomly assigned to treatment groups (control, 4 ng EE2 and 100 ng EE2). These treatments are based on environmental EE2 levels and the likely environmental exposure levels of wild birds in areas contaminated with EE2 (Calder 1964; Heberer 2002;

Kolpin 2002). EE2 was dissolved in peanut oil and fed orally to the birds using a pipette (Halldin et al. 1999). Control birds were treated with plain peanut oil. For all three of my experiments, birds were treated every other day for 3 weeks prior to behavioral trials or stress tests. During analyses of courtship and incubation behavior, males were dosed every other day until eggs hatched.

The day before mate choice trials, focal and choice birds were moved into their respective cages. All birds were visually isolated from each other before trials, but remained in auditory contact. Each focal bird underwent two choice tests, one in which the focal bird could choose between a control bird and a 4 ng EE2 treated bird and the second in which the focal bird could choose between a control bird and a 100 ng EE2 treated bird. I did not treat focal birds with EE2. On the day of the trials, I treated the choice birds one hour before removing the opaque divider between cages and videotaping the focal bird's behaviors for 20 minutes. To address the objectives of chapter II, I recorded the following focal bird behaviors directed towards the choice birds: number of beak wipes, number of hops, number of 180° turns and the amount of time birds spent on each perch in front of choice birds (Ullrich et al. 2016; Zann 1996). I scored behaviors using the Behavioral Observation Research Interactive Software (BORIS; Friard and Gamba 2016).

For courtship trials, I treated only male zebra finches with EE2. On the day of courtship trials, males were exposed to EE2 one hour before introducing them to females. Each pair was housed in a separate cage (45Wx45Dx45H cm). Immediately after introduction, I recorded behavioral interactions for 20 minutes (Lynn et al. 2010). Thereafter, I ran eight additional trials every three hours during the daylight period, over a two-day period for a total of nine courtship trials. Trials were recorded using video cameras and

scored using BORIS (Friard and Gamba 2016). To address the first objective of chapter III, I recorded the number of beak wipes and mountings for males and the number of tail flutters for females to assess courtship behaviors and I recorded the amount of time spent allopreening and clumping for both males and females to assess pair bond formation (Ullrich et al. 2016; Zann 1996).

One week after courtship trials, pairs were given a nest box and nesting material. I monitored nest boxes daily for egg laying and when an egg was found, it was weighed and labeled according to laying sequence. Once a clutch was complete, I recorded pair incubation behavior three times for each nest across the 13-day incubation period, with trials spaced at least two days apart to provide information on early, middle and late stages of incubation. Using small cameras (Hawk Eye Nature Cam X00018UD9X; Sony Pinhole Lens High Resolution CC-7HR) positioned to view inside nest boxes, I recorded behavior in 30-minute bouts. To address the second objective of chapter III, I recorded male and female parental care behaviors including, time spent in the nest box, time spent maintaining the nest (i.e., rearranging nesting material), the number of times parents turned the eggs and the time spent allopreening the mate or clumping with the mate inside the nest box (Zann 1996). To address the third objective of chapter III, I recorded clutch size, brood size, hatching success, offspring growth, fledging success and offspring survival. I recorded mass of the nestlings on their hatching day (day 0) and on days 5, 10, 17, 28, 36 and 50 post-hatch. I also measured tarsus length and wing chord on days 10, 17, 28, 36 and 50 post-hatch. The number of fledged young was assessed on approximately day 28. All young had exited the nestbox by this day, with fledging typically occurring on day 17 post-hatch (Zann 1996). The number of surviving offspring was recorded on day 50.

To address the objectives of chapter IV, I conducted stress tests in response to fasting on control and EE2 treated males and females and on the offspring of EE2 treated males (from chapter III). I first conducted a pilot fasting study to assess the duration of fasting required for zebra finches to significantly increase their CORT levels (Lynn et al. 2010). From the pilot study, I determined a fasting period of 4 hours induced an increase in CORT levels, without adverse effects on the birds. Treated birds were exposed to EE2 and control birds were exposed to peanut oil one hour before collecting a pre-fasting blood sample within 3 minutes of opening the cage door (Romero and Reed 2005). Feeders were then removed and birds were blood sampled again after 4 hours of fasting. To assess potential indirect effects of paternal exposure to EE2 on the stress responses of offspring, baseline blood samples from untreated offspring were collected within 3 minutes of opening the cage door (Romero and Reed 2005), feeders were removed and then birds were blood sampled again after a 4-hour fasting period, as in the parental generation. CORT concentrations were quantified using a corticosterone ELISA kit (Enzo Life Sciences, Inc.; ADI-901-097). Plasma was also used to analyze zinc concentrations in control and EE2 treated males and females and in the offspring of control and EE2 treated males. Zinc concentrations are highly correlated with the precursor egg yolk protein, vitellogenin, which is produced in males and females when circulating estrogen concentrations are high (Mitchell and Carlisle 1991; Wada et al. 2008; Williams 1999). To quantify zinc concentrations, I used a commercially available zinc assay kit (Sigma-Aldrich; MAKO32).

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CHAPTER II

THE EFFECTS OF 17α-ETHINYLESTRADIOL ON MALE AND FEMALE MATE CHOICE IN ZEBRA FINCHES (*TAENIOPYGIA GUTTATA*)

INTRODUCTION

In species that display pair bonding and biparental care, mate choice is considered advantageous because individuals have the opportunity to choose mates that are of good physiological quality and have the potential to be good parents (Hill 1993; Houtman and Falls 1994; Johnsen et al. 2000; Spoon et al. 2006; Trivers 1972; Zann 1996). For example, males and females may choose mates based on plumage coloration because this can be associated with good genes (Hill 1993; Trivers 1972). As another example, males may choose females based on their fecundity, as influenced by diet (Jones et al. 2001; Monaghan et al. 1996). Behavioral characteristics displayed during courtship, such as approaching a mate or performing courtship dances, are thought to be influenced by reproductive hormones, namely estradiol, which tends to increase during the mating season (Wingfield 1984).

Estradiol is essential for avian ovarian development and female reproduction (Smith 2010). Spikes in estradiol prior to ovulation can cause females to display more

sexually receptive behaviors towards males, and make individual females more attractive to males (Higham et al. 2009; Wingfield 1984). Furthermore, estradiol can influence egg fertility. Females treated with exogenous estradiol may produce the same number of eggs as control females but the number of fertile eggs is significantly lower in treated females than in control females (Rochester et al. 2008). In avian males, exposure to exogenous estrogens at varying levels (dosed orally with 1 nmol/g body mass of 1, 10 or 100 estradiol benzoate solution dissolved in canola oil) during development can feminize the gonads (Rochester et al. 2010). Estradiol exposure during post-hatching development in zebra finches (*Taeniopygia guttata*) has been shown to have long term effects on adult behavior and reproduction (Rochester et al. 2008; Rochester et al. 2010). For example, males exposed to exogenous estradiol during development showed a decrease in mounting probability as adults, but this behavior was restored when males were treated with exogenous testosterone in adulthood (Rochester et al. 2010). Testosterone can be converted into estradiol via the enzyme aromatase, and some male-typical courtship and reproductive behaviors are stimulated by estradiol, rather than testosterone (Hutchison et al. 1996). When aromatase is inhibited in males, they are less likely to approach females to initiate courtship behaviors (Tomaszycki et al. 2006). This suggests that exogenous estrogens can produce significant effects on behavior and reproduction after exposure during development and after adult exposure.

Because of the varied effects of estrogens across the lifespan on organismal function, the presence of estrogenic endocrine disrupting chemicals (EDCs) in many waterways throughout the world is an environmental concern (Herberer 2002; Kolpin et al. 2002). Many estrogenic EDCs have the capability of binding to estrogen receptors,

thereby mimicking or blocking endogenous estrogen production (Salierno and Kane 2009). Some well-known examples of estrogenic EDCs are bisphenol A (BPA), a component in plastics, the insecticide dichlorodiphenyltrichloroethane (DDT) and the synthetic estrogen in birth control pills, 17α-ethinylestradiol (EE2) (Berg et al. 1999; Frye et al. 2011; Mandich et al. 2007; Salierno and Kane 2009). Estrogenic EDCs have been demonstrated to influence reproductive morphology and behavior. Male carp (*Cyprinus carpio*) exposed to varying concentrations of BPA for two weeks produced ovarian tissue within their testes and had increased production of the egg precursor protein vitellogenin (Mandich et al. 2007). DDT has been shown to cause egg thinning in multiple bird species as well as altering developing secondary sexual characteristics in both male and female birds (Cooke 1973; Halldin 2005). DDT administered *in ovo*, has also been shown to feminize male behavior in Japanese quail (*Coturnix japonica*) (Halldin 2005). EE2 produces similar effects as endogenous estrogens because its chemical structure was designed to mimic 17β-estradiol, thus it has the ability to bind to estrogen receptors with similar affinity (Bell 2004; Berg et al. 1999; Kaspar and Witzel 1985).

EE2 is discharged into the environment via wastewater effluent with reported concentrations in the United States ranging from 1-5 ng/L (Heberer 2002; Kolpin et al. 2002). Even at these relatively low levels, EE2 can exert pronounced behavioral effects on organisms similar to those of endogenous estrogens (Bell 2004). Most behavioral research has focused on how EE2 affects aquatic organisms because water is the primary method of exposure to EE2, and aquatic animals are impacted by exposure to EE2. Adult male fathead minnows showed a decrease in reproductive behavior, scrubbing and

nibbling spawning substrate sites, when exposed to EE2 over 21 days (Salierno and Kane 2009). Additionally, aggressive behaviors tend to decrease during EE2 exposure in these same fathead minnows as well as in male sand gobies (*Pomatoschistus minutus)* and male zebrafish (*Danio rerio)* (Coleman et al. 2009; Saaristo et al. 2010; Salierno and Kane 2009). Few studies have addressed how adult behavior of terrestrial organisms, namely birds, is affected by EE2.

EE2 exposure may also influence mate preferences, and thus, the opportunity to breed. I tested whether EE2 influences mate choice preferences in male and female zebra finches. Both male and female zebra finches show preferences for mates and have well-defined behaviors associated with preference (Collins et al. 1994; Jones et al. 2001; Tomaszycki and Adkins-Regan 2005; Royle and Pike 2010; Rutstein et al. 2007). During the first stage of courtship (stage 1, Zann 1996), males typically approach females in a zig-zag pattern by hopping and turning 180° on a perch, displaying beak wipes and producing directed song (Ullrich et al. 2016; Zann 1996). Females return beak wipes, 180° turns and hops towards males, if they are interested (Ullrich et al. 2016; Zann 1996). I observed whether EE2 exposure influenced mate choice by presenting focal birds with a choice between a control, untreated bird, and an individual treated with EE2. I observed the behaviors of the focal birds to assess whether EE2 treatment influenced mate choice. I predicted that females would prefer EE2 treated males over control males because when aromatase is inhibited in males, they are less likely to approach females and engage in courtship displays. Therefore, higher circulating levels of estrogens might increase the likelihood of courtship displays (Tomaszycki et al. 2006). However, I only expected females to exhibit a preference for EE2 treated males when males were exposed to a low

dose of EE2 because high concentrations of exogenous estrogens could be detrimental to mate choice behaviors (Vandenberg et al. 2012). In other studies, female zebra finches have been shown to prefer control males over males exposed to elevated estrogen levels, therefore, I predicted females would prefer control males over males exposed to a high level of EE2 (Rochester et al. 2008). I also predicted that males would prefer EE2 treated females over control females because higher circulating levels of endogenous estrogens indicate ovulation and typically increase female receptivity towards males (Higham et al. 2009; Wingfield 1984). Specifically, endogenous estrogens play a role in the attractiveness and receptiveness of females, therefore, males were expected to prefer females with higher levels of exposure to estrogens (Wingfield 1984).

MATERIALS AND METHODS

Subjects:

Before mate choice trials, birds were housed in same sex cages with 4 (45Wx45Dx45H cm) to 8 (90Wx45Dx40H cm) individuals and provided with seed and water *ad libitum*. Bird seed was a 2:1 mixture of white millet (Stillwater Milling Company, Stillwater OK) and red millet (Jones Seed Company, Lawton OK). Once a week, birds were provided with egg food (ABBA 92A, ABBA Products, Hillside, NJ, USA), which was mixed with hard-boiled chicken eggs and avian vitamins (Avian Plus, Zoo Med Laboratories, San Luis Obispo, CA, USA). The aviary was maintained at a temperature of approximately 22°C, a humidity of 20-50% and a light:dark cycle of 14:10h.

17α-Ethinylestradiol Exposure:

I randomly assigned zebra finches to one of three treatment groups (control, 4 ng EE2 or 100 ng EE2). These treatments are based on environmental EE2 levels and the likely environmental exposure levels of wild birds in areas contaminated with EE2. The average concentration of EE2 found in waterways in the United States ranges from 1-5 ng/L, with maximum reported levels of 831 ng/L (Heberer 2002; Kolpin et al. 2002). Zebra finches drink 3-5 milliliters of water per day. If a water source were contaminated with 1-5 ng/L EE2, then EE2 exposure of the finches would range from 0.003-0.025 ng EE2/day (Calder 1964). If zebra finches drink water with the highest concentration of EE2 reported (831 ng/L EE2), then exposure would be 2.5-4.15 ng EE2/day (Kolpin et al. 2002). Thus, the 4 ng EE2 treatment represents a high level of environmental exposure. The 100 ng EE2 treatment represents exposure above currently documented environmental levels. I treated the zebra finches by pipetting 20 µl of a solution of EE2 and peanut oil into the birds' mouths (Halldin et al. 1999). Control birds were treated with 20 µl of peanut oil. Zebra finches were treated every other day for three weeks before the initiation of mate choice trials.

Mate-Choice Trials

Birds were approximately 3 years post-hatch and had not been previously paired or bred at the time of mate choice trials. Focal birds (females: n= 29; males: n=12) and choice birds (females: n=24; males: n=46) were introduced into cages used for behavioral testing to acclimate 24 hours prior to mate choice trials (Fig. 1). Choice females were used in a total of two choice tests with two different focal males. Choice males were used

in a total of three choice tests with three different focal females. Subsequent mate choice trials were separated by a one-week period, in addition to the three-week dosing period prior to trials. Each focal bird underwent two choice tests over two days; one with a choice between a control bird and a 4 ng EE2 treated bird and the other with a choice between a control bird and a 100 ng EE2 treated bird. The order of trials was randomized as well as which cage was assigned to the control bird and which cage was assigned to the treated bird. Choice birds were similar in coloration in order to control for mate choice based on plumage differences. Additionally, all birds had similar color leg bands (white or grey), which are colors that do not influence mate choice in zebra finches (Burley 1981; Burley et al. 1982). Opaque dividers were placed between cages during the acclimation period to visually separate the birds. At the beginning of trials, the dividers separating the focal bird cage from the choice cages were removed, but the dividers separating the two choice cages remained in place. This was to ensure choice birds could not see one another during the trials (Collins et al. 1994; Johnsen et al. 2000; Jones et al. 2001). Additionally, perches were aligned between the focal cage and choice cages, so that birds could interact on the same level as one another (Rutstein et al. 2007; Fig. 1). Once dividers were removed, behaviors were recorded for 20 minutes using video cameras (Sony Handycam DCR-SX40) mounted on tripods. I recorded the following focal bird behaviors directed towards the choice birds: number of beak wipes, number of hops, number of 180° turns and the amount of time birds spent on each perch in front of choice birds (Ullrich et al. 2016; Zann 1996). I scored behaviors using the Behavioral Observation Research Interactive Software (BORIS; Friard and Gamba 2016).

Statistical Analysis

I used IBM SPSS software (SPSS Inc. Chicago, Illinois, USA) to analyze mate choice data. I first tested whether focal birds showed a side preference and found birds did not preferentially choose to associate with birds on one side of the apparatus over the other (all $p>0.18$). Next I standardized the behaviors (beak wipes, hops, 180° turns and time) into Z-scores. Then I ran separate principal component analyses for male and female mate choice trials including all treatments to reduce the behaviors into principal components (PCs). I retained components with an eigenvalue greater than 1, for both males and females there was only 1 component with an eigenvalue above 1 (Table 1; Cattell 1966). For female mate choice, component 1 explained 58.92% of the variance and for male mate choice, component 1 explained 53.99% of the variance. For female mate choice tests with treated males, PCA scores were not distributed normally, therefore, I used a Wilcoxon signed ranks test to compare between control and treated groups. For male mate choice tests with treated females, I used a paired samples t-test to analyze differences in PCA scores of behaviors displayed by males to control and treated females. I subtracted control PCA scores from treated PCA scores to obtain the difference in behaviors displayed towards the two choice birds (the slope of the lines between control and EE2 PCA scores; Fig. 3; Fig. 6; Forstmeier and Birkhead 2004). I then compared these difference scores between trials with 4 ng EE2 choice birds and 100 ng EE2 choice birds using a paired samples t-test to determine if birds exhibited more pronounced preferences when presented with a choice between a control bird and a 100 ng EE2 treated bird than when presented with a choice between a control bird and a 4 ng EE2 treated bird.

Typically mate choice studies use time spent in association with each choice bird as the sole measurement of choice; therefore, I analyzed whether mate choice behaviors displayed toward a choice bird were correlated with the amount of time spent in front of a choice bird for both male and female mate choice trials. I then analyzed the difference between the amount of time focal birds spent on the side with the control bird versus the amount of time spent on the side with the EE2 treated bird. All time data were distributed normally, therefore, I used a paired samples t-test to analyze the differences between groups. To determine whether focal birds showed a preference for EE2 treated choice birds, I calculated the proportion of time spent on each side of the cage relative to the total amount of time spent interacting with the choice birds (Hill 1993; Houtman and Falls 1994; Johnsen et al. 2000; Jones et al. 2001). Proportions were distributed normally; therefore, I ran a one-sample t-test to compare the amount of time spent on the EE2 side to 0.50, which signifies no preference and an equal amount of time spent on each side (Houtman and Falls 1994; Jones et al. 2001).

RESULTS

Female Mate Choice

Standardized female choice behaviors were all significantly correlated with each other, except for the relationship between beak wipes and hops, for which there was a non-significant trend ($p=0.05$; Table 2). Specifically, the time spent on each side of the cage was significantly correlated with each of the courtship behaviors displayed (beak wipes: $p<0.001$; hops: $p=0.002$; 180° turns: $p<0.001$; Fig. 2).

Individual females varied in their mate choice PC scores, with some displaying more behaviors towards control males and others displaying more behaviors towards EE2 treated males (Fig. 3). Some individual females did not show a distinct choice during one or both of the trials. The lack of consistent preference among females was apparent in the average female behavioral PC scores, which did not differ significantly between control and EE2 treated males (Control vs. 4 ng EE2: control mean= 0.143 ± 0.226, 4 ng EE2 mean= -0.114 ± 0.164, Z=-0.724, df=28, p=0.469; Control vs. 100 ng EE2: control mean 0.044± 0.177, 100 ng EE2 mean= -0.074± 0.176; Z=-0.465, df= 28, p=0.642; Fig. 3). When testing the differences between PC scores (control PC score - treated PC score) between treatments, females on average had similar preference scores, regardless of whether they were choosing between a control male and a low dose EE2 male or a control male and a high dose EE2 male (t=0.274, df=28, p=0.786; 4 ng EE2: mean= 0.257±0.317; 100 ng EE2: mean= 0.118±0.271; Fig. 4). Again, individual females varied in mate choice preference between trials, with some choosing control males in the first trial and some choosing EE2 treated males in the second trial or vice versa (Fig. 4). Other females displayed little difference between trials, and consistently either chose the control male or the EE2 treated male (Fig. 4).

If preference was assessed only as the amount of time spent with each male, on average, females did not display a significant preference (4 ng EE2: t=-0.401, df=28, p=0.692; 100 ng EE2: t=-0.090, df=28, p=0.929). Females also did not show a significant preference for EE2 treated males compared to random association as measured by the proportion of time spent with each male (4 ng EE2: mean=0.483±0.292, t=-0.324, df=28, p= 0.749; 100 ng EE2: mean=0.495±0.305, t=-0.098, df=28, p=0.923).

Male Mate Choice

Standardized male choice behaviors were all significantly correlated with each other, except for beak wipes and hops, which were not significantly correlated ($p= 0.523$; Table 3). Specifically, the time spent on each side of the cage was significantly correlated with each of the courtship behaviors displayed (beak wipes: $p<0.001$; hops: $p=0.001$; 180° turns: $p=0.014$; Fig. 5).

Similar to the female mate choice PC scores, individual males were variable in their PC scores between mate choice trials, with some males displaying more behaviors towards control females, and others displaying more behaviors towards EE2 treated females (Fig. 6). The lack of distinct choice between females was apparent in the average male behavioral PC scores, which did not differ significantly between control and EE2 treated females (Control vs. 4 ng EE2: control mean=0.098 ± 0.281, 4 ng EE2 mean= -0.153 ± 0.286, $t=-0.499$, $df=11$, $p=0.628$; Control vs 100 ng EE2: control mean= -0.326 ± 0.189, 100 ng EE2 mean= 0.382 ± 0.365, $t=-1.48$, $df=11$, $p=0.167$; Fig. 6). When testing the differences between PC scores (control PC - treated PC) between treatments, males on average showed similar scores, regardless of whether they were choosing between a control female and a low dose EE2 treated female or a control female and a high dose treated EE2 female ($t=1.611$, $df=11$, $p=0.135$; 4 ng EE2: mean= 0.251±0.502; 100 ng EE2: mean= 0.708±0.479; Fig. 7). However, individual males varied in the PC differences they showed between trials, with some choosing control females in the first trial and some choosing EE2 treated females in the second trial or vice versa (Fig. 7). Some males were consistent in their choice, and chose the control or EE2 treated female

in both trials (Fig. 7). Finally, some individual males did not display a distinct preference in one or both trials (Fig. 7).

Males, on average, did not display a significant preference as indicated by the amount of time spent with each female (4 ng EE2: t=-1.004, df=11, p=0.337; 100 ng EE2: t=1.793, df=11, p=0.100). Overall, males did not show a significant preference for EE2 treated females compared to random choice as measured by the proportion of time spent with each female (4 ng EE2: mean= 0.433 ± 0.245, t=-0.949, df=11, p= 0.363; 100 ng EE2: mean= 0.614 ± 0.219, t=1.806, df=11, p=0.098).

DISCUSSION

I had predicted that male and female zebra finches would show preferences between control and EE2 treated choice birds. Specifically, I predicted that females would choose EE2 treated males over control males because EE2 might increase the likelihood of courtship displays by males thereby making them more attractive (Tomaszycki et al. 2006). However, for the choice between control males and males treated with 100 ng EE2, I predicted that females would prefer control males because high concentrations of exogenous estrogens could be detrimental to mate choice behaviors (Rochester et al. 2008; Vanderberg et al. 2012). For male mate choice, I predicted that males would prefer EE2 treated females over control females because increased estrogens can influence attractivity and receptivity of females (Higham et al. 2009; Wingfield 1984). I found that the attractiveness of male and female zebra finches was not significantly affected by EE2 treatment, either at low, environmentally relevant levels or at high levels. Neither males nor females showed a significant preference for

EE2 treated choice birds, and instead preference was random with respect to treatment. Average male and female mate preference was also similar whether focal birds were choosing between control and low dose birds or control and high dose birds, despite substantial individual variation. This suggests that focal birds do not perceive or respond to differences in behavior between control and EE2 treated birds, regardless of the level of exposure. This could be detrimental to the future reproductive success of a pair. For example, exogenous estradiol has been shown to increase egg infertility in female zebra finches as well as decrease mounting probability in males (Rochester et al. 2008; Rochester et al. 2010). Thus, it might be advantageous to be able to detect whether a potential mate has been exposed to elevated estrogen levels, and to exhibit a preference for unexposed individuals.

Female Mate Choice

Female mate choice has been studied extensively in zebra finches and studies have shown that females display distinct preferences for male traits. For instance, males that have darker red beaks and have higher song rates are selected as mates more often than males without those traits (Collins et al. 1994; Simons and Verhulst 2011; Tomaszycki and Adkins-Regan 2005). In other species, as well, females select mates on the basis of the elaboration of secondary sexual traits, such as comb color, which are related to testosterone levels (Zuk et al. 1995). Although estradiol is important for courtship behaviors, these behaviors may only be used secondarily in mate choice, after assessment of song and physical characteristics (Tomaszycki et al. 2006). Even if behavioral displays are influenced by exposure to EE2, female preference may ultimately not be impacted if EE2 exposure does not affect song production or beak color of zebra

finches. Song is unaffected in adult zebra finch males treated with an aromatase inhibitor (ATD) and an anti-androgen (flutamide) most likely because the song centers in the brain are organized during development and zebra finches are closed-ended song learners in which adult song is learned and crystallizes early in life (Tomaszycki et al. 2006; Zann 1996). Therefore, it is unlikely that EE2 exposure in adulthood would affect song complexity in adult males. Testosterone administered to castrated adult male zebra finches can restore and increase singing rates when males are presented with females (Arnold 1975). Male zebra finches express high levels of aromatase (i.e., the enzyme that converts testosterone into estradiol) in the brain, specifically in the song centers, which suggests that estradiol can play a role in adult song expression (Peterson et al. 2005). Thus, EE2 exposure in adulthood could influence male song rate; however, I did not record song in this mate choice experiment. Additionally, beak color of zebra finches can be influenced by hormone levels (McGraw 2006; McGraw et al. 2011) and can reflect both developmental and adult conditions (Merrill et al. 2016). Thus, dynamic changes in beak color might also reflect exposure to EE2, but I did not measure beak coloration in the current experiment.

Male Mate Choice

Male mate choice of female zebra finches is not as well studied as female mate choice but it has been documented (Burley et al. 1982; Jones et al. 2001; Monagan et al. 1996; Tomaszycki et al. 2006). Males have been shown to prefer females provided with supplementary food, which may signal egg production capability (Jones et al. 2001; Monagan et al. 1996). Perhaps any differences in behavior between control and EE2 treated females were not sufficient to signal to males that females had higher circulating

levels of estrogens. Females treated with an aromatase inhibitor (ATD) and an anti-androgen (flutamide or F) did not display significant differences in courtship behaviors when compared to control females (Tomaszycki et al. 2005). Additionally, when allowed to pair with control males, equal numbers of ATD-F females and control females formed pairs (Tomaszycki et al. 2005). Estradiol levels were not monitored in the Tomaszycki et al. (2005) study though so they could have remained at naturally circulating levels. Although male mate choice occurs in zebra finches, it may not have as much of an impact on pair formation as female mate choice; therefore, males may not express distinct preferences. Furthermore, many of the behaviors that would indicate female interest or disinterest in a male, and which could influence male choice, necessitate direct contact with the female. For example, receptive behaviors such as tail quivering, which occurs immediately prior to copulation or negative behaviors such as chasing or pecking require contact between males and females (Morris 1954; Zann 1996).

Quantifying Mate Choice in Zebra Finches

Secondary to whether EE2 influenced mate preference, I also wanted to determine whether recording courtship behaviors in addition to time spent near each choice individual provided valuable information about mate preference. Most previous zebra finch mate choice studies have only recorded time as the indicator of preference (Collins et al. 1994; Jones et al. 2001; Tomaszycki and Adkins-Regan 2005; Royle and Pike 2010). Recording preference behaviors may be most relevant for mate choice studies conducted in which multiple birds can directly interact (Banerjee and Adkins-Regan 2014; Rutstein et al. 2007; Tomaszycki et al. 2006; Tomaszycki and Zatirka 2014). In my study, I found that time spent with each choice individual was correlated with the

expression of preference behaviors; therefore, in simple three-cage mate choice designs, time spent in association is a representative indicator of choice. Rutstein et al. (2007) tested the difference between the total amount of time females spent on a perch and the total amount of "active" time females spent on perches in front of choice males. Females were considered "active" when their posture was directed towards the choice male, whereas females were considered "passive" when they faced other directions (Rutstein et al. 2007). Females were inconsistent in their preferences when the total amount of time near each male was used as the dependent variable. Fewer females displayed inconsistent preference when "active" time was used as the dependent variable instead (Rutstein et al. 2007). Recording active time, as opposed to simply time in proximity to a male, would be an improvement in future mate choice studies.

Conclusion

EE2 treatment did not influence mate preference for either male or female zebra finches. These results suggest that focal birds cannot perceive differences in choice bird behavior based on EE2 treatment. The results also suggest that individuals differ in preference and preference could be influenced by other traits I did not record such as bill color and singing for males and receptivity in females. Birds were not allowed to contact one another, and contact contributes to the courtship "dance", as well as copulation and pair bonding (Zann 1996). In the field, there are other factors that could contribute to mate choice such as mate availability and potential EE2 (or other contaminant) exposure rate. In the field, both males and females could be exposed to environmental contaminants for variable time periods. If both males and females are exposed to EE2 simultaneously, then this could influence both the expression and perception of courtship

behaviors, and thus mate choice could be affected. It would be beneficial to directly observe behavior of the EE2 treated choice birds to determine whether behavioral changes are occurring in their courtship displays.

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Table 1. Zebra finch mate choice behavioral loadings for component 1 from principal component analysis. Components with an eigenvalue greater than 1 were retained, for both males and females there was only one component with an eigenvalue above 1. Female mate choice refers to situations in which females chose between control and EE2 treated males and male mate choice refers to situations in which males chose between control and EE2 treated females.

Behavior	**Female Mate Choice Component 1**	**Male Mate Choice Component 1**
Beak Wipes	0.819	0.755
Hops	0.495	0.600
180° Turns	0.868	0.748
Time	0.829	0.819

Table 2. Correlation matrix of standardized female zebra finch mate choice behaviors. Behaviors were standardized to have a mean of zero and a standard deviation of 1 in order to produce a common scale to compare behaviors. * next to r-value indicates statistical significance (*p=0.05, **p<0.01, ***p<0.001).

	Beak Wipes	**Hops**	**180º Turns**	**Time**
Beak Wipes	1	0.182*	0.630***	0.571***
Hops		1	0.335***	0.279**
180º Turns			1	0.599***
Time				1

Table 3. Correlation matrix of standardized male zebra finch mate choice behaviors. Behaviors were standardized to have a mean of zero and a standard deviation of 1 in order to produce a common scale to compare behaviors. * next to r-value indicates statistical significance. (*p<0.05, **p<0.01, ***p<0.001).

	Beak Wipes	**Hops**	**180º Turns**	**Time**
Beak Wipes	1	0.094	0.498***	0.546***
Hops		1	0.337*	0.455**
180º Turns			1	0.354*
Time				1

Figure 1. Overhead view of zebra finch mate choice cage set-up. A) focal bird cage (90Wx45Dx40H cm). Striped box indicates neutral zone in which the focal bird was unable to see choice birds. B) Choice bird cages (32Wx27Dx33H cm), housing either control or EE2 treated birds. Dotted lines indicate opaque dividers, which visually separated choice birds during trials.

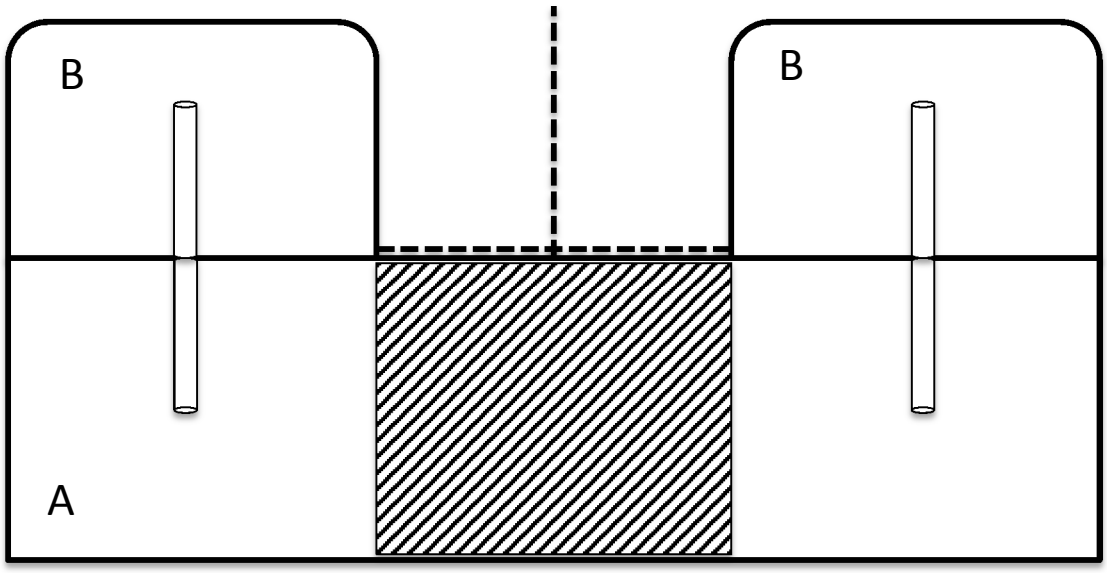


Figure 2. Correlations between the amount of time spent by focal female zebra finches in front of male choice zebra finches in relation to female mate choice behaviors (number of beak wipes, number of hops and number of 180 º turns) displayed towards a choice male. Each individual female is represented 4 times for behaviors displayed in the control (round marker) vs 4 ng EE2 (triangle marker) mate choice trials and the behaviors displayed in the control (square markers) vs 100 ng EE2 (diamond markers) mate choice trials.

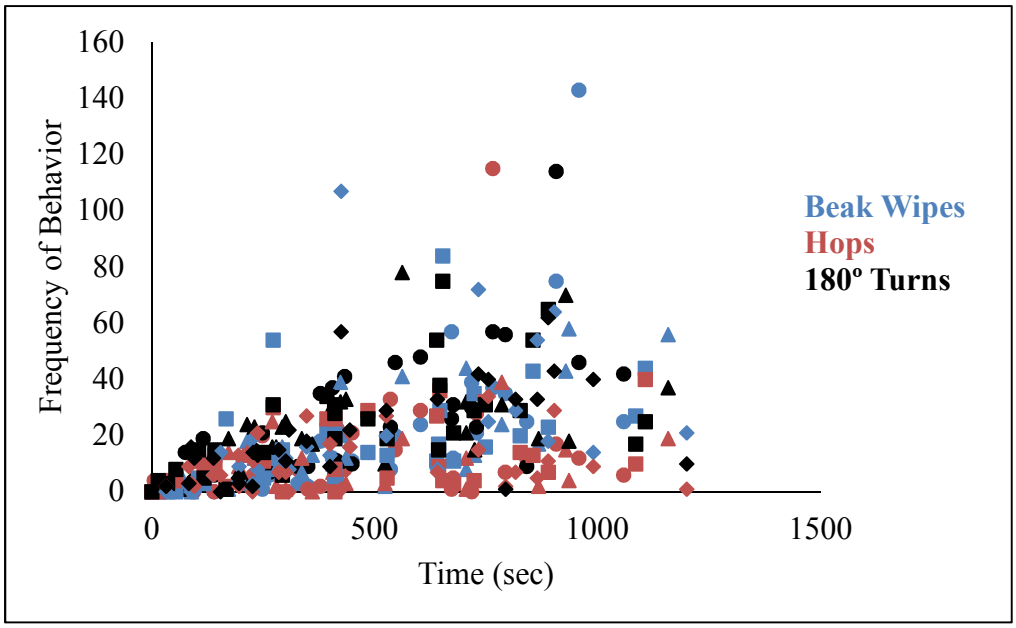


Figure 3. Principal component (PC) scores of individual female focal zebra finches (n=29) when choosing between control males and A) 4 ng EE2 males or B) 100 ng EE2 males. Each individual female is represented by the same color marker and connecting line in A and B. The black dashed line indicates the average PC scores for control and EE2 treated trials.

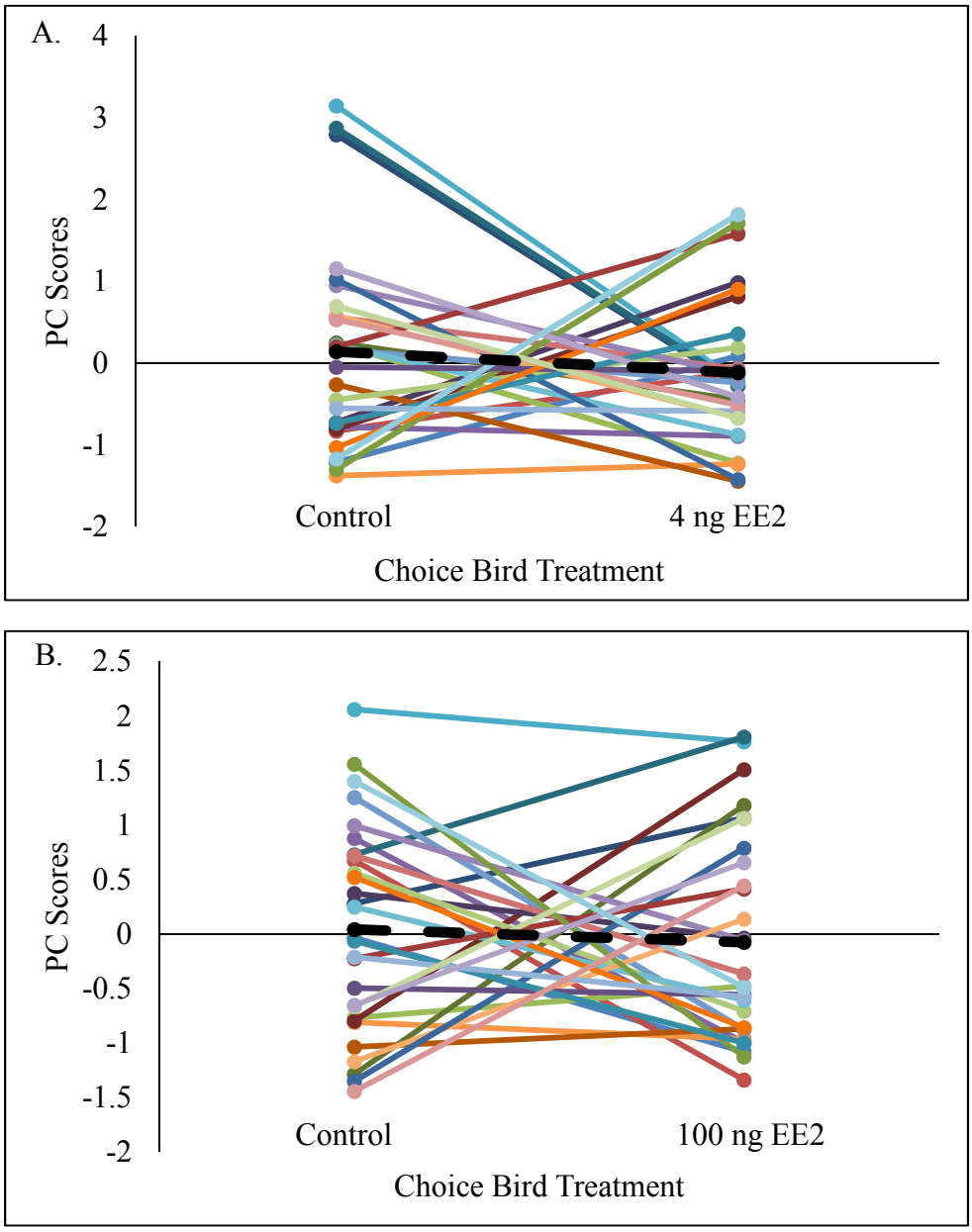


Figure 4. Difference in individual female principal component (PC) scores (calculated from the slope of the connecting lines between control PC scores and EE2 treated PC scores from Fig. 3) compared between 4 ng EE2 and 100 ng EE2 trials. The black dashed line indicates the average PC difference scores for 4 ng EE2 and 100 ng EE2 trials.

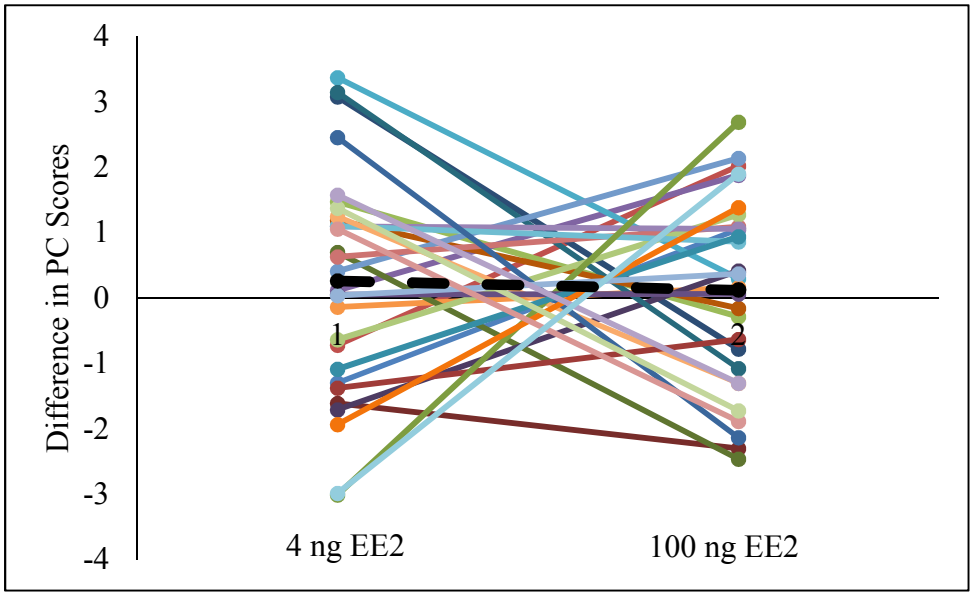


Figure 5. Correlations between the amount of time spent by focal male zebra finches in front of female choice zebra finches in relation to male mate choice behaviors (number of beak wipes, number of hops and number of 180 º turns) displayed towards a choice female. Each individual male is represented 4 times for behaviors displayed in the control (round marker) vs 4 ng EE2 (triangle marker) mate choice trials and the behaviors displayed in the control (square markers) vs 100 ng EE2 (diamond markers) mate choice trials.

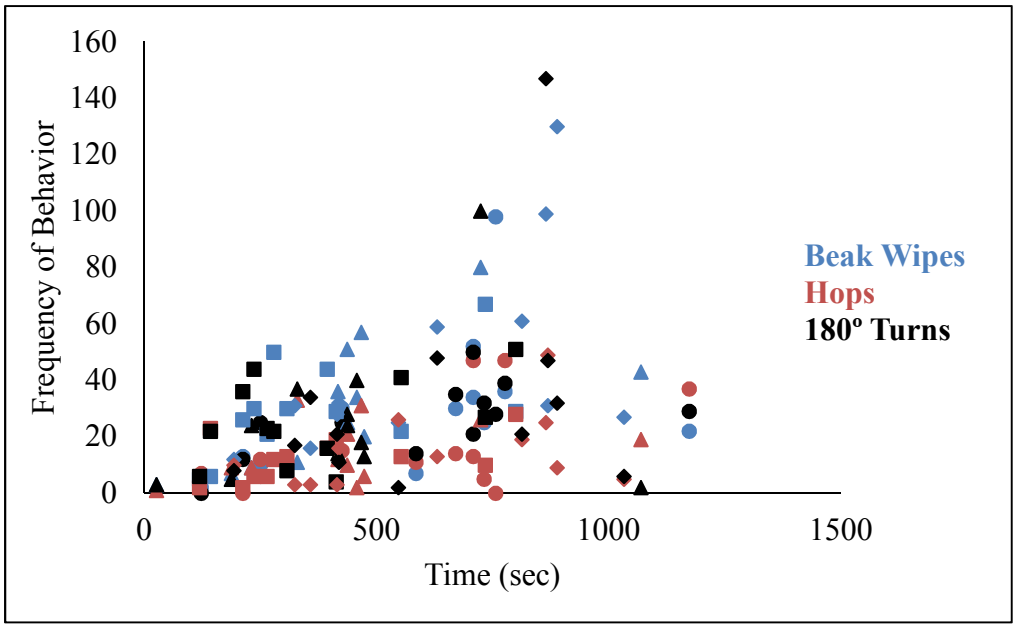


Figure 6. Principal component (PC) scores of individual male focal zebra finches (n=12) when choosing between control females and A) 4 ng EE2 females or B) 100 ng EE2 females. Each individual male is represented by the same color marker and connecting line in A and B. The black dashed line indicates the average PC scores for control and EE2 treated trials.

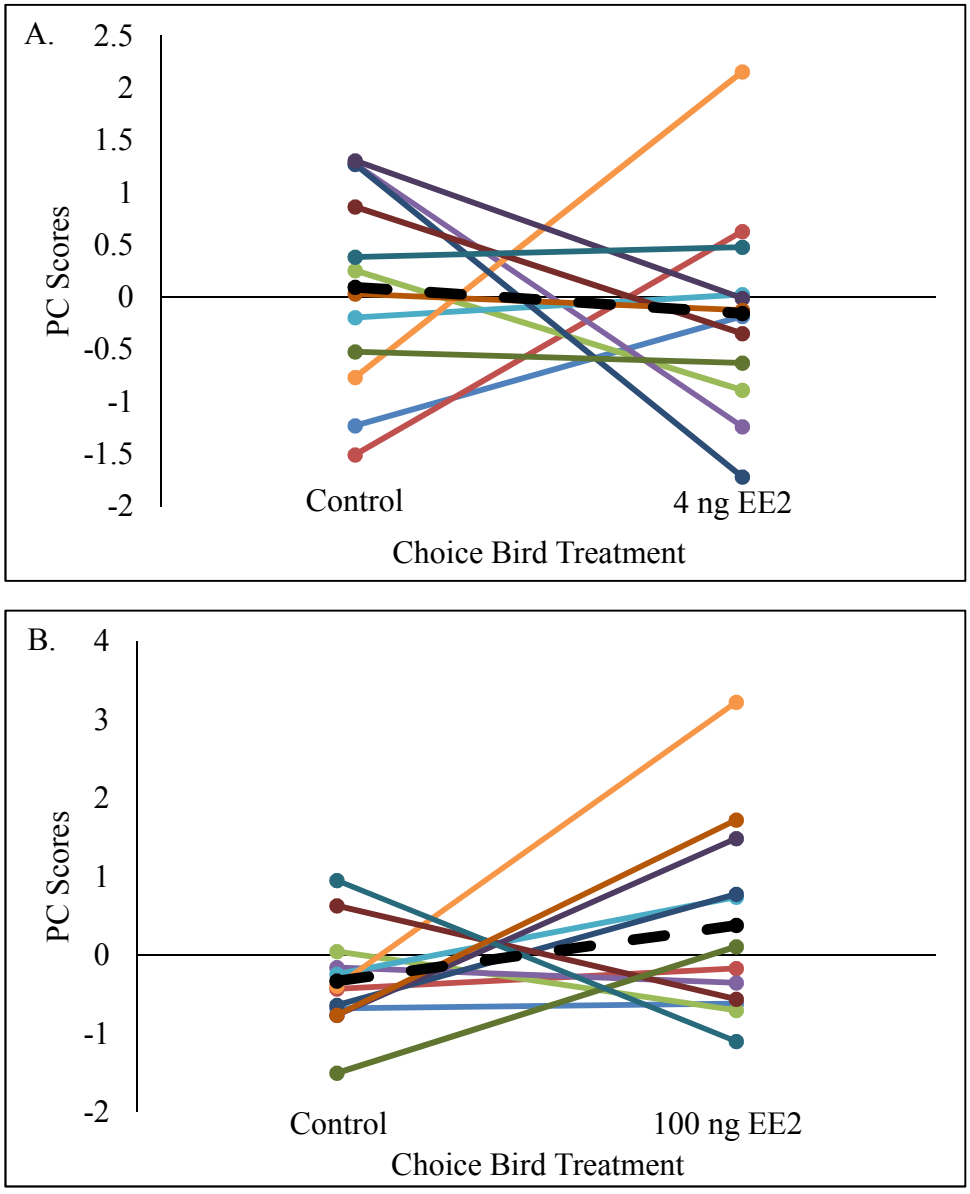
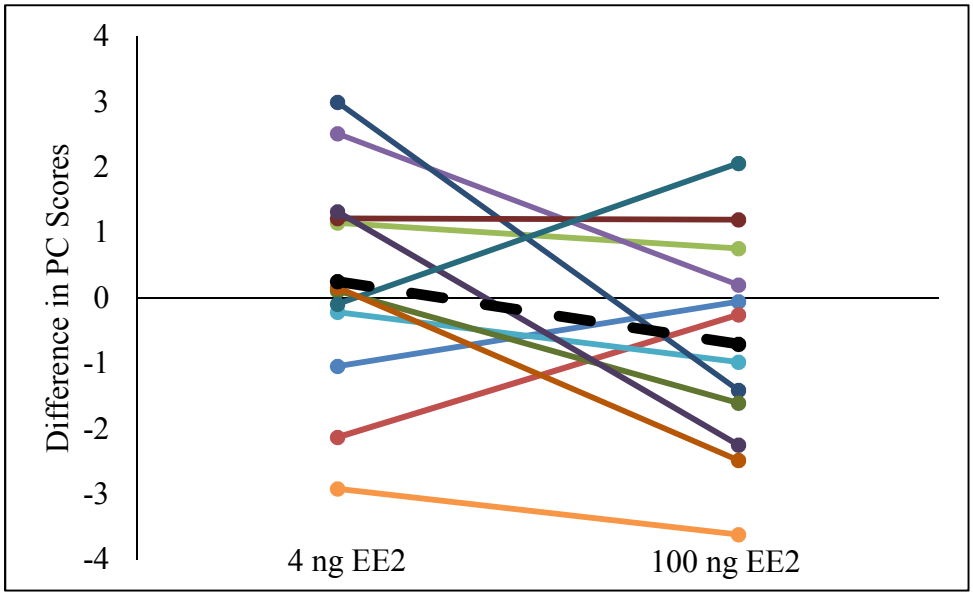


Figure 7. Difference in individual male principal component (PC) scores (calculated from the slope of the connecting lines between control PC scores and EE2 treated PC scores from Fig. 6) compared between 4 ng EE2 and 100 ng EE2 trials. The black dashed line indicates the average PC difference scores for 4 ng EE2 and 100 ng EE2 trials.



CHAPTER III

THE EFFECTS OF 17α-ETHINYLESTRADIOL ON MALE ZEBRA FINCH (*TAENIOPYGIA GUTTATA*) COURTSHIP BEHAVIORS, INCUBATION BEHAVIOR, REPRODUCTIVE SUCCESS AND OFFSPRING GROWTH

INTRODUCTION

Environmental contaminants can have profound influences on physiology and behavior (Frye et al. 2011; Hirsch et al. 2003; Love et al. 2003). Many contaminants are considered endocrine disrupting chemicals (EDCs) because they disrupt the normal function of the endocrine system by, for example, binding to hormone receptors, thereby mimicking a hormone or blocking its production (Salierno and Kane 2009). Exposure to estrogenic EDCs has been shown to feminize male gonads (Fry and Toone 1981; Hayes et al. 2010; Mandich et al. 2007), decrease male sexual behavior (Halldin et al. 1999; Panzica et al. 2005), decrease female reproductive output (Halldin 2005; Oehlmann et al. 2000) and decrease offspring growth rates (Markman et al. 2011). In this study, I analyzed the effects of one estrogenic EDC, 17α-ethinylestradiol, on male behavior and reproductive success.

17α-Ethinylestradiol (EE2) is a synthetic estrogen found in birth control pills (Berg et al. 1999; Salierno and Kane 2009). Chemically, its structure is analogous to the endogenous hormone 17β-estradiol and has the ability to bind to estrogen receptors, which are highly conserved across species, with comparable affinity, thus, producing similar effects as endogenous estrogens (Bell 2004; Berg et al. 1999; Kaspar and Witzel 1985; Welshons et al. 2003). Women taking contraceptives excrete EE2 in their urine but because EE2 is a stable hydrophobic compound it does not degrade readily and, therefore, does not filter out of contaminated water in treatment plants (Bell 2004). When EE2 enters the environment via wastewater treatment plants, it has the potential to act as an EDC (Muller et al. 2008; Saaristo et al. 2010).

Because EE2 is released into waterways via wastewater effluent, most research has addressed how EE2 influences the physiology and behavior of aquatic organisms. In some fish species, males exposed to EE2 develop ovarian tissue within the testicular tissue and produce vitellogenin, an egg protein precursor typically expressed in females in response to higher circulating estrogen levels (Kidd et al. 2007; Saaristo et al. 2010; Salierno and Kane 2009). EE2 can also reduce the expression of male secondary sexual characters, such as nuptial tubercles on fathead minnows (*Pimphales promelas*), thereby feminizing male appearance (Kidd et al. 2007; Salierno and Kane 2009). Furthermore, exposure to EE2 has been shown to affect behavior; adult male fathead minnows exposed to EE2 over 21 days showed a decrease in reproductive behavior, specifically spending less time scrubbing and nibbling spawning substrate sites (Salierno and Kane 2009). These same fathead minnows showed a decrease in aggressive behavior when exposed to EE2 (Salierno and Kane 2009). Male sand gobies (*Pomatoschistus minutus*) and male

zebrafish (*Danio rerio*) also exhibit a decrease in aggression when exposed to EE2 (Saaristo et al. 2010; Coleman et al. 2009).

Terrestrial animals, such as mammals and birds, can also be exposed to EE2 through water and food sources, such as insects, with potential consequences for physiology and behavior. Estrogenic EDC concentrations are significantly higher in insects and earthworms captured at sewage percolating filter bed sites than in insects and earthworms captured more than 2 km away from sewage sites (Markman et al. 2007; Park et al. 2009). Specifically, EE2, bisphenol A, diethylphthalate and dibutylphthalate have been extracted from insects (Park et al. 2007). Based on the typical amount of insects eaten per night and the levels of EE2 in insects near sewage treatment centers, the common pipistrelle bat (*Pipistrellus pipistrellus*), which forages near sewage treatment centers, would be predicted to ingest 28.8-57.2 ng EE2 per day (Park et al. 2009). Many insectivorous birds, such as the European starling (*Sturnus vulgaris*), have been observed feeding on contaminated insects and earthworms from active sewage treatment sites (Markman et al. 2011). This implies that terrestrial animals, specifically birds, have the potential to be naturally exposed to, and affected by, EE2.

Exposure to EE2 during avian embryonic development can cause malformations of the Müllerian duct in both sexes, with males retaining the Müllerian duct and females not fully regressing the right Müllerian duct (Berg et al. 1999). EE2 exposure during development may also affect adult reproductive behavior. For example, male Japanese quail (*Coturnix japonica*) exposed to varying concentrations of EE2 during embryonic development showed decreased expression of sexual behaviors as adults, namely a decrease in mounting attempts (Halldin et al. 1999). Additionally, female rats exposed to

varying concentrations of EE2 (both 4 ng/L and 400 ng/L) showed a decrease in receptivity and proceptivity (Seta et al. 2008). Adult exposure to EE2 may have indirect effects on offspring phenotype. For example, pregnant female mice exposed to low, environmentally relevant levels of EE2 in their drinking water gave birth to offspring with lower body weights (Pillon et al. 2012).

Although many studies have shown EE2 can directly influence development and growth in birds (Berg et al. 1999; Halldin et al. 1999: Holm et al. 2001), relatively few studies have addressed if adult exposure to EE2 can influence behavior. However, we can formulate predictions on the likely effects of adult exposure to EE2 from knowledge of the important roles of endogenous estrogens in avian courtship and reproductive behaviors of both males and females. Males typically prefer females with higher circulating levels of estrogen (Higham et al. 2009). A preference for females with higher estrogen levels is beneficial because these females are more likely to be fertile (Higham et al. 2009; Wingfield 1984). Female zebra finches (*Taeniopygia guttata*) injected with exogenous estrogen during development and implanted with estradiol as adults exhibit more courtship dancing (Adkins-Regan and Ascenzi 1987). Therefore, it could be argued that estrogen increases the proceptivity of female zebra finches by increasing courtship dancing, which could indicate to males that they are reproductively ready (Adkins-Regan and Ascenzi 1987). When aromatase, the enzyme that converts testosterone to estradiol, is inhibited, male zebra finches are less likely to approach females during courtship compared to control males (Tomaszycki et al. 2006). This suggests that estradiol, when aromatized from testosterone, influences male courtship initiation. Estrogens are also important for priming individuals for egg laying and incubation (Buntin 1996; Eisner

1960; Eisner 1969; Stern 1979). Studies have shown that an increase of estrogens is typically seen during the beginning of nesting activity (Buntin 1996). Both estrogen and progesterone treatment together can stimulate pre-laying behavior and nest building and it is thought that these hormones induce prolactin secretion, which is sustained during incubation (Buntin 1996; Eisner 1960; Eisner 1969; Stern 1979). Additionally, male birds treated with both progesterone and estradiol, displayed incubation behavior, whereas birds treated with one or the other hormone did not (Stern 1979). This suggests that estradiol can influence both male and female pre-laying, nest-building, and incubation behaviors.

To determine if exposure to EE2 might have similar effects to endogenous estrogens, I tested the effects of adult exposure to environmentally relevant concentrations of EE2 on male courtship and incubation behaviors in captive zebra finches. Additionally, I tested if adult exposure to EE2 might affect reproductive success or indirectly influence offspring growth. I predicted that EE2 would increase the expression of male courtship behaviors, with potential indirect effects on reproductive success and offspring growth. I also predicted that exposure to EE2 would induce greater investment in incubation behavior by males (Stern 1979). I predicted that both low and high dose EE2 treatments would influence behaviors, however I was unsure as to whether they would influence behavioral expression to the same degree.

MATERIALS AND METHODS

Study species and housing:

Zebra finches are an attractive model species for the potential effects of EE2 exposure on wild birds for several reasons. Zebra finches exhibit distinct courtship behaviors, biparental care and breed readily in captivity (Zann 1996). They display the greatest amount of courtship behaviors, such as mounting and beak wiping, within the first hour of introduction to opposite-sex conspecifics (Smiley and Adkins-Regan 2016). The occurrence of courtship behaviors, such as clumping and allopreening, usually observed within two days of introduction, predicts the likelihood of pair bond formation and subsequent reproductive success (Smiley et al. 2012; Zann 1996). Clutch size typically ranges from 3-5 eggs and the incubation period is approximately 13 days (Zann 1996). Both males and females incubate eggs, with females incubating 56% of the time and males incubating 40% of the time on average (Delesalle 1986; El-Wailly 1966). Offspring fledge on approximately day 17 post-hatch and develop adult plumage between day 36-50 post-hatch (Zann 1996).

At the start of the experiment, birds were housed in same sex cages of 4 (45Wx45Dx45H cm) to 8 (90Wx45Dx40H cm) individuals. When courtship trials were initiated, males and females were paired at random. Throughout the experiment, birds had *ad libitum* access to water and seed (2:1 ratio white millet: red millet). Additionally, once a week birds were given millet spray. Egg food (ABBA 92A, ABBA Products, Hillside, NJ, USA) mixed with hard boiled chicken eggs and avian vitamins (Avian Plus, Zoo Med Laboratories, San Luis Obispo, CA, USA) was provided to individuals weekly

prior to courtship trials and once a day to breeding pairs until nestlings fledged or breeding was terminated. Aviary rooms had a temperature of approximately 22°C, humidity ranging from 20-50% and a 14:10h light: dark cycle.

17α-Ethinylestradiol Exposure:

Male zebra finches were randomly assigned to one of three treatment groups (control, 4 ng EE2 or 100 ng EE2) and dosed orally, via a pipette, every other day for three weeks prior to courtship trials, throughout incubation, and until the first egg hatched. EE2 was suspended in peanut oil (20 µl) and controls received 20 µl of peanut oil. The levels of EE2 are based on documented environmental levels of EE2 in the United States and plausible exposure levels of wild birds to EE2 in contaminated areas. The average concentration of EE2 in waterways in the United States ranges from 1-5 ng/L, with maximum reported levels of 831 ng/L (Heberer 2002; Kolpin et al. 2002). Zebra finches drink 3-5 milliliters of water per day (Calder 1964). If birds drink water with 1-5 ng/L EE2, then exposure would typically range from 0.003-0.025 ng EE2/day. If birds drink water with the highest concentration of EE2 reported (831 ng/L EE2), then exposure would be 2.5-4.15 ng EE2/day. Thus, the 4 ng EE2 treatment represents a very high level of plausible environmental exposure (Kolpin et al. 2002). The 100 ng EE2 treatment represents exposure above currently documented environmental levels.

Courtship Behavior:

I recorded courtship behavior in nine, 20-minute trials over a two-day period with the first trial initiated as soon as males and females were paired. One hour after EE2 exposure, males were placed into a cage (45Wx45Dx45H cm) containing a novel female

zebra finch, and initial courtship behaviors were recorded for 20 minutes. Thereafter, I ran eight additional trials every three hours during the light period, over two days. Trials were recorded using video cameras (Sony Handycam DCR-SX40) mounted on tripods and scored using the Behavioral Observation Research Interactive Software (BORIS; Friard and Gamba 2016). I recorded the following courtship behaviors: number of beak wipes (males), number of mountings (males), number of tail flutters (females), and the amount of time spent allopreening and clumping (both sexes). Beak wiping, mounting and tail fluttering are sexual behaviors displayed by zebra finches during the early stages of courtship, whereas allopreening and clumping are social behaviors that are important for forming and maintaining a pair bond, and are not typically observed until later stages of courtship and pair bond formation (Zann 1996).

Incubation Behavior, Offspring Growth and Reproductive Success:

Each zebra finch pair was provided with a nest box and nesting material (shredded paper and cotton balls) one week after courtship trials (eight days after initial pairing). Nest boxes were then monitored daily to assess nest building and egg laying. When an egg was found, it was weighed and labeled according to laying sequence. Clutch size was recorded once the number of eggs in a nest remained the same for two days. Once a clutch was complete, I recorded incubation behavior three times for each nest across the 13-day incubation period, with trials spaced at least two days apart to provide information on early, middle and late stage incubation. Using small cameras (Hawk Eye Nature Cam X00018UD9X; Sony Pinhole Lens High Resolution CC-7HR) positioned to view inside nest boxes, I recorded behavior in 30-minute bouts. Using BORIS, I later scored male and female parental care behaviors including, time spent in

the nest box, time spent maintaining the nest (i.e., rearranging nesting material), the number of times parents turned the eggs and time spent allopreening the mate or clumping with the mate inside the nest box (Friard and Gamba 2016).

On the day of hatching (day 0), I recorded mass of nestlings with an electronic balance (to the nearest 0.01 g) and individually marked the feathers with markers. Brood size was determined when all viable eggs, established via candling, had hatched. Nestlings were weighed again on days 5, 10, 17, 28, 36 and 50 post-hatch. I measured nestling wing chord, using a ruler (to the nearest mm), and tarsus, using a caliper (to the nearest 0.01 mm), on days 10, 17, 28, 36 and 50 post-hatch. Nestlings were banded with numbered plastic bands on day 10 post-hatch. The number of fledged young was assessed on approximately day 28, all young had exited the nestbox by this measurement day, with fledging typically occurring on day 17 post-hatch (Zann 1996). The number of surviving offspring was recorded on day 50.

Statistical Analysis:

All analyses were conducted with IBM SPSS software for windows (SPSS Inc, Chicago, Illinois, USA), unless otherwise noted. Tests for normality were run on all data. If data were not distributed normally and data could not be normalized, I used non-parametric tests. I first ran correlations to determine whether courtship behaviors were correlated. As with mate choice behaviors, I standardized the behaviors (beak wipes, mountings, clumping and allopreening) into Z-scores. I used a repeated measures two-way ANOVA to determine if changes in the occurrence of beak wiping, which was distributed normally, during the courtship trials differed across treatments and over the

trials. The remaining courtship behaviors, mounting, clumping and allopreening, were binomialized (presence vs. absence) because of the low occurrence of the behaviors on day 1 of trials (Rochester et al. 2010). Mounting was assessed from courtship trial 1 because it was mainly observed within the first 20 minutes of male introduction to the female (Smiley and Adkins-Regan 2016). Clumping and allopreening were observed on both days 1 and 2 of courtship trials, because pair bond formation typically occurs within 48 hours of male and female introduction (Smiley et al. 2012; Zann 1996). I analyzed these behaviors using a Pearson's chi square test (Oehlmann et al. 2000). For post-hoc tests, I used binomial tests to analyze differences between groups.

Incubation behavior data (i.e., time spent in the nest box and time spent on maintenance of the nest) and measures of reproductive success (i.e., clutch size, brood size, hatching and fledging success) were not distributed normally and I was unable to normalize the data using common transformations. As a result, most of these data were analyzed with non-parametric Kruskal-Wallis tests with the exception of clutch size and brood size. I analyzed clutch size using a generalized linear mixed model with a Poisson distribution with paternal treatment as a fixed effect and paternal band number as a random effect. I analyzed brood size the same way, using a generalized linear mixed model with a Poisson distribution and paternal treatment as a fixed effect. Because pairs were only allowed to have one brood, the random effect of paternal band number was not included in brood size analyses. I used LSD post-hoc tests to analyze differences between groups. Additional incubation behaviors (i.e., allopreening, clumping and egg turning) and additional measures of reproductive success (i.e., the number of pairs to lay eggs and successfully hatch eggs) were binomialized (Rochester et al. 2010) and analyzed via

Pearson's chi square test (Oehlmann et al. 2000). For post-hoc tests, I used binomial tests to analyze differences between groups.

Nestling growth (i.e., mass, wing chord length, tarsus length) was analyzed using linear mixed models. Treatment, brood size, sex, and measurement day were included as fixed effects and paternal band number was included as a random effect. Brood size was found to influence nestling weight ($F_{5,25.738}$=2.798, p=0.038) so it was included in all further growth measurements. Sex did not influence nestling weight ($F_{1,79.66}$=1.364, p=0.246), tarsus length ($F_{1,201.18}$=0.823, p=0.365) or wing chord length ($F_{1,135.889}$=0.088, p=0.768); therefore, it was excluded from further analysis. To determine if paternal treatment affected initial size, asymptotic size, or maximal growth rate of nestlings, I utilized a self-starting Gompertz function in R (Version 3.3.1, Package "nlme", Sockman et al. 2008). The estimates of intercept, slope and asymptote from the Gompertz function were then analyzed using linear mixed model analyses in SPSS with treatment and brood size as fixed effects and paternal band number as a random effect.

RESULTS

Courtship Behavior

There were significant correlations between the number of male mountings, the amount of time spent clumping and the amount of time male zebra finches allopreened their mates, however, beak wiping and the amount of time females spent allopreening were not correlated with the other courtship behaviors (Table 1). The occurrence of beak wiping significantly decreased over the 9 courtship trials ($F_{8,38}$=28.701, p<0.0001) but did not differ significantly among treatment groups ($F_{16,38}$=0.958, p=0.503; Fig. 1).

Although the percentage of 4 ng EE2 treated males that mounted females was lower than the percentage of control males, there was no significant difference among treatment groups (Control= 77.7%, 4 ng EE2= 47.1%, 100 ng EE2=62.5%; chi-square= 3.53, df=2, p=0.171; Fig. 2). A higher percentage of pairs with 4 ng EE2 males displayed clumping behavior on day 1 of courtship than pairs with control males, however there was no significant difference among treatment groups (Control= 15.4%, 4 ng EE2= 38.5%, 100 ng EE2= 23.1%; chi-square= 1.88, df=2, p=0.39; Fig 3.). There was no significant difference among groups for the percentage of males and females that displayed allopreening behaviors towards their partners on day 1 of courtship trials (males: chi-square= 0.722, df =2, p=0.697; females: chi-square= 0.223, df=2, p=0.895). At the end of courtship trials, there was no significant difference among treatment groups for the percentage of pairs that displayed clumping behavior (chi-square= 2.76, df=2, p=0.252) or for the percentage of males and females that displayed allopreening behavior towards their respective mate (males: chi-square= 0.619, df=2, p=0.734; females: chi-square= 0.619, df=2, p=0.734).

Incubation Behavior

The amount of time EE2 treated males spent inside the nest box did not differ from the amount of time control males spent inside the nest box across incubation (Early incubation: chi-square=2.38, df=2, p=0.30; Middle: chi-square=0.14, df=2, p=0.93; Late: chi-square=0.91, df=2, p=0.63; Fig. 4a). The amount of time EE2 treated males spent on nest maintenance did not differ significantly from the amount of time control males spent on nest maintenance (Early: chi-square=4.21, df=2, p=0.12; Middle: chi-square=0.45, df=2, p=0.79; Late: chi-square=5.01, df=2, p=0.08; Fig. 4b)

During early incubation, more control males displayed allopreening behavior than males treated with 4 ng EE2 or 100 ng EE2 (chi-square= 8.73, df=2, p=0.013; post-hoc binomial tests: 4 ng EE2: df=8, p=0.006; 100 ng EE2: df=8, p<0.001; Fig. 5). The number of pairs that clumped during early incubation did not differ between pairs with control males and pairs with EE2 treated males (chi-square=2.167, df=2, p=0.338). Male treatment did not significantly affect egg turning behavior during incubation (Early: chi-square= 2.968, df=2, p=0.227; Middle: chi-square=1.074, df=2, p=0.585; Late: chi-square=2.12, df=2, p=0.347; Fig. 6). However, during early incubation, the control group had a higher percentage of males that turned eggs in comparison to the males in the EE2 treatment groups (control= 71.4%, 4 ng EE2 and 100 ng EE2= 33.3%; Fig. 6).

The amount of time females paired with control males spent inside the nest box did not differ when compared to females paired with EE2 treated males across all incubation stages (Early: chi-square=1.701, df=2, p=0.427; Middle: chi-square=2.257, df=2, p=0.323; Late: chi-square=0.469, df=2, p=0.791; Fig. 7a). The amount of time spent maintaining the nest box during early, middle and late incubation stages did not differ between females paired with EE2 treated males and females paired with control males (Early: chi-square=3.763, df=2, p=0.152; Middle: chi-square=3.201, df=2, p=0.202; Late: chi-square=1.060, df=2, p=0.589; Fig. 7b).

I found a non-significant trend that more females paired with control males displayed allopreening behavior toward males during early incubation when compared to females paired with EE2 treated males (chi-square= 5.63, df=2, p=0.06; Fig. 8). This trend was most likely driven by the difference between the percentage of females that displayed allopreening towards control males and the percentage of females paired with 4

ng EE2 males (Control= 40% of females; 4 ng EE2= 0% of females; Fig. 8). There was no difference in the number of times females turned their eggs throughout incubation when compared among male treatment groups (Early: chi-square=0.474, df=2, p=0.789; Middle: chi-square= 4.386, df= 2, p=0.112; Late: chi-square=0.326, df=2, p=0.850; Fig. 9).

Offspring Size and Growth:

Paternal treatment ($F_{2,26.04}$=0.162, p=0.852) did not affect nestling body mass gain. Additionally, neither nestling wing chord growth ($F_{2,23.635}$=0.847, p=0.441) nor tarsus length growth ($F_{2,23.387}$=0.052, p=0.949) were influenced by paternal treatment.

Paternal treatment did not significantly influence the intercept (initial) mass of nestlings ($F_{2,22.359}$=1.989, p=0.160), the asymptotic mass ($F_{2,25.811}$=0.369, p=0.695), or rate of mass gain over time to reach the maximal growth rate ($F_{2,21.888}$=0.711, p=0.502). Paternal treatment did significantly influence the asymptotic wing chord length of nestlings ($F_{2,18.682}$=4.056, p=0.034; Fig. 10) but did not influence the initial wing chord length ($F_{2,21.999}$=0.117, p=0.890) or the maximal wing chord length ($F_{2,22.517}$=0.291, p=0.750). The difference between wing chord lengths was found between offspring of 4 ng EE2 treated males and offspring of 100 ng EE2 treated males (df=19.322, p=0.049) and there was no difference between offspring of controls and offspring of EE2 treatments (4 ng EE2: df=18.942, p=0.112; 100 ng EE2: df=17.838, p=1.00). Paternal treatment did not significantly affect the initial tarsus length ($F_{2,17.895}$=1.305, p=0.296), the asymptotic tarsus length ($F_{2,22.112}$=1.138, p=0.339), or the maximal tarsus length of offspring ($F_{2,14.871}$=0.857, p=0.445).

Reproductive Success:

Females paired with EE2 treated males and females paired with control males were equally likely to lay eggs (chi-square= 1.66, df=2, p=0.44; Fig. 11). There was no difference in the likelihood that eggs would hatch successfully between pairs with control males and pairs with treated males (chi-square= 0.813, df=2, p=0.66; Fig. 11). Additionally, there was no difference in the average clutch size between pairs with control males and pairs with EE2 treated males ($F_{1, 99}$=3.60, p=0.061; Fig. 12). Brood size was found to be significantly different among treatment groups ($F_{1,36}$=4.98, p=0.032). Specifically, the LSD post hoc tests showed the difference was between control brood sizes and 100 ng EE2 brood sizes (p=0.038). Hatching and fledging success were not significantly different between pairs with control males and pairs with EE2 treated males (Hatching Success: chi-square=1.731, df=2, p=0.421; Fledging Success: chi-square=1.575, df=2, p=0.455).

DISCUSSION

I had predicted that EE2 would increase the expression of male courtship behavior but, overall, I found that male zebra finch courtship behaviors were not significantly influenced by EE2 treatment. However, I found some interesting trends of potential biological relevance between control and 4 ng EE2 treatment groups, specifically for mounting and clumping behaviors. Fewer males treated with 4 ng EE2 displayed mounting behavior when compared to control males and more pairs with 4 ng EE2 treated males displayed clumping behavior on the first day of pairing than pairs with control males. This suggests that sexual behavior could be dampened by exposure to EE2

at lower concentrations and that pair bonding behavior could be enhanced at environmentally relevant levels of EE2 exposure. Exogenous estrogen exposure has been demonstrated to exert similar effects on the behavior of male feral pigeons (*Columba livia*; Murton et al. 1969). Males treated with exogenous estrogen displayed fewer sexual behaviors during courtship and instead quickly expressed behaviors typically observed prior to egg laying, most notably males increased the expression of "nest demonstration" behaviors (Murton et al. 1969). Additionally, hypophysectomized males forego initial courtship behaviors and display pairing behaviors towards females when treated with exogenous estrogen (Collias 1946). Furthermore, male zebra finches treated with estradiol during development show decreased mounting probability as adults when paired with females (Rochester et al. 2010). Mounting and attempted mounting are sexual behaviors that occur soon after introduction of a male and female, within an hour in some bird species, as long as the female is receptive (Collias 1946; Murton et al. 1969; Panzica et al. 1998; Rochester et al. 2010; Smiley and Adkins-Regan 2016). Clumping and allopreening behavior are typically indicators that a pair has bonded and in zebra finches can be seen within 48 hours of introduction (Zann 1996). Clumping requires the birds to sit in direct contact, and is generally only observed after pairs have established a bond (Smiley et al. 2012; Zann 1996). Although clumping is important for pair bonding, it is also shown in other social contexts. For example, parents clump with offspring, siblings clump together and unpaired birds clump with birds of the same sex when housed together (Tomaszycki and Zatirka 2014; Zann 1996). This implies that zebra finches clump with familiar conspecifics. Hormonally, endogenous estrogens may play a role in social bonding behaviors such as clumping, and low dose exposure to EE2 could

consequently increase the likelihood of pair bond formation. By the end of the 48-hour courtship period, the same number of pairs in each treatment group displayed allopreening and clumping behavior, which indicates that EE2 exposure did not ultimately affect the likelihood of pair bond formation in captivity. However, in wild populations, earlier pair bond formation would allow a pair to begin nest building earlier and could indirectly influence reproductive success.

The amount of beak wiping males displayed was not influenced by EE2 treatment, but overall there was a decrease in beak wiping across the courtship period, which is consistent with other studies showing that zebra finches display the most courtship behaviors within the first hour of introduction (Smiley and Adkins-Regan 2016). Previous studies have documented the importance of beak wiping during the initiation of courtship (Morris 1954; Zann 1996). For example, the frequency of beak wiping is correlated with the number of song bouts produced by male zebra finches during courtship and indicates the intensity of courtship behavior displayed towards a female (Ullrich et al. 2016). Beak wiping can also occur outside of courtship and mate choice interactions, therefore, it is hard to assess whether the frequency of beak wiping is a reliable indicator of motivation during courtship (Morris 1954). The frequency of beak wiping displayed by males in my study did not correlate with the expression of other courtship behaviors. Unpaired zebra finches display beak wiping and other behaviors observed during courtship initiation towards unfamiliar birds of both sexes and have been recorded to incorporate beak wiping into displacement displays when encountering another conspecific (Morris 1954; Zann 1996). Similarly, beak wiping is observed in other bird species both when individuals encounter novel conspecifics and in the context

of courtship (Whittaker et al. 2015). Male dark-eyed juncos (*Junco hyemalis*) increase beak wiping frequency when exposed to male and female conspecifics (Whittaker et al. 2015). During male-female interactions, male beak wiping was significantly correlated with the expression of other male courtship behaviors, whereas during male-male interactions, increased beak wiping was reflective of competition between males for access to females (Whittaker et al. 2015).

Although I predicted that EE2 exposure would affect male courtship behavior, I expected the behavior of males treated with both the low and high doses of EE2 to be impacted in a similar way. Endogenous estrogens exert their effects at low concentrations. For example, behavioral and physiological effects of estradiol are induced by estradiol concentrations between 0.1-9 pg/ml in humans (Vandenberg et al. 2012). Estrogen receptors display high affinity for estrogens, which activate a response at relatively low circulating concentrations (Vandenberg et al. 2012). EE2, because it was designed to simulate endogenous estradiol, binds to estrogen receptors with similar affinity as the endogenous hormone (Kaspar and Witzel 1985). Increasing concentrations of endogenous estrogens activate negative feedback mechanisms to down-regulate continued synthesis, therefore, the high dose EE2 treatment could emulate this natural response and down-regulate hormone production (Welshons et al. 2003). Hormones increase receptor occupancy; however, at lower concentrations this increase is more substantial than at higher concentrations because there are fewer available receptors (Vandenberg et al. 2012; Welshons et al. 2003). Receptors do not have to be completely saturated to produce a response (Welshons et al. 2003). If the receptors are consistently occupied because of exogenous EE2, then individuals will decrease their responses or

rather their responses will not increase, making it necessary to create more receptors thereby requiring higher circulating levels of estrogens to produce a response (Welshons et al. 2003).

The results I observed for mounting and clumping behavior may be indicative of hormesis (Kendig et al. 2010; Vandenberg et al. 2012; Welshons et al. 2003). A response is considered hormetic when it is larger or occurs more frequently at a lower chemical dose than at a higher chemical dose (Kendig et al. 2010). In toxicology, when the response does not increase with the toxicant dose, but instead behavioral or physiological effects are more likely to be observed at lower doses than at higher doses, this has been interpreted as evidence for hormesis (Kendig et al. 2010). For example, female *Drosophila melanogaster* exposed to low levels of lead were more likely to mate within the first 20 minutes of introduction than females exposed to no lead or higher lead concentrations (Hirsch et al. 2003). Hormonal responses are linked to receptor occupancy, which is not linear when compared to hormone concentration, therefore a lower hormone concentration or EDC concentration can produce a significant response depending on receptor occupancy (Welshons et al. 2003). When exposed to the xenoestrogen octylphenol, spawning mass and egg production of the freshwater snail (*Marisa cornuarietis*) were increased compared to controls but the highest concentration showed a similar response to the second lowest concentration, whereas the middle concentration showed the highest response (Oehlmann et al. 2000). In support of the hypothesis of hormetic effects of EE2 exposure, males in the current study dosed with a high concentration of EE2 more closely resembled control males in courtship behavior, than males treated with a low EE2 dose.

Incubation behavior

I initially predicted that exposure to EE2 would induce greater investment in incubation behavior by males; however, I found that only male allopreening was influenced by EE2 treatment and none of the female behaviors were indirectly influenced by their mate's EE2 treatment. EE2 treatment did not significantly influence the amount of time males spent inside the nest box, nor did male treatment influence the amount of time that female mates spent inside the nest box. Both male and female zebra finches contribute to incubation but the amount of time individuals spend on the nest is variable even under controlled conditions (Delesalle 1986; El-Wailly 1966). It has been shown in barbary doves (*Streptopelia risoria*) that gonadectomized males exhibit incubation behavior when treated with estradiol and progesterone but do not display these behaviors when estradiol is the only treatment (Stern 1974). This could indicate that exogenous EE2 did not affect incubation behavior because males were only receiving EE2 and not added progesterone (Stern 1974). Additionally, the hormone prolactin is important for incubation and parental care as circulating concentrations steadily increase in zebra finches throughout incubation, peaking when eggs begin to hatch (Smiley and Adkins-Regan 2016). Exogenous estrogen is important for inducing nesting, while prolactin plays a central role in sustaining incubation (Buntin 1996; Rochester et al. 2008). This suggests that EE2 exposure could trigger prolactin release in nest building and incubation behavioral contexts, which may then increase nest maintenance behavior throughout the incubation period.

The amount of time spent on nest maintenance ("sprucing" sensu Zann 1996) was reduced in EE2 treated males relative to control males during early incubation. In several

bird species, estrogen exposure induces nesting activity in both males and females (Buntin 1996; Eisner 1960; Lehrman 1958). Control males spent the most time on nest maintenance at the beginning of incubation but then displayed this behavior less frequently during the middle and late stages of incubation. Conversely, EE2 treated males, particularly 4 ng EE2 treated males, exhibited little nest maintenance during early incubation and then showed an increase in the behavior during the middle, and especially the late stage, of incubation. However, these results were not significantly different between EE2 treated males and controls. Male feral pigeons exposed to exogenous estrogen increased the frequency of nest demonstration behaviors but did not increase the amount of time they spent on nest construction (Murton et al. 1969). Therefore, EE2 might influence the motivation to begin nest building, which I did not assess, rather than the maintenance of the built nest. The nest maintenance behavior of females was not affected by male treatment, suggesting that female behavior was not influenced by the behavior of their partners.

Although I expected pair bonding behaviors to be maintained throughout incubation, I only observed pair bonding behaviors during early incubation and these were primarily displayed by control males. In contrast to the effects of treatment during the courtship period, pair bond maintenance behaviors during incubation were reduced in EE2 treated males. Fewer males treated with EE2 displayed allopreening during early incubation in comparison to control males. Unsurprisingly, more females paired with control males tended to show increased allopreening behavior than females paired with EE2 treated males during early incubation but the differences were not significant. Zebra finches engage in a nest building ceremony and perhaps control males and females

display behaviors such as allopreening to strengthen pair bonds during early incubation. During nest building and incubation, exogenous EE2 may redirect male behavior towards parental care behaviors, instead of pair bonding behaviors, such that nest maintenance is increased and allopreening decreases. Estradiol, along with progesterone has been shown to induce incubation behavior in males and females, whereas prolactin takes over subsequently and increases throughout incubation (Buntin 1996; Eisner 1969; Stern 1974).

The occurrence of egg turning was not influenced by male EE2 exposure. Birds turn eggs to insure even heat transfer (Vleck 1981). When the surface temperature of an egg is cooler than normal (35-38°C), zebra finches are more likely to turn the eggs (Vleck 1981). Although not significant, I saw a trend that a higher percentage of control males turned eggs during early incubation than EE2 treated males. This suggests that either eggs were warmer in the nests of EE2 treated males during early incubation or males differed in perception of the egg surface temperature. As incubation progressed, males across the three groups were equally likely to turn eggs. This suggests that as eggs developed, any earlier difference in egg temperature or perception of egg temperature by EE2 treated males seemed to disappear. In the middle of incubation fewer females paired with EE2 treated males displayed egg turning behavior, although this difference was not statistically significant. Zebra finches display biparental care, with males and females devoting approximately equivalent amounts of time to incubation, brooding and feeding of nestlings (Zann 1996). Thus, changes in female egg turning behavior may be a response to differences among male treatments in egg turning. During the early and late

stages of incubation, the same number of females in pairs with control males and EE2 treated males displayed egg turning behavior.

Offspring Growth and Reproductive Success

I had predicted that offspring growth and reproductive success would be influenced indirectly by paternal EE2 treatment and its effects on behavior and I found in a few measurements that paternal EE2 treatment did influence offspring growth and reproductive success. Asymptotic wing length did significantly differ between offspring of 4 ng and 100 ng EE2 treated fathers, with offspring of 100 ng EE2 treated males having a lower average asymptotic wing length. Conversely, EE2 has direct effects on offspring development when young are exposed *in ovo* (Berg et al. 1999; Halldin et al. 1999). There were no significant effects of paternal EE2 exposure on clutch size, or hatching and fledging success. Brood size was significantly decreased in the pairs with 100 ng EE2 treated males compared to the pairs with control males. The number of fertile eggs laid and hatched are reduced in females treated with exogenous estradiol and in conjunction with that fewer males treated with exogenous estradiol sired offspring (Rochester et al. 2008; Rochester et al. 2010). Female zebra finches alter egg composition via carotenoid and vitamin E deposition in response to the attractiveness of their mate (Williamson et al. 2006). Although the EE2 treated male zebra finches did not have significantly different behaviors than control males, their overall appearance (i.e., beak coloration; McGraw et al. 2006) or their song (i.e., song rates; Peterson et al. 2005) could have been influenced by EE2 treatment and thus females may alter egg composition based on features other than behavior. However, EE2 treatment did not affect clutch size or hatching success, thus females paired with 100 ng EE2 treated males laid the same

number of eggs as the other pairs and on average had the same hatching success, but produced smaller broods. These differences in brood sizes should be explored further based on egg composition and other male traits.

Conclusion

Environmentally relevant EE2 concentrations have the potential to influence courtship and incubation behavior in male zebra finches. Conversely, none of the offspring growth parameters and only one aspect of reproductive success (brood size) were influenced by the highest paternal EE2 treatment; therefore, I would conclude that environmentally relevant levels of exposure to EE2 do not influence fitness of zebra finches. In the wild, birds have greater flexibility in mate choice and pair bonding, which may influence the reproductive success of EE2 treated males. Furthermore, under natural conditions, females and offspring would likely be exposed to EE2 as well as males. However, the exposure wild birds would have to EE2 or other estrogenic endocrine disrupters would be more variable, and not as consistent as every other day at specific levels. Additional factors could dilute the potential effects on wild birds such as the proximity to wastewater effluent, diet and the number of contaminated insects and/or water consumed each day. Overall the potential for environmentally relevant levels of EE2 exposure to influence the behavior of terrestrial animals is low, but for aquatic organisms, EE2 exposure continues to be of probable concern based on the current behavioral literature.

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Table 1. Correlation matrix of standardized male and female zebra finch courtship behaviors. Behaviors were standardized to have a mean of zero and a standard deviation of 1 in order to produce a common scale to compare behaviors. * next to r-value indicates statistical significance (*p<0.05, **p<0.001).

	Beak Wipes	**Mountings**	**Clumping**	**Male Allopreening**	**Female Allopreening**
Beak Wipes	1	0.139	-0.139	-0.077	-0.074
Mountings		1	0.372*	0.533**	0.166
Clumping			1	0.874**	0.231
Male Allopreening				1	-0.13
Female Allopreening					1

Figure 1. The average number of beak wipes male zebra finches (n=39) in each treatment group (control, 4 ng EE2 and 100 ng EE2) displayed towards female zebra finches over the nine 20-minute courtship trials ± standard error. The dotted line indicates the transition between day 1 and day 2 of courtship.

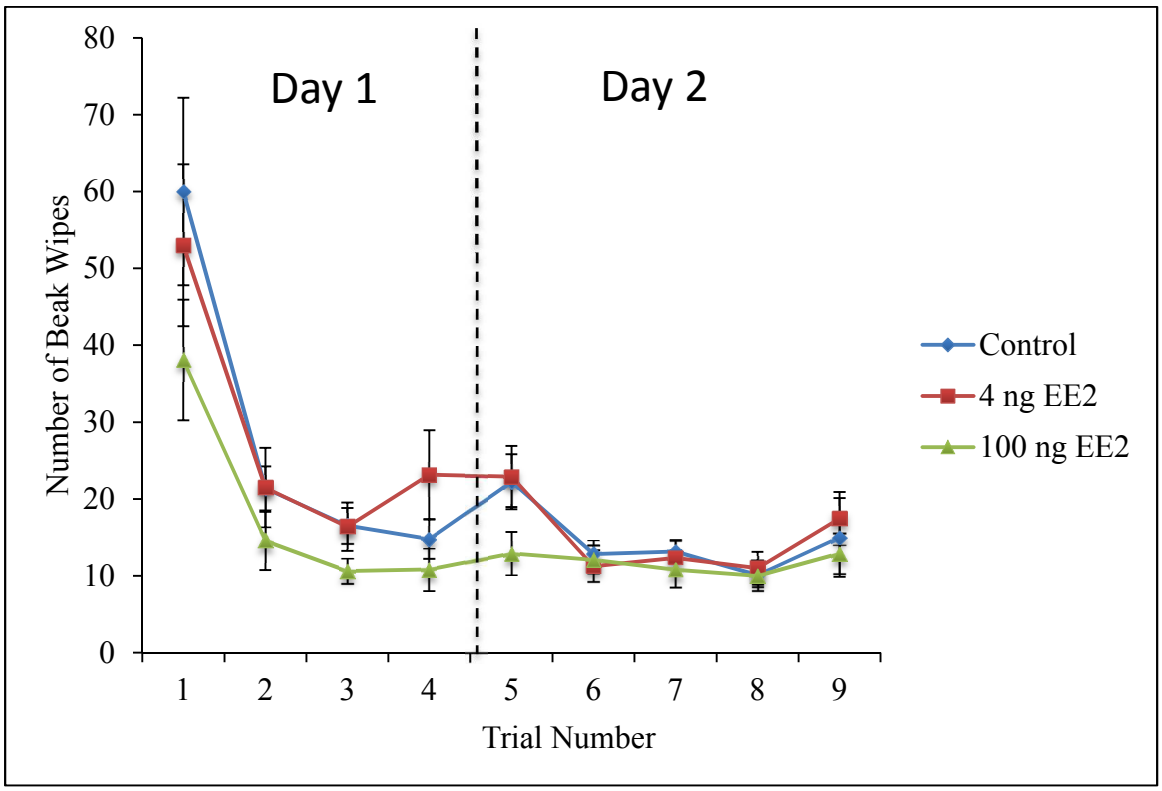


Figure 2. Percentage of male zebra finches (n=39) from each treatment group (control, 4 ng EE2 and 100 ng EE2) that mounted females during courtship trials.

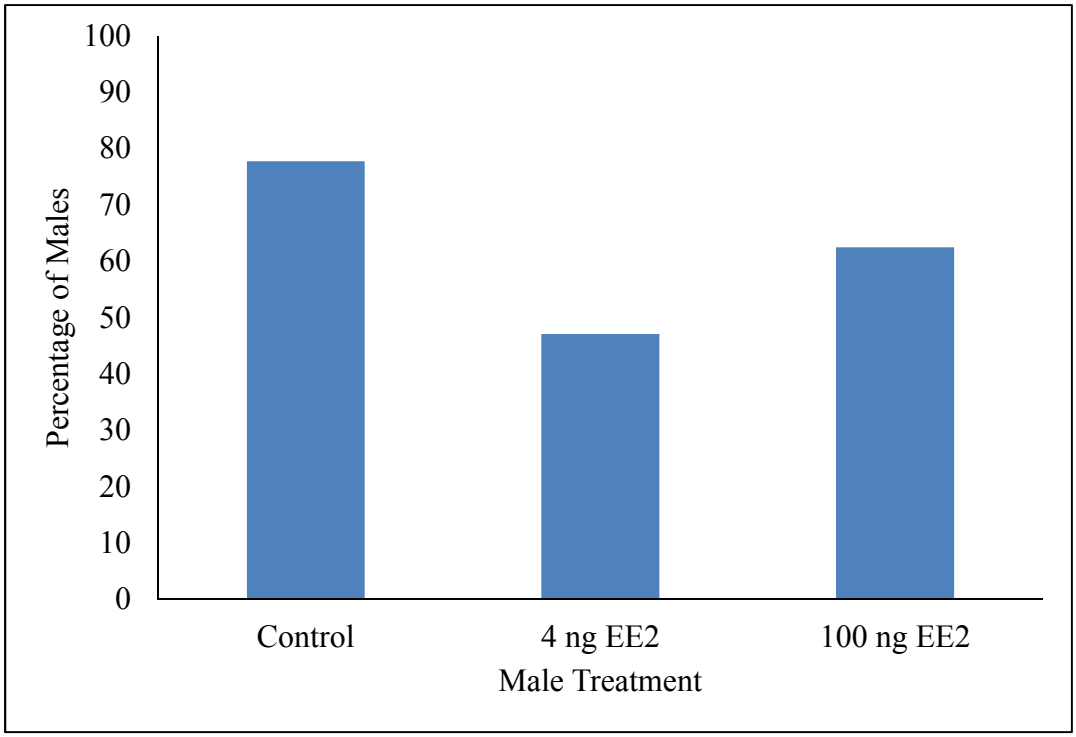


Figure 3. Percentage of zebra finch pairs (n=39) from each male treatment group (control, 4 ng EE2 and 100 ng EE2) that displayed clumping behavior on day 1 of courtship trials.

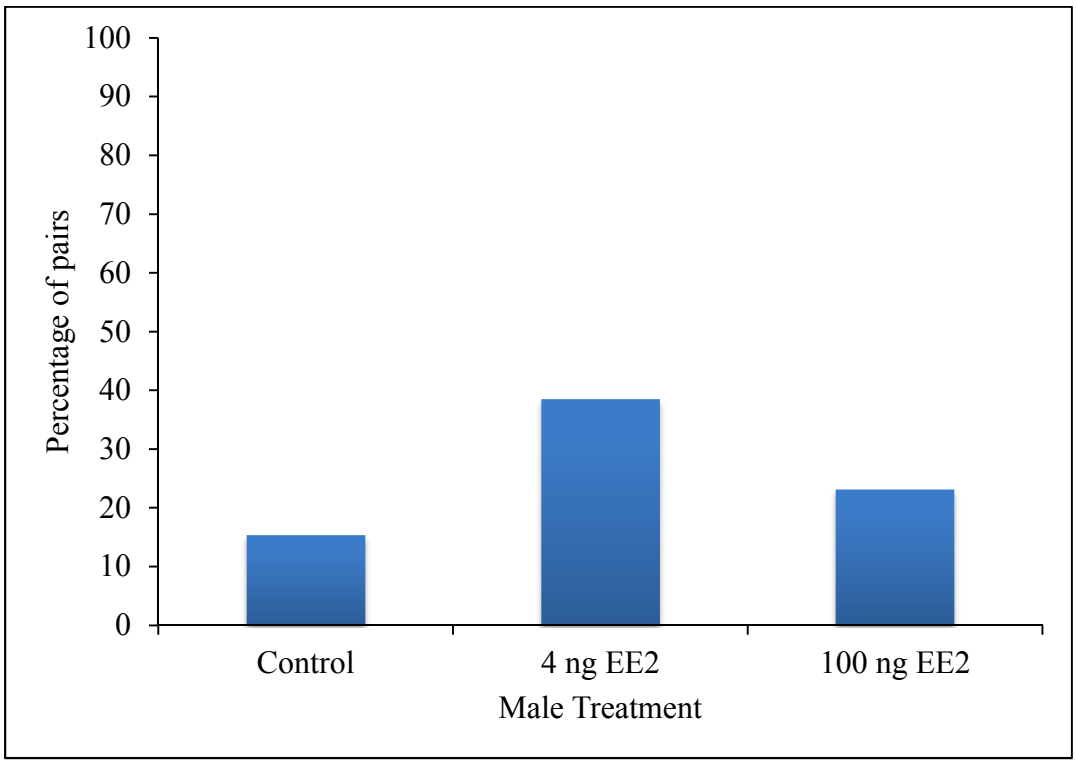
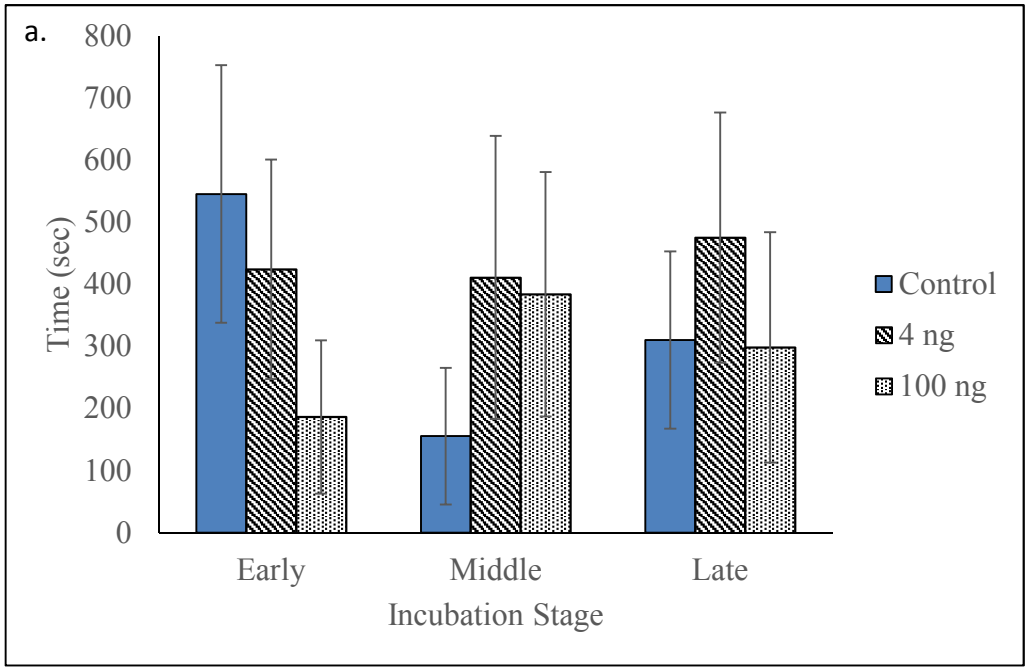


Figure 4. The average amount of time male zebra finches (n=25) from each treatment group (control, 4 ng EE2, 100 ng EE2) spent A) inside the nest box and B) maintaining the nest box during early, middle and late incubation stages ± standard error.



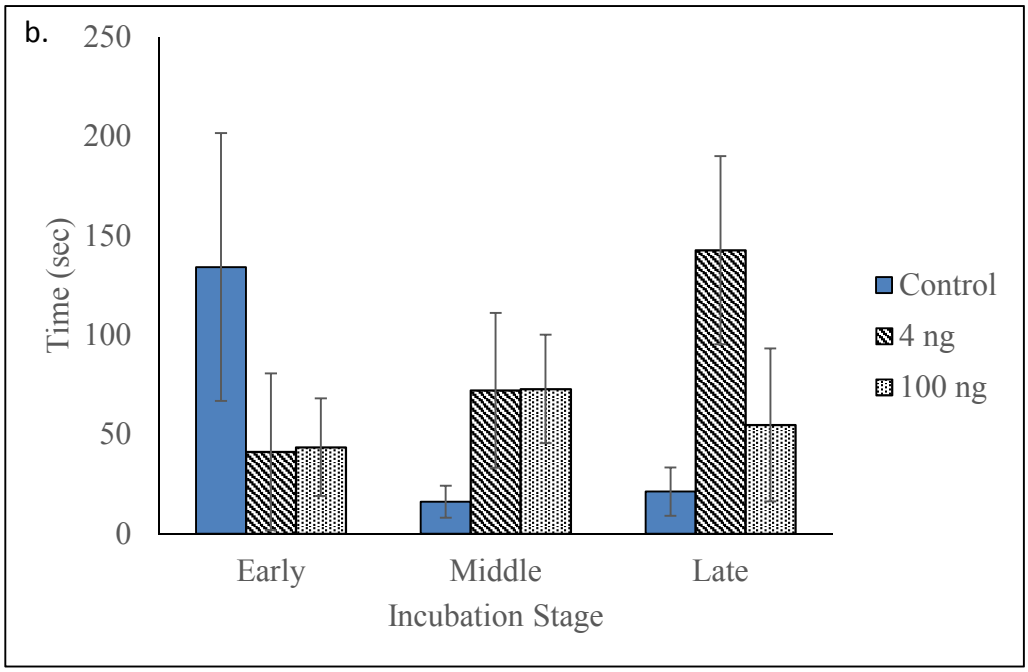


Figure 5. The percentage of male zebra finches (n=25) from each treatment group (control, 4 ng EE2 and 100 ng EE2) that displayed allopreening behavior toward female zebra finches during early incubation. (*) indicates statistically significant differences between control and treatment groups.

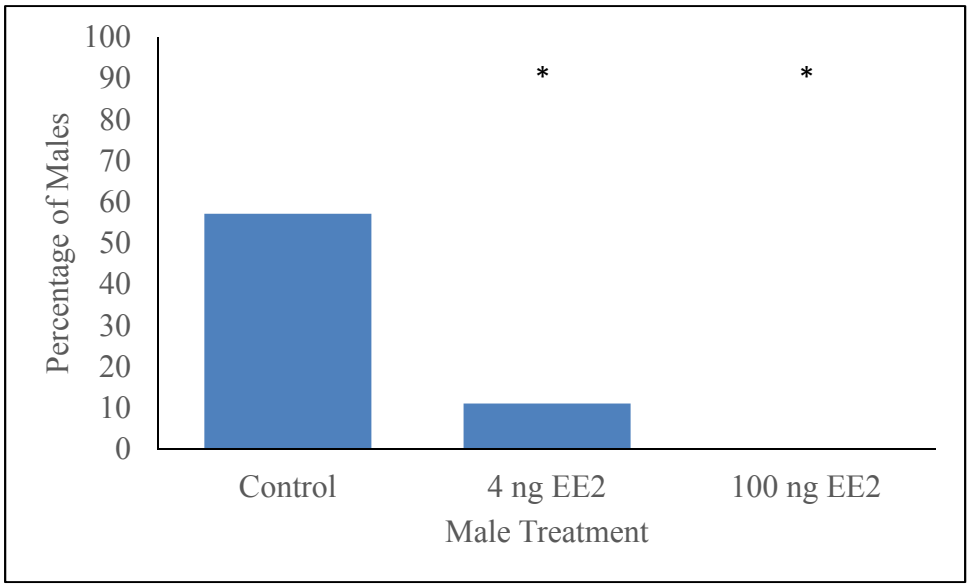


Figure 6. The percentage of male zebra finches (n=25) from each treatment group (control, 4 ng EE2 and 100 ng EE2) observed to turn eggs during early, middle and late stages of incubation.

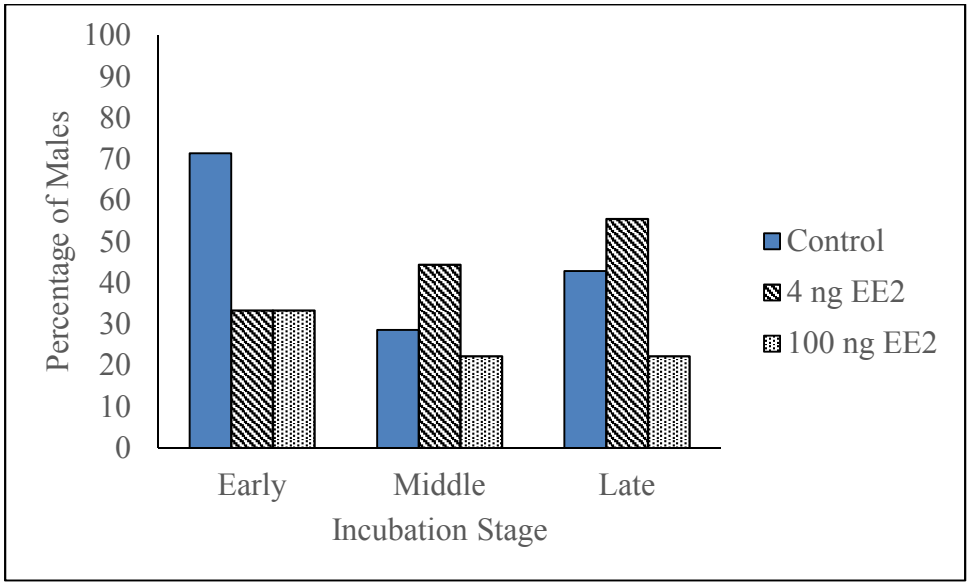
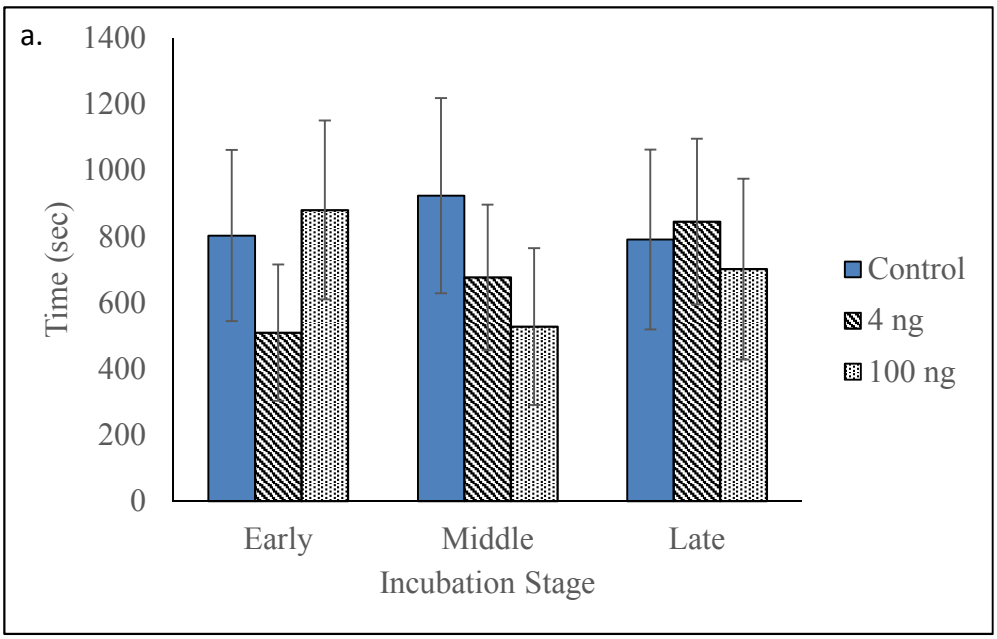


Figure 7. The amount of time female zebra finches (n=25) spent A) inside the nest box and B) maintaining the nest box during early, middle and late incubation by male treatment (control, 4 ng EE2, 100 ng EE2) ± standard error.



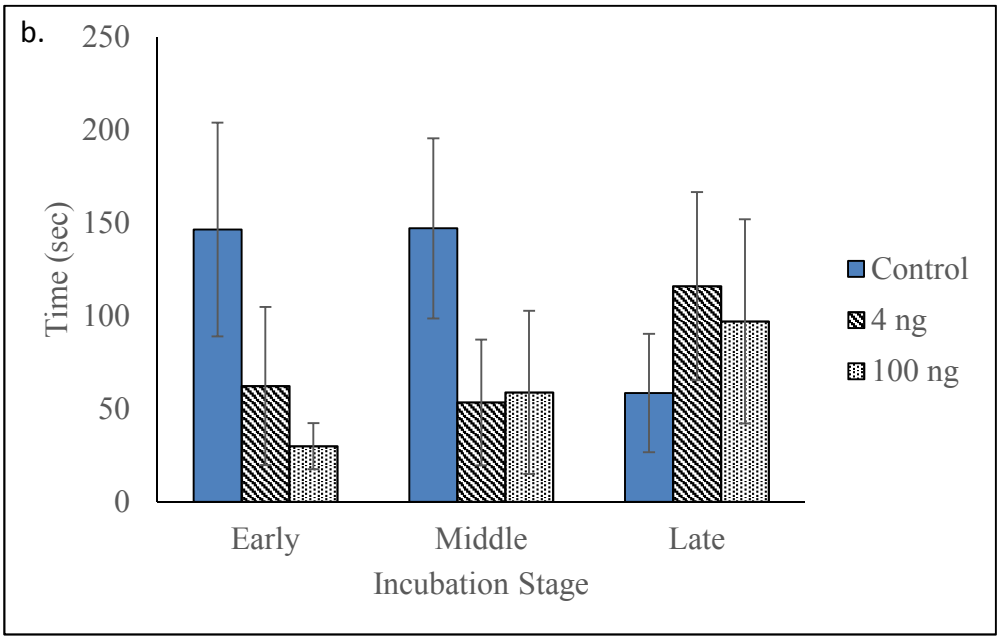


Figure 8. The percentage of female zebra finches (n=25) that were observed to display allopreening behavior towards their mate by male treatment groups (control, 4 ng EE2, 100 ng EE2) during early stage incubation.

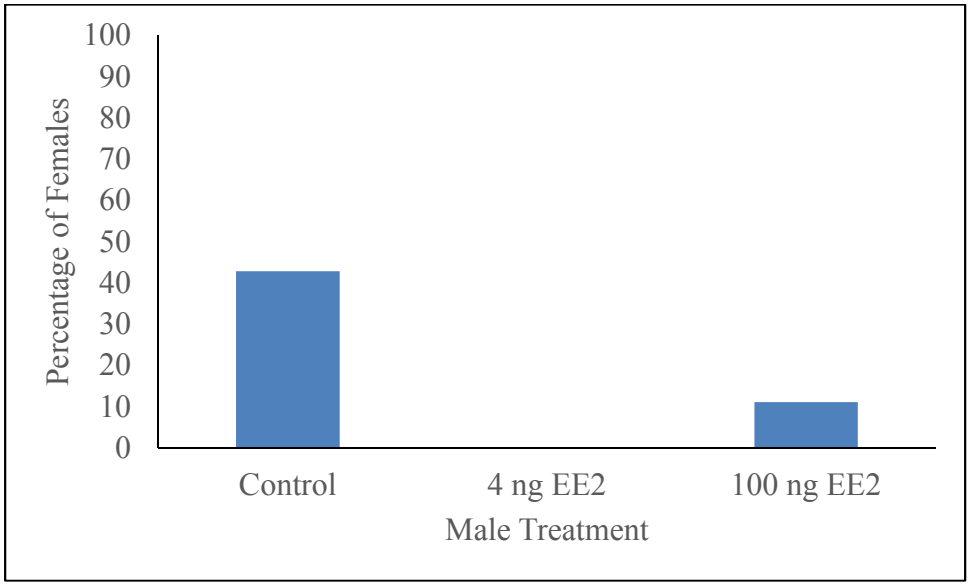


Figure 9. The percentage of female zebra finches (n=25) that were observed to turn eggs by male zebra finch treatment group (control, 4 ng EE2, 100 ng EE2) during early, middle and late incubation stages.

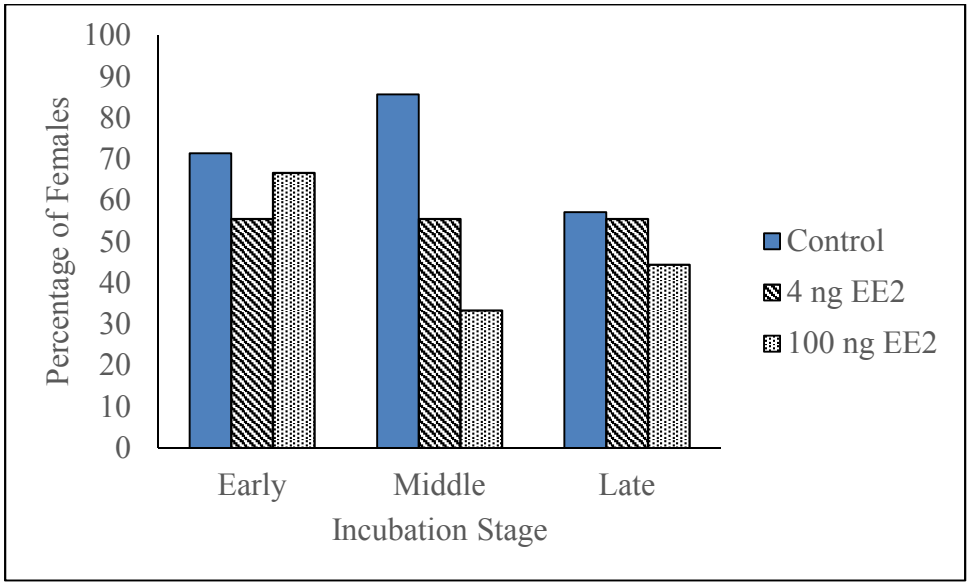


Figure 10. The average asymptotic zebra finch nestling wing chord length (n=93) by paternal treatment group (control, 4 ng EE2, 100 ng EE2) ± standard error. (*) indicates significant differences between nestlings of 4 ng EE2 treated fathers and nestlings of 100 ng EE2 treated fathers.

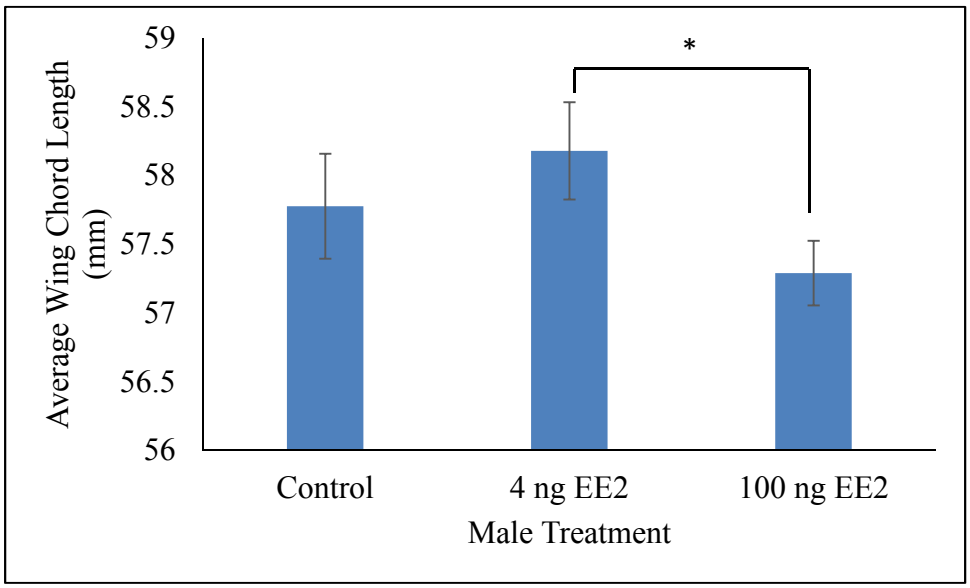


Figure 11. The percentage of zebra finch pairs that laid eggs and hatched eggs by male zebra finch treatment groups (control, 4 ng EE2 and 100 ng EE2). The percentage of pairs that laid eggs was calculated by dividing the total number of pairs (n=82) by the number of pairs that laid eggs (n=59). The percentage of pairs that hatched eggs was calculated by dividing the total number of pairs that laid eggs (n=59) by the number of pairs that hatched eggs (n=38).

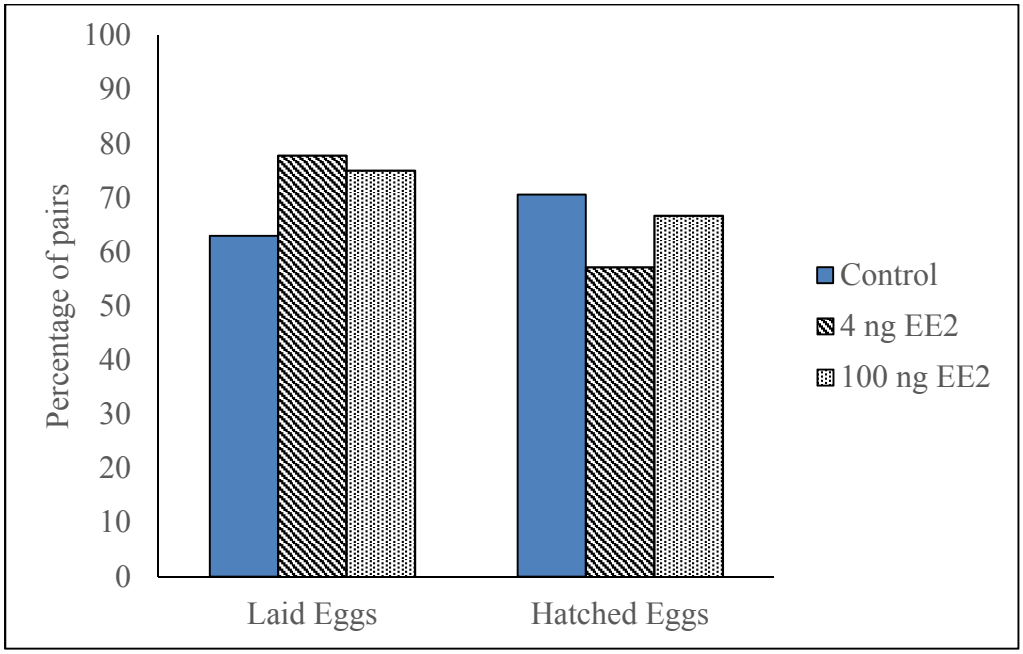
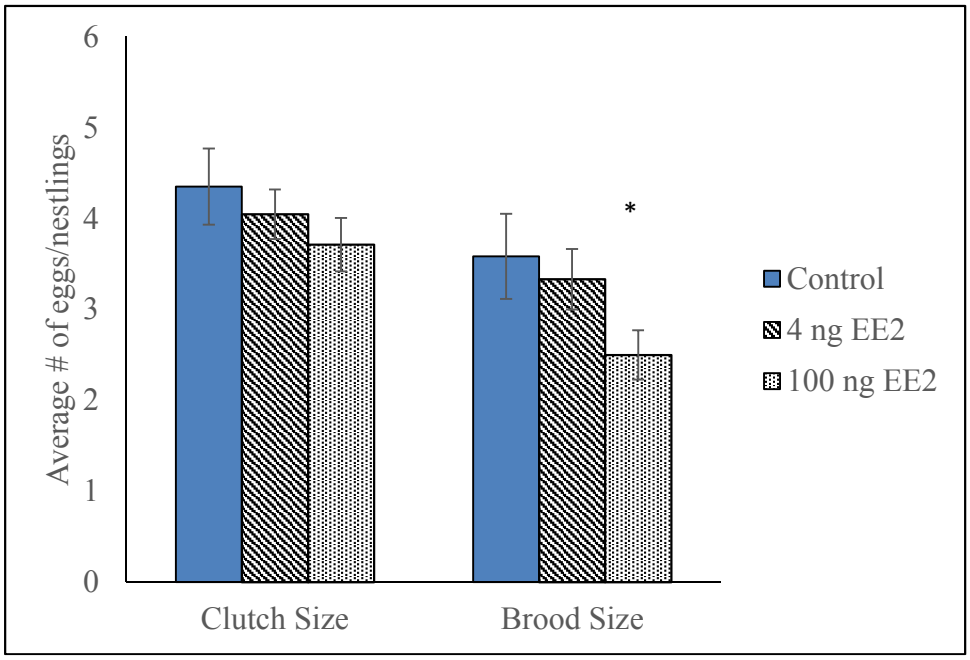


Figure 12. The average clutch size and brood size of zebra finch pairs by male treatment group (control, 4 ng EE2 and 100 ng EE2) ± standard error. The clutch size was calculated from the pairs that laid eggs (n=59). The brood size was calculated from the number of pairs that hatched eggs (n=38). (*) indicates significant differences between control and 100 ng EE2 treatment group.



CHAPTER IV

17α-ETHINYLESTRADIOL INFLUENCES FEMALE BASELINE BUT NOT STRESS-INDUCED CORTICOSTERONE LEVELS IN ZEBRA FINCHES (*TAENIOPYGIA GUTTATA*)

INTRODUCTION

Every year, the number of pharmaceuticals prescribed and taken by humans increases (Shore et al. 2014). Pharmaceuticals are often not entirely eliminated in the body and many are excreted in inactive forms that can become active during wastewater treatment or upon entering the environment (Heberer 2002; Muller et al. 2008). Additionally, most pharmaceuticals are created to have prolonged bioactivity, therefore, their effects in the environment could persist and potentially influence more than one species (Shore et al. 2014). Many studies have focused on the effects of pharmaceuticals on aquatic animals but what is not as widely studied is the effect of pharmaceuticals on terrestrial animals (Bean et al. 2014; Shore et al. 2014). Wild terrestrial animals can be exposed directly to pharmaceuticals through water consumption near wastewater treatment plants and indirectly by consuming other organisms that live near landfills, as occurs when birds or bats consume insects (Markman et al. 2007; Park et al. 2009; Shore et al. 2014). Both chronic and acute effects of pharmaceuticals on aquatic organisms have been widely studied (Kidd et al. 2007;

Mandich et al. 2007; Saaristo et al. 2010; Salierno and Kane 2009) but because terrestrial vertebrates are not consistently exposed to contaminants, the effects of pharmaceuticals could be subtler and less pronounced (Shore et al. 2014).

Many pharmaceuticals are considered endocrine disrupting chemicals (EDCs), which disrupt the normal functioning of hormones within the body (Salierno and Kane 2009). The ability of EDCs to disrupt the hypothalamus-pituitary-gonadal (HPG) axis and reproductive hormones is well established (Kidd et al. 2007; Salierno and Kane 2009). However, through both direct and indirect effects, EDCs may also impact the functioning of other critical endocrine axes, including the hypothalamus-pituitary-adrenal (HPA) axis. The HPA axis is the source of the glucocorticoid hormones, cortisol and corticosterone (CORT). CORT is a metabolic hormone and also is an important mediator of the stress response (Schoech et al. 2009). Animals exposed to pharmaceuticals with endocrine disrupting effects may have altered stress responses and glucocorticoid production. As an example, baseline and stress induced CORT levels are higher in several species of polar seabirds when they are exposed to higher levels of polychlorinated biphenyls (PCBs) (Tartu et al. 2015). House sparrows (*Passer domesticus*) exposed to crude oil have increased glucocorticoid receptor density in fat tissue and decreased density in the liver (Lattin and Romero 2014b). Crude oil exposure did not influence baseline CORT levels, but oil treated birds showed a decrease in CORT response to a stressor (Lattin et al. 2014). This suggests that crude oil decreases CORT secretion possibly by damaging adrenal cells (Lattin et al. 2014). From these studies, there is evidence that some environmental contaminants can influence the stress response, especially at high environmental concentrations.

The HPA axis and the hormones it produces can influence other hormonal axes, such as, the hypothalamus-pituitary-gonadal (HPG) axis. Elevated levels of glucocorticoid hormones are known to suppress reproductive activity by decreasing levels of sex steroids, including testosterone and estradiol, produced by the HPG axis (Lynn et al. 2010; Wingfield 1985). In female song sparrows (*Melospiza melodia*), increases in CORT levels are correlated with decreases in circulating estradiol levels (Wingfield 1985). At the level of the hypothalamus, corticotropin-releasing hormone (CRH) suppresses the release of gonadotropin-releasing hormone (GnRH), thus down-regulating the HPG axis and decreasing investment in reproductive activity (Schoech et al. 2009). Conversely, sex steroids may also impact the HPA axis, but the mechanisms underlying the effects of sex steroids on the stress response are not as well understood. Exogenous testosterone treatment in rats (*Rattus norvegicus*) decreases adrenocorticotropic hormone (ACTH) and CORT secretion, whereas exogenous estrogen treatment increases the release of these hormones from the HPA axis (Zuloaga et al. 2011). Additionally, control female rats released more CORT in response to stressors than control males, which suggests that estrogen may play a role in the release of CORT (Zuloaga et al. 2011). This provides evidence for direct effects of sex steroids on the production of CORT in response to stressors.

The synthetic estrogen in birth control pills, 17α-Ethinylestradiol (EE2), enters the environment via wastewater effluent and is considered an environmental contaminant (Bell 2004; Muller et al. 2008; Saaristo et al. 2010). EE2 has a comparable structure to the endogenous hormone 17β-estradiol (E2), and has the ability to bind to E2 receptors with similar effects as endogenous estrogens (Bell 2004; Berg et al. 1999; Kaspar and

Witzel 1985; Welshons et al. 2003). Research on women taking birth control pills containing EE2 has demonstrated the potential for EE2 to impact the HPA axis. Women using oral contraceptives concurrently with an exogenous cortisol treatment have higher overall levels of plasma cortisol than women not using hormonal contraceptives or men with an exogenous cortisol treatment (Gaffey et al. 2014). Women using hormonal birth control pills have decreased salivary CORT levels but comparable plasma CORT levels to men and women not on birth control (Kirschbaum et al. 1999). This has been linked to increased levels of corticosteroid-binding globulin (CBG), which binds to CORT and allows it to move through the circulatory system. Salivary CORT is representative of free unbound CORT concentrations, whereas plasma CORT includes both free CORT and CORT bound to CBG (Kirschbaum et al. 1999). Therefore, oral contraceptives may increase the production of CBG (Kirschbaum et al. 1999). This would result in comparable plasma CORT levels, but increased bioactivity of CORT (Kirschbaum et al. 1999). In an earlier study, women on oral contraceptives were found to have increased plasma CORT levels compared to untreated women (Carr et al. 1979). These studies tested the effects of oral contraceptives, which contain both EE2 and a synthetic progesterone, on CORT levels. Thus, it is unclear whether the effects on CORT levels are solely the result of exposure to exogenous estrogens in the oral contraceptives or to the progestins in oral contraceptives as well. Nonetheless, the authors hypothesized that EE2 is most likely influencing CORT, more than exogenous progesterone (Kirschbaum et al. 1999).

Alterations to the sensitivity of the HPA axis in parents may influence the stress responses of offspring as well. Rats (*Rattus norvegicus*) raised by stressed mothers are

typically more fearful and have higher basal corticosterone levels in comparison to rats raised by control mothers (Francis et al. 1999). Furthermore, in both guppies (*Poecilia reticulata*) and zebrafish (*Danio rerio*), transgenerational effects of EE2 exposure have been observed in anxiety and stress behaviors (Volkova et al. 2015a; Volkova et al. 2015b). In both of these studies, only the F0 generation was exposed to EE2 during development, whereas the F1 (zebrafish and guppies) and F2 (guppies) were not exposed to EE2. Nonetheless, exposure to EE2 in the F0 generation still had an effect on anxiety behaviors in subsequent generations (Volkova et al. 2015a; Volkova et al. 2015b). Although CORT was not directly measured in these studies, the persistent effects of EE2 exposure across generations suggest that there is a potential for CORT levels of offspring to be affected by parental EE2 exposure.

In this study, I tested the potential for chronic EE2 exposure to influence both baseline CORT levels and stress-induced CORT levels in zebra finches (*Taeniopygia guttata*). Other environmental contaminants and exogenous estrogens have been shown to influence baseline and stress induced CORT levels (Lattin et al. 2014; Tartu et al. 2015; Zuloaga et al. 2011); therefore, I tested whether EE2 exposure would also affect CORT levels. To quantify stress-induced CORT levels, I removed the food source for captive zebra finches, and fasted the birds for four hours (Lynn et al. 2010). I predicted that CORT levels, both pre- and post fasting, would be higher in EE2 treated birds than control birds (Burgess and Handa 1992). I also tested the indirect effects of paternal EE2 exposure on offspring CORT levels. I have found in previous experiments that adult male exposure to EE2 alters incubation behavior (Naylor unpublished data); therefore, I tested for cross-generational effects on offspring stress physiology. I also quantified plasma zinc

concentrations as an indicator of the physiological effects of EE2 exposure. Zinc concentrations are positively correlated with concentrations of the precursor egg yolk protein, vitellogenin, which is produced in both males and females when circulating estrogen levels are high (Mitchell and Carlisle 1991; Wada et al. 2008; Williams and Christians 1997; Williams 1999). Zinc concentrations have been analyzed in several species of birds, including zebra finches, as an index of vitellogenin concentrations (Wada et al. 2008; Williams and Christians 1997; Williams 1999).

MATERIALS AND METHODS

Animal housing:

For the duration of EE2 exposure, prior to fasting trials, birds were housed in same sex cages with 4 (45Wx45Dx45H cm) to 8 (90Wx45Dx40H cm) individuals and provided with seed and water *ad libitum*. Bird seed was a 2:1 mixture of white millet (Stillwater Milling Company; Stillwater, OK) and red millet (Jones Seed Company; Lawton, OK). Once a week, birds were provided with egg food (ABBA 92A, ABBA Products, Hillside, NJ, USA), which was mixed with hard-boiled chicken eggs and avian vitamins (Avian Plus, Zoo Med Laboratories; San Luis Obispo, CA, USA). The aviary was maintained at a temperature of approximately 22°C, a humidity of 20-50% and a light: dark cycle of 14:10h.

Pilot Fasting Study

I conducted a pilot study to determine the minimum amount of time necessary to induce elevated corticosterone levels in zebra finches as a result of fasting. Zebra finches can safely fast for up to 10 hours but corticosterone levels increase in as little as 4 hours

(Lynn et al. 2010). I assigned birds (males: n=20; females: n=20) to one of four different durations of fasting: one, two, three or four hours. Birds were housed in same sex cages with 2 individuals per cage. Blood samples (approximately 100 µl total) were collected within 3 minutes of opening cage doors by puncturing the brachial vein using a 26 gauge needle (Lynn et al. 2010; Romero and Reed 2005). After blood sample collection, I removed the bird feeder from the cage. One, two, three or four hours later, I obtained a second post-fasting blood sample again within 3 minutes of opening the cage door. Blood was spun down to separate plasma at 5000 rpm for 7 minutes in a centrifuge and was then stored in a -80°C freezer. I analyzed hormone concentrations in the plasma samples using a corticosterone EIA kit (Enzo Life Sciences, Inc.; ADI-901-097). Based on previous optimization assays in our lab (Merrill and Grindstaff 2015), as well as in the literature (Breuner et al. 2006; Lynn et al. 2010), with zebra finch plasma, I used a sample dilution of 1:40 and a steroid displacement reagent concentration of 1.5%. Absorbance was measured at a wavelength of 405 nm using a 96-well absorbance plate reader (BioTek ELx808).

To analyze whether the change in CORT levels from pre-fasting (one hour mean±SE: 1.82±0.62; two hour mean±SE: 1.45±0.35; three hour mean±SE: 1.46±0.28; four hour mean±SE: 0.73±0.14) to post-fasting (one hour mean±SE: 3.11±0.46; two hour mean±SE: 5.42±1.22; three hour mean±SE: 3.20±0.67; four hour mean±SE: 6.89±3.12) was significantly different from zero, which would indicate no change, I used a one-sample t-test. I found that CORT levels significantly increased after one, two, and four hours of fasting but not three hours; therefore, I fasted birds for four hours in the subsequent trials (Table 1).

17α-Ethinylestradiol Exposure:

I randomly assigned zebra finches to one of three treatment groups (control, 4 ng EE2 or 100 ng EE2). These treatments were based on environmental EE2 levels and the likely environmental exposure levels of wild passerine birds in areas contaminated with EE2. The average concentration of EE2 found in waterways in the United States ranges from 1-5 ng/L, with maximum reported levels of 831 ng/L (Heberer 2002; Kolpin et al. 2002). Zebra finches drink 3-5 milliliters of water per day. If a water source were contaminated with 1-5 ng/L EE2, then EE2 exposure of the finches would range from 0.003-0.025 ng EE2/day (Calder 1964). If zebra finches drink water with the highest concentration of EE2 reported (831 ng/L EE2), then exposure would be 2.5-4.15 ng EE2/day (Kolpin et al. 2002). Thus, the 4 ng EE2 treatment represents a high level of environmental exposure. The 100 ng EE2 treatment represents exposure above currently documented environmental levels. I exposed the zebra finches to EE2 by pipetting 20 µl of a solution of EE2 and peanut oil (Halldin et al. 1999) into the birds' mouths. Control birds were treated with 20 µl of peanut oil. Zebra finches were treated every other day for three weeks before the fasting stress tests. A three-week period has been used in previous studies to simulate chronic exposure to a contaminant or chronic exposure to a stressor (Lattin and Romero 2014a; Lattin and Romero 2014b).

Fasting Study and Blood Collection

Males (n=16) were from the same group of males used in previous female mate choice and courtship behavioral tests and females (n=18) were from the same group of females used in previous male mate choice tests (see chapters I and II). Stress tests

occurred at least 3 months after mate-choice trials and at least 6 months after courtship and incubation trials in order to ensure that there were no lingering effects of EE2 treatment on males. Males and females were treated on separate occasions for stress tests and behavioral tests. The day before fasting, zebra finches were moved to cages in same sex pairs (45Wx45Dx45H cm). On the day of testing, I treated birds with EE2 or peanut oil (control) one hour before collecting the baseline (pre-fasting) blood sample. I used the same methods for blood collection and plasma storage as in the pilot fasting study. After pre-fasting blood sample collection, I removed feeders from cages and birds were left undisturbed for 4 hours. At the end of this time, a second, post-fasting blood sample was obtained. Feeders were then returned to the cages. Additional plasma from female pre-fasting blood samples was tested for zinc concentrations. Males were blood sampled prior to courtship trials to collect plasma for zinc concentration analysis. For these samples, males had been treated every other day for 3 weeks and blood was collected one hour after EE2 treatment.

I also tested the indirect effects of paternal EE2 exposure during the pre-breeding, egg laying, and incubation stages on the CORT and zinc levels of offspring. Offspring were not directly exposed to EE2 and both male and female offspring were tested approximately 1 to 2 years after hatching so that offspring were considered adults. In brief, offspring (n=55) from 26 unique families were moved into same sex cages in pairs the day before fasting trials. As in adults, I collected pre-fasting blood samples within 3 minutes of opening cage doors, removed feeders immediately after blood sampling, and collected second blood samples four hours later, within 3 minutes of opening the cage door (Romero and Reed 2005). Additional plasma from offspring blood samples, was

tested for zinc concentrations. Because of the low plasma volumes in both pre- and post-fasting blood samples, I combined plasma so that samples from individuals were a mixture of pre- and post-fasting plasma.

As in the pilot study, I analyzed CORT in parental and offspring plasma samples using a corticosterone ELISA kit (Enzo Life Sciences, Inc.; ADI-901-097). The average intra-assay coefficient of variance was 9.8% and the average inter-assay coefficient of variance was 4.4%.

Zinc Analysis

To analyze zinc levels in adults and offspring, I used a zinc assay kit (Sigma-Aldrich; MAKO32). The zinc assay procedure suggests using a plasma sample volume of 50 µl, however, it states that if 50 µl of plasma is not available, use a standard available amount. As mentioned previously, I collected approximately 100 µl of blood from individuals for both CORT and zinc assays (except for adult males), which, once centrifuged, produced varying plasma levels, but typically left less than 50 µl of plasma for analyzing zinc. Therefore, I used 30 µl of plasma mixed with 30 µl 7% tricholoroacetic (TCA) solution, which deproteinizes the plasma (Mitchell and Carlisle 1991). The plasma and TCA mixture was centrifuged for 5 minutes at 13,000 x g, per instruction. I then added 25 µl of the supernatant (insoluble material was concentrated at the bottom of the tubes) in duplicate to a 96-well plate for each sample and brought each well to a final volume of 50 µl by adding 25 µl of deionized water to each sample well. Per kit instructions, 200 µl of zinc reagent mix was added to each well, the plate was incubated in the dark at room temperature for 10 minutes and then absorbance was

measured at 560 nm using a multi-mode plate reader (Molecular Devices Spectra Max M5[e]). The average intra-assay coefficient of variance was 4.6% and the average inter-assay coefficient of variance was 4.9%.

Statistical Analysis

All analyses were conducted with IBM SPSS software for windows (SPSS Inc, Chicago, Illinois, USA). Data were not distributed normally; therefore, all data were log transformed. For measures of parental CORT levels (i.e., pre-fasting CORT, post-fasting CORT and change in CORT) and zinc concentrations, a linear model was run with treatment and sex as fixed effects and time to collect the blood sample as a covariate (for pre- and post-fasting). The time between disturbance and blood sample collection was found to not influence parental CORT levels (pre-fasting: $F_{1,22}$=0.821, p=0.375; post-fasting: $F_{1,22}$=0.00, p=0.994), therefore, to analyze whether there was a difference in CORT between pre- and post-fasting samples, I used a paired samples t-test. Additionally, I found that CORT levels were significantly different between the sexes in parental pre- ($F_{1,22}$=6.91, p=0.015) and post-fasting samples ($F_{1,22}$=4.61, p=0.043), so I analyzed results for males and females separately using a one-way ANOVA and LSD post hoc tests. For offspring CORT and zinc concentrations, a linear mixed model was run with paternal treatment and sex as fixed effects and offspring nest as a random effect to account for the non-independence of siblings, and time to collect the blood sample as a covariate. I found that time to collect the blood sample significantly influenced pre-fasting CORT levels ($F_{1,22,71}$=10.31, p=0.004) but not post-fasting CORT levels ($F_{1,29.93}$=0.36, p=0.55), therefore, I included time to collect the blood sample in a repeated measures ANOVA to analyze the difference between pre- and post-fasting CORT levels.

RESULTS

Parental CORT and Zinc Concentrations

Pre- and post-fasting CORT levels differed by sex, with females having higher mean CORT concentrations than males (Pre-fasting CORT: female mean±SE=4.51±0.84 ng/ml; male mean±SE =1.86±0.73 ng/ml; $F_{1,22}$=6.91, p=0.015; Post-fasting CORT: female mean±SE =10.24±1.52 ng/ml; male mean±SE =4.85±1.58 ng/ml; $F_{1,22}$=4.61, p=0.043; Fig. 1). Males and females both significantly increased their CORT levels during the fasting period (males: t=-4.66, df=12, p=0.001; females: t=-6.77, df=15, p<0.001; Fig 1). However, the magnitude of increase in CORT levels in response to fasting was similar between males and females ($F_{1,29}$=1.28, p=0.269).

Treatment did not influence male pre- or post-fasting CORT levels (Pre: $F_{2,11}$=1.55, p=0.255; Post: $F_{2,10}$=0.732, p=0.505; Fig. 2a). In females, treatment significantly influenced pre-fasting CORT levels ($F_{2,14}$=3.81, p=0.048) but not post-fasting CORT levels ($F_{2,16}$=2.46, p=0.121; Fig. 2b). For pre-fasting CORT, the difference was driven by 4 ng EE2 treated females. Post-hoc analysis showed that females treated with 4 ng EE2 had increased CORT levels in comparison to control and 100 ng EE2 treated females (control mean±SE =3.51±0.97 ng/ml, 4 ng EE2 mean±SE = 7.63±1.76 ng/ml, 100 ng EE2 mean±SE =2.62±0.71 ng/ml; control vs 4 ng EE2: p=0.028; 4 ng EE2 vs 100 ng EE2: p=0.033; Fig. 2b).

Zinc levels in males differed significantly as the result of EE2 treatment ($F_{2,15}$=3.902, p=0.043). The difference in male zinc levels was primarily due to the difference between zinc levels in control males and males in the 100 ng EE2 treated

group (n=6; control mean±SE =0.62±0.08 μg/ml; n=6; 100 ng EE2 mean±SE =0.98±0.16 μg/ml; p=0.016; Fig. 3). The average zinc levels in 4 ng EE2 males (n=6; 0.68±0.04 μg/ml) was intermediate between zinc levels in the control and 100 ng EE2 treated males. Female zinc concentrations over the 3 treatments were variable because the sample size for females (n=8) was smaller than the males and the differences were not significant ($F_{2,5}$=0.17, p=0.849).

Offspring CORT and Zinc Concentrations

CORT levels in offspring did not differ between the sexes (pre-fasting: $F_{1,27.58}$=0.017, p=0.896; post-fasting: $F_{1,27.79}$=1.33, p=0.258) or by paternal treatment group (pre-fasting: $F_{2,12.94}$=1.74, p=0.215; post-fasting: $F_{2,19.418}$=0.229, p=0.798; Fig. 4). CORT levels in offspring significantly increased during the fasting period ($F_{1,32}$=7.728, p=0.01). The elevation in CORT in response to fasting was not influenced by sex ($F_{1,28}$=0.129, p=0.722) or paternal treatment with EE2 ($F_{2,28}$=1.911, p=0.167).

Zinc levels in offspring did not differ between the sexes ($F_{1, 20.36}$=0.009, p=0.924). The offspring of 4 ng EE2 treated males (mean±SE: 0.95±0.15) and 100 ng EE2 treated males (mean±SE: 1.31±0.16) had higher average zinc concentrations than offspring of control males (mean±SE: 0.89±0.10) but it was not a significant difference ($F_{2,14.71}$=0.218, p=0.806; Fig. 5).

DISCUSSION

Parental CORT and Zinc

I predicted that CORT levels, both pre- and post-fasting, would be higher in male and female EE2 treated birds in comparison to control birds. Instead, I found that only pre-fasting CORT levels in 4 ng EE2 treated females were higher than CORT levels of control females. Baseline and stress-induced CORT levels in males were not significantly impacted by exposure to EE2. Baseline CORT has been used as a biomarker for environmental change because it fluctuates to maintain homeostasis within individuals (Homberger et al. 2015; Sorenson et al. 2017). Stress-induced CORT levels indicate the responsiveness of individuals to stressors, in the case of this study, fasting (Homberger et al. 2015). All of the zebra finches tested showed an increase in CORT levels from pre- to post-fasting samples, therefore, it would seem that EE2 did not influence stress-induced CORT production. Daily unpredictable food availability (removing feeders for 4 hours at a time randomly between 8 AM and 8 PM) increases stress-induced CORT levels in comparison to control birds with *ad libitum* food access (Homberger et al. 2015). A prolonged unpredictable stressor in addition to EE2 treatment could be tested in the future to elucidate whether stress-induced CORT levels are influenced. Based on the results of this study, female baseline CORT levels could be used to indicate environmental estrogenic EDC exposure, but more research is necessary to determine if increased CORT responses in females are also observed in other species after EE2 exposure and how CORT levels after EE2 exposure compare to those induced by exposure to other environmental stressors.

Similar CORT response patterns to relatively low environmental levels of contaminants have also been found in tree swallows (*Tachycineta bicolor*) that were exposed to polychlorinated biphenyls (PCBs) in the wild (Franceschini et al. 2008). Adult females living near mid-level PCB contaminated sites had elevated stress-induced CORT levels when compared to females from higher PCB concentration sites and females from reference sites (Franceschini et al. 2008). In my study, female zebra finches exposed to the lower dose of EE2 were also the only group with significantly altered CORT levels, although these were baseline/pre-fasting CORT levels. This pattern of increased CORT at lower levels of treatment or contamination could be indicative of a hormetic effect (Kendig et al. 2010). In another study in which American kestrels (*Falco sparverius*) were exposed to PCB in captivity, baseline and stress induced CORT levels were significantly lower in PCB treated birds compared to controls (Love et al. 2003). However, among the group of birds exposed to PCB, baseline CORT levels were higher in birds with intermediate PCB concentrations in the liver (Love et al. 2003). Baseline CORT levels were lower when PCB concentrations in the liver were either very low or very high (Love et al. 2003). Further research should be done to examine the CORT response in relation to EE2 concentrations in tissues to determine if this pattern holds after EE2 exposure.

I did not find a significant effect of EE2 exposure on female post-fasting CORT levels. Additionally, there was no effect of EE2 exposure on the magnitude of the female CORT response to fasting. Females treated with 4 ng EE2 tended to increase CORT less than control females, likely because the 4 ng EE2 treated females had higher average pre-fasting CORT levels than controls and similar average post-fasting CORT levels to

control birds. 100 ng EE2 treated birds had a similar CORT response to fasting as the control females. In a human study, women on oral contraceptives had higher baseline plasma CORT concentrations in comparison to women not on oral contraceptives and men, but their salivary CORT response to stress was lower (Kirschbaum et al. 1999). Although the women in this study were treated with oral contraceptives (EE2 plus synthetic progesterone), these results are similar to the results I found, suggesting that the effects EE2 has on human CORT production could indicate how environmental EE2 exposure may influence birds in the wild. In some bird species exposed to chronic stressors, stress-induced CORT production is dampened in comparison to control birds (Lattin and Romero 2014a), but this effect is dependent upon stressor type. Furthermore, it has been shown that contaminant exposure, for example PCBs, tends to dampen the stress-induced CORT response (Franceschini et al. 2008; Love et al. 2003). Future work on the effects of EE2 on CORT levels should quantify plasma CORT and CBG capacity to determine if EE2 affects CBG in female birds.

In the parental generation, females had higher baseline and fasting-induced CORT levels than males, but there was no significant difference between the sexes in the change in CORT levels. A previous study of zebra finches also documented significant sex differences in baseline CORT levels, with non-breeding females having higher baseline CORT levels than males (Khan and Robert 2013). However, in the previous study, males produced more CORT in response to stress than females and had significantly higher post-stress CORT levels (Khan and Robert 2013). In that study, males and females were orally treated with exogenous CORT, whereas in my study, birds were fasted for 4 hours. The different stress testing methods could account for the differences seen in the post-

fasting CORT concentrations between males and females (Khan and Robert 2013). Other studies have shown that non-breeding males and females have similar baseline and stress-induced CORT concentrations but males have higher individual variation in CORT levels (Wada et al. 2008). Sex differences in CORT concentrations have also been attributed to the breeding season and whether females are reproductively active (Khan and Robert 2013). Interestingly, it has been found that incubation behavior can decrease circulating CORT levels and the CORT response to stress (Edwards et al. 2013). This is dependent on the species though and the amount of parental care provided (Edwards et al. 2013). During zebra finch fasting trials, none of the birds were in breeding pairs and they were all visually separated from the opposite sex. However, zebra finches can breed year-round so it is uncertain whether circulating CORT concentrations would have been influenced by variation in reproductive status of the birds (Zann 1996).

Zinc concentrations in males were significantly affected by EE2 treatment; specifically, males in the 100 ng EE2 treatment group had higher zinc concentrations than males in the control group. To my knowledge, zinc concentrations have been used only in female birds to assess reproductive stage and have not been used as a bio-indicator of estrogenic exposure in males or females (Mitchell and Carlisle 1991; Wada et al. 2008; Williams 1999). Typically, as EE2 exposure increases, VTG levels increase in both males and females (Kidd et al. 2007). Although, statistically, zinc concentrations were not significantly higher in the 4 ng EE2 treatment group, the elevated zinc levels in this group might be biologically relevant. Because elevated zinc levels were detected in male zebra finches in association with EE2 exposure, it would be worthwhile to further explore using zinc as a bio-indicator of EE2 (or other estrogenic contaminant) exposure in birds.

Offspring CORT and Zinc levels

Offspring of EE2 treated males showed no difference in baseline or stress-induced CORT levels when compared to offspring of control males. In contrast, in both guppies and zebrafish, treatment of the F0 generation, both males and females, had long-lasting transgenerational effects on the offspring (Volkova et al. 2015a; Volkova et al. 2015b). However, these studies did not quantify CORT responses, but instead detected effects on anxiety and stress-related behaviors (Volkova et al. 2015a; Volkova et al. 2015b). Additionally, I did not detect an effect of EE2 exposure on male CORT levels; therefore, it seems unlikely that offspring of EE2 exposed males would be impacted. In zebra finches, as well as other oviparous vertebrates, females deposit steroid hormones into the egg yolk (Boncoraglio et al. 2011; Khan et al. 2016). Other studies have shown that when females are exposed to exogenous CORT during egg formation, their offspring are affected in a number of ways including increased growth rate of exposed chicks (Khan et al. 2016). Additionally, when blood sampled at nutritional independence (30 days), the offspring of CORT exposed females had higher baseline circulating levels of CORT when compared to offspring of control females (Khan et al. 2016). The offspring that I blood sampled were 1-2 years old, and thus any parental effects from the male EE2 treatment might not have persisted in the offspring as they aged. Conducting the fasting study on offspring at an earlier age could be more beneficial to understanding whether the offspring stress response is affected indirectly by paternal EE2 treatment. Future studies should test the effects of maternal exposure to EE2 on the CORT responses of offspring; since EE2 treated females had higher baseline CORT levels.

Unlike in the parental generation, there were no sex differences in the CORT levels of offspring. This could be because offspring were housed in same-sex cages after independence and so they had never been exposed to birds of the opposite sex, therefore, they were not primed for reproductive activity. In the parental generation, males and females were older at the time of testing and both males and females had been previously housed with birds of the opposite sex (see Chapters I and II).

Zinc concentrations of the offspring of EE2 treated males were not significantly affected by paternal EE2 exposure. Further studies are needed to elucidate the role of maternal and paternal exposure to EE2 on offspring hormone levels and the potential for EE2 to indirectly influence zinc concentrations in offspring.

Conclusion

Environmentally relevant levels of EE2 can influence pre-fasting (i.e., baseline) CORT levels in female zebra finches. If exposure to EE2 increases baseline CORT concentrations, then individuals could be exposed long term to higher CORT levels. This could interfere with an individual's ability to maintain homeostasis, thereby increasing an individual's allostatic load (Wingfield 2013). Maintaining appropriate circulating CORT concentrations could take an energetic toll on the body, which could ultimately lead to early death (Haussmann et al. 2011). Conversely, paternal exposure to EE2 does not seem to have indirect effects on the hormone levels of offspring or on offspring growth (Naylor unpubl. data). This means that even if the parental generation is affected by EE2 exposure, offspring might not be impacted if they are not directly exposed to EE2.

However, this would need to be verified through field studies because there are many factors in the wild that could affect parental and offspring exposure to contaminants.

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Table 1. Summary statistics from pilot fasting study of the effect of duration of fasting (1 to 4 hours) on the change in corticosterone levels in zebra finches (n=34).

	One-Hour	**Two-Hour**	**Three-Hour**	**Four-Hour**
t	5.603	5.942	1.236	5.432
df	7	8	7	8
p-value	0.001	<0.001	0.256	0.001

Figure 1. The average log transformed pre- and post-fasting corticosterone (CORT) concentrations for male (n=14) and female (n=16) zebra finches ± standard error. Males and females significantly increased their CORT concentrations from pre- to post-fasting and both pre-fasting and post-fasting CORT levels differed significantly between the sexes.

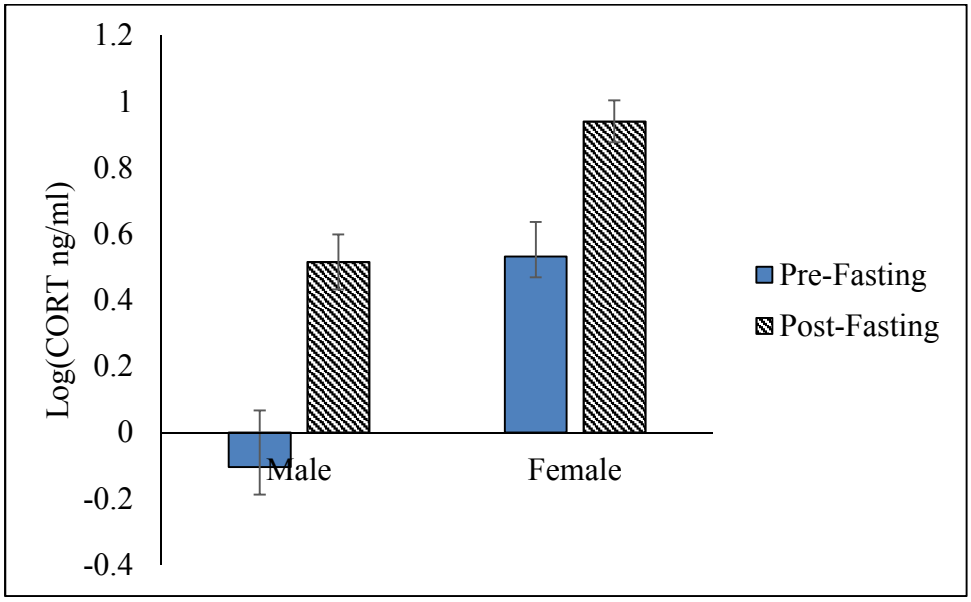


Figure 2. The average log transformed pre- and post-fasting corticosterone (CORT) concentrations for A) male (n=14) and B) female (n=16) zebra finches separated by treatment groups (control, 4 ng EE2, 100 ng EE2) ± standard error. (*) indicates a statistically significant difference among groups..

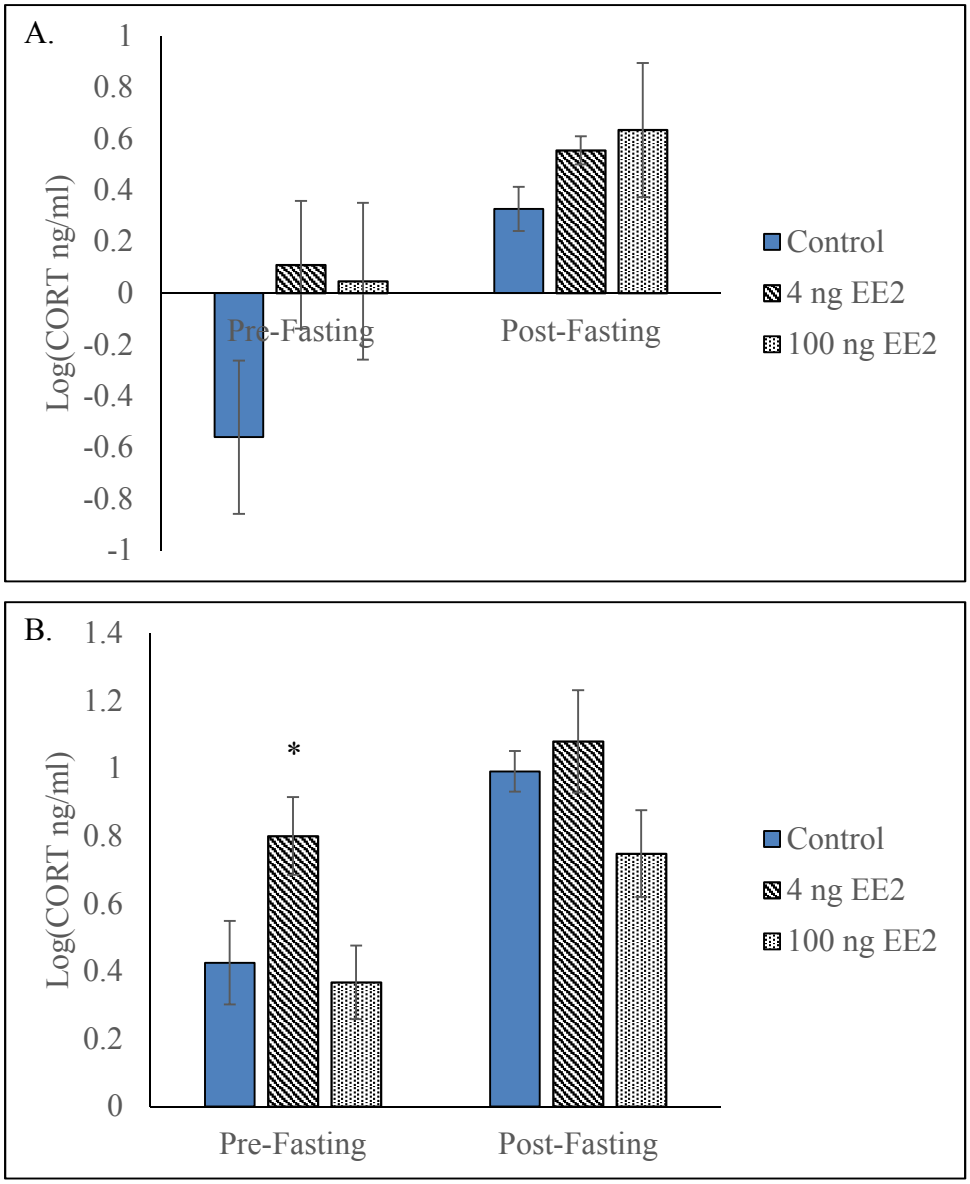


Figure 3. Average log transformed adult male zebra finch zinc concentrations (n=18) by EE2 treatment group (control, 4 ng EE2, 100 ng EE2) ± standard error. (*) indicates a significant difference between control zinc concentrations and 100 ng EE2 zinc concentrations.

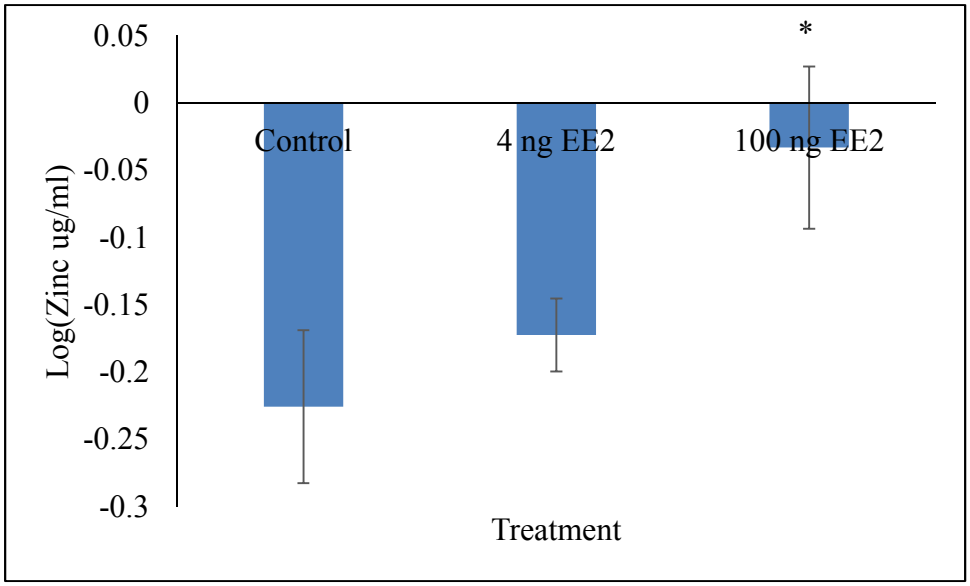


Figure 4. Average log transformed zebra finch offspring (n=36) pre-fasting and post-fasting corticosterone (CORT) concentrations by paternal EE2 treatment group (control, 4 ng EE2, 100 ng EE2) ± standard error.

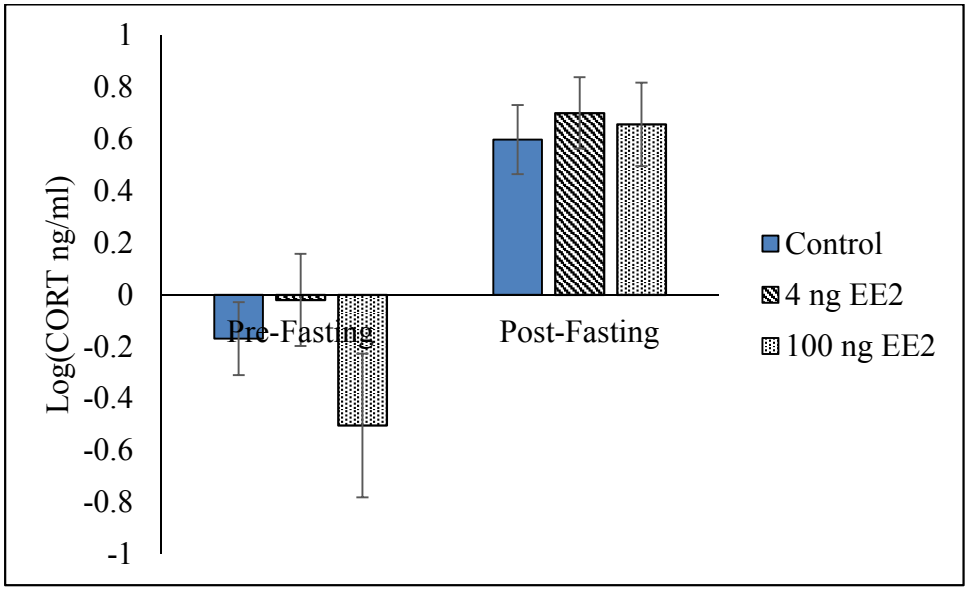
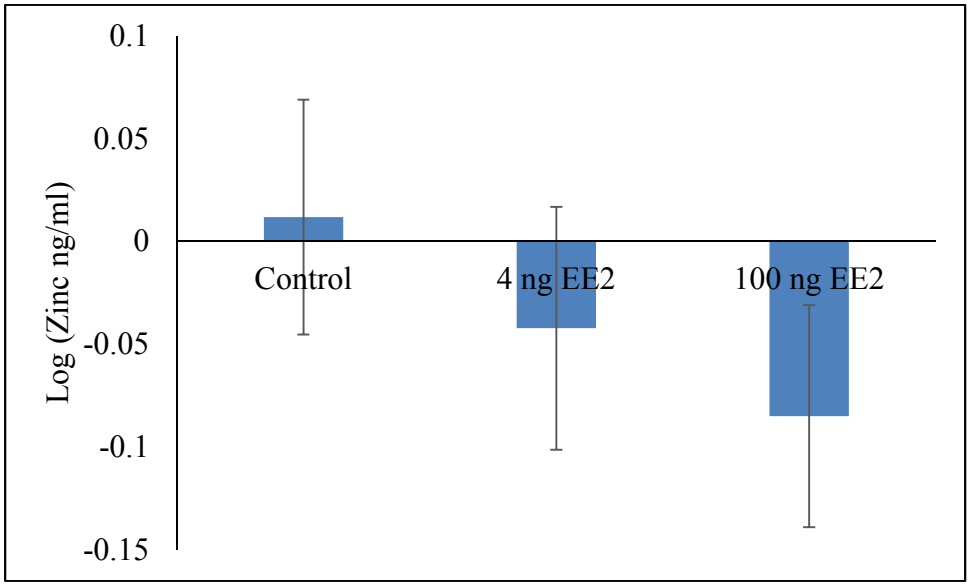


Figure 5. Average log transformed zinc concentrations of zebra finch offspring (n=26) by paternal EE2 treatment (control, 4 ng EE2, 100 ng EE2) ± standard error.



CHAPTER V

SUMMARY

SUMMARY

In chapter II, I tested the potential for EE2 to influence male and female mate choice in zebra finches (*Taeniopygia guttata*). In chapter III, I assessed the potential for EE2 to affect male courtship behavior, parental care via incubation behavior, and nest success (i.e., hatching and fledging success). I also measured offspring growth and assessed if it was influenced by paternal treatment. In chapter IV, I tested whether EE2 exposure affected baseline and stress-induced corticosterone (CORT) levels and/or zinc concentrations in males and females. Additionally, I measured these same two parameters in offspring after paternal exposure to determine whether there are transgenerational effects of EE2 exposure.

In both male and female zebra finches, exposure to EE2 did not detectably impact attractiveness to potential mates. EE2 exposure might not have influenced mate choice because traits that are important for mate choice in zebra finches but that I did not quantify, such as male song and beak color might not have been impacted by EE2 treatment (Collins et al. 1994; Simons and Verhulst 2011; Tomaszycki and Adkins-Regan 2005). Additionally, male mate choice may not have as much of an impact on pair

formation as female mate choice; therefore, males may not express distinct preferences. Furthermore, many behaviors within zebra finch mate choice and courtship necessitate direct contact with the potential mate, therefore, mate choice might be better evaluated in larger cages where males and females could assess more potential partners (Banerjee and Adkins-Regan 2014; Rutstein et al. 2007; Tomaszycki et al. 2006; Tomaszycki and Zatirka 2014).

Male courtship behavior was also not significantly influenced by EE2 exposure. However, there were interesting trends in the group of males exposed to 4 ng EE2. For example, not as many males in the 4 ng EE2 group displayed mounting behavior in comparison to males in the control and 100 ng EE2 groups. Additionally, more pairs with 4 ng EE2 treated males exhibited clumping behavior earlier in the courtship trials than pairs with control or 100 ng EE2 treated males. EE2 treatment reduced the amount of pair bonding behaviors directed towards mates because fewer males treated with EE2 displayed allopreening during early incubation in comparison to control males. This could be because exogenous EE2 redirects behavior away from pair bonding behaviors and towards nest building and incubation (Buntin 1996; Eisner 1969; Stern 1974). However, this change in behavior after EE2 exposure did not significantly influence nest success. Brood size was significantly decreased in pairs with 100 ng EE2 treated males but clutch size, hatching success and fledging success were not influenced by paternal EE2 treatment. This could be due to females manipulating egg composition based on mate attractiveness, which could ensure nestling survival (Williamson et al. 2006). EE2 could influence other male traits I did not assess, such as beak color (McGraw et al.

2006) or song rate (Peterson et al. 2005). Nestling growth, recorded via weight, tarsus length and wing chord length, was not affected by paternal EE2 treatment.

EE2 treatment significantly influenced baseline corticosterone (CORT) concentrations in female, but not male, zebra finches. Females treated with 4 ng EE2 had significantly higher baseline CORT concentrations than control females and females treated with 100 ng EE2. Baseline CORT has been used as a biomarker for environmental change because it fluctuates to maintain homeostasis within individuals (Homberger et al. 2015; Sorenson et al. 2017), therefore, it could be a useful measurement to determine whether environmental contaminants are physiologically impacting individuals.

Exposure to high levels of EE2 significantly impacted zinc concentrations in adult male zebra finches. Males in the 100 ng EE2 treatment group had significantly higher zinc levels than males in the control group. I did not find any significant effects of paternal exposure to EE2 on CORT or zinc levels in offspring.

My dissertation research shows that EE2 can influence some aspects of behavior and CORT levels in zebra finches. I cannot determine conclusively whether EE2 would have the same effects on wild birds because exposure rate could be very different in the field, males and females might be simultaneously exposed to EE2 and offspring might be exposed to EE2 as well. Additionally, in wastewater effluent there are many other contaminants that could also have profound effects on individual behavior and physiology (Kolpin et al. 2002). My study provides novel information on the potential for EE2 to influence avian behavior and physiology. If I were to continue this research, I would focus on treating females with EE2 and testing if courtship, reproductive and

parental behaviors are influenced and whether there might be indirect effects of maternal exposure to EE2 on offspring.

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