THE EFFECT OF CORTICOSTERONE NEAR MEIOTIC SEGREGATION ON OFFSPRING

PRIMARY SEX RATIO IN ZEBRA FINCHES (TAENIOPYGIA GUTTATA)

by

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(Under the Direction of Kristen J. Navara)

ABSTRACT

Chronic elevations of corticosterone have been shown to stimulate female-biased sex ratios in several avian species. Since female birds are the heterogametic sex, skews may occur prior to ovulation through non-random segregation of sex chromosomes at meiosis I. We tested the hypothesis that corticosterone can influence offspring sex ratios immediately before meiosis I completes. In zebra finches, we examined the effects of acute pharmacological and physiological corticosterone elevations near meiotic segregation on primary offspring sex ratios, as well as the relationship between natural baseline levels of corticosterone around the time of meiotic segregation and sex ratios. Females injected with pharmacological levels of corticosterone produced significantly more males. Endogenous elevations of corticosterone, both stress induced and basal levels did not influence sex ratio. These results suggest that a pharmacological dose is needed for corticosterone to induce a skew near meiosis I and that physiological corticosterone may need to be experienced chronically to induce a skew.

INDEX WORDS: primary sex ratio manipulation, corticosterone, zebra finch, stress, meiosis I, non-random segregation

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CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

Female birds exhibit a remarkable ability to manipulate offspring sex ratios. This widespread behavior exists as a striking example of maternal control since offspring genotype, rather than phenotype is being altered. Evolutionary theory predicts that offspring sex can be manipulated in a facultative manner (Trivers and Willard, 1973; Frank 1990) and indeed, three decades of research shows that sex ratio biases correlate with a variety of environmental and social factors (reviewed in Pike and Petrie, 2003; Alonso-Alvarez, 2006). Avian sex ratio manipulation is particularly intriguing because unlike in mammals, female birds are the heterogametic sex suggesting that birds have unique mechanisms for biasing offspring sex ratio. While much research has been done to identify the ecological factors influencing sex manipulation, little empirical research has been done to understand the physiological mechanisms responsible.

Understanding the mechanisms dictating sex ratio manipulation has far-reaching implications. Techniques to artificially manipulate avian sex ratios can be developed by understanding how manipulation naturally occurs. Such techniques would be economically advantageous to both commercial and exotic bird breeding operations. Captive breeding programs would also benefit from such a tool, applying it as a population management strategy for endangered species (Ewen et al., 2001; Lenz et al., 2006; Robertson et al., 2006).

Why bias sex ratios?

Trivers and Willard (1970) predicted that sex ratio adjustment should occur when the fitness benefits of producing sons and daughters differ. The benefits of producing one sex over

another should be dictated by ecological factors and female condition. The fitness benefits from adjusting sex should take the form of increased present reproductive success, future reproductive success or offspring reproductive potential (reviewed in Cockburn et al., 2000).

The first hypothesis developed to explain facultative sex ratio adjustment (Trivers and Willard 1970) is based on the premise that male reproductive potential, unlike female reproductive potential is determined by individual quality. In many mating systems, females will reproduce regardless of quality, while only high quality males will reproduce. Since offspring quality is correlated to maternal quality, the differential reproductive potential of sons and daughters of a given quality will drive sex ratio skews. Mothers in poor condition will invest in more female offspring, since low quality daughters have higher reproductive potential than low quality sons. Alternatively, high quality females should maximize reproductive success by biasing clutches in favor of males. Since offspring quality is also influence by paternal quality and sexually selected traits are heritable, mothers mated to attractive males are also predicted to produce more male offspring since attractive sons experience higher reproductive success than attractive daughters (Burley, 1981; Burley, 1986; reviewed in Cockburn et al., 2001).

While the Triver and Willard hypothesis may be used to explain sex ratio patterns under some circumstances, other life history strategies may influence sex ratio patterns as well. For instance, differential provisioning costs between sexes may act directly on maternal fitness to drive sex ratio biases (Myers, 1978; Gomendio et al., 1990; Wiebe and Bortolotti, 1992). In this case, current and future reproductive potential of the parents drive sex allocation decisions; females in poor condition should produce more of the least costly sex in order to prevent clutch failure and maximize future reproductive output. In cooperative breeding species for example, the costs and benefits of having female siblings around to help parents rear successive broods

patterns may drive sex ratio skews (Clark, 1978; Emlen et al., 1986; Lessells and Avery, 1987). Studies in the Seychelles Warbler (*Acrocephalus sechellensis*) (Komdeur, 1996; Komdeur et al., 1997; Komdeur, 1998) present strong evidence for adaptive sex ratio adjustment where by related females enhance or detract from parental fitness depending on territory quality.

While sex allocation hypotheses provide specific circumstances for facultative sex ratio adjustment, it is likely that multiple competing factors influence sex ratio. This complexity partially explains why some studies report findings that support sex allocation hypotheses, while others contradict predictions or produce biases without clear adaptive value. Further investigation of specific life history strategies will aid in understanding sex ratio adjustment as an adaptive behavior. In order to accurately interpret sex ratio manipulation as an adaptive strategy however, the physiological costs and constraints controlling sex manipulation (Pen and Weissing, 2001; Komdeur and Pen, 2002) need to be identified. Identification of physiological mechanisms will also provide greater predictive power over female sex biasing decisions in response to multiple selection pressures.

How could sex manipulation occur?

1. The Avian Female Reproductive System

The avian ovary contains thousands of small white follicles, each containing Z and W sex chromosomes. These follicles develop slowly and are recruited into the ovulatory hierarchy or undergo apoptosis (Johnson, 2000). Once recruited, deposition of concentric yolk tissue around the follicle occurs 6-11 days before ovulation and stops approximately 24 prior to ovulation (Johnson, 2000). Hierarchical follicles are ranked according to size (F1= largest, F4= smallest). For the majority of the time in the ovary, follicles are arrested in prophase of meiosis I. Half an hour to four hours prior to ovulation of the F1 follicle, meiosis I resumes, dividing the diploid

cell into a haploid ovum and polar body. Since female birds are heterogametic (maintaining Z and W sex chromosomes) allocation of the Z or W sex chromosomes to the oocyte at the completion of meiosis I determines the sex of the oocyte (allocation of Z= male, W= female). The other sex chromosome moves into the polar body with no further developmental potential. At ovulation, the F1 follicle is released from the ovary and captured by the infundibulum. Sperm present in the infundibulum fertilize the newly ovulated ovum. In chickens, the ovum then proceeds through the oviduct over a period of approximately 24 hr before being laid. During this time albumen and the egg shell are added around the ovum. Ovulation of the next hierarchical follicle will commence 15-75 minutes after oviposition (Johnson, 2000).

2. Mechanisms for Primary Sex Ratio Adjustment

Sex ratio manipulation can potentially occur between follicular growth and fledging (reviewed in Krackow, 1995a; Pike and Petrie, 2003; Alonso-Alvarez, 2006). Sex biasing that takes place at or prior to the time of fertilization is defined as primary sex ratio manipulation. Secondary sex ratio manipulation occurs after fertilization when the sex of the ovulated follicle has already been established. Proposed mechanisms for secondary sex ratio manipulation include sex-specific abortion in the oviduct, embryonic mortality and nestling mortality (Fig. 1.1). While birds do utilize secondary mechanisms of sex ratio adjustment, results from more recent studies suggests that primary mechanisms are also at work (reviewed in Pike and Petrie, 2003; Alonso-Alvarez, 2006).

Since avian females are heterogametic, they have the potential to exert fine control over sex ratio manipulation at the primary level (reviewed in Rutkowska and Badyaev, 2008; Fig.1.1). One potential mechanism involves the selective reabsorption of pre-meiotic follicles, whereby follicles bound to become the unwanted sex degenerate and are reabsorbed. Likewise, small

white follicles predestined to become a specific sex could be preferentially recruited into the follicular hierarchy. Offspring sex could also be adjusted via asynchronous development of follicles, where by the rate of follicular growth influences if the ova will become male or female (Krackow, 1995b; Young and Badyaev, 2004). For example, in house finches (*Carpodacus mexicanus*) follicles that grow faster have been shown to have a higher probability of becoming male. This potentially explains variation in sex ratio across clutch order. A third potential mechanism involves the non-random segregation of sex chromosomes at meiosis I. A bias could occur during the allocation of sex chromosomes to the oocyte and polar body during cell division at meiosis I, whereby the desired sex chromosome is allocated to the oocyte (Krackow 1995a; Pike and Petrie 2003). The meiotic drive hypothesis offers the most parsimonious explanation for primary sex ratio manipulation since no follicles are retroactively discarded after sex has been determined and the costs of producing and discarding embryos of the undesired sex is negated (Komdeur et al., 2002).

The adoption of molecular sexing methods to measure primary sex ratios, (as opposed to sexing based on sex specific plumage or phenotype characteristics) has greatly improved our ability to detect skews in primary sex ratio. Extraction of early stage embryonic DNA followed by amplification of the two homologous genes on sex chromosomes using polymerase chain reaction provides better measurements of primary sex ratio because it eliminates biases associated with sex specific embryonic and nestling mortality. Additionally, females lay eggs approximately 24h apart from each other. Studies documenting sex ratio biases generally do not observe egg laying gaps between eggs in a clutch, further suggesting that secondary mechanisms such as sex specific reabsorption of post-ovulated follicles do not account for biases observed. Common molecular sexing methods are limited however when embryonic mortality occurs at

very early developmental stages. This is because unfertilized eggs are difficult to distinguish from early embryonic death and low DNA concentrations from these samples risk contamination with maternal DNA (Arnold et al., 2003).

Steroid Hormones and Avian Sex Ratios

Offspring sex ratios in birds have been correlated to a variety of environmental and social cues, including female condition, male quality and attractiveness, food abundance, season and rainfall (reviewed in Pike and Petrie, 2003; Alonso-Alvarez, 2006). These correlations suggest that changes in the environment trigger physiological responses that cause sex ratio biases. Environmental and social cues induce a cascade of hormonal changes in order to maintain homeostasis, therefore it is likely that the integration of hormonal signals causes sex ratio biases. Reproductive and adrenal steroids are of particular interest because they respond to environmental cues and regulate reproductive processes. Three maternal steroids have been implicated as mediators of sex manipulation in birds, including testosterone (Veiga et al., 2004; Rutkowska and Cichoń, 2006; Goerlich et al., 2009), progesterone (Correa et al., 2005) and corticosterone (Pike and Petrie, 2005; 2006; Bonier et al., 2007). The involvement of 17-β-estradiol has also been examined, but no skews in primary sex ratio were found in relation to elevated concentrations of estradiol in either Japanese quail (*Coturnix coturnix japonica*, Pike and Petrie, 2006) or zebra finches (*Taeniopygia Guttata*, Engelhardt et al., 2004).

There is mixed evidence for the involvement of testosterone in the modulation of primary sex ratios. Exogenous elevations of testosterone produced a male bias in homing pigeons (*Columba livia domestica,* Goerlich et al., 2009; but see Goerlich et al. 2010), zebra finches (Rutkowska and Cichoń, 2006) and spotless starlings (*Sturnus unicolor*, Veiga et al., 2004). Pike and Petrie (2006), however, reported no effect of testosterone implants on offspring primary sex

ratio in Japanese quail. Additionally, Goerlich et al. (2010) found no bias related to elevated testosterone (see Goerlich et al., 2009), and rather a positive relationship between the residual mass of females and the number of males produced.

Evidence for the role of progesterone in sex ratio adjustment is also mixed. Correa, Adkins-Regan et al. (2005) found that a single injection of 2mg of progesterone induced a female bias in White Leghorn hens (*Gallus gallus domesticus*). However lower levels (0.25 mg), in the physiological range did not induce a sex ratio bias, and circulating concentrations of plasma progesterone produced by the high dose of progesterone have been shown to prevent ovulation entirely in both zebra finches (A. Gam, unpublished data) and chickens (Etches and Cunningham, 1976; S.B. Pinson, unpublished data). In addition, treatment of zebra finches with progesterone did not induce a similar sex ratio skew (Correa et al. 2005).

Corticosterone (CORT) is a particularly good candidate as a mediator of primary sex ratios because it is a primary homeostatic regulator, has a diurnal peak near the time of meiotic division (Westerhof et al., 1994; de Jong et al., 2001) and may be involved in ovulation (Etches and Cunningham, 1976). Evidence for the involvement of CORT in sex ratio biasing is also supported by decades of correlational studies linking female condition and food stress to sex ratio adjustments. Corticosterone is released through activation of the hypothalamic-pituitary-adrenal axis (HPA) by physiological or psychological stressors. Chronic elevations in maternal corticosterone correlate with the production of female-biased offspring sex ratios in the offspring of 3 avian species (Pike and Petrie, 2005; 2006; Bonier et al., 2007) providing strong evidence for the involvement of CORT in sex ratio manipulation. Elevations of maternal CORT with silastic implants in quail (Pike and Petrie, 2006) and white-crowned sparrows (*Zonotrichia leucophrys*, Bonier et al., 2007) stimulated females to produce significantly more daughters.

Bonier et al. (2007) also found that baseline CORT was correlated with offspring sex ratio; more females were produced by mothers with higher CORT. Supporting the hypothesized link between environmental stressors, corticosterone and sex ratio manipulation, Okekpe (2009) found that zebra finch females exposed to perceive food stress had higher baseline CORT levels and produced more female offspring. While individual hormones such as CORT and testosterone have been implicated in the sex biasing process, it is highly probable that several hormones mediate this process due do to the regulatory feedbacks between the hypothalamic-pituitary-gonadal axis and the HPA axis.

Zebra Finches as Models for Sex Ratio Studies

Zebra finches are socially monogamous, sexually dimorphic passerines (Zann, 1996) native to Australia and Indonesia (Immelman, 1965) They are practical for avian sex ratio experiments because they breed easily and show a propensity for biasing offspring sex ratios in captivity. Past research suggests that female zebra finches skew primary and secondary offspring sex ratios in relation to several variables including sequence order, male attractiveness and diet quality and quantity (Table 1.1, 1.2). All of the cues shown to stimulate sex ratio skews in zebra finches stimulate hormonal responses that could potentially modulate the observed sex ratio biases.

Proposed Studies

Chronic exposure to elevated CORT levels has been shown to influence primary sex ratios in three avian species. How CORT causes sex ratio biases however is unclear. CORT can potentially influence primary sex ratio at three different time points during follicle maturation in the ovary. The sex of ovarian follicles may be predetermined and which follicles enter the follicular hierarchy may be influenced by CORT. Secondly, follicular growth rates have been

shown to influence offspring sex and CORT influences the amount of yolk precursors deposited into follicles. Therefore CORT may control primary sex ratios by manipulating follicular growth rates. Finally, CORT may act directly on the follicle to influence meiotic segregation of sex chromosomes. None of these mechanisms have been tested empirically.

Here, I tested the hypothesis that CORT and stress can act at the time of meiotic segregation to influence primary sex ratios. To examine the effect of CORT at this critical period in sex determination, studies were designed to acutely elevate CORT levels immediately prior to the completion of meiosis I. The effects of acute pharmacological and physiological elevations of CORT on primary sex ratio were tested. Additionally, I examined the natural variation in baseline CORT levels at the time of meiotic segregation in individual females, in relation to primary sex ratio. Results from these studies shed light on the mechanism by which corticosterone acts to influence primary sex ratio in zebra finches.

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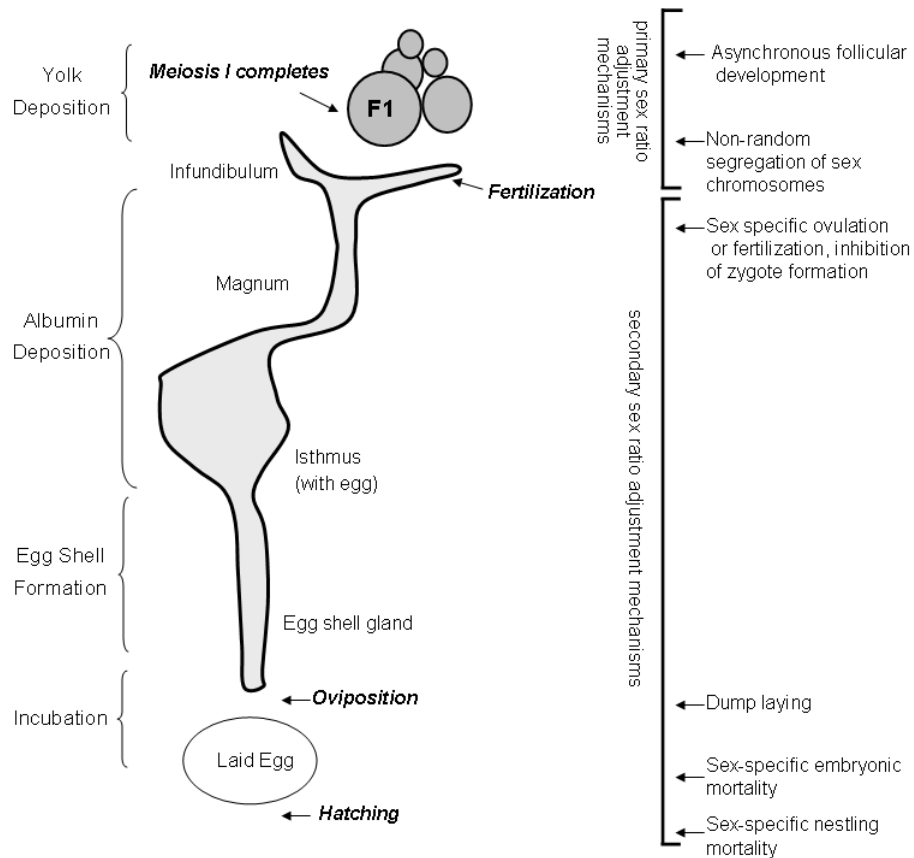
Figure 1.1. Hypothesized mechanisms for female control of primary and secondary offspring sex ratio adjustment.

Table 1.1. Previous studies showing primary and secondary sex ratio adjustments in response to food quality and perceived food abundance in zebra finch

	Effect on sex ratio	Treatment	Reference
Primary sex ratio	male bias in *ad libitum* food group	perceived food abundance with high quality diet: perceived restricted vs. unrestricted food	Okekpe (2009)
	male bias in produced by high quality food group	low vs. high quality diet	Okekpe (2009)
	more males produced by low quality food group	low vs. high quality diet	Rutstein (2004)
	no overall bias	low vs. high quality diet: mothers fed low quality diet produced males in smaller clutches and females in larger clutches	Arnold et al.(2003)
	increased number of males produced with decreasing food quality	diet quality was reduced after start of lay	Rutkowska and Cichoń (2002)
	male bias in low quality food Group	low vs. high quality diet: female biased mortality reported in high quality diet; females on low quality diet were in better condition	Bradbury and Blakey (1998)
	male bias in restricted food group	perceived food abundance: perceived restricted vs. unrestricted food (sexed at hatching)	Kilner (1998)
Secondary sex ratio	no sex ratio bias	wild population of zebra finches provided with food supplementation	Zann and Runciman (2003)

Table 1.2. Previous studies showing primary and secondary sex ratio adjustment in response to male attractiveness in zebra finch

	Effect on sex ratio	Treatment	Reference
Primary sex ratio	no sex ratio bias	manipulated male attractivenss by banding males with red or green bands; experimental unit = flight cage	Rutstein et al. (2005)
	no sex ratio bias	manipulated male attractivenss by banding males with red or green bands; paired in individual cages; female biased mortality observed in attractive group	Rutstein et al. (2005)
	no sex ratio bias	manipulated both male and female attractivness; unattractive males paired with attractive females vs attractive males paired with unattractive females	Zann and Runciman (2003)
Secondary sex ratio	Male bias produced by attractive fathers	simultaneously manipulated male and female attractiveness with band color	Burley (1981)
	Male bias produced by attractive fathers	manipulate male and female separately	Burley (1986)

CHAPTER 2

ACUTE CORTICOSTERONE TREATMENT PRIOR TO OVULATION BIASES

OFFSPRING SEX RATIOS TOWARDS MALES IN ZEBRA FINCHES [1]

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[1] Gam, A.E., M.T. Mendonça and K.J. Navara. Submitted to *Journal of Avian Biology*, 5/20/2010.

Abstract

Researchers have documented significant skews in the primary sex ratios of avian offspring in relation to a variety of environmental and social cues. Zebra finches, in particular, adjust offspring sex ratio according to both the quality and quantity of available food, as well as male attractiveness. The mechanisms behind such manipulation of offspring sex remain elusive. Recent studies suggest that females with chronically elevated corticosterone levels (both naturally and artificially) produce significantly female biased offspring sex ratios. We tested the effects of a pharmacological dose of corticosterone or progesterone administered at the time of sex chromosome segregation on the primary sex ratio of zebra finch offspring to determine whether one or both hormones act on offspring sex at this critical period. Females were injected with 20 μg of corticosterone or 20 μg of progesterone five hours prior to the predicted time of ovulation of the 3rd or 4th ovulating follicle. A third group of females were unmanipulated. The corticosterone treated group produced 72% males while the control group produced 37.5% in the 3rd or 4th ovulation of the sequence. Progesterone injections disrupted ovulation and oviposition in 90% of females. Corticosterone administration did not adversely affect oviposition or ovulation. Females injected with corticosterone had significantly elevated levels of corticosterone 20 min, 1 h and 2.5 h post-injection and produced significantly more males compared to untreated females. Our results suggest that offspring sex ratios may be influenced at the time of meiotic division by acute exposure to corticosterone and provides evidence for the timing of this effect.

Keywords   primary sex ratio manipulation · corticosterone · progesterone · maternal effects · Taeniopygia guttata

Introduction

Significant skews in the primary sex ratios of avian offspring have been documented in relation to a variety of environmental and social cues, in a range of avian species (Burley 1986, Burley et al. 1986, Ellengren et al. 1996, Svensson and Nillson 1996, Pike and Petrie 2005a, Pike and Petrie 2005b, Okekpe 2009). Adaptive theories of sex allocation stipulate that deviations from the expected 50:50 offspring sex ratio will be influenced by environmental and social factors experienced during reproduction; females will adjust offspring sex ratios in accordance with environmental conditions to maximize offspring survival and reproductive success (Trivers and Willard 1973, Lambin 1994, Wright et al. 1995, Wild and West 2007). While many studies have shown that females adjust sex ratios postnatally by varying resource allocation, offspring protection, and other behavioral variables, there is also evidence that females can negate the costly losses associated with sex-specific postnatal mortality by adjusting sex ratios in a primary manner, before fertilization (Kilner 1998, Komdeur et al. 2002, reviewed in Pike and Petrie 2003). The physiological mechanisms underlying biases in primary avian sex ratio however, remain unclear.

Female birds have the potential to control offspring sex prior to ovulation because they are the heterogametic sex, contributing either a W or Z chromosome to offspring. The sex of the offspring is set at the time of the first meiotic division when one sex chromosome is allocated to the oocyte and the other to the polar body, which occurs 2-4 hours prior to ovulation in chicken and quail (Olsen and Fraps 1950, Johnson 1996). It has been proposed that non-random segregation of sex chromosomes during meiosis is a plausible mechanism by which adjustments in offspring sex ratios occur due to this characteristic of birds (Krackow 1995, reviewed in Pike and Petrie 2003, Ruthkowska and Badyaev 2008).

Circulating hormones respond rapidly to environmental and social cues, follow annual and diurnal cycles and are important in reproductive physiology, thus it is likely that one or more hormones act as signal mediators between the environment and the physiological responses that control primary sex manipulation. Three hormones have been implicated as mediators of the sex determination process in birds, including testosterone (Veiga et al. 2004, Rutkowska and Cichoń 2006, Goerlich et al. 2009), corticosterone (Pike and Petrie 2005a, Pike and Petrie 2006, Bonier et al. 2007), and progesterone (Correa et al. 2005). Because of its local abundance at the ovulatory site and its daily peak around the time of meiotic segregation (Etches 1990), progesterone is a promising candidate. Additionally, corticosterone is of particular interest because it is the primary hormone facilitating stress responses in birds, may be functionally important during maturation and ovulation of ovarian follicles (Etches and Cunningham 1976), and has the potential to participate in sex ratio manipulation at the level of the follicle. Indeed, manipulation of both corticosterone and progesterone concentrations in birds can stimulate significant biases in primary sex ratio: previous studies showed that female biases are produced when maternal plasma levels of corticosterone are naturally high and experimentally elevated with implants (Pike and Petrie 2006, Bonier et al. 2007). Also, an acute treatment of female chickens with progesterone at the time of meiotic segregation induced a sex ratio skew towards females (Correa et al. 2005).

Our study was designed to examine the effect of exogenous corticosterone and progesterone treatment at the time of meiotic segregation on offspring sex to determine if these hormones act at this critical time to influence offspring sex. Based on previous studies in other avian species, we predicted that acute treatment of female zebra finches with either corticosterone or progesterone would stimulate the production of significantly more females. To

our knowledge, this is the first study to examine an acute injection of corticosterone on offspring sex ratios in birds.

Methods

1. General procedures

Zebra finch (*Taeniopygia guttata*) pairs obtained from a breeding colony maintained at The University of Georgia were housed in individual cages and provided feed and water *ad libidum*. Birds were kept under a 14 h L: 10 h D light schedule. Egg-laying was monitored daily and eggs marked to record sequence order. Females were injected intraperitoneally after the first or second egg of the clutch was laid with one of two treatments: 20 μg of corticosterone which was initially dissolved in 5 μl of 100% ethanol and added to 45 μl peanut oil (CORT) or 20 μg of progesterone which was also dissolved in 5 μl ethanol and added to 45 μl peanut oil. A third set of females received no injection treatment (UN). Injections were administered 5 h before the estimated time of ovulation because previous work in chickens and quail suggests that meiosis I completes between 2 and 4 h prior to ovulation (Olsen and Fraps 1950, Johnson 1996). The follicle ovulated 5 h after injection and was laid approximately 36 h after injection (Fig. 2.1). Ovulating follicles exposed to treatments were laid as the 3rd or 4th egg in the sequence (3rd egg if treatment was administered on the day the 1st egg was laid and the 4th egg in the sequence if treatment was administered on the day the 2nd egg was laid). We also collected the egg laid prior to the treated egg (pre-target) for additional comparisons with eggs laid by the same female before treatment. All eggs were allowed to incubate in the nest for 8-10 days prior to collection to allow sufficient embryonic development for DNA extraction and molecular sexing analyses. Occasionally these treatments disrupted the oviposition immediately following the injection. Females that delayed oviposition after treatment were excluded from analysis because it was not

possible to determine whether the egg collected the following day was from the ovulation event that occurred immediately following injection or from a delayed oviposition of the follicle ovulated the day prior to injection. Females that delayed oviposition were allowed to complete the clutch after which the treatment was repeated during the next clutch. Progesterone treatment resulted in delayed oviposition of 5 eggs and no visible development was observed in another 4 eggs. Only 1/10 females treated with progesterone laid successfully. For this reason, progesterone treatments were eliminated from further analyses. Delayed oviposition and/or arrested development was not more likely to occur in CORT or UN females, suggesting that the treatments employed did not trigger selective ovulation or infundibular reabsorption of ovarian follicles. The percent of undetectable development observed across treatment were as follows: 17% UN and 14% CORT.

2. Molecular Sexing

Genomic DNA was extracted from embryonic tissue using a standard salt extraction method (Lambert et al. 2000). Portions of the W-linked avian chromo-helicase-DNA binding (CHD) gene in females (CHD-W) and its non W-linked homologue (CHD-Z) were amplified using polymerase chain reaction (PCR) with primers P2 and P8 (Griffiths et al. 1998). PCR products were visualized on a 2.3% agarose gel stained with ethidium bromide. Female embryos were identified by the presence the W chromosome, visualized as two PCR products. Visualization of one band indicated embryo sex as male.

3. Radioimmunoassays

In a separate set of laying females from which offspring sex data were not collected, blood samples were taken 20 min, 1 h, and 2.5 h following the injection of CORT to verify that CORT treatment resulted in an elevation of plasma corticosterone. Because we had a limited number of

breeding birds, the effects of the injections at each time point were examined by injecting and blood sampling in the same birds on different days to avoid bias associated with repeat sampling. Blood samples were also taken from a set of untreated females at time intervals 20 min, 1 h and 2.5 h during each of these injection trials. To ensure that measurements reflect corticosterone elevations associated with the injection and not the stress of handling, samples were taken from the brachial vein within 3 minutes of capture (Wingfield et al. 1982, Romero and Romero 2002). Injections and sampling began at 2300 hours (5 h prior to ovulation, and 4 h prior to lights on) to accurately measure the blood plasma levels of corticosterone and changes in corticosterone levels in response to injections during the approximate period that meiosis occurs. Based on studies done in other avian species, it is likely that the time period from injection to blood sampling is just prior to the natural diurnal corticosterone peak that occurs at the end of the dark period (Westerhof et al. 1994, de Jong et al. 2001). The procedures for extraction and radioimmunoassay of corticosterone from plasma were followed as described by Wingfield and Farmer (1975) using a rabbit derived anti-corticosterone antibody (MP Biomedicals, Solon, OH USA, cat# 07-120016). The recovery rate of corticosterone from plasma was 91.5%. Intra-and inter-assay coefficients of variation were 6.96% and 11.78% respectively.

4. Statistical Analysis

Plasma corticosterone concentrations were analyzed among treatment groups and time points using individual unpaired t-tests. Because we performed three t-tests, we used a Bonferroni correction to account for the increased potential for Type I error. Thus results were considered significant when $p < 0.002$. All hormone data were non-normally distributed and were thus log-transformed for statistical analyses.

We sexed 16 target embryos from UN females and 18 from CORT treated females. Only eggs exposed to the treatment as ovarian follicles were used in this analysis, therefore only one egg, the 3rd or the 4th egg, depending on the day of treatment relative to the laying sequence, was used in this analysis. Differences in the proportion of males produced by birds in the two treatment groups were analyzed for both the pre-target and the target days by performing individual logistic regression analyses in StatView (SAS Institute, Cary, NC USA) using sex as the response variable (male = 1). Chi square values derived from the logistic regression model are reported here. For sex ratios from untreated females, the 3rd egg of the clutch was used for analysis when both the 3rd and 4th egg was available because most treated eggs were from the 3rd position. We were able to use either the 3rd or 4th eggs from untreated females because the sex ratios of the eggs from these two sequence positions did not differ (chi square =0.08, df =1, p =0.78). To compare how the proportion of males produced by birds changed between the pre-target and target days, individual repeated measures logistic regressions, using a generalized linear model with a logit link function in SAS 9.1 were conducted. These analyses were done when both pre-target and target samples from the same female were available. We also used a Fisher's Exact Test to compare the percentage change in number of males from the pre-target to the target eggs between the treatment groups, to see if the way sex ratios were changing between the pre-target and target eggs were different between treatment groups. Posthoc power analyses using G*power 3 show that the sample sizes used in this study are sufficient when comparing single egg samples with proportional differences in sex ratios greater than or equal to 0.33 (Erdfelter et al. 1996, Wilson and Hardy 2002).

Results

CORT injections raised plasma corticosterone levels significantly compared to untreated birds at 20 min, 1 h and 2.5 h post-injection. (t_{17} =22.57, $p < 0.0001$, t_{18} =5.06, $p < 0.0001$, t_{18} = 4.01, $p < 0.0008$; Fig. 2.2).

Females treated with CORT produced significantly more males (72%) in target eggs compared to untreated controls (37.5%), (chi square =3.955, df =1, p =0.046; Fig 2.3). There was no difference in the proportion of males produced between UN and CORT treatment groups on the pre-target day (chi square =0.117, df =1, p =0.7323), indicating that a male-bias was only stimulated following corticosterone treatment. When comparing sexes of offspring produced in the treated eggs versus the eggs laid the day before the treated eggs, the proportion of males produced in pre-target versus target eggs was not statistically different in either the UN or the CORT groups (chi square =1.12, p =0.2909; chi square =1.32, p =0.2505; Fig 2.3), however CORT females produced 28 % more males after CORT treatment while the percentage of males produced by UN females actually decreased by 7% in target eggs; this difference in the percent change between pre-target and post-target in the CORT group was significant (chi square = 5.191, df = 1, p = 0.03).

Discussion

Our results show that exogenous administration of a pharmacological dose of corticosterone just prior to the onset of sex chromosome segregation stimulates a sex ratio skew towards males. In addition, CORT-treated females increased the number of males produced by 28% following treatment while untreated females reduced the number of males produced during the same time period. The finding that there was a statistical difference in the proportion of males produced between the groups only on the target day suggests that treatment with corticosterone was responsible for stimulating the observed male-bias. The limitations of our power analyses were

arbitrarily drawn to detect proportional differences of 0.33, and this was sufficient to detect a significant difference in the proportion of males produced by CORT-treated (72%) versus untreated birds (37.5%) on the target day. While we did not see a statistical change in the proportion of males produced before and after CORT treatment, the 28% increase in the number of males that we observed may, in fact, be biologically significant, but would only be detected statistically using a larger sample size. This limitation should be considered in future experiments.

Multiple studies have pointed to the potential for hormonal control of primary sex ratio through non-random segregation of sex chromosomes during meiosis I (Krackow 1995, Pike and Petrie 2003, Rutkowska and Badyaev 2008). Even though the exact timing of meiotic segregation has not been verified in zebra finches, the fact that our injection at 5 h prior to ovulation (estimated based on the timing of chicken and quail meiotic segregation) affected sex ratios, suggests that the effects of our treatment on offspring sex may be occurring via segregation distortion during meiotic segregation. Of course, it is possible that the 14% of undeveloped eggs in the CORT group would have yielded more males than the 16% of undeveloped eggs in the untreated group if they developed. In this case, the sex ratio bias could potentially have occurred during very early stages of embryonic development. However, if corticosterone stimulated a sex-biased early mortality, we would have expected to see a higher number of undeveloped eggs produced by treated females. Given that the percentages of undeveloped embryos produced by treated and untreated females were so similar, we do not believe the sex ratio bias was occurring during early embryogenesis. This is the first study to examine the effects of acute corticosterone administration at a time point close to ovulation, when the sex chromosomes are likely segregating.

In this study, circulating corticosterone was raised via injection to levels significantly higher than levels that occur within a natural physiological range. This dose was used to see if CORT exposure can induce a sex ratio skew at the critical period of meiotic segregation, and because negative results in a lower dose would have been inconclusive. It is possible however that the pharmacological dose of corticosterone used induced this sex ratio skew via a mechanism other than that which would occur in response to a natural physiological dose of CORT. For this reason it is important to follow up this study with additional tests using physiological doses of corticosterone.

While we hypothesized that treatment with corticosterone during meiotic segregation would skew offspring sex ratios towards females, we instead saw a skew towards males. In previous cases where corticosterone stimulated female biases in sex ratios of avian offspring (Pike and Petrie 2006, Bonier et al. 2007), corticosterone elevations that stimulated sex ratio skews towards females were chronic, long-term elevations. Perhaps chronic corticosterone elevations act on offspring sex through a different mechanism compared to acute elevations. For example, acute elevations of corticosterone may act directly on the ovarian follicle, but chronic elevations may act through the modulation of other hormones, such as testosterone or progesterone, or through other factors that subsequently influence offspring sex. Over the long term, corticosterone can influence the amount of yolk precursors deposited into follicles (Salvante et al. 2003, Salvante and Williams 2003) and, as a result, the rate of follicular growth, which may influence meiotic segregation processes (Young and Badyaev 2004). Alternatively, since glucocorticoid receptors are present in the ovary (Kwok et al. 2007), acute and chronic elevations may act through similar mechanisms but effects vary due to glucocorticoid receptor dynamics on the oocyte that result from chronic corticosterone elevations. Future studies will

need to investigate the mechanisms by which chronic and acute corticosterone exposure produce opposite results on offspring sex ratios in this species.

In comparison to other studies documenting primary sex ratios, our sex ratios from untreated females were similar to those reported in untreated zebra finch colonies from other studies (40%) (Kilner 1998). It seems likely that the corticosterone dose, as opposed to the peanut oil vehicle, is responsible for the male bias we saw in the CORT-treated group, however we were not able to test this with a true control (an oil vehicle injection), because even an injection of peanut oil will induces a significant corticosterone response in the female (A. Gam, unpublished data). Peanut oil was purposefully chosen for the current experiment because of its innocuous nature, and because it does not contain the phytoestrogenic qualities of other oils (e.g. sesame oil) commonly used in similar experiments (Thompson et al. 2006). Thus while we cannot definitively rule out peanut oil as the effector here, we believe it is more likely that the CORT treatment was responsible for the sex ratio skews we saw.

This study provides further evidence for the role of corticosterone in primary sex ratio adjustment, however it adds a layer of complexity, given that acute and chronic corticosterone elevations appear to influence offspring sex differently. Whether or not these effects represent adaptive strategies remains to be determined. There are clear potential adaptive implications for an effect of chronic elevations of corticosterone on offspring sex ratio; it is well established that females of many species can influence offspring development to optimize offspring phenotype for a specific postnatal environment (Groothuis and Schwabl 2008, Navara and Mendonça 2008, reviewed in Badyaev and Uller 2009). However, the benefits of adjusting offspring sex in response to a single, acute stress event are less clear and require more study, and the adaptive nature of any physiological form of investment cannot be adequately addressed until the

mechanisms are understood. We show here that sex manipulation can occur through hormonal manipulation at the expected time of meiotic segregation, lending insight into mechanisms that may be responsible for primary sex ratio adjustment in birds.

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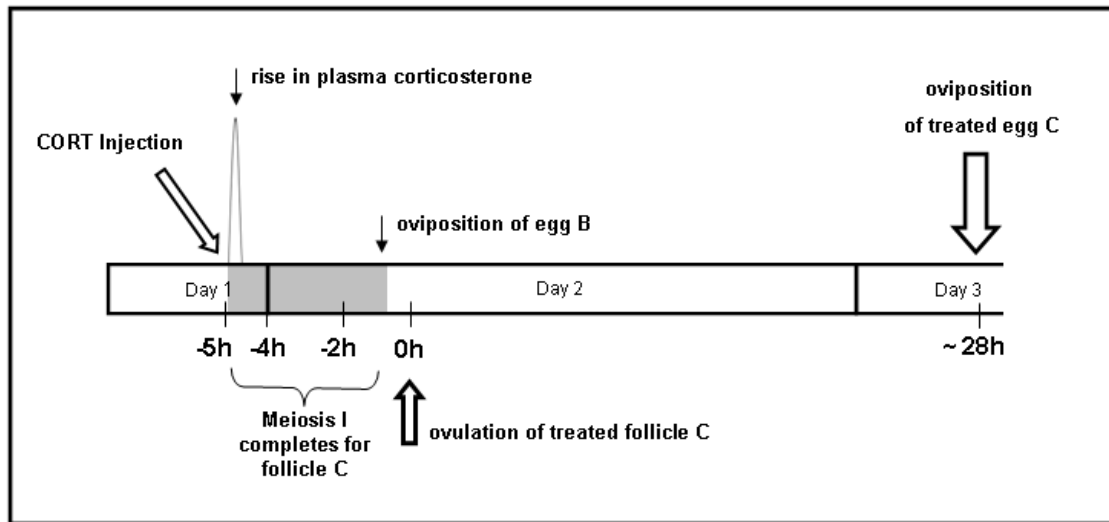
Figure 2.1. Injections were timed to raise circulating corticosterone levels just prior to the period that meiosis I occurs. Meiosis I of the target follicle C occurs 0.5-4 h before ovulation and ovulates (time 0) approximately 15-75 minutes after oviposition of egg B. The treated follicle C then precedes though the oviduct until oviposition approximately 36 h after injection.
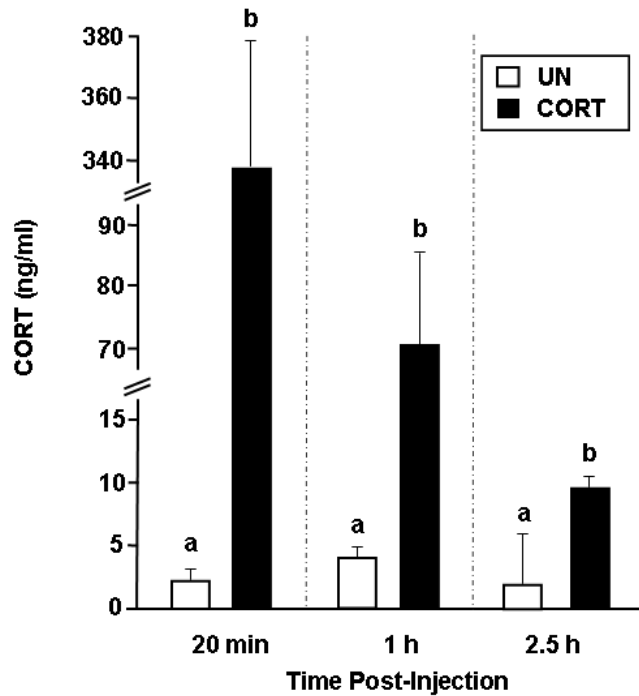
Figure 2.2. Plasma corticosterone concentrations (mean + standard error) of females injected

with 20μg corticosterone (n= 10, 10, 7) and non-manipulated females (n= 9, 10, 13). Plasma

corticosterone levels were measured 20 min, 1 h and 2.5 h after injection of CORT. Comparison

of corticosterone levels was performed between treatments at each time point and not between

time points. CORT injections significantly raised plasma corticosterone levels 20 min, 1 h and

2.5 hr after injection. All hormone measurements were log transformed for statistical analysis.

Different letters above denote values that are statistically different.
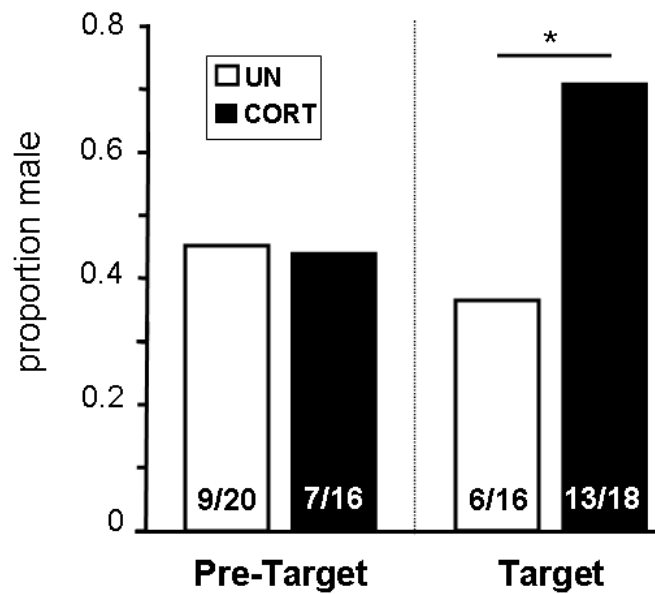
Figure 2.3. Primary sex ratio (proportion of male embryos) produced by non-manipulated and corticosterone treated females, before treatment (laid 1 d prior to target eggs) and after treatment (laid 2 d following injection). Corticosterone injected females produced significantly more male embryos than non-manipulated females after injection. * p =0.046.

CHAPTER 3

EFFECT OF ENDOGENOUS MATERNAL CORTICOSTERONE AT MEIOSIS I ON

OFFSPRING PRIMARY SEX RATIO IN ZEBRA FINCHES[1]

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[1] Gam, A.E and K.J. Navara. To be submitted to *General and Comparative Endocrinology.*

Abstract

Evidence exists for female control over offspring primary sex ratio in avian species. The underlying mechanisms controlling adjustments however remain unclear. Results from experimental and correlative research suggest that maternal corticosterone plays an important role in this process. Since females are the heterogametic sex in birds, corticosterone may potentially bias offspring sex ratios during meiosis I, through non-random segregation of sex chromosomes. In a previous study, we showed that pharmacological elevations of corticosterone near the time of meiotic segregation exerted an effect on offspring sex ratio, causing female zebra finches, *Taeniopygia guttata* to produce a male biased sex ratio. Here, we aimed to determine whether physiological elevations have a similar effect on sex ratio through two experiments. First we examined offspring sex ratio in relation to baseline corticosterone levels to determine if natural variation in circulating corticosterone near the time of meiotic segregation influences sex ratio. In the second experiment a 5 min bag handling protocol was used to induce an acute elevation in endogenous corticosterone 1 h prior to the period meiosis I completes. Maternal baseline corticosterone levels did not correlate with average clutch sex ratio. The sex ratio produced by females exposed to the bag stress did not differ from the sex ratio produced by un-manipulated females. Together these results suggest that physiological levels of endogenous corticosterone, both baseline and acutely elevated near the time of sex determination may not be involved in the adjustment of primary sex ratios in zebra finches.

Introduction

Skews in offspring sex ratio has been documented in response to a variety of ecological factors in species from many avian families (reviewed in Pike and Petrie, 2003; Alonso-Alvarez, 2006). While manipulation of offspring sex through secondary processes such as sex specific embryonic and nestling mortality accounts for some of the variation in sex ratios observed, increasing evidence exists for maternal control over sex ratio prior to ovulation. Primary sex ratio manipulation is possible in birds because females are heterogametic and maternal allocation of a Z or W sex chromosome to the ova determines offspring sex. While proximate explanations for primary sex ratio adjustment have been proposed, potential mechanisms have not been examined empirically.

Circulating hormones provide a practical connection between external factors and physiological processes that lead to primary sex ratio biases because hormones respond quickly to changes in the environment and play a regulatory role in female reproductive physiology. Three maternal steroids have been implicated as mediators of sex manipulation in birds through experimental evidence; testosterone (Veiga et al., 2004; Love et al., 2005; Rutkowska and Cichoń, 2006; Goerlich et al., 2009), progesterone (Correa et al., 2005) and corticosterone (Pike and Petrie, 2005; 2006; Bonier et al., 2007). Several other lines of evidence provide support for the role of corticosterone in particular. Corticosterone is the primary glucocorticoid that regulates physiological responses to physical and psychological stressors in birds (Holmes and Phillips, 1976) and baseline corticosterone levels correlate with many of the ecological factors associated with sex ratio biases including female condition (Marra and Holberton, 1998; Kitaysky, Wingfield, et al. 1999; Duckworth et al., 2001; Romero and Wikelski, 2001), social status (Creel, 2001) and food abundance and quality (Clinchy et al., 2004; Schoech et al., 2004; Okekpe,

2009). Additionally, corticosterone has a diurnal peak near the time of meiotic division (Westerhof et al., 1994, de Jong et al., 2001) and is required for ovulation (Etches and Cunningham, 1976).

Long term elevations in maternal corticosterone correlate with the production of female-biased offspring sex ratios in the offspring of 3 avian species (Pike and Petrie, 2005; 2006; Bonier et al., 2007) providing strong evidence for the involvement of chronic corticosterone in sex ratio manipulation. Elevations of maternal corticosterone with silastic implants in quail (Pike and Petrie, 2006) and white-crowned sparrows (Bonier et al., 2007) stimulated females to produce significantly more daughters. Bonier et al. (2007) also found that baseline corticosterone was correlated with offspring sex ratio; more females were produced by mothers with higher corticosterone.

Since chronic, long term elevations of corticosterone cause a variety of physiological changes, it is unclear whether corticosterone mediates biasing processes directly or indirectly through correlated parameters. Chronic corticosterone in starlings causes changes in blood chemistry (Awerman and Romero, 2010) and decreased body weight (Rich and Romero, 2005). In general chronic corticosterone acts to suppress reproduction (Schoech et al., 2009) and reproductive steroids such as progesterone and estrogen (Sapolsky et al., 2000; Murase et al., 2002). Additionally, exogenous corticosterone treatment through implants reduces that amount of yolk precursors and depresses follicular growth during the period of rapid yolk deposition in chickens and zebra finches (Salvante et al., 2003; Salvante and Williams, 2003) and the rate of follicular growth may influence meiotic segregation whereby more of one sex is produced in early clutch sequences (Young and Badyaev, 2004).

Corticosterone may induce primary sex ratio skews through several proposed mechanisms (reviewed in Pike and Petrie, 2003) including sex specific follicular development, abortion of the oocyte, follicular atresia or non-random segregation of sex chromosomes (Rutkowska and Badyaev, 2008). While few studies have examined the precise timing of primary sex manipulation (Komdeur et al., 2002; Correa et al., 2005; Pike, 2005; Goerlich et al., 2010) segregation distortion of sex chromosome at the time of meiotic segregation offers a parsimonious explanation. Completion of the first meiotic division yields assignment of one maternal sex chromosome to the ova and the other to the polar body with no further developmental potential, 0.5-4 hours prior to ovulation in chickens and quail (Olsen and Fraps, 1950; Johnson, 1996; Yoshimura et al., 1993). Preferential allocation of one sex chromosome to the ova (allocation of a Z $\rightarrow$ yields a male, W$\rightarrow$ yields a female) could lead to biases while circumventing costs associated with sex specific abortion and follicular atresia.

Recently, the potential relationship between circulating corticosterone levels at the time of meiosis I and offspring sex ratio adjustment was examined by administering an acute pharmacological dose of corticosterone prior to meiotic segregation in zebra finches (A. Gam in review) and chickens (S.B. Pinson in review). Females with elevated corticosterone levels produced significantly more male offspring in both species. However, since a high exogenous dose of corticosterone was used in this experiment, it is unclear whether results shed light on a natural mechanism or if the skew was pharmacologically induced.

To investigate the role of corticosterone within a physiological range on offspring sex ratio, we examined the effects of acutely elevated and natural baseline levels of endogenous corticosterone prior to meiosis I. In the first experiment, blood samples were collected from laying females 1 h prior to meiotic division to determine the relationship between baseline

corticosterone levels near the time of meiotic segregation and offspring sex ratios. In the second experiment we induced an acute rise in corticosterone 1 h prior to the onset of meiotic division in laying zebra finch females and analyzed the sex ratio of eggs exposed to the treatment as pre-ovulatory follicles. While natural maternal corticosterone level have been shown to correlate with offspring sex ratio in white-crowned sparrows, this is the first study to measure circulating baseline levels near the time of sex determination. Following the results of studies examining chronically elevated corticosterone levels and sex ratio, we predict that higher endogenous corticosterone levels will correlate with a primary sex ratio bias towards females.

Methods

1. Animals and housing

Paired zebra finches (*Taeniopygia guttata*) maintained at The University of Georgia were housed in individual cages and provided feed and water *ad libidum*. Birds were kept under a 14h L: 10h D light schedule. Egg-laying was monitored daily and new eggs marked to record sequence order.

2. Natural corticosterone experiment

To determine the relationship between natural corticosterone levels near meiosis I and offspring sex ratio, baseline blood samples were collected from laying females 1 h prior to the period meiosis I completes. Blood samples were taken after 1st, 2nd or 3rd in the clutch was laid (n= 8, 5, 4). Since sex ratios do not differ across clutch sequence in our population (A. Gam unpublished data), correlations between baseline corticosterone levels and offspring sex ratio should not be confounded by the timing of blood samples relative to clutch position.

3. Acute stress experiment

The effect of an acute endogenous rise in corticosterone on sex ratio was examined by inducing a stress response 1 h before the onset of meiotic division. A standard 5 min bag handling protocol was chosen because it has been shown to elevate corticosterone levels 20 min after initiation of the stressor (Wingfield et al., 1982). Females were randomly assigned to one of two treatments. The first group bred undisturbed. The second group received the bag handling treatment.

Previous work suggests that meiosis I completes between 0.5 and 4 h prior to ovulation in chickens and quail (Olsen and Fraps, 1950; Johnson, 1996; Yoshimura et al., 1993), and during previous experiments in zebra finches, corticosterone administered 5 h prior to ovulation induced changes in offspring sex ratios. Therefore, stress handling was administered 5 hours before the estimated time of ovulation (Fig 3.1). Treatments always took place the night the first egg of the clutch was laid. Within 5 h after administration of the handling stress, the largest preovulatory follicle (F1) exposed to the treatment would ovulate, would pass through the reproductive tract, and would be oviposited approximately 36 h later as the 3rd egg in the clutch sequence. Since exposed follicles would become the 3rd egg in the clutch, embryos from the 3rd egg in sequence were used for sex ratio analysis for both un-manipulated and bag stress groups.

Stress treatments occasionally disrupted oviposition of the egg immediately preceding treatments. Females that delayed oviposition after treatment (un-manipulated = 7, bag stress= 11) were excluded from analysis because it was not possible to determine whether the egg collected the following day was from the ovulation event that occurred immediately following treatment or from a delayed oviposition of the follicle ovulated the day prior.

In a separate set of laying females from which offspring sex data were not collected, blood samples were taken 20 and 60 min after administration of the stress protocol to verify that bag handling elicited a rise in corticosterone. Blood samples were also collected from a set of un-manipulated females at the same time. Samples were taken from the brachial vein within 3 minutes of capture (Wingfield et al., 1982; Romero and Romero, 2002) to ensure that measurements reflected corticosterone elevations associated with the treatment.

4. Molecular sexing

For both experiments, all eggs were allowed to incubate in the nest for 3-4 days prior to collection to allow sufficient embryonic development for DNA extraction and molecular sexing. For the bag stress experiment, only the 3rd egg in the clutch was analyzed from un-manipulated and bag stress groups, because that is the egg that would be affected by the treatment. For the natural corticosterone experiment, the entire clutch was sexed in order to determine average clutch sex ratio in relation to baseline corticosterone levels.

Genomic DNA was extracted from embryonic tissue using a standard salt extraction method (Lambert et al., 2000). Portions of the W-linked avian CHD (CHD-W) on females and its non W-linked homologue (CHD-Z) were amplified using polymerase chain reaction (PCR) with primers P2 and P8 (Griffiths et al., 1998). PCR products were visualized on a 2.3% agarose gel stained with ethidium bromide. The presence of the W chromosome was determined through the visualization of two bands while males were identified when only one band was visible. Eggs that showed no visible signs of development were determined to be infertile.

5. Hormone Assay

Corticosterone was extracted and separated from plasma using standard procedures described in Wingfield and Farmer (1975). In the first experiment, recovery rate of

corticosterone from plasma was 83.1%. Inter-assay and intra-assay variation was 12.97% and 3.33% respectively. For the acute stress experiment, the recovery rate of corticosterone from plasma was 91.5% and intra-assay variation was 4.7%. All hormone data were non-normally distributed and were thus log-transformed for statistical analyses.

5. Statistics

5.1. Natural Corticosterone Experiment

First, we calculated the average sex ratio for the clutch produced by each individual bird, and then compared these averages to baseline corticosterone levels using a simple linear regression. It is the standard to exclude clutches that contain less than 3 eggs, so only clutches with 3 or more fertile eggs were used in the analysis. Sex ratio averages and corticosterone measurements were normalized using arcsin and log transformation respectively. There was a concern that the act of blood sampling itself may influence the overall average sex ratio for the clutch by altering the sexes of eggs laid after the blood sampling. This does not appear to be the case, however, because the sexes of eggs laid prior to blood sampling did not differ from the sexes of eggs that would have been influenced by the blood sampling ($t_{0.05, 13}$= 0.198, p= 0.8459). However, to circumvent this possibility, we also analyzed the relationship between baseline corticosterone level and the sex of eggs produced first (n= 16) or second (n= 4) in the clutch sequence using a logistic regression analysis. We were able to combine egg sequences #1 and #2 because the natural sex ratio does not differ between clutch positions in this population (A.Gam unpublished data). The second egg in the clutch sequence was only used if the first egg was infertile.

5.2. Acute Stress Experiment

The sex ratio produced by un-manipulated and bag stress groups were compared using a logistic regression analysis. We sexed 31 target embryos from un-manipulated females and 33

from bag stress treated females. Differences in the number of females that produced males was analyzed among treatment groups by performing a logistic regression in StatView (SAS Institute, Cary, NC USA) using sex as the response variable (male =1). Chi square values derived from the logistic regression model are reported. Plasma corticosterone concentrations were analyzed between treatment groups using an unpaired t-test.

Results

1. Natural Corticosterone Experiment

Baseline corticosterone levels were not related to average offspring sex ratio ($r^2 = 0.117$, p= 0.1656; Fig 3.2): females with higher corticosterone levels near meiosis I were not more likely to produce more of one sex over the other. Comparison of corticosterone levels to the sex of eggs laid first or second in the clutch (before blood sampling), yielded no significant relationship ($\chi 2 = 2.117$, p= 0.1456).

2. Acute Stress Experiment

Holding birds in cloth bags for 5 minutes raised corticosterone levels significantly 20 min and 60 min after initiation of handling compared to un-manipulated birds ($t_{0.05, 15} = 2.726$, p= 0.0156; $t_{0.05, 14} = 4.482$, p= 0.0005; Fig. 3.3).

The stress treatment did not influence offspring sex ratios: females exposed to bag handling stress produced a similar offspring sex ratio (54.4%) compared with un-manipulated birds (64.5%) ($\chi 2 = 0.656$ p= 0.412; Fig 3.4).

Discussion

In this study we conducted two experiments to examine the effect of endogenous corticosterone elevations at the critical period in sex determination on offspring sex ratio. We found that acute elevations of corticosterone within a physiological range as well as natural

baseline corticosterone levels experience by laying females immediately prior to meiosis I are not related to offspring sex ratios. These results suggest that physiological elevations of circulating corticosterone at the time of meiosis I may not be a factor that directly influences sex ratio adjustment. The female biases observed after long-term exposure to corticosterone in other species may have been facilitated by effects related to chronic corticosterone exposure to corticosterone and not directly by the elevated levels of corticosterone at meiotic segregation.

Bag handling induced a significant rise in corticosterone to 13.9 ng/ml on average, 20 min after handling. We do not know the precise timing of meiotic segregation in zebra finches, however stress protocols were timed to coincide with the injection time used in the acute pharmacological corticosterone experiments, which did yield a significant sex ratio skew. In addition, the treatment was designed to stimulate a corticosterone elevation within the period during which meiotic segregation was observed in all other avian species examined to date. More work needs to be done to determine when during the five hours prior to ovulation meiotic segregation occurs, however this is the first experiment to take a more targeted approach to this time period.

Observing no effect of physiological elevations on sex ratio provides incite into potential mechanisms by which pharmacological corticosterone induces a sex ratio bias. Pharmacological exposure to corticosterone could override the natural conversion of corticosterone to $11\beta-$ dehydrocorticosterone, allowing for increased binding and activation of mineralocortiocoid receptors (MR) by corticosterone (Farman and Rafestin-Oblin, 2001). Since corticosterone has a high binding affinity to MRs (Dibattista et al., 1985; 1989), the binding specificity of mineralocorticoids to MRs is normally maintained through the enzymatic conversion of corticosterone to $11\beta-$dehydrocorticosterone by 11-hydroxysteroid dehydrogenase type 2(11β -

HSD2) which leaves a metabolite unable to bind to MRs or GRs (Edwards, 1990; Funder, 1993). Exposure to high levels of corticosterone may have saturated 11β -HSD2 conversion capacity, allowing for increased binding and activation of MRs. Alternatively, pharmacological elevations could stimulate the release or inhibition of secondary factors that play a role in the sex biasing mechanism. For example, testosterone another steroid implicated in the sex biasing process has been shown to fluctuate in response to acute stress (Chichinadze and Chichinadze, 2008; Deviche et al., 2010; Van Hout et al., 2010).

While previous studies have examined the relationship between natural corticosterone levels and offspring sex ratio (Bonier et al., 2000), this was the first study to examine offspring sex ratio in relation to baseline levels sample near meiosis I. The time of sampling may be critical when examining this relationship since circulating plasma corticosterone levels follow a diurnal cycle (Johnson, 2000). The observed lack of correlation between baseline corticosterone and sex ratios could be due to the time of sampling, however we think this is unlikely, because even though corticosterone levels fluctuate throughout the day, corticosterone levels remain relatively stable during the 5 h window prior to ovulation when meiotic segregation is occurring (A.Gam, unpublished data), so we are relatively confident that we have a representative sample of levels during meiotic segregation.

To date, the involvement of corticosterone in sex ratio manipulation has been examined using chronic elevations of corticosterone. While these studies have been useful in identifying potential hormones involved in sex ratio manipulation, the pathway by which corticosterone acts to influence sex ratio remains unclear. In particular, it is unknown whether circulating hormones act directly or indirectly on the follicle to induce skews. Our results suggest that elevation of corticosterone within the physiological range at the time of meiotic segregation is not enough to

stimulate sex ratio biases in zebra finches. Whether this result is species-specific, or whether

chronic corticosterone elevations act via a more chronic effect of corticosterone across avian

species remains to be determined.

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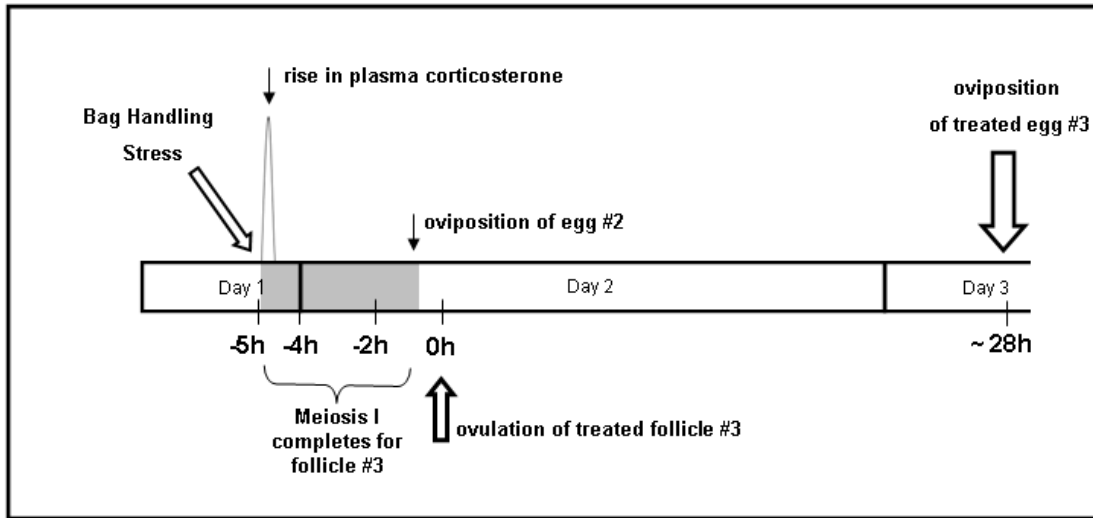
Figure 3.1. Bag handling stress protocol was timed to raise circulating corticosterone levels just prior to the period that meiosis I completes. Meiosis I of the target follicle #3 occurs 0.5-4 h before ovulation and ovulates (time 0) approximately 15-75 minutes after oviposition of egg #2. The treated follicle #3 then precedes though the oviduct until oviposition approximately 36 h after treatment and is laid as egg #3 in the clutch sequence.
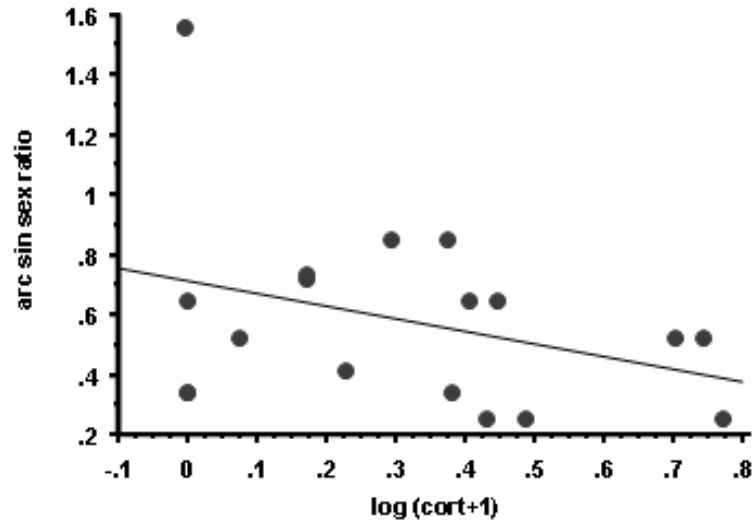
Figure 3.2. Baseline corticosterone levels sampled from laying females near the completion of meiosis I were not related to offspring sex ratio ($r^2 = 0.117$, p= 0.1656).
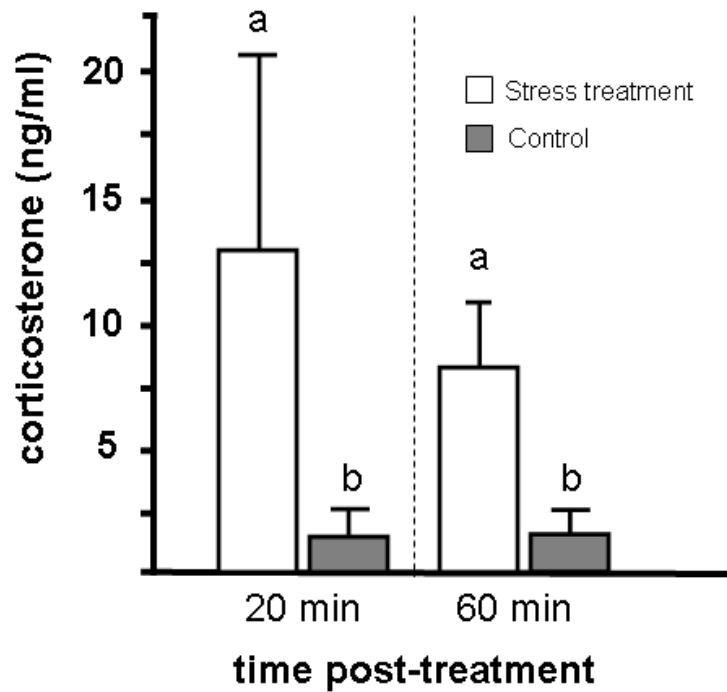
Figure 3.3. Plasma corticosterone concentrations (mean + standard error) from females exposed

to a 5 min bag handling stress protocol (n= 9, 8) and un-manipulated females from which blood

samples were taken at the same time (n=8, 8). Stress treatment elevated circulating

corticosterone levels significantly compared to un-manipulated birds 20 min and 60 min after

initiation of stress in females ($t_{0.05, 15}$ = 2.726, p= 0.0156; $t_{0.05, 14}$ = 4.482, p= 0.0005).

Corticosterone levels were analyzed between treatments at each time point and not between time

points. All hormone measurements were log transformed for statistical analysis to achieve

normality. Different letters above denote values that are statistically different.
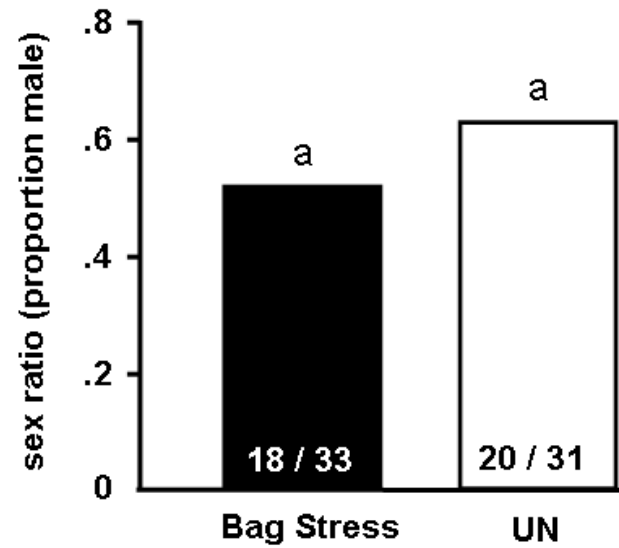
Figure 3.4. Females exposed to bag handling stress produced a similar offspring sex ratio (54.4%) compared to the sex ratio produced by un-manipulated birds (64.5%) ($\chi2 = 0.656$ p= 0.412). Different letters above denote values that are statistically different.

CHAPTER 4: CONCLUSION

A mechanistic understanding of sex ratio manipulation would be advantageous to commercial breeding industries, conservation biology and evolutionary biology. Increased awareness of theses advantages has led to a recent influx of research in this field. Currently, the mechanisms involved in sex ratio manipulation have yet to be determined. Chronic exposure to corticosterone however, the primary glucocorticoid produced in response to stress has been shown to consistently induce a female bias in previous studies. My research focused on elucidating the role that maternal corticosterone plays in offspring sex adjustment by studying the effect of corticosterone exposure specifically at the time of sex determination in relation to offspring sex ratio.

Our initial study examined the effect of pharmacological corticosterone elevations near the time of meiotic segregation. We found that exposure to high exogenous levels of circulating corticosterone induced a male biased offspring sex ratio. These results suggested that near meiosis I, corticosterone can act in a very acute manner to influence offspring sex. Since high exogenous doses can induce a cascade of changes not stimulated by natural corticosterone levels, we followed up this study by investigating the effect of corticosterone elevations within physiological ranges on offspring sex ratio. We examined the effects of both basal and stress induced elevations of corticosterone close to meiosis I. Neither, acute physiological elevations nor natural baseline corticosterone levels predicted offspring sex ratio. These studies together show that for physiological elevations of corticosterone to induce a skew, chronic exposure is necessary as has been shown in previous studies (Pike and Petrie, 2005; 2006; Bonier et al.,

2007). Offspring sex can be manipulated artificially, however, right before meiotic division with an acute pharmacological dose of corticosterone.

The finding that corticosterone elevations in the physiological range near meiosis I are not sufficient to induce skews in the sex ratio lends insight into how chronic exposure influences sex ratio can be investigated. Potentially, chronic corticosterone influences sex ratios by mediating changes in follicular growth rate. During rapid yolk deposition, chronic corticosterone depresses yolk precursor acquisition and follicular growth (Salvante et al., 2003; Salvante and Williams, 2003). Work by Young and Badyaev (2004) shows that growth rate influences sex determination in house finches; female oocytes were produced in slower growing follicles. Chronic corticosterone may therefore cause female biased sex ratios through mechanisms involving decreased follicular growth rates. Alternatively, chronic corticosterone may mediate biases through the accumulation of corticosterone and other steroid hormones in the yolk.

It is also important that only pharmacological doses of corticosterone induced a sex ratio skew. Perhaps pharmacological exposure activated a separate signaling pathway involved in sex ratio manipulation. Since acute exposure to corticosterone has been shown to cause a rapid elevation testosterone in mammals (Oconnor, et al. 1985; reviewed in Chichinadze and Chichinadze, 2008) and European starlings (Van Hout, et al. 2010), testosterone, another candidate steroid hormone involved in sex ratio manipulation could have caused the male bias. Validating the effect of pharmacological corticosterone injections on testosterone levels in female zebra finches will be useful in testing this hypothesis. Alternatively, pharmacological exposure to corticosterone could have caused a skew by overriding the natural conversion of corticosterone to 11 β –dehydrocorticosterone, allowing for increased binding and activation of mineralocortiocoid receptors (MR) by corticosterone (Farman and Rafestin-Oblin, 2001). Since

corticosterone has a high binding affinity to MRs (Dibattista et al., 1985; 1989), binding specificity of mineralocorticoids to MRs is normally maintained through the enzymatic conversion of corticosterone to 11 β –dehydrocorticosterone by 11-hydroxysteroid dehydrogenase type 2(11β -HSD2) which leaves a metabolite that is unable to bind to MRs or GRs (Edwards, 1990; Funder, 1993). Exposure to high levels of corticosterone may have saturated 11β -HSD2 conversion capacity, allowing for increased binding and activation of MRs. Future studies should examine the direct effects of testosterone elevations at meiosis I and mineralocorticoid receptor binding to further investigate the mechanisms facilitating sex ratio adjustment.

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