

Current Biology

An Acoustic Password Enhances Auditory Learning in Juvenile Brood Parasitic Cowbirds

Highlights

- Exposure to cowbird chatter calls (“the password”) enhanced song learning in male cowbirds
- Exposure to chatter calls in early life induced neurogenomic signatures of plasticity
- Songs paired with chatter calls triggered neurogenomic signatures of familiarity
- The password provides a mechanism to guide learning of specific songs

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In Brief

How obligate brood parasites, which are raised by different host species, recognize and learn phenotypes of their own species remains unknown. Louder et al. demonstrate that a specific non-learned vocalization (the “password”) serves as a cue to initiate the onset of perceptual learning for other socially relevant signals in parasitic cowbirds.



An Acoustic Password Enhances Auditory Learning in Juvenile Brood Parasitic Cowbirds

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SUMMARY

How does a naive, young animal decide from which adults to learn behavior? Obligate brood parasitic birds, including brown-headed cowbirds (*Molothrus ater*), face a particular challenge in learning species-specific behaviors; they lay their eggs in the nest of another species, and juveniles are raised without exposure to adult conspecifics. Nevertheless, male cowbirds need to learn a conspecific song to attract appropriate mates, and female cowbirds need to learn to identify conspecific males for mating. Traditionally, it was thought that parasitic bird species rely purely on instinctual species recognition [1–4], but an alternative is that a species-specific trait serves as a “password” [5], a non-learned cue for naive animals that guides decisions regarding from whom to learn. Here, we tested the hypothesis that the adult “chatter call” enhances the learning of specific songs in juvenile cowbirds. We exposed acoustically naive juvenile male and female cowbirds to songs paired with chatter calls and found that the chatter call enhanced song production learning in males and induced a neurogenomic profile of song familiarity in females, even for heterospecific songs. Thus, a combination of experience-independent and -dependent mechanisms converges to explain how young cowbirds emerge from another species’ nest yet learn behaviors from conspecifics. Identifying whether such password-based mechanisms relate to perceptual and behavioral learning in non-parasitic taxa will contribute to our general understanding of the development of social recognition systems.

RESULTS AND DISCUSSION

Young animals learn diverse behaviors and preferences from adults of their own species, yet how naive juveniles determine

suitable referents for learning remains unclear [6]. Despite being raised exclusively by other species, obligate brood parasitic birds reliably recognize and reproduce with their own species. Although juvenile brood parasites are known to learn behaviors and social preferences from conspecifics [7–9], the mechanisms that guide decisions when to learn and from whom to learn remain unclear. The password hypothesis [5], which proposes that a specific non-learned cue is used to guide the learning of phenotypes associated with that cue, provides a plausible yet untested explanation for how brood parasitic cowbirds learn their species-specific behaviors, including their vocal repertoire [5, 10].

For the brown-headed cowbird, the “chatter call” is proposed as a password for song learning [5]. Adult female brown-headed cowbirds produce the chatter call in a variety of social contexts, including in response to conspecific intruders [5] and following preferred male songs [11]. The chatter calls are produced throughout the year and do not show geographic variation in acoustic structure [12]. In courtship contexts, males modify their own songs in response to female chatter calls [7] and females, who do not sing, use other females’ chatter calls as a guide for preferred male songs [11]. Nestling and juvenile cowbirds, which lack prior exposure to adult cowbird vocalizations, display phonotactic behaviors consistent with the early preferences for conspecific chatter calls [5]. In turn, young cowbirds exposed to adult conspecifics (i.e., captured in cowbird flocks) exhibit greater neural activity, as measured by expression for the immediate-early gene *EGR1*, in response to the chatter call relative to control heterospecific calls [13]. Thus, the chatter call appears a plausible behavioral and neuro-developmental candidate to serve as a password, yet the effects of the chatter call on song learning in juveniles have not been tested in naive cowbirds.

Here, to test the hypothesis that the chatter call enhances the learning of two key behaviors, song production in males and song recognition in males and females, we compared behavioral and neural responses for songs paired with and without the chatter call. We demonstrate that, relative to controls, hearing songs coupled with chatter calls increased song acquisition in males and recognition learning in both sexes, even when the song is heterospecific and for juveniles that lack previous experience with the chatter call.



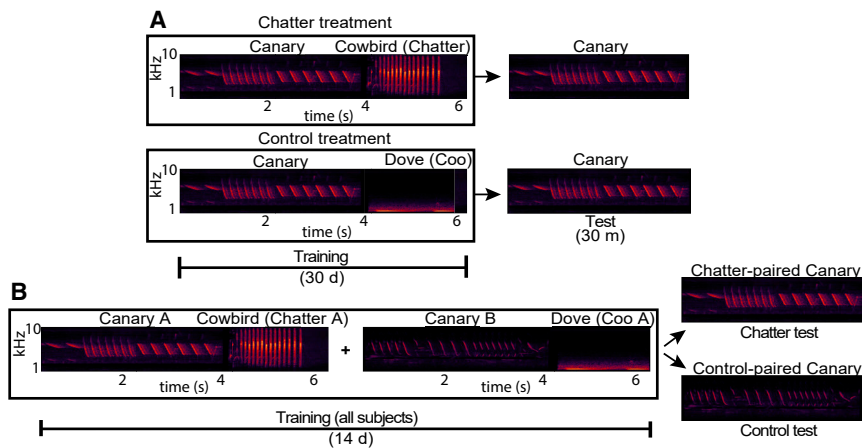


Figure 1. The Experimental Stimuli and Design

(A) Naive juvenile male brown-headed cowbirds (70–80 days post-hatching) received either chatter treatment (canary song paired with cowbird chatter call) or control treatment (canary song paired with a dove coo); then, all males were tested with the respective canary song only.

(B) Naive juvenile female cowbirds (40–50 days post-hatching) were trained with both a canary song paired with a cowbird chatter call and a second canary song paired with a dove coo and then tested with either the chatter-paired or control-paired song-only playback. Spectrograms are displayed from one group of randomly selected females. A second group heard different exemplars of canary songs, dove coos, and cowbird chatters.

Chatter Call Biases the Acquisition of Song in Naive Juvenile Male Cowbirds

To test whether hearing the female chatter call influences acquisition of specific song structure in males, we exposed song-naive juvenile males, hand-raised without contact with adult birds, to playbacks of either a canary (*Serinus canaria*) song immediately followed by a conspecific chatter call (chatter treatment; $n = 3$) or the same canary song followed by a mourning dove (*Zenaida macroura*) coo (control treatment; $n = 4$; Figure 1A) for 60 min a day starting at age 70–80 days post-hatching. We chose to pair the chatter call with a heterospecific song because separating the effects of hearing cowbird songs paired with or without chatter calls may have been more biologically and statistically challenging, given that isolate-raised cowbird males' songs already have some similarity to the social (typical) conspecific songs [4]. Furthermore, previous experiments demonstrated that vocally naive male cowbirds can learn to produce canary-like songs (repeated notes structurally similar to canary songs or calls) after 150–300 days post-hatching [14, 15]. Juvenile cowbirds in the wild generally do not join conspecific cowbirds flocks until at least 40–60 days post-hatching [16] and, in some populations, may not interact with adults and learn songs for up to 1 year post-hatching [17]. Female cowbirds produce chatter calls in response to male song in autumn conspecific flocks and influence the development of male songs [18], thereby supporting the ecological validity of the age of juveniles used for this experiment.

After 30 days of playback training, we recorded males singing and analyzed 5 distinct song bouts per subject. Using a spectrogram-based acoustic similarity index [19], we determined that chatter treatment males produced songs with significantly greater similarities to the canary song than did control treatment males (Figure 2B; $F_{1,5} = 6.98$; $p = 0.04$). Furthermore, when examining representative bioacoustic features of subjects' songs in the chatter versus the control treatment, frequency range and duration features were not significantly different ($p > 0.10$; data not shown), but chatter subjects had songs with consistently lower entropy (i.e., less disorder and greater tonality [20]; mean = 1.97 ± 0.08 SE) relative to control subjects (mean = 2.46 ± 0.07 ; $F_{1,5} = 23.68$; $p = 0.005$). Although statistically different, the songs of chatter treatment males remained only

modestly correlated to canaries (Figure 2A). Genetically heritable mechanisms and whether juveniles learned from song playbacks versus live tutors can strongly limit the similarity to tutor songs [21]. Therefore, the imperfect imitation of canary song by cowbirds could be attributed to inherent limitations in producing heterospecific songs imposed by the cowbird vocal tract, the young age of the juvenile cowbirds, the short duration of the experimental exposure (30 days), and/or the lack of a live song tutor.

Chatter Call Induces Molecular Markers of Neural Plasticity in Juvenile Male Cowbirds

Because hearing chatter calls significantly increased the degree to which males copied playback songs, we then tested for a neural basis of that effect. We focused on the auditory forebrain as it is critical to auditory memory and song learning in songbirds [22–24] and song experience alters chromatin states that regulate transcriptional responses [22, 25] that mediate the sensory component of song learning [26], including conspecific auditory recognition in brood parasites [13, 27]. We predicted that, if the chatter call served as a signal for what to learn, then birds that had been exposed to the chatter call would display neurogenomic responses indicative of greater learning in the auditory forebrain compared to birds that had not been exposed to the chatter call.

To test for transcriptomic signatures of chatter-induced neuroplasticity, we examined brain gene expression in males from the previous song-acquisition experiment. We exposed each male cowbird to the training canary song alone for 30 min (without coupling to the chatters or dove calls) and immediately extracted the auditory forebrain for subsequent RNA sequencing [28]. We aligned RNA reads to a reference-guided genome assembly we constructed for the brown-headed cowbird (see STAR Methods), quantified read abundances, and tested for differential expression between chatter and control treatments (Figure 1A). On average, 76% of the 26.5 million reads (after trimming) aligned to the cowbird reference-guided genome assembly. Of the 12,045 total genes with above-threshold levels of RNA, 32 were considered significantly differentially expressed (adjusted $p < 0.10$; Table S1).

Several differentially expressed genes demonstrate support for the prediction that exposure to the chatter call influences

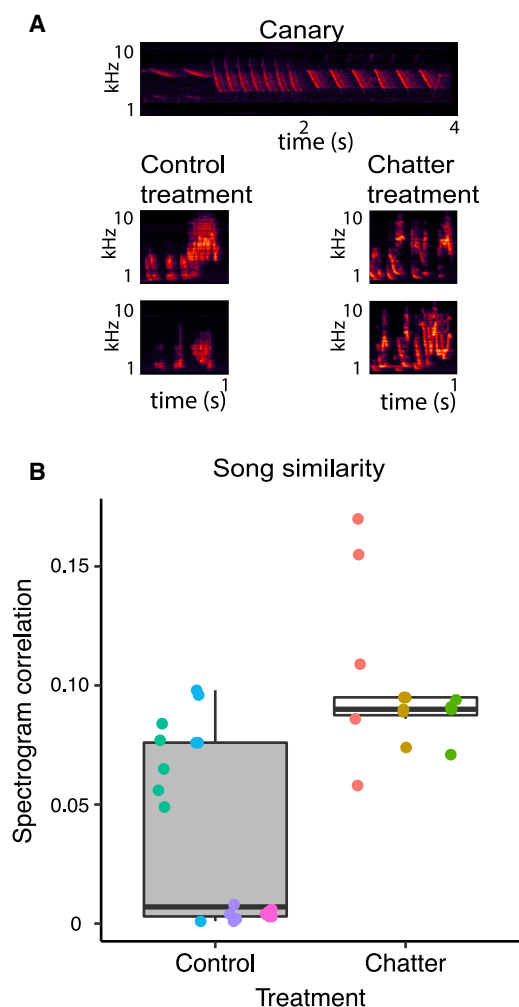


Figure 2. Males Exposed to Chatter Treatment Sing with Greater Similarity to Canary Songs

(A) Representative spectrograms for songs from two individuals from each treatment.

(B) Boxplots for song correlation for each male from control and chatter treatments are presented (dot color represents each subject; 5 songs per subject). Despite some overlap in song correlation between treatment groups, male brown-headed cowbird songs are more similar to canary song in chatter versus control treatment using a spectrogram-based acoustic similarity index ($F_{1,5} = 6.98$, $p = 0.04$).

neuroplasticity. The top-ranked differentially expressed gene, annotated as a probable glutamate receptor (LOC102072152; Figure 3B), was significantly lower in the chatter treatment subjects compared to the control (rank = 1; Wald = 6.94; adjusted $p < 0.0001$; Table S1). This gene is orthologous to KBP (kainite binding protein), which in chickens (*Gallus gallus*) shows declining expression levels throughout the brain with increased age [29]. Further, the glutamate class of receptors has well-known effects on synaptic transmission, plasticity, and development [30]. We also found that aromatase RNA was significantly less abundant in male cowbirds exposed to the chatter treatment relative to controls (rank = 2; Wald = 6.79; adjusted $p < 0.0001$; Figure 3A;

Table S1). Aromatase is a steroidogenic enzyme that converts androgens into estrogens and is involved in auditory processing and tutor song memorization during developmental song learning in the zebra finch (*Taeniopygia guttata*) auditory forebrain [31, 32]. Additionally, TENM1 (teneurin-1) and CASP6 (caspase-6) were significantly differentially expressed (rank = 3 and 4; Wald > 4.75; adjusted $p < 0.006$; Table S1). TENM1 is developmentally regulated in brain areas involved in sensory information processing in chicken [33], and CASP6 induces neuronal degradation and age-dependent memory function in the rodent hippocampus [34].

To determine which functional categories of differentially expressed RNAs were overrepresented, we performed rank-order-based Gene Ontology (GO) analysis [35]. Rank-based approaches do not require that individual genes are significantly differentially expressed but rather test for functional cohesion among genes with lower p values. We identified several significant GO categories (adjusted $p < 0.001$) indicative of neural plasticity, such as “regulation of nervous system development,” “regulation of axonogenesis,” and “regulation of neuron differentiation” (Figure 3C). Genes that contribute to these GO terms had greater mRNA abundance in the chatter treatment, and several have known associations with the development of auditory learning in songbirds (Figure 3D). For example, hearing conspecific songs activates mTOR (mechanistic target of rapamycin) signaling in the auditory forebrains of male zebra finches during the sensory song learning period, but not in younger males [36]. Similarly, GPER1 (G protein-coupled estrogen receptor 1) has increased relative expression here in male cowbirds exposed to the chatter call and is known to mediate neuroestrogen signaling while increasing developmentally during the sensory song learning period in male zebra finches [37].

Overall, juvenile cowbird males that experienced chatter calls sang songs with greater resemblance to canary playback songs and with more tonal structure (lower entropy) relative to controls. These male cowbirds also exhibited greater functional neurogenomic signatures of neuroplasticity upon re-exposure to the canary song alone. This is consistent with the password hypothesis for conspecific song learning in parasitic cowbirds. We next tested whether the chatter call enhances auditory learning for a specific canary song when all subjects experienced both chatter-paired canary songs and controls, as predicted by the password hypothesis.

Familiarity Increases for Specific Canary Songs that Had Been Paired with the Chatter Call

Female cowbirds do not produce songs, but they prefer particular male songs previously paired with chatter calls [11]. We therefore hypothesized that the chatter call served as a specific cue for selective song recognition learning, even without prior experience with chatter calls. To test this hypothesis, we examined whether canary songs that had been paired with the chatter call resulted in neurogenomic responses that, based on a prior literature of the molecular biology of learning and memory in songbirds, would indicate enhanced learning when compared to controls.

Juvenile females (40–50 days post-hatching) naive to adult cowbird vocalizations were each trained on two pairs of stimuli for 14 days: a canary song paired with a chatter call (e.g., canary

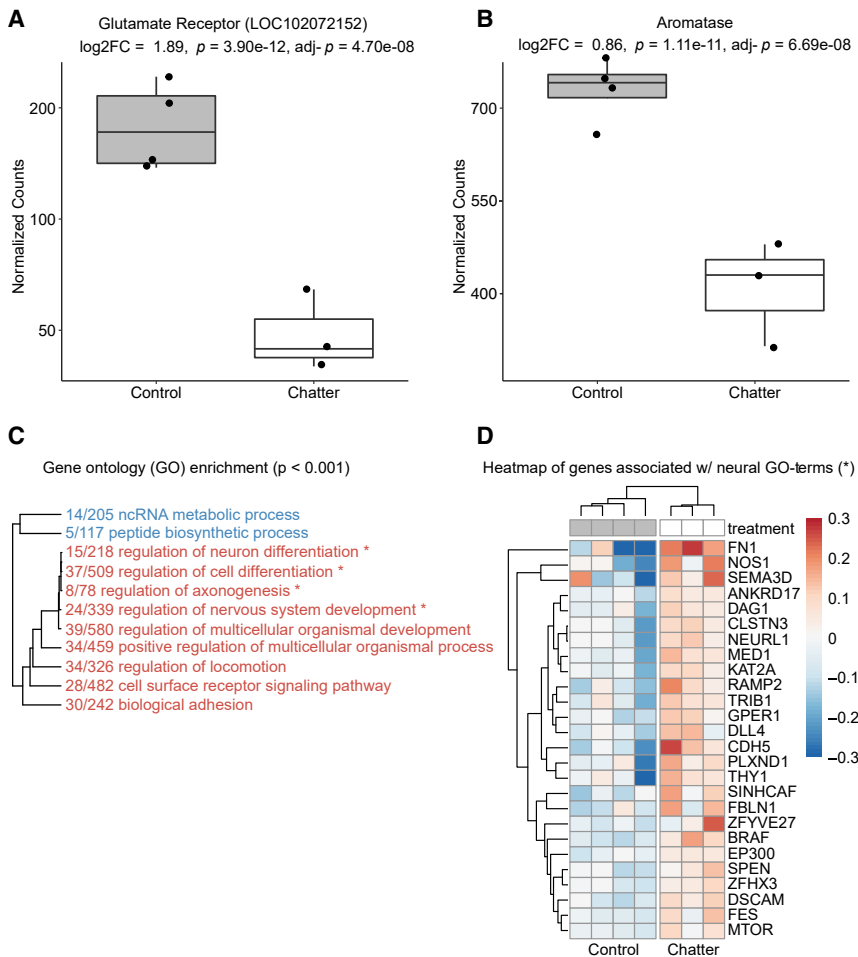


Figure 3. Males Exposed to Chatter Treatment Show Signatures of Neuroplasticity

(A and B) Boxplots for the two top-ranked differentially expressed genes, (A) glutamate receptor and (B) aromatase, both showing lower RNA levels in the chatter treatment than the control condition. All p values are relative to chatter vs. control treatments.

(C) Results of significant Gene Ontology (GO) terms (false discovery rate [FDR] $p < 0.001$). Colors in GO term list indicate enrichment of GO categories with either red (upregulated) or blue (downregulated) genes. The numbers of significant genes ($p < 0.05$) relative to the total numbers of genes belonging to each GO category are listed. The hierarchical tree indicates the level clustering of GO categories based on the number of shared genes.

(D) The heatmap of genes from GO terms associated with neural plasticity (denoted with * in C) ranked high on the global list (raw $p < 0.05$), with Z score normalized expression values in red for upregulated genes in the chatter treatment. See also Table S1.

A; Figure 1B) and a different canary song paired with a dove coo (e.g., canary song B; Figure 1B). Distinct canary songs, dove coos, and cowbird chatters were used between two groups of randomly selected females ($n = 4$ in each group) such that one group was exposed to canary A and B pairings and the other group heard different canary songs (i.e., canary C and D) paired with a different chatter and coo, respectively. After 2 weeks, during which stimulus pairs were played daily (two sessions of 30 min), we compared the females' auditory forebrain neurogenomic response to hearing 30 min of either chatter-paired or control-paired canary-song-only playbacks using RNA sequencing (RNA-seq). These females were still too young for mating assays, such as copulation solicitation displays; thus, we could not collect behavioral or song preference data on female subjects [4].

On average, 75% of the 31.9 million RNA-seq reads (after trimming) aligned to the cowbird reference-guided genome assembly. Of the 12,100 total transcripts with above-threshold levels of expression, 25 were significantly differentially expressed (adjusted $p < 0.10$) between hearing chatter-paired or control-paired playbacks (Table S2).

Across species and experimental paradigms, recognition of a previously learned stimulus can be characterized by diminished genomic, molecular, and electrophysiological responses when compared to novel stimulus responses in the relevant brain areas

identified as the top-ranked markers for song habituation in the zebra finch auditory forebrain [38]. As would be predicted if chatter-paired songs were more familiar, all 6 of these genes were expressed at lower levels in response to the chatter-paired canary song compared to controls; 4 were significantly different at $p < 0.05$ (without adjusting for multiple testing because each candidate gene was considered individually; Wald > 2.83 ; $p < 0.004$; Figure 4). Critically, these same 4 genes, NR4A2, NR4A3 (nuclear receptor subfamily group A), EGR1 (early growth response 1), and FOSL2 (FOS-related antigen 2), are highly expressed after playbacks of novel song but are expressed at lower levels after a song becomes familiar in zebra finch auditory forebrain, a molecular signature of learning [40, 43]. All 4 of these genes are involved in long-term memory formation across species [44, 45], and of note, NR4A2 and NR4A3 were among the top significantly differentially expressed genes here (rank = 2 and 3; Wald > 4.73 ; adjusted $p < 0.001$; Table S2).

Several more differentially expressed genes in our datasets provided additional support for greater familiarity of the chatter-paired canary song in female cowbirds. For example, RELN (reelin), which plays a critical role in synaptic plasticity, dendritic morphogenesis, and associative learning in mice, was significantly less abundant after hearing playback of the chatter-paired canary song compared to controls (rank = 15; Wald = 3.99; adjusted $p = 0.05$; Table S2) [46]. Additionally, KCND2

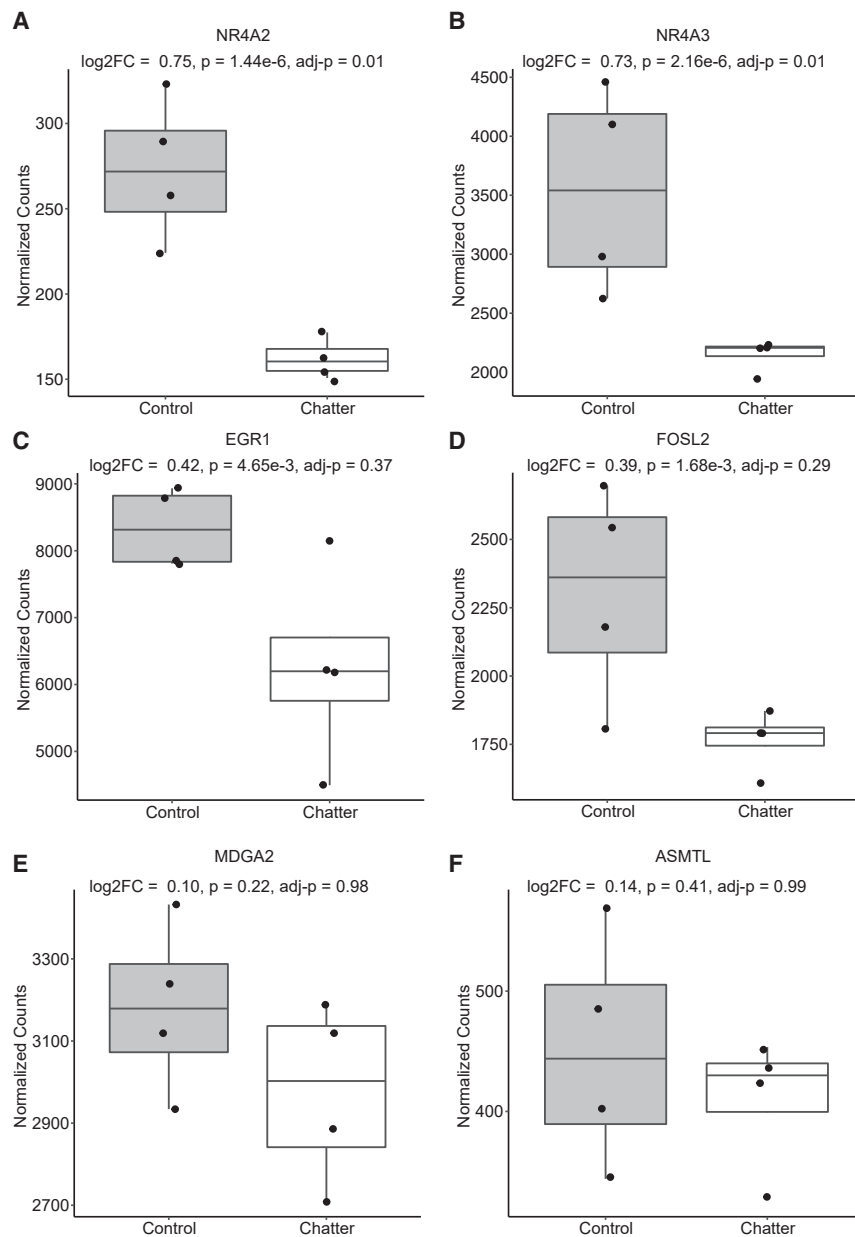


Figure 4. mRNAs Diagnostic of Song Familiarity Show Expected Relative Abundances in Female Cowbirds

(A–F) Boxplots of RNA levels (normalized counts) in female cowbirds for the 6 known song familiarity genes [43], (A) NR4A2, (B) NR4A3, (C) EGR1, (D) FOSL2, (E) MDGA2, and (F) ASMTL, annotated in the cowbird genome assembly. All p values are relative to chatter vs. control treatments. See also Table S2.

parasite, even when the stimulus is a heterospecific song. Here, the password appears to not only affect neurogenomic signatures for neuroplasticity but also serves as a specific cue for learning certain songs for juveniles that lack previous experience with the password. Given the importance of songs in cowbirds' social interactions, such as mate choice and species recognition, password-based learning is predicted to have long-term implications on parasitic preferences and behavior.

Instead of simply advocating for “innate” recognition as a feasible mechanism for avian brood parasite's behavioral development [1–4], these experiments expand our understanding of what triggers the vocal, ontogenetic, and neural basis of socially guided auditory learning in these species. Furthermore, experience-independent cues that initiate learning may be characteristic of other systems where “innate” processes have been invoked: for example, the recognition of specific vocal signals early in development [50–52] and password-like learning [53] are also observed in non-parasitic songbird lineages. Thus, password-based learning potentially represents a more broadly relevant pattern and feasible mechanism of socially cued species recognition learning

(potassium voltage-gated channel subfamily d member 2) is linked to dampened excitatory neuronal responses to novel stimuli in mice and mRNA was less abundant in response to the chatter-paired song (rank = 25; Wald = 3.71; adjusted p = 0.09; Table S2) [47].

The known neuroplasticity genes that showed lower relative expression levels after birds heard chatter-paired canary song compared to control-paired canary song are consistent with the interpretation that the chatter call enhanced learning of the associated songs. Similar responses in the auditory forebrain would be expected in female and male cowbirds, as found in zebra finches [48, 49], although further tests are required to confirm this.

Overall, the experiments reported here support the hypothesis that the cowbird chatter call serves as an acoustic password cue to enhance the learning of a coupled song in this obligate brood

in birds and other social taxa in general. If widespread across taxa, identifying the underlying molecular and neural mechanisms that contribute to password-based learning will improve our understanding of the evolution of social behavior.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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- **QUANTIFICATION AND STATISTICAL ANALYSIS**
- **DATA AND CODE AVAILABILITY**

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.cub.2019.09.046>.

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AUTHOR CONTRIBUTIONS

M.I.M.L., C.N.B., and M.E.H. designed the experiments; M.I.M.L., M.E.H., and R.J.D. collected field samples; M.I.M.L., C.N.B., A.A.N.L., R.J.D., and M.E.H. raised the subjects; M.I.M.L. collected all recordings and tissue samples; A.A.N.L. conducted molecular lab work; M.I.M.L., C.N.B., and M.E.H. analyzed the data; M.I.M.L., C.N.B., S.E.L., and M.E.H. wrote the first draft; and all authors provided critical feedback and reviewed and edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological Samples		
Frozen tissue for DNA from <i>Molothrus ater</i>	Museum of Southwestern Biology	MSB:Bird:39518
Fresh tissues for RNaseq from <i>Molothrus ater</i>	nature	N/A
Deposited Data		
Cowbird raw RNaseq reads	NCBI SRA	BioProject: PRJNA565489
Software and Algorithms		
DEseq2	[54]	V1.2
Trim Galore	http://www.bioinformatics.babraham.ac.uk	V0.3.7
HiSat2	[55]	V2.1
HTSeq-count	[56]	V0.9.1
BWA	[57]	V0.7.1
Genome Analysis Toolkit	[58]	V3.8
R	https://www.r-project.org	V3.5.1
Audacity	https://www.audacityteam.org	V2.2.0
Raven Pro	[20]	V1.5
GO_MWU	https://github.com/z0on/GO_MWU	N/A
Other		
formula for nestling songbirds	[59]	N/A

LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and recordings should be directed to, and will be fulfilled by, the Lead Contact, Matthew Louder (mckimlouder@gmail.com). This study did not generate new unique reagents or software.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Subject rearing and housing

Cowbird nestlings were collected from the wild at 3–5 days post-hatching in Champaign and Pulaski Counties, Illinois, USA. We hand-reared cowbirds in isolation from conspecific and heterospecific adults to ensure that they would not hear adult male songs or female chatter calls prior to experimental treatments. Nestlings were raised with cowbird nestmates in a ‘simulated nest’, in which plastic bowls were lined with paper towels to provide nest lining. Nestlings were kept within an incubator/brooder until chicks were fully fledged and mobile (~10 days post hatching). Nestling cowbirds were fed the FoNS diet (formula for nestling songbirds) via syringe every 30–60 min from 600 until 1900 daily [59]. Once subjects could feed from a bird seed/dog-food mixture independently, they were moved to a large indoor aviary and housed together until experimental playback trials. Birds were provided water, cuttle bone, and food mixture *ad libitum* with supplemental fresh spinach, carrots, blueberries, crickets, and hard-boiled eggs.

During the period of experimental playback trials, subjects were individually housed in cages within sound attenuation chambers (Med Associates) each installed with a fan and a light. On average, sound is attenuated 44 dB at 100 Hz between two chambers. Furthermore, we lined the walls with acoustic foam to increase sound attenuation. We provided subjects the same food and water as above *ad libitum*. To reduce potential adverse effects of social isolation, a female zebra finch was included in each sound attenuation chamber as a companion. Female zebra finches do not sing songs [60], and therefore are unlikely to influence song learning of male or female cowbirds.

Ethical Note

This research was approved under University of Illinois animal care and use permit (IACUC #18052) and the U.S. Fish and Wildlife Service (permit # MB08861A). The study was designed to minimize the number of subjects used terminally and potential effects of prior stress.

METHOD DETAILS

Experimental playback trials

Playback treatments were performed within sound attenuation chambers. Songs were broadcast at ~65 dB at 0.5 m from the speaker. We filtered playback stimuli above 10000 Hz and below 500 Hz and normalized for mean amplitude of all stimuli with Audacity (v 2.2.0). For males at age 70–80 days post hatching, we broadcast a canary song (4 s) followed by a chatter call (2 s) (Chatter treatment, $n = 3$) or the same canary song followed by a mourning dove coo (Control treatment, $n = 4$). Stimulus pairs were played 4 times a minute for 30 minutes twice daily (at 800 and 1700); for a total of 60 min each day. After 1 month, males were recorded singing within the sound attenuation chambers for ~1 hr. Prior to tissue extraction at 1 month, males were exposed to the canary song only for 30 min (4 songs per min).

For females at age 40–50 days post hatching ($n = 8$), for 14 days we broadcasted a canary song (e.g., Canary song A) immediately followed by a chatter call (4 per m) followed by a different canary song (e.g., Canary song B) followed by a dove coo. The stimulus pairs were played for 30 min twice daily as above. Prior to tissue extraction at 14 days, females were exposed to 30 min of experimental playbacks of either Chatter-paired canary song only (4 songs per min) or Control-paired canary song only (4 songs per min). Furthermore, we used distinct canary songs, dove coos, and cowbird chatters for two groups such that one group was exposed to Canary A and B pairings ($n = 4$ each for each group) and the other group heard different canary songs (i.e., Canary C and D) paired with a different chatter and coo respectively.

Song analysis

To analyze the bioacoustic distances [e.g., 19] between the playback canary song and the juvenile male cowbird's developing songs, we recorded each subject in their home cage and sound proof chamber for ~60 min. We obtained recordings for the analyses from $n = 3$ Chatter-treatment males and $n = 4$ Control-treatment males. For each recording, we created a spectrogram of each male's vocalizations using Raven Pro, version 1.5 [20], by applying discrete Fourier transforms calculated using a Hann window of 235 samples with 50% overlap and 256 frequency bins. We then subsampled each recording to generate the spectrogram of $n = 5$ distinct song-like bouts from each male to capture the diversity of their vocalizations. We also generated the spectrogram for the canary song used in the playbacks.

Raven Pro [20] includes a sound correlation feature that contrasts two sounds simultaneously and computes a similarity score, based on either their waveforms or their spectrograms. This technique finds the best correlation coefficient by sliding one spectrogram past another so that different time lags are tested. For each possible time lag the correlation coefficient is calculated, and the highest coefficient is retained as the response metric. Here we calculated spectrogram correlation coefficients between each male's song bouts and its respective playback canary song, yielding $n = 35$ data points across $n = 7$ individuals. Finally, to assess the bioacoustic features in which Chatter versus Control juvenile male songs differed, we used Raven Pro to collect bandwidth, duration, and average entropy data on each of the 5 songs per subjects.

RNA-sequencing

After the experimental playback (30 min), subjects were immediately sacrificed via decapitation and the right hemisphere's auditory forebrain was extracted with a dental trowel (as in [28]). The tissues were placed in 250 μ L of Tri-Reagent (Molecular Research Company) and then immediately homogenized, followed by RNA extraction following the manufacturer's protocol. The total RNA was then DNase treated and purified (Promega), followed by cleanup with QIAGEN RNeasy (Valencia, CA, USA) mini kit. We assessed the quality of purified RNA on a Bioanalyzer (Agilent, Wilmington, DE, USA) (RIN > 7.0). All library preparations and sequencing were performed at the University of Illinois at Urbana-Champaign Roy J. Carver Biotechnology Center. A library for each sample was prepared with an Illumina TruSeq Stranded RNA sample prep kit. Libraries from each experiment (males and females) were pooled separately, quantitated by qPCR, and the pools were sequenced on two lanes of an Illumina HiSeq 4000 (prepared with a HiSeq 4000 sequencing kit version 1), producing single-end 100 bp reads; resulting in ~27 million reads per individual in males and ~31 million reads for females.

Reference-guided genome assembly

Lacking a reference genome for the brown-headed cowbird, we created a reference-guided genome assembly. Briefly, we extracted DNA from liver and muscle tissue of a female brown-headed cowbird (cataloged at the Museum of Southwestern Biology MSB:Bird:39518) and performed paired-end (200 bp) whole-genome sequencing on one lane of HiSeq (2500) at the Duke Genome Center. We then removed Illumina adapters from reads with Trim Galore! v0.3.7 (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) which incorporates Cutadapt v1.7.1 [61]. Following the "pseudo-it" pipeline (<https://github.com/bricesarver/pseudo-it>), we aligned whole-genome DNA reads to the closest phylogenetically related species whose reference genome was publicly available at the time, the white-throated sparrow (*Zonotrichia albicollis*) [62] with BWA-mem [57]. We then identified cowbird SNPs and inserted into the sparrow reference genome with the Genome Analysis Toolkit (UnifiedGenotyper) [58]. This was followed by a second iteration of alignment and inserting SNPs, resulting in the annotated cowbird reference-guided genome.

Differential expression and GO analysis

We removed Illumina adapters from RNaseq reads with Trim Galore! v0.3.7 (as above). We aligned the reads to the reference-guided assembly with HiSat2 [55] and quantified read counts with HTSeq-count [56]. After removing genes with low expression (< 1 count per million in at least 3 subjects), we normalized for read-depth and analyzed for differential expression with DEseq2 (Tables S1 and S2) [54]. We then performed ranked-based Gene Ontology (GO) analysis with adaptive clustering [35], following the “GO_MWU” pipeline (https://github.com/z0on/GO_MWU). We ranked our gene list by negative decimal logarithms of the raw (uncorrected) p value for each gene from the differential expression analyses. We multiplied by -1 if the gene was downregulated to indicate directionality. We used the gene ontology of the chicken and used the default for cutTreeHeight (0.25) for merging GO term clusters.

QUANTIFICATION AND STATISTICAL ANALYSIS

We analyzed the correlation of juvenile male songs to canary and bioacoustic features using a linear mixed model in R (lme4), with treatment (Chatter versus Control) as the predictor and male subject identity as the random effect.

For differential expression tests we used DEseq2, which employs the Wald test to calculate p values between pairwise comparisons [54]. DEseq2 is generally conservative in classifying differentially expressed genes [63], therefore, we considered genes differentially expressed if the adjusted p value (Benjamini & Hochberg false discovery rate) was < 0.10 [54].

To test for enriched GO terms, we determined whether genes were significantly located near the top of the ranked gene list, versus spread evenly throughout with the Mann-Whitney U test. We presented significantly enriched GO term clusters that were adjusted p value < 0.001.

DATA AND CODE AVAILABILITY

The raw RNA-seq data reported in this paper are available at NCBI SRA BioProject: PRJNA565489.