Effects of Song Experience and Song Quality on Immediate Early Gene Expression in Female Canaries (*Serinus canaria*)

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ABSTRACT: Female songbirds are thought to make mate choices based on aspects of male song quality. Male canaries (*Serinus canaria*) produce songs with "special" syllables that have been shown to be highly salient to female listeners – eliciting high rates of sexual displays and enhanced immediate early gene (IEG) expression. Immunohistochemistry for the IEG ZENK was used to examine the effects of experience with these syllables on activity in the caudal mesopallium (CMM) and nidocaudal mesopallium (NCM), two auditory areas important in processing conspecific song. Photostimulated female canaries were housed in sound attenuated chambers and played pseudosongs containing either three special syllables or three non-special syllables, an intro, and an outro sequence. Females that heard special syllable pseudosongs exhibited higher ZENK expression in CMM. To assess the effects of experience, photostimulated females were pair housed and exposed to playback of song with or without special syllables for 14 days. After transfer to individual housing, birds were played one of the aforementioned stimuli or silence. ZENK expression in CMM and NCM was equivalent for song with and without special syllables, but significantly lower for silence. Females who experienced song with special syllables had lower plasma estradiol concentrations after final song playback. This study indicates that CMM exhibits an IEG response bias to special syllables in limited acoustic contexts, but not in full song, which may contain additional biologically relevant information. Furthermore, estradiol concentrations may mediate changes in song responses, serving as a mechanism for modulating mate choice in differing song environments.

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Keywords: **immediate early gene; auditory perception; bird song; female choice; sexual selection; canary; *Serinus canaria***

INTRODUCTION

When songbirds are making mating decisions, song quality serves as an essential factor in the decision process (Searcy, 1986; Kroodsma and Byers, 1991). Different songbird species have particular song features that are of preeminent importance for mate choice, such as song length, repertoire size, or special syllables (Searcy, 1992; Vallet *et al.*, 1998; Gentner and Hulse, 2000). Females choose males with songs containing these particular features, since they can serve as an honest signal of mate quality (Gil and Gahr, 2002). However, it is unclear to what extent these decisions are due to an innate preference for specific song features or due to preferences determined by experience of a particular social environment.

One way to examine the relative weights of these factors at the cellular level is by measuring differences in expression of immediate early genes (IEGs). IEGs are transcription factors that are rapidly induced following the activation of a neuron and are, therefore, useful molecular markers of neuronal activation (Farivar *et al.*, 2004). In particular, ZENK

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((an acronym for zif-268 (Christy *et al.*, 1988), egr-1, (Sukhatme *et al.*, 1988), ngfi-a, (Milbrandt, 1987), and krox-24, (LeMaire *et al.*, 1988)) has been shown to be a robust example of a song-inducible gene (Mello *et al.*, 1992; Mello and Clayton, 1994; Mello and Ribeiro, 1998; Leitner *et al.*, 2005). In several songbird species, the magnitude of the ZENK response in auditory processing areas has been found to correlate with song attractiveness and behavioral measures of female mate preferences. For example, white-crowned sparrows exhibit greater ZENK expression in the caudal mesopallium (CMM) and dorsal nidocaudal mesopallium (NCMd) in response to playback of song in their hatch dialect, which is correlated with copulation solicitation displays and other preference behaviors (Maney *et al.*, 2003). Wild-caught house finches have an enhanced induction of ZENK in CMM, NCMd, and NCMv following playback of conspecific song versus heterospecific song (Hernandez and MacDougall-Shackleton, 2004). In addition, activity in the caudocentral nidopallium (NCC) has been shown to be higher when female zebra finches hear female-directed song rather than undirected song (Van Ruijssevelt *et al.*, 2018). Therefore, differences in ZENK expression following presentation of a variety of song types can indicate the relative levels of preference for these songs.

In addition to serving as a marker of neuronal activation, song-induced expression of ZENK may also represent a molecular mechanism necessary for the cellular plasticity that produces experience-dependent variation in auditory processing. The induction of ZENK is subject to habituation, as repeated playback of the same song results in diminished ZENK expression (Jarvis *et al.*, 1995). ZENK induction has also been implicated in the molecular cascade responsible for the formation and stability of long-term memories (Clayton, 2000; Jones *et al.*, 2001). Differences in song quality in the social environment could lead to repeated differences in song-induced ZENK expression, which could then influence later ZENK expression at the time of mate selection through this signaling cascade. In one songbird species, European starlings (*Sturnus vulgaris*), females prefer males that produce long-bout songs compared to short-bout songs, as assessed by both behavioral preference and IEG expression in the auditory telencephalon (Gentner and Hulse, 2000; Gentner *et al.*, 2001). However, female starlings exposed to only short-bout songs for one week exhibited a reduction in the selectivity of ZENK response to long-bout songs in comparison to female starlings exposed to long-bout songs, who continued to exhibit a selective response to long-bout songs (Sockman *et al.*, 2002). Repeated exposure to especially salient song features, such as long-bout songs in starlings, may lead to increased selectivity through repeated activation of ZENK and its downstream targets, promoting synaptic plasticity.

Another possible mechanism for this experience-dependent plasticity and the consequent variation in song preferences are catecholamine-mediated changes in neuronal response properties. The catecholamines dopamine (DA) and norepinephrine (NE) are influential in determining response properties of the auditory system in a context-dependent manner (Cirelli *et al.*, 1996; Dave *et al.*, 1998; Bao *et al.*, 2001; Cardin and Schmidt, 2004; Cirelli and Tononi, 2004; Castelino and Ball, 2005). Furthermore, behavioral and neural selectivity for attractive song is reduced following noradrenergic denervation (Appeltants *et al.*, 2002; Lynch and Ball, 2008). Female starlings exposed to long songs exhibited elevated levels of the norepinephrine metabolite, 3-Methoxy-4-hydroxyphenylglycol (MHPG), the dopamine metabolite, 3,4-Dihydroxyphenylacetic acid (DOPAC), and had an increased probability of fibers immunoreactive for dopamine beta hydroxylase (DBH) in NCM (Sockman and Salvante, 2008). Therefore, in environments with an abundance of high quality song, increased catecholamine innervation in auditory areas may support increased female selectivity.

Estrogenic effects on auditory processing may also be involved in this process. Estradiol is important for modulating selectivity for specific song features. Selective ZENK responses for conspecific song depend on circulating estradiol above non-breeding baseline levels (Maney *et al.*, 2006). In addition, hearing song leads to increases in estradiol concentrations in NCM (Remage-Healey *et al.*, 2008). Repeated exposure to song could lead to increases in estradiol that promote selectivity in subsequent responses to song.

These proposed mechanisms – ZENK-induced cellular plasticity, catecholamine innervation, and estrogenic-mediated selectivity – are all highly intertwined. Estradiol treatment increases the density of fibers immunoreactive for tyrosine hydroxylase (TH) or DBH (Sanford *et al.*, 2010). Additionally, estradiol can induce ZENK expression (Tremere *et al.*, 2009). Therefore, experience-dependent variation in song selectivity may be due to some combination of all three.

In the present study, we examine if these mechanisms of song selectivity are also efficacious in song selectivity canaries (*Serinus canaria*). Canary males produce songs with "sexy" or "special" syllables that are composed of two simultaneous high-frequency notes with a high-repetition rate (Vallet, 1995; Vallet *et al.*, 1998). They are difficult to produce because they require rapid sequential use of both sides of the syrinx (Suthers *et al.*, 2012). These special syllables

are highly salient to female listeners and elicit high rates of sexual copulation solicitation displays (Vallet *et al*., 1998). Canaries have a distinct perception of these special syllables, perceiving these syllables as similar, regardless of the male that produced them, and exhibiting an enhanced perception of increases in their tempo (Fishbein *et al*., 2019). As is the case with the preference for long over short-bout songs in European starlings, canaries do exhibit an IEG response bias for these syllables. Females that hear these special syllables exhibit higher ZENK expression in CMM, but not in NCM (Leitner *et al*., 2005). Female canaries also exhibit increases in circulating levels of testosterone and estradiol after hearing these syllables (Marshall *et al*., 2005). We hypothesized that experience of song with or without special syllables can modify IEG responses to variation in song quality. In order to investigate the relationship of ZENK expression in CMM with behavioral and endocrine responses to these syllables, we performed one experiment playing female canaries pseudosong stimuli composed simply of intro notes, special or non-special syllables, and outro notes. In order to determine the effects of experience on these mechanisms, we performed a second experiment exposing female canaries to two weeks of full songs that either contained or did not contain these special syllables. We then tested behavioral, neurochemical, and physiological responses to a final song playback with or without special syllables. We predicted that females that heard song containing special syllables would exhibit selective IEG responses to song containing these special syllables, while experience of song without special syllables would attenuate this effect.

MATERIALS AND METHODS

Experimental Animals

Adult female canaries of the American Singer strain were obtained from a local breeder (Maryland Exotic Birds). Upon arrival, birds were placed on a short day photoperiod (8L:16D) to induce a state of photosensitivity. Birds were housed in 49 × 95 × 51 cm cages, with six birds of the same sex per cage, in a colony room containing both males and females at the University of Maryland, College Park, MD and provided canary food and water ad libitum. After the start of each experiment, birds were moved to 48 × 48 × 42 cm cages and maintained on a 12L:12D (light:dark) long day light cycle (9:00 a.m.–9:00 p.m.) for at least six weeks in order to induce a state of photostimulation. All procedures were approved by the University of Maryland, College Park Animal Care and Use Committee.

Experiment 1: Pseudosong Experiment. For the experiment examining differences in response to special or non-special pseudosong playback, fourteen females were housed in groups of two or three in sound-attenuated chambers for the six-week period of photostimulation. These females were then transferred to individual sound-attenuated chambers. The subsequent day we exposed females to a playback of special pseudosongs (n = 6), playback of non-special pseudosongs (n = 6), or silence (n = 2) for 90 min and collected brains. Playback and brain collection was conducted in two sessions, with playback condition counterbalanced across session.

Experiment 2: Full Song Experiment. To examine the effects of experience utilizing full song playbacks, females were housed in group aviaries on long day lengths (12L) for six weeks until photostimulated. Females were then transferred to sound-attenuating chambers in groups of two or three. The extra bird in three-bird chambers would later serve as a silent control for playback. After a 72-h acclimation period, blood samples were collected from the wing vein to assay baseline circulating estradiol concentrations. Twenty-four hours later, females were exposed to song playback of either special (n = 14) or non-special (n = 14) full song for 14 days via speakers (PUI Audio, Inc.) connected to an 8-channel digital router (Fireface UC, RME) to simulate song environment experience conditions. Special or non-special songs were played for five hours per day at partially randomized 30 min intervals during the photophase (9:00 a.m.–9:00 p.m.). The first 30-min playback of each day always began 10 min following the onset of the photophase and the last 30-min playback always ended 10 min prior to the end of the photophase. This dispersal of song throughout a long-day photoperiod was an attempt to mimic the experience of free-living females during the breeding season (Slagsvold, 1977; Leitner *et al.*, 2001). In order to mask inter-chamber sounds, white noise was played in the room housing the chambers.

Shortly after the onset of the photophase on the 15th day, females were transferred to identical, individual sound-attenuation chambers and held in isolation on the same long day photoperiod. On the subsequent day, females were exposed to either playback of novel special song, playback of novel non-special songs, or silence for 90 min and brains were collected. Conditions of final playback were counterbalanced across groups in the fourteen-day experience playback such that there were six females per experimental condition (special experience, special stimulus; special experience, not special stimulus; not-special experience, special stimulus; not-special experience, not-special

stimulus) and two females per control group (special experience, silence; not-special experience, silence). Playback and brain collection was conducted in two cohorts and experimental conditions were counterbalanced across the cohorts to ensure all samples were collected during the same time period post final stimulus playback.

Brain and Blood Collection

Twenty-five minutes after the end of stimulus playback, brains were collected from females in steps. The first step was rapid decapitation, followed by brain extraction and fixation for 2 h in 5% acrolein in phosphate buffered saline (PBS) (pH of 7.4). Brains were then washed in PBS for 15 min four time and cryoprotected in 30% sucrose. After 24 h at 4°C, brains were flash frozen on dry ice and stored at −80°C. Trunk blood samples were collected in heparinized microcentrifuge tubes and centrifuged for 5 min at 9000xg. Plasma was decanted and stored at −80°C until assayed for estradiol (E2) via enzyme immunoassay (EIA). Oviduct length, oviduct mass, follicle volume, and ovary mass were also measured.

Song Recordings

Songs used in this experiment were recorded from five male American Singer canaries that were implanted subcutaneously with T-filled silastic implants (Dow Corning; outside diameter = 1.65 mm, inside diameter = 0.76 mm; 12 mm in length filled with 10 mm of T, Sigma-Aldrich T-1500) to ensure high rates of singing, in the same fashion as in previous studies (Alward *et al*., 2013; 2016; 2017). Recordings from these males were taken over several different days (at least three days per male). Females for both experiments were never in contact with, or heard the songs of, the males used for stimuli recordings. Spectrogram examples for each category of stimulus can be seen in Figure 1.

Experiment 1. To produce pseudosong stimuli, we extracted 100 special syllables and 100 non-special syllables from the song files used in the full song experiment. From these syllables, we created 965 special pseudosongs and 965 non-special pseudosongs. Each pseudosong was composed of an intro note sequence (A), three syllables - either special (S) or non-special (N), and a closing note sequence (C) in an

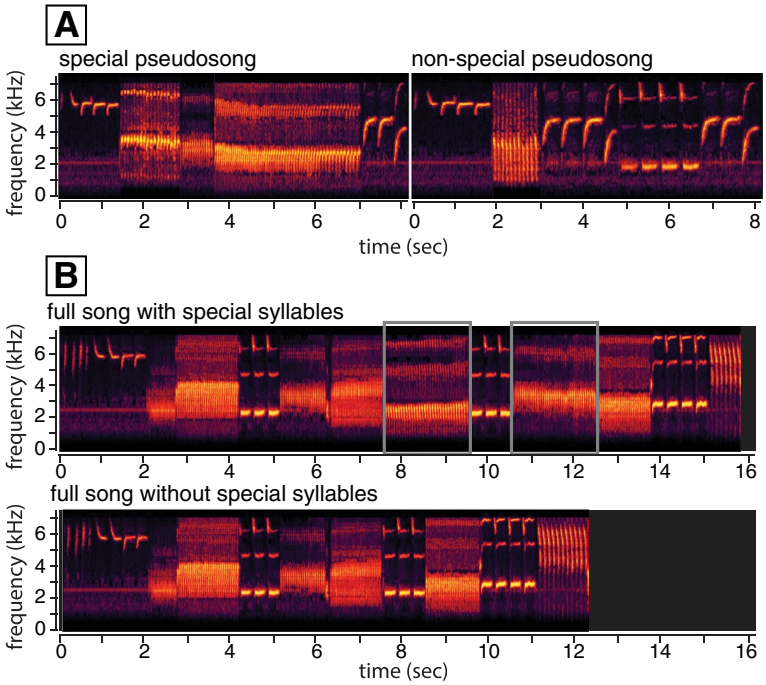

Figure 1 Examples of stimuli used in experiment 1 (a) and experiment 2 (b). (a) Pseudosongs were composed of the same intro note sequence and outro note sequence surrounding either three special or non-special syllables. (b) Special full song stimuli were unedited full songs recorded from testosterone-treated males that contained special syllables (surrounded by gray rectangles). Non-special full song stimuli were the same songs with special syllables removed. [Colour figure can be viewed at wileyonlinelibrary.com]

A-SSS-C or A-NNN-C pattern, depending on condition. Special or non-special syllables were selected randomly from the syllables available, such that each pseudosong had three unique syllables of that type. Therefore, the three syllables within a pseudosong may have originated from the song of the same male or different males. Construction of these pseudosongs was similar to stimuli for previous special syllable playback studies (Monbureau *et al.*, 2015). For the final playback, each female was exposed to a unique set of special pseudosongs or non-special pseudosongs based on experimental group. Each female heard 90 min of pseudosong playback, selected randomly from the 965 we had created, as described above. On average, each female heard 723 pseudosongs during the 90 min playback. Random selection of the pseudosongs occurred independently for each bird, so no two females heard the exact same stimulus set.

Experiment 2. In order to create non-special full song stimuli, each of the special song clips was examined in Adobe Audition and any syllables resembling special syllables, with a two-note structure and rapid frequency modulation, were clipped from the recording and the pieces of the recording on either side of the special syllable were joined. There were a total of 530 full song clips (265 special and 265 non-special) with 400 used in the experience playback and 130 used for the final stimuli playback. Songs from all five individuals were represented in both experience and stimulus playback to control for differences due to individual identity. However, none of the song clips were used in both types of playback (experience and stimuli) so that the songs in the stimuli playback would be novel for all birds regardless of experimental condition. During each 30-min playback, song clips (either special or non-special, depending on condition) were randomly selected to play until the 30-min mark was reached. Although non-special songs were slightly shorter than special songs, due to the removed special syllable, each female head the same total amount of song (30 min per each epoch during the experience playback and 90 min during the final playback). On average, each female in the non-special song experience condition heard 43 songs per 30 min epoch while females in the special song experience condition heard 42 songs per 30 min epoch. For the final 90 min playback, females in the non-special song condition heard an average of 135 songs while females in the special song condition heard an average of 130 songs. In a similar fashion to experiment 1, random selection of the songs occurred independently for each chamber, so females in the same chamber during the experience heard the same experience song set, distinct set from females in other chambers, but during the final playback no two females heard the exact same song set. Playback volume varied due to natural variation in the male's song, but ranged from 51 to 82 dB SPL with an average of 72 dB.

Quantification and Statistical Analysis

Videos were recorded of females during the final pseudosong or full song playback and behavior was quantified. Previous studies have shown that the ZENK protein expression is more protracted than mRNA expression and peaks in response to song playback between 1 and 2 h following the stimulus onset (Mello and Ribeiro, 1998). We therefore quantified behavior for the first thirty minutes of playback so that our behavioral measures would correspond with the IEG expression quantified from females sacrificed 90–120 min following the stimulus start. Behavioral measures were collected in 1-min time bins. For each bin, the number of vocalizations, CSDs, and perch hops were counted. The vocalizations quantified were short calls, short trills, and female specific trills, which have been previously identified as typical responses of female canaries to male songs (Amy *et al.*, 2015). Since most females did not perform CSDs, the video for the entire playback was observed to verify that CSDs did not occur later in the playback.

Plasma Estradiol Enzyme Linked Immunoassay (EIA)

Heparinized capillary tubes were used to collect baseline blood samples from the brachial vein and experimental blood samples were collected from severed carotid arteries at time of tissue collection and placed in heparinized microcentrifuge tubes. The volume of whole blood collected during sampling was 75–300 µl. To minimize the effects of stress hormone release on E2 concentrations, blood samples were collected within 3 min after removing the bird from its home cage. Samples were centrifuged at 9000*g* for 5 min at 4°C and the supernatant plasma was collected and stored at −80°C. Plasma concentrations of E2 were measured using an enzyme-linked immunoassay from Cayman Chemical (Estradiol ELIA Kit, cat # 582251, Ann Arbor, MI). The assay was validated for use in canary plasma via standard validation protocol (as described in Gill *et al.*, 2015). Briefly, a test for parallelism and recovery of added mass were performed to assess assay sensitivity and specificity. Samples were run in duplicate and the mean intra-assay coefficient of variance was 5.64%.

Immunocytochemistry

Brain tissue was sectioned with a cryostat (Microm HM 500 OM) at 30 μm in the sagittal plane and immunocytochemistry was performed to either visualize the ZENK protein or to double-label ZENK and tyrosine hydroxylase (TH). Assays were run using well established immunohistochemical protocols (Lynch *et al*., 2012). Briefly, for double labeling with TH followed by ZENK, free-floating sections were washed in phosphate-buffered saline (PBS, 0.01M, pH 7.5) and treated with 0.5% H_2O_2 for 30 min to block endogenous peroxidases. After three rinses in PBS containing 3% Triton-X (PBST), sections were placed for 1 h in blocking solution (20% normal horse serum (NHS) in PBST). Sections were then incubated for 48 h at 4°C in 2% NHS and primary antibody (1:10,000, mouse, Tyrosine hydroxylase, Immunostar, cat#: 22941) in PBST. Sections were then incubated for 1 h in biotinylated horse anti-mouse secondary antibody (1:250, Vector, cat#: BA-2000) in PBST. Antibody bound to ZENK protein was visualized using Vectastain ABC Elite kit (Vector Laboratories) and 3,3′-diaminobezidene tetrahydrochloride chromagen, yielding a brown reaction product (Vector, cat#: SK-4100). To double-label for ZENK, sections were then washed in PBS, incubated at 4°C in 20% normal goat serum (NGS) in PBST for 1 h, and incubated in 5% NGS and primary antibody (1:2,000, rabbit, Egr-1, Santa Cruz, cat#: sc-189) in PBST for 48 h. Sections were washed in PBS and incubated in biotinylated goat anti-rabbit secondary antibody (1:250, Vector, cat#: BA-1000) in PBST for 1 h. Sections were again incubated in avidin-biotin horseradish-peroxidase complex (Vectastain ABC Elite Kit, 1:200) for 1 h, and visualized using a diaminobenzidine peroxidase substrate kit (Vector, cat#: SK-4100), with nickel enhancement for a black reaction product. Sections were subsequently mounted onto gelatin-coated microscope slides. The immunocytochemistry protocol for only labeling ZENK was the same as described above, with the steps from NHS blocking through the first ABC visualization removed.

The specificity of the staining observed with the anti-ZENK and anti-TH antibodies employed in the present study have been previously validated for used in several avian species, including the canary (Bailhache and Balthazart, 1993; Mello and Ribeiro, 1998; Lynch *et al*., 2013).

Quantification and Statistical Analysis

All quantification procedures were conducted blind to the experimental condition of each animal. We sampled from CMM and NCM using a method previously described (Sockman *et al*., 2002). Photomicrographs (2600 pixel × 2060 pixel RGB images) were captured with a video camera (AxioCam HRc, Zeiss Instruments) mounted to a microscope (Axioskop EL-Einsatz, Zeiss Instruments) every fourth section from the midline to approximately 1200 μm bilaterally. For all areas sampled, a grid was placed in accordance to anatomical markers at 10× magnification before switching to 20× magnification to ensure that the sampling area was consistent across sections. For CMM, the grid was aligned to the lamina mesopallium ventralis in the most dorsal position possible. For dorsal NCM, the grid was aligned along the lateral ventricle in the most dorsal, posterior point. For ventral NCM, the grid was aligned as ventrally as possible. (See Fig. 2 for example photomicrographs, Fig. 2a for window placement.) For NCC, the grid was aligned just above the most dorsal tip of the dorsal acropallium in sections with a visible dorsal section of the lateral mesencepalic nucleus (MLd).

From these photomicrographs, the number of ZENK-immunoreactive cells were counted using an automated Fiji (Schindelin *et al*., 2012) macro routine. The macro, written for this analysis, subtracted background noise in the image, performed an automatic thresholding algorithm (ImageJ-default, Yen), and ran the analyze particles routine to count the number of cells. A different macro routine was used for quantifying the percentage of each photomicrograph covered by TH-immunoreactive fibers. First, color deconvolution was performed on the image using user-inputted regions of interest to separate out areas where there was a high density of brown staining. Next, the image was automatically set to a threshold (Yen) and the ImageJ "Measurement" function was performed to measure the percentage area with such staining.

For the pseudosong experiment, we analyzed ZENK-ir, estradiol concentrations, and behavior measures using Welch two-sample *t*-tests. For the full song experiment, we analyzed ZENK-ir in CMM, NCMd, NCMv, and NCC using a two-way ANOVA with the factors of experience and stimulus. For ZENK expression, analysis was performed on the log-transformed mean count for each animal (figures depict untransformed data) due to a high positive skew. We then conducted a post-hoc Tukey multiple comparisons of means to distinguish effects between stimulus treatments. Behavioral measures (perch hops, total calls, short calls, simple trills, and female specific trills) were analyzed via two-way ANOVA design. One sample was excluded from analysis of final estradiol levels due to low volume of plasma collected. The remaining estradiol data were log transformed and analyzed via two-way ANOVA as ZENK and behavior measures.

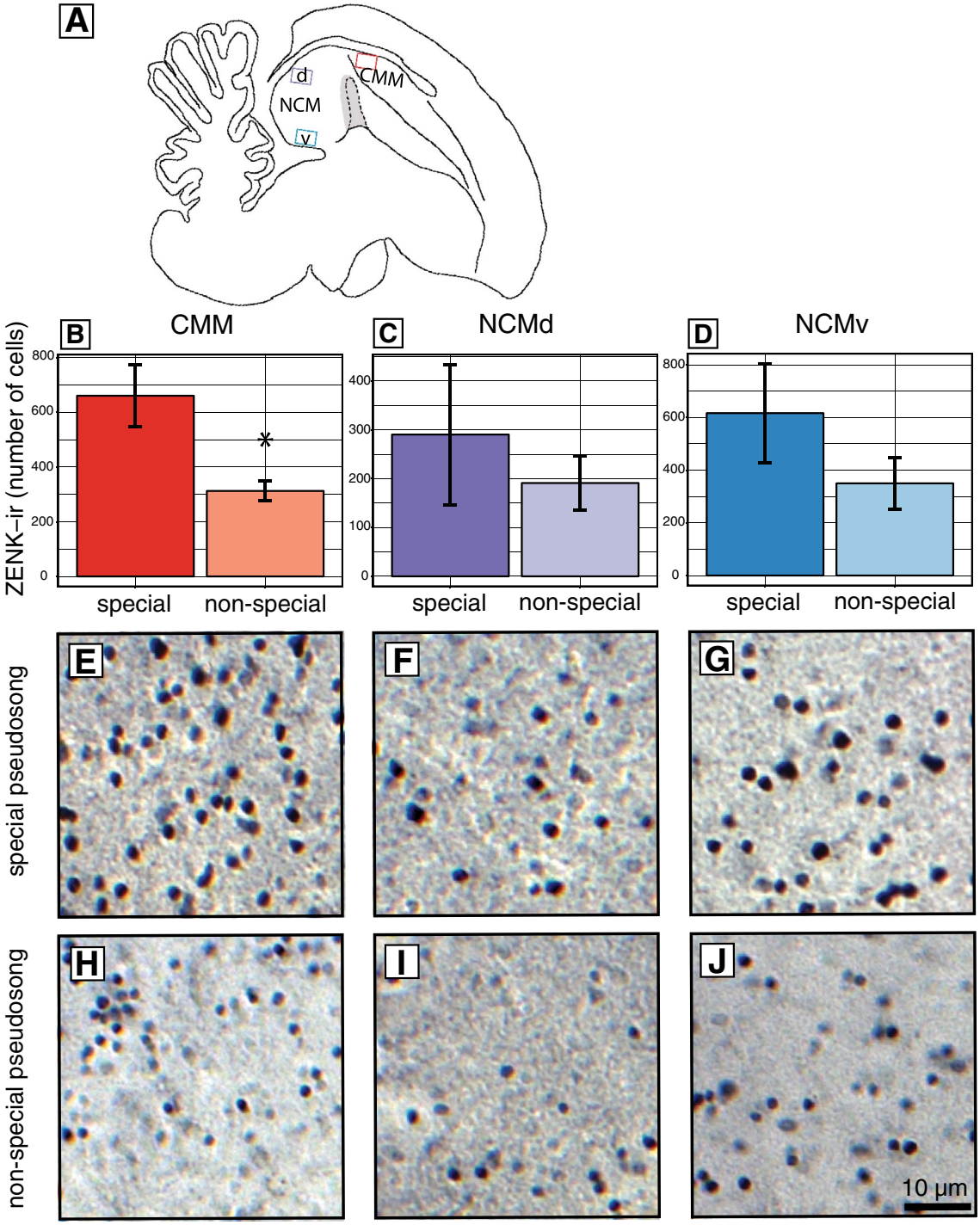

Figure 2 Effect of pseudosong playback on ZENK expression. (a) Rectangles indicate the locations of photomicrographs collected for quantification of ZENK-ir. (b–c) Bar graphs depict ZENK-ir cells (mean cell count ± standard error of the mean) in 855 × 678 µm photomicrographs taken from (b) CMM, (c) NCMd, and (d) NCMv. Bars with an asterisk (*) are significantly different from other bars in that brain region. (e–j) Examples of 855 × 678 µm photomicrographs showing ZENK expression in CMM (e,h), NCMd (f,i), and NCMv (g,j). [Colour figure can be viewed at wileyonlinelibrary.com]

We analyzed TH-ir innervation and physiological variables (follicle volume, ovary mass, oviduct mass, oviduct length) by Welch two sample *t*-tests.

RESULTS

Experiment 1. The effect of pseudosong containing special syllables on IEG expression

IEG Expression. Consistent with previous studies, we observed a difference in CMM IEG expression between special pseudosong and non-special pseudosong. CMM ZENK-ir was greater in females exposed to playback of pseudosongs containing special syllables ($t(7) = 3.49$, $P = 0.01$) (Fig. 2). Furthermore, the effect size for this analysis ($d = 1.88$) indicates a large effect. In NCMd and NCMv, ZENK-ir did not differ between females exposed to playback of pseudosong containing special syllables or pseudosong not containing special syllables ($t(7) = 0.28$, $P = 0.78$ and $t(7) = 0.90$, $P = 0.40$, respectively). The effect sizes for these analyses were small ($d = 0.16$ and $d = 0.48$).

For pseudosongs, the correlation among ZENK expression levels in auditory processing areas was limited. ZENK expression in CMM was not significantly correlated with expression in NCMv ($r(7) = 0.66$, $P = 0.05$) or expression in NCMd ($r(7) = 0.34$, $P = 0.37$) or NCC ($r(7) = 0.73$, $P = 0.49$). In addition, there was not a significant correlation between ZENK-ir in the dorsal and ventral segments of NCM ($r(7) = 0.29$, $P = 0.45$).

ZENK-ir was also measured in NCC with no significant effects based on stimulus type ($t(7) = -0.05$, $P = 0.96$) and a negligible effect size ($d = -0.03$).

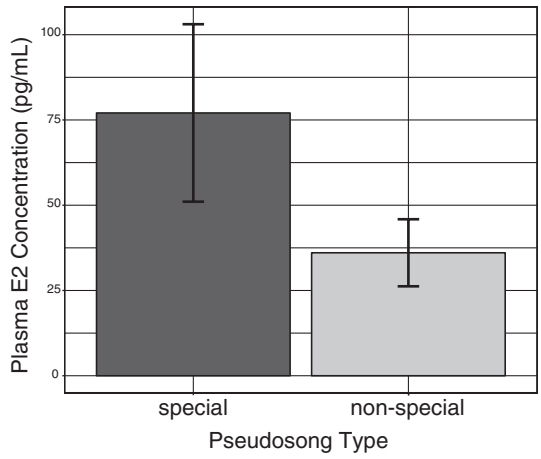

Figure 3 Circulating plasma estradiol levels (pg/mL ± standard error of the mean) at the time of brain collection, shortly after pseudosong playback.

Estradiol. Although females exposed to playback of special syllable pseudosongs tended to have higher estradiol concentrations (M = 77.06 pg/mL) than females exposed to playback of psuedosongs without special syllables (M = 36.06 pg/ML), this difference did not reach statistical significance ($t(5) = 1.47$, $P = 0.20$) (Fig. 3). However, the effect size of this analysis ($d = 0.62$) exceeded Cohen's estimate for a medium effect. Estradiol concentrations were significantly correlated with ovary mass ($r(7) = 0.807$, $P = 0.008$) and with ZENK-ir in NCMv ($r(7) = 0.815$, $P = 0.007$).

Physiological Measures. There were no significant differences in physiology measures between the two stimulus groups. Both groups were similar in oviduct length ($t(7) = -0.46$, $P = 0.66$), oviduct mass ($t(7) = -0.50$, $P = 0.64$), follicle length ($t(6) = -0.23$, $P = 0.82$), and ovary mass ($t(7) = -0.70$, $P = 0.51$).

Behavior. We did not observe a difference in behavior between females exposed to special pseudosong or non-special pseudosong. However, several behavioral variables did correlate with some measures of ZENK-ir. ZENK expression in CMM was significantly correlated with the number of short trills a female produced in response to pseudosong playback ($r(7) = 0.820$, $P = 0.01$). In addition, ZENK expression in NCC was significantly correlated with the number of female specific trills produced ($r(7) = 0.724$, $P = 0.04$).

Three females performed copulation solicitation displays (CSDs) in response to pseudosong playback (two in response to special syllable pseudosong, one in response to non-special pseudosong). The expression of ZENK in NCC was higher for females who performed CSDs (M = 675.50 cells) than females who did not (M = 411.89 cells).

Experiment 2. The effect of experience with full song containing special syllables on IEG response to later song playback

IEG Expression. We did not observe a difference in IEG expression between full song containing special syllables and full song without these special syllables (see Fig. 4 for example photomicrographs). There was a main effect of the final song stimulus on ZENK expression in CMM, NCMd, and NCMv ($F_{2,22} = 17.50$, $p < 0.001$; $F_{2,22} = 6.59$, $P = 0.006$; and $F_{2,22} = 8.93$, $P = 0.001$, respectively) with small to medium effect sizes (CMM $\eta^2 = 0.61$, NCMd

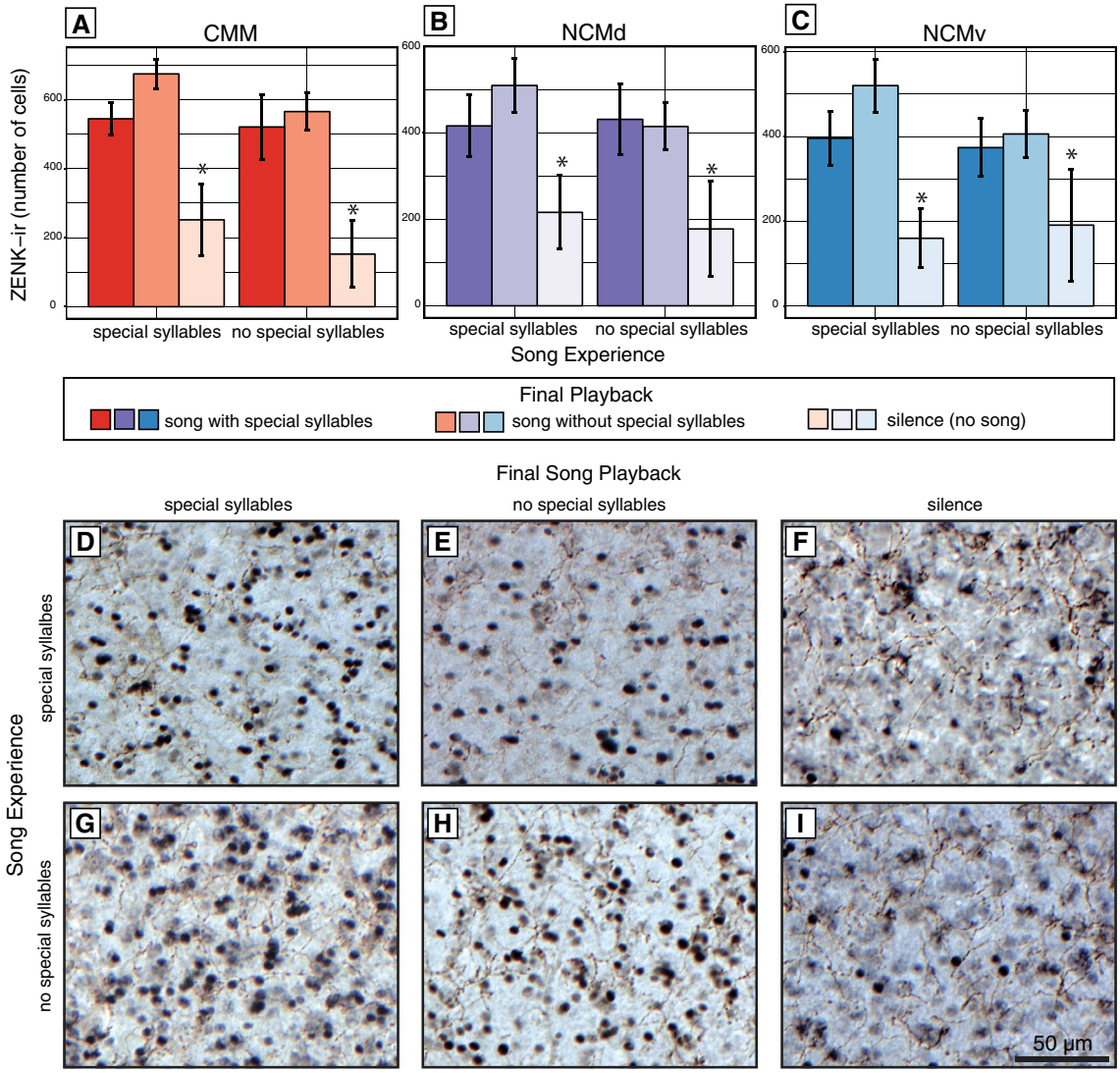

Figure 4 Effect of final full song playback on ZENK expression. (a–c) ZENK expression (mean cell count ± standard error of the mean) in 855 × 678 µm photomicrographs taken from (a) CMM, (b) NCMd, and (c) NCMv. Bars with an asterisk (*) are significantly different from other bars in that brain region. (d–i) Examples of 855 × 678 µm photomicrographs showing ZENK expression and TH fiber innervation in CMM. Females were in one of six conditions: (d) special experience, special stimulus, (e) special experience, not-special stimulus, (f) special experience, silence, (g) not-special experience, special stimulus, (h) not-special experience, not-special stimulus, or (i) not-special experience, silence. [Colour figure can be viewed at wileyonlinelibrary.com]

$\eta^2 = 0.38$, and NCMv $\eta^2 = 0.45$). However, a post-hoc Tukey multiple comparisons of means revealed that this difference was driven by significant differences between silent controls and hearing song (Fig. 4). In addition, song experience had no main effect on ZENK expression. ZENK-ir in CMM, NCMd, and NCMv did not differ between females exposed to 14 days of song with special syllables and females exposed to 14 days of songs without special syllables ($F_{1,22} = 2.38$, $P = 0.14$, $\eta^2 = 0.09$; $F_{1,22} = 0.57$, $P = 0.46$, $\eta^2 = 0.03$; and $F_{1,22} = 0.70$, $P = 0.41$, $\eta^2 = 0.03$, respectively). The interaction between song experience and final playback stimulus was not significant in any of the brain regions measured (CMM: $F_{2,22} = 0.79$, $P = 0.47$, $\eta^2 = 0.06$; NCMd: $F_{2,22} = 0.07$, $P = 0.74$, $\eta^2 = 0.03$; NCMv: $F_{2,22} = 0.14$, $P = 0.87$, $\eta^2 = 0.01$).

In general, ZENK-ir was higher in CMM than NCM, but the effects reported above did not differ between these regions. For full song stimuli, ZENK expression was highly correlated among auditory processing areas. ZENK expression in CMM was

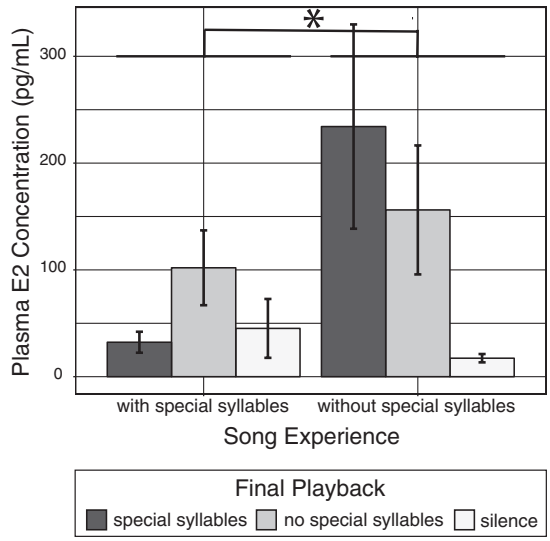

Figure 5 Plasma estradiol levels (pg/mL ± standard error of the mean) at the time of brain collection, shortly after final song playback. Females who had experience with special syllables had significantly lower estradiol concentrations than females who had experience without special syllables (denoted by asterisk).

significantly correlated with expression in NCMd ($r(26) = 0.92$, $p < 0.001$) and in NCMv ($r(26) = 0.91$, $p < 0.001$). ZENK-ir was also highly correlated between the dorsal and ventral segments of NCM ($r(26) = 0.95$, $p < 0.001$).

ZENK-ir was also measured in NCC and nucleus taeniea (TnA) with no significant effects (NCC experience: $F_{1,21} = 1.01$, $P = 0.33$, $\eta^2 = 0.04$, stimulus: $F_{1,22} = 0.38$, $P = 0.69$, $\eta^2 = 0.04$, interaction: $F_{1,21} = 0.307$, $P = 0.74$, $\eta^2 = 0.03$; TnA experience: $F_{1,22} = 0.09$, $P = 0.76$, $\eta^2 = 0.01$, stimulus: $F_{2,22} = 0.95$, $P = 0.40$, $\eta^2 = 0.08$, interaction: $F_{2,22} = 0.11$, $P = 0.90$, $\eta^2 = 0.01$).

Tyrosine Hydroxylase. There was no difference in the percent of photomicrograph area covered by TH fibers between females who experienced song with special syllables and females who experienced song without special syllables in any of the brain regions analyzed (CMM: $t(18) = 0.99$, $P = 0.34$, $d = 0.14$; NCMd: $t(24) = 0.47$, $P = 0.64$, $d = 0.01$; NCMv: $t(22) = 0.52$, $P = 0.61$, $d = 0.01$; NCC: $t(21) = -0.13$, $P = 0.89$, $d = -0.05$; TnA: $t(20) = 0.48$, $P = 0.63$, $d = 0.04$).

Estradiol. There was a main effect of song experience on final estradiol levels ($F_{1,21} = 5.25$, $P = 0.03$, $\eta^2 = 0.18$) (Fig. 5). The effect of final stimulus playback and the interaction between song experience and final stimulus was not significant (stimulus: $F_{2,21} = 2.93$, $P = 0.07$, $\eta^2 = 0.08$; interaction: $F_{2,21} = 3.18$, $P = 0.06$, $\eta^2 = 0.12$). A post-hoc Tukey multiple comparisons of means showed that females who heard songs with special syllables during the final playback differed significantly by experience ($P < 0.04$).

Physiological Measures. There were no significant differences in follicle volume ($t(20) = 1.54$, $P = 0.14$, $d = 0.58$), ovary mass ($t(21) = 0.95$, $P = 0.35$, $d = 0.36$), oviduct mass ($t(24) = 0.78$, $P = 0.44$, $d = 0.29$), and oviduct length ($t(25) = 0.53$, $P = 0.60$, $d = 0.20$) between females that experienced song with special syllables and females that experienced song without special syllables. Seven of the females had much larger follicular volumes than the average, five of these females had experienced song with special syllables and two had experienced song without.

Behavior. We did not see significant differences in behavior during the final song playback between females based on male song quality heard during experience or final playback. There was no main effect of song experience on total number of calls ($F_{1,20} = 0.47$, $P = 0.50$, $\eta^2 = 0.02$), short calls ($F_{1,20} = 0.17$, $P = 0.68$, $\eta^2 = 0.01$), simple trills ($F_{1,20} = 0.38$, $P = 0.55$, $\eta^2 = 0.02$), female specific trills ($F_{1,20} = 0.01$, $P = 0.95$, $\eta^2 = 0.01$), or perch hops ($F_{1,22} = 0.63$, $P = 0.44$, $\eta^2 = 0.03$). Final playback stimulus had no main effect on total calls ($F_{2,20} = 0.83$, $P = 0.45$, $\eta^2 = 0.08$), short calls ($F_{2,20} = 1.72$, $P = 0.20$, $\eta^2 = 0.15$), simple trills ($F_{2,20} = 0.86$, $P = 0.44$, $\eta^2 = 0.08$), female specific trills ($F_{2,20} = 2.07$, $P = 0.15$, $\eta^2 = 0.17$), or perch hops ($F_{2,22} = 0.64$, $P = 0.54$, $\eta^2 = 0.06$). There was no interaction effect between experience and playback stimulus on total calls ($F_{2,20} = 0.38$, $P = 0.69$, $\eta^2 = 0.04$), short calls ($F_{2,20} = 0.29$, $P = 0.75$, $\eta^2 = 0.03$), simple trills ($F_{2,20} = 0.93$, $P = 0.41$, $\eta^2 = 0.08$), female specific trills ($F_{2,20} = 1.62$, $P = 0.22$, $\eta^2 = 0.14$), or perch hops ($F_{2,22} = 1.26$, $P = 0.30$, $\eta^2 = 0.10$). In addition, there was no significant correlation between any behavior measured and IEG expression.

DISCUSSION

This study demonstrated a robust effect of hearing song on IEG expression in the specialized auditory processing areas of CMM, NCMd, and NCMv. It also replicated previous results indicating that special

syllables enhance ZENK expression in CMM when played back in a limited acoustic context. However, we did not observe a difference in ZENK expression between full songs containing special syllables and those without these syllables. We also did not observe differences in catecholamine innervation of these auditory processing areas due to differing song experience. Song quality experience did have a significant effect on plasma estradiol levels upon later exposure to song, with females who experienced songs with special syllables displaying significantly lower E2 levels. These effects did not extend to physiological development or behavior during final song playback.

Selective Processing of Song Quality

We observed an enhanced ZENK response in CMM for special syllables in a context with limited additional acoustic information, as has been previously demonstrated (Leitner *et al*., 2005). However, this effect did not extend to full song containing special syllables. These results demonstrate that song context is more important than previously realized. A key difference between the two experiments is the acoustic information surrounding the presentation of these syllables. For the pseudosong experiment, stimuli were composed of an intro note sequence, three special or non-special syllables, and an outro note sequence. Playback from this pseudosong experiment more closely resembled those used in previous studies, such as Leitner *et al*.(2005) – where syllables of interest (special or not special) were presented surrounded by synthetic tone-based syllables. Stimuli for our full song experiment were actual songs performed by males with high circulating testosterone levels, with the special syllables removed in the non-special songs. Therefore, all females in the full song study were exposed to complex acoustic stimuli that may contain biologically significant information in other song features, such as temporal windows and fine structure. Perhaps specific syllables, such as special syllables, are not as influential in auditory processing in these areas as the overall song context. There is evidence that IEG expression in songbird auditory processing areas is involved in extracting information from auditory signals and translating this information into the appropriate behavioral response (McMillan *et al*., 2017). For example, conspecific mobbing calls and calls from a predator, which both contain threat information despite being acoustically different, result in increased IEG expression (Avey *et al*., 2011). Since our full songs were all produced by males in breeding condition, regardless of their special syllable content, this IEG expression in CMM and NCM may simply indicate that these songs are all produced by males. Furthermore, we found that ZENK induction in response to full song playback was highly correlated among CMM, NCMd, and NCMv, while ZENK expression in response to pseudosong playback was less correlated among these areas. This indicates that auditory processing of less complex song or syllables may rely on CMM, but processing of full song is highly interconnected between secondary auditory processing areas. Therefore, it is unlikely that CMM alone is responsible for processing information about song quality and salience.

IEG Response

Generally, short songs composed primarily of special syllables would not be found in nature. Therefore, these pseudosongs could be thought of as supernormal stimuli. Animals often respond more strongly to supernormal stimuli than natural stimuli – such as when male silver-washed fritillary butterflies court more aggressively in response to very high frequency flickering stimuli compared to the flickering frequencies actually produced by females in the wild (Staddon, 1975). Therefore, the supernormal feature of frequent special syllables in these pseudosongs may have contributed to the enhanced ZENK expression in CMM that we observed.

The full song experiment was conducted with the expectation of an increase in ZENK selectivity for songs with special syllables, however another possibility is that experience habituates ZENK induction, rather than stimulates it. Song sparrows played songs that they had heard previously exhibit lower ZENK activation in vNCM compared to song sparrows played novel songs (McKenzie *et al*., 2006). In addition, house finches that have been isolated during development, and therefore had no experience with song, have higher ZENK induction in response to song than house finches that experienced song during development (Hernandez and MacDougall-Shackleton, 2004). Since we did not observe differences between females who experienced specials syllables and females that experienced song without special syllables, the effect of experience in either stimulating or habituating ZENK expression is unclear.

Although we did not observe a difference in auditory telencephalon ZENK expression between full songs with or without special syllables, it is possible that experience with these syllables did influence activity in these regions. Experience treatments may need to be longer in duration or have more pronounced

differences in order to produce a significant effect. In addition, while IEGs can provide us with a sense of overall activity of a brain region, we cannot determine the specificity of this activity. It is possible that experience with special syllables influences the connectivity of neurons in these regions, promoting synapses between particular neurons in order to enhance their response to song that contains these syllables. Future studies are needed to determine if experience with particular song types can alter synaptic density or recruitment of specific neurons.

Catecholamine Innervation

We did not observe a difference in tyrosine hydroxylase innervation in auditory processing areas between females who had experienced song with or without special syllables. Unlike the increased probability of DBH-ir fibers in the NCM of female starlings exposed to long-bout songs observed by Sockman and Salvante (2008), there was no increase in the density of TH-ir fibers following exposure to song containing special syllables. Since TH is an enzyme earlier than DBH in the molecular pathway converting L-tyrosine to epinephrine, perhaps changes in catecholamine innervation to auditory areas are restricted more specifically to fibers involved in norepinephrine transmission.

Estradiol Concentration

Estradiol has been shown to induce changes in physiological state that influence responses to acoustic communication (Maney and Pinaud, 2011). This study demonstrates that the reverse process may also be true. Hearing particular acoustic features can induce variation in circulating estradiol. Repeated exposure to these acoustic features, and the consequent endocrine responses, may lead to changes in response to acoustic communication. Females who heard pseudosong composed of special syllables tended to have higher estradiol concentrations, although there was not a significant difference. Final estradiol levels of females that experienced two weeks of song without special syllables were significantly higher than females who had experienced song with special syllables. In addition, estradiol levels of the females who heard song were higher than the females who did not hear song during the final playback. Based on these results, song with special syllables may induce increased circulating estradiol concentrations. Playback of conspecific song to female canaries can stimulate the gonadotropin-releasing hormone system that leads to release of gonadotrophins such as luteinizing hormone (LH) from the pituitary, followed by an increase in sex steroids such as estradiol (Bentley *et al.*, 2000). However, chronic elevated levels of estradiol can lead to negative feedback at the pituitary, inhibiting the release of LH and any consequent increases in estradiol (Shaw *et al.*, 2010; Ubuka *et al.*, 2013). Therefore, repeated exposure to these special syllables, and the consequent chronic elevated circulating estradiol, may prevent an increase in estradiol concentration in response to later song playback – explaining the overall decreased concentrations of circulating estradiol in females who heard song containing special syllables for two weeks.

Estradiol levels differed between the pseudosong experiment (range: 12.39–142.95 pg/mL) and full song experiment (range: 13.41–361.76 pg/mL). One potential explanation is the added two weeks of long day exposure that females in the full song experience received. Perhaps females in the pseudosong experiment required further photostimulation to exhibit similar increases in estradiol. In addition, females in the long day experiment had two weeks of song exposure prior to the final playback and estradiol quantification, while females in the pseudosong experiment had not heard any song for the six weeks prior to final playback and estradiol quantification. As our results support the idea that song exposure influences circulating estradiol, this is a likely explanation of the differences in estradiol between the two experiments.

Conclusions

Our results indicate that hearing song induces IEG expression and that individual salient syllables within the context of a full song are not completely responsible for variation in the magnitude of the IEG response. In addition, we provide initial evidence that, although experience with these syllables does not significantly influence IEG expression, it may influence later endocrine responses to song of varying quality. Females who heard special syllables exhibited reduced circulating estradiol concentrations upon later exposure to song, possibly due to habituation or negative feedback. Determining how repeated exposure to special syllables influences the hypothalamic–pituitary–gonadal axis could provide further insight into the role of the social environment in driving hormone-mediated plasticity during the breeding season. Additionally, to address more precisely the relative roles of special syllables and song context on IEG expression, future studies should be performed to investigate the biological significance of different acoustic features in overall song composition.

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CONFLICT OF INTEREST

The authors have nothing to disclose.

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