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Research report

Vocal production and playback of altered song do not affect ZENK expression in black-capped chickadees (*Poecile atricapillus*)

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HIGHLIGHTS

- We examined neural response to playback of typical and altered song in chickadees.
- Altering song did not reduce ZENK response in NCM and CMM of either sex.
- Eliminating the frequency change between song notes decreased vocal response to songs.
- Increased vocalizing was not associated with increased ZENK in HVC.

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ABSTRACT

The two-note *fee bee* song of the black-capped chickadee (*Poecile atricapillus*) is sung at many different absolute frequencies, but the relative frequencies between the start and end of the *fee* note (the glissando) and between the *fee* and the *bee* notes (the inter-note ratio) are preserved regardless of absolute frequency. If these relative frequencies are experimentally manipulated, birds exhibit reduced behavioural responses to playback of altered songs both in field studies and laboratory studies. Interestingly, males appear to be sensitive to alterations in the glissando, while females appear to be sensitive to alterations in both the glissando and the inter-note ratio. In this study, we sought to determine whether the behaviour of male and female chickadees corresponds to differences in *zenk* protein immunoreactivity (ZENK-ir) in auditory perceptual regions following playback of *fee bee* songs with typical and altered pitch ratios. Overall, there was a small but significant sex difference in ZENK-ir (females > males), but altering relative frequencies did not reduce ZENK-ir compared to typical song. Birds did vocalize less in response to playback of songs that lacked an inter-note interval, but amount of singing *fee bee* song, *chick-a-dee* calls, or *gargles* was not correlated with ZENK-ir in perceptual regions (caudomedial nidopallium, NCM and caudomedial mesopallium, CMM) or in HVC, which is part of the song system. Our results confirm that ZENK-ir in NCM and CMM is not involved in fine-grain perceptual discrimination, however it did not support the idea that increased vocalizing increases ZENK-ir in HVC.

1. Introduction

Birdsong plays a vitally important role in the breeding behaviour of both males and females [7]. Among male songbirds, it is a key aspect of territorial defense against male conspecifics and plays a role in aggressive interactions between rival males [48,44]. In the case of females, male song is used as an indicator of the singer's quality, reflecting factors such as age and dominance, and is thus used in mate selection (e.g., [1,12]).

Black-capped chickadees (*Poecile atricapillus*) are a typical temperate songbird, where male song operates in both territory defense and mate choice [30]. As part of their large vocal repertoire, they produce a simple, two-note song, phonetically called *fee bee* [20]. Sung primarily by males, it is used in aggressive and sexual contexts as described above, and sung extensively during the dawn chorus. While varying in absolute frequency, the *fee bee*'s relative frequency structure is governed by two pitch ratio rules

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that are uniform among the vast majority of North American chickadee populations [24]. Specifically, the pitch difference between the start and end of the initial *fee* note (a downwards inflection referred to as the "glissando") and between the end of the *fee* and the start of the *bee* (referred to as the "inter-note interval" as in previous studies) are governed by highly consistent pitch ratios (1.056 and 1.134, respectively) ([49], see Fig. 1A). Socially dominant, high-ranking males have greater reproductive success than subordinate, low-ranking males [33,31], and this difference may relate to female assessment of male quality via song structure: dominant males are better able to achieve the species-typical pitch ratio between the *fee* and *bee* notes [9].

Previous behavioural studies have demonstrated the specific importance of the pitch ratio rules characterizing the *fee bee* song via playback experiments using songs with species-typical pitch ratios and songs where those pitch ratios have been altered experimentally. In a field playback study comparing behavioural response to typical and altered song, male chickadees displayed a weaker territorial response to songs lacking the glissando change in the *fee* note compared to intact song, indicating that the glissando is important for male recognition of conspecific song [45]. In contrast, male chickadees did not show different levels of territorial response to songs with the inter-note pitch interval altered [41,45]. In a lab experiment, Ratcliffe and Otter [40] found that estradiol-implanted females responded more strongly to normal songs than to songs with either the inter-note pitch interval altered or the glissando inflection removed. Thus, in terms of behavioural response, it appears that females attend to both of the pitch ratio rules characterizing male song, whereas males attend only to the glissando. However, this does not appear to be due to a deficit in the ability to perceive the difference: Njegovan and Weisman [32] used an operant discrimination paradigm to demonstrate that male chickadees are capable of discriminating among note pairs with different inter-note pitch ratios. In that study, males were able to successfully discriminate between rewarded stimuli featuring an inter-note ratio of 1.12 (close to the species-typical ratio of 1.134) and non-rewarded stimuli with ratios of 1.00 and 1.26. We wondered, then, if the behavioural differences and perceptual performance described above would be reflected by changes in responsiveness within avian brain regions associated with the perception and recognition of conspecific vocalizations.

Acoustic information is processed in the songbird brain via an ascending auditory pathway that is analogous to the brain circuitry governing human auditory perception, and which makes connections with the pathways that control vocal learning and production in songbirds, intersecting at the nucleus HVC [38]. Within the ascending auditory pathway, caudomedial nidopallium (NCM) and caudomedial mesopallium (CMM) appear to play particularly important roles in classifying acoustic information [4]. For example, electrophysiological studies show these areas respond more to conspecific vocalizations than to heterospecific vocalizations [8] or other, non-avian sounds [21]. Similarly, lesions to CMM in zebra finches (*Taeniopygia guttata*) interfere with their ability to discriminate between conspecific and heterospecific vocalizations [26].

Activity in NCM and CMM in response to acoustic stimuli is most commonly measured by quantifying the number of cells that express the immediate-early gene *zenk* and its protein (referred to as ZENK to distinguish it from the *zenk* gene; [25]). This neural response, in black-capped chickadees and other songbirds, varies depending on a number of factors related to characteristics of both the vocalization and the listener. For example, ZENK expression in response to conspecific vocalizations is typically higher in NCM of male chickadees than in female chickadees [34,2]. Sex of the singer also affects neural response, with greater ZENK expression to chickadee songs produced by males compared to those produced by females [2]. Breeding condition is also important: *zenk* protein immunoreactivity (ZENK-ir) within the dorsal NCM of male chickadees was higher in response to conspecific compared with heterospecific vocalizations, but only when the chickadees were in breeding condition [37]. In a recent experiment by Avey et al. [3], ZENK expression in NCM and CMM did not differ in response to conspecific calls compared to heterospecific calls of similar acoustic characteristics, suggesting that differences in reactivity may primarily result from differences in the structural characteristics of the vocalizations, regardless of whether or not they happen to be conspecific.

In contrast, ZENK expression in HVC increases not in response to hearing song, but to producing it. In zebra finches and canaries (*Serinus canaria*) singing increases both *zenk* mRNA [22] and protein [29], and the amount of expression increases with number of songs produced [22]. However, this linear relationship between the number of songs and amount of ZENK expression in HVC may not occur in all species; for example, although male house sparrows (*Passer domesticus*) that sang had ZENK labelling in HVC, there was no correlation with amount of labeling and amount of singing [42].

The objective of this study was to examine the behavioural and neural responses of black-capped chickadees to songs with typical pitch ratios compared to songs with altered pitch ratios. In particular, we wanted to see if ZENK-ir in NCM and CMM corresponds with the behavioural differences observed in response to normal and altered songs in previous research. Further, in light of the sex differences suggested by the behavioural studies outlined above, we sought to determine whether males and females differed with respect to amount of ZENK-ir in response to these songs. Specifically, we expected ZENK-ir in males to be higher in response to playback of normal songs compared to songs with the glissando eliminated, and in females to be higher in response to playback of normal songs compared to songs with either of the pitch ratios altered. We also examined the relationship between vocalizing and ZENK-ir in both the perceptual areas (NCMd, NCMv and CMM) and HVC.

2. Methods

2.1. Animals

Adult black-capped chickadees ($n = 26$ males, 23 females) were captured within the Halifax Regional Municipality during winter months between November 25, 2011 and February 16, 2012 (CWS Permit # ST2779). Individuals were attracted via playback of chickadee vocalizations (including calls and songs, recorded in Ontario at least a decade prior to this study) and captured using potter traps baited with sunflower seeds. Sex of the bird was assessed at time of capture based on mass, wing chord and tail length [15], and confirmed by examination of gonads at perfusion. Age of the bird was determined by the extent and shape of white in the outer retrices [39]. Non-adult birds (birds born in the same year of capture or spring previous to capture) were released immediately; the age of adult birds was confirmed at perfusion via observation of skull pneumatization. In order to minimize stress, birds were transported to individual cages at Dalhousie University's animal care facilities within 1 h. of capture. In addition, all birds were caught prior to noon in order to provide adequate habituation time in animal care prior to the end of the first day. All methods and procedures in this study were approved by Dalhousie University's University Committee on Laboratory Animals (Protocol # 08-020).

2.2. Housing, feeding, photoperiod

Upon arrival to the laboratory, birds were placed in individual cages (91 cm wide × 41 cm high × 46 cm deep) made of

galvanized-after-welding steel mesh with a stainless steel pan for collecting waste. Each cage contained wooden perches, a swing and a water bath, as well as evergreen boughs, and birds had *ad libitum* access to drinking water, grit and food (a mix of husked sunflower seeds and Mazuri Small Passerine diet feed, with unhusked sunflower seeds on top). Birds were housed together in rooms containing 10–15 birds each, and the initial light cycle in each room was set to match natural day length at time of capture. Room temperatures were maintained at approximately 18 °C. A minimum of seven days after capture (to allow for habituation to the laboratory setting), all birds were photostimulated: the light cycle was extended to 15 h light: 9 h dark to reflect day length during the early spring and bring the birds into breeding condition [35]. Each bird was photostimulated for 5–6 weeks before being transferred to the playback chamber.

2.3. Playback chamber

The playback chamber consisted of a standard budgerigar cage (47 cm wide × 30 cm high × 30 cm deep) containing drinking water, grit and food (same mix as above), as well as three wooden perches positioned at equal height and distributed equidistantly along the longest dimension of the cage. The cage was on a shelf within a sound-attenuating booth (Eckel Industries, Model #AB2000). The shelf also held a speaker (Cambridge Audio Atom V.5; frequency response range 5–20,000 Hz) aimed directly at one end of the cage and connected to an iPod nano (Apple) via an amplifier (Cambridge Audio Azur 340A/340A SE), both of which were located outside the booth. A video camera (Sony Model # DRC-HC28), attached to an adjustable steel rod suspended near the top of the booth and connected to an iMAC (equipped with iMovie for video/audio recording during playback) outside the booth, was pointed directly down on the cage for a "bird's-eye" view. The speaker and the video camera were connected to their external components through a panel located on the interior wall of the booth. Prior to a bird being transferred to booth, volume of the selected playback recording was adjusted to 70 dB (at the perch closest to the speaker) using a decimeter (Extech Instruments, Model #407750). The booth was lit by four cool-white 20-watt fluorescent light bulbs (Sylvana) located against the outer surface of a window in the booth's wall that was cased in so that light within the booth could be controlled. The light cycle in the booth matched schedule for photostimulation (15 h light: 9 h dark) and the temperature averaged 18 °C.

2.4. Stimulus vocalizations

Stimulus vocalizations for playback consisted of *fee bee* songs of four different types: (1) Normal, with species-typical frequency ratios between the start and end of the *fee* note (1.056) and between the end of the *fee* and start of the *bee* (1.134); (2) Flat Fee (FF), with frequency ratio from start to end of *fee* note reduced to 1.0; (3) Flat Inter-note Pitch Interval (FI), with frequency ratio between end of *fee* and start of *bee* reduced to 1.0; and (4) Flat Fee/Flat Inter-note Pitch Interval (FFFI), with both frequency ratio alterations. We began with unaltered recordings of six *fee bee* songs taken from a set of recordings originally made in Ontario, Canada between February 1999 and May 2000 that was used in previous studies (see Refs. [34,37]). They ranged in absolute frequency (measured one quarter of the way into the *bee* note) from 3100 to 3350 Hz, reflecting the normal range of *fee bee* song frequencies [49]. We prepared the three other stimulus types by taking each of the six normal songs and altering the frequency ratios in each of the three ways described above using the audio editing program Audition 2.0 (Adobe Systems Inc., San Jose, CA). This gave us a total of 24 song stimuli (six Normal, six FF, six FI, six FFFI) so that every potential subject of a given sex would hear a unique playback stimulus—a normal or altered song beginning at a particular frequency. Fig. 1 shows sound spectrograms of an original stimulus (A) and the three altered stimuli (B–D) made from that stimulus.

2.5. Playback protocol

Following photostimulation, each bird was transferred to a standard budgerigar cage inside one of four sound-attenuated booths (described above) during the morning of the day prior to hearing playback stimuli. Although the booths were located in the same room, sounds from one could not be heard inside another when both doors were closed. The door of each booth was kept open during the day of transfer to minimize stress during habituation and then closed overnight. The next day, following a total habituation time of approximately 24 h, each bird was videotaped for 90 min:30 min each of pre-playback, playback and post-playback. Prior to the 90 min period, the door of the booth was opened briefly and quietly in order to turn on the video camera (more than one booth was never open at the same time). Following the 30 min pre-playback period, we presented the bird with 30 min of playback from one of the four stimulus groups described above, during which

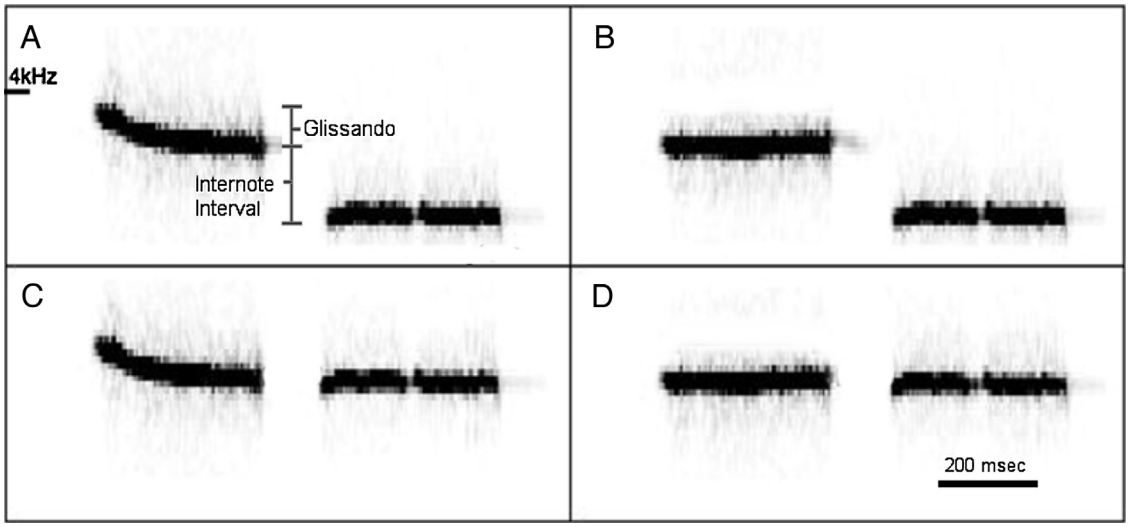

Fig. 1. Spectrograms showing the playback stimuli used for each group of birds: (A) Normal, with species-typical pitch ratios labeled, (B) Flat Fee, with glissando in fee note removed, (C) Flat Interval, with inter-note pitch interval removed, and (D) Flat Fee / Flat Interval with both glissando and inter-note pitch interval removed.

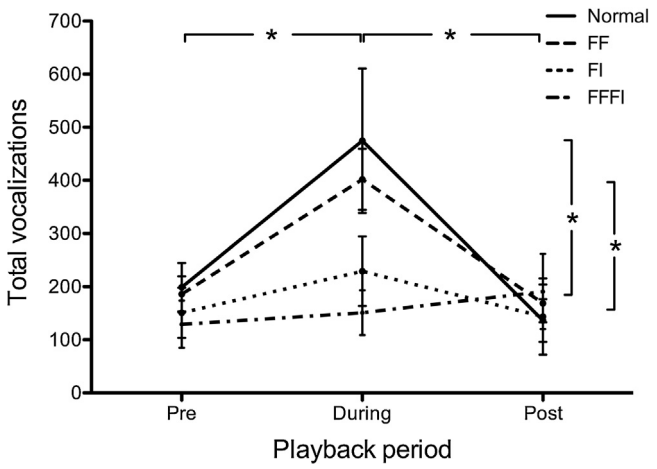

Fig. 2. Average of total vocalizations sung (including all vocalization types) produced during each playback period in response to Normal, Flat Fee (FF), Flat Interval (FI), and Flat Fee/Flat Interval (FFFI) stimulus playback. Asterisks indicate significant differences at $p < 0.05$; error bars represent standard errors of the means.

the one stimulus song was repeated every 10 s for 30 min. Assignment of birds to playback groups was random with the restriction that no two birds of either sex had the same stimulus. Playback was scheduled to take place after 0900 and before 1200 for all birds. Video recording continued during a 30 min post-playback period following the end of playback.

2.6. Perfusion

In keeping with previous protocols for *zenk* protein immunohistochemistry following playback [34,37], the bird was removed from the booth 60 min after the end of stimulus presentation and deeply anaesthetized using 0.15 cc of Euthanyl. Following this, each bird was transcardially perfused with 0.1 M phosphate-buffered saline (PBS) and then with 4% paraformaldehyde for 3–5 min each. The brain was removed and post-fixed in 4% paraformaldehyde for 24 h, followed by cryoprotection in 30% sucrose solution until saturated, about 24 h. Brains were flash frozen using dry ice and stored at −80 °C until sectioning. At the end of each perfusion, sex was confirmed by examination of the gonads. For each male, the length and width of the left testis was measured to the nearest 0.1 mm using dial calipers. The ovaries of each female were examined to confirm breeding condition; we assessed stage of ovary using an arbitrary scale described in Phillmore et al. [35]. We also confirmed the bird was an adult through examination of pneumatisation of the skull.

2.7. ZENK immunohistochemistry

Each brain was divided into separate hemispheres and sectioned sagittally at 30 μm using a cryostat. Every third section from the midline was used for ZENK immunohistochemistry; if being processed immediately sections were collected into PBS, if not being processed right away, sections were collected into cryoprotect (30% sucrose and 30% ethylene glycol in buffer) and stored at −20 °C. Immunohistochemistry was performed on the free-floating sections, beginning with washes in 0.1 M PBS, incubation in 0.5% hydrogen peroxide (15 min), and additional washes in 0.1 M PBS. Sections were incubated in 10% Normal Goat Serum (Vector) and then incubated in a commercially available primary antibody (anti Egr-1, Santa Cruz Biotechnology, catalog # sc-189) at 1:20,000 dilution in PBS containing 0.3% Triton-X for approximately 20 h. Sections were then washed in PBS containing 0.1% Triton-X and incubated in biotinylated goat anti rabbit antibody (Vector; 1:200 dilution in PBS containing 0.3% Triton-X) for 1 h. Sections were washed again in PBS containing 0.1% Triton-X and then incubated in avidin-biotin horseradish peroxidise (Vector ABC Vectastain Elite kit) for 1 h. After washing again in 0.1 M PBS, labeling was made visible using SigmaFast DAB tablets (3,3-diaminobenzidine tetrachloride) (Sigma). Sections were mounted on gelatine-coated slides, dehydrated, and coverslipped using Permount.

2.8. Behavioural analyses

For each bird, we analyzed vocal responses, proximity to the sound source (speaker), and activity levels (time spent moving) during the entire 90 min video recording, separated into the 30 min before, during, and after playback. For vocal responses, we listened to the entire video and quantified the type and number of vocalizations emitted during each of the three recording periods. Location within the cage was measured throughout the 90 min recording using the arena mode in EthoVision (Noldus). For the purpose of quantifying each bird's location, the arena (recorded from above) was divided into three equally sized zones along the length of the cage with Zone 1 being the third of the cage closest to the speaker and Zone 3 being the third furthest from the speaker. Using EthoVision, we calculated the time spent in each of the zones during each of the three recording periods. EthoVision was also used to calculate the activity level of each bird during the 90 min recording, quantified as the total distance the bird flew in cm.

2.9. ZENK-ir quantification and analyses

We measured the level of *zenk* protein immunoreactivity (ZENK-ir) in three perceptual brain regions: the dorsal and ventral sections of the NCM, and the CMM (Fig. 3A) and in HVC (Fig. 5A). These regions were identified using borders and neuroanatomical landmarks used in previous papers [34,2]. We attempted to select at least three sections per hemisphere that were located within 1080 μm of the midline (the first twelve sections collected). We chose sections in which the NCM was still attached to the rest of the brain in order to ensure that orientation of Field L and these regions were correct, and used the same sections for CMM. Images of each brain region were captured at 20x magnification using a Q-Imaging Fast 1394 digital camera mounted to an Olympus BX51 microscope. Analysis of the image was conducted using the software package Image-Pro Plus. An area of interest was drawn to demarcate the region being quantified. The area of interest for CMM was placed ventral of the lateral ventricle and rostral of Field L. The area of interest for NCM was placed caudal of Field L: above the midpoint of Field L for NCMd and below the midpoint of Field L for NCMv. Then, the detection threshold was adjusted manually so that only ZENK-ir cells were being counted. In order to avoid counting selected debris and/or overlapping cells, a size range restriction (9.07 μm^2 to 27.21 μm^2) was set as in Avey et al. [2]. We then divided the number of ZENK-ir cells by the area of interest, subtracting the area of any holes from the area used in the ZENK-ir. The same methods, equipment, and settings applied for quantification of ZENK-ir in HVC, except counts were done using Image-J instead of Image-Pro Plus.

3. Results

3.1. Behaviour—vocalizations, proximity, and activity

For each bird we counted instances of each vocalization type separately, but found that the overwhelming majority of vocalizations (97.1% across all birds) were " *tseets* "; this is consistent with another playback study performed in our lab [37]. Only seven out of 26 males produced a *fee-bee* song, these males were in the Normal

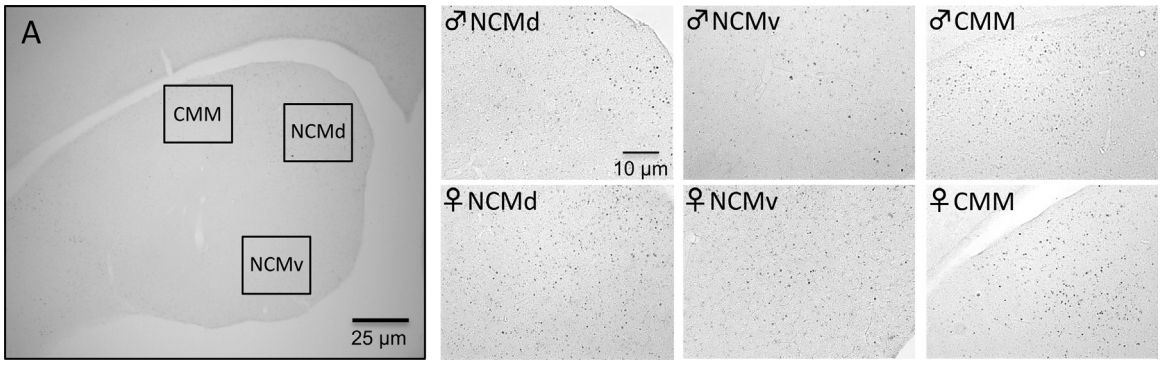

Fig. 3. Panel A: Sagittal image (taken with 4x objective) of a section stained with 3,3-diaminobenzidine for the protein of the immediate early gene *zenk*, showing where the three perceptual regions of interest were sampled. The other panels show representative images taken with the 20x objective in the three regions of interest for a male (top row) and a female (bottom row).

(3), FI (1), and FFFI (3) playback groups. Twelve out of 26 males produced *chick-a-dee* calls; there were at least two males that called in each playback group. Three out of 22 females produced *fee bee* song; they were in the Normal (2) and FI (1) playback groups. Ten females produced *chick-a-dee* calls; at least one was in each playback group. Therefore there were no obvious patterns with respect to playback group and vocal response to stimulus type. In light of this, we pooled the counts of vocalizations together across types for each bird prior to analyses. Fig. 2 shows the average total number of vocalizations produced by birds in each playback group. To determine if there were differences in the amount of vocalizing among the birds, we ran a sex × playback group × playback period mixed ANOVA. Because the vocalization data failed Mauchly's Test of Sphericity ($p < 0.05$), a Greenhouse-Geisser correction was applied. The mixed ANOVA revealed no main effect of sex ($F(1,40) < 1$) or playback group ($F(3,40) = 1.50$, $p = 0.23$). There was a significant main effect of playback period ($F(1.43,57.06) = 14.02$, $p < 0.001$) and a significant interaction between playback group and playback period ($F(4.28,57.06) = 3.40$, $p = 0.01$), which we explored further by doing separate one-way ANOVAs for each level of these two factors.

One-way ANOVAs for each playback group revealed a significant effect of playback period on the total number of vocalizations in the 'Normal' ($F(1.14,12.57) = 8.85$, $p = 0.01$) and 'Flat Fee' ($F(1.18,12.93) = 13.41$, $p = 0.002$) groups; in each case, post-hoc tests revealed that birds hearing these stimuli vocalized more during playback than either before or after. There was no effect of playback period for the 'Flat Inter-note Pitch Interval' and 'Flat Fee-Flat Interval' groups. One-way ANOVAs for each playback period revealed no differences among playback groups in the periods before or after playback, but a significant effect of playback group during playback ($F(3,44) = 3.86$, $p = 0.02$). Post-hoc tests revealed that, during playback, birds that heard 'Normal' stimuli vocalized more than birds that heard 'Flat Interval' or 'Flat Fee/Flat Interval' stimuli, and birds that heard 'Flat Fee stimuli' vocalized more than birds that heard 'Flat Fee/Flat Interval' stimuli. In other words, bird vocalized more to stimuli that had the inter-note ratio intact compared to stimuli with inter-note ratio removed.

To test whether birds showed a preference for a particular stimulus type based on time spent near the speaker, we conducted a sex × playback group × zone mixed ANOVA on time spent in each third of the cage from nearest the speaker (Zone 1) to farthest (Zone 3). We used only the data from the period when stimuli were actually being played to the bird. There were no main effects of sex ($F(1,40) = 1.73$, $p = 0.20$) or playback group ($F(3,40) < 1$) but a significant main effect of zone ($F(2,80) = 7.75$, $p = 0.001$) and a significant sex × zone interaction ($F(2,80) = 3.86$, $p = 0.03$). Separate

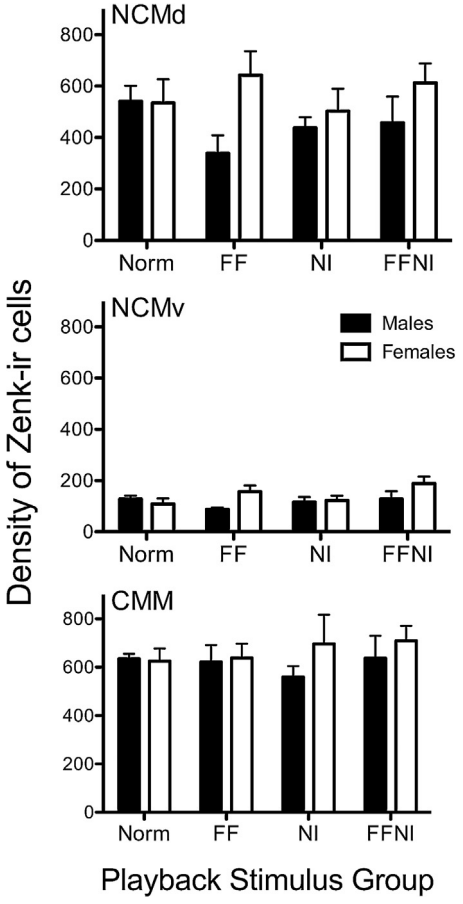

Fig. 4. Average density of ZENK-ir cells in NCMd, NCMv, and CMM of male and female chickadees in response to playback of four different types of stimuli: Normal *fee bee* song (Norm), Flat Fee (FF), Flat Interval (FI), and Flat Fee/Flat Interval (FFFI). Error bars represent standard errors of the means.

one-way ANOVAs on playback group × zone for each sex revealed a main effect of zone for each; males: $F(1.56,34.28) = 6.70$, $p = 0.006$), females: $F(2,36) = 5.16$, $p = 0.011$) but no playback × zone interactions. Post hoc tests showed males and females spent most of their time in Zone 1; this combined with no playback × zone interaction indicates that location in the cage did not vary with type of stimulus being played. We also examined whether playback affected how active the birds were; we used EthoVision to measure the total distance flown (in cm) before, during, and after playback. We

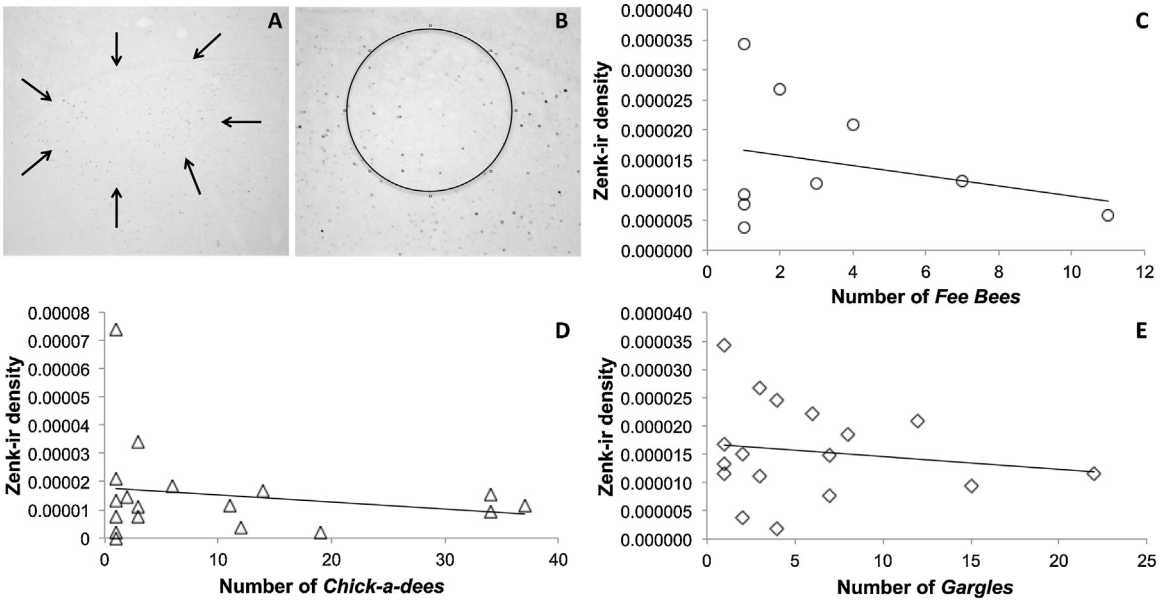

Fig. 5. Sagittal image of a section stained with 3,3-diaminobenzidine for the protein of the immediate early gene *zenk* showing HVC (A, 10x objective) and the area of interest within HVC (B, 20x objective). Remaining panels show ZENK-ir densities in HVC plotted as a function of number of vocalizations of each type: *fee bees* (C), *chick-a-dees* (D), and *gargles* (E).

conducted a sex × playback group × playback period mixed ANOVA but there were no significant main effects of sex ($F(1,39) = 1.27$, $p = 0.27$), playback group ($F(3,39) = 2.51$, $p = 0.07$) or playback period ($F < 1$), nor were there any significant interactions.

3.2. Physiology

Gonads were examined at time of sacrifice in order to confirm the sex of the birds and to assess breeding condition. For males, we removed the left testis and measured its length and width to the nearest 0.1 mm with dial calipers, and then used this data to estimate the testis volume using the formula for an ellipsoid ($4/3\pi$ a ^{2} b; where a = width/2 and b = length/2). The average testes volume across all birds was 67.40 mm^3 (SD = 25.69), which is comparable to the testes sizes of breeding condition chickadees reported by other studies in our lab (e.g., [36,37]). A one-way ANOVA indicated that there was no difference in testes volume across the four playback groups ($F(3,22) = 0.89$, $p = 0.46$). All of the female birds had stage 2 hierarchical ovaries, indicating that they were in breeding condition.

3.3. Brain

3.3.1. ZENK-ir and playback

Fig. 3A shows an example of a section from which cell counts were taken in NCM and CMM and representative images of each area from males (top panels) and females (bottom panels). Fig. 4 shows average density of ZENK-ir in NCMd, NCMv and CMM in males and females. We ran a sex × playback group × brain region (NCMd, NCMv, and CMM) × hemisphere mixed ANOVA. This revealed a main effect of sex ($F(1,47) = 4.11$, $p = 0.049$, partial eta squared = 0.09): overall, females had more ZENK-ir than males. There was also a main effect of brain region ($F(2,46) = 270.91$, $p < 0.001$, partial eta squared = 0.87); post-hoc comparisons revealed that CMM exhibited significantly more ZENK-ir than both NCMd and NCMv, and that NCMd showed greater ZENK-ir than NCMv. In contrast to our prediction, there was no main effect of playback group ($F(3,45) < 1$). We also found no main effect of hemisphere ($F(1,48) < 1$), nor were there any significant interactions. Separate two-way (sex × playback group) ANOVAs were then conducted for each of the three brain regions examined, and because in the previous analyses there were no differences in cell counts between hemispheres, data from the left and right hemispheres were averaged for these analyses. In NCMd, there was no main effect of playback group ($F(3,41) < 1$) but there was a main effect of sex ($F(1,41) = 5.19$, $p = 0.03$, partial eta squared = 0.11); females had higher ZENK-ir than males. In both NCMv and CMM, two-way ANOVAs revealed no main effects of sex (NCMv: $F(1,47) = 3.67$, $p = 0.06$; CMM: $F(1, 47) = 1.23$, $p = 0.27$) or playback group (NCMv: $F(3, 45) = 1.61$, $p = 0.20$; CMM: ($F(3,45) < 1$). Thus, the overall main effect of sex in the previous analysis was driven mainly by higher ZENK-ir in NCMd of females compared to males. There was no sex × playback group interactions for any region (NCMd: $F(3,41) = 1.37$, $p = 0.27$; NCMv: $F(3,41) = 1.85$, $p = 0.15$; CMM: $F(3,41) < 1$).

3.3.2. ZENK-ir and vocalizing

For each bird we calculated ZENK-ir density in left and right hemispheres and counted the number of *fee bee* songs, *chick-a-dee* calls, *gargles*, and *tseets* (as explained above, the majority of vocalizations were *tseets*). We first compared ZENK-ir in left and right hemispheres of HVC and in each playback group for males and females. Two birds in particular were outliers in terms of amount of expression in HVC—one male and one female were 4 and 4.7 SDs above the mean HVC density of all birds in the study. Without the outliers, a sex × playback group × hemisphere mixed ANOVA revealed no effect of sex, playback group, or hemisphere (Fs < 1), nor were there any interactions. The outliers did affect the analyses, as when they were included there was a hemisphere × playback group interaction ($F(3,32) = 3.27$, $p = 0.04$; partial eta squared = 0.24), likely because both outliers were in the FFFI group. However, we are confident in the analyses that exclude the outliers. Because there was no main effect of hemisphere, we used the average of ZENK-ir in the two hemispheres in subsequent analyses.

We then performed correlations on vocalization counts and ZENK-ir densities. Because both Pearson and Spearman correlations are affected by extreme outliers (although Spearman

less so), we excluded birds that exceeded the upper or lower outer quartile ranges [23] on variables in each correlation matrix. Results from these correlations are presented in Fig. 5.

We first tested whether the number of vocalizations totalled over the pre-, during-, and post-playback periods was correlated with ZENK-ir densities in HVC (see Fig. 5a), NCMd, NCMv, and CMM; none were significantly correlated (all $r_s < 0.1$). Interestingly, ZENK-ir densities among perceptual areas were correlated (NCMd and NCMv: $r_s(42) = 0.76$, $p < 0.001$; NCMd and CMM: $r_s(42) = 0.53$, $p < 0.001$; NCMv and CMM: $r_s(42) = 0.48$, $p = 0.001$), but density in HVC was not correlated with density in any perceptual region (HVC and NCMd: $r_s(42) = 0.17$, $p = 0.27$; HVC and NCMv: $r_s(42) = 0.23$, $p = 0.13$; HVC and CMM: $r_s(42) = 0.10$, $p = 0.50$).

We then investigated the relationship between ZENK-ir densities and rates of vocal production in subsets of our data, looking separately at birds that produced at least one *fee-bee*, birds producing at least one *chick-a-dee* call, and birds producing at least one *gargle* call. For each vocalization group, we performed correlations with the number of appropriate vocalizations (either songs, calls, or gargles), the number of *tseets*, left, right and average HVC ZENK-ir densities, and average NCMd, NCMv, and CMM densities. ZENK-ir density in HVC of singers was not correlated with number of *fee bees* ($rs(7) = -0.45$, $p = 0.27$) or number of *tseets* ($rs(7) = -0.18$, $p = 0.64$), and neither was ZENK-ir densities of NCMd, NCMv or CMM (Fig. 5b). ZENK-ir density in HVC of callers was not correlated with number of *chick-a-dees* ($rs(16) = -0.435$, $p = 0.07$) or number of *tseets* ($rs(16) = -0.20$, $p = 0.43$), and neither was ZENK-ir densities of NCMd, NCMv or CMM (Fig. 5c). Finally, ZENK-ir density in HVC of garglers was not correlated with number of *gargles* ($rs(15) = -0.37$, $p = 0.14$) or number of *tseets* ($rs(15) = -0.20$, $p = 0.44$), and neither was ZENK-ir densities of NCMd, NCMv or CMM (Fig. 5d).

4. Discussion

Our main goal was to determine whether neural responses in chickadees to playback of normal and altered *fee bee* songs parallel previously reported differences in how males and females respond behaviourally to songs with species-typical pitch ratio rules and those with pitch ratios altered. ZENK-ir in NCMd, NCMv, and CMM, regions dedicated to processing conspecific vocalizations, did not vary in response to songs with typical pitch ratios altered compared to songs with ratios intact. Further, contrary to expectations, we did not find a sex difference in how males and females responded to particular alterations, either in the brain or in behaviour. However, the negative results are not due to lack of statistical power (i.e. n of each sex in each playback group), but rather because the effect sizes appear to be subtle at best. We did find that females had more ZENK-ir in perceptual regions than males. Again, the effect size was rather small but because the number of birds used to detect this effect were relatively large ($n = 26$ males, 23 females), it was statistically significant. Behaviourally males and females vocalized more in response to songs with the inter-note pitch ratio intact compared to songs with no pitch change between the *fee* and the *bee* notes.

We also looked at activity in HVC, a region typically associated with vocal production, and found that playback, as expected, did not affect ZENK-ir. However, contrary to previous research in zebra finches and canaries, amount of ZENK-ir in HVC was not positively correlated with number of songs produced.

4.1. Behavioural response to playback

Previous behavioural studies conducted in the field and laboratory suggest that chickadees attend to experimental alterations to the normal *fee bee* song pitch ratios [41,45]; specifically, that females attended to both ratios, while males attend only to the glissando. In our study, both males and females vocalized more in response to *fee bee* songs with the inter-note pitch ratio intact than to those with that ratio eliminated; altering the glissando did not affect response in either sex.

One explanation for the differences in male responsiveness between those studies and the current one is methodological: the previous findings resulted from fieldwork conducted in the birds' territories, whereas the current study took place in a laboratory under more artificial conditions. Songbirds and other animals, including chickadees, differ in behaviour and physiology between laboratory and field studies [6,27,36,47]. Captivity can have profound influences on songbird breeding behaviour, possibly via altered stress responses [16]. In European starlings (*Sturnus vulgaris*), for example, fluorescent lighting affects stress levels and, following from that, behaviour [18,19]. It is possible that the captive males in this study, although physiologically in breeding condition but in an entirely different and somewhat unfamiliar environment, may have attended to and assessed the song stimuli differently than they would have in a familiar territorial context. In particular, it is possible that the males in this study did not associate the *fee bee* songs as potential threats as they would have in the more natural context of their territories, and thus did not show, or could not show, typical behavioural responses. Instead, we must consider that the birds were motivated to respond to the playback because they had been visually and acoustically isolated for at least 14 h, and playback provided a possibility of interaction with conspecifics in this social and interactive species [46]. Therefore, lack of response to *fee bees* without the inter-note pitch interval may indicate lack of recognition of those stimuli as conspecific, rather than a preference for *fee bees* with the interval intact.

Our results are similar to those of Ratcliffe and Otter [40] in terms of female attendance to the inter-note interval, but we did not find that females attend to the glissando. Our study was fairly similar in methodology to Ratcliffe and Otter [40] in that it involved catching chickadees from the wild, photostimulating them over several weeks, and testing them using sound-attenuated booths. However, it differed in some respects. Their behavioural measure was the number and duration of copulation displays by the estradiol-implanted females; we instead relied on natural vocal response to playback. Another difference was that Ratcliffe and Otter [40] presented each female with a male chickadee model prior to presentation of the stimuli. It may be that, without such visual stimulation in captivity, the females in this study were not stimulated enough by playback alone and therefore did not assess male quality or respond to it as they would in the field. However, given that the inter-note interval in particular may be an indicator of male dominance status used by females in mate selection [9] and that the inter-note interval is relatively impervious to degradation due to broadcast through the forest, it is good candidate for females to assess as an accurate indicator of adherence to species-typical pitch ratios and, therefore, male quality [10].

4.2. Neural response and vocal perception

There are several potential explanations for the lack of differences in ZENK-ir to normal and altered *fee bees*. One possibility is that there are in fact differences in neural activity within CMM and NCM in response to these stimuli, but they cannot be detected using ZENK-ir. Avey et al. [3] suggested there might be particular subgroups of neurons within these regions that are selective for particular song features; in our study it would be relative pitch ratios in song. Such expression differences within regions would not be detected by the quantification used here, since *zenk* protein may be expressed by all subgroups, or only one subgroup. Similarly, there may be subtle changes in the activity of neurons within NCM and CMM that can only be seen in the instantaneous response mea-

sured by electrophysiological recordings. For example, in European starlings, neurons in CMM fire differentially to different conspecific motifs [28]. Thus, it may be that brain activity related to discrimination of normal and altered *fee bee* songs is happening in these regions, but was not detected using this study's methods.

It is also possible that the relatively small changes in song structure distinguishing the normal and altered song stimuli in this study fall outside the discriminatory range of NCM and CMM. Indeed, while abundant research has demonstrated the important roles of these regions in processing and extracting information from sounds, it has mostly been shown for gross, rather than fine, features of stimuli, such as conspecific versus heterospecific (e.g., song sparrows, [43]; chickadees, [37]) degrees of complexity (starlings, [17]), or even vocalization types (chickadees, [34]). Recent research by Avey et al. [3] supports the idea that NCM and CMM may not be selective for fine structural features; ZENK-ir did not differ in response to conspecific call notes and call notes of related species (chestnut-backed chickadee *Poecile rufuscens* and tufted titmouse *Baeolophus bicolor*) with similar acoustic structure. These findings suggest that NCM and CMM are not so much selective for heterospecific versus conspecific as they are selective for structural differences of a larger magnitude, but the point in the range of stimulus features that the brain changes from processing gross to fine features is not clear.

It may be then, that subtle features of stimuli are processed in other areas within the avian auditory pathway. HVC, centrally important to song learning and production, may also contribute to fine discrimination among conspecific songs. In female songbirds, HVC lesions interfere with female assessment of male song quality in canaries [5,13], and HVC neurons alter their firing patterns in response to so-called sexy syllables performed by male canaries [14]. However, using ZENK-ir to look for these differences is not likely to be fruitful: indeed, we did not find differences in HVC ZENK-ir related to playback of our altered stimuli, but considering that *zenk* (and its protein) is purported to be important for memory formation and species recognition rather than fine grain perceptual discrimination [25], this is not surprising.

Our finding that females had more ZENK-ir than males, mainly driven by a sex difference in NCMd, contrasts with the findings of previous studies in adult chickadees in which ZENK levels in CMM and NCM have typically been higher in males than in females (e.g. [34,2]. However, the size of the effect we report here was weak (less than the 0.10 defined by Cohen [11], even though it was statistically significant. This could be because all of our stimuli were relatively similar *fee bee* songs (whether normal or altered) and therefore may have had the same robust effect on ZENK-ir in NCM and CMM, inducing maximum expression and obscuring the typical sex difference in chickadee neural reactivity. The amount of sexual dimorphism in chickadees varies with neural measure (for example, there is a large difference in FoxP2 expression and Area X volume, but a small difference in HVC volume [37]), and our results here could simply reflect a small sex difference that was previously undetectable with smaller sample sizes.

4.3. Neural response and vocal production

Contrary to research on zebra finches and canaries showing a strong correlation between number of songs produced and HVC ZENK-ir [22], we found no such relationship in between *fee bee* songs and HVC ZENK-ir in chickadees, similar to results for another wild-caught species, house sparrows [42]. We also tested two other, complex types of vocalizations: *chick-a-dee* calls, used for maintaining contact between conspecifics and as an alarm call, and *gargles*, used primarily in aggressive contexts [20]. Numbers of calls and *gargles* were also not correlated with HVC ZENK-ir. The amount of ZENK-ir in HVC was extremely low compared to that in perceptual regions; some birds only had one or two cells per section counted. It could be that if we designed an experiment to elicit maximal vocal responses from the birds, we would see a strong linear relationship between singing and HVC response. Although our results are "negative", they provide evidence that not all avian species behave the same way, and thereby encourage researchers not to take results from typical model species (i.e. zebra finches and canaries), as representative of all avian neurobiology.

5. Summary

Although behavioural responses to the normal and altered songs in this study partly matched the findings of previous field and lab studies in chickadees, ZENK-ir did not vary between normal and altered *fee bee* songs. ZENK-ir in NCM and CMM is capable of indicating differential response of gross acoustic features necessary for species recognition, a task that relies on memory. However instantaneous discrimination of more subtle acoustic features, such as among conspecific songs with varying pitch ratios, may occur elsewhere in the avian auditory pathway and, more likely, not rely on memory (and therefore protein) formation. Our results confirm that HVC ZENK-ir is not involved in fine-grain perceptual discrimination, however it did not support the idea that increased vocalizing increases ZENK-ir in HVC. Further research is required to understand the importance of pitch ratios in the *fee bee* song to both males defending territories and females selecting mates, and the underlying neural mechanism driving behavioural responses to song.

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