# Early Life Conditions that Impact Song Learning in Male Zebra Finches also Impact Neural and Behavioral Responses to Song in Females

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**ABSTRACT:** **Early life stressors can impair song in songbirds by negatively impacting brain development and subsequent learning. Even in species in which only males sing, early life stressors might also impact female behavior and its underlying neural mechanisms, but fewer studies have examined this possibility. We manipulated brood size in zebra finches to simultaneously examine the effects of developmental stress on male song learning and female behavioral and neural response to song. Although adult male HVC volume was unaffected, we found that males from larger broods imitated tutor song less accurately. In females, early condition did not affect the direction of song preference: all females preferred tutor song over unfamiliar song in an operant test. However, treatment did affect the magnitude of behavioral response to song: females from larger broods responded less during song preference trials. This difference in activity level did not reflect boldness per se, as a separate measure of this trait did not differ with brood size. Additionally, in females we found a treatment effect on expression of the immediate early gene ZENK in response to tutor song in brain regions involved in song perception (dNCM) and social motivation (LSc.vl, BSTm, TnA), but not in a region implicated in song memory (CMM). These results are consistent with the hypothesis that developmental stressors that impair song learning in male zebra finches also influence perceptual and/or motivational processes in females. However, our results suggest that the learning of tutor song by females is robust to disturbance by developmental stress.** Develop Neurobiol 00: 000–000, 2018

***Keywords:*** **immediate early gene; song learning; song preference; social behavior network; auditory forebrain**

## INTRODUCTION

The developmental stress hypothesis proposes a mechanism by which variation in male song might influence female mate choice in songbirds (Nowicki et al., 1998, 2002a). Because early life stressors can impair brain development and song learning, the

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hypothesis suggests, song quality should reflect early life conditions that might also affect the singer's quality as a potential mate. The idea that developmental stress affects male song has largely been supported: early life stressors impair male song quality (Nowicki et al., 2000; Buchanan et al., 2003; Spencer et al., 2003, 2004, 2005; Soma et al., 2006; Zann and Cash, 2007; Schmidt et al., 2013b), the accuracy of song learning (Nowicki et al., 2002a; Buchanan et al., 2003; Holveck et al., 2008; Brumm et al., 2009; though see Kriengwatana et al., 2014; Crino and Breuner, 2015), and in some cases the volume of the brain nuclei that regulate song acquisition and production (Nowicki et al., 2002a; Buchanan et al., 2004; MacDonald et al., 2006; but see Gil et al., 2006).

Evidence suggests that female songbirds choose their mates based in part on features of the learned songs produced by conspecific males. Females of various species have been found to prefer longer songs (Eens et al., 1991; Wasserman and Cigliano, 1991), more complex songs (Clayton and Pröve, 1989; Spencer et al., 2005), larger song repertoires (Searcy, 1984; Hasselquist et al., 1996), and songs that were copied more accurately from tutors (Nowicki et al., 2002b). Females also have been shown to prefer songs similar to those they heard early in life (Clayton, 1990; Nagle and Kreutzer, 1997; Riebel, 2000; Hernandez and MacDougall-Shackleton, 2004; Hernandez et al., 2009; Anderson et al., 2014; Lachlan et al., 2014), suggesting that females learn songs when young and reference those memories when making mate choice decisions (Catchpole, 1980; Wasserman and Cigliano, 1991; Gentner and Hulse, 2000; Gil and Gahr, 2002; Riebel et al., 2002; Riebel, 2003). Thus, female songbirds likely undergo a learning process that could be impacted by developmental conditions, ultimately influencing their mate choice behavior (MacDonald et al., 2006; Riebel et al., 2009; Woodgate et al., 2010; Schmidt et al., 2013; Farrell et al., 2015).

Studying the mechanisms underlying song preferences in female birds is critical for predicting the strength and direction of female mate choice and the resulting sexual selection on male traits (Riebel et al., 2009). If stress experienced early in life impairs female brain development and behavior, as it does in males, preferences for particular song traits could be dampened and the strength of sexual selection on male song and on song learning correspondingly weakened (Endler and Basolo, 1998; Riebel et al., 2009). Adult female zebra finches (*Taeniopygia guttata*) show strong, repeatable preferences for songs similar to those they heard early in development (Clayton, 1990; Riebel, 2000). We therefore examined the impact of developmental stress on female finches' responses to song and on brain activation associated with these responses.

Several previous studies have examined effects of developmental stress on the direction and strength of female preference for male song using operant conditioning assays, with mixed results. Farrell et al. (2015) found that developmental stress had no impact on preference for song length in female European Starlings (*Sternus vulgaris*). Similarly, Riebel et al. (2009) reported that developmental stress had no effect on the direction of female zebra finches' preference for tutor song relative to unfamiliar song (i.e., females preferred tutor song over unfamiliar song regardless of nutritional history). The same study found that developmentally stressed females have a lower overall strength of preference. Two other studies testing preferences based on song complexity rather than familiarity, however, found no impact of developmental stress on the direction or strength of female preference (Woodgate et al., 2011; Schmidt et al., 2013).

Fewer studies have directly examined the impact of developmental stress on the female songbird brain. Developmental stress has been shown to reduce the volumes of some brain regions, including the HVC (proper name) and hippocampus, in both female and male birds (Pravosudov et al., 2005; MacDonald et al., 2006). Neither of these brain regions, however, is thought to play a role in female perception, memory, or preference for conspecific song (Brenowitz, 1991; Chew et al., 1996; Del Negro et al., 1998; MacDougall-Shackleton et al., 1998; Bolhuis and Gahr, 2006). Rather, two regions of the auditory forebrain, the caudal medial nidopallium (NCM) and the caudal medial mesopallium (CMM), are thought to regulate the perception of song quality and the formation and storage of song memory in female songbirds (Bolhuis and Gahr, 2006; Terpstra et al., 2006). Though both sub-regions of the auditory forebrain respond selectively to song over tones or silence, NCM may be especially responsive to variation in song quality and novelty, with dorsal NCM (dNCM) and ventral NCM (vNCM) showing differential response to features such as song duration (Gentner et al., 2001; McKenzie et al., 2006; Terpstra et al., 2006). In contrast, CMM is reported to respond more selectively to familiar songs and is proposed by some researchers to represent the neural substrate of song memory in female birds (Ribeiro et al., 1998; Eda-Fujiwara et al., 2003; Gentner and Margoliash, 2003;

Gentner et al., 2004; Leitner et al., 2005; Terpstra et al., 2006). Additionally, several nodes of the social behavior network, a highly conserved and interconnected group of brain regions involved in regulating social behaviors such as flocking and mate choice (Goodson, 2005, 2008), show selective response to conspecific song and could therefore be involved in regulating female preferences (Maney et al., 2008). One study examining the effects of developmental stress on neural response to song in NCM and CMM found that stressed females showed equal neural activation (measured as immediate early gene expression) in response to heterospecific and conspecific song, in contrast to reduced neural activation in response to heterospecific song as seen in controls (Schmidt et al., 2013). A second study found that developmentally stressed females had lower neural activation (again measured as immediate early gene expression) throughout the auditory forebrain, in response to unfamiliar conspecific song, compared to controls (Farrell et al., 2015). To our knowledge, however, no study has yet examined the effects of developmental stress on neural responsiveness to tutor song in female songbirds nor have neural responses in the social behavior network been examined in the context of developmental stress. We examined the effects of developmental stress on the brains and behavior of both male and female zebra finches in the same experimental treatment, in order to explore whether conditions that negatively impact male song learning also impact female birds' neural and behavioral responses to tutor song.

## MATERIALS AND METHODS

We examined the effects of developmental stress on the behavior and brains of both male and female zebra finches by manipulating brood size. Breeding pairs of zebra finches do not fully compensate for the nutritional needs of larger broods (Naguib et al., 2004), and we chose brood sizes such that our experimental parameters remained within the natural range of early conditions and ecologically relevant variation in phenotypic outcomes (Naguib et al., 2004). Once birds reached adulthood, we assessed boldness by measuring latency to approach a novel object in a food cup, to test for differences in behavioral phenotype as a function of brood size. Then, to test the effects of early rearing conditions on males, we measured the similarity of subjects' adult songs to those of their tutors and the stereotypy (similarity across renditions) of their song production. We then collected their brains and measured the volumes of the song control nucleus HVC and the telencephalon. To test for the effects of early rearing conditions on female song preferences at maturity, we presented each female a choice between tutor song and unfamiliar song in an operant test. To resolve how perceptual and motivational processes might contribute to differences in female responses to song at maturity, we re-exposed them to playback of tutor song and measured immediate early gene immunoreactivity (ZENK-ir) in three subdivisions of the auditory forebrain that are involved in song processing, in four regions of the social behavior network that are implicated in pair bonding and response to song, and in a control brain region, the hippocampus (HP). We exposed all females to their tutors' song so that both control and stressed females experienced the same treatment, for two reasons: (1) to optimize the chances of detecting differences across treatments, and (2) because prior work already documented the responsiveness (quantified as the expression of the immediate early gene ZENK) of the brain regions we examined to conspecific song relative to silence, tones, or heterospecific song (Terpstra et al., 2006; Maney et al., 2008; Schmidt et al., 2013).

### Subjects

The original research reported herein was performed under guidelines established by Duke University IACUC (A290-10-11). A total of 64 zebra finches were reared by 15 zebra finch pairs housed in a single room in the Duke University Biological Sciences Animal Facility. When housed with a live male tutor, young zebra finches (of both sexes) preferentially learn his song over those of other males, even if those other males are singing nearby, making such group housing appropriate for this experimental design (Riebel et al., 2002). We housed breeding pairs and families in individual cages (46 × 23 × 25 cm) and held them on a 16L:8D photoperiod at ∼25°C with *ad libitum* access to food, water, and grit. Nestlings were moved among synchronously breeding pairs within 4 days of hatching (except in two cases when fostering occurred 6 days after hatching) to create two rearing treatments: 34 birds were reared in enlarged broods of 5–7 nestlings (average of 5.67, standard deviation of 0.82) and 30 birds were reared in reduced or un-manipulated broods of 1–4 nestlings (average of 3.00, standard deviation of 1.00). On average, two nestlings (standard deviation of 0.866) were moved into enlarged nests or removed from reduced nests. Moving nestlings among nests at days 4–6 should not affect song learning because the sensory phase of song learning does not begin until day 25 in zebra finches (Immelmann, 1969). Of the experimentally reared birds, 20 females and 14 males were reared in large broods and 14 females and 16 males were reared in small broods.

Previous studies (Naguib et al., 2004) and our data (see "Results," Fig. 3) show that birds from larger broods gain mass more slowly and weigh less at independence, indicating that they experience some nutritional limitation, in addition to the possible stressors associated with crowding and sibling competition. We weighed experimental birds to the nearest tenth of a gram approximately twice a week until birds reached independence and took morphological measures to assess body condition at 50–60 days post-hatch. We separated young from their family groups and housed them in sex-specific, mixed treatment groups when fledglings were ~50 days post-hatch (near the end of the sensory period of song learning).

## Assessment of Boldness

The day prior to the start of boldness testing, we moved birds into individual cages (46 × 23 × 25 cm) in a separate behavioral testing room such that they were visually but not acoustically isolated from other birds. The next morning at 08:00 each subject had its food removed until the assay began at 14:00. To assess behavioral phenotype (or "personality"), we presented each bird with a novel food cup in the center of its cage and recorded the latency in seconds for the bird to eat from the novel cup (Sewall et al., 2013). These novel cups were 5 cm in diameter and had three brightly colored plastic disks (2.5 cm diameter red, blue, and green) affixed to the sides. After a 5-min time out, birds were presented with their familiar cup (which had no plastic disks), again placed in the middle of the cage, and the latency to eat was recorded. The difference in latency to eat from the novel cup relative to the familiar cup was used as a measure of boldness. Birds were returned to their tutor flocks as soon as this single-trial assay was completed.

## Male Song Recording and Analysis

We recorded songs from all experimental male birds (after 120 days of age) and their tutors (the actual or foster father) by temporarily housing them in individual cages within sound attenuation chambers (IAC acoustics, Bronx, NY). We captured spontaneously produced songs using cardioid microphones (Shure SM57) and Sound Analysis Pro (SAP) bioacoustics recording software (Tchernichovski and Mitra, 2001). We compared spectrograms of the songs of sons to those of their tutors in order to assess song imitation.

To compare the songs of experimental males with those of their tutors, one experienced researcher who was blind to treatment (J. Soha) selected single motifs from 5–6 (average of 5.7 ± 0.2 standard error) songs of each tutor and from 3–6 (average of 5.7 ± 0.1 standard error) songs of each son. We always selected the most common motif from tutors and, from sons, a motif that best matched (by eye) the tutor's motif. One experimental bird (small brood) was omitted because we were unable to record sufficient song for analysis. We normalized all motif samples to the same RMS (root mean square) amplitude using Signal sound analysis software (version 5.05.03, Engineering Design, Berkeley, CA). We then compared tutor and son motifs using the batch similarity process (with asymmetric comparison) in SAP to examine how much of the tutor's song was represented in each son's song (Tchernichovski and Mitra, 2001). We set the amplitude at 21.6 dB and all other settings at default. This analysis produced three measures: (1) *Percent Similarity* is the percentage of the tutor song that is matched in pupil song based on significant similarity in five acoustic features (pitch, FM, AM, Wiener entropy, and goodness of pitch) assessed over 70-ms intervals, (2) *Accuracy* is the frame-by-frame similarity of these matching segments, and (3) *Sequential Match* compares the sequences of the matching segments in the tutor and pupil songs. Additionally, to assess the stereotypy with which experimental birds produced their songs, we selected five motifs from each bird in a within-subject analysis. Each motif came from a different recording; two recordings were from one day and three were from another, and same-day recordings were typically separated by at least 10 min. We compared the five motifs using the batch similarity process (with symmetric comparisons) in SAP, then averaged the results from all pairwise comparisons ($N = 10$) for each bird to generate two measures of stereotypy for each experimental subject: within-individual Percent Similarity and within-individual Accuracy.

## Male Brain Collection and Histology

To examine the effects of rearing conditions on the brains of male birds, we euthanized birds with an overdose of sodium pentobarbital after all song recording was completed (ca. 300 days post-hatch). We rapidly removed the brain, separated the hemispheres, flash froze the tissue on dry ice and stored all tissue at −80°C until processing. We sectioned one hemisphere from each bird in the sagittal plane at 40 $\mu$m using a cryostat (set to −22°C), alternating hemispheres between subjects and across treatments. We collected frozen tissue sections on glass microscope slides (superfrost plus, Fischer) and Nissl stained every third section. Specifically, we immersed the frozen slides in 4% paraformaldehyde and then serially dehydrated the tissue in ethanol before staining with thionin, removing excess stain in an ethanol wash, clearing the tissue in xylenes, and affixing coverslips. We captured images of the entire telencephalon (0.63× objective) and of song control nucleus HVC (6.3× objective) using an Olympus MVX 10 microscope connected to a DP 71 camera (Olympus) and DP Controller software (Olympus) and referencing an avian brain atlas (Nixdorf-Bergweiler and Bischof, 2007). We used Fiji software (Schindelin et al., 2012) to trace the boundaries and calculate the area of the telencephalon and song control nucleus HVC in each photomicrograph in which the brain region of interest was present. We calculated the volume of each brain region (in $mm^3$) by summing each area measurement multiplied by the distance between measured sections. We then calculated the relative volume of HVC as a proportion of the volume of the telencephalon for each subject.

## Female Operant Training and Testing

We tested females for song preferences at adulthood (ca. 120 days post-hatch) using an operant assay that takes advantage of the reinforcing qualities of song and that has been used successfully with female zebra finches in previous studies (Riebel, 2000; Riebel et al., 2002). We first trained the females to hop on perches to trigger song playback (either heterospecific or conspecific song) and then tested their preference (described below) for their tutor's song relative to the song of an unfamiliar male. We followed established methods for determining song preference by taking the total number of hops in response to tutor song divided by the total number of hops on both perches (referred to as "preference level" herein; (Collins, 1999; Riebel, 2000; Riebel et al., 2002; Leitão et al., 2006).

The day prior to the start of training, we moved females into individual cages (46 × 23 × 25 cm) inside individual sound attenuation chambers with a 16L:8D light cycle. At this time, we removed all regular perches and installed two operant perches and one regular perch. We placed these perches (8 cm long and 8 cm high) 19 cm apart and equidistant from an audio speaker (Altec Lansing iM-237 Orbit) located above the cage. Landing on an operant perch triggered playback of one song at an amplitude of 76 ± 2 dB SPL (re 20 $\mu$Pa at 1 m) measured with a RadioShack 33–2055 sound level meter, fast response, A-weighting. No additional song was played as long as the female remained perched; a female had to leave a perch and hop onto it again to trigger another song playback. A computer running SAP software automated the operant training by logging perch hops and playing song stimuli.

Training sessions occurred each day from 04:00 to 18:00 h. During this time, landing on one perch triggered playback of an unfamiliar male zebra finch's song ("unfamiliar song"), while landing on the other perch triggered playback of a heterospecific song (song sparrow, *Melospiza melodia*, recorded in the Nowicki laboratory from wild-caught birds). Each female heard a novel combination of unfamiliar song and heterospecific song on each training day. We switched the perch that triggered unfamiliar song from one side to the other at the beginning of each day. Half of the females began training with unfamiliar song on the left perch and half began with heterospecific song on the left perch. We trained all females for at least two days. A bird finished training once it hopped on both operant perches on two consecutive days. This ensured that each bird had an opportunity to learn that both perches triggered song and that the song treatments switched between the perches. Of the 34 experimental females, 26 (14 from large broods and 12 from small) progressed through training (requiring 2.96 ± 3 days).

After training, we tested the females' responses to their tutor's song relative to unfamiliar male songs during four test days (04:00–18:00 h). Tutor's songs were recorded as described above, and unfamiliar songs were drawn from a pool of songs obtained from the laboratory of Dr. Heather Williams at Williams College. Each female received unique sets of training songs and test songs that were matched for duration, and no female heard the same unfamiliar song during training and test periods. For each female, we paired her tutor's song with four unfamiliar songs matched for song duration. Each female heard four dyads of stimuli, a new dyad each day, with the song treatments switched between perches each day.

## Female Song Re-Exposure and Immunohistochemistry

To assess the effects of rearing conditions on brain activation in response to song in females, we exposed subjects to playback of tutor song before collecting their brains to quantify the expression of the immediate early gene ZENK (the protein product of egr-1) in eight brain regions. Specifically, we transferred each female to an individual sound attenuation chamber just prior to lights off the day before playback was started. Beginning two hours after lights on the next morning, we played females 30 min of recorded tutor songs, repeated at a rate of six songs per min. Playback was staggered over the day (08:00–14:00 h) to allow us to collect up to 14 brains per day over 3 days, with treatment and tutor group balanced across days. We held females in silence for 60 min after the end of playback before deeply anesthetizing them with sodium pentobarbital, transcardially perfusing them with PBS followed by 4% paraformaldehyde, and collecting their brains. We separated the two hemispheres and immersion fixed the brains in paraformaldehyde for 24 h, saturated them in 30% sucrose until they sank (ca. 48 h) and flash froze them on dry ice. All tissue was stored at −80°C. We sectioned one brain hemisphere in the sagittal plane and one in the coronal plane at 40 $\mu$m and collected the tissue as floating sections, alternating which hemisphere was sectioned in each plane among subjects to balance treatments and tutors.

We carried out immunohistochemistry for ZENK in every third section from both hemispheres following established protocols for application in bird tissue (Sockman et al., 2002; Sewall and Davies, 2017). Briefly, we blocked the tissue in 1% hydrogen peroxide followed by 2% normal goat serum and an Aviden Biotin blocking kit (Vector Labs, SP-2001, Burlingame, CA) before incubating in Egr-1 primary antibody (raised in rabbit, Santa Cruz Biotechnology, SC-189, Dallas, TX) at a ratio of 1–8,000 for 48 h at 4°C. Then, after washing the tissue in PBS with triton, we incubated in biotinylated secondary goat anti-rabbit IgG antibody (Vector Labs, BA-1000) at a ratio of 1–250 for 1 h. We visualized the reaction using a aviden-biotin horseradish-peroxidase and DAB peroxidase kit (Vectastain ABC, Elite Kit, Vector PK-6100; Vector SK-4100), washed and float mounted the tissue on coated microscope slides, and cover slipped after a serial dehydration in ethanol and clearing with xylenes. We processed the sagittal and coronal sections in two separate batches, with treatment and tutor balanced among immunohistochemistry batches.

In sagittal sections, we examined ZENK-ir in three subdivisions of the auditory forebrain that are involved in song processing, are selectively activated by song playback, and show different responses to subtle aspects of song: vNCM, dNCM, and CMM (Mello et al., 1992; Mello and Ribeiro, 1998; Gentner et al., 2001; Leitner et al., 2005; Woolley and Doupe, 2008; Schmidt et al., 2013). In coronal sections, we examined four regions of the social behavior network that are implicated in social motivation, pair bonding, and response to song by female birds: nucleus taeniae of the amygdala (TnA), the ventrolateral subdivision of the caudal

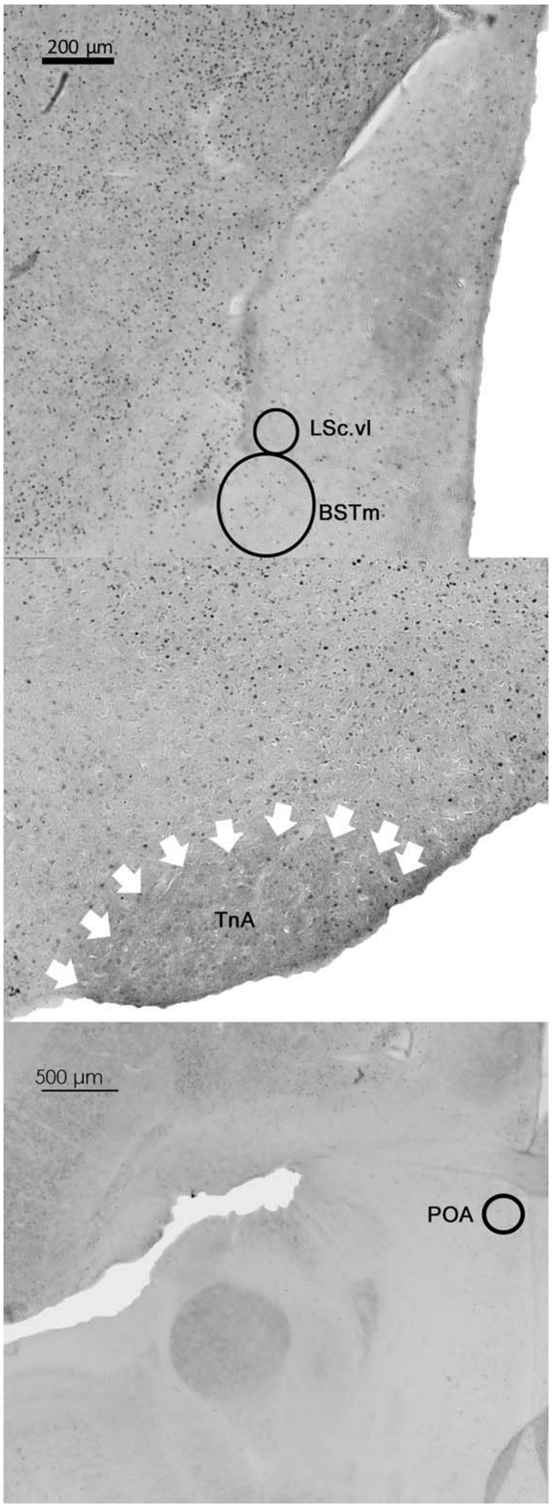

**Figure 1** Placement of counting frames within the LSc.vl, BSTm, TnA, and POM. See Introduction for abbreviations.

lateral septum (LSc.vl), the medial bed nucleus of the stria terminalis (BSTm) and the medial preoptic area (POM; Goodson, 2005, 2008; Maney et al., 2008), and a control brain region (HP). All brain regions except POM and HP are reported to show selective responses to conspecific song over silence or tones, as measured by ZENK expression (Terpstra et al., 2006; Maney et al., 2008).

All quantification of immunoreactivity for ZENK was carried out by a research assistant (S. Campbell) blind to the experimental condition of each subject. We captured gray scale images of each region using an AxioCam MR camera attached to a Zeiss Axioimager microscope (Zeiss). We imaged all auditory forebrain regions using the 20× objective (200× total magnification), and social behavior network regions using the 10× objective (100× magnification), except for the POM, which was imaged with the 5× objective (50× magnification). The control region, HP, was imaged with the 2.5× objective (25× magnification).

We located the social behavior network regions following Maney et al. (2008) and references therein (see also Sewall and Davies, 2017). Specifically, to identify BSTm, we followed Aste et al. (1998) and Maney et al. (2008) and placed a 0.2-mm$^2$ counting circle dorsal to the anterior commissure in two tissue sections. We used Cheng et al. (1999) and Stokes et al. (1974) to locate TnA and traced the entire boundary in three sections (Fig. 1). For LSc.vl, we referenced Goodson et al. (2004) and placed a 0.03-mm$^2$ counting circle medial to the lateral ventricle and beginning rostrally at the level of the anterior commissure in two tissue sections. We located the POM with reference to Alger and Riters (2006) and quantified the region medial to the septomesencephalic tract by placing a 0.2-mm$^2$ counting circle in three tissue sections. As a control region we placed a 0.13-mm$^2$ circular counting frame in the ventral subdivision of the HP in two tissue sections.

In every region, we used an automated routine in Image J software (ver. 3.1, National Institutes of Health) to count immunoreactive cells that were visible within the counting frame or traced region and were above a set threshold value (selected as 110 based on comparison with manual counts). Quantification for CMM, dNCM and vNCM were made within the image area, with counting frames (400 × 300 $\mu$m) placed as described in Sockman et al. (2002) and measurements summed across the six most medial tissue sections (Fig. 2). All data were calculated as the total whole number of immunoreactive cells per mm$^2$, to standardize counts regardless of the size of the counting frame or number of tissue sections sampled.

## Statistical Analysis

To assess treatment effects on mass, boldness, song learning by males, brain volumes in males, and song perception in females, we used separate generalized linear mixed effects models (GLMMs). Specifically, we examined the response variables of mass, boldness, each measure of song learning, HVC volume relative to telencephalon volume, female preference for tutor song relative to unfamiliar song, and female activity (number of hops) during operant tests, including brood identity (the family in which a subject was reared) as a random factor. To analyze the treatment effects on ZENK expression across brain regions in

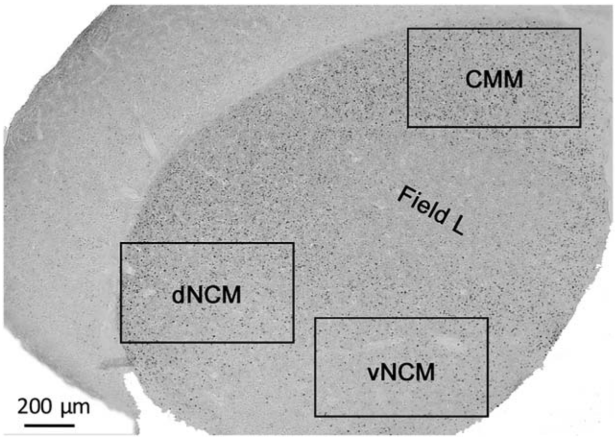

**Figure 2** Placement of counting frames to quantify ZENK immunoreactivity within three subregions of the auditory forebrain of adult female zebra finches.

females, we conducted a single GLMM with treatment and brain region coded as fixed factors, mass at fledging as a covariate, and individual coded as a random factor to account for non-independence of cell counts (Crawley, 2007; Bolker et al., 2009). We specified a Poisson distribution for all count data (number of hops in operant assays and cell counts) based on model residual deviance (Crawley, 2007). All statistical analyses were conducted using R statistical software (R Development Core Team, 2013) with the packages GLMER and LME4 (Bates et al., 2015).

## RESULTS

### Body Mass and Boldness

At independence (ca. 25 days post-hatch), birds reared in larger broods had lower mass (mean: 11.8 g, range: 9.4–13.5 g) than those in smaller broods (mean: 12.7 g, range: 10.8–14.6 g; GLMM, effect of treatment, $t = 2.522$, $P = 0.015$; Fig. 3). However, birds subsequently compensated for early life conditions and showed no difference in mass at adulthood (GLMM, effect of treatment, $t = 0.997$, $P = 0.322$). There was no effect of our brood size manipulation on our measure of boldness at adulthood (GLMM, effect of treatment, $t = 1.533$, $P = 0.131$).

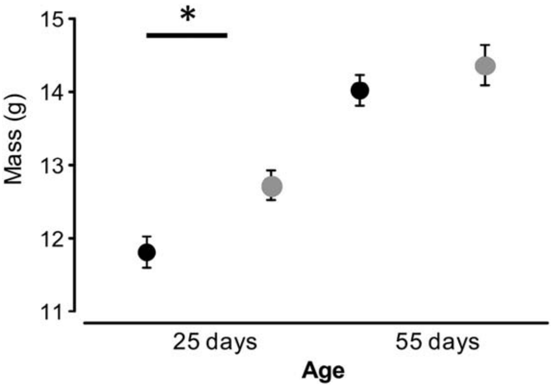

**Figure 3** Effect of brood size treatment on mean fledgling and adult mass in grams at independence (ca. day 25 post-hatch) ± standard error in male and female zebra finches. Birds from large broods in black and small broods in gray circles.

### Male Behavior and Brain Measures

Male zebra finches from larger broods showed impaired song learning. Males from large broods imitated their tutors less accurately than males from small broods, based on the SAP Accuracy measure (GLMM, $t = 2.730$, $P = 0.013$; Fig. 4). Song stereotypy was also marginally lower in males from larger broods based on the SAP within-individual Accuracy measure, although not significantly so (GLMM, $t = 1.179$, $P = 0.088$). We found no effect of experimental treatment on the volume of HVC relative to telencephalon volume (GLMM, treatment, $t = -1.288$, $P = 0.222$).

### Female Behavior and Brain Measures

Brood size manipulation did not affect the strength of females' preferences for tutor song relative to unfamiliar zebra finch song in the operant test (GLMM, effect of treatment, $t = -0.449$, $P = 0.659$; Fig. 5). Females from both treatments triggered the perch that played tutor song more than 50% of the time, and thus showed preference for tutor song over unfamiliar song. However, females from the larger broods were less active during operant trials and triggered

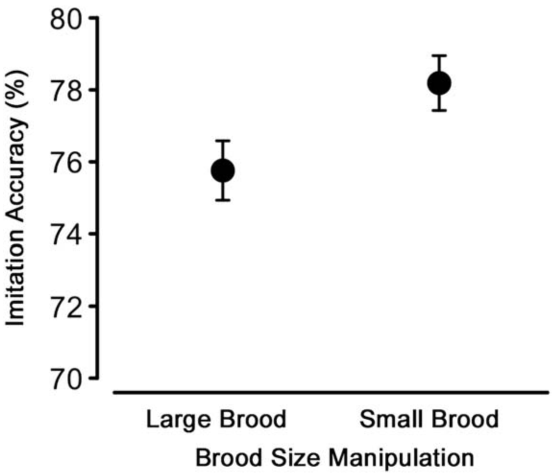

**Figure 4** Effect of brood size treatment on mean imitation accuracy ± standard error of adult male zebra finch song learning.

song fewer times overall, relative to females from small broods (GLMM, treatment, $z = -17.15$, $P < 0.001$; Fig. 5). Additionally, females from larger broods exhibited higher ZENK immunoreactivity in one region of the auditory forebrain and three regions of the social behavior network in response to hearing playback of tutor's song: females from larger broods had more immunopositive cells per mm$^2$ in dNCM, TnA, LSc.vl, and BSTm (GLMM, treatment × brain region interactions, all $P < 0.001$; Table 1; Fig. 6). There were no differences in the immediate early gene response in CMM, vNCM, POM, or the control region (HP).

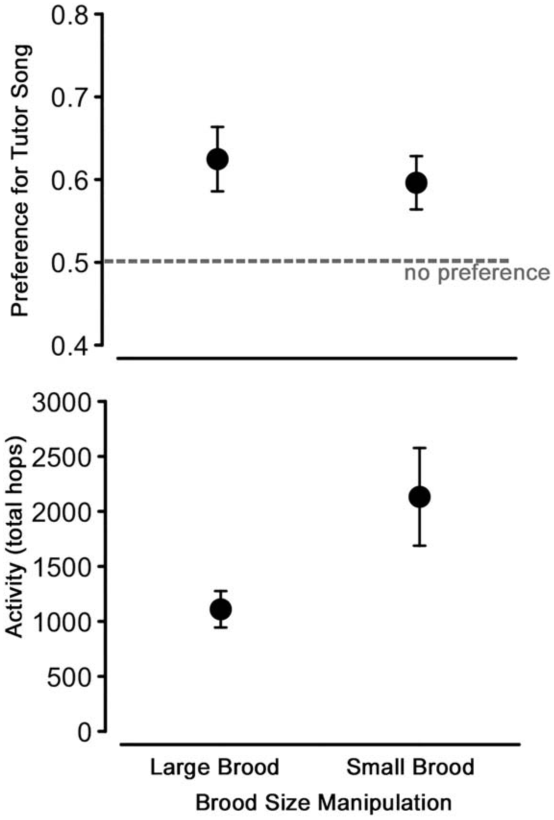

**Figure 5** Effect of brood size treatment on (top) adult female zebra finch mean preference for tutor song relative to novel song ± standard error and (bottom) females' mean response activity (number of songs triggered) during operant testing ± standard error.

**Table 1 Effects of Brood Size Treatment on ZENK-ir (Cells per mm$^2$) in Response to Tutor Song Within Eight Brain Regions of Female Zebra Finches**

| Term | Estimate | Standard Error | *z* value | *p* value |
|---|---|---|---|---|
| Intercept | 6.262 | 1.016 | 6.16 | <0.001 |
| Treatment | −0.055 | 0.212 | −0.26 | 0.796 |
| CMM | −0.113 | 0.019 | −5.76 | <0.001 |
| vNCM | −1.426 | 0.030 | −47.11 | <0.001 |
| dNCM | −0.969 | 0.025 | −37.97 | <0.001 |
| TnA | −0.933 | 0.026 | −36.21 | <0.001 |
| LSc.vl | −0.095 | 0.022 | −4.38 | <0.001 |
| BSTm | 0.003 | 0.021 | 0.15 | 0.877 |
| POM | −2.441 | 0.053 | −46.13 | <0.001 |
| HP | 2.441 | 0.053 | 46.13 | <0.001 |
| Mass at fledging | −0.047 | 0.087 | −0.55 | 0.5843 |
| Treatment × CMM | 0.056 | 0.074 | 0.76 | 0.448 |
| Treatment × vNCM | 0.075 | 0.047 | 1.57 | 0.116 |
| Treatment × dNCM | −0.363 | 0.044 | −8.16 | <0.001 |
| Treatment × TnA | −0.137 | 0.044 | −3.16 | 0.002 |
| Treatment × LSc.vl | −0.520 | 0.039 | −13.51 | <0.001 |
| Treatment × BSTm | −0.568 | 0.038 | −14.90 | <0.001 |
| Treatment × POM | 0.070 | 0.078 | 0.90 | 0.367 |
| Treatment × HP | −0.070 | 0.078 | −0.90 | 0.366 |

Results from a GLMM with Poisson distribution specified for count data. Females from large broods coded as 0 and small broods coded as 1.

## DISCUSSION

Our results show that the brains and behavior of female zebra finches can be impacted by the same developmental stress that affects males. As in previous studies (Nowicki et al., 2002a; Buchanan et al., 2003; Holveck et al., 2008; Brumm et al., 2009; Honarmand et al., 2010; though see Gil et al., 2006), the mild food restriction and other stressors potentially imposed by being reared in a large brood negatively impacted nestling and fledgling mass (Fig. 3) as well as male song learning (Fig. 4). Also consistent with previous work (Riebel et al., 2009; Woodgate et al., 2011; Schmidt et al., 2013), this level of

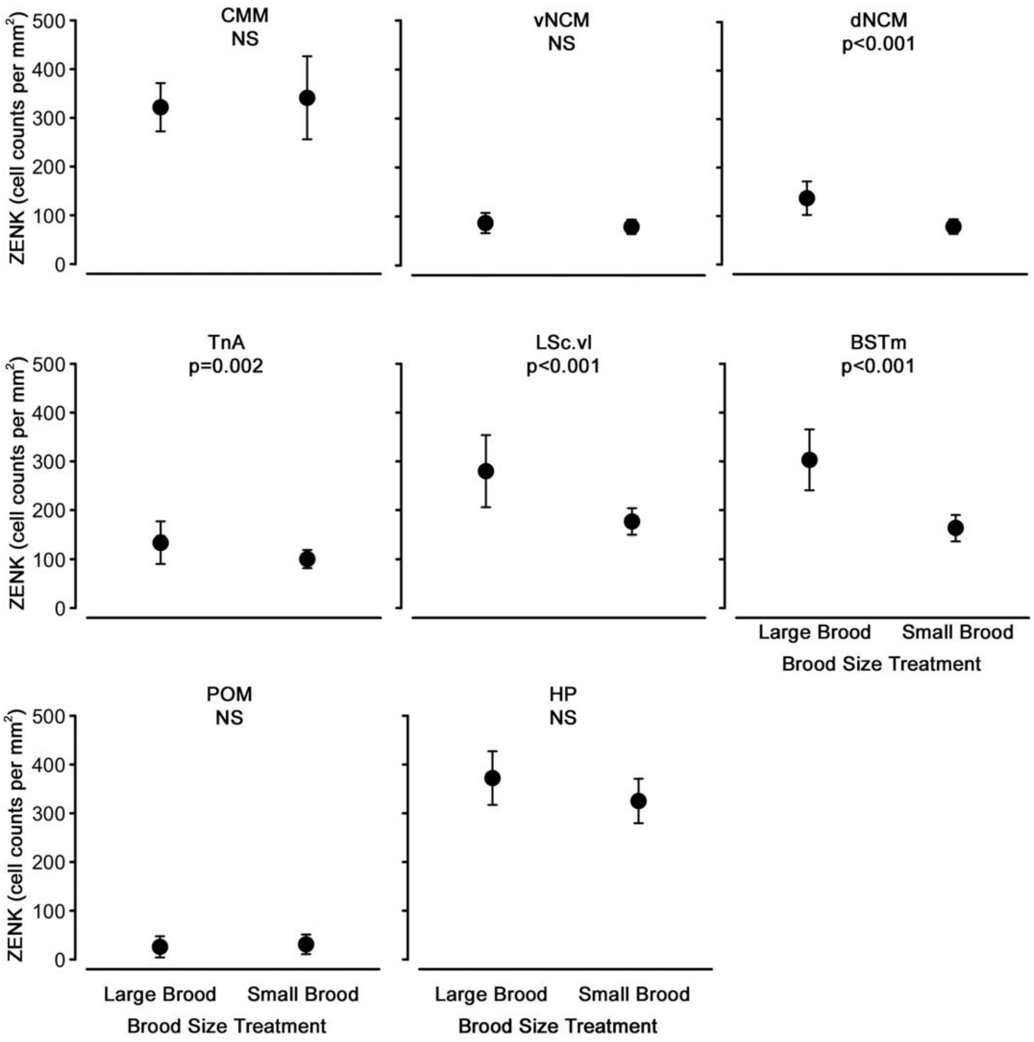

**Figure 6** Effects of brood size on mean ZENK expression (number of immunopositive cells per mm$^2$) in response to hearing tutor song in nodes of the auditory forebrain (CMM, vNCM, and dNCM), the social behavior network (TnA, LSc.vl, BSTm, POM), and a control region (HP) of adult female zebra finches. NS = not significant. Error bars represent standard error not adjusted for the specified distribution (Poisson).

developmental stress did not affect the preference for tutor song over novel song in adult females (Fig. 5). We did find, however, that female activity during the song choice tests was affected by the brood size manipulation: females from larger broods triggered song playback less than half as many times as did females from small broods (Fig. 5). This result is similar to that of a previous study showing reduced activity in developmentally stressed females during mate choice trials in an open arena (Woodgate et al., 2010; see also Schmidt et al., 2013). However, birds from larger broods were equally active in a separate boldness assay. Thus, our results together with prior work suggest that developmental stress might negatively impact female songbirds' mate choice behavior, but that this impact might be mediated by shifts in perception or motivation to respond to song rather than impairment of the formation or referencing of song memories.

In species in which only males sing, sex-specific effects of developmental stress on adult behavior are to be expected because male and female behaviors result from different learning processes and underlying neural systems. Male imitation of song proceeds through two stages: sensory learning, which is the memorization of song, and sensorimotor learning, in which males match their own vocal output to the memorized model using auditory feedback (Immelmann, 1969; Catchpole and Slater, 1995). Female zebra finches acquire song preferences from tutors they are exposed to when young, but do not replicate tutor song and thus do not experience sensorimotor learning. However, female songbirds do reference the song memories they form early in life when assessing songs they hear as adults (Clayton, 1990; Nagle and Kreutzer, 1997; Riebel, 2000; Hernandez and MacDougall-Shackleton, 2004; Hernandez et al., 2009; Anderson et al., 2014). Developmental conditions can impact brain structure and function underlying these learning processes, resulting in life-long effects on behavior that are correlated with the relative vulnerability of underlying neural systems (Nowicki et al., 2002a; Pravosudov et al., 2005; Buchanan et al., 2013). Sex differences in response to developmental stressors are expected when the particular brain processes that are vulnerable to developmental conditions are active only in one sex. For example, aspects of song production measured in males in the present work depend on the song control nuclei of the brain (Nottebohm et al., 1976; Brainard and Doupe, 2000) and, though we and others using brood size manipulations (Gil et al., 2006) failed to find a significant treatment effect in HVC, other studies have found negative impacts of developmental stress on HVC volume (Nowicki et al., 2002a; Buchanan et al., 2004). It is possible that the stress imposed by increased brood size is not sufficient to reduce overall song nucleus volumes, and more fine-scaled measures of neurogenesis, neuron density, neuron morphology, or neurophysiological response may be needed to detect treatment effects on male brains. In contrast, female response to song depends upon perceptual sensitivity to variation in song quality, the referencing of memories of tutor song, and the regulation of motivation to respond to song (Riebel, 2003; Vyas et al., 2009). Thus, variation in female behavioral response to song could result from underlying differences in any of multiple brain mechanisms. Our research design aimed to identify which brain and learning processes might be impacted by developmental stress in females.

We found that females from both treatment groups preferred tutor song (Fig. 5) and we found no differences in ZENK response in CMM, a region of the auditory forebrain argued by some to play a central role in song memory and recognition in female birds (Terpstra et al., 2006; Table 1, Fig. 6). These results are consistent with the interpretation that females from different treatment groups memorized tutor song equally well and were equally good at referencing those memories. We cannot rule out the possibility that females from larger broods have a dampened perceptual sensitivity to song, as is suggested by other studies (Farrell et al., 2016), but the finding that females from large broods had lower overall response levels during operant trials suggests birds in this study had reduced motivation to respond to song. Given that our brood size manipulation had no impact on boldness per se, it is unlikely that such reduced response reflects a treatment effect on behavioral phenotype or “personality”.

Regions of both the auditory forebrain and the social behavior network showed treatment differences in ZENK response to tutor song, suggesting at least two hypotheses about the specific processes impacted by developmental stress. First, rearing condition impacted the immediate early gene response to tutor song in a sub-region of the auditory forebrain, dNCM. Both subdivisions of NCM are selective for song over tones and are specifically ascribed with the detection of novelty (Gentner et al., 2001; Terpstra et al., 2006), suggesting that developmental conditions impacted perception of song salience (Fig. 6). The biological relevance of immediate early gene expression is not well understood, and the relationship between ZENK-ir and female behavior differs across brain regions (Maney et al., 2003, 2008). In general, however, ZENK levels are reliably higher in response to socially salient cues and decrease with repeated exposure to signals (Chew et al., 1995; Mello et al., 1995; Maney et al., 2003; Vignal et al., 2008; Woolley and Doupe, 2008; Maney, 2013). Repeated exposure to a single song decreases ZENK-ir and this rapid habituation underlies sensitivity to novel songs because new signals trigger dishabituation and renewed neural response (Mello et al., 1995; Terpstra et al., 2006). Our finding of elevated ZENK-ir in dNCM of females from larger broods could indicate that developmental stress dampens the relatively higher response to novel acoustic stimuli. Our data cannot directly address this hypothesis because of our experimental design, but similar studies by Schmidt and colleagues (2013a) and Farrell and colleagues (2015; 2016) support this conclusion.

In addition to elevated ZENK-ir in dNCM, three nodes of the social behavior network of the brain, LSc.vl, BSTm, and TnA, showed increased ZENK-ir in females from larger broods (Fig. 6). Though we did not expose females within treatments to different stimuli, prior work has found that LSc.vl., BSTm, and TnA show higher ZENK-ir in response to song than to either tones or silence while POM and HP do not (Maney et al., 2008). Thus, of the social behavior network regions we examined, the three in which we found a treatment effect on ZENK-ir are known to exhibit selectivity for song, whereas the two in which we failed to find a treatment effect do not (Maney et al., 2008). Though the biological relevance of

ZENK-ir in the social behavior network of female birds is not fully understood, elevated ZENK-ir in response to repeated song exposure is again consistent with some alteration of the normal habituation process. Decreased neural selectivity in brain regions involved in social bonding and mate choice could lead to equal responsiveness to all signals, and compromised behavioral discrimination among signals based on their salience (Maney, 2013).

Taken together, our behavioral and IEG data are consistent with the hypothesis that developmental stress experienced by female birds impairs the motivation to respond to song. Future work should test the hypothesis that female mate choice behavior may be impacted through effects on motivation rather than on the early acquisition of tutor song. Additionally, the possibility that sensorimotor learning is especially vulnerable to developmental stress, making males (in species in which only males produce song) particularly vulnerable to early life stressors, should be pursued.

## CONFLICT OF INTEREST STATEMENT

The authors certify that they have no affiliations with or involvement with any organization or entity with any interest in the subject matter or materials presenting in this manuscript.

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