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The Nature of Beauty: How Environmental Conditions Impact Sexual Selection in the Zebra Finch

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The Nature of Beauty: How Environmental Conditions Impact Sexual Selection in the Zebra Finch

DISSERTATION

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for the degree of

DOCTOR OF PHILOSOPHY

in Ecology and Evolutionary Biology

by

Kerianne Murphy Wilson

Dissertation Committee:
Professor Nancy T Burley, Chair
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ABSTRACT OF THE DISSERTATION

The Nature of Beauty: How Environmental Conditions Impact Sexual Selection in the Zebra Finch

By

Kerianne Murphy Wilson

Doctor of Philosophy in Ecology and Evolutionary Biology

University of California, Irvine, 2019

Professor Nancy T Burley, Chair

Development and reproduction are life history stages during which the demand for resources is especially high, so individuals are expected to be particularly vulnerable to environmental conditions during these times. Thus, the way in which an individual allocates resources to different life functions during these two life stages should be meaningful in shaping natural and sexual selection. In a series of breeding experiments, my research examined the effects of diet quality on development and reproductive patterns of zebra finches. I focused on the following questions: (1) How does developmental diet influence the adult expression of male secondary sexual traits and male reproductive success via social mates vs. extra-pair partners? (2) How do females adjust reproductive allocation in response to the diet history of their social and extra-pair mates? (3) How do early diet and breeding diet jointly influence parental investment patterns and reproductive success of social mates during the incubation phase? To address these questions, I manipulated zebra finch developmental and/or breeding diet quality and tracked founders and offspring from multiple breeding colonies over three successive generations. Throughout the birds' development and reproduction, I quantified traits and behaviors expected to contribute to survival and fitness. The first chapter of my thesis found that males raised under high-quality nutritional conditions had higher adult expression of visual, but not acoustic, secondary sexual traits as well as higher fitness via production of sons and extra-pair paternity. Chapter 2 demonstrates that females differentially allocate resources in response to male quality (both based on a natal diet manipulation and extra-pair success) and to extra-pair offspring, and that females obtained direct fitness benefits from pairing with attractive males. Results from Chapter 3 indicate that birds exposed to a high-quality diet during development and a low-quality diet during reproduction struggle to invest in both reproduction and somatic maintenance during the incubation phase, suggesting this diet history combination may be detrimental to survival and reproduction.

INTRODUCTION

Sexual selection plays a significant role in shaping animal phenotypes (Darwin 1871). Across taxa, both intrasexual and intersexual competition lead to sexual dimorphisms in male appearance and behaviors that are associated with higher fitness. Sexual selection pressures typically impact females differently, favoring traits associated with high reproductive investment (Trivers 1972). Sexually selected trait expression is typically y highly variable and reflects both environmental and heritable influences. A major focus of the research herein is to improve understanding of how environmental conditions lead to variation in secondary sexual trait expression and what the consequences are for resource allocation and fitness.

Resource allocation is the keystone of life history trade-offs: theory predicts there is a trade-off between investing in somatic maintenance versus reproduction. Thus, in order to maximize fitness, allocation towards each of these life functions must be optimized within the limits of phenotypic plasticity (Stearns 1989). During development, individuals must trade off investing in traits needed for future reproduction versus investing in their immediate needs for survival and growth. During reproduction, individuals must balance the immediate needs of dependent offspring versus their own maintenance and future needs (ex: feather molt slows or stops in most avian species during this time --Ashmole 1962). Based on the importance of developmental and reproductive periods for understanding life history trade-offs, I focused my research on these life stages to explore the relationships between food quality and resource allocation.

This research captive birds gives greater insight into how animals cope with environmentally imposed constraints and helps to identify possible sources of environmental stress. By carefully eliminating variables like predation and nest site location, captive studies test ecological and evolutionary hypotheses in isolation from confounding variables. These experiments explore how environmental conditions, specifically food quality, impact life history trade-offs and what the consequences are for reproductive success. The following questions are addressed: How do environmental conditions impact avian resource allocation? How does developmental stress impact sexual selection and what are the reproductive consequences? What traits meet nutritional stress hypothesis predictions? How do variable environmental conditions (good or poor food quality) at key life stages (development and reproduction) impact allocation towards reproduction and somatic maintenance?

Environmental conditions experienced early in life have lasting consequences for both survival and reproduction (Metcalfe & Monaghan 2001; Spencer & MacDougall-Shackleton 2011) such that poor conditions are expected to result in lower resource allocation to both somatic maintenance (small body type, poor immunity) as well as reproduction (less developed secondary sexual traits, fewer offspring) (Metcalfe & Monaghan 2001). Thus, traits that are determined early in life may signal information about quality in terms of one’s ability to cope with nutritional stress. The nutritional stress hypothesis (NSH) posits that the honesty of a sexually selected trait can be maintained if trait expression is indicative of an individual’s resilience to nutritional stress endured during development. This hypothesis was originally developed and applied to passerine song, as successful acquisition of learned song traits is thought to require conditions favorable to brain development (Nowicki et al. 2002). Recently, Spencer and MacDougall-Shackleton (2011) convincingly argued that NSH should also be applicable to other sexually selected traits that are determined during development. A key NSH prediction is that individuals with less nutritional stress during development will have a fitness advantage. For Chapter 1, I sought to identify elements of song in a model passerine that meet NSH predictions as well as assess whether other secondary sexual traits such as beak color or cheek patch size also meet NSH predictions.

Until relatively recently, socially monogamous species were thought to also be genetically monogamous (Lack 1986). However, through the use of molecular techniques, it is now well understood that there is a difference between social and genetic monogamy with most socially monogamous species actually being genetically promiscuous (Avise 1996). Thus, males may not always be sure of their paternity of the offspring they are raising, which may establish conflict over the reproductive allocation decisions of a pair (Kempenaers and Schlicht 2010).

The benefit of Extra-pair offspring for males is fairly obvious- males are able to sire an offspring that he doesn't have to raise. However, there has been much debate over what benefits, if any, females gain from producing extra-pair offspring (Griffith et al. 2002; Kempenaers and Schlicht 2010; Forstmeier et al. 2014). While genetic benefits are often suggested, apparent differences in quality between within- and extra-pair offspring may be confounded by greater parental allocation towards extra-pair offspring (Jennions and Petrie 2000; Griffith et al 2002; Kempenaers and Schlicht 2010; Schmoll 2011). Ascertaining allocation differences to offspring may be difficult in species with bi-parental care, however, since both female extra-pair success and male attractiveness may influence how much each partner invests in a clutch attempt.

Theory predicts that extra-pair mates should possess preferred or attractive traits because they are indicators of genetic quality (Forstmeier et al. 2002; Kempenaers and Schlicht 2010; Wilson et al. 2019; but see Tschirren et al. 2012). However, the extent to which preferred traits are heritable is usually unknown, and environmental conditions may often influence expression of such traits (Griffith et al. 1999; Cornwallis and Uller 2010). Here we consider two measures of male quality/attractiveness that have been previously considered in zebra finches: extra-pair mating success (Houtman 1992; Bolund et al. 2009) and early diet quality. The aim of Chapter 2 was to understand how female zebra finches adjust reproductive allocation in response to their extra-pair success and the attractiveness of their partner (as measured by male extra-pair success and natal diet quality).

Two major competing hypotheses have been proposed regarding the fitness consequences of environmental conditions that organisms experience during early development and reproduction. The silver spoon hypothesis predicts that individuals raised on a high quality diet will have higher fitness than those raised on a poor quality diet regardless of the food environment in which they reproduce (Grafen 1988; Lindstrom 1999). Alternatively, the thrifty phenotype hypothesis (aka: environmental matching) predicts that individuals will have higher fitness when their adult diet quality matches the one in which they were raised (Gluckman et al. 2005; Spencer et al. 2006). Monaghan (2008) proposes two additional alternatives: 1) fitness may increase for all individuals when breeding conditions are favorable, but when breeding conditions are poor, those that developed under poor conditions will have the fitness advantage and; 2) individuals raised in favorable conditions always have higher fitness, and those that developed under poor conditions have higher fitness in poor relative to good adult conditions. The aim of Chapter 3 was to determine the combined reproductive consequences of early life and adult breeding environmental (nutritional) conditions and evaluate which of these hypotheses is most applicable to captive zebra finches.

Avian incubation has recently begun to receive recognition as a costly phase of reproduction (Heaney and Monaghan 1996; Reid et al. 2000), as parents not only face increases to energetic demands (Reid et al. 2000) but also time demands (Drent et al. 1985; Jones 1989; Zann & Rossetto 1991). Furthermore, zebra finches both share in incubation duties (Zann 1996) and their individual contributions to clutch success can be quantified in multiple ways. Thus, an experiment focused on the incubation phase was predicted to shed light on the impact that early life and breeding nutritional conditions may have for reproductive investment. In Chapter 3, I investigated the consequences of nutritional conditions during development and breeding on incubation behaviors and reproductive outcomes.

My thesis research will help advance the study of evolution of behavior and life histories by determining how resource allocation varies in response to environmental conditions during key stages in which resources are known to be scarce. Since environmental conditions are in constant flux, it is important to reconcile various trade-off predictions in order to better understand how allocation strategies change in response to these extremes in order to predict species outcomes. This research provides tests of several key contemporary hypotheses in the literature, including the nutritional stress, silver spoon and thrifty phenotype hypotheses. It will further investigate areas of resource allocation between developmental and reproductive stages that have been largely neglected.

CHAPTER 1:

Early Life and Transgenerational Stressors Impact Secondary Sexual Traits and Fitness

INTRODUCTION

Environmental conditions experienced in early life may have lasting consequences for both survival and reproduction (Lindstrom 1999; Metcalfe and Monaghan 2001; Spencer and MacDougall-Shackleton 2011), with unfavorable conditions resulting in lower resource allocation to both somatic maintenance (small body dimensions, poor immunity) and reproduction (less-developed secondary sexual traits, fewer offspring) (Metcalfe and Monaghan 2001; Blount et al. 2003; Naguib and Nemitz 2007; Tschirren et al. 2009; Tilgar et al. 2010). Developmental stress may result from a variety of challenges, including poor early nutrition (Nilsson and Gardmark 2001) resource competition among siblings and other conditions that result in elevated stress hormone levels (Mousseau and Fox 1998; Crino et al. 2017). Traits whose adult expression is significantly influenced by early life conditions may signal information about an individual's history and/or ability to cope with developmental stress. Secondary sexual traits in particular appear to be good candidates as indicators of male mating quality (Andersson 1994) if their expression meaningfully reflects the effects of early life conditions on male contributions to either direct fitness (e.g., via parental caregiving) and/or indirect fitness (by signaling heritable quality) of their mates.

The idea that adult secondary sexual trait expression reflects early life conditions forms the basis of the nutritional stress hypothesis, which posits that passerine song is an indicator of dietary conditions experienced during development (Nowicki et al. 1998). This hypothesis is based on the premise that successful acquisition of learned song traits requires conditions favorable to brain development (Nowicki et al. 2002). A key prediction is that individuals that experience less nutritional stress during development have a fitness advantage. To date, only one empirical study has investigated fitness consequences arising from diet-induced variation in song traits (Woodgate et al. 2012), although several studies have reported relationships between repertoire size and reproductive success not tied specifically to diet (Eens et al. 1991; Hasselquist et al. 1996; Gil and Slater 2000; Reid et al. 2005).

The developmental stress hypothesis (Spencer et al. 2000; Buchanan et al. 2003; Spencer and MacDougall-Shackleton 2011), an expansion of the nutritional stress hypothesis, more broadly considers how secondary sexual traits other than song may reflect developmental conditions and impact fitness. Several experiments that manipulated avian brood size have found that natal clutch traits can influence developmental stress: enlarged brood size (De Kogel and Prijs 1996; De Kogel 1997; Nicolaus et al. 2009) and late hatch order (Zach 1982; Saino et al. 2001; Bowers et al. 2011; Gilby et al. 2012) can result in reduction of body size and secondary sexual trait expression, which in turn can have fitness consequences (Drummond and Rodriguez 2013; Dey et al. 2014). To date, however, possible effects of natural (unmanipulated) brood size and hatch order variation have not been studied in this context (MacDougall-Shackleton 2015). We expected to find correspondence with results of brood manipulation studies in that birds from larger broods and later hatch order would experience higher developmental stress. Brood composition – the number of male versus female siblings – may also have consequences for individual quality, because resource allocation to the sexes often differs (Clutton-Brock et al. 1985; Badyaev et al.; 2002; Nicolaus et al. 2009) and the consequences of sibling competition may differ for males and females (Bowers et al. 2011; Braasch et al. 2014; Stauffer et al. 2018).

While inter-generational parental effects resulting from environmental conditions that breeders experience as adults have long been known (Mousseau and Fox 1998; Badyaev 2005), there is growing awareness that an individual's early life conditions can also impact its offspring, which implies the occurrence of multi-generational (or "transgenerational") effects (Burton and Metcalfe 2014; Krause and Naguib 2014). Thus, for example, studies across a range of taxa have shown that parental early life experiences can affect offspring growth (Lummaa and Clutton-Brock 2002; Naguib and Gil 2005; Taborsky 2006; Alonso-Alvarez et al. 2007; Saastamoinen 2013). However, relatively few studies to date have followed offspring into adulthood to investigate impacts on adult phenotype and fitness (but see Naguib et al. 2006). Also, compared to maternal effects, paternal effects have received little attention (Uller 2008; McAdam et al. 2014), but are likely important, especially in species that exhibit bi-parental care (see Moreno et al. 1997 for example in pied flycatchers).

To obtain a more complete understanding of the impact of stressors and secondary sexual traits on sexual selection, we measured extra-pair reproductive success as well as within-pair success. While a few previous studies have assessed extra-pair paternity in a caged 'choice' test setting (e.g., Houtman 1992; Forstmeier 2007), our study was conducted in a colony setting more natural for this species (Zann 1996). This design is superior, because conditions such as density and sexual conflict may influence tendencies for birds to seek and accept extra-pair mates (Petrie and Kempenaers 1998).

In sum, using a captive population of zebra finches (Taeniopygia guttata castanotis), a socially monogamous species, we sought here to investigate consequences of an experimentally applied stressor (diet quality of male subjects during development), as well as the unmanipulated natal brood traits of males and their parents on male adult phenotype and reproductive success. The secondary sexual traits included in our study had previously been shown to have roles in mate choice: song (Holveck and Riebel 2007), beak color (Burley and Coopersmith 1987; Price and Burley 1994; Simons and Verhulst 2011), and cheek patch size (Naguib and Nemitz 2007; Tschirren et al. 2012). Given that parents often invest differentially in male and female offspring (Trivers and Willard 1973; West 2009) and that the sexes may respond differently to early life conditions (Clutton-Brock et al. 1985; Metcalfe and Monaghan 2001; Tilgar et al. 2010), we included consideration of separate effects of male and female siblings. We predicted that greater expression of acoustic and visual secondary sexual traits would have a positive effect on reproductive success components (Trivers 1972; Andersson and Iwasa 1996; Kokko et al. 2002) and that the expression of these traits would be negatively affected by developmental stressors, in accordance with the developmental stress hypothesis predictions. By comparing the effects of multiple early life stressors on multiple secondary sexual traits and considering the implications for both genetic and social reproductive success, we are able to illustrate how developmental stress can shape sexual selection.

METHODS

Founder Rearing Conditions and Experiment Initiation

The 64 adult founders (32 of each sex) of this breeding experiment (generation 1) were produced in 2012 in one of three outdoor aviary populations (each also composed of 32 birds of each sex), each of which was supplied a different diet (generation 0). The aviaries were of identical size and physical layout. To reduce the potential for unintended differences between populations to influence the results, these three populations were established from a pool of birds derived from three aviaries in the previous generation (generation -1). A stratified random design was used to balance assignment of birds across flights while minimizing opportunities for double-first-cousin pairings. Within generations, all populations reproduced over the same time course and the age range of founders was standardized; across generations, founder density was held constant and effective population size was standardized as closely as possible. In generation 0, male founders of the current experiment (generation 1) were reared in aviaries held on either the HI (daily hen's egg supplement) or LO (no hen's egg supplement) diet, while females were reared on the typical diet used in previous generations (LAB diet: thrice weekly egg supplement). All birds received green vegetables thrice weekly and ad libitum supplies of water, a commercial mix of ripe grass seed for estrildines, cuttlefish bone, and ground oyster shell. To prevent developmental compensation for short- and medium- term exposure to a particular early diet (Arnold et al. 2007; Krause and Naguib 2015), all birds were kept on their natal diet until they were selected as breeders for generation 1.

Apart from diet variation, the rearing protocol for all founders and their parents was the same: populations were founded at standard densities (64 adults), and once offspring in these populations reached 45(+ 3) days of age (when sexual dichromatisms become apparent), they were caught and housed in single-sex cages at standard densities within their natal flights until they reached 100 days of age. This procedure was implemented to provide developing birds visual and acoustic contact with adults; such contact is important for imprinting on visual and acoustic traits (Immelmann 1975; Bolhuis 1991; Bischof et al. 2002). During this time span, males learn songs and both sexes develop mate preferences (Eales 1989; ten Cate et al. 1993; Zann 1996). Data were collected for the unmanipulated candidate stressors considered: hatch order and number/sex of surviving siblings for all male founders and their social parents. After 100 days of age, birds were housed indoors in cages until selected for this study.

In spring 2013, 16 males from each of the HI and LO diet treatments and 32 females reared on the LAB diet were selected for the study. All founders had wild-type plumage and were judged to be in excellent overall condition. No more than two siblings per family ($N_{families}$ = 23 for males, 24 for females) were selected. All birds were previously unmated; age varied between 6 and 13 months at the start of the experiment and did not differ between treatments ($P > 0.5$).

Six weeks prior to the start of breeding, all founders were placed on the LAB diet. Birds were uniquely color-banded for identification with colors previously established not to impact mate preference. Prior to release, several size traits and secondary sexual traits were measured: mass, tarsus length, head width, cheek patch size, and beak color. Song recordings were also made at this time. In order to assign genetic parentage to offspring, a 25 μL blood sample was collected from the brachial vein of each founder and suspended in DMSO solution (Seutin et al. 1991).

Founders were housed in a single (80 m3) aviary flight and allowed to freely pair and breed. Approximately 2.5 nest cups were available for each breeding pair; nesting material (grass and feathers) was provided daily. At the end of the experiment, breeding was suspended by removing clutches initiated more than 150 days after release and before eggs could have hatched. The population was then kept intact until the last-hatched offspring reached 100 days of age.

Secondary Sexual Trait Measures

Both cheek patches were measured for each male founder. Birds were hand-held so that a cheek patch was parallel to and on the same plane as a measuring ruler and photographed when feather posture was relaxed. Photographs were taken under standard illumination using a Canon® EOS camera. Cheek patch area was measured using ImageJ software (U.S. National Institutes of Health, Bethesda, MD, USA) by a person not aware of male diet treatment. In order to further standardize measurements, one individual held all birds, and another person took all photographs and made measurements. Analyses were based on the average size of both cheek patches, as well as the negative absolute size difference (- | left – right |) of cheek patches ("cheek patch symmetry").

Beak color was measured using the Munsell® Book of Color, Glossy Finish Collection (X-Rite, Inc., Grand Rapids MI, U.S.A.). This color system describes the hue, value and chroma of beak color. Males were held in a standard position and their beak color was compared to the color chips. When beak color fell between two chips, scores were interpolated. These scores (hue, value and chroma) were then used to generate a single index of beak color (Burley et al. 1992) in which the highest score is assigned to beaks that are perceived by humans as the reddest, darkest, and brightest. Females prefer males with high beak color scores (Burley and Coopersmith 1987; Simons and Verhulst 2011).

(Ultraviolet reflectance of male beaks is minimal, and spectrophotometer-based scores have been found to correlate well with Munsell® scores [Bolund et al. 2010].)

Zebra finches produce a single, highly stereotyped song motif (alternatively referred to as “song phrase” [Riebel 2009]) that shows considerable variation among individuals and is the unit of song in our analyses. The number of motifs delivered in any given bout of song is highly variable (Zann 1996). Songs were recorded using the following procedures: individually caged males were placed in a sound attenuation chamber containing a separate cage of two unfamiliar stimulus females. Sound was recorded on Mac OS X using an Audio Technica model AT 2020 condenser microphone and Garageband software. Males were recorded until they produced at least 3 songs and 10 motifs. The silence between songs was spliced from recordings, and MP4 files were converted to WAV files using Free Convert (XillSoftware Company, New York, NY, U.S.A.); motifs were visualized in Sound Analysis Pro 2011 (SAP11) (Tchernichovski and Mitra 2004).

For each male, we selected a total of five recorded motifs for analysis, using several guidelines to maximize the number of songs sampled. The selected motifs came from at least three different songs, with no more than two motifs from any one song. Songs that were too noisy to permit syllable identification were excluded. In order to avoid classifying introductory syllables as part of a male’s motif, the first motif of every song was also excluded.

All motifs were initially scored to identify the range of syllable types present in the population. No syllable type was found to be unique to a specific diet treatment. Following criteria previously established for syllable classification (Williams and Staples 1992; Leadbeater et al. 2005), syllable boundaries were identified by silence surrounding a unit of sound or by abrupt changes in amplitude or harmonic qualities (sound morphology). Through these criteria, 12 unique syllable types were identified (Figure 1.1). The two observers that identified these syllable types then independently assessed male motifs using this classification and no inter-observer discrepancies occurred.

Two measures of song complexity (total syllable number [Nowicki et al. 1998; Spencer et al. 2003] and proportion of unique syllables [Holveck and Riebel 2007]) and one measure of song performance (stereotypy [Holveck and Riebel 2007]) were used to assess male song quality. (While several studies have analyzed song rate as a potential metric of male zebra finch quality [ten Cate and Mug 1984; Houtman 1992; Collins et al. 1994; Birkhead et al. 1999; Forstmeier 2007; Riebel 2009; but see David et al. 2012], this measure varies with female attractiveness and male satiation [Riebel 2009; Ritschard and Brumm 2012], which were not controlled for in this experiment.) The number of syllables in each selected motif was averaged across the five motifs for each male in order to calculate total syllable number. The "proportion of unique syllables" was calculated as the number of unique syllables divided by total number of syllables. In order to account for individual male differences in syllable morphology, syllables were scored as unique if their between-motif variation was visually assessed to be less than their within-motif variation, as determined by two scorers.

Variation across each male's motifs was assessed using the stereotypy coefficient described by Holveck and Riebel (2007). All five motifs were compared in a pairwise fashion, and syllable changes (additions, deletions and substitutions) between them were quantified. The resulting stereotypy coefficient describes the overall variation among motifs on a scale of 0 to 1, with 1 indicating that all motifs have exactly the same syllable number and order, and 0 indicating that all syllables of all motifs are different (equation below). Stereotypy coefficient values varied from 0.727 to 1 for this population.

Stereotypy coefficient= 1- [(Σ changes)/(Σ motifs-1)(Σ syllables)]

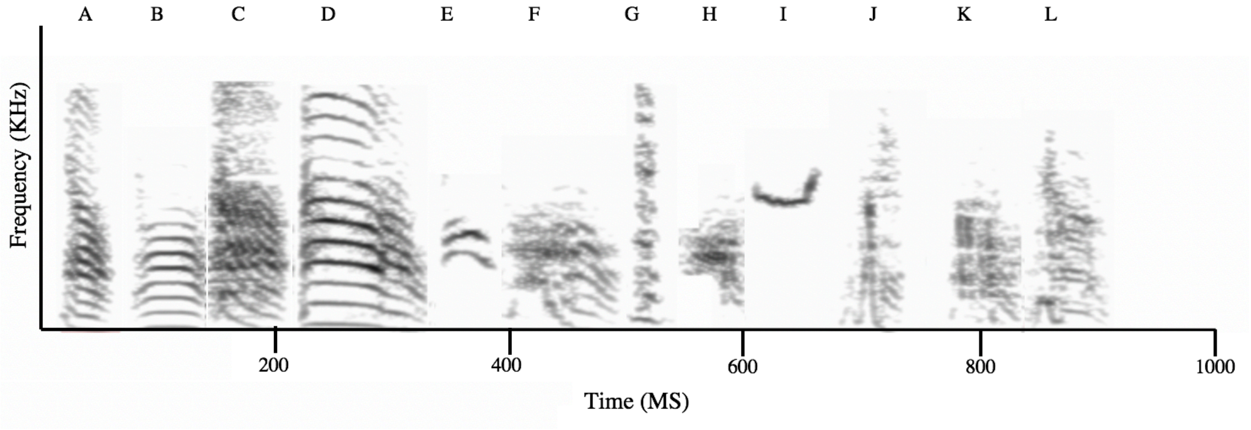


Figure 1.1: Unique syllable types identified in male zebra finch songs.

Breeding and Reproductive Success Measures

Throughout the experiment, nests were censused each morning, at which time egg and hatchling numbers were recorded and every new egg and hatchling were individually marked in order to track lay order and hatch order. If two offspring hatched on the same day, the heavier hatchling was considered older, on the assumption it had been provisioned over a longer interval. Each nestling received a uniquely numbered metal leg band before fledging. Active nests were observed weekly in order to assign social parents to each clutch. When offspring reached 45(+/- 3) days of age, a single 25 µL blood sample was collected from the brachial vein. Measures of male reproductive success were based on offspring that survived to at least 45 days of age and included the number of reared offspring ("social parentage") and the number of offspring sired ("genetic parentage"), and extra-pair paternity.

Genetic Parentage Assignment

Genotyping was performed for founders and offspring using eight highly polymorphic microsatellite loci (Tgu1, Tgu3, Tgu4, Tgu5, Tgu8, Tgu9, Tgu10 and Tgu12) that have been established for zebra finches (Forstmeier et al. 2007). For each sample, DNA was extracted using phenol-chloroform-isoamyl extraction and ethanol precipitation (Milligan 1998). DNA was then amplified for each locus using PCR. Each PCR sample had a volume of 10 µL and included the specific forward and reverse primers (one of which was fluorescently labeled) corresponding to each microsatellite locus (Integrated DNA Technologies, Iowa, USA). PCR began with denaturing for five minutes at 95°C, followed by 32 cycles at 95°C (40 s denaturing), 53°C (40 s annealing) and 72°C (60 s extension) and ended with an extension step of seven minutes at 72°C. Analyses were completed using an ABI 3100 Genetic Analyzer (Applied Biosystems). Data were analyzed using Genemapper 4.0 (Applied Biosystems).

Allele frequencies, non-exclusion probabilities, heterogeneity and parentage were calculated and assigned using CERVUS 3.0 (Kalinowski et al. 2007). The mean observed heterozygosity was 0.846, which does not differ significantly from the mean expected heterozygosity (0.854). There were no departures from Hardy-Weinberg equilibrium at any of the eight loci, or for all loci combined. The mean number of alleles per locus was 14.25 (SD = 3.06), and the non-exclusion probability for the first parent (mother) was 0.001 and for the second parent (father) was < 0.0001.

The founders and offspring included in genotyping results had signals for at least six of the eight loci. Out of 192 offspring that were genotyped, 19 were excluded from further analyses. Six offspring were excluded because fewer than six loci could be determined and the birds were no longer available for blood re-sampling. Another six offspring were excluded because they were reared by same-sex pairs. Lastly, seven offspring were excluded because they were attended by only one social parent. A total of 28 offspring were not genotyped because they could not be fit on 2 plates (192 specimens) allotted for genetic analyses and were assumed to be genetic offspring of their social parents. Extra-pair paternity (EPP) rates were estimated from a sample of 173 offspring, which is considered an appropriate sample size for such analysis (Griffith et al. 2002). Offspring were classified as conspecific brood parasites when neither social parent was found to be a genetic parent.

Statistical Analyses

Pearson's correlations were performed to assess occurrence of phenotypic correlations in male trait expression. The same tests were used to quantify relationships among candidate developmental stressors (parents' natal clutch traits as well as male subjects' natal clutch traits, and male natal diets), male adult traits (head width, tarsus length, mass, song stereotypy, total syllable number, proportion of unique syllables, beak color, cheek patch size and symmetry), and reproductive success (genetic/ social/extra-pair production of sons and daughters). Potential developmental stressors were coded for analysis such that higher values were assigned to states predicted to impose greater stress (LO diet, greater number of siblings of each sex for males and their parents, and later hatch order of males and their parents). Four males (three HI and one LO) were excluded from analyses of reproductive success because they formed same-sex pairs.

The effect of select candidate stressors on male adult traits and reproductive success was further analyzed when correlation coefficients generated from Pearson analyses were significant at $\alpha < 0.10$. In order to reduce the influence of correlations among dependent variables, all variables were first z-transformed and male identity was included in models as a random effect. Linear mixed effect models were then performed to assess the effect of candidate stressors on male adult traits and reproductive success ($\alpha = .05$). Linear mixed effect models were also used in analyses in which phenotype was partitioned into three components (acoustic traits, visual traits, size traits), and reproductive success was partitioned into son and daughter production.

The same approach was used to evaluate influences of male phenotypic traits on reproductive success. Here, the a priori expectation was that higher trait values (generating positive effects) would contribute to reproductive performance.

Two-sample t-tests with equal variance were used to assess differences in reproductive success between males that did or did not sire extra-pair offspring.

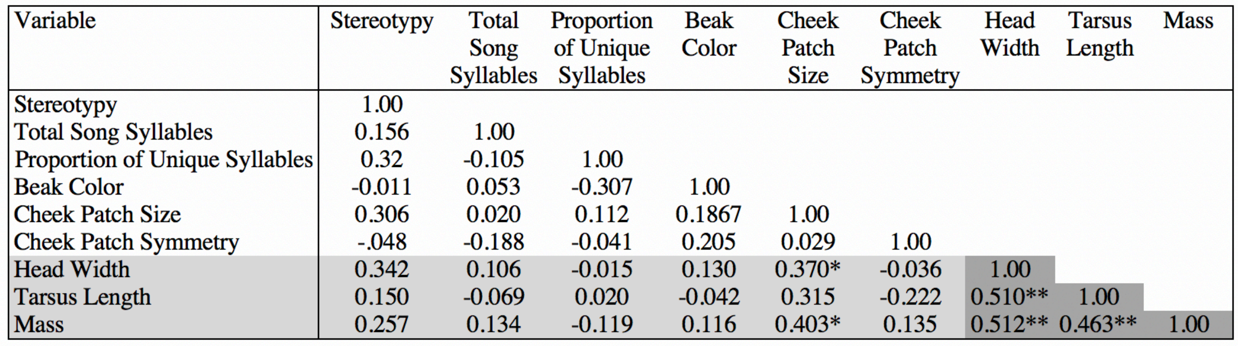
Based on Shapiro-Wilks tests and visual assessment of quantile-quantile plots, five variables were transformed prior to analyses: syllables per motif (squared), the proportion of unique syllables (raised to the fourth power), cheek patch size (z-transformed), cheek patch symmetry (square root prior to being multiplied by -1) and beak color (z-transformed, then cubed). All measured variables are reported here. Analyses were performed in STATA 14 (StataCorp LP, College Station, Texas, USA).

RESULTS

The three male size traits were positively inter-correlated, but the six secondary sexual traits were not inter-correlated (Table 1.1).

Of the 173 genotyped offspring, 145 had the same genetic and social parents and 23 were categorized as extra-pair offspring (EPP rate = 13.3%). Five offspring were scored as conspecific brood parasites and these offspring were produced by five different genetic pairs. The 23 extra-pair offspring were sired by ten males (range: 1-7 offspring), four of which lost paternity to another male.

Table 1.1: Correlations among secondary sexual traits and size traits of males. Unshaded boxes indicate correlations among secondary sexual traits; light gray shading indicates correlations between secondary sexual traits and size traits; and dark gray shading indicates correlations among size traits. Pearson's correlation coefficient values reported for N = 32; family N = 23. P < 0.05 indicated by *, P < 0.01 indicated by ** .



Variable	Stereotypy	Total Song Syllables	Proportion of Unique Syllables	Beak Color	Cheek Patch Size	Cheek Patch Symmetry	Head Width	Tarsus Length	Mass
Stereotypy	1.00								
Total Song Syllables	0.156	1.00							
Proportion of Unique Syllables	0.32	-0.105	1.00						
Beak Color	-0.011	0.053	-0.307	1.00					
Cheek Patch Size	0.306	0.020	0.112	0.1867	1.00				
Cheek Patch Symmetry	-.048	-0.188	-0.041	0.205	0.029	1.00			
Head Width	0.342	0.106	-0.015	0.130	0.370*	-0.036	1.00		
Tarsus Length	0.150	-0.069	0.020	-0.042	0.315	-0.222	0.510**	1.00	
Mass	0.257	0.134	-0.119	0.116	0.403*	0.135	0.512**	0.463**	1.00

Stressor Effects on Male Phenotype

Two candidate stressors had negative overall effects on male phenotype expression: diet and male hatch order (Figure 1.2, Table 1.2). Tests to assess which aspects of the male phenotype (acoustic, visual, and/or size traits) were impacted by candidate stressors indicated that diet, paternal hatch order and the number of sisters in the natal brood of male's father negatively impacted visual traits, and the numbers of sisters in the natal broods of male's mothers positively impacted visual traits (Figure 1.3B). Hatch order negatively impacted male body size (Figure 1.3C) and no candidate stressors impacted acoustic traits (Figure 1.3A).

Table 1.2: Correlations among developmental stressors and male traits (secondary sexual traits and size traits). Pearson's correlation coefficient values reported for N = 28 (family N = 23). $P < 0.05$ indicated by *, $P < 0.01$ indicated by ** .

Male Trait	Diet	Male Hatch Order	Male Brothers	Male Sisters	Maternal Hatch Order	Maternal Brothers	Maternal Sisters	Paternal Hatch Order	Paternal Brothers	Paternal Sisters
Stereotypy	-0.226	-0.368*	-0.221	0.135	0.007	-0.001	-0.118	0.244	-0.259	-0.145
Total Syllables	-0.263	0.013	-0.073	-0.137	0.065	-0.277	0.177	0.116	-0.308	0.302
Proportion of Unique Syllables	0.128	0.137	-0.265	0.191	-0.031	0.361	-0.146	0.112	-0.117	0.247
Cheek Patch Size	-0.476**	-0.255	-0.033	0.416*	-0.316	0.069	0.466*	-0.241	-0.004	-0.354
Cheek Patch Symmetry	-0.187	-0.092	0.216	-0.042	0.040	0.257	0.287	-0.237	0.154	-0.113
Beak Color	-0.473**	-0.250	0.165	-0.123	0.196	-0.054	0.206	-0.357	-0.156	-0.453*
Head Width	-0.244	-0.418*	0.259	0.321	-0.196	-0.135	0.155	0.003	0.010	-0.075
Tarsus Length	0.010	-0.067	0.139	0.126	-0.287	-0.209	0.151	0.091	-0.117	0.272
Mass	-0.551**	-0.241	0.130	0.256	0.290	-0.163	0.011	0.207	-0.242	0.033

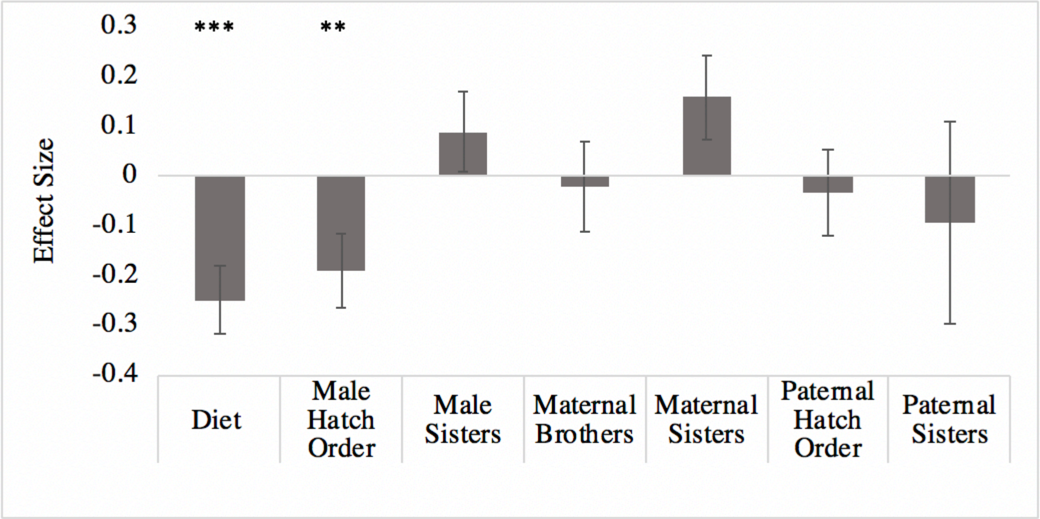


Figure 1.2: Effect sizes (+ SE) of z-transformed candidate developmental stressors on z-transformed overall male trait expression, including acoustical secondary sexual traits (stereotypy, total syllables, proportion of unique syllables), visual secondary sexual traits (cheek patch size and symmetry, beak color), and body size traits (head width, tarsus length, and mass). * ($P\leq .05$); ** ($P\leq .01$); *** ($P\leq .001$); **** ($P<.0001$). Data from Table 2.

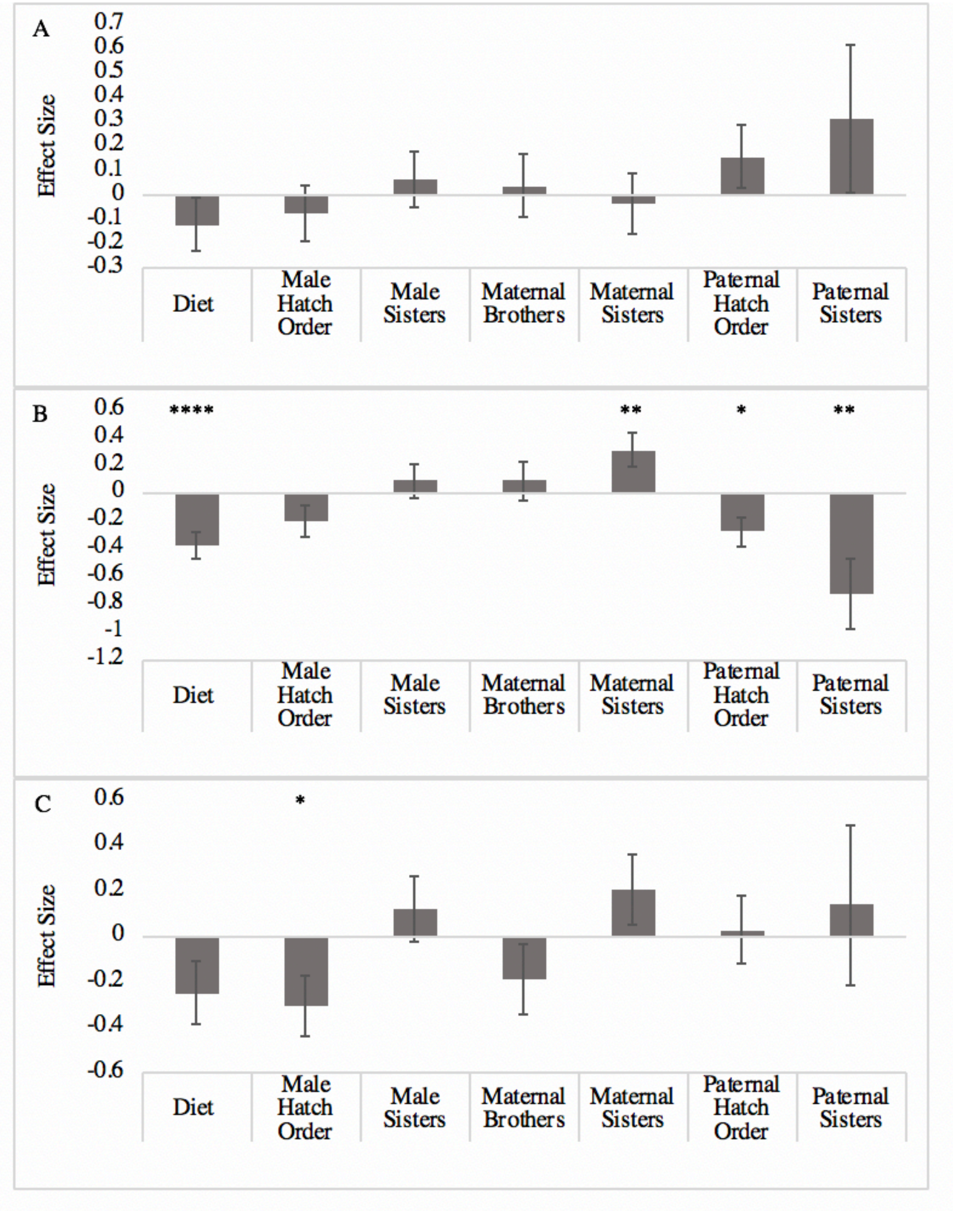
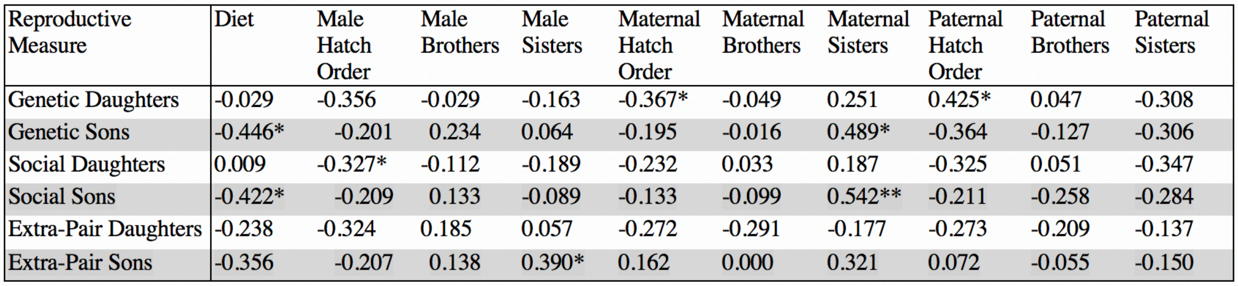


Figure 1.3: Effect sizes (+ SE) of z-transformed candidate developmental stressors on z-transformed adult male expression of A) acoustical secondary sexual traits (stereotypy, total syllables, proportion of unique syllables), B) visual secondary sexual traits (cheek patch size and symmetry, beak color), and C) body size traits (head width, tarsus length, and mass). * (P≤ .05); ** (P≤ .01); *** (P≤ .001); **** (P<.0001). Data from Table 2.

Stressor Effects on Male Reproductive Success

Two potential stressors were found to have overall negative effects on reproductive success: diet and male hatch order (Figure 1.4, Table 1.3). Male hatch order and that of his father negatively impacted daughter production (Figure 1.5A), while diet quality negatively impacted son production (Figure 1.5B).

Table 1.3: Correlations among developmental stressors and reproductive measures. Pearson's correlation coefficient values reported for N = 28; family N = 23. P < 0.05 indicated by *, P < 0.01 indicated by ** .



Reproductive Measure	Diet	Male Hatch Order	Male Brothers	Male Sisters	Maternal Hatch Order	Maternal Brothers	Maternal Sisters	Paternal Hatch Order	Paternal Brothers	Paternal Sisters
Genetic Daughters	-0.029	-0.356	-0.029	-0.163	-0.367*	-0.049	0.251	0.425*	0.047	-0.308
Genetic Sons	-0.446*	-0.201	0.234	0.064	-0.195	-0.016	0.489*	-0.364	-0.127	-0.306
Social Daughters	0.009	-0.327*	-0.112	-0.189	-0.232	0.033	0.187	-0.325	0.051	-0.347
Social Sons	-0.422*	-0.209	0.133	-0.089	-0.133	-0.099	0.542**	-0.211	-0.258	-0.284
Extra-Pair Daughters	-0.238	-0.324	0.185	0.057	-0.272	-0.291	-0.177	-0.273	-0.209	-0.137
Extra-Pair Sons	-0.356	-0.207	0.138	0.390*	0.162	0.000	0.321	0.072	-0.055	-0.150

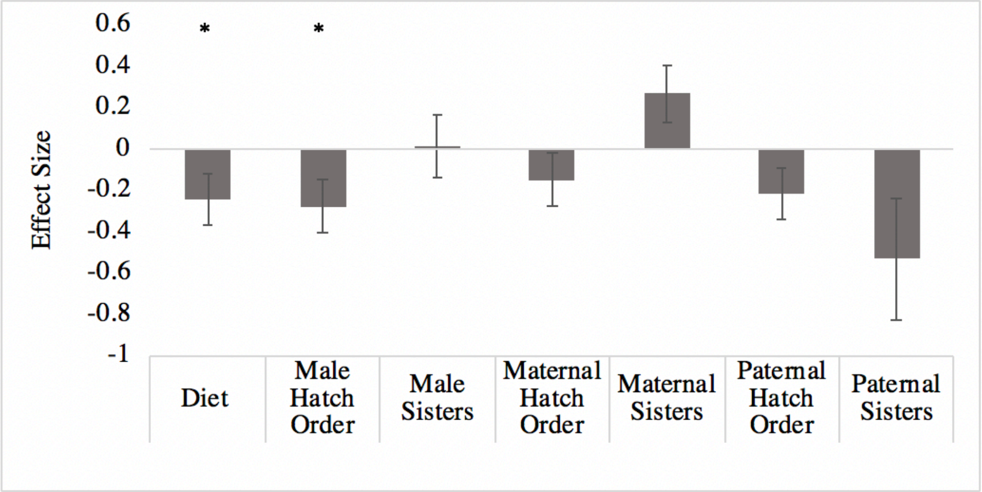


Figure 1.4: Effect sizes (+ SE) of z-transformed candidate developmental stressors on all z-transformed male reproductive success measures (genetic, social and extra-pair production of sons and daughters). * (P≤ .05); ** (P≤ .01); *** (P≤ .001); **** (P<.0001). Data from Table 3.

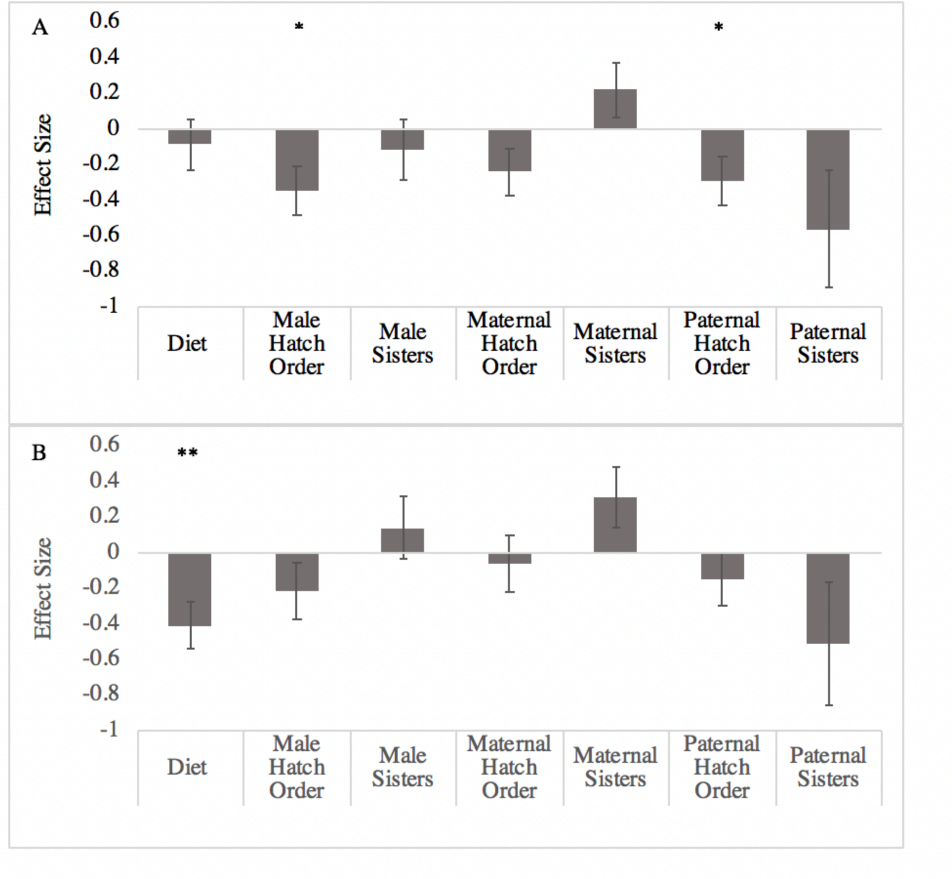
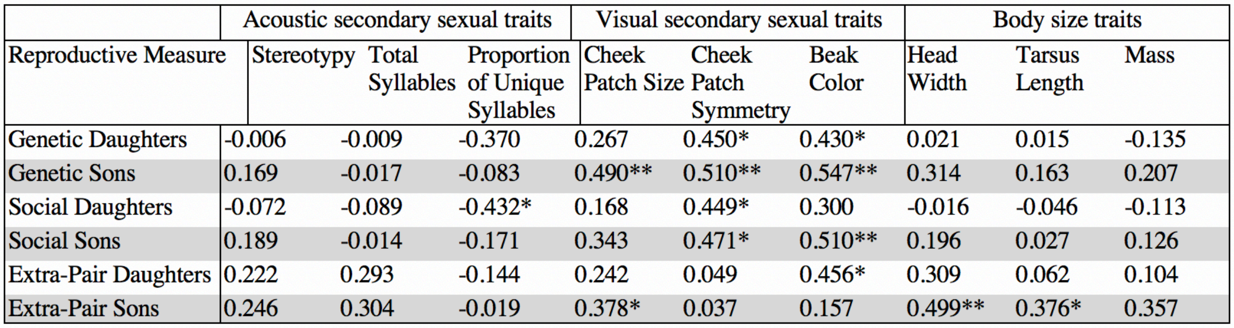


Figure 1.5: Effect sizes (+ SE) of z-transformed candidate developmental stressors on z-transformed A) daughter production (genetic, social and extra-pair) and B) son production (genetic, social and extra-pair). * (P≤ .05); ** (P≤ .01); *** (P≤ .001); **** (P<.0001). Data from Table 3.

Effects of Male Traits on Reproductive Success

Three of the nine phenotypic traits measured in males were found to be positive predictors of the collective measures of male reproductive success cheek patch size, cheek patch symmetry and beak color (Figure 1.6, Table 1.4). Cheek patch symmetry and beak color positively predicted both daughter and son production while cheek patch size and head width predicted production of sons only (Figure 1.7). The proportion of unique syllables in a male's song was a negative predictor of the production of daughters; that is, males with a lower proportion of unique syllables produced more daughters (Figure 1.7A).

Table 1.4: Correlations among male traits (secondary sexual traits and size traits) and reproductive measures. Pearson's correlation coefficient values reported for N = 28; family N = 23. P < 0.05 indicated by *, P < 0.01 indicated by ** .



	Acoustic secondary sexual traits			Visual secondary sexual traits			Body size traits		
Reproductive Measure	Stereotypy	Total Syllables	Proportion of Unique Syllables	Cheek Patch Size	Cheek Patch Symmetry	Beak Color	Head Width	Tarsus Length	Mass
Genetic Daughters	-0.006	-0.009	-0.370	0.267	0.450*	0.430*	0.021	0.015	-0.135
Genetic Sons	0.169	-0.017	-0.083	0.490**	0.510**	0.547**	0.314	0.163	0.207
Social Daughters	-0.072	-0.089	-0.432*	0.168	0.449*	0.300	-0.016	-0.046	-0.113
Social Sons	0.189	-0.014	-0.171	0.343	0.471*	0.510**	0.196	0.027	0.126
Extra-Pair Daughters	0.222	0.293	-0.144	0.242	0.049	0.456*	0.309	0.062	0.104
Extra-Pair Sons	0.246	0.304	-0.019	0.378*	0.037	0.157	0.499**	0.376*	0.357

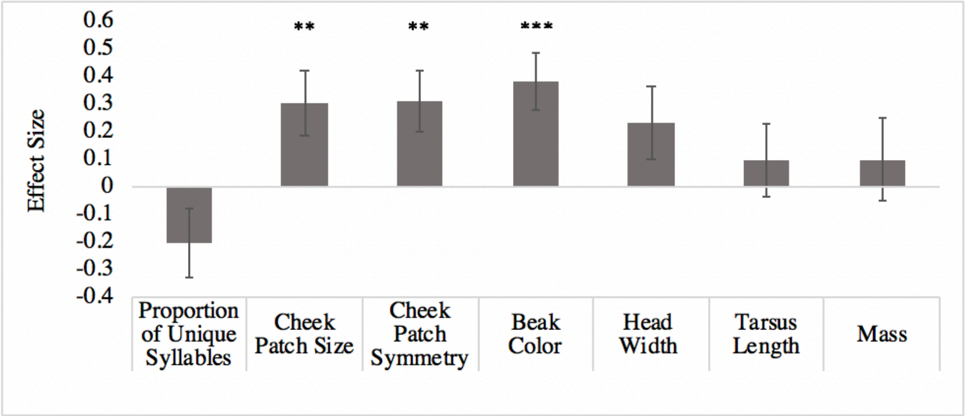


Figure 1.6: Effect sizes (+ SE) of z-transformed influential male body size and secondary sexual traits on all z- transformed male reproductive success measures (genetic, social and extra-pair production of sons and daughters). * (P≤ .05); ** (P≤ .01); *** (P≤ .001); **** (P<.0001). Data from Table 4.

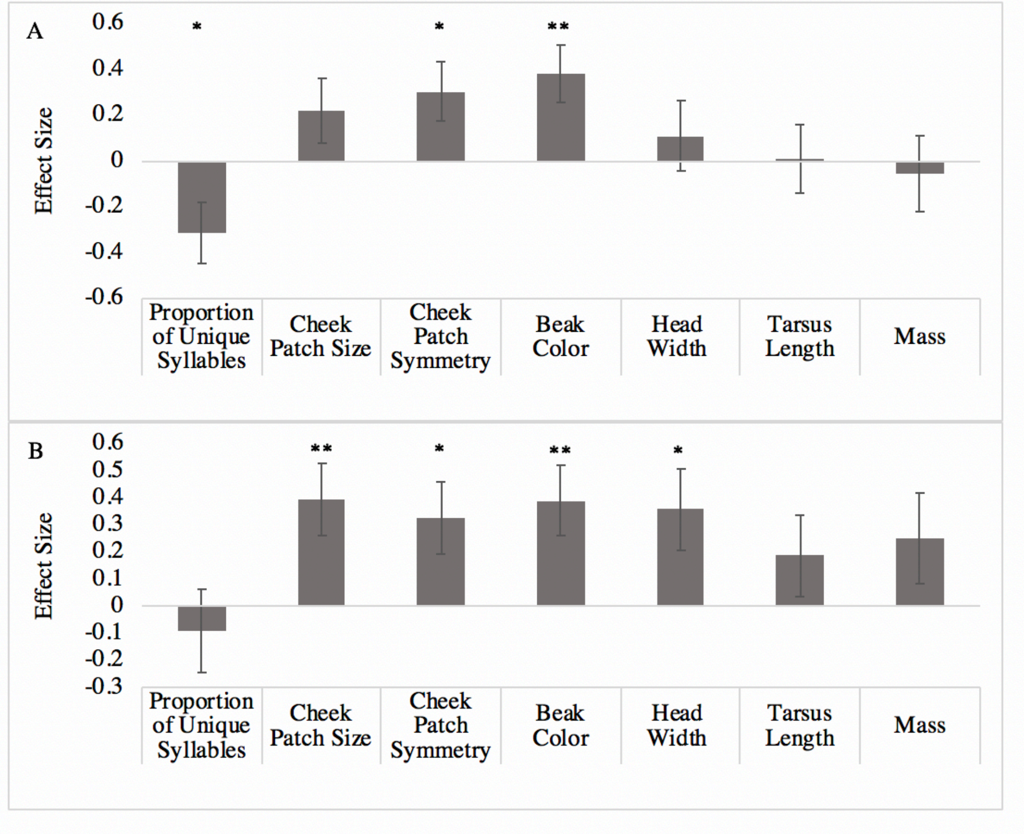


Figure 1.7: Effect sizes (+ SE) of z-transformed influential male body size and secondary sexual traits on z- transformed A) daughter production (genetic, social and extra-pair) and B) son production (genetic, social and extra-pair). * (P≤ .05); ** (P≤ .01); *** (P≤ .001); **** (P<.0001). Data from Table 4.

Additional Reproductive Success Results

The correlations among genetic and social reproductive measures (total offspring [r = 0.91], sons [r = 0.92] and daughters [r = 0.90]) were all highly significant (PC: P's < 0.005). Males that sired extra-pair offspring tended to produce more offspring overall (Two-sample t-test with equal variance: t = -2.05, df = 26, P = 0.051); this pattern resulted from their siring a greater number of sons (t = -3.16, df = 26, P = 0.004), but not more daughters (t = -0.67, df = 26, P = 0.52).

DISCUSSION

A strong focus of recent research on developmental stress centers on the influence of early nutritional conditions on the adult expression of bird song (e.g., MacDougall-Shackleton and Spencer 2012; Woodgate et al. 2012; Schmidt et al. 2013; Kriengwatana et al. 2014), under the rubric of the nutritional stress hypothesis (Nowicki et al. 1998; 2002). Our findings support the profitability of broadening the scope of this hypothesis to consider stressors other than nutrition and secondary sexual traits other than song. Results here and elsewhere (e.g., Gorissen et al. 2005; Holveck et al. 2008) indicate that the early life conditions of individuals (notably hatch order) and perhaps natal conditions experienced by their parents (hatch order and brood composition) can serve as early stressors with significant impacts on offspring fitness.

Qualitatively, there was little difference in the tendency of males' secondary sexual traits to influence patterns of social and genetic reproductive success (Table 1.4). Thus, while accounting for genetic reproductive success is important for understanding the evolutionary significance of developmental stressors, social parentage can often serve as an acceptable proxy. We found that visual secondary sexual traits were more consistent predictors of components of reproductive success, including production of both daughters and sons, than were acoustic traits (Table 1.4; Figures 1.6 and 1.7). In addition, since daughter production was not influenced by males' early life diet, this result suggests differential survival of male offspring of the diet treatments and possible sex allocation by HI males and their mates (Burley 1986b; Booksmythe et al. 2015).

The level of production of extra-pair offspring found here (13%) is very similar to that reported (12%-15.3%) in other laboratory studies in which male variation in attractiveness was not manipulated outside the naturally occurring range (Tschirren et al. 2012); wild populations have lower rates (1.7%-2.4%; Birkhead et al. 1990; Griffith et al. 2010). Size traits predicted son production, especially via extra-pair paternity, suggesting that females may choose extra-pair partners using male traits different from those employed in social mate choice. In conjunction with the finding of sex allocation (that HI-diet males and males with extra-pair reproductive success produced more sons), results support the conclusion that multiple sets of male traits are under sexual selection in this species.

Given that it has long been known for a wide range of species that parental effects (Mousseau and Fox 1998) and –for birds – hatch order (Both et al. 1999; Bowers et al. 2011) can have long-term influences on phenotype and fitness, it is surprising that intergenerational influences and early life experiences on adult sexual signal expression have not been widely considered (but see, Soma et al. 2006; Gil et al. 2006; Holveck et al. 2008). Though confounding aviary effects are possible, we found that diet and male hatch order had negative impacts on both male traits and reproductive success (Figures 1.2-1.5). Also, male hatch order appears to have transgenerational fitness consequences, influencing the number of granddaughters produced (Figure 1.5A). In contrast to the significant hatch order effects, no equivalent patterns were found for male brood size/composition, suggesting that – for studies in which clutch/brood size is not experimentally manipulated – zebra finches are more readily able to reduce variation in offspring quality through tactical adjustment of clutch/brood size (Roff 2002; Vedder et al. 2017) than they can offset costs that accrue specifically to offspring of late hatch order (Figures 1.3 and 1.4).

A paradoxical finding was that the number of sisters in the natal broods of males' mothers had a positive effect on male visual trait expression, while the number of sisters in the natal broods of males' fathers had a negative effect (Figure 1.2). A possible explanation for contrasting grand-parental influences is exemplified by results of an experiment in which condition of half of the female zebra finches breeding in a large aviary was manipulated by clipping of flight feathers after pair formation, while condition of their mates was not (Foster and Burley 2007). In that study, control (unclipped) females tended to produce more female offspring than did females whose feathers were shortened, largely as a result of lower daughter mortality (although unclipped females also had larger clutch sizes). In this species, female hatchlings generally appear more vulnerable to mortality resulting from early stressors including food (Burley et al. 1989; deKogel 1997); also, female fecundity is proportional to body mass at fledging (Martins 2004), perhaps because adult females are capital breeders (draw protein from pectoral muscle for breeding [Jones and Ward 1976; Houston et al. 1995]). Parental feeding rates collected during Foster and Burley's experiment indicated that control females, which maintained higher body mass, provisioned female-biased broods more. Collectively, findings suggest that the positive impact of number of female brood mates in the males' maternal lineage results from females having been in relatively good physiological condition at the time they produced the mothers of the males in the current experiment. That the opposite pattern was found in the paternal lineage is consistent with evidence indicating that daughters benefit relatively more from high maternal physical condition than do sons. This study further contributes to this idea since the number of sisters, but not the number of brothers, were found to contribute to male developmental stress.

The suggestion that early life stressors may exert greater viability/fecundity selection on females does not conflict with the result found here that male early diet failed to influence daughter production. Rather, the logic developed here suggests that – other things being equal – females in very good condition make greater investments in daughters because of positive effects on their survival and fecundity; investigation of this possibility would require manipulation of the diet in the maternal lineage. By contrast, the current study suggests that high paternal condition as revealed by secondary sexual trait expression leads to enhanced investment in son production because of the sexually selected benefits sons may accrue. Of course, the extent to which both members of a breeding pair share similar sex allocation tactics will likely vary among circumstances (Burley 1988; Foster and Burley 2007; Mainwaring et al. 2011); predicting consequences of manipulating reproductive quality of both sexes simultaneously would be more difficult. Additional studies of transgenerational effects on both sexes are clearly needed to develop a picture of their overall impact on individual fitness across a range of taxa. We suggest that investigation of transgenerational effects on the developmental trajectories of male zebra finches, and the impact of trajectories on fitness, might help clarify and extend the findings reported here.

Developmental Stress and Secondary Sexual Traits

Acoustic Traits

Contrary to nutritional stress hypothesis predictions, rearing diet did not influence song traits (Table 1.2; Figure 1.3). As discussed by MacDougall-Shackleton (2015), previous studies on zebra finches have been inconsistent in their findings regarding diet effects on song: a number of studies have reported that several song traits are negatively affected by poor early diet (syllable number: Spencer et al. 2003; peak frequency: Spencer et al. 2003; Zann and Cash 2008; song duration: Spencer et al. 2003; song rate: Zann and Cash 2008; copy precision: Brumm et al. 2009), while some found no effect (syllable number: Zann and Cash 2008; Brumm et al. 2009; Kriengwatana et al. 2014; unique syllable number: Kriengwatana et al. 2014; motif duration: Brumm et al. 2009; song rate: Birkhead et al. 1999; Spencer et al. 2003), or positive effects (song duration: Zann and Cash 2008).

Kriengwatana and colleagues (2014) addressed the problem of inconsistencies among study results by suggesting that song traits play a smaller role in mate choice than visual traits in this species and that low intensity of selection on song results in substantial song trait variability among populations. Our findings are consistent with this idea, in the qualified sense that song quality may not be important in social mate choice, although several other functions of song are recognized (species [Zann 1996; Riebel 2009], mate [Miller 1979a], and kin [Miller 1979b] recognition; stimulation of female to pair [Dunn and Zann 1997, Tomaszycki and Adkins-Regan 2006] and coordination of pair activities [Elie et al. 2010]). The strongest support for the nutritional stress hypothesis has been found in species with few visual sexual dimorphisms, and for which song plays a major role in mate choice (swamp sparrows: Nowicki et al. 2002; European starlings: Buchannan et al. 2003; song sparrows: MacDonald et al. 2006; Schmidt et al. 2013; but see Müller et al. 2010 for no effect in canaries), suggesting that the relationship between song and nutritional stress may be more complicated for visually ornamented species than originally proposed. Additional studies that assess both acoustic and visual sexual dimorphisms will further our understanding of the role developmental stress plays in shaping sexually selected traits in general, and song specifically. Since the effects of undernutrition (from caloric restriction) can vary from those of malnutrition (from nutrient deficiencies or imbalances -- Morgane et al. 1993, 2002; Besson et al. 2016), it will be important going forward to address effects of qualitatively different types of food stressors. To date, almost all studies of nutritional stress in zebra finches have focused on undernutrition (except Birkhead et al. 1999 and this study).

While diet has been the focus of most studies of the nutritional stress hypothesis, a study by Holveck and colleagues (2008) manipulated brood size in zebra finches in order to influence male quality. They found that male song stereotypy was higher for males that hatched earlier in their broods; similar effects have been reported in another estrildine, the Bengalese finch (Lonchura striata domestica; Soma et al. 2006, 2009). We did not find an overall effect of hatch order on male song traits, although we did find a similar correlation to theirs between song stereotypy and hatch order (Table 1.2).

Interestingly, the proportion of unique syllables negatively predicted reproductive success via daughter production (Figure 1.7A), suggesting that this trait may have a detrimental effect on male fitness, which is the opposite of conventional expectations (but see Byers and Kroodsma 2009). The only prior study to explicitly test reproductive outcomes of the developmental stress hypothesis for zebra finches also found that the proportion of unique syllables in a male's song was negatively associated with reproductive success (Woodgate et al. 2012: Table A1). Perhaps there is a trait not yet considered or categorized as perceived by zebra finches that informs the cost associated with the proportion of unique syllables. In any case, the relationship between song attractiveness and fitness remains unclear, since female song preference was neither examined here nor by Woodgate et al. (2012), and studies that have reported female preference for songs with greater number of unique syllables (Spencer et al. 2005; Holveck and Reibel 2007) did not assess male reproductive success. Future studies should assess female preferences for song traits and fitness effects of these traits for males of the same population.

Visual Traits

Expression of visual secondary sexual traits was sensitive to both diet quality and parental clutch traits. Collectively, visual traits contributed significantly to all measures of fitness (Table 1.4) and exerted the largest impact of the three phenotype categories on reproductive success (Figures 1.6 and 1.7). These results are consistent with previous studies that found both female mate preferences for beak color (Burley and Coopersmith 1987; deKogel 1997; Simons and Verhulst 2011) and cheek patch size (Naguib and Nemitz 2007; Tschirren et al. 2012; Burley et al. 2018) as well as impacts of these traits on social and/or genetic parentage (Price and Burley 1994; Tschirren et al. 2012). To our knowledge, the impact of cheek patch size symmetry on attractiveness of male zebra finches has not yet been studied.

Previous research has shown that beak color is heritable (Price and Burley 1993; Price 1996; Birkhead et al. 2006; Schielzeth et al. 2012) and that it reflects breeding state (Burley et al. 1992) and immune function (McGraw and Ardia 2003; Birkhead et al. 2006), such that redder beaks generally indicate better condition and immune response, at least in males. (However, in wild zebra finches, male beak color expression is positively correlated with ectoparasite load [Burley et al. 1991]; this may reflect high circulating corticosteroid levels in response to stress [Roberts et al. 2007; McGraw et al. 2011]). Overall, then, male beak color appears to be a general quality indicator.

Despite its prominence in the visual phenotype and display repertoire of male zebra finches, the cheek patch has received little attention by researchers. Nevertheless, results of several previous studies do indicate that cheek patch size is a sexually selected trait (Price and Burley 1994; Naguib and Nemitz 2007; Tschirren et al. 2012), and females in the population from which birds used in this experiment were derived have been found to prefer males with large cheek patches (Burley et al. 2018). Findings here suggest that this secondary sexual trait is also a reliable signal of developmental stress in males, since it, along with other visual secondary sexual traits, showed sensitivity to diet quality (Table 1.2, Figure 1.3B) and predicts offspring production (Table 1.4; Figure 1.7).

Our conclusion that cheek patch size is a meaningful indicator of developmental stress is supported by a small literature on the significance of the production of pheomelanin, the pigment that produces chestnut-colored feathers (McGraw and Wakamatsu 2004). For species that normally express both pheomelanin and eumelanin (which produces grey and black feathers), eumelanin expression increases when levels of glutathione, an important intracellular antioxidant (Wu et al. 2004), are experimentally reduced, suggesting that pheomelanin-based plumage ornaments serve as honest indicators of male quality under circumstances that generate high oxidative stress (Galván and Solano 2009; Galván et al. 2015). Moreover, the developing central nervous system competes with pheomelanogenesis for the essential amino acid cysteine (reviewed by Galván and Møller 2011), for which glutathione is the main physiological reservoir (Benedetto et al. 1981). Theory predicts that developing organisms selectively shunt limiting resources to tissues more critical to survival (Stearns 1989), so we would expect large cheek patch expression only when cysteine availability is high. In this context, it is notable that hen's egg supplementation increases the availability of methionine, a precursor to cysteine (Allen and Hume 1997), suggesting that diet may influence allocation between nervous tissue and cheek patch plumage. In a previous study (Bonaparte et al. 2011), males cage-reared on the HI diet were found to have larger head widths than those reared on the LO diet, but that result was not found here (Table 1.2, Figure 1.3C). Investigation of signal function of cheek patch size and the potential trade-offs in developmental allocation to cheek patch size versus nervous system function are promising directions for future research.

Cheek patch symmetry appears less sensitive to early life stressors than other visual traits (Table 1.2). This result counters conventional expectations that early life conditions are predicted to influence trait asymmetry (Swaddle 2003; Møller and Pomiankowski 1993). However, we do not contend that variation in cheek patch symmetry meets the exacting criteria to be considered fluctuating asymmetry (Palmer and Strobeck 1992; Swaddle 2003). Indeed, given lateralization of brain function and display orientation during courtship (Templeton et al. 2014), it may well be that deviations from perfect symmetry in display traits are non-random. Nonetheless, future studies should not ignore cheek patch symmetry since it was a predictor of reproductive success in this study. Overall, results of this experiment reinforce the view that visual secondary sexual traits are sexually selected traits in zebra finches. Because their expression shows sensitivity to multiple developmental stressors, beak color and cheek patch size appear to serve as indicators of male quality. Based on evidence from two populations that their expression is not phenotypically inter-correlated (Table 1.1; Burley and Price 1994; Burley et al. 2018), these traits provide information on different aspects of male quality. Additional research will be needed to clarify the range of stressors, including the relative contribution of transgenerational influences, that impact secondary sexual trait expression in this species.

CHAPTER 2:

Extra-Pair Offspring and Male Attractiveness Impact Parental Investment and Reproductive Success in the Socially Monogamous Zebra Finch

INTRODUCTION

While males can clearly benefit from producing offspring outside of their social pair, the possible benefits to females from extra-pair mating are widely debated (summarized in: Griffith et al. 2002; Kempenaers and Schlicht 2010; Forstmeier et al. 2014). A well-cited hypothesis is that females may benefit by gaining indirect genetic benefits in the form of higher-quality offspring. Studies on birds have generated some support for the indirect fitness benefit hypothesis (Houtman 1992; Hasselquist et al. 1996; Forstmeier et al. 2002; Fossøy et al. 2008; Kawano et al. 2009), but meta-analyses indicate that genetic benefits may be small or absent (Westneat and Stewart 2003; Arnqvist and Kirkpatrick 2005; Akay and Roughgarden 2007; Eliassen and Kokko 2008). More recently, the potential for maternal effects to confound genetic benefits has gained attention (Jennions and Petrie 2000; Griffith et al 2002; Kempenaers and Schlicht 2010; Schmoll 2011): Just as females often invest differentially in offspring sired by preferred or attractive males (Burley 1986a; Sheldon 2000; Horvathova et al. 2012), they may differentially invest resources towards their own extra-pair offspring (EPO) (Schmoll et al. 2011; Tschirren et al. 2012).

Theory predicts that extra-pair mates should possess preferred or attractive traits because they are indicators of genetic quality (Forstmeier et al. 2002; Kempenaers and Schlicht 2010; Wilson et al. 2019; but see Tschirren et al. 2012). However, the extent to which preferred traits are heritable is usually unknown, and environmental conditions may often influence expression of such traits (Griffith et al. 1999; Cornwallis and Uller 2010).

Two measures of male quality/attractiveness that have been previously considered in zebra finches, the species under study here, are extra-pair mating success (Houtman 1992; Bolund et al. 2009) and early diet quality. Early diet can impact adult secondary sexual trait expression (Wilson et al. 2019; de Kogel 1997; Naguib and Nemitz 2007), and female zebra finches prefer males raised under higher diet-quality conditions (Spencer et al. 2005; Burley et al. 2018). Phenotypic variation resulting from experimentally manipulated natal diet reflects environmentally induced variation in male quality, while male extra-pair success is thought to reflect genetic as well as environmental determinants of variation (Jennions and Petrie 2000; Neff and Pitcher 2005). Since a female's mate choice and reproductive allocation decisions are likely to depend on her mate's developmental history as well as his genetic quality, use of these two measures may allow us to better understand the relative importance of a strictly environmental component of male attractiveness and one that likely reflects both genetic and environmental effects.

Studies of maternal investment in response to extra-pair offspring have often focused on primary reproductive allocation, notably egg size (Krist et al. 2005; Bolund et al. 2009; Tschirren et al 2012) and egg contents (Bolund et al. 2009). While egg size has been found to be a positive predictor of several measures of offspring success for birds (Wagner and Williams 2007; Krist 2011), purported findings of differential investment in eggs fertilized by extra-pair males have been questioned on the grounds that observed effects can be more parsimoniously attributed other clutch traits, especially lay order (Magrath et al 2009; Ferree et al 2010; Griffith and Buchanan 2010a; Krist 2011; Forstmeier et al. 2014), which can systematically influence both egg size and hatch order. Thus, studies of avian EPO quality should to include effects of lay order.

Parental investment during the incubation and provisioning phases -- often contributed by both social parents -- can also impact offspring success (Ardia et al. 2010; DuRant et al. 2013;Wada et al. 2015; Grüebler et al. 2018) and can be costly to care-givers (Owens and Bennett 1994; Monaghan and Nager 1997; Alonso-Alvarez et al 2004; Nord and Williams 2015; Williams 2018). Furthermore, since the sexual conflict that results from extra-pair behavior varies with reproductive phase (Chaine et al. 2015), parental investment decisions are likely to vary throughout a reproductive attempt. Thus, these reproductive phases are additional, but typically overlooked, sources of variation in allocation of resources to within-pair offspring (WPO) versus EPO. In order to accurately assess how extra-pair success of both sexes impacts parental investment, studies should consider post-hatching parental allocation in addition to pre- hatching patterns.

In species with bi-parental care, males may also adjust their allocation to offspring in response to the presence of EPO in their clutch (Birkhead and Møller 1992; Houston and McNamara 2002; Arnqvist and Kirkpatrick 2005). Since male zebra finches cannot, to our knowledge, decipher between individual WPO and EPO, their ability to assess paternity has been questioned (Kempenears and Sheldon 1997; Sheldon 2002; Kempenears and Schlicht 2010; but see Johnstone 1997). A male is unlikely to make paternal care adjustments directly in response to the presence of EPO in a brood, but may adjust his investment in response to changes in his social mate's behavior prior to egg-laying or to her subsequent investment patterns (Gilby et al. 2011; Mariette and Griffith 2012).

A bird's own extra-pair success is also expected to affect its parental investment patterns. Males that sire more EPO may lower parental investment in their social mates' clutches (Sheldon 2002; Ball et al. 2017; Crouch and Mason-Gramer 2018), while females that produce EPO may increase investment (Schmoll et al. 2011). However, many avian species, including the zebra finch, exhibit a high degree of pair coordination in which mates display behaviors such as nest relief of an incubating partner and synchronized visits to provision nestlings. The interplay between paternal and maternal investment in response to both partners' extra-pair activities has been underexplored but is likely to be complicated by pair coordination effects.

By manipulating diet quality of males in early life and measuring reproductive investment, within-pair and extra-pair reproductive success, we address the following questions: How do female zebra finches adjust reproductive allocation in response to their extra-pair success and the attractiveness of their partner (as measured by male extra-pair success and natal diet quality)? Do females invest more in EPO and/or offspring sired by attractive males? Do males invest differentially in offspring care based on their own quality and their level of paternity in a given clutch? We predicted, in line with the differential allocation hypothesis, that females with high-quality mates would invest more in offspring at all phases of reproduction, and that females would invest more in EPO regardless of the quality of their social mates.

METHODS

Founder Rearing Conditions and Flight Initiation

Founders of this breeding study were raised in one of four outdoor aviary flights (80 m3), each of which was founded by 64 birds (32 of each sex); females and males were produced in separate flights (two flights for each sex). Access to boiled hen's egg differed among flights, but in other respects (size, resources, microclimate) all flights were virtually identical. Females were reared in flights in which hen's egg was made available three times a week (LAB diet), while males were reared in flights in which egg was provided either daily (HI diet) or never (LO diet). Hen's egg has an amino acid profile similar to that of half-ripe grass seed (Allen and Hume 1997), which is seasonally available to zebra finches in the wild and has a higher protein content than ripe grass seed. All rearing flights provided breeders and fledged offspring with ad libitum access to a commercial mix of ripe grass seed for estrildines, cuttlefish bone, ground oyster shell and water, and green vegetables three times a week.

Founders were banded with numbered, closed metal leg bands when they were 7-14 days old. Once founders reached 45 + 3 days of age, they were housed in same-sex cages at standard densities inside their natal flights and on their natal diets Birds remained in same-sex cages until they were selected for use in the present study. Additional details on founder rearing conditions and the history of this experimental colony can be found elsewhere (Wilson et al. 2019).

In total, 32 females and 32 (16 HI and 16 LO) males founded a single breeding colony for the current experiment. Founders varied from 6 to 13 months of age at the start of breeding. All birds were judged to be in excellent overall condition and no more than 2 siblings from each family were employed. Once selected, birds were held in single-sex flights and maintained on the LAB diet for six weeks before they were released into the breeding flight. During this time, male phenotype data were collected (see Wilson et al. 2019 for details), and a 25 μL blood sample was collected from the brachial vein of each bird. Lastly, all birds were banded for identification during observations using colors for which zebra finches show no preference (Burley 1985).

Breeding Conditions and Reproductive Measures

Founders of both sexes were released simultaneously into a single (80 m3) outdoor aviary flight and allowed to freely pair and breed for 5 months. The flight was maintained on the LAB diet. Ample nest sites (~2.5/ breeding pair) and nest material (dried Bermuda grass and feathers) were provided. All nest sites were checked daily: new eggs and nestlings were individually marked for identification, and new eggs were weighed on an electronic balance sensitive to one hundredth of a gram. Occasionally fresh eggs were not found on the day they were laid, as evidenced by the discovery of two or more unmarked eggs in a nest on the same day; such eggs were not included in analyses due to ambiguity of laying order. “Clutch position” was assigned to each egg based on whether it was produced the first, middle or last third of a clutch. Nestlings were banded between 7 and 14 days of age. A 25 μL blood sample was collected from the brachial vein of offspring once they reached 45 + 3 days of age at which time they were also removed from the flight and housed in same-sex cages for use in other studies.

Social and Genetic Parentage Assignment

Social parentage was assigned to each clutch through regular observations of active nests beginning when eggs first appeared in a nest and ending once the last nestling had fledged. Genetic analyses of blood samples collected from all founders and 196 of the 205 surviving offspring were used to assign genetic parentage using procedures described in detail elsewhere (Wilson et al. 2019). Briefly, DNA was isolated then amplified through PCR with fluorescently labeled primers corresponding to eight highly polymorphic microsatellite loci previously established for zebra finches (Forstmeier et al. 2007). CERVUS 3.0 (Kalinowski et al. 2007) was used to assign parentage using similarities and differences at these eight loci. In order to be included in the final sample, offspring blood samples needed to yield six or more unambiguous loci. This criterion excluded six offspring. An additional six offspring were excluded because they were raised by same-sex parents, and seven more were excluded because only one social parent could be identified during observations. Offspring for which genetic parentage assignment was not available were assumed to be within-pair offspring.

Based on results of genetic testing, female extra-pair success was quantified on a clutch-by-clutch basis. Since male extra-pair success was used as a measure of male quality/attractiveness, results of genetic testing were used only to determine whether or not a male produced any surviving EPO throughout the breeding span. We acknowledge that clutches may have contained EPO that died before independence, resulting in the mis-classification of male or female parental investment in response to their own or their mate's extra-pair behavior and clutches for which surviving offspring were genotyped. The chances of this occurring were minimized by focusing analyses on only offspring that survived to independence and the clutches that contained these offspring.

Parental Nest Attendance

Nests were selected for parental attendance observations if they contained at least one egg and no nestlings. Nests that were not actively attended by either parent during two successive observation periods were assumed to have been abandoned and were not included in analyses. Observers recorded data from inside the aviary and were careful to minimize their disturbance of incubating birds. Observers conducted 30-minute, all accounts sampling of the amount of time each parent spent inside its nest. Thus, “nest attendance time” included time devoted both to incubation, nest construction and possibly nest defense. Nests included in analyses were observed on two to six days ($\bar{X}$ + SD: 5.26 + 1.13) during the incubation phase (observation day $\bar{X}$ + SD: 6.93 + 3.85, with day 1 being the day the first egg was laid; the first egg hatched on about day 14) and included offspring that survived to independence. Nest observations were discontinued after the first egg hatched.

Parental Hen’s Egg Consumption and Nestling Provisioning

Sampling of parental hen’s egg consumption and nestling provisioning were conducted on days the colony received hen’s egg supplements. First, founders were provided with a bowl of egg placed on the aviary floor (the normal location in which food and other resources are provided to these ground-feeding birds). After birds began to arrive at the food bowl, a stopwatch was set and two observers called out the band combinations of birds that took egg from the bowl while a third observer recorded these data. Birds were scored feeding for three minutes; if feeding bouts were interrupted by birds being startled and leaving the floor, the clock stopped and was re-started after birds resumed feeding. After birds displayed 3 minutes total of active feeding, observers removed the food bowl and left the flight. After about five more minutes, experimenters entered the aviary and scored crop contents of all nestlings, a routine that required 10 to 15 minutes. For a given day, all founders received a score for hen’s egg consumption sample that ranged between 0-3; this score reflected the number of trials in which they took egg at least one time with each trial worth 0.5. Crop contents were recorded for nestlings ranging in age from 0 (hatching) to 15 days (X̄ + SD: 5.36 + 3.66 days of age); such nestlings have translucent skin through which contents can be unambiguously scored. The amounts of egg and seed present in the crop were each assessed using 0.5-point increments on a scale of 0-3, with a higher score reflecting greater crop contents. On each sampling day, 6 founder feeding samples and 3 nestling crop content samples were collected. Data were averaged across samples of a given sampling day.

Analyses

Analyses included data from 26 females and 27 males that produced at least one surviving offspring. Of these founders, 12 males were raised on the HI diet and 15 were raised on the LO diet. Nine females and 9 males produced EPOs. Analyses of egg mass and total offspring included only offspring that survived at least to independence (45 + 3 days of age). Analyses of female extra-pair success for egg mass and total offspring compares the production of individual EPO and WPO. Other analyses of female extra-pair success (nest attendance time, nestling provisioning) compared clutches containing EPO to those with only WPO. Nest attendance and nestling provisioning are reported for clutches for which extra-pair status could be assigned (i.e., those that contained at least one offspring that survived to independence).

The effects of female extra-pair success, male attractiveness (extra-pair success and diet history) and their interactions were assessed for egg mass, nest attendance time, parental hen’s egg consumption and nestling provisioning using repeated measure, linear mixed-effect models (RM LMMs), with female identity included as a random effect. An LMM with these same effects was used to assess the total number of offspring that females produced. Clutch position was included as a covariate in the RM LMM used to assess egg mass. Offspring sex was included as an additional fixed effect in the RM LMMs used to assess nestling provisioning. Founder sex was included as an additional fixed effect in the RM LMMs used to assess nest attendance time and parental hen's egg consumption. ANOVA was used to analyze the effects of male attractiveness on female production of EPOs. Non-significant interaction effects ($\alpha > 0.05$) were removed from models in a reverse step-wise process beginning with four-way interactions, followed by three-way and then two-way interactions. No fixed effect was removed from any analysis.

Analyses were found to meet normality assumptions by visual inspection of quantile-quantile plots and Shapiro-Wilks tests. Egg mass was log-transformed in order to meet assumptions. Marginal means and delta standard errors are reported. Statistical tests were performed in STATA 14 (StataCorp LP, College Station, Texas, USA).

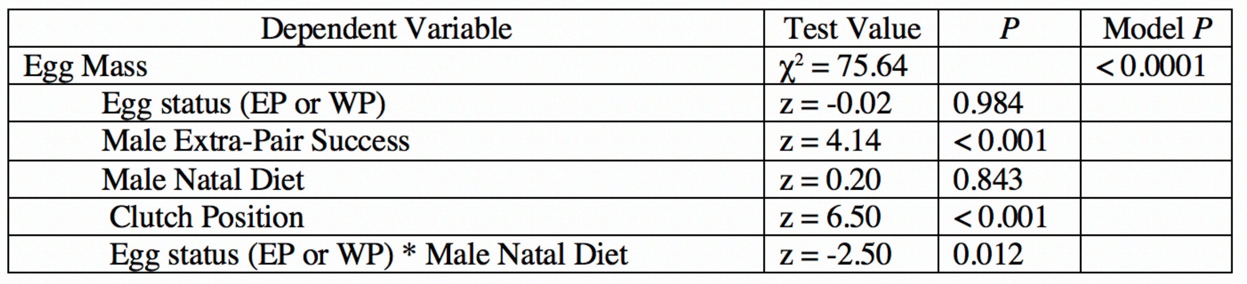
RESULTS

Egg Mass and Clutch Position Effects

Egg mass was influenced by clutch position, male extra-pair success and the interaction between male diet history and female extra-pair success. Egg mass increased significantly with lay order (P's < 0.001 - early-laid: 1.06 g; middle-laid: 1.12 g; late-laid: 1.15 g) (Table 2.1). Females mated to males that produced EPOs laid heavier eggs than those mated to males with WPOs only (Table 2.1, Figure 2.1). EP eggs produced by females with LO diet mates weighed less than those females' WP eggs ($z = -2.47$, $P = 0.013$) or eggs laid by females with HI diet mates (WP eggs: z = -3.45, P < 0.001; EP eggs: z = -2.97, P = 0.003) (Table 2.1, Figure 2.2).

EP eggs were equally likely to be laid across clutch positions (Fisher's Exact: P = 0.722).

Table 2.1. Egg mass of offspring surviving to independence. Female identity contributed significantly to the model. Repeated measures linear mixed-effects model, N = 188.



Dependent Variable	Test Value	*P*	Model *P*
Egg Mass	$\chi^2 = 75.64$		< 0.0001
Egg status (EP or WP)	z = -0.02	0.984	
Male Extra-Pair Success	z = 4.14	< 0.001	
Male Natal Diet	z = 0.20	0.843	
Clutch Position	z = 6.50	< 0.001	
Egg status (EP or WP) * Male Natal Diet	z = -2.50	0.012	

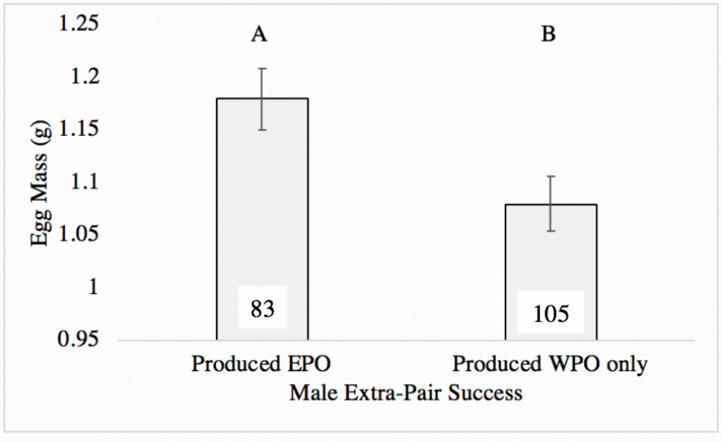


Figure 2.1: The effect of male extra-pair success on egg mass. Data corresponds to Table 1. Letters indicate location of significant differences between groups. Sample size (number of eggs) for each group is listed on its corresponding bar.

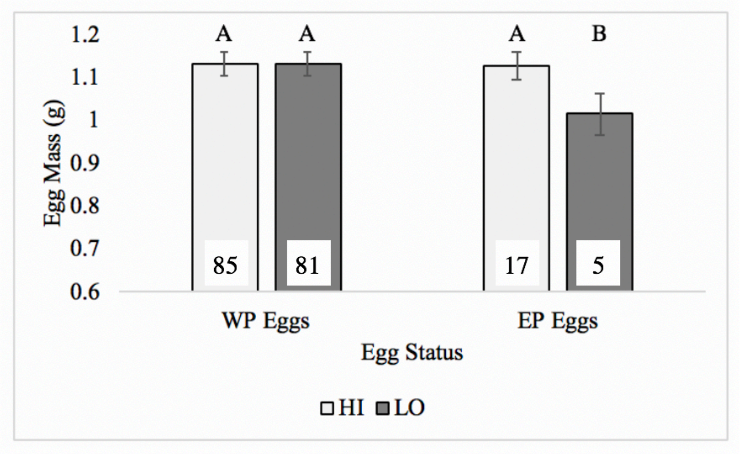


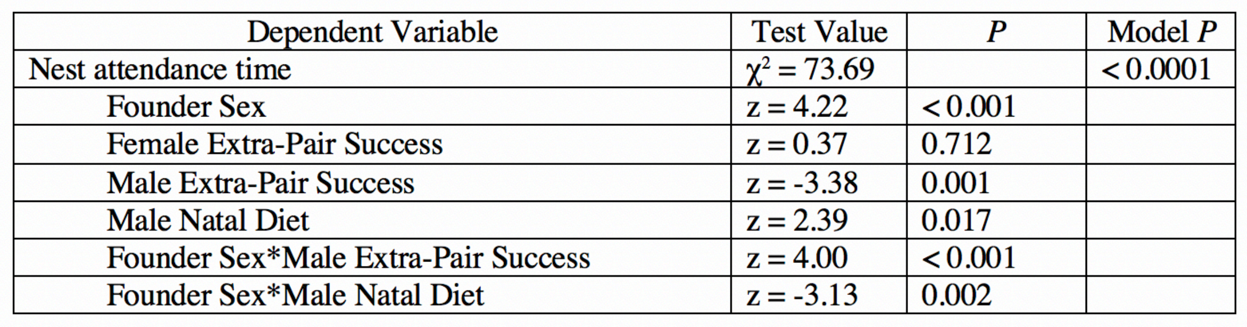
Figure 2.2: The effects of male natal diet and egg status (EP or WP) on egg mass. Data correspond to Table 1. Letters indicate location of significant differences among groups (P ≤ 0.021) based on post-hoc analyses. Sample size (number of eggs) for each group is listed on its corresponding bar.

Incubation Phase Reproductive Investment

Nest attendance time was affected by founder sex, male extra-pair success and male diet history. The effects of male extra-pair success and male diet history were driven by their interactions with sex. Males spent more time than females attending their nests (Table 2.2; X + SE - males: 18.50 + 0.71 min; females: 11.22 + 0.71 min). Males that sired EPOs spent the most time attending their nests (P's < 0.028), while their mates spent the least (P's < 0.001) (Table 2.2, Figure 2.3). Males that sired only WPOs spent less time attending their nests than males that sired EPOs (z = 2.20; P = 0.028), but still spent more time incubating than their mates (z = 2.46; P = 0.014) (Table 2.2, Figure 2.3). LO diet males spent the most time attending their nests (P's < 0.044), while their mates spent the least (P's < 0.017) (Table 2.2, Figure 2.4); HI diet males spent more time attending their nests than their mates (z = 3.08; P = 0.002) (Table 2.2, Figure 2.4).

The total amount of time that nests were attended did not vary based on founder sex, male or female extra-pair success or male diet history (χ2 =1.11 ; P =0.77).

Table 2.2. Time spent (min/30-min sample) by caregivers in their nests during the incubation phase. Repeated measures linear mixed-effects model, N = 392.



Dependent Variable	Test Value	*P*	Model *P*
Nest attendance time	$\chi^2 = 73.69$		< 0.0001
Founder Sex	z = 4.22	< 0.001	
Female Extra-Pair Success	z = 0.37	0.712	
Male Extra-Pair Success	z = -3.38	0.001	
Male Natal Diet	z = 2.39	0.017	
Founder Sex*Male Extra-Pair Success	z = 4.00	< 0.001	
Founder Sex*Male Natal Diet	z = -3.13	0.002	

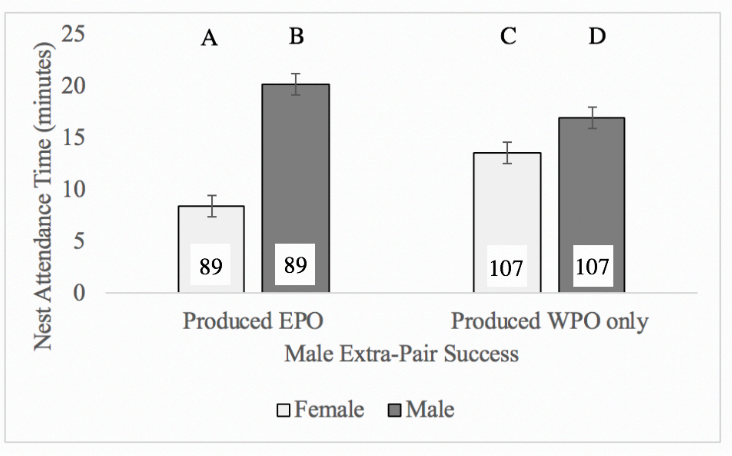


Figure 2.3: Effects of the interaction between founder sex and male extra-pair success on average nest attendance time during 30-minute samples. Data corresponds to Table 2. Letters indicate location of significant differences among groups (P ≤0.028) based on post-hoc analyses. Sample size (number of samples) for each group is listed on its corresponding bar.

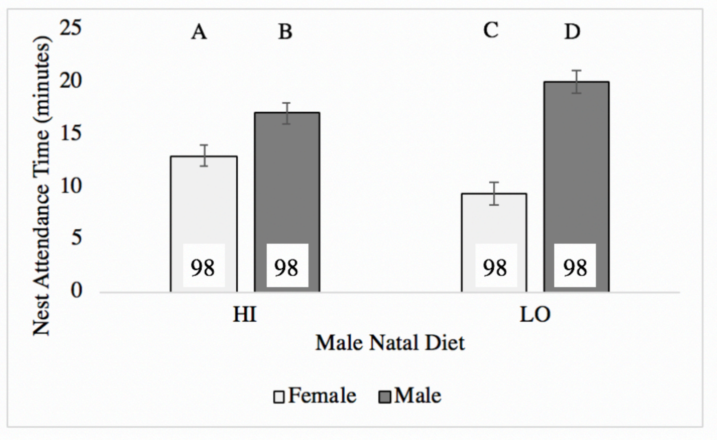


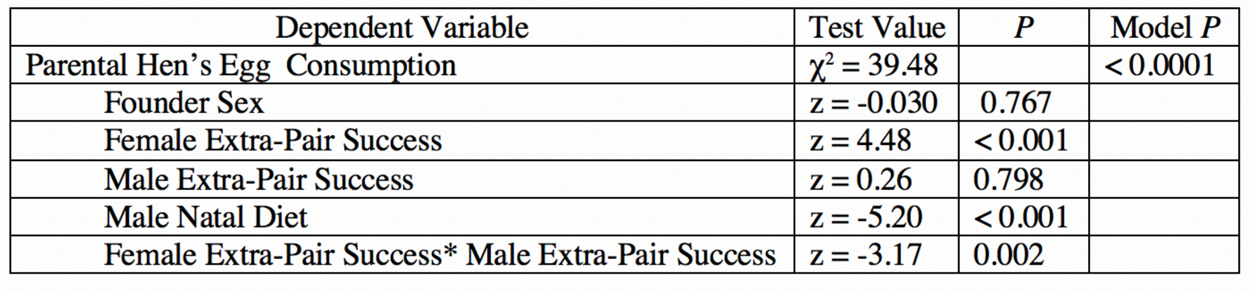
Figure 2.4: Effects of the interaction between founder sex and paternal natal diet on average nest attendance time during 30-minute samples. Data corresponds to Table 2. Letters indicate location of significant differences among groups ($P \leq 0.044$) based on post-hoc analyses. Sample size (number of samples) for each group is listed on its corresponding bar.

Nestling Phase Reproductive Investment

Parental hen's egg consumption during the nestling phase was affected by female extra-pair success, male natal diet and the interaction between female and male extra-pair success. Parents with clutched containing EPOs consumed more egg than those with WPO only clutches (Table 2.3; X + SE – EPO: 1.70 + 0.15; WPO: 1.11 + 0.08). Pairs that included a male raised on the HI diet consumed more egg than those including a male raised on the LO diet (Table 2.3; X + SE -HI: 1.74 + 0.12; LO: 0.83 + 0.11). Pairs in which females produced EPO in their clutch and males produced WPO only consumed more egg than any other combination (Table 2.3, Figure 2.5). Parent sex did not impact egg consumption patterns during the nestling phase.

Offspring sex and female extra-pair success influenced nestling provisioning. Female offspring were provisioned with higher amounts of egg than male offspring (Table 2.4; Figure 2.6). Nestlings from clutches containing EPOs were provisioned with higher amounts of egg compared to WPO-only clutches (Table 2.4; Figure 2.7). Seed provisioning did not vary based on male or female extra-pair success or male diet history (Table 2.4).

Table 2.3: Average consumption of hen's egg during the nestling phase. Repeated measures linear mixed-effects model, N = 220.



Dependent Variable	Test Value	*P*	Model *P*
Parental Hen's Egg Consumption	$\chi^2 = 39.48$		< 0.0001
Founder Sex	z = -0.030	0.767	
Female Extra-Pair Success	z = 4.48	< 0.001	
Male Extra-Pair Success	z = 0.26	0.798	
Male Natal Diet	z = -5.20	< 0.001	
Female Extra-Pair Success* Male Extra-Pair Success	z = -3.17	0.002	

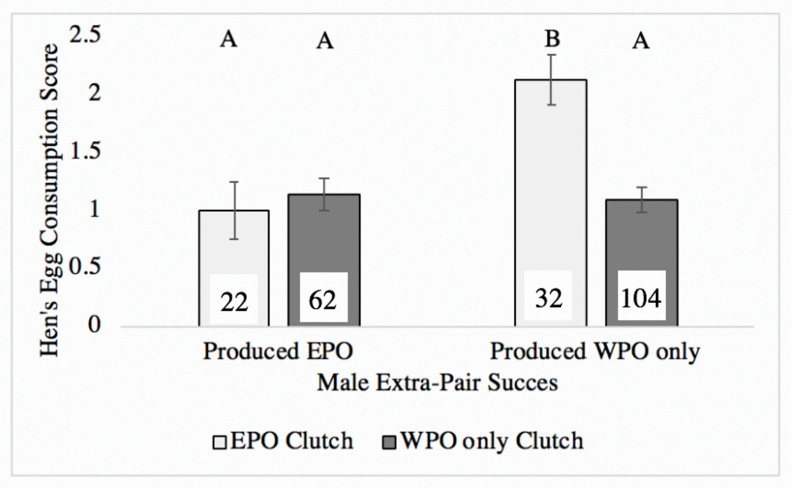


Figure 2.5: The effects of male and female extra-pair success on hen's egg consumption during the nestling phase. Data correspond to Table 3. Letters indicate location of significant differences between groups. Sample size (number of samples) for each group is listed on its corresponding bar.

Table 2.4: Mean crop content scores of nestlings sampled after hen's egg was provided to parents (seed was available ad libitum). Repeated measures linear mixed-effects model, N = 110.

Dependent Variable	Test Value	*P*	Model *P*
Hen's Egg	$\chi^2 = 21.81$		0.0002
Offspring Sex	z = -2.67	0.008	
Female Extra-Pair Success	z = 2.81	0.005	
Male Extra-Pair Success	z = 1.83	0.068	
Male Natal Diet	z = -1.37	0.172	
Seed	$\chi^2 = 1.04$		0.9035
Offspring Sex	z = -0.28	0.783	
Female Extra-Pair Success	z = -0.59	0.553	
Male Extra-Pair Success	z = 0.68	0.500	
Male Natal Diet	z = 0.56	0.574	

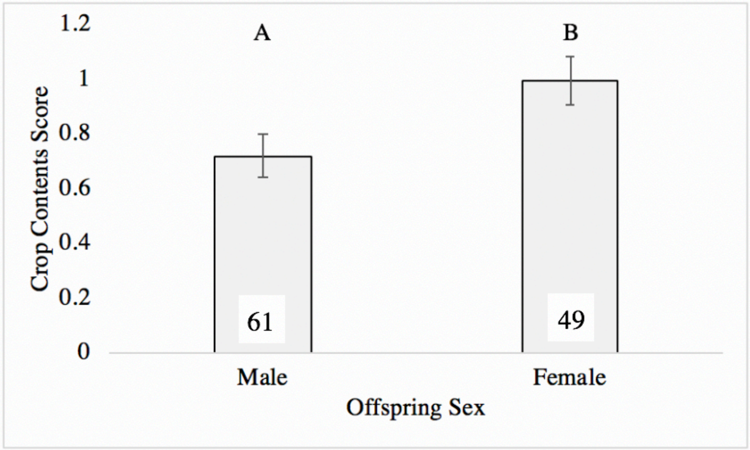


Figure 2.6: The effect of offspring sex on nestling provisioning of hen's egg. Data correspond to Table 4. Letters indicate location of significant differences between groups. Sample size (number of samples) for each group is listed on its corresponding bar.

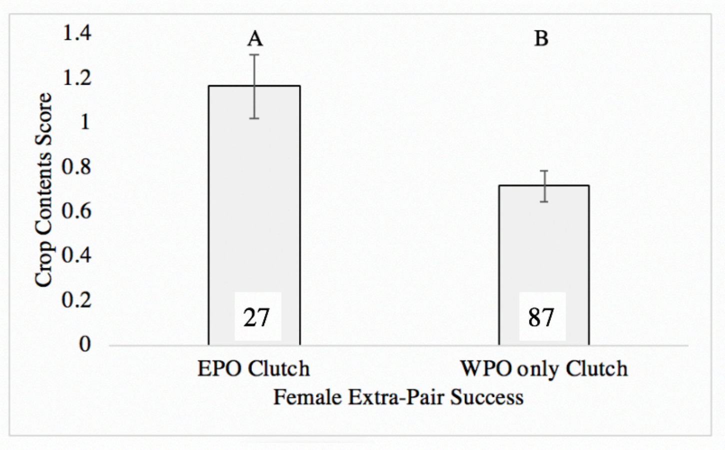
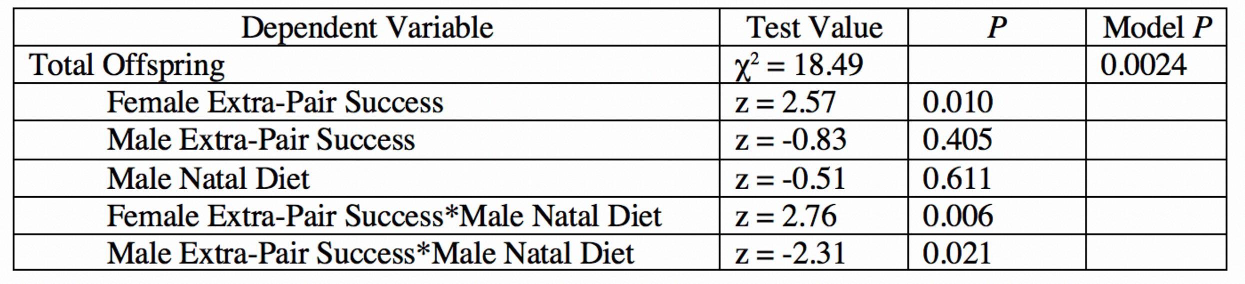


Figure 2.7: The effect of female extra-pair success on nestling provisioning of hen's egg. Data correspond to Table 4. Letters indicate location of significant differences between groups. Sample size (number of samples) for each group is listed on its corresponding bar.

Reproductive Success

A female's production of offspring was affected by her extra-pair success and the interactions between her extra-pair success and her mate's diet history, as well as her mate's extra-pair success and diet history. Across all females, those that produced EPOs had higher reproductive success compared to females with only WPOs (Table 2.5; X + SE: 8.09 + 1.33 vs. 6.68 + 0.89). Among females mated to HI diet males, females that produced EPOs produced more offspring (z = 2.57, P = 0.01) (Figure 2.8). Females mated to LO diet males that sired only WPOs produced fewer offspring than females mated to LO diet males that sired EPOs (z = 2.82, P = 0.005) and females mated to HI diet males that sired only WPOs (z = -2.35, P = 0.019) (Figure 2.9). Lastly, male attractiveness (male extra-pair success and natal diet) did not influence the number of EPOs females produced (ANOVA P = 0.48) and females did not produce more EPOs of one sex (Fisher's exact P = 0.39).

Table 2.5. Total number of offspring produced by each female that survived to independence. Linear mixed-effect model, N = 26.



Dependent Variable	Test Value	*P*	Model *P*
Total Offspring	$\chi^2 = 18.49$		0.0024
Female Extra-Pair Success	z = 2.57	0.010	
Male Extra-Pair Success	z = -0.83	0.405	
Male Natal Diet	z = -0.51	0.611	
Female Extra-Pair Success*Male Natal Diet	z = 2.76	0.006	
Male Extra-Pair Success*Male Natal Diet	z = -2.31	0.021	

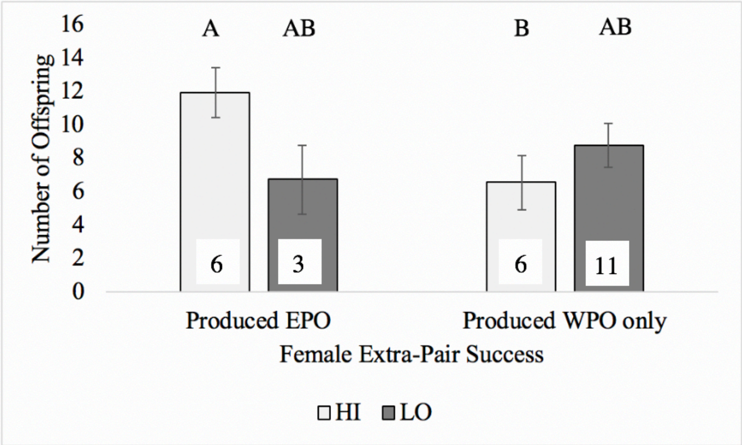


Figure 2.8: Effects of the interaction between male natal diet and female extra-pair success on total female reproductive success. Data corresponds to Table 5. Letters indicate significant differences among groups (P < 0.01) based on post-hoc analyses. Sample size (number of female founders) for each group is listed on its corresponding bar.

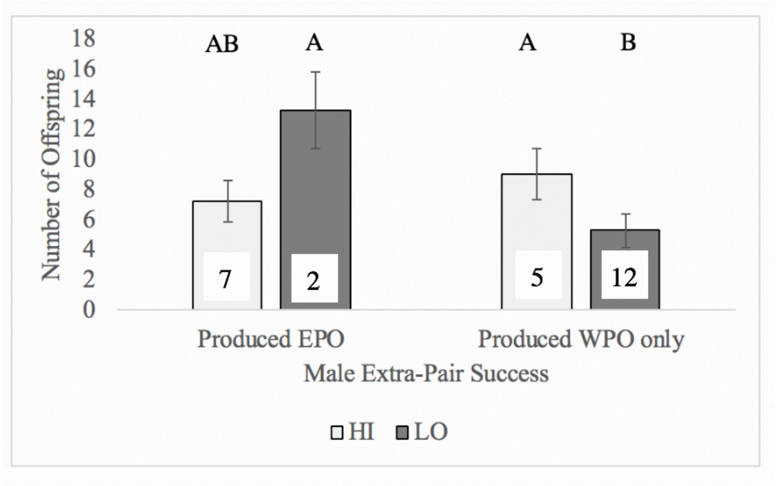


Figure 2.9: Effect of the interaction between male natal diet and male extra-pair success on total female reproductive success. Data correspond to Table 5. Letters indicate significant differences among groups (P < 0.019) based on post-hoc analyses. Sample size (number of male founders) for each group is listed on its corresponding bar.

DISCUSSION

The influence of female extra-pair success and male quality on parental investment patterns differed across the reproductive phases studied here, which highlights the challenge of attributing differences between WPOs and EPOs to genetic factors and further complicates the question of whether females benefit from extra-pair mating. Zebra finches increased investment towards clutches containing EPOs during the nestling phase, as evidenced by greater hen's egg provisioning of clutches containing EPOs (Table 2.4, Figure 2.7). Also, one measure of male quality, extra-pair success, was linked to a reduction in female and increase in male allocation towards nest attendance during the incubation phase (Table 2.2, Figure 2.3). Ultimately, it seems females likely did benefit from extra-pair matings since those that produced EPOs raised overall more offspring to independence (Table 2.5, Figure 2.8). These results suggest that, while a female may benefit from gaining good genes for her offspring through extra-pair mating, differential investment of her and her mate towards EPO clutches can also lead to greater reproductive success.

Egg Phase

Egg mass increased with laying-order, but unlike previous reports on other species (Cordero et al. 1999; Krist et al. 2005; Magrath et al. 2009), EP eggs were not heavier than WP eggs, perhaps because they were not disproportionately likely to be laid near the end of the clutch order, where eggs are heaviest. The most compelling evidence for female differential allocation during the egg phase resulted from differences in investment based on her mate's quality: females mated to males that produced EPOs produced heavier eggs. Differential allocation of egg mass in response to male quality has been demonstrated several times previously, both in this species (Arnold et al. 2016; but see: Bolund et al. 2009) and other avian species (Cunningham and Russell 2000; Horathova et al. 2012). The effects of greater allocation toward offspring sired by attractive males often last into adulthood (Cunningham and Russell 2000; Gilbert et al. 2006; Arnold et al. 2016) and confounds demonstration of possible genetic benefits to offspring.

An alternative interpretation, which is not mutually exclusive (Holveck and Riebel 2010), is that pairs mated assortatively based on male attractiveness and female fecundity. Since egg mass is heritable for females (Potti 1999; Christians 2002) and a can be influenced by female early life environment -- with better environments associated with heavier eggs (Potti 1999; Griffith and Buchanan 2010a) -- high quality males may have secured mates that were more fecund. There is support for assortative pairing in zebra finches (Burley 1986a; Burley and Foster 2006; Holvek and Riebel 2009), but that assortment may not extend to fecundity since males may be unable to assess female fecundity (Wang et al. 2017). Whether differential allocation by females and/or assortative mating explains egg mass patterns remains unclear. However, results do indicate that maternal investment during the egg phase is not likely to explain differences in the quality of EPOs and WPOs.

Unexpectedly, females paired to LO diet males invested less in EP eggs relative to WP eggs, which is contrary to expectations, since males raised on the LO diet had lower expression of secondary sexual traits (Burley et al. 2018; Wilson et al. 2019), while extra-pair males are expected to be high-quality. While this finding could be reasonably attributed to type-I error, given the small sample size, it may be premature to discount this result without subsequent replication since extra-pair mating behaviors are often complex (e.g., Ihle et al. 2013; Baran and Adkins-Regan 2014; Yuta et al 2018). Indeed, previous studies have identified differences in relative EPO condition or fitness in good versus poor environmental conditions. However, even these results are mixed, with one study reporting a benefit to EPOs under good conditions (O'Brien and Dawson 2007) and others reporting a benefit under poor conditions (Schmoll et al. 2005; Gravin et al. 2006; Arct et al. 2013). Taken together, it is clear that the effects of extra-pair paternity on primary resource allocation are likely to vary with environmental conditions; a better understanding of how allocation varies is needed in order understand the consequences of different allocation decisions.

Incubation Phase

While wild zebra finch pairs split parenting duties, including incubation, evenly (Zann and Rossetto 1991; Zann 1996; Gilby et al. 2013), the a priori expectation was that females would allocate more time than males to nest attendance since females tend to incubate more than males in captive populations (El-Wailly 1966; Delesalle 1986; Gorman and Nager 2003; Morvai et al. 2016; Wilson et al. 2017), and because incubation is possibly less costly to females (Vleck 1981a; Zann 1996; Deeming 2008; Hill et al. 2014). However, nest attendance time measured here included other activities such as time spent nest building, which is a male-dominated task (Zann 1996). Thus, it is likely the high male nest attendance here is attributable to differences in methodologies. Nevertheless, zebra finch pairs do exhibit plasticity in their incubation behaviors (Zann and Rossetto 1991; Gilby et al. 2013; Wilson et al. 2017), and this plasticity likely reflects differences in individual condition or quality as well as intensity of sexual conflict (e.g., Wilson et al. 2017).

Since female-only incubation is likely the ancestral avian state (Burley and Johnson 2002; Tullberg et al. 2002; Moore and Varricchio 2016), incubation is typically considered to be female-led. Nonetheless, if males increase their nest attendance time, a comparable decrease in female nest attendance time would be expected since pairs are likely aiming for some optimal total amount of incubation effort (Jones 1989; Wilson et al. 2017). Here and elsewhere (Hill et al. 2011), males that sired EPOs displayed greater incubation effort to their own clutches. Attractive males that can obtain extra-pair mates easily likely devote less much time to extra-pair courtship. Thus, they may not face a trade-off between nest attendance and extra-pair activities. Mates of males that have high nest attendance early in the clutch cycle may then have greater time to feed, which may contribute to the greater egg mass of these females. Male extra-pair success may indicate direct benefits as well as indirect genetic quality to females.

Assortative mating and/or differential allocation may also impact patterns of reproductive investment during the incubation phase. The inequality in nest attendance was greatest between LO diet males and their mates (Figure 2.4), suggesting that females paired to LO diet males may have been unable to invest more in incubation based on their own condition (Weibe and Martin 2000; Gorman and Nager 2003; Wilson et al. 2017) or unwilling to invest more based on the quality of their mate (Burley 1986a, Sheldon 2000; Arnold et al. 2016). Indeed, females are expected to invest in incubation such that they finish the incubation period without dipping below a critical condition level (Weibe and Martin 2000; Gorman and Nager 2003). Furthermore, males raised on the LO diet are not expected to be resource-restricted when breeding under improved nutritional conditions (Gorman and Nager 2003; Wilson et al. 2017), so they would be capable of compensating for their mate’s reduced incubation effort.

Nestling Phase

Support for differential allocation between EPOs and WPOs was observed during the nestling phase of reproduction: parents provided clutches containing EPOs more nutrient-rich egg. Parents provision offspring at comparable rates to one another in both wild and captive populations of zebra finches (Gilby et al 2011; Morvai et al. 2016) and our finding that egg consumption was similar between sexes suggests that this pattern existed in the current population as well. Early nutrition has lasting effects, with greater resources associated with higher offspring survival, better adult condition and greater expression of secondary sexual traits (Birkhead et al 1999; Lindstrom 1999; Nowicki et al. 2002; Spencer et al. 2003; Fitze et al 2003; Grüebler et al. 2018, Wilson et al. 2019). Thus, differential provisioning of EPO likely associated with the often-reported patterns of increased quality of EPOs.

Pairs show a high degree of synchrony in provisioning by often foraging and returning to the nest simultaneously to feed offspring in the wild. (Mariette and Griffith 2012; 2015). While captive populations have not been observed to the same extent, egg consumption rates were comparable between the sexes and nestling phase pair synchrony has been reported and pairs do evenly share parental care duties during this phase (Gilby et al 2011; Morvai et al. 2016). Thus, increased female provisioning would conceivably result in an increase in their mate's provisioning as well even though it would be maladaptive for males to increase provisioning for unrelated nestlings. Patterns of male reproductive investment in response to female infidelity likely depend on several factors including the costs and benefits of current and future reproductive investments (Kempanaers and Sheldon 1997; Whittingham and Dunn 2001; Holen and Johnstone 2007; Alonzo 2010). While an increase in provisioning towards unrelated young is clearly maladaptive, the benefit of close pair synchronization across breeding attempts would likely outweigh the costs since rates of extra-pair paternity are relatively low for this species (13% for this population; 12–15.3% for other captive studies [Tschirren et al. 2012]; and 1.7–2.4% for wild populations [Birkhead et al. 1990; Griffith et al. 2010]). This finding highlights the need to consider pair coordination in addition to other forms of sexual conflict (or lack thereof) in interspecific patterns of extra-pair behavior.

Daughters may have been provisioned more than males because of sex differences in nutritional demands. Previous work in zebra finches has shown that clutches are more female-biased when mothers are in better condition (Foster and Burley 2007), and that female offspring suffer higher mortality with nutritional conditions are poor (Burley et al. 1989; deKogel 1997). This result adds to the growing body of evidence for this species that daughters are more costly to raise than sons.

Offspring Survival

Male quality and female extra-pair success played meaningful roles in female offspring production. Females -- especially those mated to HI diet males -- benefited by producing EPOs because females that produced one or more EPOs produced more offspring overall and this pattern was more pronounced between HI-mated females that did and did not produce EPOs. Several contemporary frameworks of the evolution of extra-pair behavior have highlighted the potential for this behavior to be maladaptive for females (Arnqvist and Kirkpatrick 2005; Kempenaers and Schlicht 2010; Forstmeier et al 2011; Forstmeier et al. 2014) but persist in populations because extra-pair tendencies have a heritable component (Forstmeier et al. 2011). However , here we show that in some instances females do benefit from producing EPOs.

Additionally, in some contexts, male extra-pair success may not be maladaptive to females since females may be able to gain both direct and indirect benefits. LO diet males that produced EPOs were particularly good partners since they invested more in nest attendance and their mates produced more offspring. Since these males do not express traits that reflect good early nutritional conditions, it is possible that they have particularly good genes since they are still successful at producing extra-pair offspring, which would result in their female partners having high reproductive success in terms of both offspring quality and quantity. However, these results must be considered with caution since the sample size was very low. Nevertheless, it may be adaptive for females to produce extra-pair offspring themselves, and females paired to males with high extra-pair success may gain an advantage.

CHAPTER 3:

Diet History Effects on Zebra Finch Incubation Performance: Nest Attendance, Temperature Regulation, and Clutch Success

INTRODUCTION

Until relatively recently, the incubation phase of avian reproduction was not considered to be a resource-limited phase. However, we now understand it to be energetically on par with demands of the offspring provisioning phase (Monaghan and Nager 1997, Marasco and Spencer 2015, Nord and Williams 2015), which suggests that birds should practice tactical allocation of resources to this stage of reproduction (Stearns 1989, Heaney and Monaghan 1996). Major parental activities during the egg phase involve incubation and nest building/maintenance, both of which contribute to keeping eggs within their thermoneutral zone (Ar and Sidis 2002). Most incubation-phase studies have focused on the cost of nest attendance (Vleck 1981a, Weibe and Martin 2000, Coe et al. 2015) or on nest quality (Reid et al. 2002, Hilton et al. 2004, Heenan 2013), but the two are rarely considered simultaneously (Grubbauer and Hoi 1996). Integration of these two activities is necessary in order to understand how birds respond to competing demands during the incubation phase and prioritize activities based on resource availability.

Most incubating birds need to leave the nest periodically for somatic maintenance, especially feeding, which results in nest temperature fluctuation even in species with bi-parental incubation (Zann 1996, Deeming 2002). Tactics that minimize such fluctuation are advantageous, because low or fluctuating developmental temperatures can cause embryo mortality and may have lasting deleterious effects on surviving offspring (Olsen et al. 2006, Ardia et al. 2010, DuRant et al. 2013, Wada et al. 2015). In general, embryos are vulnerable to temperature fluctuation (Deeming and Jarrett 2015) and, in some species, they become increasingly more vulnerable over the course of development (Cooper and Voss 2013). Parental cooperation is thus important and can lead to greater allocation to incubation by the partner in better condition (Gorman and Nager 2003). In most species studied, however, females typically contribute the greater share of incubation (Deeming 2002), which likely reflects deep historical constraints (female-only incubation is likely the ancestral avian state – Burley and Johnson 2002, Tullberg et al. 2002, Moore and Varricchio 2016) and may reflect the presence of sexual conflict (Trivers 1972, Andersson 1994, Chapman 2006).

In addition to direct heat transfer, birds also construct nests that provide insulation to eggs and young. Such insulation can decrease the cost of incubation (Vleck 1981a, Grubbauer and Hoi 1996, Reid et al. 2002, Hilton et al. 2004), especially at lower temperatures (El-Wailly 1966, Biebach 1986, Reid et al. 2000). The design of nests and the materials used in their construction vary greatly among species (Collias and Collias 1984). Materials used in different portions of the nest of any given species may vary in their insulative and conductive properties (Hansell and Deeming 2002, Hilton et al. 2004, Heenan 2013, Healy et al. 2015), with the result that different parts of a nest have distinct roles in microclimate maintenance (Deeming 2012). However, few studies have investigated this possibility (Deeming and Mainwaring 2015).

Zebra Finches (Taeniopygia guttata castanotis) are gregarious, socially monogamous granivores native to the semi-arid zone of Australia. In the wild (Zann 1994) and in captivity (Burley 1988, Zann and Rossetto 1991), both parents participate in incubation, brooding and feeding of offspring. They build a domed nest of grass stems and line it with plant material and feathers (Zann 1996). Their readiness to breed in captivity under a range of photoperiods, combined with striking similarities in the behavior of captive and free-living birds (Zann and Rossetto 1991, Griffith and Buchanan 2010b, Perfito 2010), make them ideal for controlled laboratory experimentation on diet effects.

In order to explore costs and parental tactics during this under-studied phase of avian reproduction, here we examine how the nutritional state of breeders during development and breeding - two periods in life in which resources are scarce (Martin 1987, Nowicki et al. 2002) - may influence allocation to nest attendance and nest building. Others have focused on varying natal diet (Birkhead et al. 1999), pre-breeding diet (Monaghan et al. 1996, Selman and Houston 1996, Gorman et al. 2005) or breeding diet (Rutstein et al. 2004) as a means of manipulating Zebra Finch condition, since birds provided with a higher quality diet are generally reported to display better "condition", although the measure of condition varies widely (e.g., muscle condition (Selman and Houston 1996), fecundity (Monaghan et al. 1996), immunity (Birkhead et al. 1999)) and is sometimes not defined (Rutstein et al. 2004, Gorman et al. 2005). Yet it seems likely that dietary characteristics at each of these points of life may interact to influence resource allocation during incubation. For example, if individuals develop costly phenotypes in response to early good nutrition (e.g., greater skeletal size in Zebra Finches: Boag 1987, Bonaparte et al. 2011), those phenotypes may impair reproduction if resources become limited later in life (Monaghan 2008). We also assess the effects of nest attendance and nest structural components on nest temperatures and temperature fluctuation and determine the reproductive consequences for these variables. We hypothesized that a consistently high-quality food environment throughout life would result in a higher allocation of resources towards incubation behaviors (nest attendance and nest quality), and that individuals faced with food limitation would invest less. Based on prior results (Boag 1987, Bonaparte et al. 2011), we expected that high-natal-diet birds would experience greater stress when breeding on a low-quality diet. We also predicted that both incubation patterns and nest structural components would influence the nest microclimate and clutch fate (i.e., whether or not a clutch attempt produced at least one hatchling that survived 24 hours).

METHODS

Natal Diet Conditions and Founder Selection

In 2013, the 120 parents of the subjects of this study were allowed to breed in two large (160 m3) outdoor flights. Birds in one flight had access to the HI diet (daily supplements of hens egg, and thrice weekly supplements of green vegetables), while those in the other flight received the LO diet (thrice weekly supplements of green vegetables, but no egg supplements). All birds were provided water, cuttlefish bone, oyster shell and commercial finch seed mix ad libitum. After offspring reached nutritional independence (X ± SE= 45 ± 3 days), they were caught and housed within their natal flights in single-sex cages at standard densities until they were ~100 days old. This housing protocol ensured that young birds had continual access to social parents and other adult conspecifics throughout development. Such contact is important for sexual imprinting on visual traits (Immelmann 1975, Bolhuis 1991, Bischof et al. 2002) as well as for song learning (Eales 1989; ten Cate et al. 1993). After reaching adulthood, birds were moved indoors and maintained on their natal diet until selected for this experiment. Only birds in good condition with wild-type plumage and conformation were selected. No pairs involved siblings and no pairs had the identical set of parents.

Once selected for participation in this study, subjects were uniquely color-banded for identification purposes (using colors previously determined not to influence attractiveness – Burley, 1985) and released in same-sex groups into outdoor flights for two weeks in order to strengthen their flight muscles. During this period, all birds were fed an intermediate (LAB) diet, with egg supplements three times a week, alternating with thrice weekly vegetable supplements.

Pairing and Breeding

After the two-week-long flight-conditioning interval, birds were sorted and released into natal-diet-specific outdoor flights to promote assortative pairing. Pair bonds were determined via observations of courtship, allopreening and joint nest site searches made during the second week of assortative housing. After two weeks all identified pairs were randomly assigned to breed on either the HI or LO diet and moved to the appropriate outdoor flight. Pairs were kept on their assigned diets for the remainder of the study. This procedure generated four treatment groups: Pairs in which both individuals were raised and bred on the HI diet (HI/HI); those raised on the HI diet and bred on the LO diet (HI/LO); those raised on the LO diet and bred on the HI diet (LO/HI); and those raised and bred on the LO diet (LO/LO). Seventy-one percent of all birds subsequently re-paired with new partners; of these, 51% were non-assortative with respect to natal diet. Those that re-paired with an individual from the opposite rearing diet were allowed to remain in the flight, but were not included in this study. The age of birds at the start of breeding ranged from 92 to 340 days and did not differ between breeding flights (Linear mixed-effects model (LMM), z=0.71, P >0.45).

Birds were provided with nest sites (2.25 sites/pair) and allowed to breed for five months during the summer and fall of 2014. Resources for nest-building (grass and feathers) were provided in a bin on the aviary floor; this was refreshed daily. All nest sites were censused daily and contents recorded. Eggs that appeared since the previous day's census were uniquely marked; fresh eggs were also weighed. Eggs that failed to hatch within 20 days from their lay date were dissected to determine their contents (no development or dead embryo). Social parentage at each nest was assigned on the basis of frequent observations on all active nests.

Duration of nest attendance by members of a breeding pair, nest quality scores and nest temperature data were collected at focal nests as specified below. All focal nests met the following criteria at the time they were selected: They contained 2-4 fresh eggs laid on consecutive days in a nest under active construction as determined by the presence of fresh nesting material.

Effects of natal and rearing diet treatments on condition and mortality of breeders, as well as other components of reproductive success, will be explored in a paper in which the birds that bred with partners from the opposite natal diet are included (authors, in preparation).

Measurement of Incubation Temperatures

During the 2014 breeding season, ambient temperatures ranged from highs of 22-38°C (X ± SE= 28 ± 3) to lows of 13-24°C (X ± SE= (19 ± 2). Incubation is thought to be more costly outside of the thermoneutral zone (28 - 40 °C) (Calder 1964, Vleck 1981b). Each focal nest contained a thermal sensor from the time it was selected until the day that the second hatchling appeared. Focal nests were selected at the beginning of the incubation phase (X ± SE = 0.3 ± 1.7 days, where day 0 was the day the last egg was laid). Initiation of nest temperature data collection did not differ based on natal or breeding diet (LMM P>.35 for fixed effects).

iButton® devices (Maxim Integrated, San Jose, CA, U.S.A.) served as thermal sensors to record nest temperature during the incubation period of each clutch and were placed directly under nest contents so that the sensor made physical contact with eggs. Preliminary observations established that the presence of these devices did not disrupt Zebra Finch activity, and that there was high inter-sensor agreement. Inter-sensor agreement was assessed by placing all sensors in the same location and allowing them to record the temperature every minute for 36 hours. The average temperature over this period was calculated for each of the 20 sensors used. Variation in average temperature for all devices was low (X ± SD=24.4 ± 0.28°C). Sensors recorded temperature every minute and reported at 0.5 °C intervals. To retrieve data, all sensors were collected each morning and data downloaded; then sensors were reset and randomly re-distributed to nests. Temperatures recorded by sensors during the daily data retrieval period (~20 minutes) were not included in analysis.

Previous observations have shown that incubation activity displays a diurnal pattern (Zann and Rossetto 1991). During daylight hours, the parents alternate incubation duties in time blocks ranging from a few minutes to over an hour. In the morning, the nest is regularly left unattended for short periods while both parents forage, while in the afternoon, the nest is usually continuously attended. At night, only the female typically attends the nest, but sometimes both sexes are present (Zann and Rossetto 1991). Here, temperature data were grouped to reflect this rhythm. To ensure grouping properly aligned with Zebra Finch activity, we determined when birds first came off the nest in the morning by identifying a sudden drop from the steady nighttime nest temperature. Analysis of a random subsample of 5 days per pair showed that the drop occurred at an average of 96 (±36) minutes before sunrise each day. Accordingly, the "morning" sampling interval began 90 minutes before sunrise (determined from records of the Astronomical Applications Department of the US Navy) on the median day of incubation for each nest; "afternoon" and "evening" samples began 8 and 16 hours after the morning sample, respectively. For each sampled nest, morning, afternoon, and nighttime temperature averages were used as estimates of egg temperature, with their respective standard deviation used to estimate temperature fluctuation (Table 3.1). Temperature data was later aligned with nest attendance observations to determine the mean nest temperature and fluctuation that occurred during nest attendance.

Parental Activity Observations

Throughout the two-week span of incubation, 30-minute focal samples (Altmann 1974) of parental activity were collected at each focal nest by observers who were blind to the test hypotheses and to birds' natal diets, but were inadvertently aware of breeding diet of birds in flights under observation. The focal sampling method has been previously used to estimate variation in individual contributions to parental care in this species (Burley 1988). During each trial, two observers recorded the total amount of time each parent attended their nest and the total amount of time the nest was unattended. At least four focal samples (X ± SE = 5.2 ± 0.3) were taken for each nest.

Nest Scores

In the wild, Zebra Finches build domed nests from grass stems, typically in thorny shrubs or other structures that provide a suitable substrate (Immelmann 1965). In the lab, plastic cups are used as substrates. The nest of grass consists of a base ("platform") that supports the dome. After egg-laying is complete, parents line the nest interior with a variable number of feathers. Parents continue to add to the nest until the first egg hatches, at which time nest quality begins to deteriorate (authors, unpublished data). Here, natural materials (grass stems and other plant material; feathers) were provided for nest building. Observers scored the platform, lining and dome of each focal nest twice per week during the incubation period; scores were based on a five-point (platform, lining) or six-point (dome) rubric. Scoring was initiated at the beginning of the incubation period (X ± SE = -0.9 ± 2.4 days where day 0 was the day the last egg was laid) and stopped when the first hatchling appeared.

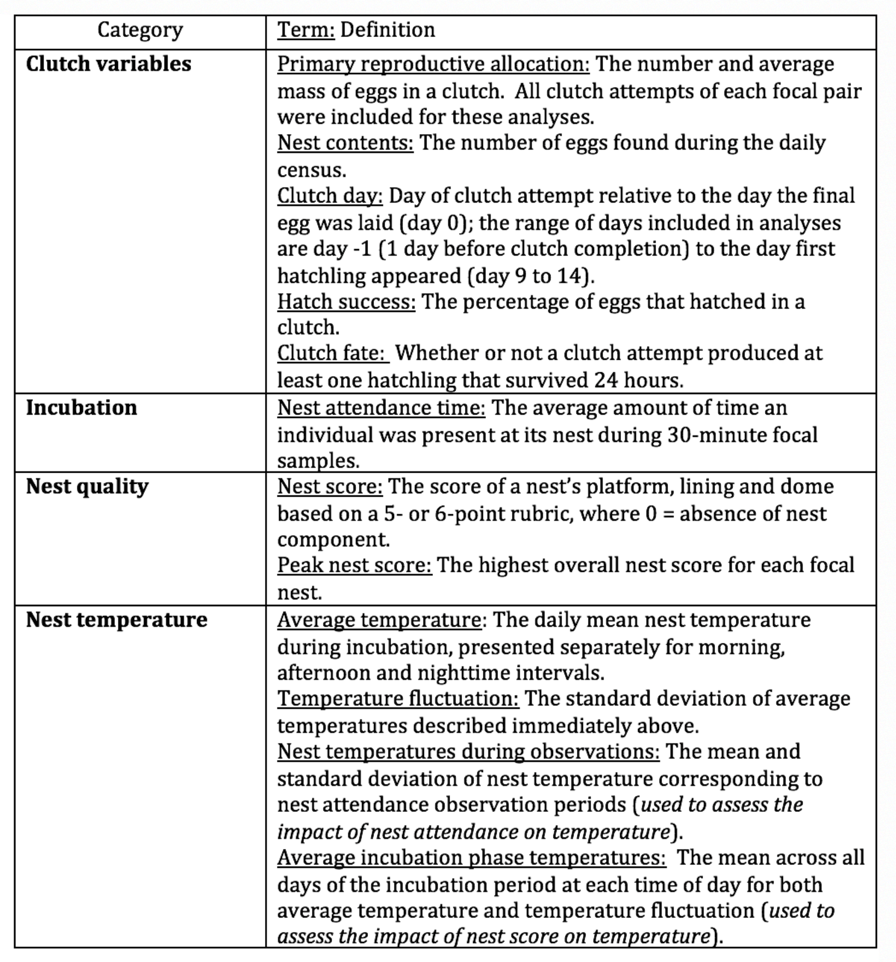
Statistical Analysis

Linear mixed-effects models (LMM) were used to examine the fixed effects of natal diet, breeding diet, and treatment interactions on primary reproductive allocation, nest attendance, nest quality, nest temperature, and hatch success. (Terms used to describe clutch, temperature and nest quality variables are defined in Table 3.1.) For all repeated measures analyses, pair identity was included as a random effect. Analysis of predictors of nest attendance patterns additionally included sex as a fixed effect and included possible interaction effects among sex and diet treatments. Analyses of nest temperature included time of day as a fixed effect and interactions among time of day and diet treatments. Nest contents and clutch day were included as fixed effects in models for nest attendance, nest temperatures and nest quality (Table 1). LMMs were also used to assess which incubation-phase measures were predictive of clutch size and hatch success. Clutch fate was analyzed using logistic regression.

All significant interaction effects were further assessed using contrasts. Non-significant interactions, followed by non-significant fixed factors other than diet treatments, were removed in a reverse stepwise procedure (P to remove = .15). Residuals were determined to meet normality assumptions.

The following components of the study were grouped for sequential Bonferroni correction: Primary reproductive allocation, nest quality, and nest temperatures. Marginal means and Delta-method standard errors are reported in tables. Statistical tests were performed in STATA 14.

Table 3.1: Definitions of terms



Category	<u>Term:</u> Definition
Clutch variables	<u>Primary reproductive allocation:</u> The number and average mass of eggs in a clutch. All clutch attempts of each focal pair were included for these analyses. <u>Nest contents:</u> The number of eggs found during the daily census. <u>Clutch day:</u> Day of clutch attempt relative to the day the final egg was laid (day 0); the range of days included in analyses are day -1 (1 day before clutch completion) to the day first hatchling appeared (day 9 to 14). <u>Hatch success:</u> The percentage of eggs that hatched in a clutch. <u>Clutch fate:</u> Whether or not a clutch attempt produced at least one hatchling that survived 24 hours.
Incubation	<u>Nest attendance time:</u> The average amount of time an individual was present at its nest during 30-minute focal samples.
Nest quality	<u>Nest score:</u> The score of a nest's platform, lining and dome based on a 5- or 6-point rubric, where 0 = absence of nest component. <u>Peak nest score:</u> The highest overall nest score for each focal nest.
Nest temperature	<u>Average temperature</u>: The daily mean nest temperature during incubation, presented separately for morning, afternoon and nighttime intervals. <u>Temperature fluctuation:</u> The standard deviation of average temperatures described immediately above. <u>Nest temperatures during observations:</u> The mean and standard deviation of nest temperature corresponding to nest attendance observation periods (*used to assess the impact of nest attendance on temperature*). <u>Average incubation phase temperatures:</u> The mean across all days of the incubation period at each time of day for both average temperature and temperature fluctuation (*used to assess the impact of nest score on temperature*).

RESULTS

Results are reported for 29 breeding pairs, including seven HI/HI, eight HI/LO, seven LO/HI, and seven LO/LO pairs.

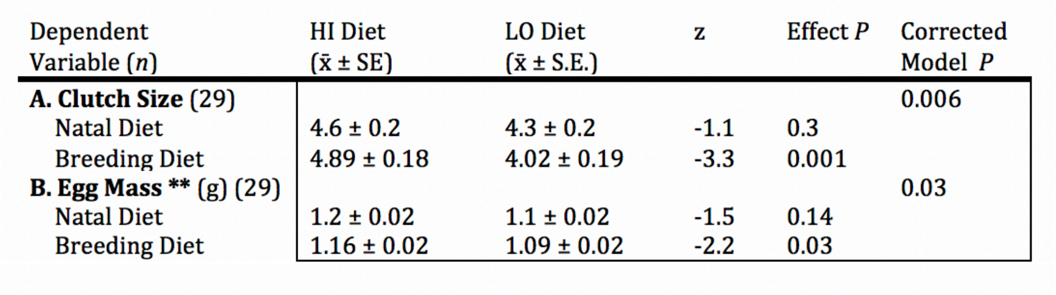
<u>Primary Reproductive Allocation and Clutch Traits</u>

Primary reproductive allocation varied by breeding diet (Table 3.2). Pairs that bred on the HI diet had significantly larger clutches (repeated measures LMM: Wald $\chi2$ =12.4, df= 2, corrected model P=0.006; Table 3.2, Model A) and heavier eggs (repeated measures LMM:

Wald χ2 = 7.0, df= 2, corrected model P=0.03; Table 3.2, Model B). Egg mass differed among pairs, as indicated by the significant random term in the model.

Diet treatments did not affect the number of clutches that pairs attempted during the study (P>0.3) or the inter-clutch interval (number of days between first egg dates of successive clutches) (P>0.2).

Table 3.2: Primary reproductive allocation as a function of diet treatment. Traits are evaluated across all clutch attempts of focal pairs. Linear fixed effects models; sample size (n) = number of pairs, df= 2 for both models. Asterisks denote significant pair effects (**P<0.001).

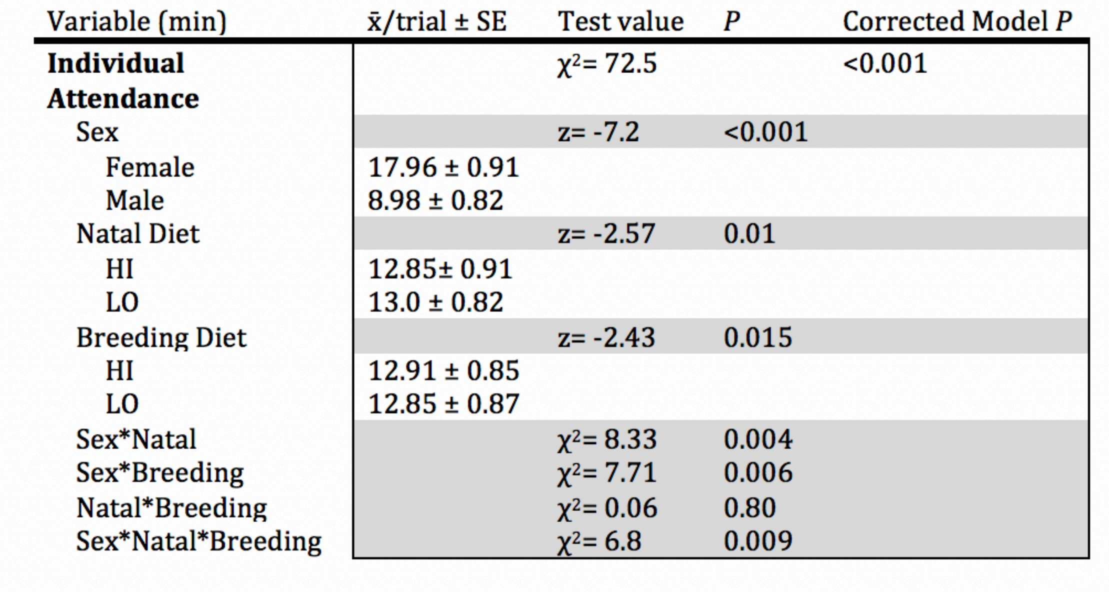


Dependent Variable (*n*)	HI Diet (x̄ ± SE)	LO Diet (x̄ ± S.E.)	z	Effect *P*	Corrected Model *P*
A. Clutch Size (29)					0.006
Natal Diet	4.6 ± 0.2	4.3 ± 0.2	-1.1	0.3	
Breeding Diet	4.89 ± 0.18	4.02 ± 0.19	-3.3	0.001	
B. Egg Mass ** (g) (29)					0.03
Natal Diet	1.2 ± 0.02	1.1 ± 0.02	-1.5	0.14	
Breeding Diet	1.16 ± 0.02	1.09 ± 0.02	-2.2	0.03	

<u>Nest Attendance</u>

Nest attendance time differed by sex and all interactions involving sex (LMM: Wald χ2=72.9, df=7, model P<0.001) (Table 3.3). Across all treatments, females displayed significantly greater nest attendance time than males (P < 0.001). The significant results for natal and breeding diet are driven by the strong effect of the three-way interaction among diet conditions and sex and reflect the large sex differences in nest attendance in the HI/HI treatment: HI/HI females had the highest nest attendance time of any group while HI/HI males had the lowest (Figure 3.1). The amount of time nests were left unattended did not differ among diet treatment groups (repeated measures LMM: Wald χ2=0.1, P>0.9).

Table 3.3: Nest attendance as a function of sex and diet treatments. The average number of minutes the nest was attended by each parent individually during 30-minute observation trials. Linear mixed-effects model, n =29, df=7.



Variable (min)	$\bar{x}$/trial ± SE	Test value	*P*	Corrected Model *P*
Individual Attendance		χ^2= 72.5		<0.001
Sex		z= -7.2	<0.001	
Female	17.96 ± 0.91			
Male	8.98 ± 0.82			
Natal Diet		z= -2.57	0.01	
HI	12.85± 0.91			
LO	13.0 ± 0.82			
Breeding Diet		z= -2.43	0.015	
HI	12.91 ± 0.85			
LO	12.85 ± 0.87			
Sex*Natal		χ^2= 8.33	0.004	
Sex*Breeding		χ^2= 7.71	0.006	
Natal*Breeding		χ^2= 0.06	0.80	
Sex*Natal*Breeding		χ^2= 6.8	0.009	

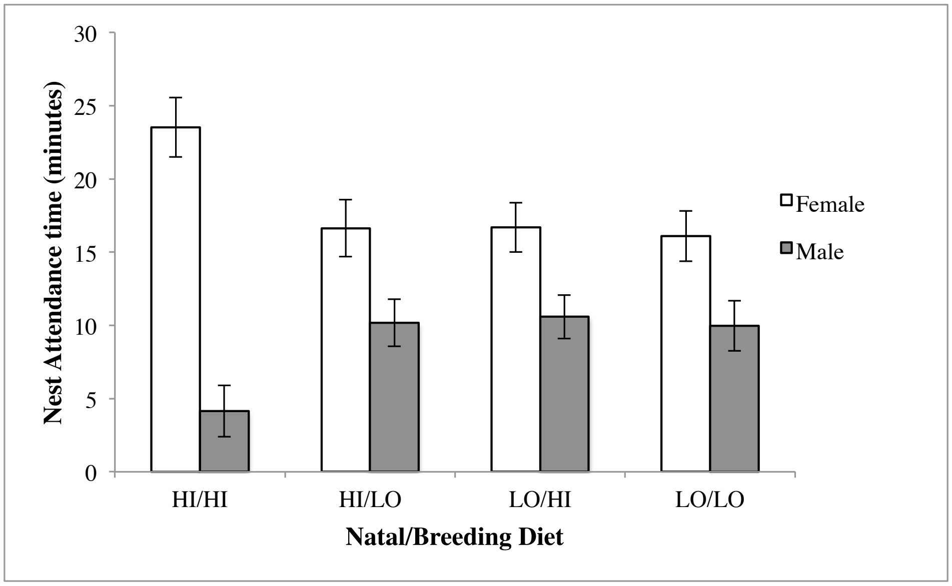
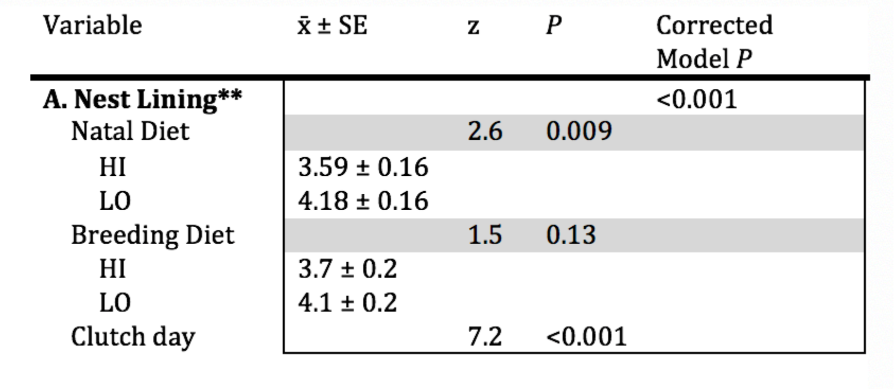


Figure 3.1: Nest attendance during 30-min focal samples. Females incubate more than males, HI/HI (natal diet/breeding diet) females incubate the most and HI/HI males incubate the least (repeated measures LMM: Wald $\chi2$=72.9, corrected model P<0.001, P<0.02 for all comparisons).

Nest Quality

Nest lining was the only measure of nest quality influenced by diet treatment (repeated measures LMM: Wald χ2=84.0, df=4, corrected model P<0.001). Birds raised on the LO diet had higher nest lining scores than birds raised on the HI diet (Table 3.4). Nest lining score also increased with clutch day (z= 7.2, n =28, P<0.001), and was influenced by pair identity, as indicated by the significant random term in the model. Diet treatments did not affect nest platform and nest dome (repeated measures LMM: platform: Wald χ2=0.1 corrected model P>0.9; dome: Wald χ2=3.3 corrected model P>0.3).

Table 3.4: Nest quality as a function of diet treatments and clutch day. Scores of nest traits measured during incubation period of clutch attempts for which focal nest attendance samples were taken. Linear mixed-effects models, n =29, df=4. Asterisks denote significant pair effects (**P<0.001).



Variable	x̄ ± SE	z	*P*	Corrected Model *P*
A. Nest Lining**				<0.001
Natal Diet		2.6	0.009	
HI	3.59 ± 0.16			
LO	4.18 ± 0.16			
Breeding Diet		1.5	0.13	
HI	3.7 ± 0.2			
LO	4.1 ± 0.2			
Clutch day		7.2	<0.001	

<u>Nest Temperature</u>

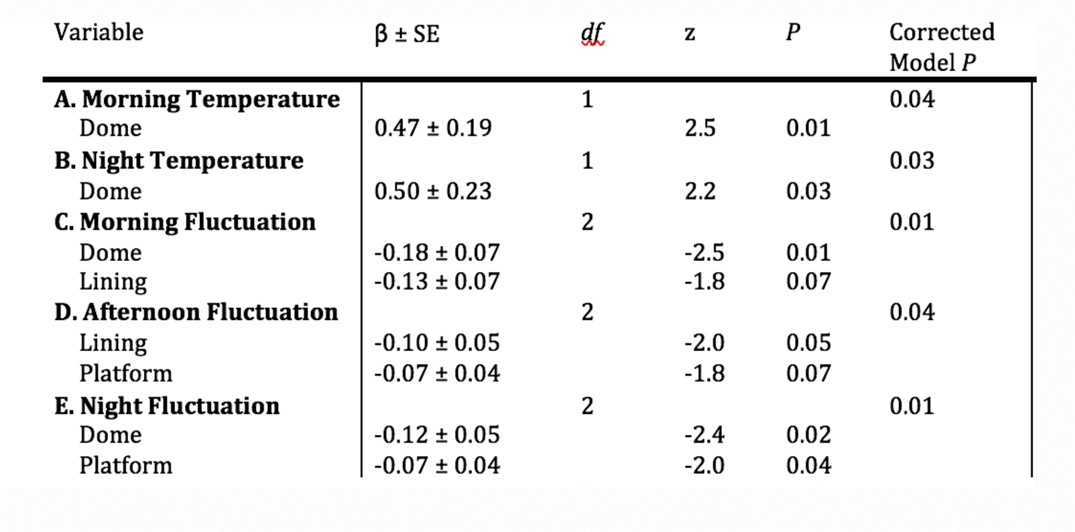
Impact of nest attendance

Temperature data collected during focal samples were analyzed to determine whether nest attendance patterns impacted temperature measures (Table 3.1). Nest attendance time predicted nest temperature patterns. More nest attendance time resulted in higher nest temperatures (repeated measures LMM: Wald χ2=10.1, df =2, β ± SE= 0.10 ± 0.03, uncorrected model P=0.002) and lower temperature fluctuation (repeated measures LMM: Wald χ2=14.1, df =2, β ± SE= 0.04 ± 0.01,uncorrected model P=0.003).

Impact of nest quality

Peak nest score measures predicted nest temperature patterns during the incubation period. Higher dome scores predicted higher nest temperatures and lower temperature fluctuation during the morning and at night (LMM: morning temperature: Wald χ2=6.3, df = 1, corrected model P=0.036; morning fluctuation: Wald χ2=11.2, df = 2, corrected model P=0.014; night temperature: Wald χ2=4.9, df = 1, corrected model P=0.027; night fluctuation: Wald χ2=12.2, df = 2, corrected model P=0.012; Table 3.5, Models A, B, C and E). Higher lining scores predicted lower temperature fluctuation in the afternoon (Wald χ2=8.1, df = 2, corrected model P=0.036; Table 3.5, Model D), and higher platform scores predicted lower temperature fluctuation at night (Table 3.5, Model E).

Table 3.5: Nest Temperature as a function of nest score. Linear mixed-effects models, n =29.



Variable	β ± SE	*df*	z	*P*	Corrected Model *P*
A. Morning Temperature		1			0.04
Dome	0.47 ± 0.19		2.5	0.01	
B. Night Temperature		1			0.03
Dome	0.50 ± 0.23		2.2	0.03	
C. Morning Fluctuation		2			0.01
Dome	-0.18 ± 0.07		-2.5	0.01	
Lining	-0.13 ± 0.07		-1.8	0.07	
D. Afternoon Fluctuation		2			0.04
Lining	-0.10 ± 0.05		-2.0	0.05	
Platform	-0.07 ± 0.04		-1.8	0.07	
E. Night Fluctuation		2			0.01
Dome	-0.12 ± 0.05		-2.4	0.02	
Platform	-0.07 ± 0.04		-2.0	0.04	

Average nest temperature

Nest temperature during the incubation period differed with time of day, nest contents, and clutch day; interaction effects involving diet treatment also contributed significantly to the model (repeated measures LMM: Wald χ2=278.7, df= 13, corrected model P<0.001; Table 3.6, Model A). Nest temperatures were lower in the morning (X ± SE = 35.5 ± 0.06°C) and higher in the afternoon (X ± SE = 36.4 ± 0.06°C) and at night (X ± SE = 36.2 ± 0.06°C) (Figure 3.2). Nest temperature increased with nest contents and clutch day. Nests of LO/LO birds had higher average temperatures in the morning compared to birds breeding on the HI diet (HI/HI and LO/HI), while nests of HI/LO birds had higher nest temperatures at night than those of all other diets (Figure 3.3A). Pair identity also contributed significantly to the model.

Nest temperature fluctuation

Temperature fluctuation during the incubation period was predicted by time of day, clutch day, nest contents and by the two-way interaction between natal diet and breeding diet (repeated measures LMM: Wald χ2=523.3, df=7, corrected model P<0.001; Table 3.6, Model B). Fluctuation was highest in the morning (X ± SE = 1.76 ± 0.03°C), intermediate in the afternoon (X ± SE = 1.08 ± 0.03°C) and lowest at night (X ± SE = 0.93 ± 0.03°C) (Figure 3.3B). Fluctuation decreased with nest contents and clutch day. Nests of HI/LO birds had greater temperature fluctuation than all other diet treatment combinations (Figure 3.3B). Lastly, fluctuation was significantly impacted by pair identity.

Table 3.6: Nest Temperature as a function of diet treatments and clutch traits. Linear mixed-effects models, n =29, average temperature df= 13, temperature fluctuation df=7. Test values are reported for contrasts between stated times of day. Asterisks denote significant pair effects (**P<0.001).

Dependent Variable	x̄ ± SE	Test value	*P*	Corrected Model *P*
A. Average Temperature (°C)**		$\chi^2 = 287.7$		<0.001
Natal Diet		z = -0.1	0.9	
HI	36 ± 0.05			
LO	36 ± 0.05			
Breeding Diet		z = 1.0	0.3	
HI	35.8 ± 0.05			
LO	36.3 ± 0.05			
Time of Day				
Morning → Afternoon		z = 3.4	<0.001	
Afternoon→ Night		z = -1.8	0.07	
Night → Morning		z = -5.2	<0.001	
Time of Day*Natal		χ^2=13.6	0.001	
Time of Day*Breeding		χ^2=7.52	0.02	
Time of Day*Natal*Breeding		χ^2= 7.5	0.001	
Nest contents		z = 2.4	0.02	
Clutch day		z = 8.9	<0.001	
B. **Temperature Fluctuation (°C)****		$\chi^2 = 523.3$		<0.001
Natal Diet		z = 0.6	0.54	
HI	1.2 ± 0.02			
LO	1.3 ± 0.02			
Breeding Diet		z = -1.0	0.30	
HI	1.2 ± 0.02			
LO	1.3 ± 0.02			
Time of Day				
Morning → Afternoon		z = -16.8	<0.001	
Afternoon → Night		z= -3.3	0.001	
Night → Morning		z = 20.6	<0.001	
Natal*Breeding		χ^2= 5.1	0.02	
Nest contents		z = -3.7	<0.001	
Clutch day		z = -4.8	<0.001	

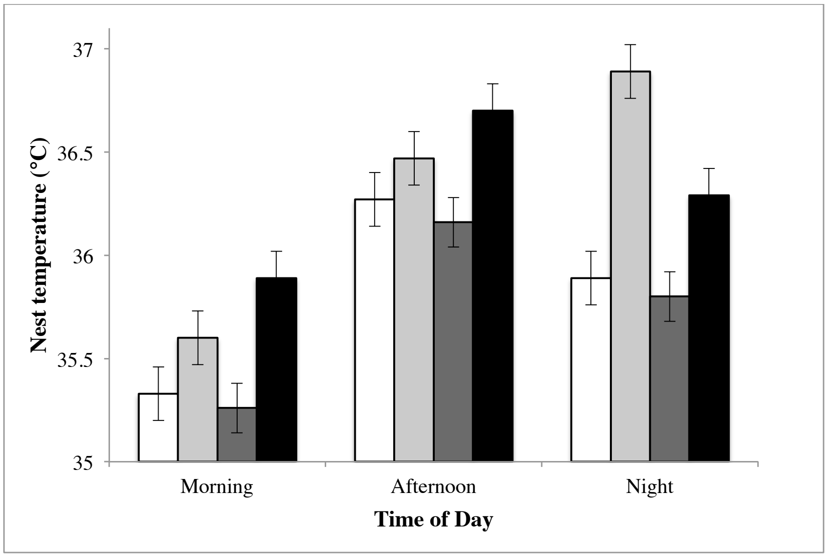


Figure 3.2: Average nest temperatures by time of day and treatment. Temperatures were lower in the morning than other periods (P<0.001 for all significant comparisons). In the morning, LO/LO (black) nest temperatures were higher than HI/HI (white) and LO/HI (dark grey) temperatures; at night, HI/LO (light grey) nest temperatures are the highest (three-way ANOVA used to generate comparisons: P<0.02 for all comparisons).

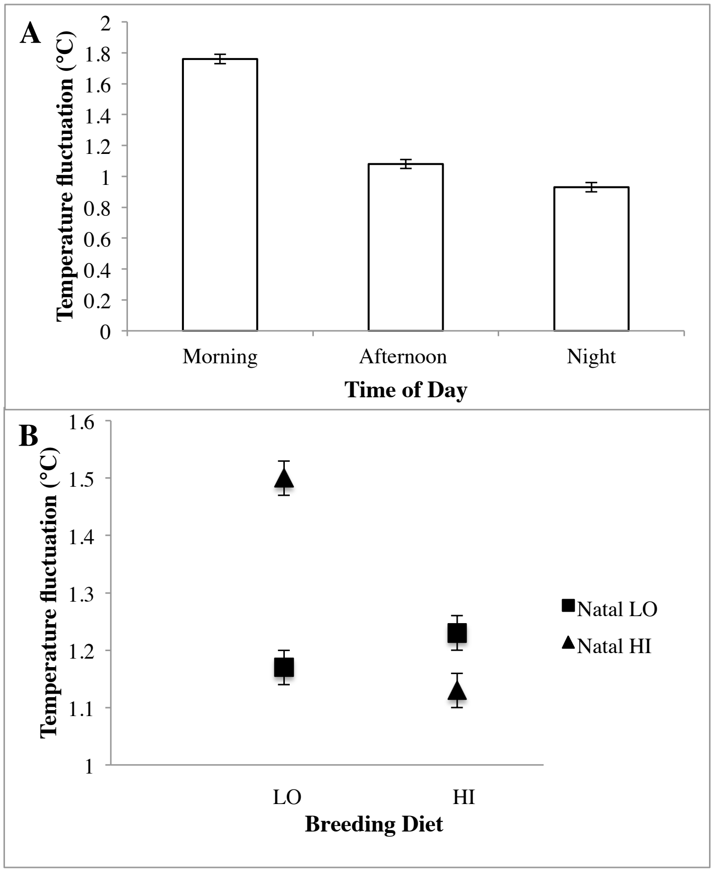


Figure 3.3: Nest temperature fluctuation by time of day and treatment. A) Fluctuation was highest in the morning, intermediate in the afternoon and lowest at night (P <0.001 for all comparisons). B) HI/LO nests had the most temperature fluctuation (three-way ANOVA used to generate comparisons: P<0.001 for all comparisons).

<u>Hatch Success</u>

All clutch attempts by each focal pair were included in the analyses of hatchling number and clutch hatch success, but only focal nest data were available for analyses of variables predicting hatch success. The number of hatchlings per clutch was impacted by breeding diet (Wald χ2=7.1, df=2, uncorrected model P=0.03). As found for clutch size (Table 3.2, Model A), pairs that bred on the HI diet had significantly more hatchings (X ± SE = 2.7 ± 0.26) than those that bred on the LO diet (X ± SE = 1.7 ± 0.27). Hatch success (Table 3.1) was not predicted by diet treatments (Wald χ2= 3.5, df=2, uncorrected model P>0.15; natal diet: P> 0.7 HI: X ± SE = 0.57 ± 0.05, LO: X ± SE = 0.55 ± 0.05; breeding diet: P> 0.06, HI: X ± SE = 0.63 ± 0.05, LO: X ± SE = 0.49 ± 0.05) or by other variables (temperature measures: P>0.4; incubation time: P>0.6; nest score: P>0.9). Furthermore, diet treatments did not affect the proportion of eggs that failed to develop (repeated measures LMM: Wald χ2 =0.5, df=2, uncorrected model P>0.7) or the proportion of embryo mortalities (repeated measures LMM: Wald χ2 =1.2, df=2, uncorrected model P>0.5).

Clutch Fate

For all clutch attempts, clutch fate was not impacted by natal or breeding diet (logistic regression (LR), LR χ2=2.8, df=2, natal diet β ± SE= -0.21 ± 0.55, breeding diet β ± SE= 0.90 ± 0.56, uncorrected model P=0.24). For focal nests, clutch fate did reflect nest temperature fluctuation: clutches that produced at least one viable hatchling experienced lower temperature fluctuation than clutches that failed to do so (LR χ2 =4.9, df=1, β ± SE= -5.4 ± 2.7, uncorrected model P= 0.03). Clutch fate was not dependent on nest attendance time, nest score or average nest temperature.

DISCUSSION

A major aim of this study was to further our understanding of how diet quality influences reproductive investment during the incubation stage. We found that breeding diet affected primary reproductive allocation, while nutrition during natal and breeding periods jointly affected investment patterns of both sexes, including nest attendance, nest traits and nest temperature. Furthermore, clutches were more likely to yield hatchlings when temperature fluctuation was low, which was likely due to the negative impact fluctuation has on embryo development (Cooper and Voss 2013). Temperature fluctuation varied with both nest attendance and nest scores, such that greater nest attendance and higher nest scores predicted less temperature fluctuation. Thus, the interaction between developmental and reproductive nutritional states influences the outcome of incubation via effects on nest temperature fluctuation. Results that bear on additional aims of the study are discussed below.

Condition-Dependent Sexual Conflict

Among songbird species with bi-parental incubation, the total amount of time that nests are attended during incubation varies with male contribution to attendance, but not with that of females (Matysioková and Remeš 2014). This implies that males incubate to provide females their needed recess and ensure that total attendance time meets some threshold or target hatch success (Jones 1989), with the common result of less total incubation by males than females, as seen for captive Zebra Finches (El-Wailly 1966, Delesalle 1986, Gorman and Nager 2003, but see Gilby et al. 2013). The idea that females choose to incubate whenever circumstances are permissive is superficially contradictory to predictions of sexual conflict theory (Trivers 1972, Andersson 1994, Chapman 2006). In many birds, however, including Zebra Finches, only females possess brood patches, so they may experience greater incubation efficiency (Vleck 1981a, Zann 1996 Deeming 2008, Hill et al. 2014). Under such circumstances, the benefit/cost calculation may favor greater female attendance whenever their foraging needs are met (Marasco and Spencer 2015).

This logic can be extended to address patterns of intraspecific variation in incubation. Specifically, the observation that HI/HI pairs had the greatest sex difference in incubation time implies that HI/HI females experienced lower somatic costs of incubation (Smith et al. 1989, Cresswell et al. 2003). Since female Zebra Finches are capital breeders (Houston et al. 1995), HI/HI females may have higher capacity to store reserves as a result of their early history as well as by residing in a permissive, energy-rich food environment during reproduction. While our prediction that individuals encountering a high-quality food environment throughout life would result in higher allocation towards nest attendance was met only for females, this logic indicates that the low attendance by males was not evidence for sexual conflict, but rather of its relative absence: The potential for sexual conflict during incubation is low when females experience low foraging needs.

Nest temperature results suggest that pairs with a good developmental, but poor reproductive, environment struggle to allocate resources to both somatic and reproductive demands. HI/LO pairs had the highest nighttime incubation temperatures, which is counterintuitive since incubation is more costly at low ambient temperatures. This suggests that males in this treatment group joined females in the nest to mitigate thermal regulation costs associated with solitary perching; this possibility is supported by the finding that, in nature, joint nocturnal incubation is more common in winter (Zann and Rossetto 1991). The rarity of joint incubation suggests that is has costs, which could include increased egg damage (Spoon et al. 2006) and, especially at night, increased mortality from predation. This line of reasoning suggests that male Zebra Finches elect to participate in nocturnal nest attendance only when somatic demands are so costly that they offset costs of joint incubation. Future studies could further investigate this finding by manipulating male somatic costs and monitoring nest attendance during the night.

Temperature fluctuation patterns of HI/LO pairs were also informative of condition. HI/LO pairs showed greater nest temperature fluctuation than other groups, suggesting that they took longer parental recesses (Vleck 1981b). Given the high somatic investment found for birds reared on the HI diet in previous studies (Boag 1987, Bonaparte et al. 2011), these birds were expected to be the most challenged by the energetic demands of breeding under a low resource base. Unfortunately, our nest attendance observations were not designed to capture larger incubation trends, so we were unable to directly document these longer recesses. Nevertheless, somatic demands may have regularly compelled an incubating bird to leave the nest and forage before his/her mate returned to incubate, leading to longer gaps in incubation and greater temperature fluctuation. This, in turn, likely forced the more satiated partner to assume incubation responsibilities before he/she was ready to do so, suggesting increased sexual conflict over incubation duties in this treatment. Thus, sexual conflict may vary with parental foraging needs during this phase.

Clutch fate was tied to nest temperature fluctuation, with clutches that failed at the hatching stage showing higher fluctuation than others. Although not studied here, poor temperature maintenance may also have had long-term consequences for offspring performance (such as lower hatching mass and reduced immunity -- Olsen et al. 2006, Ardia et al. 2010, DuRant et al. 2013) and may additionally have transgenerational consequences (Krause and Naguib 2014). So despite the finding that HI/LO birds had overall similar reproductive performance to other groups, this pairing type may experience lower lifetime fitness as a result of poor offspring performance.

Collectively our results appear consistent with a reproductive allocation pattern hypothesized by Monaghan (2008), who suggested that good food conditions experienced during breeding yield higher fitness for all individuals regardless of conditions they experienced in early life, and that individuals raised under good conditions suffer the most when food conditions are poor during breeding. This pattern may be due to greater somatic investment by individuals raised under good conditions. Future research will be needed to establish a direct link between somatic investment and resource allocation during incubation.

Tradeoffs Involved in Incubation

Nest thermal patterns provided insight into how parents generally cope with competing demands during incubation, both on a daily and phase-wide basis. In the morning, high fluctuation and low temperatures were consistent with the occurrence of foraging by both parents, while decreased fluctuation and increased nest temperatures later in the day aligned with more stable incubation and less time off the nest (Zann and Rossetto 1991). Consistent with the presumed needs of older embryos for a stable thermal environment (Cooper and Voss 2013), we found that as the incubation period progressed, temperature fluctuation decreased and that nest temperature and nest quality increased. This suggests that birds adjust their behavior in strategic ways throughout the incubation period to ensure the survival of their offspring.

Additionally, we found that thermal patterns were affected by clutch size. Clutch size is known to affect heat loss during recess (Boulton and Cassey 2012), but we further found that it had a notable impact on temperature regulation throughout the whole incubation period, as larger clutches had lower temperature fluctuation and higher average temperatures. This finding supports reports that larger clutches are more energy-efficient because the thermal properties of larger clutches partially offset the additional cost of incubation (Hepp et al. 2015, Nord and Williams 2015). While larger clutches incur greater absolute incubation costs (Moreno and Carlson 1989, Monaghan and Nager 1997, Weibe and Martin 2000, Nord and Nilsson 2012), birds may be able hatch more offspring at a relatively lower per capita cost by increasing clutch size (Weibe and Martin 2000, Reid et al. 2002). This is likely to be especially profitable in species with relatively high adult mortality, such that the probability of surviving to re-nest is often low. The ability of birds to capitalize on the physical principles driving the effect of clutch size on temperature maintenance is likely predicated by their condition.

Influence of nest components on temperature regulation

Consistent with previous studies, we showed that nests function to maintain a stable egg microclimate (Deeming and Mainwaring 2015) by insulating eggs (Healy et al. 2015). In addition, we demonstrated the functional importance of different nest components. Domes were most helpful in temperature maintenance when eggs were most susceptible to cooling: In the morning when parents were off the nest and at night when ambient temperatures were lowest (Table 3.5). A likely mechanism by which domes conserve heat is by creating a still-air environment (Turner 1987, 1988). Thus, consistent with the sentiments of Mainwaring et al. (2015), dome construction may evolve not only for predator avoidance, but also as a way to reduce costs of incubation and minimize temperature fluctuation.

Finally, rearing diet influenced nest quality: Nest lining scores were higher for birds with a low-quality natal diet. These birds may have relied more on nest lining to mitigate temperature fluctuation, which suggests that elements of nest variability may be linked to environmental conditions faced during development. In this study, the daily allotment of feathers was much smaller than that of grass, and many birds stole feathers from nests of other birds. So, the effort of competing for feathers and defending against feather thieves was probably costly for LO natal diet birds. Collectively, our findings indicate that additional research on factors that influence intraspecific variation in nest quality would be a profitable direction to pursue.

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