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The neural response of female zebra finches (*Taeniopygia guttata*) to conspecific, heterospecific, and isolate song depends on early-life song exposure

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ABSTRACT

The auditory forebrain regions caudo-medial nidopallium (NCM) and caudo-medial mesopallium (CMM) of songbirds exhibit differential expression of the immediate-early gene ZENK in response to playback of different song stimuli, and dependent on early-life auditory experience. Similarly, song preferences depend both on auditory experience and unlearned biases for particular song features. We explored the contributions of early-life auditory experience and the type of song stimuli on the Zenk response in the auditory forebrain of female zebra finches. Females were raised in three different early tutoring conditions: conspecific tutors that sang isolate song, heterospecific tutors, or conspecific tutors that sang wild-type song. At maturity, these females were exposed to one of five different playback conditions: wild-type song, isolate song, tutor song, heterospecific song, or white noise. Subsequently, the number of cells immunoreactive for ZENK in CMM and NCM was measured. We predicted that birds exposed to conspecific song early in life, and during the song playback in adulthood, would have the highest neural response. Instead, we found that the Zenk response varied across playback conditions with the highest response to conspecific wild-type and conspecific isolate song. In addition, we found a main effect of tutoring, with the lowest overall Zenk response in females tutored by males singing isolate song. Most importantly, there was a significant interaction in that females tutored by wild-type conspecific or heterospecific songs showed a similar increased response to zebra finch songs (wild-type or isolate), but females tutored by isolate song showed no differential response to conspecific song and only showed elevated Zenk response to the particular songs they were tutored with. Combined, our results indicate that unlearned response biases to conspecific song elements depend on previous auditory experience. That is, early experience appears to modulate the expression of innate biases.

1. Introduction

Acoustic communication requires that receivers perceive and differentially respond to signals. In songbirds, the neural processing and behavioural responding to conspecific vocalizations, similar to song production learning, depends on both innate biases and on early experience. Adult female zebra finches (*Taeniopygia guttata*) prefer songs heard early in life compared to unfamiliar songs (Miller, 1979; Riebel et al., 2002), even if the unfamiliar song resembled their tutor song (songs of unfamiliar brothers; Riebel and Smallegange, 2003). This preference extends to songs of tutors that female zebra finches were exposed to at various stages of development (Holveck and Riebel, 2014). The influence of the early acoustic experience goes beyond song familiarity and also affects preferences for song types or acoustic features of song. For example, female zebra finches reared in the absence of tutors did not prefer normal tutor songs more than abnormal songs from isolate reared males (Lauay et al., 2004). Similarly, neural responses in the auditory forebrain of cross-fostered (by Bengalese finches, *Lonchura striata domestica*) female zebra finches were similar in response to songs of the foster father compared to unfamiliar male Bengalese song. This similarity was also found in comparison with unfamiliar conspecific and Bengalese finch song, but the response was greater to unfamiliar songs of the foster species over a third species of finches (Woolley et al., 2010; Hauber et al., 2013).

However, early acoustic experience is not the only factor shaping adult female song preferences. Non-learned, or innate, biases guide

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song preferences toward species-specific typical song features. Female zebra finches raised by conspecifics, or in the absence of a tutor, prefer their own species song even if the song is unfamiliar, over heterospecific unfamiliar songs (Braaten and Reynolds, 1999; Lauay et al., 2004; Campbell and Hauber, 2009). The fact that this preference is present even in the absence of tutoring, suggests that females have non-learned biases towards their conspecific song (Woolley, 2012), similar to biases that guide song learning by male songbirds (e.g., Fehér et al., 2009; Nelson and Marler, 1994). Nonetheless, in cross-fostering experiments, when female zebra finches were raised by Bengalese finches, the adult preference for their own species song decreased (Campbell and Hauber, 2009). Similarly, not being exposed to heterospecific song in early life decreased preferences for conspecific song in zebra finches (Campbell and Hauber, 2010). These studies indicate that experience can interact with innate biases to guide song pReferences

In addition to behavioral responses, the neural responses of the auditory forebrain in female songbirds also depends on the interaction of non-learned biases and early-life auditory experience (reviewed by Hernandez et al., 2008). Variation in the amount of induction of the immediate early gene Zenk in the caudomedial mesopallium (CMM) and caudomedial nidopallium (NCM) in response to sound playback depends on both the type of sound heard in adulthood, and on early auditory memories, and thus likely reflects a combination of long- and short-term auditory memories, and the biological salience of the song (Tomaszycki et al., 2006). In contrast, neuronal firing in the primary auditory region Field L is greater in response to conspecific song regardless of whether zebra finches were raised in the presence or absence of male song (Hauber and Woolley, 2007). In juvenile brood parasitic brown-headed cowbirds (*Molothrus ater*) the Zenk response in CMM is greater to conspecific songs, but only after they have recently experienced conspecific songs (Lynch et al., 2017). These examples illustrate that, as with behavioural preferences, differential neuronal responding to conspecific signals depends on an interaction of innate biases and auditory experience.

In this study, we explore the interaction of early acoustic experience with song exposure in adulthood in regulating neural activity (assessed by immediate-early gene induction) in NCM and CMM. We expand on earlier work by raising female zebra finches with conspecific tutors, heterospecific tutors, and with tutors singing an isolate song, thereby expanding the range of early-learned songs to include species-typical songs with high biological salience, low salience songs that retain some species-typical features (isolate song), and songs with fewer species typical features (heterospecific song). In adulthood we played back all of these categories of tutor song as well as white noise, hypothesizing that variation in the Zenk response to conspecific, heterospecific, and isolate song would vary depending on which of these stimuli were experienced early in life. In particular, we predicted the highest Zenk response in birds that were tutored with conspecific wild type song, and that were exposed to conspecific wild type and tutor songs.

2. Materials and methods

2.1. Study subjects and tutoring conditions

All the experimental procedures, care, and housing conditions followed the guidelines of the Canadian Council on Animal Care and were approved by the University of Western Ontario's Animal Use Subcommittee (protocol 2007-089).

The zebra finches used in this study were sourced from a colony maintained at the Advanced Facility for Avian Research (AFAR) at the University of Western Ontario. We paired females and males in individual breeding cages with a nest cup and access to multi-vitamin seeds, grit, cuttlefish bone, and water *ad libitum*. One tablespoon (14.8 g) of egg-food mix (blended hard-boiled egg and bread) was provided daily during the breeding process. When the chicks hatched the amount of egg food mix was increased to one tablespoon per chick until the offspring were moved to the experimental groups. During the entire experiment, the rooms were kept at 24 °C and on a 14 h:10 h light:dark photoperiod.

The breeding pairs produced 56 female zebra finch offspring in 26 clutches. Both parents reared the young until day 10 post-hatch of the oldest chick. At day 10 after hatching we removed the father from the cage to avoid the chicks hearing their father's song and we moved the mothers and the chicks to a nursery room with other females (who do not sing) and their chicks. Although zebra finch embryos and hatchlings can hear, zebra finch males typically do not learn songs heard prior to day 25 after hatching, and they require at least 10 days of interaction with the father to make an accurate copy of the song (Roper and Zann, 2006). Additionally, parental recognition seems to be complete by day 16 after hatching (Bischof and Lassek, 1985). Therefore, our protocol minimized the likelihood that females would learn their genetic father's song. These females would have been potentially exposed to the subsong of their brothers prior to day 35 when the male offspring were removed from the cage.

To determine the sex of the birds, we collected small blood samples and extracted DNA around day 20 post-hatch. We then determined sex through PCR amplification of genes located on the sex chromosomes (Griffiths et al., 1998).

The mother raised the chicks until the median age of the clutch was 35 days after hatch, when offspring could feed independently (Clayton, 1987). At that day, females were randomly assigned to one of three tutoring condition groups, and their male brothers were moved to different rooms and used for another experiment not described here. As such, females belonging to each group were exposed to different early auditory experiences from day 35 after hatching until maturation after day 90 (see below), when the playback experiment was performed.

At 35 days of age we randomly assigned female zebra finches to a tutoring group in one of three conditions. Each tutoring group was composed of 1 adult tutor male and 2 or 3 young females housed in the same cage. Birds from the same group had visual and auditory contact with other tutoring groups within the same condition. The distance between the cages was at least 50 cm. It is therefore unlikely that the proximity to other cages interfered with tutor song selection because the lack of free social interaction reduces the suitability of the tutor (Eales, 1987). Moreover, male zebra finches copy the song from tutors that they have social interaction with, and that behave more aggressively towards them, rather than birds in adjacent cages (Clayton, 1988). During the experiment there was no visual or auditory contact between conditions as different tutoring groups were housed in different rooms in nonadjacent parts of the facility. The rooms were identical with respect to ambient noise, temperature, lighting and other conditions. Birds within tutoring groups remained together until females reached maturity (age 90 days) at which point playback studies were conducted.

2.2. Early-life tutoring conditions

2.2.1. Group 1. wild-Type conspecific tutored

Subjects in this condition were exposed to wild-type zebra finch songs (Fig. 1a). This condition contained seven tutoring cages that each had an adult male zebra finch (conspecific) tutor that sang a wild-type song, and two or three of 18 young zebra finch females in total.

2.2.2. Group 2. isolate conspecific tutored

Subjects in this condition were exposed to isolate zebra finch songs (Fig. 1b). This condition contained seven tutoring cages that each had an adult male zebra finch that sang an isolate song, and two or three of 19 young zebra finch females in total. Isolate song develops when males are kept in social and acoustic isolation throughout their song-learning period (day 10–90; Williams et al., 1993). Isolate songs are simpler and more uniform in structure than wild-type songs, and contain fewer notes per syllable and per song.

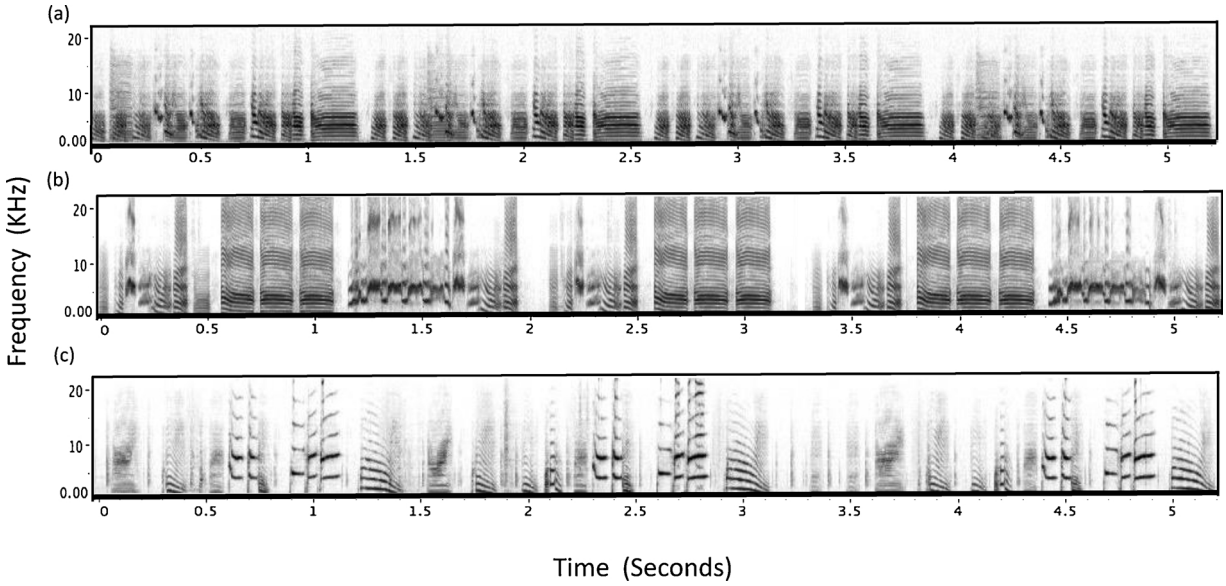

Fig. 1. Examples of song stimuli used in this study. Spectrograms illustrate the songs of **(a)** one wild type zebra finch song, **(b)** one isolate zebra finch song and **(c)** one Bengalese finch song.

To obtain the isolate song tutor males, we bred seven males from the wild-type zebra finch colony. The breeding conditions were the same used to obtain the females for this experiment, however, when the young males reached independency around day 35 after hatching, they were individually housed in a soundproof attenuation chamber until day 140 after hatching. Song development in zebra finches is completed by four months after hatching (Price, 1979). Our manipulations resulted in males that sang isolate songs (e.g. Fig. 1b).

2.2.3. Group 3. wild-Type heterospecific tutored

Subjects in this condition were exposed to wild-type heterospecific (Bengalese finch) songs (Fig. 1c). This condition contained five tutoring cages that each had one adult male Bengalese finch tutor, and three or four of 19 young female zebra finches in total. Bengalese finches were selected as heterospecific tutors because male zebra finches tutored by Bengalese finches are able to imitate Bengalese finch song (Clayton, 1988). However, Bengalese finch syllable syntax and syllable types rarely occur in normal wild-type zebra finch songs (Funabiki and Konishi, 2003).

2.3. Playback experimental procedure

When females reached maturity, and after being exposed to different tutoring conditions (90–110 days of age), they were randomly exposed to one of five playback conditions (Table 1): (1) Tutor song (the song of the male they were tutored with), (2) unfamiliar wild-type conspecific song (zebra finch song from a non-tutor male), (3) unfamiliar wild-type heterospecific song (Bengalese finch song), (4) unfamiliar isolate conspecific song, or (5) white noise (control stimuli).

All the songs used as stimuli were obtained from the different tutor groups (isolate conspecific, wild-type conspecific, wild-type heterospecific) described above. To record the stimuli, each tutor male was isolated in a sound attenuation chamber for around 20 h. Following this isolation period a conspecific female in a different cage was introduced to the chamber to stimulate the male to sing. Therefore, all songs used for playback were female-directed songs. Songs were recorded for 10 min using an omnidirectional microphone (Sennheiser ME62/K6P) and a digital audio recorder (MARANTZ PMD671) with a sampling rate of 44.1 kHz and 16-bit resolution. Birds were recorded twice with an interval of at least 7 days and all the birds were older than 4 months, so their song was already crystallized. Using sound analysis software Raven pro (Cornell Lab of Ornithology) the recordings of each bird were examined and one of the directed songs was selected for use as a stimulus. For playback, this song was repeated in 30-s intervals, having one-second intervals between song repetitions, followed by 30 s of silence. Therefore, each minute of playback contained 30 s of exposure to the song playback and 30 s of silence. Depending on the song duration, the song was repeated two, three or four times until reaching the 30 s of song playback. This process was repeated to produce 19 playback song stimuli (7 wild-type zebra finch, 7 isolate zebra finch and 5 wild-type Bengalese finch)

For the white noise playback, a white noise static mp3 file from an

Table 1
Sample sizes of early-life tutoring conditions and playback conditions. Sample sizes for main effects are indicated in bold. Total sample size = 56.

		Early-tutoring condition		
Playback condition	Total Number of Birds per playback condition	Wild Type Conspecific Tutored	Isolate Conspecific Tutored	Wild Type Heterospecific Tutored
Total Number of Birds per early tutoring condition		**18**	**19**	**19**
Wild Type Conspecific Song (Non-tutor)	**12**	4	4	4
Isolate Conspecific Song (Non-tutor)	**11**	3	4	4
Tutor Song	**11**	4	4	3
Wild Type Heterospecific Song (Non-tutor)	**12**	4	4	4
White Noise	**10**	3	3	4

The bold numbers signifies the total sample per condition.

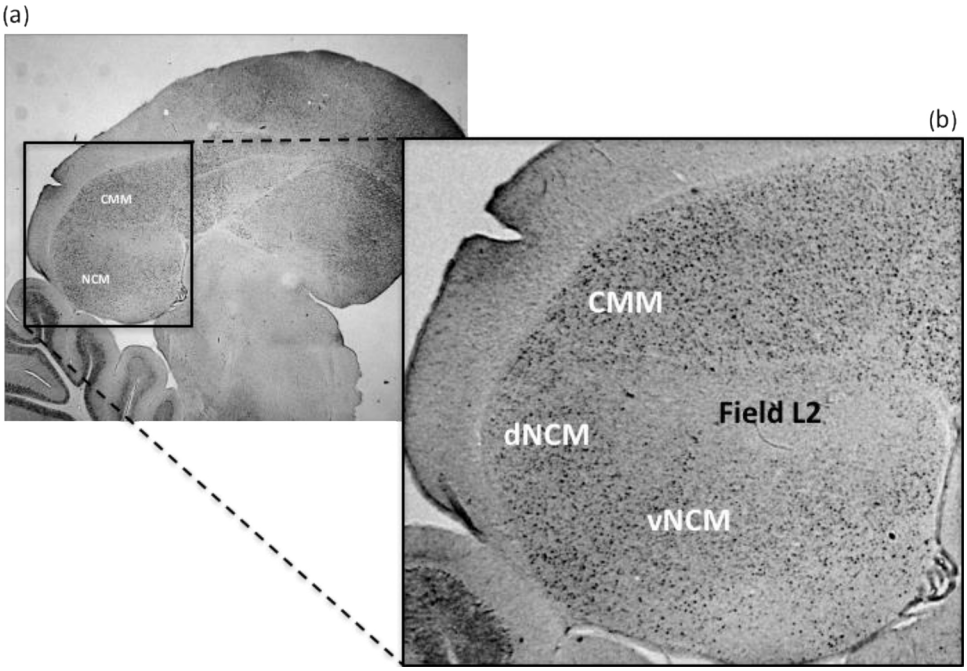

Fig. 2. Photomicrographs of **(a)** a sagittal section of a female zebra finch brain with magnified inset **(b)** showing the auditory forebrain regions where ZENK immunoreactivity was quantified. The locations sampled are indicated in white font. CMM = caudomedial mesopallium, dNCM = dorsal caudomedial mesopallium, and vNCM = ventral caudomedial mesopallium. Rostral is to the right, and dorsal is to the top.

Internet website (http://whitenoisemp3s.comhttp://whitenoisemp3s.com) was downloaded and played in the same isolation chamber. The white noise playback was then recorded with the same equipment as above. The stimulus was then treated as the other playbacks. White noise segments were 30 s in duration and arranged in two repetitions of 15 s, having one-second intervals between white noise repetitions, followed by 30 s of silence.

Prior to the playback exposure each female bird was moved into a 36.5 × 24 × 30 cm cage that contained one seed cup, one water bottle, one grit cup, a cuttlebone and two perches (food and water were provided *ad libitum*) inside a sound attenuation chamber. Each attenuation chamber was equipped with 2 playback speakers (KOSS HDM/111 BK) used to broadcast the stimuli. Speakers were placed in front and at the side of the cage. The volume of the speakers was adjusted to produce an average sound intensity of 75 dB SPL at the position of the perch in the cage.

Birds were kept in complete isolation for approximately 24 h. Fifteen minutes before the stimulus was played the lights inside the attenuation chambers were turned off to avoid as much as possible any movement or vocalization by the female bird that might affect IEG expression. The randomly assigned song playback was then played for 30 min while the bird was in the dark. One hour after the stimulus playback ended, while still in the dark, birds were given an overdose of isoflurane anesthetic, decapitated and the brains were rapidly dissected from the skulls for further analysis. Birds did not vocalize during the playback, but we could not monitor whether the birds were actively attending to the playback.

2.4. Immunohistochemistry

Once brains were collected they were immediately immersed in 4% paraformaldehyde for at least 4 d to fix them. Fixed brains were then immersed in 30% sucrose in phosphate-buffered saline (PBS) for 48 h at 4 °C to cryoprotect them, then the brains were frozen rapidly in powdered dry ice before storage at −80 °C until processing. One brain hemisphere was randomly chosen and processed for this experiment.

Brains were sliced in 40 µm thickness in parasagittal sections starting from the midline on a cryostat. Every second section was collected into tissue culture wells containing 0.1 M PBS. Sections were immunolabeled to localize ZENK (egr-1) protein as follows: free floating sections were washed twice in 0.1 M PBS, incubated for 15 min in 0.5% H_2O_2 in PBS at room temperature, then washed three times in 0.1 M PBS. Sections were then blocked using 10% normal goat serum (Vector labs, catalog # S-1000) in 0.3% Triton-X 100 (PBST) for 1 h incubation at room temperature. The normal goat serum was then removed and the sections were incubated for approximately 20 h at 4 °C in the primary antibody, a polyclonal antibody reared in rabbit (Egr-1, Santa Cruz Biotechnology, catalog # SC-189) at a concentration of 1:2000 in 0.3% PBST. Sections were then washed three times in 0.1% PBST and incubated for 1 h at room temperature in the secondary antibody, biotinylated goat anti-rabbit, (IgG, Vector labs, catalog # BA-1000) 1:250 diluted in 0.3% PBST. After that, sections were washed in 0.1% PBST three times and incubated in avidin-biotin horseradish peroxidase complex (ABC Vectastain Elite kit, Vector labs, catalog # PK-6100) at room temperature for 1 h. Sections were then washed in 0.1% PBST and visualized using 3′,3-diaminobenzidine tetrahydrochloride chromagen (SigmaFast DAB) and washed three times in PBS. Brains were processed in groups of 3–4 and each immunohistochemistry run had brains from the different treatment conditions. Finally, sections were mounted on gelatin-coated microscope slides, dehydrated in ethanol and cleared in solvent (Harleco Neo-Clear, EMD Chemicals) and protected with coverslips affixed with Permount (Fisher Scientific).

2.5. ZENK quantification

We quantified the level of ZENK immunoreactivity (ZENK-ir) in three forebrain auditory areas within the telencephalon: the dorsal caudal medial nidopallium (dNCM), the ventral caudal medial nidopallium (vNCM) and the caudal medial mesopallium (CMM). Dorsal, ventral and caudal areas of NCM boundaries were defined taking the lateral ventricle as a point of reference (Fig. 2). The NCM rostral border area was defined using Field L that was visible as an area without immunoreactivity. CMM was delineated by the most caudal area bounded by the lateral ventricle and the caudo-ventral border of the mesopallial lamina. Six sections of one hemisphere of each zebra finch were measured. Quantification started with the first section, moving medial to lateral in which NCM was attached to the rest of the brain. Therefore, six photomicrographs (515 µm by 386 µm) per brain region, per bird were taken. For dNCM the photomicrographs were taken from the most dorso-caudal part of NCM. vNCM photomicrographs were obtained

Table 2
Results of linear mixed model analysis to determine the effects of early-life tutoring and playback stimulus on Zenk immunoreactivity in the auditory forebrain of zebra finches. Significant fixed effects are indicated in bold font.

Variable	*F*	*df*	*P*
Early-tutoring condition	5.46	2, 106.9	< 0.01
Playback condition	10.44	4, 116.1	< 0.01
Auditory forebrain region	36.03	2, 94.1	< 0.01
Early-tutoring condition X playback condition	4.99	8, 113.0	< 0.01
Early-tutoring condition X auditory forebrain region	0.155	4, 94.05	0.96
Playback condition X auditory forebrain region	1.58	8, 94.05	0.14
Early-tutoring condition X playback condition X auditory forebrain region	0.47	16, 94.05	0.93

from the center of the ventro-rostral area. CMM photomicrographs were acquired from the most caudal part of the structure. This sampling procedure replicates that used in several previous studies (e.g., (Gentner et al., 2001; Avey et al., 2005; Hernandez and MacDougall-Shackleton, 2004; Schmidt et al., 2013); Fig. 2).

Photomicrograph images (0.515 by 0.386 mm) were captured using a Leica Digital CCD camera mounted on a Leica DM5000 B light microscope through a 20X objective lens. We used Leica Application Suite to compile each picture as a z-stack from a series of images taken at a regular interval (0.63 μm) throughout the focal depth of the section using a Leica 420D camera. Compiling these photomicrographs created an image in which all cells were in focus (Hall and MacDougall-Shackleton, 2012). The observer was blind to the tutoring condition and to the playback broadcasted to the bird.

For each image, we used ImageJ64 (NIH) software to count the number of ZENK-ir cells in the whole image. First we converted the images to 8-bit gray scale, then the number of particles with an optical density above a threshold value were counted using the threshold tool. This threshold was set manually in every image due to the variability in the background staining, in a way that the group of pixels emphasized by the software were equivalent with what a blind observer considered labeled nuclei. To set exclusion limits for cell size (2.0–56 μm^2) we randomly selected 6 birds and from the 18 photomicrographs per bird (6 x each area) and choose a subset of 20 cells. From these 360 measurements per bird, 2160 measurements in total, we determined the minimum and maximum sizes of the cells and established a minimum and maximum. Exclusion limits for sphericity were set at 0.45. The observer was blind to the bird's tutoring condition and to the song playback broadcast to the subject.

2.6. Statistical analysis

We analyzed all data with linear mixed models using SPSS 19.0 (SPSS Inc., Chicago, IL, USA). The number of ZENK-ir cells in the three different auditory areas (CMM, dNCD, vNCM) was the dependent variable. For each brain region, we calculated the average cell count per field of view for each bird, such that only one value per bird per brain area was used as the dependent measure. Early-life tutoring condition, playback condition, and auditory forebrain region were entered as fixed effects. Nest identity (the natal nest of the subjects), bird identity (nested within nest identity) and brain hemisphere (left or right) were added as random effects.

Fully loaded models were run including all predictors and interactions of interest. Therefore, the model included the main effects of early-life tutoring condition, playback condition and auditory forebrain region, with all higher order interactions, including nest identity and bird ID, as random effects. Preliminary analyses indicated no significant main effect or interactions of brain hemisphere, thus brain hemisphere as a random effect was excluded from further analyses. Models including both nest identity and bird ID as random effects fully converged, but did not have a positive Hessian matrix. This likely resulted from these random effects being redundant. Removal of bird ID as a random effect resulted in a positive Hessian matrix. Regardless, all results discussed below were the same whether or not bird ID was included as a random effect. Holm-Bonferroni corrections were used for all post-hoc pair-wise comparisons.

Significant interactions were further examined by plotting data separately for each early-life tutoring condition. The sample sizes for each playback group within each tutoring condition was thus reduced (Table 1) and this precluded statistical analyses for each condition separately.

3. Results

The linear mixed model included, as significant predictors of the number of ZENK-ir cells, the main effects of early-life tutoring condition, playback condition, and auditory forebrain region, as well as the interaction between early-life tutoring condition x playback condition (N = 56 birds; Table 2). This significant interaction indicates that variation in how birds responded to the playback stimuli depended on which early acoustic environment they developed in. The interactions between early-life tutoring condition and auditory forebrain region, playback condition and auditory forebrain region, and the three-way interaction of early-life tutoring condition x playback condition x auditory forebrain region were not significant (Table 2). Because there were no interactions between brain region (CMM, dNCM,vNCM) and the other factors in the model, we did not run subsequent analyses separately for each brain region.

3.1. Main effect of early-life tutoring

Bonferroni post-hoc analysis of the significant main effect of early-life tutoring condition revealed that female zebra finches tutored by isolate conspecific song had significantly fewer ZENK-ir cells than those tutored by wild-type heterospecifics ($p = 0.03$) and wild type conspecifics ($p = 0.04$) regardless of song playback (Fig. 3a). There was no significant difference in the number of ZENK-ir cells between females tutored by wild-type heterospecifics and those tutored by wild-type conspecifics. Thus, being tutored by a male singing isolate song reduced overall responsiveness of the auditory forebrain compared to being tutored by conspecific or heterospecific wild-type song.

3.2. Main effect of playback stimuli

Bonferroni post-hoc analysis of the significant main effect of playback condition showed significant variation in response to the different playback stimuli (Fig. 3b). As noted in many prior studies, white noise exposure resulted in the fewest Zenk-ir cells. Playback of conspecific song, whether wild-type or isolate song, lead to the greatest number of Zenk-ir cells. Unexpectedly, there were significantly more Zenk-ir cells in birds hearing isolate zebra finch song than in those hearing tutor song ($p = 0.04$). However, it is important to note that the group labelled "tutor song" here consisted of a mixture of birds tutored with all three types of tutor songs (conspecific, heterospecific, isolate). Overall, the results are consistent with prior studies that the greatest Zenk response was to conspecific song (wild-type or isolate), the lowest response was to white noise, and there was an intermediate response to heterospecific song.

3.3. Main effect of brain region

Bonferroni post-hoc analysis of the significant main effect of brain region revealed that CMM had significantly more ZENK-ir cells than dNCM ($p < 0.001$; Fig. 3c) and vNCM ($p < 0.001$). dNCM also had significantly more ZENK-ir cells than vNCM ($p < 0.001$). Because the effect of brain region did not significantly interact with any of the other factors we did not analyze these regions separately.

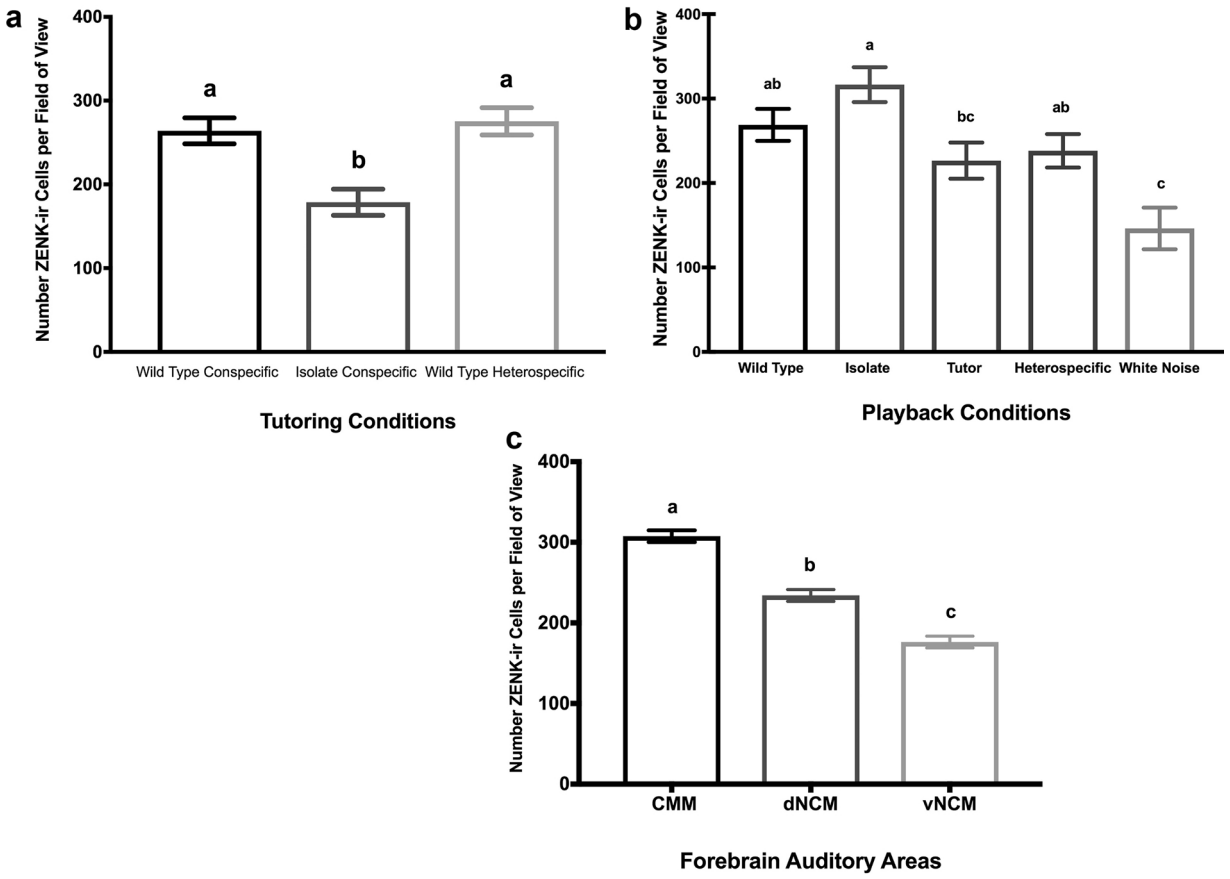

Fig. 3. Zenk immunoreactivity (Zenk-ir) in auditory regions of female zebra finches raised in different early tutoring environments and exposed to five playback stimuli in adulthood. The number of cells indicated are per 515 μm by 386 μm field of view photomicrograph. Bars indicate estimated marginal means (± 95% CI) for each group. Groups that share the same lower-case letter did not significantly differ from each other. **(a)** Main effect of tutoring conditions. Females tutored with isolate conspecific song had overall lower Zenk-ir than those tutored with wild-type conspecific or heterospecific song. **(b)** Main effect of playback condition. Females exposed to white noise had the lowest Zenk-ir, and those exposed to isolate song had the highest. In general, playback of conspecific song (wild-type, isolate, or tutor) resulted in higher Zenk-ir than playback of heterospecific song. **(c)** Main effect of brain region on the number of Zenk-ir in female zebra finches.

3.4. Interaction of early-life tutoring and playback stimuli

To explore the significant interaction between early-life tutoring conditions and playback song, we plotted data separately for each tutoring condition (Fig. 4). For birds tutored by conspecific wild-type song and those tutored by wild-type heterospecific (Bengalese finch) song the number of ZENK-ir cells appears lower in birds exposed to white noise than in those exposed to isolate song and wild-type song, but the responses to wild-type and isolate song do not appear to differ (Fig. 4). Thus, these two groups of birds showed the same pattern of results as those observed when all tutoring conditions were analyzed together (Fig. 3b). For birds tutored with isolate song there was a different pattern than the other two tutoring groups (Fig. 4). In this tutoring condition birds played back their tutor song had the greatest Zenk response, and the other groups had low responses that were similar to white noise playback (Fig. 4).

3.5. Sample size and power analysis

This experiment had similar sample sizes to previous research that detected significant differences between groups of birds exposed to different songs (e.g., Kruse et al., 2004; Mello et al., 1992; Park and Clayton, 2002; Hernandez and MacDougall-Shackleton, 2004). Sensitivity power analysis using G power analysis (Faul et al., 2007) showed that the general model reported above could detect an effect size $f = 0.35$, which is considered a medium to large effect (Cohen, 1969). Thus, the null results reported above indicate either no difference or only a small difference between groups.

4. Discussion

Our objective was to determine whether variation in the response of auditory forebrain regions of female zebra finches to conspecific, heterospecific and isolate song depends on early experience. To do this, we measured the number of ZENK-ir cells in three forebrain auditory regions (CMM, dNCM, vNCM), in response to playback of different acoustic stimuli with birds that had early exposure to different tutoring conditions; wild-type zebra finch (conspecific), wild-type Bengalese finch (heterospecific), and isolate zebra finch (conspecific). Overall, we found that females tutored by wild-type conspecifics and heterospecifics showed greater ZENK-ir levels than females tutored by isolate song (Fig. 3a). We also found that conspecific song playback, whether wild-type or isolate song, led to highest levels of Zenk-ir compared to heterospecific song or white noise (Fig. 3b). Finally, variation in the Zenk response to these stimuli significantly interacted with early life-experience (Table 2, Fig. 4). Below we discuss each of these findings in turn. The differential levels of neuronal activation as a result of tutoring conditions and playback conditions suggest that neural activation in forebrain auditory regions is modulated by developmental acoustic experience (Hernandez et al., 2008). Thus, exposure during ontogeny to different kinds of natural song vocalizations seems to shape song perception in adulthood and possibly also affects the information coding capacity of auditory neurons (Woolley et al., 2010; Woolley, 2012).

The main effect of early experience is consistent with some studies,

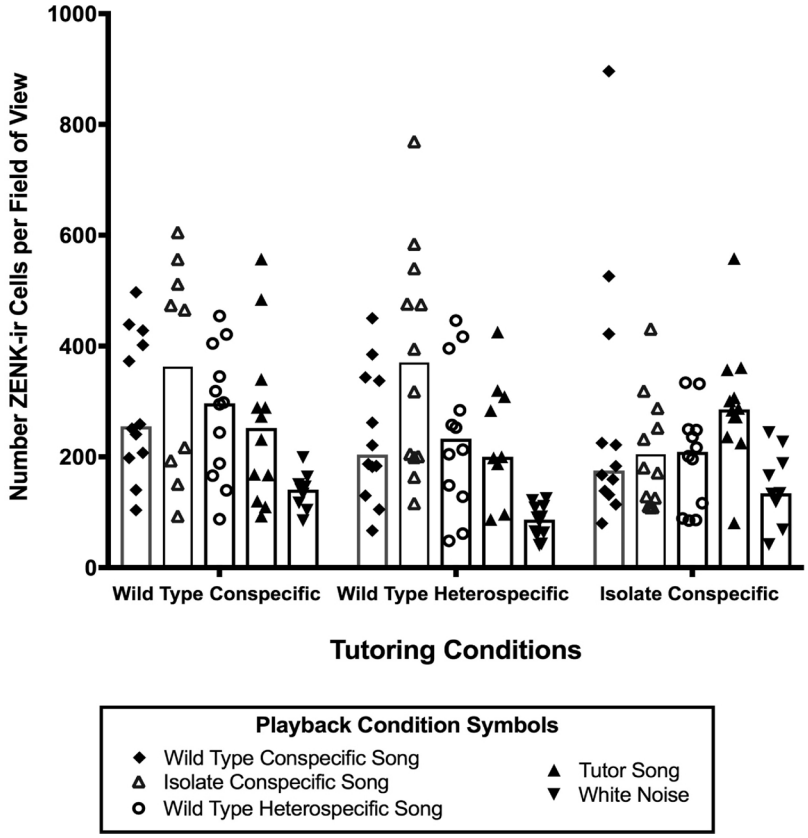

Fig. 4. Zenk immunoreactivity (Zenk-ir) in auditory regions of female zebra finches raised in different early tutoring environments and exposed to five playback stimuli in adulthood. Bars indicate estimated marginal means for each group, and symbols indicated individual values for each brain region for each bird. That is, each bird is represented three times, once for each brain region. Repeated observations per bird were controlled for in the statistical analyses by including brain region as a fixed effect and bird as a random effect. The data are separated by tutoring condition to illustrate the significant interaction between tutoring condition and playback treatment. Data are pooled across brain regions as there was no significant interaction between brain region and the other factors. Females tutored by wild type conspecific or wild type heterospecific song had higher Zenk-ir in response to conspecific song playback than to white noise; females tutored by isolate conspecific song did not.

in that early life experience has been shown to modulate Zenk response to song in adulthood (Hernandez et al., 2008). Being reared with a male singing isolate song should have preserved most social interactions (although tutor-offspring interactions were not measured in our study), but would have created an acoustic environment with fewer well-formed song syllables compared to normal zebra finch or Bengalese finch song. It was unexpected that even a heterospecific song led to higher Zenk responsiveness overall than being raised with a male singing isolate song. These results suggest that normal development of CMM and NCM likely depend on early life exposure to a broad range of sounds that characterize song in these species. Further work with synthetic sound stimuli could clarify what sound parameters are most important for this process. Prior work has shown that being reared in acoustic isolation can have life-long effects on sound perception (Njegovan and Weisman, 1997; Sturdy et al., 2001). Our experiment here suggests that auditory processing can be affected not only by being raised in isolation, but being reared with isolate songs.

The results above indicate that being raised by a heterospecific results in an overall Zenk-response similar to being raised by a conspecific (Fig. 3a). However, with respect to the Zenk response to specific categories of songs we found that conspecific songs, whether normal wild-type or isolate songs, resulted in the greatest Zenk response. Heterospecific song resulted in a response intermediate to that of conspecific song and white noise (Fig. 3b). Thus, in contrast to the long-term effects of early experience, isolate song in exposure in adulthood resulted in greater Zenk responses than heterospecific song. This suggests that the species-typical features in isolate song engage auditory processing by CMM and NCM more than the acoustic features of Bengalese finch song. That is, non-learned biases towards species-typical song features appears to influence auditory processing in addition to experience-dependent effects.

We included a group of birds that were played back the exact songs they were tutored with ("Tutor Song" in Figs. 3 and 4) to test whether re-exposure to early learned song in adulthood would induce the highest levels of Zenk response in adulthood. Instead we found intermediate responses to these songs in all but one group (discussed below). It is important to note, however, that this group contained a mixture of birds being played conspecific, heterospecific, and isolate songs. With the exception of females tutored with isolate song, there was no overall increase in Zenk response in birds hearing tutor song in our study. This does not contradict prior studies that have found greater Zenk response to tutor song when compared tutored and untutored wild-type conspecific songs (e.g., Bolhuis and Eda-Fujiwara, 2003; Terpstra et al., 2006; Gobes et al., 2010) because our experiment included a much broader range of tutor groups.

Perhaps our most important finding was a significant interaction between early-life tutoring and adult song playback condition (Table 2, Fig. 4). This interaction appears to depend primarily on the responses of the females raised with males singing isolate song. Variation in the Zenk response of females raised with conspecific or heterospecific males singing normal wild-type songs (Fig. 4) are more-or-less similar to the variation in response observed overall (Fig. 3b). However, for females raised with males singing isolate song, the pattern was very different. These females had low Zenk responses overall (Fig. 3a) and Zenk was elevated only in the birds played back the particular isolate songs they were tutored with (Fig. 4). The species-typical features in non-tutored isolate song or in wild-type conspecific songs did not lead to elevated Zenk response like they did in the other two tutor groups. Thus, being reared by a male singing isolate song led to both an overall reduction in Zenk responses to all stimuli (Fig. 3a), and a reduced response to non-tutored conspecific songs in particular (Fig. 4).

Further work is required to confirm this significant interaction between early-life tutoring and playback stimulus, and to determine why birds raised with isolate song showed such low levels of Zenk response. Our experimental protocol also did not allow us to control for some potential confounding effects. For example, we kept birds in the dark during and following playback to reduce activity-driven gene expression. However, we are not able to determine if birds were awake, or not,

during this period. In addition, our isolate rearing protocol did not control for very early exposure to subsong by brothers prior to day 35 post-hatch. Further work is required to determine if early subsong exposure contributed to the effects we observed.

Combined, our results are consistent with earlier work indicating that auditory processing of conspecific songs in NCM and CMM depends on both auditory experience and on unlearned responses to species-typical song features. Unexpectedly, isolate song was sufficient to induce high levels of Zenk response in females that were tutored by either conspecific male zebra finches or heterospecific male Bengalese finches. However, if females were reared by male zebra finches singing isolate song, the elevated Zenk response to unfamiliar conspecific song disappeared, and only playback of tutor songs in particular led to any increase in Zenk response. It thus appears that the auditory biases to species-typical songs in adulthood depend on an early life environment with a wide range of acoustic stimuli found in both zebra finch and Bengalese finch song, but not in zebra finch isolate song. More simply, the expression of innate biases in the Zenk response appears to depend on early learning.

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