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Original Article

Early life and transgenerational stressors impact secondary sexual traits and fitness

Kerianne M. Wilson, Andrey Tatarenkov, and Nancy Tyler Burley
Department of Ecology and Evolutionary Biology, University of California, Irvine, 321 Steinhaus Hall, Irvine, CA 92697-2525, USA

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Developmental stress from early life challenges impacts adult phenotype across a range of species. However, the potential transgenerational consequences for adult phenotype are largely unknown. Additionally, the possible impacts of natural hatch/birth order and natal brood composition in unmanipulated broods/litters on adult performance has been understudied. This experiment takes a novel approach to studying developmental stress by integrating and assessing multiple potential stressors and multiple secondary sexual traits simultaneously in order to determine how these influence both social and genetic reproductive success. Male zebra finches were colony-reared on high- or low-quality diets; as adults, they reproduced competitively on an intermediate diet. Male visual ornaments (beak color and cheek patch size) were found to be reliable signals of developmental stress, since they showed high sensitivity to multiple early conditions and predicted reproductive success. Contrary to the nutritional stress hypothesis, early diet did not impact song traits investigated. Male reproductive success was impacted by diet history, male hatch order, and natal brood traits of males' fathers, with daughter and son production sensitive to different subsets of identified reproductive stressors. Notably, diet influenced only son production and the hatch orders of males and their fathers influenced only daughter production. Findings suggest that the sexes respond differently to early life conditions, which may influence subsequent sex allocation patterns. Despite good general correspondence in patterns of social and genetic reproductive success, males that sired 1 or more extra-pair offspring achieved higher fitness through greater son production.

Key words: **developmental stress hypothesis, extra-pair paternity, *Taeniopygia guttata*, transgenerational fitness effects, visual ornamentation**

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).

Address correspondence to K. M. Wilson. E-mail: kkmurphy@uci.edu.

The developmental stress hypothesis (Spencer et al. 2003; 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 intergenerational 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 multigenerational (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 (EPP) 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 1993b; 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 1 of 3 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 3 populations were established from a pool of birds derived from 3 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, whereas 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 2 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 m^3) 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) 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). 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 2 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 2 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); motifs were visualized in Sound Analysis Pro 2011 (SAP11) (Tchernichovski and Mitra 2004).

For each male, we selected a total of 5 recorded motifs for analysis, using several guidelines to maximize the number of songs sampled. The selected motifs came from at least 3 different songs, with no more than 2 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). The 2 observers that identified these syllable types then independently assessed male motifs using this classification and no interobserver discrepancies occurred.

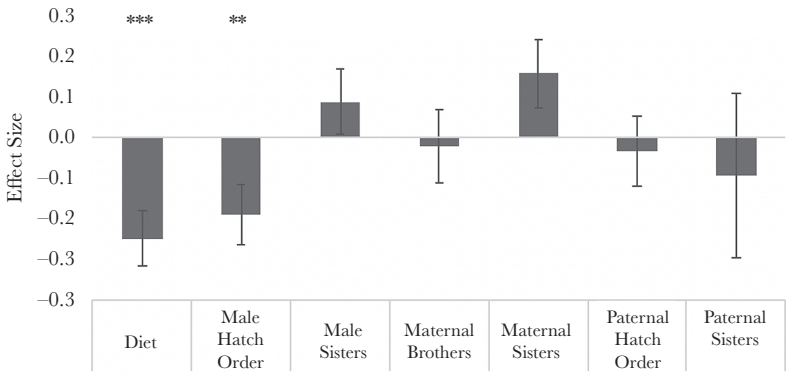

Figure 1
Effect sizes (±SE) of z-transformed candidate developmental stressors on z-transformed overall male trait expression, including acoustical secondary sexual traits (stereotypy, total syllables, proportion of unique syllables), visual secondary sexual traits (cheek patch size and symmetry, beak color), and body size traits (head width, tarsus length, and mass). *$P \leq 0.05$; **$P \leq 0.01$); ***$P \leq 0.001$; ****$P < 0.0001$). Data from Table 2.

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. 2013), 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 5 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 2 scorers.

Variation across each male's motifs was assessed using the stereotypy coefficient described by Holveck and Riebel (2007). All 5 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.

$$\text{Stereotypy coefficient} = 1 - [(\Sigma \text{ changes}) / (\Sigma \text{ motifs} - 1)(\Sigma \text{ syllables})]$$

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 2 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 EPP.

Genetic parentage assignment

Genotyping was performed for founders and offspring using 8 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, Coralville, IA). PCR began with denaturing for 5 min 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 7 min 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 8 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 6 of the 8 loci. Out of 192 offspring that were genotyped, 19 were excluded from further analyses. Six offspring were excluded because fewer than 6 loci could be determined and the birds were no longer available for blood resampling. Another 6 offspring were excluded because they were reared by same-sex pairs. Lastly, 7 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. 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 (3 HI and 1 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 $\alpha \leq 0.10$. 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 ($\alpha = 0.05$). Linear mixed effect models were also used in analyses in which phenotype was partitioned into 3 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, 5 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, TX).

RESULTS

The 3 male size traits were positively intercorrelated, but the 6 secondary sexual traits were not intercorrelated (Table 1). Results for analyses using residual cheek patch can be found in the supplement (Supplementary Table S1).

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 5 different genetic pairs. The 23 extra-pair offspring were sired by 10 males (range: 1–7 offspring), 4 of which lost paternity to another male.

Stressor effects on male phenotype

Two candidate stressors had negative overall effects on male phenotype expression: diet and male hatch order (Figure 1; Table 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 2b). Hatch order negatively impacted male body size (Figure 2c) and no candidate stressors impacted acoustic traits (Figure 2a).

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 3; Table 3). Male hatch order and that of his father negatively impacted daughter production (Figure 4a), whereas diet quality negatively impacted son production (Figure 4b). Results for the effects of candidate stressors on the total number of offspring produced can be found in the supplement (Supplementary Table S2 and Figure S3).

Effects of male traits on reproductive success

Three of the 9 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 5, Table 4). Cheek patch symmetry and beak color positively predicted both daughter and son production whereas cheek patch size and head width predicted production of sons only (Figure 6). 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 6a). Results for the effects of adult male traits on the total number of offspring produced can be found in the supplement (Supplementary Table S3 and Figure S4).

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: Ps < 0.005). Males that sired extra-pair offspring tended to produce more offspring overall (2-sample *t*-test with equal variance: $t = -2.05$, df = 26, $P = 0.051$) (Supplementary Figure S2); 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

Table 1
Correlations among secondary sexual traits and size traits of males

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	−0.048	−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

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 $N = 23$.
*$P < 0.05$. **$P < 0.01$.

Table 2

Correlations among developmental stressors and male traits (secondary sexual traits and size traits)

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

Pearson's correlation coefficient values reported for $N = 28$ (family $N = 23$).
*$P < 0.05$. **$P < 0.01$.

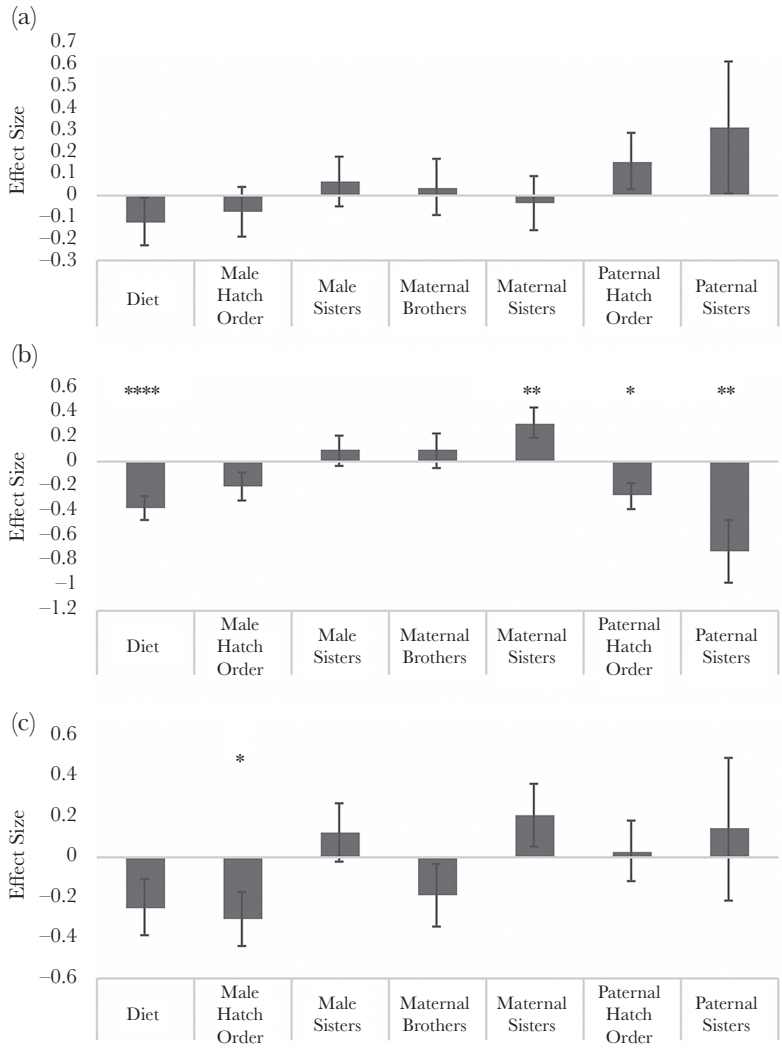

Figure 2

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). *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ****$P < 0.0001$. Data from Table 2.

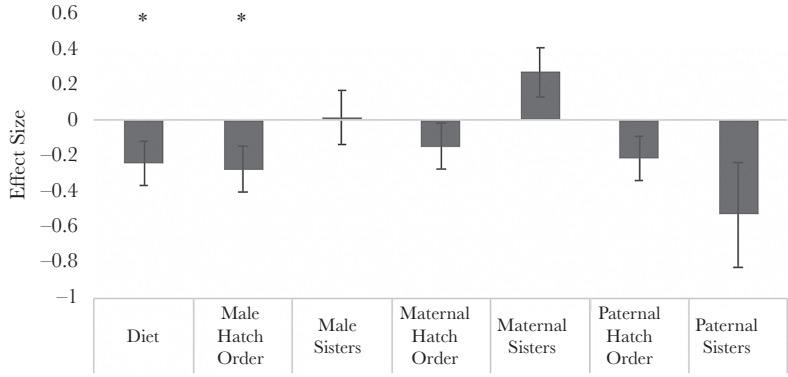

Figure 3
Effect sizes (±SE) of *z*-transformed candidate developmental stressors on all *z*-transformed male reproductive success measures (genetic, social and extra-pair production of sons and daughters). *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ****$P < 0.0001$. Data from Table 3.

Table 3
Correlations among developmental stressors and reproductive measures

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

Pearson's correlation coefficient values reported for $N = 28$; family $N = 23$.
*$P < 0.05$. **$P < 0.01$.

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 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 4; Figures 5 and 6). 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 1986; Booksmythe et al. 2017).

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 EPP (Supplementary Figure S2), 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 1–4). Also, male hatch order appears to have transgenerational fitness consequences, influencing the number of granddaughters produced (Figure 4a). 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 2 and 3).

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, whereas the number of sisters in the natal broods of males' fathers had a negative effect (Figure 1). 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, whereas condition of their mates was not (Foster and Burley 2007). In that study, control (unclipped) females tended to produce more female offspring

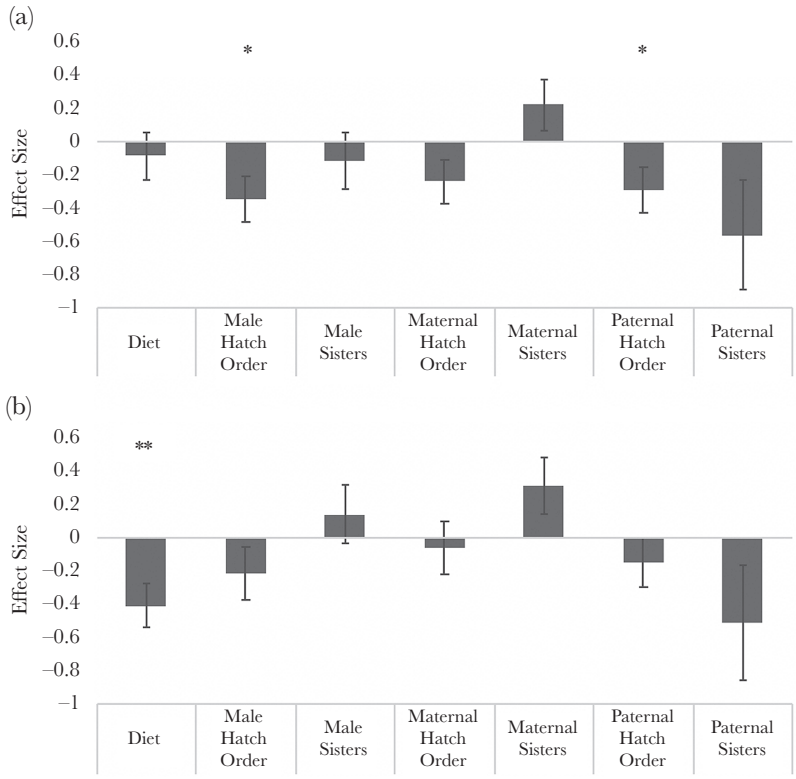

Figure 4
Effect sizes (±SE) of *z*-transformed candidate developmental stressors on *z*-transformed (a) daughter production (genetic, social and extra-pair) and (b) son production (genetic, social and extra-pair). $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$; $****P < 0.0001$. Data from Table 3.

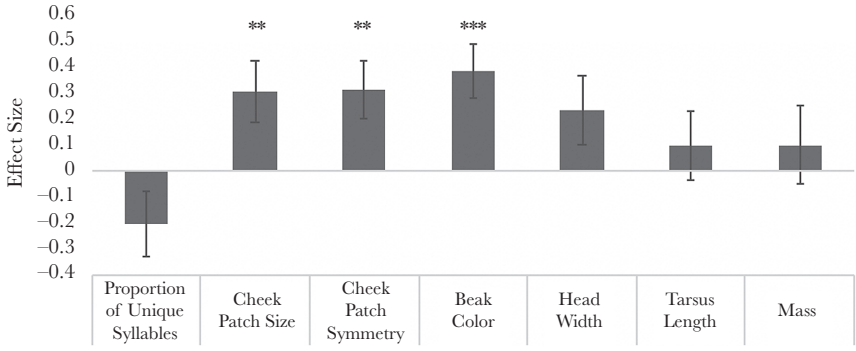

Figure 5
Effect sizes (±SE) of *z*-transformed influential male body size and secondary sexual traits on all *z*-transformed male reproductive success measures (genetic, social and extra-pair production of sons and daughters). $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$; $****P < 0.0001$. Data from Table 4.

Table 4
Correlations among male traits (secondary sexual traits and size traits) and reproductive measures

	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

Pearson's correlation coefficient values reported for $N = 28$; family $N = 23$.
$*P < 0.05$. $**P < 0.01$.

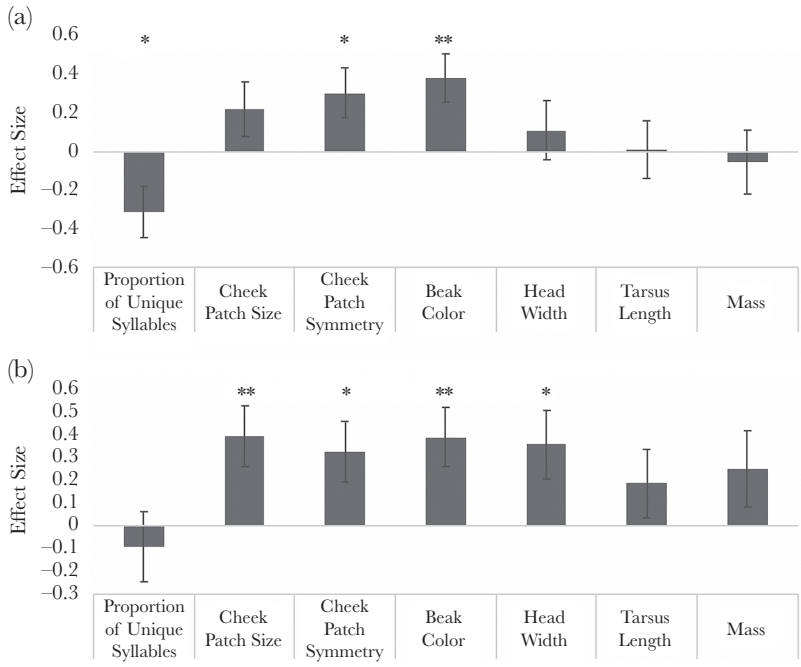

Figure 6
Effect sizes (±SE) of *z*-transformed influential male body size and secondary sexual traits on *z*-transformed (a) daughter production (genetic, social and extra-pair) and (b) son production (genetic, social and extra-pair). $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$; $****P < 0.0001$. Data from Table 4.

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 2; Figure 2). 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), whereas 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 2).

Interestingly, the proportion of unique syllables negatively predicted reproductive success via daughter production (Figure 6a), 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 4) and exerted the largest impact of the 3 phenotype categories on reproductive success (Figures 5 and 6). 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 1993b; 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 1993b; 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 2; Figure 2b) and predicts offspring production (Table 4; Figure 6).

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 2, Figure 2c). 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 2). This result counters conventional expectations that early life conditions are predicted to influence trait asymmetry (Møller and Pomiankowski 1993; Swaddle 2003). 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 nonrandom. 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 2 populations that their expression is not phenotypically intercorrelated (Table 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.

SUPPLEMENTARY MATERIAL

Supplementary data are available at *Behavioral Ecology* online.

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Data accessibility: Analyses reported in this article can be reproduced using the data provided by Wilson et al. (2019).

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