



Effects of nutritional stress during different developmental periods on song and the hypothalamic–pituitary–adrenal axis in zebra finches

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ABSTRACT

In songbirds, developmental stress affects song learning and production. Altered hypothalamic–pituitary–adrenal (HPA) axis function resulting in elevated corticosterone (CORT) may contribute to this effect. We examined whether developmental conditions affected the association between adult song and HPA axis function, and whether nutritional stress before and after nutritional independence has distinct effects on song learning and/or vocal performance. Zebra finches (*Taeniopygia guttata*) were raised in consistently high (HH) or low (LL) food conditions until post-hatch day (PHD) 62, or were switched from high to low conditions (HL) or vice versa (LH) at PHD 34. Song was recorded in adulthood. We assessed the response of CORT to handling during development and to dexamethasone (DEX) and adrenocorticotrophic hormone (ACTH) challenges during adulthood. Song learning and vocal performance were not affected by nutritional stress at either developmental stage. Nutritional stress elevated baseline CORT during development. Nutritional stress also increased rate of CORT secretion in birds that experienced stress only in the juvenile phase (HL group). Birds in the LL group had lower CORT levels after injection of ACTH compared to the other groups, however there was no effect of nutritional stress on the response to DEX. Thus, our findings indicate that developmental stress can affect HPA function without concurrently affecting song.

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Introduction

Growth and developmental processes are sensitive to environmental factors, and early life adversity can have profound effects on behavior and physiology of animals (Barnes and Ozanne, 2011; Lindström, 1999). Birdsong is a trait that appears particularly sensitive to stressful developmental conditions (Spencer and MacDougall-Shackleton, 2011), which indicates that it may be a reliable indicator of developmental history and this may contribute to birdsong being a sexually selected trait (Nowicki et al., 1998, 2002). For songbirds such as the zebra finch (*Taeniopygia guttata*), development of a specific network of interconnected brain nuclei mediates song learning and production. Development of these brain regions and song learning behaviors begin soon after hatching and conclude at sexual maturity (Brainard and Doupe, 2002; Kirn, 2010). Because zebra finches do not modify their song elements in adulthood, any stress-induced deficits in song learning during early life may reliably indicate how well an individual was able to develop when confronted with stress. While there seems to be

some support for this hypothesis, the results are not uniform across studies (Table 1). These inconsistencies may partially be caused by the variation in types of stressors used and measures used to quantify song. To help resolve these inconsistencies, further studies are required that replicated previously used developmental stress protocols and include a comprehensive analysis of song parameters.

One way in which external environments can affect development is through glucocorticoids via activation of the hypothalamic–pituitary–adrenal (HPA) axis. Glucocorticoid receptors (GR) are present in the song control nuclei HVC and RA (robust nucleus of the arcopallium), implying that these areas are potential targets for glucocorticoids such as corticosterone (CORT; Shahbazi et al., 2011; Suzuki et al., 2011). Artificially increasing CORT can affect song-related behaviors and neural structures. For example, oral administration of CORT to hatchlings for approximately 30 and 50 days reduced song complexity of zebra finches and song sparrows (*Melospiza melodia*), respectively (Schmidt et al., 2013; Spencer et al., 2003), and HVC volume (proper name) of zebra finches (Buchanan et al., 2004). Administration of CORT via silastic implants for a longer duration (approximately 85 days) also reduced song learning accuracy of zebra finches (Shahbazi et al., 2011). Furthermore, song sparrows with larger song repertoires had larger HVC volumes and lower CORT concentrations in response to

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Table 1

Summary of studies of developmental stress on zebra finch song. Values for the duration of treatment represent days post-hatch, where 0 corresponds to the hatch date.

	Manipulation	Duration	Song complexity	Learning accuracy	Song rate
de Kogel and Pijls (1996)	Brood size reduction	0–50			↑
Tschirren et al. (2009)	Brood size enlargement	0–90			↑
Gil et al. (2006)	Brood size enlargement	0–90	=	=	
Holveck et al. (2008)	Brood size enlargement	0–30	=	↓	
Birkhead et al. (1999)	Protein reduction	0–30			=
Spencer et al. (2003)	Food accessibility	5–30	↓		=
Zann and Cash (2008)	Food accessibility	0–30	↓	=	
Brumm et al. (2009)	Food accessibility	5–30	=	↓	

stress (MacDougall-Shackleton et al., 2009; Pfaff et al., 2007; Schmidt et al., 2012). Collectively, these findings suggest that CORT may be a mechanism by which developmental stress affects song.

The timing of developmental stress may be particularly important because stressors will likely have stronger or weaker effects on traits depending on the timing of trait development relative to the timing of the stressor (Spencer and MacDougall-Shackleton, 2011). For instance, altering nutritional conditions during the age when cheek patches of male zebra finches are developing affected cheek patch expression, but altering nutritional conditions before that age did not (Honarmand et al., 2010; Naguib and Nemitz, 2007). Previous experimental studies on developmental stress and song using zebra finches have used either brood size or nutritional manipulations (see Table 1). Those that manipulate nutritional conditions directly usually terminate treatment once birds reach nutritional independence, around post-hatch day (PHD) 30 (e.g. Gil et al., 2006; Holveck et al., 2008; Spencer et al., 2003). However, zebra finch song-control regions HVC, RA and Area X develop and mature from approximately PHD 10–50 (Bottjer et al., 1985), coinciding with the behavioral development of song learning, which extends well past PHD 30. Thus, developmental stress after PHD 30 should likely affect zebra finch song development, but no studies to date have confirmed this.

Stress during different periods of development may also have differential effects on song. In zebra finches, stress during the sensory phase can affect aspects of song learning, presumably by influencing neural representation of tutor songs (e.g. Holveck et al., 2008; Spencer et al., 2003; Zann and Cash, 2008). On the other hand, stress during the sensorimotor phase may have more potent effects on vocal performance because during this phase birds must learn to precisely coordinate motor movements to match neural representations of song (Podos et al., 2009; Sakata and Vehrencamp, 2012). Vocal performance includes aspects of song that are physically difficult to produce because it places great demands on birds' ability to perform rapid modulations of the syrinx, respiratory, and vocal tract motor systems (Podos et al., 2009). The ability to sing consistent or stereotyped renditions of a song could also be affected by developmental stress if it limits the amount of time young zebra finches are able to practice singing (see Pytte and Suthers, 2000). As described above, previous work shows that zebra finch song learning may be affected by developmental conditions, but whether vocal performance is also affected remains unresolved. So far, song rate has been the principle measure of the effects of developmental stress on vocal performance in zebra finches (Birkhead et al., 1999; Brumm et al., 2009; Gil et al., 2006; Spencer et al., 2003; Tschirren et al., 2009), but this measure may not be representative of true vocal performance capabilities (Podos et al., 2009).

Here, our aim was to determine the relationship between timing of developmental stress and adult song complexity and song performance in zebra finches, and to determine whether CORT mediates the effects of stress on song. More specifically, we addressed the following questions: (1) Does nutritional stress at different developmental periods have distinct effects on HPA axis? (2) Does nutritional stress during later juvenile development (PHD 30–60, when young are feeding independently) affect adult song learning and/or vocal performance and if so, can it be distinguished from the effects of early stress? We defined

nutritional stress as conditions that made finding and/or obtaining food more difficult without inducing a calorie deficit (Lemon, 1991), and followed a nutritional stress protocol previously used by Spencer et al. (2003), Zann and Cash (2008) and Brumm et al. (2009). An important advantage of our approach is that we can see how diverse aspects of song (including a relatively unexplored component, song stereotypy) are affected by nutritional stress during early periods, compared to later periods of development.

Materials and methods

Animals and manipulation

This study was conducted over two years (2011 and 2012). Growth and body mass of birds used in these experiments have been previously reported elsewhere (Kriengwatana et al. in review). Randomly paired adult male and female zebra finches from our breeding colony were each housed in a 36 × 43 × 42 cm cage with access to an external nest box (20 × 13.5 × 13.5 cm) and kept on a 14 h:10 h light:dark cycle at 22 °C. Pairs received grit, cuttlefish bone, seed (Living World premium finch seed; 11.0% protein, 5.9% lipid), and water ad libitum, and were supplemented with daily portions of hardboiled chicken eggs, cornmeal, and bread. All animal care and husbandry protocols were approved by the Animal Use Subcommittee at the University of Western Ontario (protocol #2007-089), and followed guidelines of the Canadian Council on Animal Care. Only broods with 4 or 5 nestlings at the start of treatment were included in our experiment. Nests were monitored daily for nesting activity and randomly assigned to treatment conditions after the first egg hatched.

Treatments began on PHD 5–6 and ended on PHD 61–62 (date of hatch is PHD 1). We manipulated food accessibility similar to Spencer et al. (2003), whereby birds in the high treatment (H) were given access to 65 g seed and 13.5 g egg-food daily, while birds in the low treatment (L) were given access to 50 g total of seed in a mixture containing a 1:3 ratio of seeds and woodchips (by volume), and 6.5 g egg-food daily. This manipulation forces zebra finches in the L treatment to search longer for seeds, but still allows them to obtain a similar amount of seeds per day as the H treatment (Lemon, 1991). This manipulation has been shown to negatively affect body mass, adult song control brain regions, and song characteristics of zebra finches (Buchanan et al., 2004; Lemon, 1993; Spencer et al., 2003; Zann and Cash, 2008).

Nutritional manipulation was separated into two phases: pre-nutritional independence (PHD 5 or 6–PHD 34 or 35) and post-nutritional independence (PHD 35 or 36–61 or 62). Birds experienced either the same conditions (H to H or L to L) for both phases, or different conditions during the pre- and post-nutritional independence phases (i.e. switched from H to L or L to H). This resulted in four treatment groups: HH, HL, LH, LL. After the treatment period, all birds were given ad libitum seed, and offspring were kept with their parents until PHD 90 to ensure that young males learned song from their fathers exclusively (Adret, 1993). After PHD 90, birds were kept in single sex groups of four to five individuals. Potential year effects were controlled statistically (see below). A total of 9 and 13 breeding pairs produced 33 and 58 experimental offspring in the first and second year of the study,

respectively. Sample sizes for each group after combining both years were: HH = 20 (10 males), HL = 27 (13 males), LH = 23 (9 males), and LL = 21 (13 males).

Song recording and analysis

Songs were recorded from males after song had crystallized at approximately PHD 200 (mean \pm SD = 215.86 ± 9.97) and PHD 100 (mean \pm SD = 107.29 ± 12.39) in the first and second year of the study, respectively. Males were isolated in sound attenuation chambers for 24 h, then an unfamiliar female was placed in a cage next to the male and his directed song was recorded for 10 min using a Marantz PMD 670 recorder and an omnidirectional microphone (Sennheiser ME 62). After the recording session ended, birds were returned to their home cages and the same procedures were repeated again approximately one week later. Songs were digitized with a sample frequency of 44.1 kHz and a bit rate of 32 bits per second and sound spectrograms were generated using Raven Pro 1.4 software.

Zebra finch song is composed of a stereotyped sequence of syllables, usually preceded by repetitions of a simple introductory syllable. Syllables are separated by periods of silence of less than 5 ms (Sossinka and Böhner, 1980), and syllables are themselves composed of elements that can be separated by silence or by abrupt changes in frequency modulation or amplitude (Williams and Staples, 1992). Zebra finches may also sing many songs consecutively (song bouts) and these bouts of singing are defined as periods of singing with less than 2 s of silence between songs (Sossinka and Böhner, 1980).

Song complexity

Zebra finches may sing variations of their song by adding, repeating, or skipping elements or syllables in their song (Sturdy et al., 1999), so we chose the most common variant among 10 randomly selected songs as the birds' primary song. Using the primary song we visually identified the number of elements and syllables as well as the number of unique elements and syllables. We did not count the number of introductory elements towards the total number of elements in a bird's song.

Learning accuracy

We also calculated three measures of learning accuracy. The first measure was the similarity index (Böhner, 1990), which quantifies the number of elements the tutor and pupil song have in common. The value of the similarity index varies between 0 and 1, where 1 indicates that the number of elements in the two songs is identical. We calculated the similarity index once for each tutor–pupil pair by comparing the most common variant (i.e. primary song) of the tutor song to the most common variant of the pupil song. The second measure of learning accuracy was degree of syllable match (i.e. the degree of similarity between a syllable copied by the pupil and the original syllable produced by the tutor). After visually identifying which syllables a pupil had copied from their tutor, we isolated these syllables from 10 renditions of the pupil's primary song and cross-correlated them with the corresponding syllable in 10 renditions of the tutor's primary song. Syllables in the pupil song that were not copied from the tutor song were not included in the cross-correlation analysis. Cross-correlations were performed using Raven Pro 1.4 batch correlations function. The last measure of learning accuracy was precision of syntax learning used by Holveck et al. (2008). By examining transitions between elements, this measure quantifies how accurately pupils were able to arrange elements they had learned in the same order as the tutor's song. The total number of shared transitions between the tutor and pupil primary songs was divided by the total number of shared elements minus 1 to obtain a value between 0 and 1 that indicates the proportion of shared transitions (see Appendix B in Holveck et al., 2008 for the complete algorithm). A value of 1 indicates that all the transitions between the same elements in the two songs were shared,

thus indicating that the pupil was able to perfectly replicate the sequence of copied elements.

Song stereotypy

We calculated four measures of song stereotypy to assess vocal performance. First, we measured sequence consistency, which expresses the frequency with which a common sequence appears and is calculated as the proportion of syllable transitions that conform with the most frequent transition for a given syllable (Kao and Brainard, 2006; Scharff and Nottebohm, 1991). For example, if a bird's primary song consists of syllables ABCD, but in one randomly selected song he sings BACD, then the sequence consistency score for this comparison would be 0.33 (only the transition from C to D match). Second, we measured sequence linearity, which quantifies the number of different possible transitions that can be observed after each unique syllable. Sequence linearity was calculated as the number of unique syllables minus one, divided by the sum of syllable transitions (Foster and Bottjer, 2001; Kao and Brainard, 2006). For instance, if a bird sings a song with the syllables ABCD, then the four unique syllables and three unique transitions generate a sequence linearity score of 1. If the bird then sings ABCC, then the three unique syllables and three unique transitions generate a score of 0.66. Both sequence consistency and linearity yield a score between 0 and 1, with 1 indicating complete consistency or linearity. However, unlike sequence consistency, sequence linearity is not affected by variability at the end of songs (i.e. a song with syllables ABCD will yield the same score as a song with syllables ABC). Therefore, our third measure of song stereotypy was the percentage of syllable types that terminated songs (Kao and Brainard, 2006). Because these three measures are sensitive to the number of songs analyzed, we limited analyses to 10 randomly selected songs per bird. Lastly, we measured syllable consistency, which we defined as the similarity between all renditions of a syllable produced. We cross-correlated all renditions of a syllable produced using the batch correlation function in Raven Pro 1.4.

Song output

We calculated three measures of song output: total number of songs, latency to sing, and maximum song bout duration (duration of the longest song bout). Total number of songs included the bird's primary song and any variants.

HPA axis function

Effects of nutritional stress on HPA axis function were examined during both pre- and post-nutritional independence phases and in adulthood. Consequently we have corticosterone (CORT) samples for two time points during development (PHD 30 and 60) while birds were still on treatment, and one sample for a time point after treatment was terminated (PHD 240).

Stress series

Elevation of CORT in response to a standardized capture and handling stressor was measured in birds in both the first and second year of the study (2011 and 2012). The samples were collected when the average age of all the offspring in the nest was PHD 30 and 60. Between 09:00 and 11:00, blood samples were collected from the brachial vein within 3 min of disturbance to obtain baseline CORT levels. Birds were then placed in opaque bags and blood was collected again after 15 and 30 min. In total, approximately 75 μ L of blood was collected from an individual during one series of blood sampling.

ACTH and DEX challenges

Adrenocorticotrophic hormone (ACTH) and dexamethasone (DEX) measures of HPA axis function were obtained only from birds in the first year of study (2011). At approximately PHD 240, we tested whether nutritional stress during development had long-term effects

on HPA-axis functioning by measuring maximum CORT output and the strength of the negative feedback using standardized doses of ACTH and DEX, respectively (following Dickens et al., 2009). Doses of 25 IU/kg ACTH (Sigma Aldrich, A6303) dissolved in Ringers solution and 1000 µg/kg DEX (Sandoz Canada Inc., 2301) were injected into the pectoralis muscle using a 300 µL insulin syringe. The appropriate injection volume to achieve the desired dose was calculated based on individual body mass measured on the previous day. Similar concentrations have been successfully used by Schmidt et al. (2012) to increase and suppress CORT in song sparrows (*M. melodia*). Our own pilot studies in zebra finches indicate that there were minimal differences in plasma CORT between 25 IU/kg and 100 IU/kg ACTH, so we used the lower dose. For DEX, our pilot studies indicated that a dose of 1000 µg/kg was more effective at suppressing CORT than a dose of 500 µg/kg (unpublished results).

ACTH and DEX challenges were conducted on separate occasions, with two weeks between the challenges. For both ACTH and DEX challenges, a baseline blood sample was collected within 3 min of entering the room where the birds were housed and a stress-induced sample was collected after 30 min of restraint stress prior to injections. Immediately after the second blood sample, birds were injected with ACTH or DEX and then released into individual cages. Post-injection samples were collected 30 min after injections (i.e. 60 min after initial disturbance). Therefore we had CORT samples from three different time points: within 3 min (baseline), 30 min (pre-injection), and 60 min after disturbance (post-injection) from both ACTH and DEX challenges. In total, approximately 100 µL of blood was collected from an individual during each of the challenges. Plasma was separated from all samples and stored at –20 °C until assayed.

Corticosterone assay

Samples from 2011 and 2012 were assayed separately. CORT was quantified in unextracted plasma using a radioimmunoassay (MP Biomedicals, 07-12013) that has been previously validated in zebra finches (Schmidt and Soma, 2008). Plasma was diluted 1:10 with steroid diluent (5 µL plasma + 45 µL diluent) and samples were run in duplicate (50 µL assay volume). Samples were analyzed randomly with respect to treatment in 8 assays. We included a sample from a pool of zebra finch plasma in every assay to measure inter-assay variability. The lowest point on the standard curve was 1.56 pg/tube and the highest point on the standard curve was 250 pg/tube. All samples fell within the range of the standard curve (1.56–250 pg/tube). The intra-assay coefficient of variation was 11.53% for a low control (12.5 pg/tube) and 4.78% for a high control (125 pg/tube). The inter-assay coefficient of variation was 8.62% for the low control and 10.54% for the zebra finch plasma pool.

Statistics

Statistical analyses were conducted using SPSS 20.0 and R. We used linear mixed models with restricted maximum likelihood (REML) to determine the effect of treatment on all our dependent measures (i.e., song and CORT). This analysis is appropriate for our data because we can control for the potential nonindependence of our samples (i.e. relatedness of siblings in each nest) to avoid pseudoreplication. In all of the analyses described below we first tested the significance of the random effect of broods and individuals nested in broods by using maximum likelihood theory to compare fitting of data into a similar model without the random effect. All two-way and higher-order interactions were included in the full model and stepwise deletion of non-significant terms was applied to obtain the most parsimonious model of the data. Pairwise comparisons between treatment groups were adjusted using Sidak corrections. Effect size is given as Cohen's *d* for pairwise comparisons (calculated with an online calculator at <http://www.cognitflexibility.org/etaeffectsize/>) and as eta-squared for linear mixed models (η^2 , calculated using the lsr package in R).

Song learning and vocal performance

We used factor analysis to reduce the number of song variables because many of the variables within each parameter (i.e. complexity, learning accuracy, stereotypy, and output) were correlated. Table 2 shows the results of the principal components analyses (PCA) and their factor loadings for each song parameter. For song complexity, we first obtained residuals from the regression of pupil elements and syllables against tutor elements and syllables (and similarly for unique elements and syllables). PCA of these four residuals extracted one component that explained 75.61% of the total variance. All song measures had high positive loadings on this component. PCA of measures of song learning accuracy also extracted one component that explained 64.01% of the total variance. Proportion of shared transitions and similarity index showed high positive loadings while degree of syllable match showed modest positive loading on this factor. PCA of song stereotypy and output measures also resulted in one component each that explained 55.21% and 65.22% of the total variance, respectively. For both components, measures showed high positive loadings except for % syllables terminating song and latency to sing, which had negative loadings on stereotypy and output, respectively.

To analyze effects of our manipulation on song learning and vocal performance, separate linear mixed models were used with each component obtained from the PCAs as the dependent variable. Group was entered as a fixed effect, with age at time of song recording as a fixed effect covariate. Year of the study (2011 or 2012) was entered as an additional random effect. The total sample size was 44 because we were unable to obtain any songs from one male in the HH group despite multiple recording attempts.

HPA axis function

We calculated three different measures of HPA axis function: baseline CORT, integrated CORT response, and HPA challenge. The measure of baseline CORT consisted of samples collected within 3 min at pre-nutritional independence, post-nutritional independence, and within 3 min (before injections) of the ACTH challenge and DEX challenge. Integrated CORT response was calculated from samples collected at the pre- and post-nutritional independence phases. Integrated CORT response was defined as the area under the curve created by plotting values of the baseline, 15 min, and 30 min samples and provides information on CORT increase and clearance rates (Breuner et al., 1999). Area under the curve was calculated using Prism 5.0 (Graphpad software). HPA challenge was defined as CORT levels in response to ACTH and DEX injections. Maximal adrenal CORT secretion in response to ACTH and the strength of negative feedback in response to DEX were calculated as the difference between the 60 min and 30 min sample (we refer to this difference as ACTH change and DEX change). We also calculated the change in CORT between the 60 min and 30 min samples as a ratio (percentage), but results were similar whether we used the former or

Table 2

Principal components analysis (PCA) for song complexity, learning accuracy, stereotypy, and output.

	% total variance	Factor loadings on extracted component
Song complexity	75.61	# elements (0.928)
		# syllables (0.915)
		# unique elements (0.892)
		# unique syllables (0.728)
Song learning accuracy	64.01	Shared transitions (0.968)
		Similarity index (0.951)
		Syllable match (0.280)
Song stereotypy	55.21	Sequence consistency (0.879)
		Sequence linearity (0.762)
		% Terminating syllables (–0.540)
		Syllable consistency (0.751)
Song output	65.22	Total # songs (0.825)
		Latency to sing (–0.759)
		Max. song bout duration (0.836)

latter method of measuring ACTH and DEX changes. Consequently, in the **Results and Statistics** sections we analyze data using ACTH and DEX differences (not percent change).

Separate linear mixed models were used for baseline CORT, integrated CORT, ACTH change, and DEX change. The binary variable, year of study (2011 or 2012), was entered as an additional random effect for all models. For baseline CORT, baseline values from all sampling sessions (i.e. pre-nutritional independence, post-nutritional independence, ACTH challenge, and DEX challenge) were entered as the dependent variable, with group (HH, HL, LH, LL), sex, and sampling session as fixed effects. For integrated CORT, separate linear mixed models were used for integrated CORT responses at the pre- and post-nutritional independence phases. For both models, integrated CORT (ng/mL per min) was entered as the dependent variable, with group and sex as fixed effects. For HPA challenges, ACTH change and DEX change were entered as the dependent variable, with group (HH, HL, LH, LL) and sex as fixed effects. Sample sizes for ACTH change and DEX change were HH = 6, HL = 10, LH = 9, LL = 8.

Results

Song learning and vocal performance

Nutritional stress during development did not significantly affect any measure of song learning or vocal performance (Fig. 1). Year of study was not a significant random effect in any of the models. There was no significant main effect of treatment group on principal component factors of song complexity ($F(3, 40) = 0.80, p = 0.502, \eta^2 = 0.056$), learning accuracy ($F(3, 40) = 0.36, p = 0.780, \eta^2 = 0.026$), song stereotypy ($F(3, 40) = 0.084, p = 0.968, \eta^2 = 0.0063$), or song output ($F(3, 13.62) = 1.87, p = 0.183, \eta^2 = 0.18$).

HPA axis function

Baseline CORT

Diet manipulations had significant effects on baseline CORT only during development (Fig. 2). Analyses indicated that there was no significant main effect of group (overall means \pm SEM expressed as ng/mL for HH = 5.86 ± 1.32 ; HL = 6.35 ± 1.34 ; LH = 4.98 ± 1.38 ; LL = 7.13 ± 1.39), however, there was a significant main effect of sampling session ($F(3, 179.35) = 14.99, p < 0.001, \eta^2 = 0.11$) and a significant interaction of group \times sampling session ($F(9, 186.76) = 5.61, p < 0.001, \eta^2 = 0.16$). Pairwise comparisons of the interaction indicated that during the pre-nutritional independence phase, HL had significantly lower baseline CORT than LL ($p = 0.037, d = 1.31$), and almost significantly lower baseline CORT than LH ($p = 0.052, d = 1.44$; Fig. 2). During the post-nutritional independence phase, HH had significantly lower baseline CORT than HL ($p = 0.015, d = 2.28$) and LL ($p = 0.029, d = 1.14$; Fig. 2). LH also had significantly lower baseline CORT than HL ($p = 0.017, d = 2.23$) and LL ($p = 0.030, d = 1.12$; Fig. 2). There were no significant group differences between baseline CORT in adulthood (ACTH and DEX baseline samples). This suggests that L treatment increased baseline CORT during both pre- and post-nutritional independence (irrespective of previous nutritional conditions), but these effects did not persist after diet manipulations ended.

Integrated CORT response

Diet manipulations significantly affected integrated CORT responses at the post-nutritional independence phase only (Fig. 3). Analyses of integrated CORT responses at the pre-nutritional independence phase revealed that there were no significant main effects of group or sex ($F(3, 16.93) = 1.53, p = 0.243, \eta^2 = 0.082$ and $F(1, 79.63) = 3.82, p = 0.054, \eta^2 = 0.025$, respectively), or significant interaction of group \times sex ($F(3, 78.31) = 2.12, p = 0.104, \eta^2 = 0.056$; Fig. 3a). Analyses of the integrated CORT responses at the post-nutritional independence phase indicated that there was a significant main effect

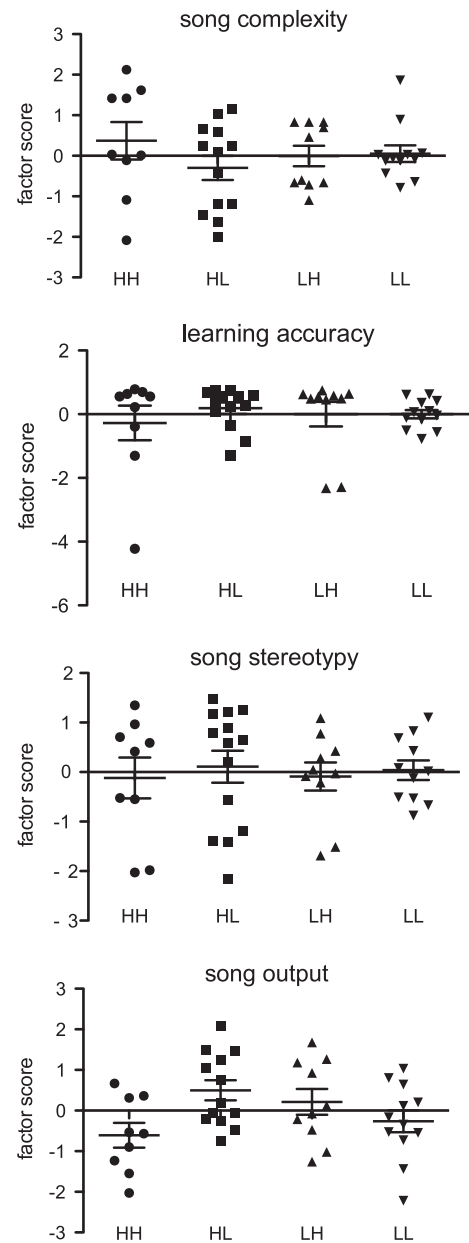


Fig. 1. Nutritional manipulations did not significantly affect measures of song learning and vocal performance. Horizontal lines indicate mean, error bars represent \pm SEM. Points indicate raw individual data (uncorrected for brood size or year of study effects).

of group ($F(3, 78) = 4.32, p = 0.007, \eta^2 = 0.16$), but no significant main effect of sex or interaction of group \times sex. Pairwise comparisons of the significant main effect indicated that the HL group had significantly higher integrated CORT responses than the HH group during the post-nutritional independent phase ($p = 0.005, d = 1.24$; Fig. 3b). This result suggests that L conditions during the post-nutritional independence phase lead to higher rates of CORT secretion in birds that had previously (i.e. in the pre-nutritional independence phase) experienced H conditions.

HPA challenges

Diet manipulations significantly affected CORT concentrations in the ACTH, but not DEX challenge (Fig. 4). Analysis of ACTH change revealed a significant main effect of group ($F(3, 22) = 4.64, p = 0.012, \eta^2 = 0.23$; Fig. 4). Pairwise comparisons indicated that the LL group had a significantly lower change in CORT following ACTH injection than HH and LH ($p = 0.025, d = 1.56$ and $p = 0.033, d = 1.32$,

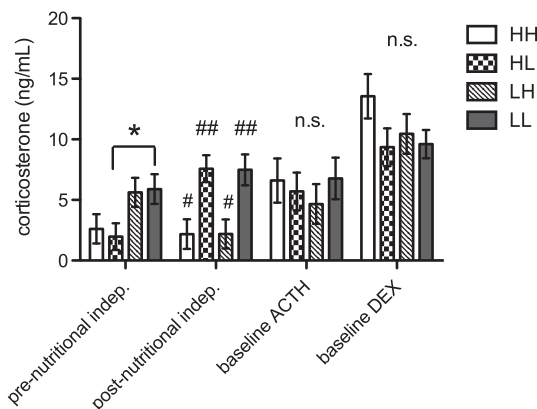


Fig. 2. Interaction of group \times sampling session for baseline CORT. At the pre-nutritional independence phase (PHD 5–34), HL had significantly less CORT than LL ($p < 0.05$; indicated by *). At the post-nutritional independence phase (PHD 35–62) HH had significantly less CORT than LL and HL ($p < 0.01$; indicated by #s). Groups did not significantly differ in baseline CORT when sampled as adults (ACTH and DEX sessions).

respectively). This indicates that LL conditions reduced adrenal cortex sensitivity to ACTH and/or capacity to secrete CORT. DEX change was not significantly affected by diet manipulations (group $F(3, 25) = 0.56$, $p = 0.648$, $\eta^2 = 0.084$; Fig. 4).

Discussion

The current experiment investigated the effects of nutritional stress at different developmental periods on HPA axis function, song learning and vocal performance. Our results indicate that nutritional stress can temporarily elevate baseline CORT, and that switching from H to L

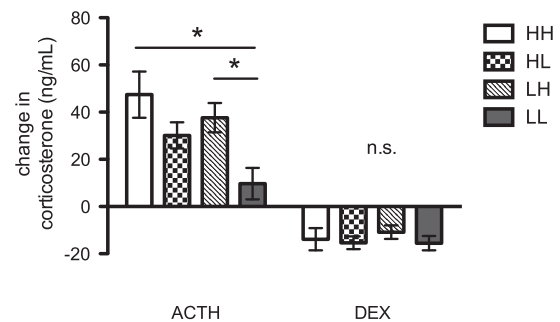


Fig. 4. Main effect of group for HPA axis regulation in response to DEX and ACTH challenges. LL groups produced significantly less CORT compared to HH and LH in response to ACTH injections ($p < 0.05$; indicated by *). However, there were no differences between groups in response to DEX injections.

conditions increased rates of CORT secretion. However, these increases did not appear to affect song learning or vocal performance. Furthermore, there was no difference between early and juvenile nutritional stress on song learning or vocal performance, even though the nutritional stress throughout early and juvenile development had long-term effects on adult HPA axis regulation.

Our study provides a comprehensive analysis of how developmental stress can affect zebra finch song, and is unique and informative for three reasons. First, it determines whether song can be affected by nutritional stress after nutritional independence, when zebra finch neural and behavioral development of song is still occurring — previous studies that directly manipulated nutrition have not extended treatment past nutritional independence (see Table 1). Second, we have measured song stereotypy, which is an aspect of song that has previously not been examined in studies of developmental stress in zebra finches. Third, we have various song measures for each bird. This gives a clearer picture of how developmental stress can affect song as a whole, which complements existing research on how developmental stress can affect components of song. Moreover, because we manipulated nutritional conditions, our study is a more direct test of the effects of nutritional stress on song and HPA function compared to other studies that have used brood size or CORT manipulations.

Surprisingly nutritional stress during development did not significantly affect any measures of song learning or vocal performance used in the current experiment, which is not consistent with the developmental stress hypothesis that song is an honest indicator of developmental history (Nowicki and Searcy, 2004; Nowicki et al., 1998, 2002). We conducted a sensitivity power analysis using G*Power 3.0 software (Faul et al., 2007) which indicated that our sample size would have allowed us to detect treatment effects on song with 95% certainty if there was a large effect (Cohen's $f = 0.656$) for any of the dependent measures. Consequently, if our treatment had any effect on song it would have to have been a small or moderate effect size. Next, we computed Cohen's f for each of the song measurements according to Chen and Chen (2010). Cohen's f estimates a standardized average effect size across all the levels of an independent variable. This value ranged between values considered small to medium (0.10–0.25; Chen and Chen, 2010) for all of our song measures: song complexity (0.17), learning accuracy (0.18), song stereotypy (0.16), and song output (0.19). Combined, these analyses suggest that the null results we observed are not due to inability to detect a moderate to strong effect given our sample size.

In zebra finches, experimental studies on whether developmental stress affects song have yielded inconsistent results (Table 1). In a previous study, both food restriction and artificial elevation of CORT in nestlings until PHD 30 reduced song complexity and HVC volume (Buchanan et al., 2004; Spencer et al., 2003). The same food restriction manipulation was used by Zann and Cash (2008) and Brumm et al. (2009). Like Spencer et al. (2003), Zann and Cash (2008) also found

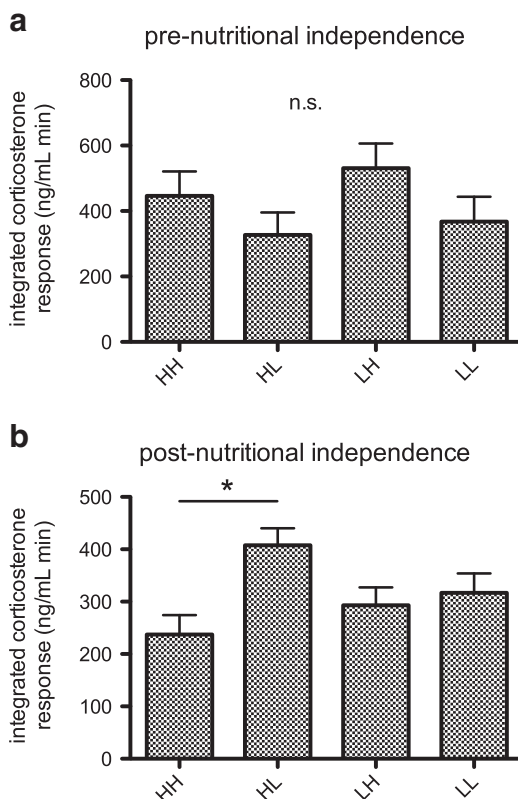


Fig. 3. Main effect of group for integrated CORT responses during the pre-nutritional independence phase (a) and post-nutritional independence phase (b). Diet manipulations affected integrated CORT responses during the post-nutritional independence phase, with the HL group showing greater responses than the HH group ($p = 0.005$).

that stress reduced song complexity. At the same time, they reported that stress had no effect on a measure of learning accuracy. However, Brumm et al. (2009) reported that stress had no effect on song complexity or song amplitude, but reduced the accuracy of syllable sequence matching. Reduced syntax matching but no effect on song complexity was also reported by Holveck et al. (2008), who used differences in brood size as the developmental stress manipulation. Gil et al. (2006) also manipulated brood size, but found that it had no effect on song complexity, learning accuracy, or volumes of song control nuclei HVC, RA and LMAN (lateral part of nucleus mesencephalicus anterioris).

Effects of developmental stress on song rate are just as perplexing, as song rate was not affected by diet quality in two experiments (Birkhead et al., 1999; Spencer et al., 2003), but was increased by both enlargement and reduction of brood sizes in two separate experiments (de Kogel and Pijns, 1996; Tschirren et al., 2009). Even current nutritional conditions have been reported to affect song rate (Ritschard and Brumm, 2012). In addition to replicating Gil et al.'s (2006) report of a lack of effect of developmental stress before nutritional independence on song, our findings indicate that nutritional stress after nutritional independence, when song development is still occurring, also may not affect song learning or vocal performance. We acknowledge that the age that we chose to manipulate juvenile stress corresponded to an age where there was an overlap between the sensory and sensorimotor phase, thus effects of juvenile stress on song performance might have been clearer if stress was experienced later in development (i.e. PHD 80–90), such as the one used by Pytte and Suthers (2000). In light of the inconsistencies in the literature mentioned above, more studies are almost certainly needed to confirm these findings.

Collectively, it seems that the effect of developmental stress on zebra finch song are highly variable — even though identical nutritional manipulations were used by Spencer et al. (2003), Zann and Cash (2008), Brumm et al. (2009), and in the present study, the results still varied considerably between the experiments. In particular, the birds in the LH group experienced conditions in the same order and similar duration compared to the experimental groups of the other three studies. Despite this, the effects of nutritional stress in this group still do not match those of Spencer et al. (2003), Zann and Cash (2008), or Brumm et al. (2009). It is possible that the variation in song is caused by individual differences in strategies used to respond to poor nutritional conditions during development. This idea is supported by Bolund et al.'s (2010) findings that the mass of offspring zebra finches at PHD 8 was not significantly correlated with adult song rate, song complexity, or song length.

The inconsistent effects of developmental stress on zebra finch song do not discredit the hypothesis that song may be an accurate indicator of developmental aspects of male quality in other species (Nowicki and Searcy, 2004; Nowicki et al., 1998, 2002). Instead, it suggests that the reliability of song as an indicator may be species-specific due to the way song is used. Zebra finch courtship involves a combination of song and displays from the male. Song and courtship displays are produced in close proximity to females, which allow them to visually assess other traits such as beak color and chest symmetry in addition to song characteristics (Collins et al., 1994; Swaddle and Cuthill, 1994; Zann, 1996). As females likely assess many traits in order to gain information about multiple aspects of male quality (Keagy et al., 2012), male song may be a supplementary, less important factor in mate assessment. In fact, females seem to show stronger preference for redder beak color than high song rate, and these two traits are not always correlated (Birkhead et al., 1998; Collins et al., 1994; Simons and Verhulst, 2011). In the absence of visual cues, however, female zebra finches may turn to using song rate, structure, and complexity, which may explain why they can show preference for these aspects of song (Riebel, 2009).

Song repertoire size of song sparrows appears to be a much more reliable indicator of male quality (correlating positively with body condition, survival and reproduction, and cognitive abilities) and is reduced in birds that experience developmental stress (e.g. MacDonald

et al., 2006; MacDougall-Shackleton et al., 2009; Pfaff et al., 2007; Schmidt et al., 2013; Sewall et al., 2013). This could be because song sparrows' songs are broadcast over long distances. Consequently, male song should contain enough information to offset the costs for a receiver journeying to investigate a male in his territory. The more robust effects of developmental stress on song repertoire size in song sparrows may also indicate that sexual selection of song characteristics is greater for wild or first-generation hand-reared song sparrow than for domesticated zebra finches. Indeed, the relaxed sexual selection pressures on domesticated zebra finches may have reduced the strength of the relationship between song and signaler quality, leading to large individual differences in how song development is affected by developmental stress (perhaps especially nutritional stress, as discussed earlier). Moreover, behavioral variation between domesticated populations may subsequently contribute to the inconsistent results observed by different research groups (e.g. Crabbe, 1999; Wahlsten et al., 2003).

Elevated baseline CORT in response to nutritional stress during development has been reported by some (e.g. Honarmand et al., 2010), but not all previous studies (e.g. Costantini, 2010; Spencer et al., 2003). In the present study, the L treatment increased baseline CORT during development, which suggests that our manipulation was indeed perceived as stressful. The body mass results support the claim that our manipulation was stressful because birds that experienced the H treatment tended to be heavier than birds that experienced the L treatment (Fig. 5). We also found that L conditions during the post-nutritional independence phase increased integrated CORT response of the HL group. However, even though L treatment effectively altered CORT secretion during development, it seemed to have limited long-term effects on song and HPA function. Specifically, elevated CORT during development did not seem to affect song in adulthood, and only the LL group that experienced persistent developmental stress showed altered adult HPA regulation (i.e. birds in the LL group secreted significantly less CORT in response to adrenal stimulation by ACTH). Chronic stress can decrease HPA axis reactivity to ACTH by down-regulating adrenal ACTH receptors, or diminishing capacity of adrenals to produce CORT (Rich and Romero, 2005), and either of these may have occurred in our study. The fact that there was no difference between groups in response to DEX suggests that our treatment did not affect negative feedback mechanisms, such as altered neural glucocorticoid receptors. The observed decreased response to ACTH observed in our and other studies likely helps to mitigate the detrimental effects of constantly elevated plasma CORT. In summary, our results suggest that prolonged nutritional stress during development can have programming effects on HPA axis function, but further work is required to determine which HPA axis regulatory mechanisms are affected.

Conclusions

In conclusion, the effects of developmental stress on zebra finch song are highly variable depending on the type of stressor, timing of stress, and the parameters used to define song learning and/or vocal performance. Our results suggest that nutritional stressors that have short-term effects on HPA axis function may not affect song development in zebra finches. Moreover, even though adult HPA axis function was affected if nutritional stress was experienced throughout development (PHD 5–62), this was not reflected in adult song characteristics. This indicates that adult song quality may not accurately reflect HPA axis development and function in this species.

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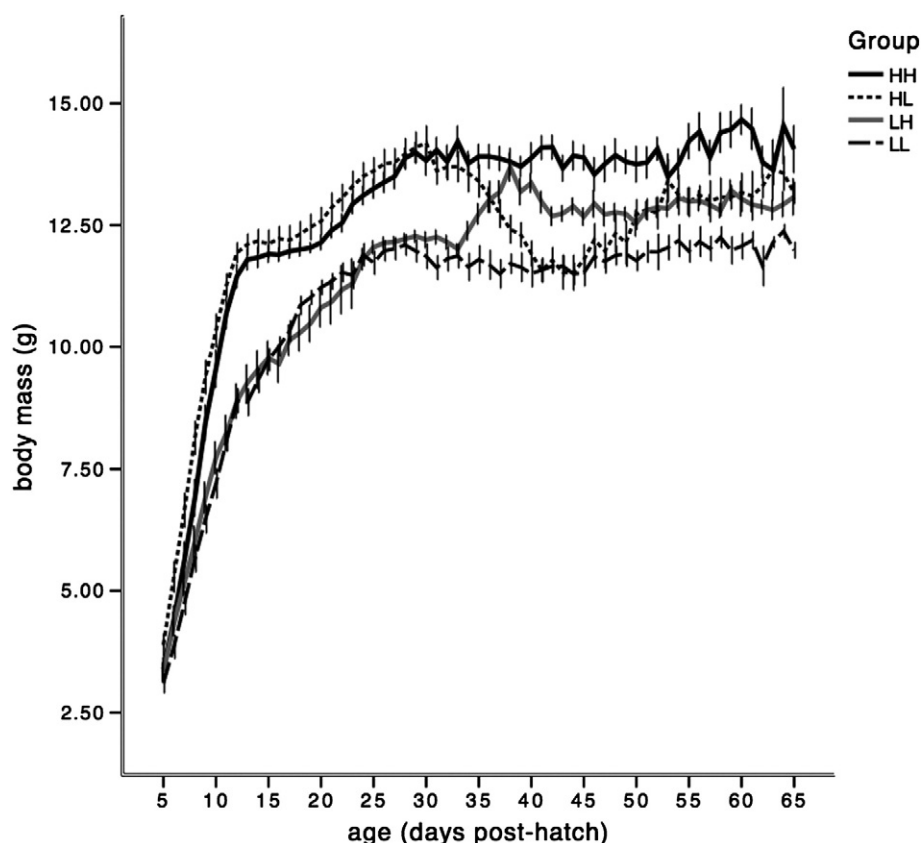


Fig. 5. Daily change in body mass of the four nutritional treatment groups. The pre-nutritional independence phase was from PHD 5–35 and the post-nutritional independence phase was from PHD 36–62. L treatment coincided with lower body mass compared to H treatment. Error bars indicate \pm SEM.

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