RESEARCH ARTICLE

A neural basis for password-based species recognition in an avian brood parasite

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ABSTRACT

Obligate avian brood parasites are raised by heterospecific hosts and, therefore, lack crucial early exposure to relatives and other conspecifics. Yet, young brood parasites readily recognize and affiliate with others of their own species upon independence. One solution to this social recognition paradox is the ontogenetic 'password' mechanism used by obligate parasitic brown-headed cowbirds (*Molothrus ater*), whereby conspecific identification is initially mediated through the cowbird chatter: a non-learned vocal cue. We explored the neural basis of such password-based species recognition in juvenile and adult male cowbirds. We found that cowbird auditory forebrain regions express greater densities of the protein product of the immediate-early gene *ZENK* in response to the password chatter call relative to control sounds of mourning dove (*Zenaida macroura*) coos. The chatter-selective induction of *ZENK* expression occurs in both the caudal medial nidopallium (NCM) and the caudal medial mesopallium (CMM) in adults, but only within the NCM in juveniles. In contrast, we discovered that juvenile cowbirds exhibit neural selectivity to presentations of either conspecific or heterospecific songs, but only in CMM and only after recent experience. Juvenile cowbirds that did not have previous experience with the song type they were exposed to during the test period exhibited significantly lower activity-dependent gene expression. Thus, in juvenile male cowbirds, there is early onset of species-specific selective neural representation of non-learned calls in NCM and recently experienced song in CMM. These results suggest that NCM is evolutionarily co-opted in parasitic cowbirds to selectively recognize the password chatter, allowing juvenile cowbirds to identify adult conspecifics and avoid mis-imprinting upon unrelated host species. These ontogenetic comparisons reveal novel insights into the neural basis of species recognition in brood parasitic species.

KEY WORDS: Species recognition, Brood parasite, Avian auditory forebrain, Immediate early gene

INTRODUCTION

The ability to discern conspecifics from other species is the foundation upon which most complex social interactions are built.

Recognition of conspecifics can be inherent or learned during early development (or both). Under genetically guided communication systems, inherent preferences for conspecific signals are engaged to recognize one's own species (Ryan, 1998; Ryan and Cummings, 2013). Alternatively, species recognition may rely on early social experiences with conspecifics, including early song learning from 'tutors' in juvenile male and female songbirds, as opposed to relying on innate preferences (Irwin and Price, 1999). Examining mechanisms of conspecific recognition is crucially relevant in obligate brood parasites, which are species in which offspring are exclusively raised by heterospecifics, and include lineages in fishes, insects and birds (Göth and Hauber, 2004; Manna and Hauber, 2016).

Approximately 1% of avian species are obligate brood parasites that do not build a nest, incubate eggs or provision their young (Payne, 1977). Instead, parasitic females leave their eggs in the nest of a host species. Although brood parasitism imparts fitness benefits through increased reproductive output without the costs associated with parental care, it presents a set of ontogenetic challenges for the developing young compared with parental birds in which sexual imprinting leads to the recognition of conspecifics. One of the earliest developmental challenges faced by juvenile brood parasites is to avoid mis-imprinting on the species of unrelated parents and siblings (Slagsvold and Hansen, 2001). Imprinting on the song or visual cues of the foster parent would result in inaccurate social recognition well into adulthood, which in turn would lead to substantial fitness costs through misdirected reproductive effort. Cross-fostered songbirds from non-parasitic species imprint on heterospecific foster parents, resulting in an adult that displays inaccurate mate choices, song production and signal preferences (Slagsvold et al., 2002; ten Cate and Vos, 1999). Thus, the young brood parasite in a foreign nest is faced with a unique challenge regarding species recognition and therefore must possess a means to identify conspecifics. Consequently, avoidance of mis-imprinting may be a two-part problem: (1) the young bird should recognize a subset of birds as their own species; and (2) possess a means or mechanism to avoid mis-imprinting on the familiar birds that surround them.

One theory that addresses the paradox of species recognition in brood parasite young is the 'password' hypothesis. This hypothesis suggests that social recognition processes in brood parasites are initiated by exposure to a password, which is defined as any unique identifier such as a species-specific vocalization or other phenotypic attribute (Hauber et al., 2001). The password not only identifies conspecifics but also initiates social learning, particularly with respect to conspecific traits (Hauber et al., 2001). The password alone may evoke species recognition, whereas additional signals, such as song or physical attributes, may enhance social decision-making or discrimination tasks. An ontogenetic behavioral study that supported the password hypothesis focused on juvenile brown-headed cowbirds [*Molothrus ater* (Boddaert 1783)], an obligate brood parasite ubiquitous across North America (Hauber et al.,

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2001). Brown-headed cowbirds (hereafter, cowbirds) are oscine Passeriformes within the Icteridae family (i.e. blackbirds). The cowbird is an excellent brood parasitic species in which to examine this issue because it is a host generalist and is known to parasitize over 200 different species (Lowther, 1993). Consequently, juvenile cowbirds experience stark differences in early social cues, including a diversity of host vocalizations, that they could mis-imprint upon. Behavioral tests of the password hypothesis in cowbirds demonstrated that even in the earliest developmental stages, cowbird nestlings already express greater begging responses to a non-learned conspecific vocalization, called the chatter, relative to a suite of other conspecific and heterospecific vocalizations, including songs (Hauber et al., 2001). Additionally, cowbird fledglings both in the lab and in the wild preferentially approach speakers broadcasting the chatter call compared with conspecific and heterospecific songs, as well as chatter-like (control) vocalizations (Hauber et al., 2001; Hauber, 2002). One of the key ideas behind the password hypothesis is that signals serving as a password must be non-learned and salient from an early age, whereas other conspecific signals used in courtship or other communication contexts are only learned after the password has been used to correctly identify conspecifics. This suggests that password signals evoke specific neural responses in early developmental periods that may be malleable across an ontogenetic timeline.

The auditory forebrain is a prominent neural substrate for social and species recognition in many oscine bird species, including brood parasitic species (Louder et al., 2016). Common neural substrates that have emerged as being crucial for social recognition in passerines in the auditory forebrain regions are referred to as the caudomedial nidopallium (NCM) and the caudomedial mesopallium (CMM), regions that are homologous to the mammalian auditory cortex (Jarvis et al., 2005). Just as the auditory cortex is vital to perceptual processing of auditory information in mammals (Kanwal and Rauschecker, 2007), the NCM and CMM are vital to social perception and recognition in songbirds, and exhibit selective responses to biologically meaningful auditory stimuli (Mello et al., 2004). Studies of activity-dependent gene induction, such as expression of immediate-early genes (IEGs), demonstrate that the NCM and CMM exhibit specific neural responses to conspecific songs as opposed to heterospecific songs in both parental (Mello et al., 1992) and brood parasitic songbirds (Louder et al., 2016), attractive conspecific songs as opposed to less attractive songs (Leitner et al., 2005; Monbureau et al., 2015), recently experienced songs as opposed to novel songs (Sockman et al., 2002), songs that include the birds' local dialect as opposed to a foreign dialect (Maney et al., 2003), as well as non-learned calls versus silence (Gobes et al., 2009). The NCM and CMM also play distinct roles in song recognition, as some songbirds exhibit greater IEG induction in the CMM in response to preferred song, whereas IEG induction in the NCM reflects song familiarity (Woolley and Doupe, 2008). Thus, the NCM and CMM have emerged as key brain regions for social recognition of acoustic cues in many species of parental and parasitic oscine birds. Here, we examine activity-dependent gene induction in the NCM and CMM across juvenile and adult cowbirds in response to calls and songs of conspecifics versus calls and songs of heterospecifics. We measure induction of the IEG *ZENK* (an acronym for *zif268*, *egr-1*, *ngfi-a*, *krox24*) within the NCM and CMM to determine whether a chatter-specific response occurs across juvenile and adult stages, and whether this response is altered in the NCM or CMM across these two stages.

In experiment 1, we examine neural responses in the auditory forebrain in response to the chatter calls, the putative password for

species recognition in brown-headed cowbirds. We examine auditory responses across juvenile and adult stages, with the prediction that neural responses to the chatter in young cowbirds are more salient than other calls young cowbirds may be exposed to in their social environment. We also predict that neural responses to the password will vary across juvenile and adult stages. Such malleability in neural responses has been described in forebrain vocal circuits in brood parasites but not in related parental taxa (Liu et al., 2016). In experiment 2, juvenile cowbirds were either exposed to recent experience or no recent experience with either conspecific or heterospecific songs, which are both learned vocalizations. We examine whether the NCM and CMM respond to learned vocalizations in young cowbirds and the role of recent experience in shaping these responses with the prediction that the same auditory regions examined in response to chatter calls also respond to learned songs in juvenile male cowbirds. By examining the neural basis of a possible password and the effect of recent song experience on juvenile brood parasites, we can begin to unravel the paradox of social recognition and mis-imprinting avoidance in an animal not raised by or with related family members.

MATERIALS AND METHODS
Housing, aging and acoustic exposure
Experiment 1

Twenty-seven male brown-headed cowbirds were captured in August–September 2015 via bait traps at John F. Kennedy airport in NY, USA, with scientific collecting permission granted to K.S.L. from the Federal and New York State Fish and Wildlife Department (Federal permit, MB96705A; NY state permit, 1181). All birds were removed from the trap on the day of capture and placed in semi-natural outdoor aviaries at Hofstra University for no longer than 2 weeks. Males were photographed and aged independently by K.S. L. and M.I.M.L. according to plumage patterns described by Ortega et al. (1996) (Fig. 1A–C). Mottled birds that had not yet molted into adult plumage (Fig. 1A) or still possessed juvenile primary and secondary coverts (Fig. 1B) were considered juvenile birds. Birds with brown heads and black primary and secondary coverts were considered adults (Fig. 1C). It is likely these males had been exposed to the chatter call for at least 1 year. Because our study examines neural responses in wild-caught birds, we cannot fully ascertain the extent of exposure to chatters prior to capture in either of our juvenile and adult classes.

Males were randomly placed into treatment categories to be exposed to 1 h of chatter calls or mourning dove (*Zenaida macroura*) coos (Fig. 2 shows sonograms; $N=9$ adults for chatter; $N=6$ adults for coos; $N=5$ juveniles for chatters; $N=6$ juveniles for coos). The mourning dove coo was chosen because it is a representative, ubiquitous sympatric heterospecifc call of a sympatric forest-edge granivorous bird that should not evoke a functional response in cowbirds. By choosing this call, we are not testing whether juvenile cowbirds find chatters more salient than spectrally or temporally similar heterospecific vocalizations. Rather, we are examining neural responses to this call to determine whether it exhibits greater IEG induction relative to other call types the juvenile cowbird will frequently encounter in its social environment, thus providing information about the salience of the chatter call relative to calls the juvenile may encounter in its social surroundings. Indeed, cowbirds were exposed to mourning dove vocalizations while housed in the large outdoor aviary as mourning doves routinely ate spilled bird seed on the ground surrounding these cages during our studies. Accordingly, the mourning dove serves as a positive control. We did not include a negative control,
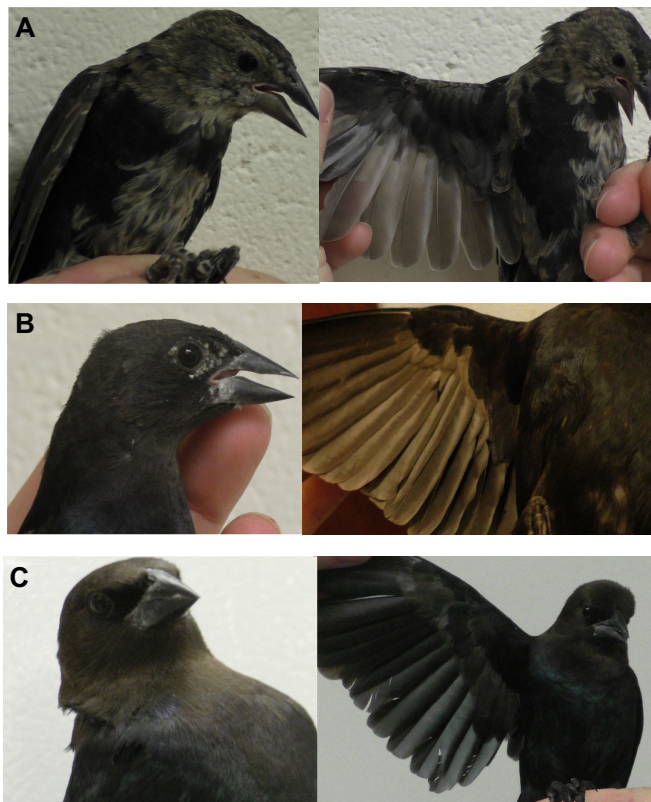
Fig. 1. Photos of feather characteristics that identify the age of males. (A,B) Juvenile males are either extremely mottled (A) or have recently lost the mottled pattern but their primary and secondary coverts are still non-uniform in color and/or speckling appears around the eyes (B). (C) Males display full adult plumage with matching color in primary and secondary coverts, and the typical brown head that appears in adult males of this species. Birds in stages represented by A and B were placed into the same category for analysis, as both these males have less experience with conspecifics relative to adults.

which reduced the number of subjects sacrificed for this study. Many previous studies have repeatedly demonstrated that sounds, including those that lack biological relevance, induce greater activity-dependent gene expression in the perceptual processing regions (the NCM and CMM) when compared with silence in many avian species (Bailey and Wade, 2003, 2005; Gentner et al., 2004; Gobes et al., 2009; Mello et al., 2004; Tomaszycki et al., 2006), including in brood parasitic songbirds (Louder et al., 2016). Furthermore, we did not record vocalizations, other behaviors or physiological responses (e.g. heartrate; Hauber et al., 2002) in response to the playbacks, as vocalizing is not known to be involved in differential activity-dependent gene induction in perceptual processing regions in songbirds (Roach et al., 2016). For example, in field- and isolate-reared black-capped chickadees (*Poecile atricapilla*), both calls and songs resulted in significant *ZENK* induction in the NCM and CMM (Phillmore et al., 2003), but production of both calls and songs had no effect on *ZENK* induction in these perceptual brain regions (Roach et al., 2016).

Subjects were placed individually into an acoustic isolation chamber to habituate to the chamber for 30 min. After 30 min, chatter or coo vocalizations (Fig. 2) were broadcast to the males for 1 h using speakers controlled via Bluetooth from an Apple iPod. Songs were broadcast at 65 dB, as measured by an SPL meter 0.5 m from the speaker. Five independent examples of chatter and coo sounds were acquired from the Macaulay Library, Cornell University (Ithaca, NY, USA). Each sound was filtered above

2000 Hz and below 500 Hz, and all sounds were normalized for mean amplitude using Audacity. In order to match the amount of stimulation between experimental and control stimuli, we matched these signals in peak amplitude and duration, following Hauber et al. (2001) and Louder et al. (2016). Vocalizations were synthesized with 20 s of vocal stimulus per minute and arranged so that one or two chatters or coos from each male recorded was presented in each minute of presentation. This sound arrangement avoided auditory habituation to the stimulus. Sound presentations were followed by 30 min of silence as described previously (Lynch and Ball, 2008; Lynch et al., 2012). Ninety minutes after the onset of the auditory stimulus, birds were deeply anesthetized using isoflurane and perfused using 4% paraformaldehyde. Following perfusion, brains were post-fixed in 4% paraformaldehyde for 24 h at 4°C followed by cryoprotection in 30% sucrose for 48 h. In addition to collecting brains, we also recorded gonad size. All males in juvenile category had completely regressed gonads that were unmeasurable. Adult male gonad size ranged from 0.8 to 1.5 mm in length, indicative of male birds in non-breeding condition. All procedures were approved by the Institutional Animal Care and Use Committee at Hofstra University.

Immunocytochemistry, quantification and analysis

Brain tissue was sectioned into four series of 40 μm coronal sections on a Leica CM1950 cryostat, placed into cryoprotectant and stored at −20°C until free-floating immunocytochemistry (ICC) was conducted to label ZENK as described previously (Lynch et al., 2008, 2012, 2013; Lynch and Ball, 2008). Briefly, tissue was placed in 0.5% hydrogen peroxide with 20% normal goat serum to block non-specific binding. Additional blocking was carried out using avidin-biotin vector blocking solution (Vector Laboratories, Peterborough, UK) prior to incubation with 1/2000 dilution of ZENK primary antibody (sc-189, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 48 h at 4°C. Tissue was then incubated in biotinylated secondary antibody (goat anti-rabbit, 1/250, Vector Laboratories) for 1 h at room temperature prior to incubation in avidin-biotin horseradish-peroxidase (Vectastain ABC kit, Vector Laboratories) for 1 h at room temperature. Tissue was treated with 3,3′-diaminobenzidine (DAB) with nickel enhancement (Vector Laboratories) to visualize ZENK immunoreactivity. Sections were mounted onto positively charged slides and dehydrated in a series of increasing concentrations of ethanol prior to being coverslipped using Permount (Sigma-Aldrich, St Louis, MO, USA).

The density of ZENK immunoreactivity (Fig. 3A) was quantified in the NCM and CMM under bright-field illumination on an Olympus Bx53 microscope equipped with an Olympus DP73 camera. Photomicrographs were taken using CellSens Standard software. Four representative photomicrographs were taken by an observer blind to the experimental treatments. These four photomicrographs were taken from the left and the right hemisphere. Because NCM and CMM are relatively large forebrain areas, we narrowed our quantification to a 1.66×1.24 mm sampling frame systematically placed ~100 μm from the midline, as illustrated in Fig. 3B. The level along the rostrocaudal axis at which the sampling frame was placed is illustrated in Fig. 3C–E. The landmarks that separate the NCM and CMM were just as described previously (Lynch and Ball, 2008; Lynch et al., 2013). For example, activity-dependent gene expression does not occur in Field L, which serves as a major landmark separating the two regions. As illustrated in Fig. 3A,B, ZENK-immunoreactive cells were quantified in the dorsal regions of the NCM.

The average ZENK immunoreactivity density was calculated from these four representative photomicrographs for each subject
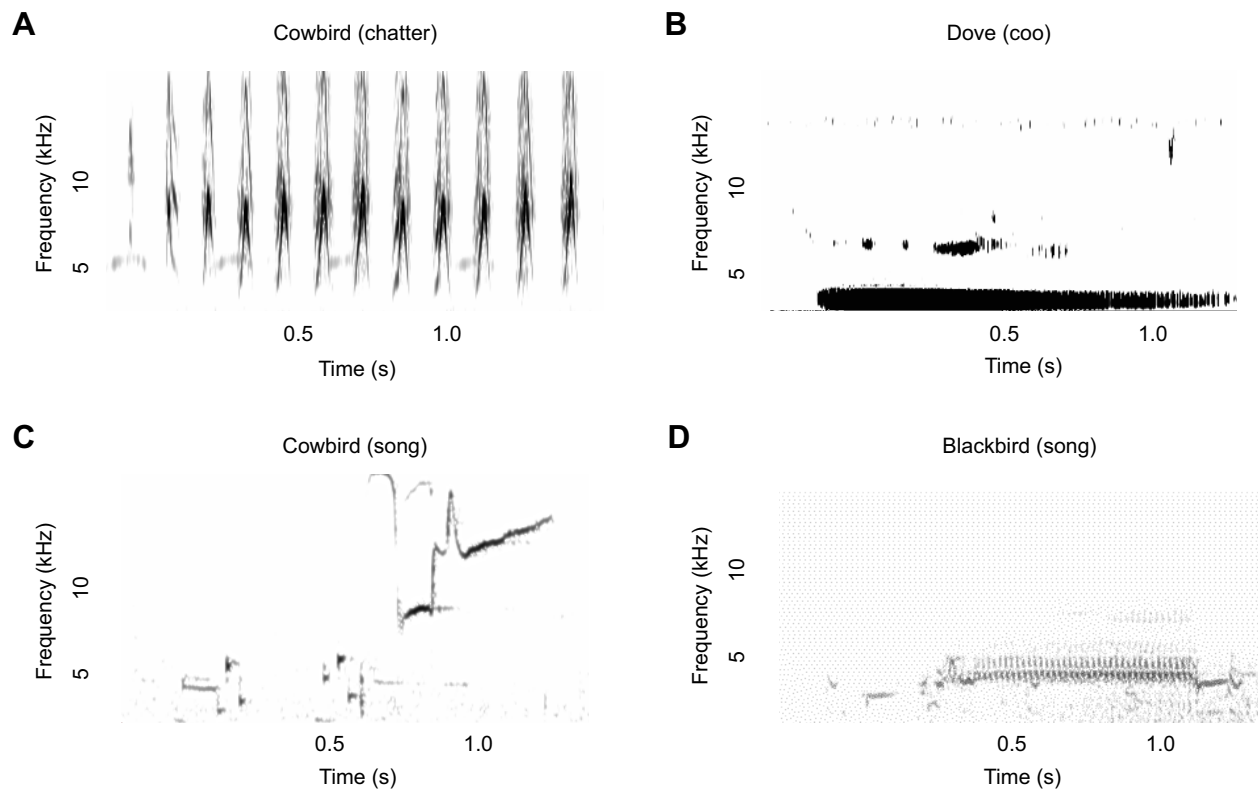
Fig. 2. Sonograms of all vocal signals used in the present study. (A,B) Experiment 1 tested immediate-early gene (IEG) induction in the auditory forebrain in response to cowbird chatters and dove coos. (C,D) Experiment 2 tested IEG induction in the auditory forebrain in response to cowbird song and red-winged blackbird songs.

that were from both the left and the right hemisphere. For all brain regions, automated cell counts of nuclei containing ZENK immunoreactivity was carried out using ImageJ (National Institutes of Health, Bethesda, MD, USA), as described previously (Lynch et al., 2008, 2012, 2013; Lynch and Ball, 2008). Briefly, photomicrographs were transformed into grayscale in ImageJ, which removed much of the background, leaving mostly ZENK-filled nuclei. The average pixel density of nuclei was calculated after ImageJ assigned values to each nucleus. This value was similar across photomicrographs. Using average pixel density, we were able to remove image artefacts that were substantially greater than the average pixel density of a ZENK-filled nucleus. We set the threshold for object counting to be 20% below the average value of the ZENK-filled nucleus, so that small artifacts would be removed. ImageJ then automatically counted the number of nuclei. We chose the outline particle function in ImageJ to ensure that counted particles corresponded to labeled cells from the original photomicrograph. Clumped nuclei were estimated by finding the average nucleus size so that pixel number for the average ZENK-containing nuclei could be determined. The number of pixels in the average ZENK cell was then divided by the number of pixels in the clump to obtain an estimate of how many cells were in the clump. These were added to the total number of non-clumped cells.

All statistical analyses were performed in SAS 9.4 (SAS Institute, Cary, NC, USA). To account for non-normality in count data from the mean ZENK immunoreactivity density of each subject, we used negative binomial regression, separate for the NCM and CMM data. We analyzed ZENK immunoreactivity expression in these two brain regions with age, auditory treatment and their interaction as potential explanatory factors.

Experiment 2

Twenty-three juvenile male cowbirds were collected in August–September 2016, while still young enough to display mottled plumage only (Fig. 1). Juvenile birds with mottled plumage are at least 38 days post-hatch (Brackbill, 1976). Only juvenile males with mottled plumage were used in experiment 2. Males were housed in semi-natural conditions in outdoor aviaries so they could be isolated from adult cowbirds for a minimum of 2 weeks, after which time they were habituated to one of two indoor aviaries. Juveniles were visually isolated from each other while housed individually in 610 mm×610 mm cages while inside. In aviary 1, individually housed juvenile birds (N=12) were exposed to 8 days of adult male brown-headed cowbird songs, whereas juvenile birds (N=11) in aviary 2 were exposed to adult male red-winged blackbird *Agelaius phoeniceus* songs. Red-winged blackbird songs were chosen in this study because they are a common host and geographically sympatric species for the brown-headed cowbird (Lowther, 1993). Both cowbirds and blackbirds are oscines, and learn to produce their songs (but not their calls). The amplitude of song being received at each cage ranged between 65 and 70 dB, and broadcasts ran 8 h a day starting at 06:00 h followed by 16 h of silence. Songs were constructed as described above. Briefly, five independent examples of song were recorded from different male brown-headed cowbirds or red-winged blackbirds. Each sound was filtered above 2000 Hz and below 500 Hz, and all sounds were normalized for mean amplitude. Vocalizations were synthesized with 20 s of vocal stimulus per minute and arranged so that one or two songs from each male recorded was presented in each minute of presentation.

Juvenile males were placed into acoustic isolation boxes following 8 days of song exposure. After habituation to the box, either songs of the same species or songs of the other species were presented as
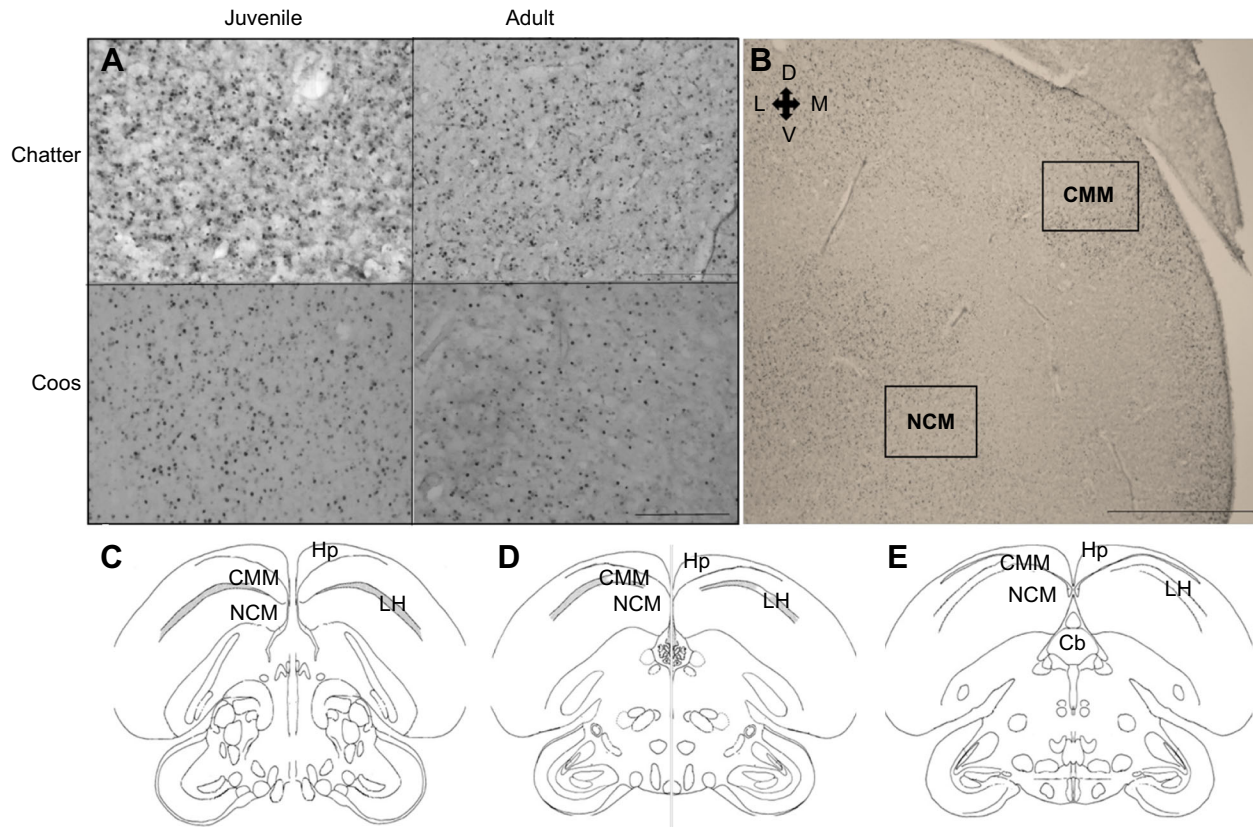
Fig. 3. Photomicrographs illustrating quantification methods. (A) Photomicrographs of ZENK immunoreactivity in the NCM in each of the four conditions: juvenile and adults exposed to chatters or coos. Scale bar: 100 μm. (B) Photomicrograph of ZENK immunoreactivity across two auditory forebrain regions in which expression of the IEG *ZENK* was quantified: the caudomedial nidopallium (NCM) and caudomedial mesopallium (CMM). Sections were cut in the coronal plane. Scale bar: 100 μm. (C–E) Illustration of sections in which ZENK immunoreactivity was quantified along the rostrocaudal extent. NCM, caudomedial nidopallium; CMM, caudomedial mesopallium; Hp, hippocampus; LH, lamina hyperstriatica; Cb, cerebellum.

above (see Fig. 2 for sonogram; exposed to brown-head songs/tested on brown-head songs: *N*=6; exposed to brown-head songs/tested on red-wing songs: *N*=6; exposed to red-wing songs/tested on red-wing songs: *N*=6; exposed to red-wing songs/tested on brown-head songs: *N*=5). Thus, some juvenile males were previously exposed to the song they were tested with and others were hearing the song for the first time since capture at roughly 38 days old (as measured by plumage; Brackbill, 1976). Ninety minutes after the onset of song, juvenile male birds were removed from the acoustic isolation boxes and sacrificed. ZENK immunoreactivity was identified and quantified as described above. Songs presented during testing were not songs from the same adult males used during the pre-trial exposure period. Song stimuli were composed the same way as described above, but test stimuli were constructed with five different adult males and the context of song presentation was altered in order to avoid habituation to song presentation. As in experiment 1, we analyzed ZENK immunoreactivity expression in these two brain regions with exposure song type, test stimulus and their interaction as potential explanatory factors.
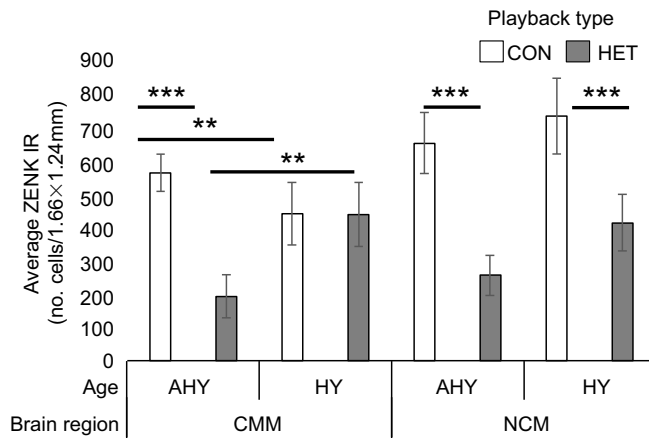
RESULTS
Experiment 1
All ZENK data are reported as the mean±s.e. number of ZENK-immunoreactive cells/sampling frame (1.66×1.24 mm). With respect to the NCM, we detected a greater density of ZENK immunoreactivity in response to conspecific chatter (mean=693.42±102.51) versus heterospecific sound exposure (mean=335.18±79.30 cells; negative

binomial regression, $F_{1,22}$=20.8, P=0.0001; Fig. 4). No difference in ZENK-immunoreactivity densities within the NCM was detected between first-year juveniles (mean=573.85±99.56 cells) versus adults (mean=454.75±76.25 cells; $F_{1,22}$=3.2, P=0.085), and there was no interaction between the type of sound exposure and the age category ($F_{1,22}$=1.16, P=0.292).

With respect to the CMM, mean ZENK-immunoreactivity density was also found to be greater in response to conspecific chatter (mean=502.51±75.29 cells) versus heterospecific sound exposure (mean=315.60±80.36; negative binomial regression, $F_{1,22}$=7.9, P=0.009; Fig. 4). No difference in ZENK-immunoreactivity densities within the CMM was detected between first-year juveniles (mean=439.95±95.94) versus adults (mean=378.15±59.7; $F_{1,22}$=2.29, P=0.144). However, there was a significant interaction between auditory treatment and age ($F_{1,22}$=7.81, P=0.01), whereby adults exhibited a greater response to conspecific chatter (mean=563.74±55.56) versus heterospecific sound exposure (mean=192.56±63.86), yet juveniles were observed to have nearly identical ZENK-immunoreactivity densities in conspecific chatter (mean=441.27±95.02) versus heterospecific sound exposure (mean=438.63±96.86).

Experiment 2
ZENK immunoreactivity did not significantly differ in the NCM as a consequence of recent song exposure ($F_{1,19}$=1.16, P=0.295; Fig. 5) or stimulus type ($F_{1,19}$=1.13, P=0.30; Fig. 5). There was no significant interaction between previous song experience and song

Fig. 4. Results of IEG induction detected by ZENK immunoreactivity within the auditory forebrain of wild-caught male brown-headed cowbirds after exposure to cowbird chatters or dove coos. AHY, after hatch year; HY, hatch year; CMM, caudomedial mesopallium; NCM, caudomedial nidopallium; CON, conspecific (chatter); HET, heterospecific (mourning dove coo). AHY/CON, $N=5$; AHY/HET, $N=6$; HY/CON, $N=9$; HY/HET, $N=6$. NCM ZENK immunoreactivity across age category, $P=0.08$; CON/HET category, $P=0.001$; interaction between main effects, $P=0.29$. CMM ZENK immunoreactivity across age category, $P=0.14$; CON/HET category, $P=0.009$; interaction between main effects, $P=0.01$. Data are means±s.e.m.; **$P<0.01$; ***$P<0.001$.

type ($F_{1,19}=2.4$, $P=0.13$; Fig. 5). In contrast, the density of ZENK immunoreactivity exhibited a significant interaction between previous song experience and song type in the CMM ($F_{1,19}=14.1$, $P=0.001$; Fig. 5), whereas there was no significant difference in ZENK immunoreactivity in relation to previous song experience ($F_{1,19}=0.3$, $P=0.58$; Fig. 5) or song type ($F_{1,19}=0.3$, $P=0.58$; Fig. 5). Results of post-hoc analysis for each comparison in this experiment are listed in Table 1.

DISCUSSION

The results presented here assess the neural basis of acoustically cued brood parasitic species recognition in cowbirds and identify a neural representation of the chatter call, which serves as a signal that reliably identifies conspecifics. Specifically, we demonstrate that auditory forebrain regions that respond selectively to learned
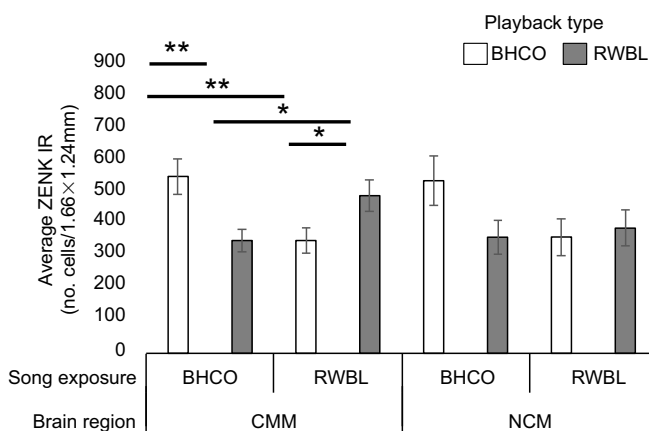
Fig. 5. Results of IEG induction in juvenile cowbirds only. ZENK immunoreactivity in the auditory forebrain was measured in juvenile male birds with and without prior song exposure. BHCO, brown-headed cowbird; RWBL, red-winged blackbird; CMM, caudomedial mesopallium; NCM, caudomedial nidopallium. Data are means±s.e.m.; *$P<0.05$; **$P<0.01$.

vocalizations, such as conspecific songs in parental (Woolley et al., 2010) and brood parasitic (Louder et al., 2016) songbirds, also respond selectively to the non-learned chatter in parasitic cowbirds. The chatter-selective IEG induction occurs in both the NCM and CMM regions in adults and within the NCM in the juveniles. These results reveal a selective neural representation of this non-learned vocalization within the NCM in juvenile male cowbirds that is malleable across juvenile and adult stages (Table 2). In contrast, we demonstrate that juvenile cowbirds do not exhibit song-selective responses in these auditory forebrain regions unless they are provided with recent experience of songs. However, the song that the juvenile cowbird hears during playback appears to have little effect on activity-dependent gene induction in the auditory forebrain, as juvenile cowbirds provided with recent prolonged exposure to conspecific and heterospecific songs exhibit elevated gene induction in response to the song with which they are familiar (Table 2). Consequently, we see a neural signature of imprinting in juvenile cowbirds with prolonged exposure to songs from unrelated species but without recent song exposure; these regions do not exhibit a neural response to either song type in juvenile male cowbirds. Together, our findings from both experiments indicate that the chatter is more salient than other non-password conspecific vocalizations in juvenile cowbirds. In addition, our findings support previous behavioral experimental evidence that juvenile cowbirds use chatter calls to initially identify groups of conspecifics then learn and adopt the songs of the conspecifics and the acoustic mate-choice preference cues only after long-term exposure (Freeberg et al., 1995; Freed-Brown and White, 2009).

The chatter was initially identified as a likely candidate to serve as a password that aids in species identification for brown-headed cowbirds because the chatter: (1) is vocalized frequently by females and occasionally by males; (2) is produced throughout the breeding season when young cowbirds are hatching and fledging; and (3) has no dialects throughout the large species range, implying it is not a vocalization that is learned from local conspecifics (Burnell and Rothstein, 1994). Behavioral ontogenetic experiments have confirmed that young cowbirds have a perceptual bias towards chatter calls, and upon independence they preferentially approach these vocalizations relative to other calls, including chatter-like vocalizations of other species (Hauber et al., 2001). Furthermore, female adult cowbirds use the chatter as a cue to assess the song potency of males (Freed-Brown and White, 2009). As a potential password, the chatter should be used to identify conspecifics and to initiate social learning with respect to conspecific traits (Hauber et al., 2001). This is consistent with the time frame of song learning reported in cowbirds, in which cowbirds memorize song in their second year but delay production until the third year (Brenowitz and Beecher, 2005). Thus, the chatter call may be the first social cue the young cowbird uses for social recognition before song learning can occur in the second year.

Previous behavioral studies of the chatter demonstrated that juvenile cowbirds were more responsive to the chatter, as opposed to learned vocalizations such as flight whistles, courtship songs and heterospecific songs (Hauber et al., 2001), which is similar to behavioral responses of adults (Hauber, 2002; Rothstein et al., 2000). By pairing the known behavioral and the novel neural response results here in response to chatter calls versus songs, we can begin to understand social recognition in brood parasitic species. For example, juvenile cowbirds depart from hosts during the first few weeks after fledging, potentially assisting with the avoidance of mis-imprinting (Louder et al., 2015). Upon locating flocks of other juvenile and adult conspecifics by attending to the

Table 1. *Post hoc* results for experiment 2

Exposure song type	Test stimulus	Exposure song type	Test stimulus	Estimate	s.e.	d.f.	*t*	*P*
BHCO	BHCO	BHCO	RWBL	0.45	0.144	19	3.12	0.0056*
BHCO	BHCO	RWBL	BHCO	0.4499	0.1511	19	2.98	0.0078*
BHCO	BHCO	RWBL	RWBL	0.1151	0.1436	19	0.8	0.4325
BHCO	RWBL	RWBL	BHCO	−0.0001	0.1517	19	0	0.9995
BHCO	RWBL	RWBL	RWBL	−0.3349	0.1442	19	−2.32	0.0315*
RWBL	BHCO	RWBL	RWBL	−0.3348	0.1513	19	−2.21	0.0393*

The comparisons between each song type juveniles were exposed to and the song type presented on test day.
BHCO, brown-headed cowbird; RWBL, red-winged blackbird.
*$P<0.05$.

chatter, they are then exposed to additional species-specific signals, including plumage, display and acoustic traits (Freeberg et al., 1995). Additional behavioral, field and neural studies are still needed to further discern the role of the chatter as a password that cues species recognition. Such future neural studies should also reveal whether the chatter is more salient than spectrally or temporally similar heterospecific vocalization. To date, these specific acoustic comparisons have only been conducted at the ontogenetic-behavioral level (Hauber et al., 2001).

The brain regions involved in the neural representation of the chatter are frequently involved in the recognition of learned vocalizations used in courtship or territorial defense in adult songbirds. A long history of studies demonstrate that the NCM and CMM display greater activity-dependent gene expression when measured by three different IEGs (*ZENK*, *C-fos* and *Arc*) in birds exposed to conspecific songs when compared with control sounds (Mello et al., 2004; Velho et al., 2005). The amount of expression of these genes in these two auditory regions is not simply related to whether the bird is exposed to conspecific song or some other sound, rather IEG induction also reflects biologically relevant characteristics of the song, such as: regional dialects (Maney et al., 2003), song quality (Leitner et al., 2005; Monbureau et al., 2015), recent experience with song (Sockman et al., 2002), associative and non-associative song learning (Gentner et al., 2004; Jarvis et al., 1995), and even whether the song is directed to a conspecific or undirected (Woolley and Doupe, 2008). These patterns of gene induction hold true across sexes (Lynch and Ball, 2008; Lynch et al., 2012; Mello et al., 2004; Monbureau et al., 2015; Woolley and Doupe, 2008) and various passerine species (Gentner et al., 2004; Louder et al., 2016; Maney et al., 2003; Mello et al., 1992; Schubloom and Woolley, 2016). Interestingly, our results not only reveal that the NCM also represents non-learned vocalizations in juvenile cowbirds but we also report that the NCM and CMM are responding differently to this vocalization in juvenile birds but not adults. The mean ZENK count data shows that the CMM might be used differentially among juveniles and adult cowbirds, as there is a

significant interaction for age and stimulus-type in the CMM, but not the NCM. This is also consistent with other studies that have found differential ontogenetic and functional roles in these auditory forebrain regions. For example, female zebra finches exhibit greater *ZENK* induction in the CMM in response to preferred song (i.e. song directed to a conspecific), as opposed to undirected song (Woolley and Doupe, 2008). In contrast, the number of *ZENK*-expressing cells in the NCM depends on whether the song is familiar or unfamiliar (Woolley and Doupe, 2008). Thus, the CMM and NCM serve different functions in these birds, as the CMM is sensitive to category or quality of song, whereas the NCM is sensitive to familiarity with the song.

Here, we find that the NCM is responding to a non-learned vocalization in which previous experience with the sound may have little to do with responses in this region, at least in juvenile birds. On the other hand, the CMM in juvenile cowbirds does not exhibit differential *ZENK* induction in response to the chatter. It is possible that the NCM in juveniles and adults provides a neural representation of a password for species recognition, whereas discrimination tasks that require finer tuning and greater subtlety, especially with songs that require learning, may be within the domain of the CMM (Jeanne et al., 2011). Thus, juvenile birds that have less experience with song exposure or various song qualities compared with adults would express less *ZENK* induction in this region after exposure to chatter. A recent study of the neural responses to song in the NCM and CMM of the pin-tailed whydah (*Vidua macroura*), an African obligate brood parasitic finch, supports this hypothesis, as those results reveal that the CMM does display a greater increase in *ZENK* expression in response to conspecific songs when compared with heterospecific songs (Louder et al., 2016). Finally, our own results from experiment 2 confirm that experience-dependent species-specific responses to songs in juvenile male cowbirds is also limited to the CMM, and imply the persistence of a potential neural mechanism of mis-imprinting in parasitic cowbirds at early developmental stages. To avoid such experience-driven neural selectivity, cowbird young must locate and socialize with flocks of conspecifics from an early age onwards. Behavioral evidence through chatter-based password (Hauber et al., 2001) and habitat selectivity-based (Louder et al., 2015) mechanisms are in support of this developmental trajectory of conspecific recognition in juvenile cowbirds.

These results support the hypothesis that the auditory forebrain responds to signals that may serve as a species recognition password. Specifically, this neural representation exists within the NCM throughout juvenile and adult stages. The NCM is a region that is also involved in the representation of more-complex learned vocalization in many adult passerine species. The neural representation of a non-learned vocalization acting as a password for brood parasitic species identification appears to have evolved by using existing neural mechanisms. It is possible that part of the

Table 2. Comparing the outcomes of experiments 1 and 2

Brain region Age	CMM HY	NCM HY	CMM AHY	NCM AHY
Experiment 1: calls	No	Yes	Yes	Yes
Experiment 2a: conspecific	Yes	Yes	n/a	n/a
Experiment 2b: heterospecific	No	No	n/a	n/a

There is greater activation of brain regions in response to conspecific over heterospecific stimuli. In experiment 1, juvenile (HY) and adult (AHY) male cowbirds were exposed to non-learned vocalizations (calls). Experiments 2a and 2b are part of the same experiment; however, the song type listed in each row refers to the song that juvenile male cowbirds were recently exposed to for 8 days for 8 h day^{-1}.

auditory forebrain was co-opted in parasitic cowbirds to represent non-learned vocalizations, so as to avoid mis-imprinting upon unrelated host species. Furthermore, as brood parasitic songbirds evolved from non-parasitic ancestors (Sorenson and Payne, 2002), auditory forebrain-based neural processing of passwords may also play a prominent role in the initiation and guidance of the learning process and social function in non-parasitic species (Soha and Marler, 2000; Whaling et al., 1997).

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