EVIDENCE THAT MATERNAL DIET ALTERS STEROID LEVELS AND PRIMARY OFFSPRING SEX RATIO IN THE ZEBRA FINCH

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EVIDENCE THAT DIET ALTERS MATERNAL STEROID LEVELS AND PRIMARY OFFSPRING SEX RATIO IN THE ZEBRA FINCH

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VITA

Chioma Camille Okekpe, daughter of Joseph and Shelia Okekpe was born July 24, 1983 in Atlanta, Ga. She graduated with honors from Morrow High School, Morrow, Georgia, in June 2001. She received a Bachelors of Science in Biomedical Sciences with a concentration in Physical Therapy from Auburn University, Auburn, Alabama in August 2005. That same year she returned to Auburn University to attend graduate school in August 2005. While attending graduate school, she worked as a Graduate Teaching Assistant in the Department of Biological Sciences, teaching Anatomy and Physiology I and II labs. Additionally, during her graduate career at Auburn she was a NSF-Bridge to the Doctorate Fellow 2005-2006, received Honorable Mention for the NSF Graduate Research Fellowship Award in 2006, was the recipient of the Best Student Poster Award in the Division of Comparative Endocrinology at the 2009 Society of Integrative and Comparative Biology annual meeting, and served as president of the Future Science, Engineering and Mathematics Faculty Club from 2005-2008.

THESIS ABSTRACT

EVIDENCE THAT DIET ALTERS MATERNAL STEROID LEVELS AND PRIMARY OFFSPRING SEX RATIO IN THE ZEBRA FINCH

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The growing interest in the reproductive benefits of biasing offspring sex ratios in avian species has generated curiosity and provided much insight into the potential factors and mechanisms controlling sex-ratio adjustment. Differing offspring sex ratios often occur in response to changes in the environment. Of particular interest, but less understood, are the mechanisms regulating primary (pre-ovulatory) sex-ratio adjustment. Recently, a number of bird studies have suggested that females (the heterogametic sex) are capable of using primary mechanisms to adjust offspring sex ratios; however the mechanism responsible for this is unknown.

Sex of offspring is determined in the first meiotic division when one sex chromosome is retained in the oocyte while the other segregates to the polar body. During this time, follicular steroid production is limited primarily to progesterone (P_4) and so it has been suggested that maternal steroids, which are sensitive to environmental perturbations, could also influence sex chromosome segregation. Additionally, primary offspring sex ratios have been shown to be affected by some of the same environmental factors that are known for inducing changes in an individual's hormonal milieu. Researchers studying the effects of elevated levels of maternal steroids on primary sex ratio have mainly done so by administering pharmacological doses of exogenous hormones to mothers, but none have examined endogenous hormones at meiosis I (critical time in sex determination).

We manipulated both diet quality and perceived availability in breeding female zebra finches (*Taeniopygia guttata*) so that we could examine the effect this changing factor would have on natural levels of P_4, corticosterone (CORT), and testosterone (T) in circulation during meiosis I and throughout the day. We found that females fed the high quality diet produced significantly more male offspring (81%) and exhibited moderately low to very low levels of these steroids. Females fed the low quality diet produced 38% males and had the highest P_4 levels, but relatively low levels of CORT. When the high quality diet was perceived to be restricted females produced 46% males and had the highest CORT levels, while P_4 remained relatively low. All three steroids reached their peak during the period of meiosis I; however T levels were very low (below 1ng/ml) in all diet treatments throughout the day. Our results suggest that no one hormone is responsible for primary sex ratio adjustment in this species. It appears that natural levels

of maternal steroids, particularly P_4 and CORT, in circulation during meiosis I play an integral yet complex role in the mechanism.

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TABLE OF CONTENTS

LIST OF TABLES

TABLE PAGE

LIST OF FIGURES

FIGURE PAGE

CHAPTER ONE:

Effect of diet quality and availability on maternal condition and primary offspring sex ratio in the zebra finch

INTRODUCTION

Early evolutionary biologists hypothesized that natural selection favored those parents who produced an equal number of male to female offspring (Fisher, 1930). However, several studies have revealed that, under certain ecological and social conditions, offspring sex ratios can depart from parity (Hardy, 1997). The Trivers-Willard hypothesis posits that these deviations are favored by natural selection and are actually expected when the costs of rearing offspring of different sex differ, and/or when the relative fitness benefits expected from having sons or daughters differ (1973). In such cases theory predicts that parents will adjust the sex of their offspring in a manner that would minimize production costs and parental investment and maximize future fitness returns (Trivers and Willard 1973; Charnov 1982). This theory has been applied to explain offspring sex ratio adjustment in a wide range of organisms in response to a variety of environmental and social factors.

Studies of social insects (Godfray and Hardy 1993), mammals (Clutton-Brock and Iason 1986), and birds (Howe 1977) have shown a number of factors contributing to sex

ratio adjustment in response to both natural and experimental changes in environmental and social conditions. For instance, field studies have revealed biases in sex ratio with rainfall events (Zann et al. 1995), clutch size (Arnold et al. 2003), birth/laying order (Erickson, 1976; Velando et al. 2002), and mate quality (Collins et al. 1994; Grindstaff et al. 2001; Korsten et al. 2006). Likewise, empirical studies investigating the effects of food availability (Kitaysky, et al. 2001; Kitaysky et al. 2007), habitat quality (Marra and Holberton 1998), diet quality (Kitaysky et al. 1999), maternal condition (Love et al. 2004; Pike and Petrie 2005), and local mate competition (Werren and Simbolotti, 1989) have demonstrated offspring sex ratios that deviated from 50:50 in several species.

While many of these studies show biased offspring sex ratios at independence, these biases have often been due to a post-ovulatory (secondary) mechanism such as differential resource allocation resulting in sex-specific offspring mortality. More recently, however, the idea of sex ratio adjustment via pre-ovulatory (primary) mechanisms has garnered much attention. Primary sex ratio adjustment has been implicated as the optimal method for altering offspring sex ratios because the resources being provisioned to the young are not being wasted as they would in cases of secondary adjustment (Pike and Petrie 2005). Although there have been several studies looking into the potential factors influencing primary sex ratio adjustment, the mechanism by which skewed sex ratios are produced in response to ecological and/or social cues remains largely unknown.

Birds have particular aptitude for influencing primary sex ratios because female birds are the heterogametic sex (bearing both sex chromosomes, Z and W), and thus have

the potential for direct control over offspring sex and potentially primary sex ratio adjustment (Sheldon, 1998). As a result, an increasing number of studies in several species have focused on and provided evidence for primary sex ratio adjustment (Komdeur et al. 2002).

Studies of captive zebra finches (*Taeniopygia guttata*) have shown that adjustments in primary offspring sex ratio do occur in this species in response to changes in maternal condition or diet (Bradbury and Blakey 1998; Kilner 1998; Rutkowska and Cichon´ 2002; Arnold et al. 2003). Though not always statistically significant, there has been a trend for mothers in or breeding under poor conditions to produce male-biased offspring sex ratios. However, results have not always been consistent within this species, showing both male and female-skewed offspring sex ratios in response to a decline in environmental conditions or exhibiting no skews at all (Table 1). Additionally, some studies have neglected to include measurements of sex ratio at laying (primary sex ratio) while others report sex ratio skews that do not correspond with the predictions made by the Trivers-Willard Hypothesis (male-biased sex ratios produced by mothers in good condition) or the results exhibited by similar studies that used different avian species (Table 1).

Despite the discrepancies that appear to exist among these studies, to date all studies show that females have a differential sensitivity to unfavorable environmental conditions during breeding and will make adjustments to offspring sex ratios in response to these conditions (Kalmbach et al. 2001). In this study, we investigated the relationship between breeding diet, maternal condition, and primary offspring sex ratio in a captive

colony of zebra finches. We manipulated both diet quality and availability in breeding female zebra finches and analyzed the ensuing offspring sex ratios at laying. Our experiment was designed to test the Trivers-Willard hypothesis as well as address the conflicting results exhibited by previous studies of this species.

METHODS

General Methods

We obtained zebra finches from commercial breeders (Armchair Safari, Auburn, Al; Exotic Finch Loft, Miamisburg, OH) were housed and bred in captivity in an indoor aviary in individual cages which remained in the same room at Auburn University but in visual isolation from one another. All birds were kept on a 13L:11D photoperiod in a temperature-controlled environment (24 ± 2°C). Cages were equipped with food, water, perches, nests and nesting material to promote breeding activity. Each cage was inspected at least once a day so that we could assess nest status and account for the presence of eggs, nestlings and fledges. All birds were banded at fledging for identification. Breeding pairs were grouped such that no two closely related adults were paired together. Using manual dial calipers, we collected measurements of females' tarsus length (to the nearest 0.01mm) and mass using a digital scale (to the nearest 0.01g). The residuals of mass:tarsus length were calculated as a measure of female body condition (Reist 1985) while on each diet regimen.

Diet

In past studies, dietary manipulation for zebra finches have often included commercial seed finch diets supplemented with fresh fruits and vegetables, egg, vitamins,

or calcium as a higher quality diet and white millet as a low quality diet and (Bradbury 1998; Arnold et al. 2003). In those studies that restricted the amount of food available to these birds (via feeders that manipulated adult perception of food availability), a mixture of white and pancium millet and a rearing mix were used (Kilner 1998). In our study all breeding pairs were fed one of three breeding diet treatments: 1) ad libitum high quality (HQ) commercial seed containing vitamin supplement: Kaytee Supreme Finch Fortified Daily blend (Central Garden and Pet Company), 2) ad libitum low quality (LQ): plain white millet diet supplemented with cuttlebone, and 3) a restricted HQ diet. This restricted HQ diet is the same as the aforementioned HQ diet, but was restricted in perceived availability through the use of opaque bird feeders with a small opening for feeding. Previous HQ diets have been composed of up to 44% protein; our HQ diet contains up to 14% protein, 4% crude fat, and 10% crude fiber. Our LQ diet contains 11.6% protein, 3.5% crude fat, and 6.5% crude fiber compared to previous LQ diets with a protein composition ranging from 11.6% to 22% protein.

Total sample size for each diet treatment were as follows: HQ ad libitum – 40 pairs, HQ restricted – 20 pairs, and LQ ad libitum – 20 pairs. All subjects were provided with water ad libitum and remained on their respective breeding diets for approximately 7 months (enough time for pairs to rear up to 4 clutches). The first clutch provided information on offspring sex ratio only while each subsequent clutch allowed us to gather hormone data for a separate, but related study as well as additional sex ratio data.

Molecular sexing

DNA samples for molecular sexing were collected from either blood samples or from embryonic tissue of eggs that failed to hatch and skeletal muscle of chicks that died before blood samples could be taken. All eggs that failed to hatch 14-21 days post incubation were opened and their contents screened by eye for embryonic tissue for sampling (Brommer et al. 2003). We treated all tissue samples with SDS detergent and proteinase K for tissue digestion.

Next, we extracted DNA from these digested samples using a series of phenol-chloroform-amyl washes. The extracted DNA was then amplified using a polymerase chain reaction (PCR). PCR amplified part of the W-linked CHD gene (CHD-W) in females and its non W-linked homologue (CHD-Z) in both sexes. Universal primers P2 (5'-TCTGCATCGCTAAATCCTTT-3') and P8 (5'CTCCCAAGGATGATRAAYTG-3') were used to amplify the genes (Griffiths et al. 1998). Additionally, FideliTaq Master Mix (USB Corporation), $MgCl_2$ (USB Corporation), and 200-500ng of our template DNA were incorporated to make up a 10ul reaction for PCR. Amplification of our product was performed under the following thermal profile: denaturing step at 94 ºC for 1 min 30 s, followed by 30 cycles of 48 ºC for 45 s, 72 ºC for 45 s and 94 ºC for 30 s, and a final run of 48 ºC for 1 min and 72 ºC for 5 min. Products were separated on 1% agarose gel incorporated with ethidium bromide. Photo copies of gels were analyzed using the Chemi Imager program. The sex of the sampled individuals was determined by the presence of either one (CHD-Z; male) or two (CHD-W and CHD-Z; female) bands.

Statistical analysis

Maternal body condition was examined in relation to diet treatment using the residuals of body mass regressed on tarsus length; the effect of diet on maternal body condition was analyzed by diet treatment using a one way ANOVA. Clutch size was analyzed by diet treatment using one way ANOVA. We defined sex ratio as the number of males within a clutch divided by the total number of offspring within that clutch and presented as proportion male. Offspring sex ratios (proportion male) were arcsin transformed and analyzed using a general linear model. We tested the effects of dietary treatment and clutch size on primary offspring sex ratio as well as their interactions using a general linear model. Clutch sequence had no effect on offspring sex ratio so primary offspring sex ratio was determined by analyzing the largest clutch from each female because it is difficult to see sex ratio differences in small clutches. Clutches of one were considered abnormal and excluded from all sex ratio analysis. Departures from 50:50 were analyzed for each dietary treatment using a G-test. All analyses were performed in Statview (SAS Institute, Inc v.5.0.10.) unless stated otherwise. Sample sizes vary among analyses because not all data were available for all mothers.

RESULTS

Maternal Body Condition

We observed no significant difference in maternal body condition based on diet quality or availability ($F_{2, 49} = 1.85$, $p = 0.17$), though females fed the LQ ad lib diet exhibited, as expected, the lowest average body condition index (Fig. 1).

Clutch size

General linear model revealed a significant effect of diet treatment on average clutch size ($F_{2, 41} = 7.25$, $p < 0.01$) (Fig. 2). On average, mothers fed the HQ ad lib diet had clutch sizes of 4.05 eggs, mothers fed the restricted diet produced clutches of 2.61 eggs, and those fed the LQ diet had clutches of 3.12 eggs. Fishers PLSD post hoc analysis revealed significant differences in clutch size between the HQ ad lib and HQ restricted diets ($p < 0.01$); there was no significant difference in clutch size between mothers fed the HQ restricted and LQ ad lib diets ($p = 0.32$) or the HQ and LQ ad lib diets ($p = 0.11$).

Primary offspring sex ratio

We did observe an overall effect of dietary treatment on primary offspring sex ratio ($F_{2, 45} = 3.59$, $p = 0.04$). Fishers post hoc analysis revealed that mothers fed the HQ ad lib diet

produced significantly more male offspring (81% male) when compared to mothers fed the LQ ad lib (38% male) and HQ restricted (46% male) diets ($p = 0.03$ and $p = 0.04$, respectively); however mothers fed the LQ ad lib and HQ restricted diets did not differ significantly from one another ($p = 0.71$) (Fig. 3). There was no interaction effect between breeding diet and clutch total on primary offspring sex ratio ($F_{2, 42} = 1.61$, $p = 0.21$).

DISCUSSION

By manipulating parental breeding diet quality and perceived food availability, we were able to successfully alter clutch size and primary offspring sex ratio, independently of maternal condition in our colony of zebra finches. Mothers fed the HQ ad lib diet had larger average clutch sizes and skewed their offspring sex ratio towards males (81%). Data from the restricted HQ and LQ ad lib treatment groups show that female zebra finches responded to declines in food quality and perceived availability by producing smaller clutches of fewer male offspring (46% and 38%, respectively). Others have suggested that when parents are constrained by poor dietary conditions it is in their best interest to rear the less costly sex in small broods and clutches (Arnold et al. 2003). In our study, mothers breeding under poorer nutritional conditions yielded smaller female-skewed clutches when compared to those mothers breeding under optimal dietary conditions. The outcome of our study corroborates the predictions made by Trivers and Willard (1973) and suggests that zebra finch mothers that breed under favorable conditions will skew the primary sex ratio of their offspring towards males.

In the past, studies have manipulated maternal condition as a means of investigating its potential role in offspring sex ratio adjustment (Bradbury and Blakey, 1998; Kilner, 1998; Nager et al. 1999). Much of the focus of factors influencing sex ratio adjustment has centered on the maternal condition hypothesis which posits that offspring sex ratios are a

reflection of maternal condition during breeding (Trivers and Willard, 1973). Prevailing environmental conditions, which can have an effect on body condition (Merila 1997; Moss and Croft 1999), have been suggested to be an accurate predictor of the ensuing offspring sex ratio (Myers et al. 1985; Burley et al. 1989). In such situations, one would predict an overproduction of the more costly sex when mothers have high quality resources readily available to them. Contrary to those studies that report significant effects of diet restriction (Kilner, 1998) or quality (Bradbury and Blakey, 1998) on maternal condition, we show no such effect. It is possible that the disparity seen between our results and the results of previous studies is a result of diet quality. The protein content of our HQ seed diet appears to be less than what has been documented in prior zebra finch studies which report female biases. These studies supplemented their diets with items such as egg or fresh fruit and vegetables, thus raising the protein content considerably. Though typically, in the wild, zebra finches do not have access to such items and so their diets, like the individuals in our colony, consist primarily of grass seed. Furthermore, since we did not supplement our HQ ad lib diet with these food items the protein content between our three diet treatments did not vary greatly and as a result, were not extreme enough to have an effect on the average body conditions of the mothers (though the body condition measure of females fed the LQ ad lib diet did trend lower than those on the other diets).

Despite the lack of difference in maternal body condition, the adjustments made to diet quality and availability had a significant effect on clutch size and apparently played a significant role in biasing offspring sex ratios at laying. In our study, clutches from mothers fed the HQ ad lib diet were significantly larger than those mothers fed the HQ restricted but not the LQ ad lib diet, yet the sex ratios differed significantly. Arnold et al. (2003) reported changes in offspring sex ratio based on clutch size. Mothers fed a low quality pre-breeding diet produced an excess of female offspring in smaller clutches and an excess of sons in larger clutches; while the opposite effect was observed in mothers fed a high-quality pre-breeding (Arnold et al. 2003). The average clutch sizes we report (LQ ad lib-3.12, HQ restricted- 2.61, and HQ ad lib - 4.05) are smaller than those reported in previous studies of primary sex ratios in zebra finches (LQ-4.5 and average diet-5.4 (Rutkowska and Cichon 2002) and the modal size LQ-4.07, MQ-4.29, and HQ-4.25 (Arnold et al. 2003). Although we report smaller average clutch sizes, the observed difference in primary sex ratio in our colony of zebra finches was not a result of differences in clutch sizes among dietary treatments (sex ratio*clutch size interaction effect, $p=0.21$). Offspring sex ratio in this species has also been shown to be significantly related to laying order, in which skews in sex ratio tend to mirror the sex of earlier laid eggs (Clotfelter 1996; Kilner 1998). Therefore, although it remains a possibility that our male skew is because of an order effect (which we did not track), the lack of the interaction effect supports the idea that the effect was due to diet rather than laying order.

This difference in our results with those presented in previous zebra finch studies continues the controversy. Earlier reports of zebra finch sex ratios have also provided conflicting results. For example, some studies have reported male biased sex ratios are produced as a result of poor maternal or dietary conditions (Kilner 1998; Foster and Burley 2007)**,** others have found no significant difference in sex ratio is seen when diet is altered (Zann and Runciman 2003), still others observe male-skews when mothers are in good condition, but were fed a LQ breeding diet (Bradbury, 1998). Contrary to past studies of this species, our results show that mothers tended to lay a significantly higher proportion of male eggs when fed a HQ ad lib diet compared to the restricted and LQ diet. The results we present are consistent with earlier reports for a variety of other avian species that show male skews when mothers are breeding under favorable conditions (Nager et al. 1999; Whittingham and Dunn 2000; Clout et al. 2002; Pike and Petrie 2005). We observed a significant effect of maternal diet on primary offspring sex ratio that first, corroborates with previous studies of varying species and second, produces the appropriate sex biases predicted by the Trivers-Willard hypothesis. There are differential costs and benefits to producing sons and daughters and the fitness returns gained from producing one sex over the other are dependent on maternal and environmental conditions (Lindström 1999; Whittingham and Dunn 2000). Studies in which male biases are seen as a result of poor dietary or maternal conditions have explained these biases by arguing that females are more vulnerable to nutritional stress and are therefore

considered to be the more costly sex to rear (Bradbury and Blakey 1998; Kilner 1998; Rutstein et al. 2004). Here we show that mothers breeding under better dietary conditions produced male-biased clutches and contend that males are actually the more costly sex because in zebra finches because males exhibit faster growth rates (von Engelhardt et al. 2006) and are slightly larger than females at hatch(Rutkowska and Cichon 2002, Zann 1994). This occurrence of male biased sex ratios to mothers breeding under optimal condition may have an adaptive significance because successful sons will benefit more from good conditions than daughters (Cameron and Linklater 2002). These sons are also more likely to produce more offspring than a daughter from the same mother (Trivers and Willard 1973). Thus it would prove more beneficial for mothers breeding under good conditions to increase fitness returns from their offspring by investing more into quality sons (Cameron and Linklater 2002). Therefore, our results are more in keeping with the predictions of Trivers and Willard as well as in line with those seen in other avian species

It has been proposed that maternal diet and perception thereof, rather than maternal body condition per se, plays a directive role in controlling sex ratio **(**Rosenfeld and Roberts 2004). Our study also indicated that despite no significant difference in body condition of females fed the different diet treatments, mothers fed the HQ ad lib diet produced significantly more male-skewed clutches than mothers fed the LQ ad lib and perceived restricted diet. These results suggest that the manipulations we made to breeding diet could potentially be inducing changes in some physiological factor that causes mothers to produce skewed sex ratios. For example, researchers interested in the

mechanism controlling primary sex ratio adjustment have found maternal corticosterone, the avian stress hormone, to have a significant effect on sex ratio adjustment in avian species (Pike and Petrie 2006; Bonier et al. 2007). Thus, it may be that diet quality and or availability is sufficient to trigger some change in hormonal milieu rather than some intrinsic change in body condition, and this change in hormonal environment can influence sex ratio by causing a change in maternal physiology during the period of sex determination. Given our contradictory results with those observed in other studies, it is clear that further investigation of maternal effects and environmental factors are still required to truly disentangle the factors (if any) controlling primary sex ratio adjustment in this species. Still our results are in line with the predicted Trivers-Willard outcome and with other avian species. An assessment of these factors and the resulting offspring sex ratio will provide a better understanding of both the adaptive significance and the proximate mechanisms associated with primary sex ratio adjustment in this species.

Table 1
Previous studies showing primary sex ratio adjustment in response to maternal condition and diet in zebra finches and other avian species.

Species name	Bias related to	Effect	Reference
Zebra Finch *Taneniopygia guttata*	Diet availability	Clutch sex ratios at hatch were male-biased on a restricted diet	Kilner (1998)
	Diet quality and maternal condition	Mothers fed the low quality diet were in better condition and produced male biases at hatch	Bradbury and Blakey (1998)
	Diet quality and subsequent maternal investment in eggs	Increase in the proportion of male eggs produced when diet quality was reduced after the start of laying	Rutkowska and Cichoń (2002)
	Diet quality	No overall bias, but mothers fed the low quality diet produced males in small clutches and females in large clutches	Arnold et al. (2003)
	Diet quality and maternal breeding condition	No bias in primary sex ratio in the wild or laboratory	Zann and Runciman (2003)
	Diet quality	More male eggs on low quality diet	Rutstein et al. (2004)

Table 1
Previous studies showing primary sex ratio adjustment in response to maternal condition and diet in zebra finches and other avian species.

Species name	Bias related to	Effect	Reference
American Kestrel *Falco sparverius*	Diet availability and parental condition	Proportion of male offspring at hatch increased as food supply and parental condition declined	Wiebe and Bortolotti (1992)
Lesser-Black Backed Gull *Larus fuscus*	Maternal condition via continuous egg removal	As female condition declined, the sex ratio of her eggs was skewed towards females	Nager et al. (1999)
Tree Swallow *Tachycineta bicolor*	Maternal condition	Male biased sex ratios were associated with mothers in better condition	Whittingham and Dunn (2000)
Kakapo *Strigops habroptilus*	Diet quality	Mothers receiving supplementary feeding produced an excess of male offspring in subsequent clutches	Clout et al. (2002)
House Wrens *Troglodytes aedon*	Maternal condition	Overall sex ratio of the population was not biased, but more sons were produced by mothers in better condition	Whittingham et al. (2002)
Yellow-Legged Gull *Larus cachinnans*	Maternal condition as indicated by plasma cholesterol	Brood sex ratio of mothers in poor condition was female biased	Alonso-Alvarez and Velando (2003)

Table 1
Previous studies showing primary sex ratio adjustment in response to maternal condition and diet in zebra finches and other avian species.

Species name	Bias related to	Effect	Reference
Pigeon *Columba livia*	Maternal condition via continuous egg removal	Mothers in poor condition produce a female bias in first and second eggs	Pike (2005)

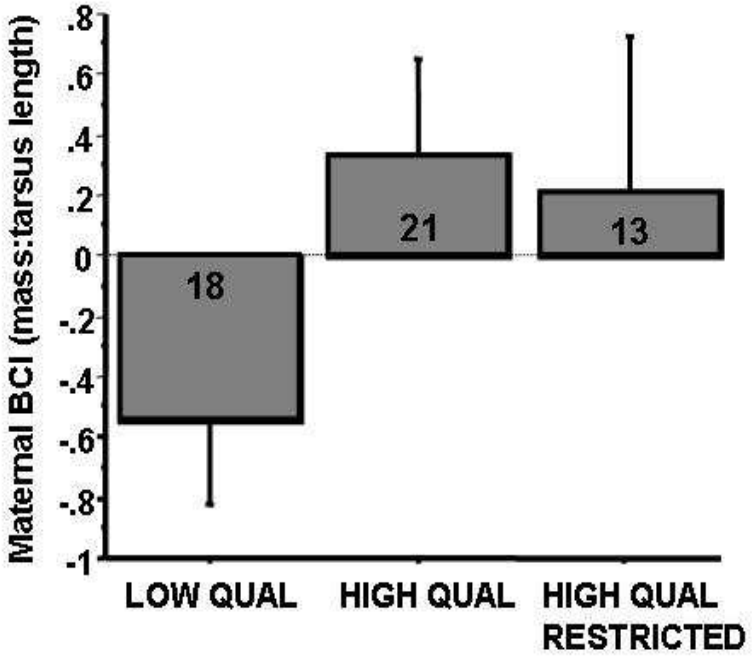

Figure 1. Maternal Body Condition Index (BCI) by diet treatment ($F_{2, 49} = 1.85$, $p = 0.17$).

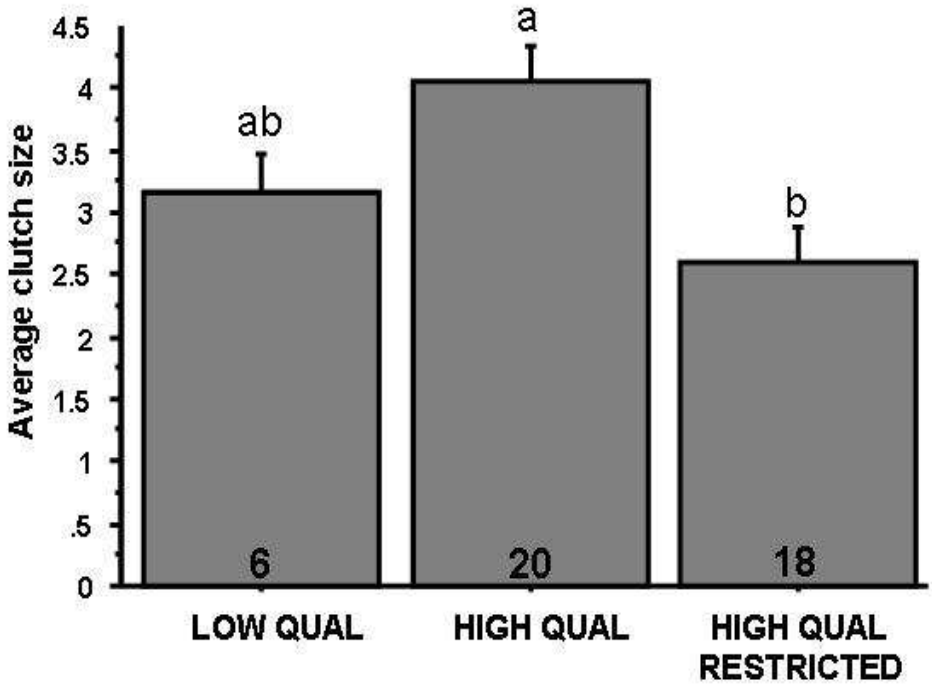

Figure 2. Average clutch size by diet treatment ($F_{2, 41} = 7.25$, $p < 0.01$). Mothers on the LQ *ad lib* diet produced average clutch sizes of 3.12, HQ *ad lib* diet had an average clutch size of 4.05, and HQ restricted diet produced an average clutch size of 2.61. Numbers located inside the bars indicate the total number of clutches sampled within that particular diet treatment.

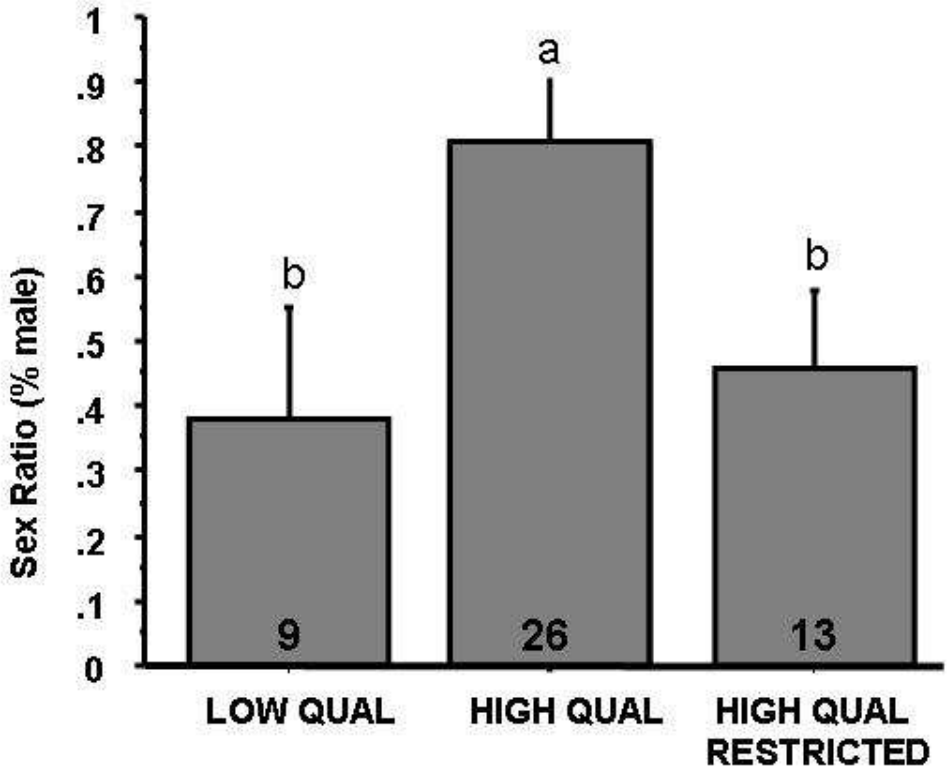

Figure 3. Mean primary offspring sex ratios (proportion of sons) produced by mothers on different diet treatments ($F_{2, 45} = 3.59$, $p = 0.04$). Numbers located inside the bars indicate the total number of clutches sampled within that particular diet treatment.

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CHAPTER TWO:

Effect of diet on the preovulatory levels of maternal steroid hormones and primary sex ratio the zebra finch

INTRODUCTION

Biases in offspring sex ratios have been reported to occur in many species in response to a number of social and environmental factors (Godfray and Hardy, 1993; Hasselquist, 2002; Sheldon and West, 2004). In avian species, which have chromosomal sex determination, the predominant hypothesis of the mechanism responsible for skewing primary sex ratios is segregation distortion during meiosis I (Rutkowska and Badyaev, 2008). Birds have a particular aptitude for sex ratio adjustment because female birds are the heterogametic sex, bearing sex chromosomes Z and W. Therefore, they may have direct control over determination of offspring sex, potentially manipulating offspring sex ratios during meiosis I (4-6 hours before ovulation), the suggested critical time for sex determination (Krackow, 1995; Ankney, 1982). If birds adjust sex ratios in this manner, avian sex ratios should be especially susceptible to maternal modifications. Despite the collection of evidence supporting theories of primary sex ratio adjustment, the mechanism behind primary sex ratio adjustment remains unknown.

The labile nature of the endocrine system in response to environmental and social changes accompanied by its intimate relationship with reproductive processes would allow maternal steroids to be the chief player in the sex biasing mechanism (Krackow, 1995). Evidence is emerging that suggests a causal relationship between maternal hormones and offspring sex ratio. The exogenous administration of certain sex steroids to breeding females successfully affects their subsequent offspring sex ratios. For example, Correa et al. (2005) reported female biases following progesterone injection in domestic chickens (*Gallus gallus domesticus*), while testosterone administration in zebra finches (*Taeniopygia guttata*) and spotless starlings (*Sturnus unicolor*) resulted in male biased offspring sex ratios (Rutkowska and Cichon, 2005; Veiga et al., 2004; Rutkowska et al., 2005; Pike and Petrie, 2006). The effects of these sex steroids could also be mediated by changes in the females' levels of circulating glucocorticoids, especially since glucocorticoids are associated with the stress response and their levels are easily altered by changes in the external environment. For example, exogenous corticosterone administration stimulated a female biased sex ratio produced by quails (Pike and Petrie, 2006) and starlings (Love et al., 2005), while naturally elevated levels of corticosterone in white-crowned sparrows (*Zonotrichia leucophrys*) have also resulted in the production of more daughters when compared to mothers having low levels of corticosterone (Bonier *et al.*, 2007).

However, many of these studies have manipulated maternal hormone levels through exogenous administration of hormones using silastic implants, usually at pharmacological levels (Graey et al. 1990; Criscuolo et al., 2005). Additionally, many of

these treatments were not synchronized with the natural timing of reproductive or ovulatory cycles. While these experiments have provided tremendous insight into the possible role played by maternal steroids in the sex biasing mechanism, it is unclear whether or not the levels of hormones administered to these birds represents realistic hormonal fluctuations during the period of meiosis I. Furthermore, we do not know how these exogenously elevated levels of hormones relate to modification in the mothers' natural levels when she is experiencing stress imposed by diet or body condition.

In the current study, we manipulated diet quality as well as its perceived availability and documented the effect of these treatments on endogenous levels of maternal steroids during the critical time for sex determination. Plasma progesterone (P_4) and corticosterone (CORT), two steroids that previously stimulated female-skewed sex ratios in other species, were analyzed. We also investigated the effects of these treatments on levels of maternal testosterone (T), previously shown to stimulate male-skews in bird species including the zebra finch. Finally, we documented the daily cycle of these steroids outside the critical meiosis I time interval under the different dietary regimens.

METHODS

Study Species

Zebra finches (*Taneiopygia guttata*) are small, nomadic, seed-eating passerines that inhabit the arid and semiarid regions of Australia and Indonesia (Zann 1994). These sexually dimorphic birds are socially monogamous and breed opportunistically in response to rare rainfall events when resources are adequately available (Zann et al., 1995). These birds have been extensively studied in the wild as well as captivity and several lines of evidence suggest that deviations in offspring sex ratio occur in relation to a number of factors; i.e., maternal condition, diet quality, and food availability (Kilner, 1998; Bradbury, 1998). (Bradbury and Blakey, 1998; Kilner, 1998; Arnold et al., 2003; Rutstein et al., 2003).

General Methods

We housed the zebra finches in individual cages within the same room at the Auburn University aviary. Birds were kept on a 13L:11D photoperiod in a temperature-controlled environment (24 ± 2°C) and all cages were equipped with food, water, perches, nests and nesting material to promote breeding activity. Cages were inspected daily to assess nest status and account for the presence of eggs, nestlings and fledges. All birds were given a metal leg band containing a unique sequence of numbers at fledging for

identification. Breeding pairs were grouped so that no two closely related adults were paired together. Using manual dial calipers we measured female tarsus (accuracy = 0.01mm) and weight (accuracy = 0.01g) using a digital scale after females had been on the experimental diets (described below) for 3 months. We used the residuals of mass:tarsus length as a measure of body condition (Reist, 1985).

Diet

We fed breeding pairs one of three diet treatments: 1) *ad libitum* high quality (HQ): Kaytee Supreme Finch Fortified Daily blend (Kaytee Products, Inc., Chilton, WI), a commercial seed containing vitamin supplements and up to 13.5% protein, 4% crude fat, and 10% crude fiber, 2) *ad libitum* low quality (LQ): plain white millet diet containing 11.6% protein, 3.5% crude fat, and 6.5% crude fiber supplemented with cuttlebone, and 3) a restricted HQ diet. This restricted HQ diet is the same as the aforementioned HQ diet, but was restricted in perceived availability through the use of opaque bird feeders with a small opening for feeding.

Total sample sizes for each diet treatment were as follows: HQ *ad libitum* – 40 pairs, HQ restricted – 20 pairs, and LQ *ad libitum* – 20 pairs. We provided all subjects with water *ad libitum* and all pairs remained on their respective breeding diets for approximately 7 months (enough time to rear at least 4 clutches). First clutches produced under the dietary treatments were used to collect preliminary (Chapter 1) data on sex ratio. Each subsequent clutch allowed for the collection of data on maternal steroid levels during meiosis I as well as additional sex ratio information.

Blood Sampling

We inspected all cages on two-hour intervals beginning at 0600 hours and ending at 1700 hours for one month to determine the approximate time of oviposition and ovulation in our colony. The time of meiosis I was predicted by monitoring oviposition, which occurs 15-30 minutes before ovulation of the next follicle (Liu et al., 2002; Yoshimura et al., 1994), and approximately15-20 minutes after sunrise (McMaster et al., 1999). Blood samples were collected from females on the day the first egg of their clutch was laid. We removed the females from their cages for blood sampling at 0100 hours, approximately 5 hours before ovulation, during the period of meiosis I (Johnson, 1998), when steroid production by the F1 (most mature) ovarian follicle is primarily P_4 (Etches and Duke, 1984).

Blood samples were also collected from sub-samples of non-laying females (so as not to disrupt the laying cycle) to determine daily basal profiles of maternal hormones. Samples were collected on six hour intervals, beginning 2 hours after the lights came on: 0700, 1200, and 1700 hours.

All blood samples were taken within 3 minutes of capture using a 26-gauge half inch needle to puncture the brachial vein and microhematocrite tubes for collection. Blood samples were then placed into microcentrifuge tubes and plasma was separated using centrifugation at 1300 rpm for 8 minutes. Plasma samples were stored at -20˚C until assay.

Molecular sexing

We collected DNA samples for molecular sexing of offspring from blood or skeletal muscle of offspring that died before blood samples could be collected. All eggs that failed to hatch 14-21 days post incubation were opened and their contents screened by eye for embryonic tissue for sampling (Brommer et al., 2003). All tissue samples were treated with SDS detergent and proteinase K for tissue digestion.

DNA was extracted from blood and tissue samples using phenol-chloroform-isoamyl-alcohol based extractions. Genetic sexing of DNA was then amplified using a polymerase chain reaction (PCR). PCR amplified part of the W-linked CHD gene (CHD-W) in females and its non W-linked homologue (CHD-Z) in both sexes. Universal primers P2 (5'-TCTGCATCGCTAAATCCTTT-3') and P8 (5'CTCCCAAGGATGATRAAYTG-3') were used to amplify the genes (Griffiths *et al.*, 1998). Additionally, FideliTaq Master Mix (USB Corporation, Cleveland, OH), $MgCl_2$ (USB Corporation, Cleveland, OH), and 200-500ng of our template DNA were incorporated to make up a 10ul reaction for PCR. Amplification of our product was performed under the following thermal profile: denaturing step at 94 ºC for 1 min 30 s, followed by 30 cycles of 48 ºC for 45 s, 72 ºC for 45 s and 94 ºC for 30 s, and a final run of 48 ºC for 1 min and 72 ºC for 5 min. Products were separated on 1% agarose gel incorporated with ethidium bromide. Photo copies of gels were analyzed using the

Chemi Imager program. The sex of the sampled individuals was determined by the presence of either one (CHD-Z; male) or two (CHD-W and CHD-Z; female) bands.

Radioimmunoassay

Tritiated radioimmunoassays were conducted to assess the amount of circulating hormones prior to ovulation, and in association with varying diet quality and availability. P_4, CORT, and T were separated by celite column chromatography in accordance with methods described by Schwabl (1993). We quantified all hormones using a competitive binding radioimmunoassay. These assays were performed following techniques previously described in Mendonça et al. (1996). The mean inter- and intra-assay variations for progesterone and corticosterone were 19.5 and 10.6% and 17.3 and 12.6%, respectively. The mean inter- and intra-assay variation for testosterone was 11.8 and 14%. The sensitivity of all three assays was 10 pg/ml.

Statistical analysis

Plasma hormone values were log-transformed to achieve normality. We then tested for heterogeneity of variances using the F_{max} test (Sokal and Rohlf, 1995). Data found to be homoscedastic were analyzed using parametric analyses while heteroscedastic data were analyzed using nonparametric analyses. The effects of diet treatment on maternal hormone levels were analyzed using a one-way ANOVA and a Fisher's PSLD used for post-hoc comparisons. First we analyzed the effect of diet on

maternal hormones in mothers that were able to complete a clutch. Using a one-way ANOVA with clutch sequence as the covariate, we found no significant interaction between clutch sequence and diet on the levels of maternal P_4, CORT, or T ($p = 0.20$, $p=0.44$, and $p=0.95$, respectively). Because maternal hormone levels did not vary with clutch sequence (females were able to rear at most 4 clutches), and it is difficult to detect sex ratio differences in small clutches, and to also avoid pseudoreplication, we used the hormone data from the largest clutch size produced by each female to determine the effect of diet on maternal hormone levels in relation to the sex ratios produced in those clutches. Additionally, to determine the *overall* effect of diet on hormone levels, we also included, in a separate analysis, those females who were bled, but who failed to complete a clutch following sampling. Again, all females used in this analysis were only used once. We defined a complete clutch as clutches that were completed after blood samples were taken; that is, females were able to lay additional eggs following blood sampling. Complete clutches ranged from 2-6 eggs. Clutches of 1 were not considered complete because we could not rule out the fact that our nightly blood sampling might have interrupted the females' laying sequence, leaving her with only 1 egg. One-way ANOVA analysis with clutch sequence as the covariate resulted in no significant interaction between clutch sequence and diet on *overall* levels of P_4, CORT, or T ($p = 0.32$, $p=0.78$, and $p=0.78$, respectively). Kruskal-Wallis analyses were employed to compare basal plasma hormone samples from females to compare the daily cycle of hormones.

Maternal body condition was calculated as the residuals of body mass regressed on tarsus length (Reist, 1985) . It was then analyzed in relation to diet treatment by a one way ANOVA. We then examined the relationship between maternal condition and hormone levels using linear regression analysis of P_4 and Spearman correlation analysis for CORT. This analysis was performed using measurements of maternal body condition taken three months into their respective diet when females had laid at least one clutch.

We defined primary sex ratio as the percentage of males present in each clutch at laying; the number of males was used as the dependent variable and the total number of sexed offspring within the clutch as the denominator (number of males/clutch total). Sex ratio departures from 50:50 were analyzed for each diet treatment using a G-test. Binary data (offspring sex) were arcsin transformed and analyzed using a general linear model. We also tested the effects of maternal hormone levels on primary sex ratio using linear regression analysis. Clutch sex ratio was arcsine transformed to achieve normality and analyzed against log transformed hormone data. Finally, we analyzed the effect of maternal hormone levels on clutch size were analyzed using linear regression. All analyses were performed in Statview (SAS Institute, Inc v.5.0.10.) unless stated otherwise. Sample sizes vary among analyses because not all data were available for all mothers.

RESULTS

Maternal body condition

Maternal body condition did not differ by diet treatment (refer to chapter 1 (Fig. 1)). There was no significant correlation between maternal body condition and levels of CORT (rho = -0.16, p = 0.48) or P_4 (r = 0.07, p = 0.76) at the time of meiosis I (i.e. the 0100 sample period).

Hormone levels

Mean P levels at 0100 hr sample period (the approximate time period of meiotic segregation) differed significantly between diet treatments ($F_{2, 21}$ = 4.92, p = 0.02) in females that were able to complete a clutch following blood sampling and for whom we have complete sex ratio data. P_4 levels of mothers fed the LQ *ad lib* diet exhibited significantly higher levels of P_4 than mothers fed the HQ *ad lib* and HQ restricted diets (Fisher's PLSD analysis, Fig. 4a). P_4 levels ranged from 2.14 ± 1.63 (HQ restricted, N =6) to 9.31 ± 1.48 ng/ml (LQ *ad lib*, N = 7). Mean P_4 levels at the period of meiosis I (0100 hr) collected for all females (i.e. whether they completed a clutch or not) also differed significantly by diet treatment ($F_{2, 28}$ = 9.35, p < 0.01) and ranged from 0.65 ± 1.90 (HQ restricted, N =8) to 7.12 ± 1.86 ng/ml (LQ *ad lib*, N = 8). P_4 levels of females on the LQ *ad lib* diet were significantly higher than those of females fed the HQ *ad lib* and HQ restricted diets (p = 0.01, respectively). There was also a significant difference

between females fed the HQ *ad lib* and HQ restricted diets ($p = 0.02$), with HQ *ad lib* being higher (Fisher's PLSD analysis).

There was no significant difference in the hormonal profile of P_4 based on the time of day ($p = 0.17$). In these females, P_4 levels did not vary significantly over the course of the day (0700, 1200, and 1700 hours); ranging from 1.90 ± 0.59 at 1700 hours to 1.98 ± 1.07 ng/ml at 1200 hours. Females fed the LQ *ad lib* diet tended to have the highest P_4 levels the majority of the day, followed by the HQ *ad lib* treatment group, while the HQ restricted females had the lowest levels (Fig 4b). Peak P_4 levels were observed at 0100 hours.

For those females for which we have complete sex ratio and hormone data, mean levels of maternal CORT, like P_4, differed significantly between diet treatments ($F_{2, 19} = 4.14$, $p = 0.03$) at the 0100 sample point (Fig. 5a). In this instance, mothers fed the HQ restricted diet displayed significantly elevated levels of CORT when compared to those mothers fed the HQ *ad lib* diets (Fisher's PLSD). Levels of CORT at this time ranged from 5.50 ± 01.00 (LQ *ad lib*, N =4) to 19.36 ± 6.87 ng/ml (HQ restricted, N =5). When we compared CORT levels of all females (i.e. whether they completed a clutch or not), mean CORT levels collected during the period of meiosis did not differ significantly by diet treatment ($F_{2, 27} = 1.39$, $p = 0.39$).

CORT levels differed significantly based on the time of day ($p < 0.01$). These levels rose over the course of the day, from 1.78 ± 0.35 at 0700 hours to 5.82 ± 1.25ng/ml at 1700 hours; peak levels were observed at 0100 hours. Females fed the HQ restricted

diet tended to have the lowest levels of CORT during the day, then rose significantly between 1700 and 0100 hours. Females fed the HQ *ad lib* and LQ *ad lib* had relatively similar levels of CORT throughout the day (Fig 5b).

Testosterone levels of females were low (in the picogram range) and in some treatments at the edge of the detectability of our assays. Mean testosterone levels did differ significantly by diet treatment, both *overall* ($F_{2, 26} = 6.79$, $p < 0.01$) and in mothers who were able to complete a clutch ($F_{2, 18} = 12.05$, $p < 0.01$) (Fig. 6a). In both cases, females fed the LQ diet exhibited significantly higher levels of T when compared to those fed the HQ *ad lib* and restricted diets. ($p < 0.01$ in all cases). Overall mean T levels ranged from 0.001± 0 ng/ml (HQ restricted, n = 8) to 0.46 ± 0.19 ng/ml (LQ *ad lib*, n = 5). In those mothers that completed their clutch we observed a range of 0.001± 0 ng/ml (HQ restricted, n = 6) to 0.54 ± 0.17ng/ml (LQ *ad lib*, n = 5). Testosterone levels did not differ by the time of day ($p = 0.96$) (Fig. 6b). Peak levels (0.10 ± 0.07 ng/ml) were observed only at 0100 hours; at 0700, 1200, and 1700 hours the average T levels were 0.001 ± 0 ng/ml.

Clutch size

There was no significant correlation between clutch size and levels of P_4 ($r = 0.28$, $p = 0.13$), CORT ($r = 0.31$, $p = 0.10$), or T ($r = 0.01$, $p = 0.96$). However, clutch size did differ significantly by diet treatment (refer to chapter 1 (Fig. 2)).

Sex ratio

Individual clutch sex ratios were not significantly correlated with maternal P_4 ($r = 0.18$, $p = 0.43$) (Fig. 7a), CORT ($r = 0.14$, $p = 0.55$) (Fig. 7b), or T ($r = 0.06$, $p = 0.80$) (Fig. 7c), levels. However, we did observe an overall effect of diet on sex ratio (refer to chapter 1). Mothers fed the HQ *ad lib* diet produced significantly more male skewed offspring sex ratios when compared to mothers fed the LQ *ad lib* and HQ restricted diets, but offspring sex ratio did not differ significantly between the LQ *ad lib* and the HQ restricted diets. There was no interaction effect between breeding diet and clutch size on primary offspring sex ratio (refer to chapter 1 (Fig. 3)).

DISCUSSION

We found that diet quality had a significant effect on circulating levels of maternal P_4 and CORT during a time period that approximately corresponds to when meiosis I is occurring, and when the sex of offspring can potentially be influenced. We observed that, at this proposed critical period, mothers fed the HQ restricted diets exhibited elevated CORT levels and the lowest P_4 levels when compared to females maintained on the LQ and HQ ad lib diets. In contrast, females fed the LQ *ad lib* diet, exhibited the opposite pattern: significantly elevated P_4 levels and, though not statistically significant (HQ restricted vs. LQ *ad lib*, $p = 0.06$), lower CORT levels. Females fed the HQ *ad lib* diet displayed a pattern that fell between the others, exhibiting moderate levels of P_4 and low levels of CORT. We analyzed sex ratio data for these same individuals in a concurrent study and found that females fed the HQ *ad lib* diet produced significantly more male offspring when compared to females fed the LQ and restricted diets. The observed hormone profile and sex ratio pattern results of our study correspond with past studies where elevation of P_4 and CORT (by exogenous administration of these hormones) skewed offspring sex ratios away from male biases (Correa et al., 2005; Pike and Petrie, 2006). In our study, the groups that previously exhibited low proportion of male-skewed clutches (e.g. LQ *ad lib* X = 38% and HQ restricted, X= 46%) were the same groups that had either significantly higher CORT than the HQ *ad lib* treatment group (i.e., 19.36 $\pm$ 6.87 vs. 5.50 $\pm$ 1.00 ng/ml) or significantly higher P_4 (9.31 $\pm$ 1.49 vs. 4.05 $\pm$ 1.48

ng/ml). Our study is the first to demonstrate, at the critical time of sex chromosome segregation, an endogenous elevation in CORT or P_4, two of the hormones that have been suggested to influence offspring sex ratio in birds, occurs in response to a diet manipulation.

A majority of the focus in studies of mechanisms of primary sex ratio in birds has been placed on the potential role played by preovulatory steroids due to their involvement in and close proximity to the timing of meiosis I. However, many studies have only examined levels of these steroids collected outside this critical period (von Engelhardt et al., 2004; Pike and Petrie, 2006; Bonier et al., 2007). For example, in several bird species steroid levels are relatively low and consistent during the day; peak levels of P_4 and estrogens have been observed to occur 4-6 hours prior to ovulation, during the period of meiosis I, and T peaks 10-6 hours prior to ovulation (Johnson, 1998). Similarly, basal CORT levels have been observed to remain low throughout the day and increases considerably during the dark hours of the daily cycle (Etches, 1979; Breuner et al., 1999; Rich and Romero, 2001). Here we report that maternal steroid levels were at their lowest and did not differ significantly between sampling at 0700, 1200, and 1700 hours. It was not until 0100 hours (5 hours prior to ovulation) that we observed both peak levels of maternal steroids as well as significant changes in these levels by diet. Thus, if elevated levels of maternal steroids are, in fact responsible for sex ratio biases, based on our findings, these steroids should be collected during the period of meiosis I when levels of these hormones would have the most proximate impact.

In this study we observed the effect that diet quality had on levels of maternal T. In all cases (mothers who were and were not able to complete a clutch), T levels were significantly elevated in females on the LQ *ad lib* diet. Additionally, results from blood samples taken throughout the day revealed that T levels were at their peak at 0100 hours. Elevated levels of maternal T have been correlated with the increased production of male offspring (Veiga et al., 2004; Rutkowska and Cichon, 2005), but the levels we observed, though at their highest during the time period corresponding to meiosis I, were extremely low and the females in the diet treatment with the highest T levels produced 38% females, rather than showing a male bias. Therefore our data do not correspond with those studies that have shown significant male-biased offspring sex ratios when females were given high levels of exogenous T. Additionally, males from mothers with these high T levels suffered from lower hatching success while their female counterparts exhibited higher survival prospects (Rutkowska and Cichon, 2005). The difference in effect seen on male and female offspring makes T an unlikely candidate for being the chief hormone responsible for primary offspring sex ratio adjustment. Our results lend support to this idea since endogenous female T levels were very low overall and those with the highest T levels during meiosis I produced predominantly female offspring.

The change we observed in maternal steroid levels at the time approximating the period of meiosis I was independent of a change in maternal body condition. There was no significant difference in maternal body condition among the diet treatments nor did we observe a significant correlation between maternal hormones at the potential critical time of meiosis 1 and body condition. Our diet regimes were such that maternal body

condition did not differ significantly between diet treatments thus suggesting that these diets were not severe enough to impose physical stress on these mothers. However, we did observe a significant elevation in CORT levels in mothers fed the HQ restricted diet at the 0100 sample period. Even though the perception of reduced availability of food did not affect maternal body condition, this treatment appeared to impose a *psychological* stressor that resulted in an increase in CORT levels.

We also expected to see an increase in maternal CORT levels as an indicator of stress due to feeding under conditions of low quality diets as in other studies (Hao et al., 2000). In the past, related stressful conditions, both acute and chronic, have almost consistently resulted in an increase in baseline levels of CORT (Clinchy et al., 2004; Kitaysky et al., 1999; Kitaysky et al., 2001b; Lynn et al., 2003; Schoech et al., 2004). However, CORT levels exhibited by mothers fed the LQ *ad lib* diet closely resembled that of mothers fed the HQ *ad lib* diet, and, thus, were significantly lower than the levels seen from mothers in the restricted treatment group. These results indicate that in this study perceived availability, and not quality per se, had a much stronger effect on the physiological condition of these mothers. Apparently, the perception of an abundant food resource, albeit of a somewhat lower quality than that of the other treatments, was not perceived as a stressor.

Although CORT levels in those fed the LQ *ad lib* diet did not differ from those on the HQ *ad lib* diets, individuals on the LQ *ad lib* diet did exhibit a significant elevation in levels of P_4 at the 0100 sample when compared to those on both of the HQ diets. One possible explanation for the variable levels of P_4 seen at this time could be attributed to a

shift in the timing of the preovulatory surge of P_4 in mothers fed the LQ diet. Poor diet quality (Neumann et al., 2002), has been found to have the potential to affect the duration of the ovulatory cycle or the timing of oviposition (Rattner et al., 1982). Although the composition of our diets were fairly similar, they were different enough to cause females in this diet treatment to have the lowest average condition index (albeit not significantly different) and to produce lower clutch sizes (although not significant) again indicating some nutritional deficiency in these females. It could be that the timing of preovulatory surge of P_4 (which typically occurs 4-6 hours prior to ovulation (Etches and Cheng, 1981; Johnson and Van Tienhoven, 1980; Liu et al., 2002; Proudman et al., 1984; Yang et al., 1997) was altered by the diet quality. Therefore, it is possible that the levels we report in mothers fed the LQ diet were taken represent a shift in the timing of the P_4 peak, and we captured it as it was near its highest point rather than when it was approaching or leaving its peak.

Based on previous studies, there seems to be a propensity for mothers to produce female-biased offspring sex ratios when CORT or P_4 levels are elevated. The levels we report are comparable to these studies. For example, Bonier *et al*. (2007) and Love et al. (2005) reported female-biased sex ratios to be correlated with elevated levels of maternal CORT similar to ones we report (14.32 ± 3.2 and 18.3 ± 3.5 ng/ml, respectively, versus our value of 19.37 ± 6.87 ng/ml). Correa et al. (2005) showed that exogenous administration of P_4 to leghorn hens resulted in female-skewed sex ratios, and their reported P_4 levels measured at the time corresponding to meiosis I (≤6 ng/ml) were similar to our value of 9.31 ± 1.48 ng/ml. However, we do not show a direct correlation

between these individual hormones and sex ratio of the clutch. This is not surprising because blood samples were only collected on one day of the laying cycle and the sex of the egg corresponding to that day was not determined. Furthermore, it is difficult to extrapolate information taken from an individual hormone collected at one point in time that would be a comprehensive representation of maternal hormonal profile for the entire clutch.

Although we show a significant effect of diet quality on maternal P_4 and CORT during meiosis I, this does not translate into a direct correlation between these individual hormones and the resulting primary offspring sex ratio. This lack of direct correlation does not automatically imply that sex ratio manipulation in response to naturally circulating levels of maternal steroids cannot occur in this species, but that maybe the required causal hormones are not appearing in the correct ratio to induce a sex bias in offspring (Pike and Petrie, 2003). It is clear that additional studies focusing on the interactions of natural levels of a combination of maternal steroids in circulation during the critical time in sex determination are needed to elucidate the mechanism and hormones responsible for primary offspring sex ratio in this species. Our results agree with the hypothesis that mothers having elevated levels of either CORT or P_4 in circulation during the period of meiosis I will produce more females than males per clutch. This study, in conjunction with previous studies investigating the potential hormonal mechanisms involved in primary sex ratio adjustment, have yielded interesting results linking dietary conditions, maternal steroids, and sex-ratio that warrant further investigation. To our knowledge this the first study to analyze a response to diet in

naturally circulating levels of maternal steroids during the diel cycle and the potentially critical meiosis I period in the zebra finch.

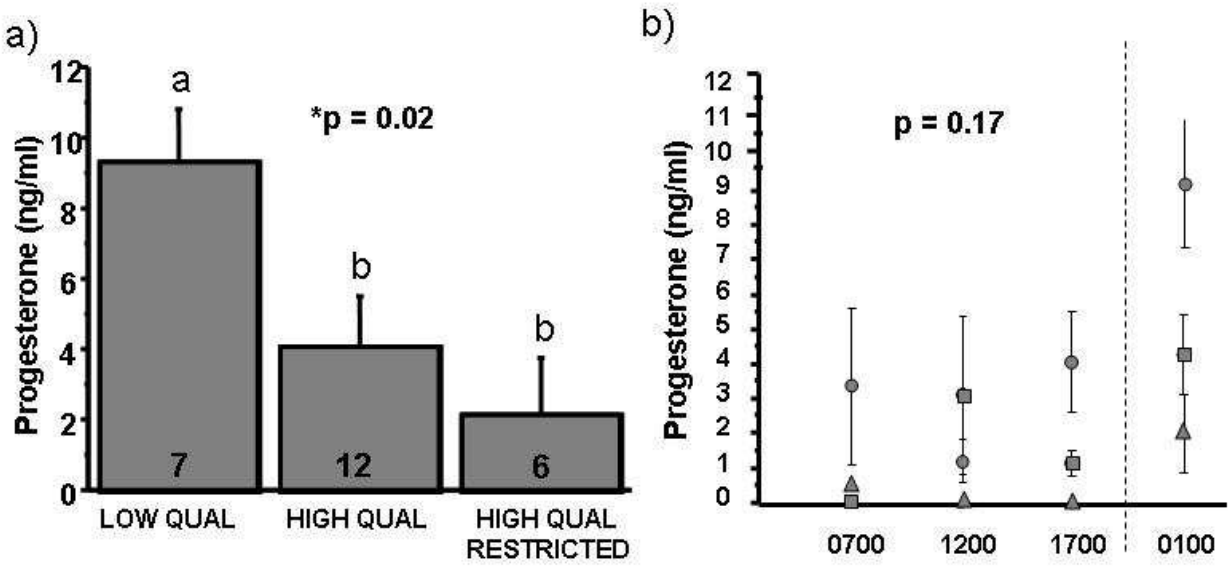

Figure 4. Mean plasma progesterone (P_4) levels by (a) during the period of meiosis I by diet treatment and (b) by the time of day ● LQ, ■ HQ, ▲ HQ restricted; 0700 (N= 9), 1200 (N= 13),1700 (N= 14), and 0100 (N = 25). The dashed line separates females who completed a clutch (0100 period) from females that were not laying eggs at the time of sampling.

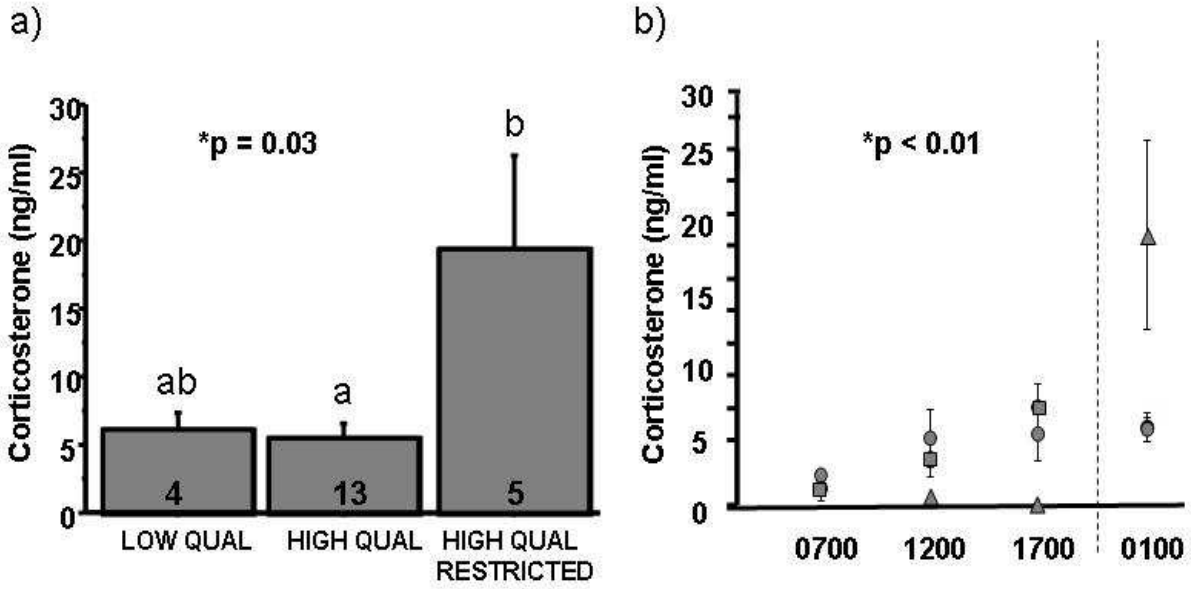

Figure 5. Mean plasma corticosterone (CORT) levels (a) during the period of meiosis I by diet treatment and (b) the time of day ● LQ, ■ HQ, ▲ HQ restricted; 0700 (N= 9), 1200 (N= 13), 1700 (N= 13), and 0100 (N = 22). The dashed line separates females who completed a clutch (0100 period) from females that were not laying eggs at the time of sampling

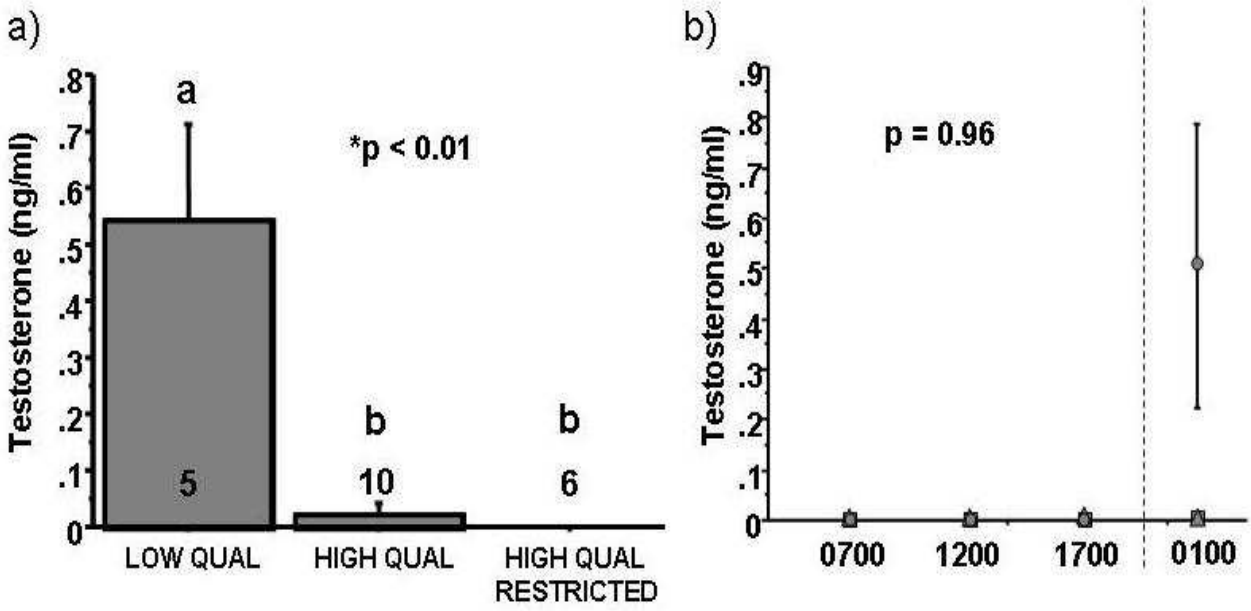

Figure 6. Mean plasma testosterone (T) levels (a) during the period of meiosis I by diet treatment and (b) the time of day ● LQ, ■ HQ, ▲ HQ restricted; 0700 (N= 9), 1200 (N= 5), 1700 (N= 6), and 0100 (N = 20). The dashed line separates females who completed a clutch (0100 period) from females that were not laying eggs at the time of sampling

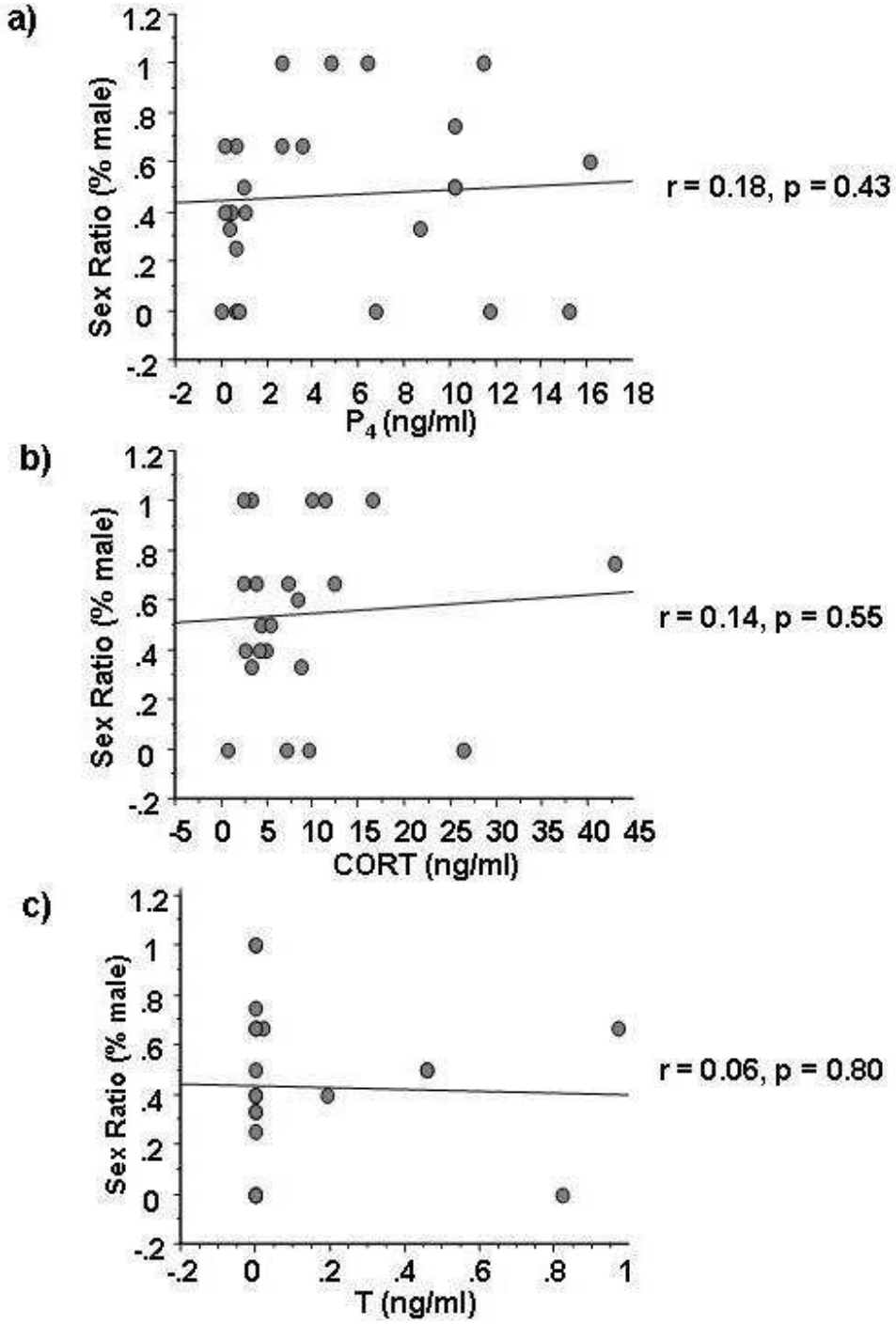

Figure 7. Relationship between the concentration of maternal (a) P_4, (b) CORT, and (c) T of laying females and their primary offspring sex ratio.

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