Vocal Matching and Intensity of Begging Calls Are Associated with a Forebrain Song Circuit in a Generalist Brood Parasite

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ABSTRACT: Vocalizations produced by developing young early in life have simple acoustic features and are thought to be innate. Complex forms of early vocal learning are less likely to evolve in young altricial songbirds because the forebrain vocal-learning circuit is underdeveloped during the period when early vocalizations are produced. However, selective pressure experienced in early postnatal life may lead to early vocal learning that is likely controlled by a simpler brain circuit. We found the food begging calls produced by fledglings of the brown-headed cowbird (*Molothrus ater*), a generalist avian brood parasite, induced the expression of several immediate early genes and early circuit innervation in a forebrain vocal-motor pathway that is later used for vocal imitation. The forebrain neural activity was correlated with vocal intensity and variability of begging calls that appears to allow cowbirds to vocally match host nestmates. The begging-induced forebrain circuits we observed in fledgling cowbirds were not detected in nonparasitic passerines, including species that are close relatives to the cowbird. The involvement of forebrain vocal circuits during fledgling begging and its association with vocal learning plasticity may be an adaptation that provides young generalist brood parasites with a flexible signaling strategy to procure food from a wide range of heterospecific host parents. © 2015 Wiley Periodicals, Inc.
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INTRODUCTION

The evolutionary origin and adaptation of complex behavioral traits, such as vocal learning, can be better understood by examining the developmental origin of behavior and its neural circuits with a comparative approach (Katz, 2011). Food begging calls (hereafter begging calls) are the earliest vocalizations produced by neonatal altricial birds, and these calls are used to compete with nestmates and to procure parental care (Godfray, 1991, 1995; Kilner and Johnstone, 1997; Budden and Wright, 2001). It was initially thought that the early emergence of begging calls, their simple acoustic structure, and the underdeveloped vocal

learning circuits observed in young birds made it unlikely that begging calls could be learned by imitation (Simpson and Vicario, 1990). Moreover, song imitation in oscine passerine birds involves a protracted song ontogeny (Marler, 1997; Tchernichovski et al., 2001) that gradually integrates auditory input and vocal motor output through auditory feedback (Konishi, 1965) and a specialized, complex forebrain neural circuit that is not fully innervated and functional until the age of independence (Mooney and Rao, 1994; Aronov et al., 2008). However, recent studies show vocalizations produced by hatchlings can be learned or modified from social or auditory experience. For example, vocal matching of begging calls during the nestling stage has been observed in vocal learning species (e.g., songbirds, parrots) as well as vocal non-learners (i.e., cuckoos) (Davies et al., 2006; Langmore et al., 2008; Berg et al., 2011; Colombelli-Negrel et al., 2012). In addition, begging calls given by young birds after they leave the nest (i.e., fledglings) can also be modified by auditory feedback (Liu et al., 2009), presumably because selective pressures experienced during early postnatal life may induce learning that allows individuals to adjust begging calls to enhance feeding by caregiving parents (Berg et al., 2011).

The early vocal learning observed in some birds suggest that the neural pathway that underlies the early learning of begging calls might differ from that used in song learning. Learning of vocalizations in songbirds involves two forebrain circuits that are necessary for acquisition and production of learned song: an anterior forebrain pathway with a basal ganglia relay that is essential for song learning, and a posterior pathway, consisting of nuclei HVC and RA, that is required for the production of learned vocalizations [Fig. 1(a)]. These forebrain vocal circuits (also known as the "song system") are absent in birds that lack vocal learning and are thought to be inactive when songbirds produce unlearned vocalizations (Wild, 1997; Farries, 2001, but see Ter Maat et al., 2014). Production of unlearned sounds is presumably controlled by midbrain and brainstem vocal pathways in both oscines and non-learning species (Simpson and Vicario, 1990 but see Ter Maat et al., 2014). In songbirds, the forebrain song system is fully developed for song learning when juveniles become independent and start producing "babbling" subsong (Aronov et al., 2008; Liu et al., 2009; Veit et al., 2011), and it takes a minimum of 3 months to develop a stabilized learned song (Tchernichovski and Mitra, 2002). It remains unclear, however, whether early vocal learning can occur without a pro-

tracted ontogeny and a forebrain vocal circuitry that is not fully established.

In this study, we assessed the potential for vocal learning plasticity and the underlying neural circuits involved with begging calls in a generalist brood parasite, the Brown-headed Cowbird (*Molothrus ater*, hereafter cowbird). This species is one of the most generalized brood parasites in the world, using hundreds of host species across its geographic range (Ortega, 1998) with >20 hosts parasitized within a single community (Rivers et al., 2010). Importantly, some female cowbirds appear to be host generalists at the individual level, so cowbird offspring originating from a single mother may be raised by different host species within a community and experience markedly different rearing environments (Woolfenden et al., 2003; Strausberger and Ashley, 2005). We hypothesized that the diversity and unpredictability of early rearing environments experienced by young parasitic cowbirds impose strong selective pressure to evolve specialized behavioral and neural circuit adaptations for obtaining food from heterospecific host "parents," and that such adaptations are lacking in nonparasitic species that are always raised by genetic parents in nature. We examine the learning plasticity of begging call and begging induced forebrain gene expression patterns by comparing the brood parasitic cowbirds, their non-parasitic close relatives (some of which are also hosts), and more distantly related songbird species. This approach will allow us to identify whether the food begging call and its induced vocal circuits of parasitic cowbirds are different from those of nonparasites.

MATERIALS AND METHODS

Animals and Study Site

We collected offspring (eggs and nestlings) of cowbirds and eight host species for hand-rearing in the laboratory. Our study species can be placed into five groups (1) Parasitic cowbirds: cowbirds were collected from the Field Research Center of Rockefeller University in Millbrook, New York ($n = 15$ individuals; 6–9 days posthatch [dph]) and from Konza Prairie Biological Station near Manhattan, Kansas ($n = 8$ individuals, 11–15 dph); all birds fledged from artificial nests 11–15 dph, when most wild cowbirds fledge. (2) Closely-related nonparasitic members of the family Icteridae (Klicka et al., 2015): Red-winged Blackbird (*Agelaius phoeniceus*; $n = 3$ individuals from three nests) and Common Grackle (*Quiscalus quiscula*; $n = 3$ individuals from two nests). (3) Distantly-related natural host young: For each parasitized nest at the Rockefeller Field research center and Institute of Ecosystem Studies in

Millbrook, New York, we collected 1 to 2 host nestlings from each brood in addition to the parasitic cowbird (parasitized nests at this location typically harbor only a single cowbird). Host nestlings included three oscine species,

which included the Ovenbird (*Seiurus aurocapilla*; $n = 4$ individuals from three nests); the Chipping Sparrow (*Spizella passerina*; $n = 5$ individuals from four nests); the Blue-winged Warbler (*Vermivora cyanoptera*; $n = 4$
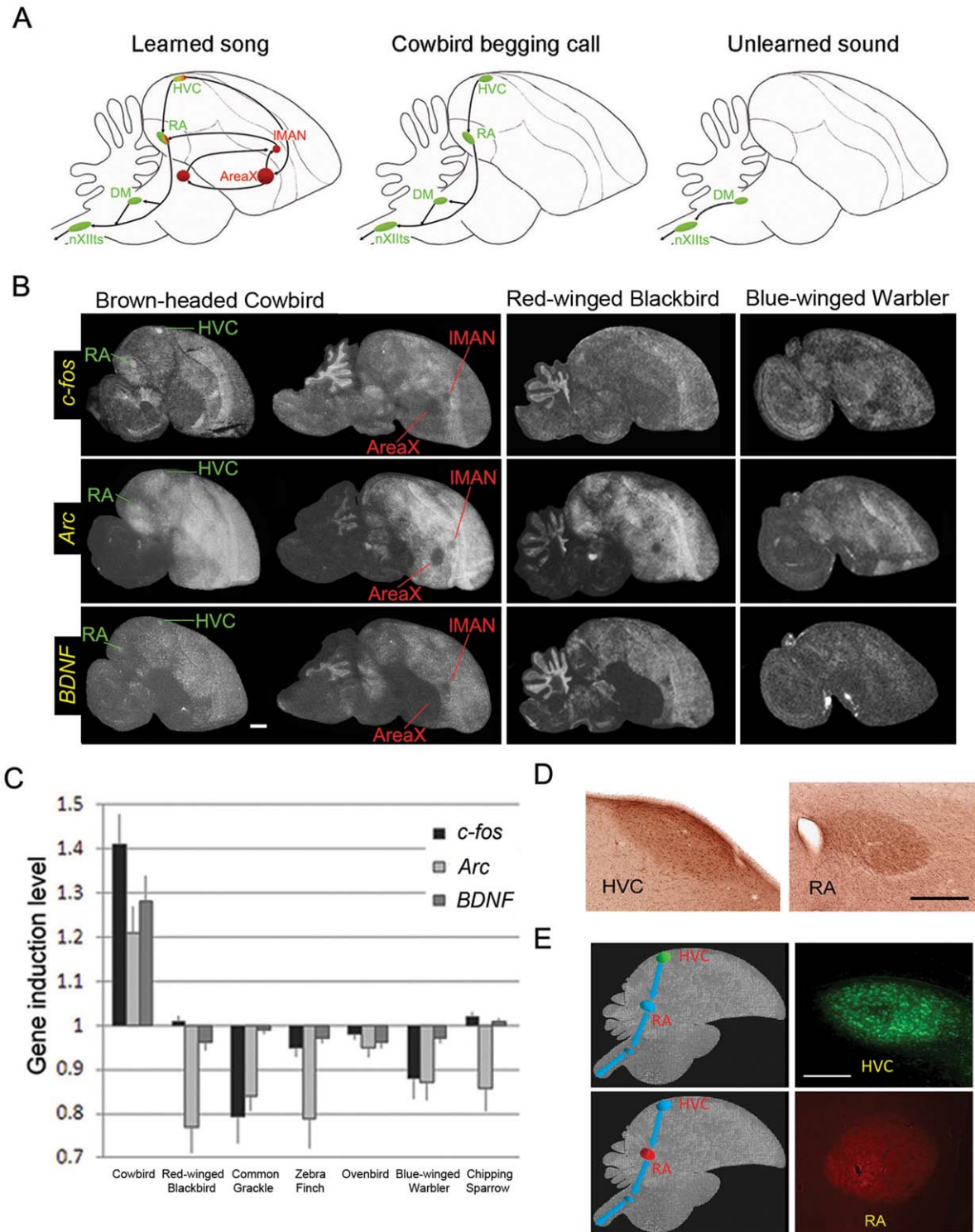

Figure 1.

individuals from three nests), and a single suboscine species, the Eastern Phoebe (*Sayornis phoebe*, n = 4 individuals from four nests). (4) Laboratory-bred domesticated Zebra Finch (*Taeniopygia guttata*; n = 4 individuals from four nests) hosts were sourced from a breeding colony at the Rockefeller University Field Research Center. (5) Laboratory-bred domesticated Canaries (*Serinus canaria*) hosts: eight breeding pairs of canaries were used as foster parents; each pair was housed in a single breeding cage. In each canary nest, two fertile cowbird eggs and two canary eggs were placed together. Cowbird eggs (n = 36 eggs) were collected from an outdoor aviary at the University of Pennsylvania, of which 20 eggs (55% of eggs) were fertile. Of these, nine eggs hatched and five of them were successfully reared by foster canaries.

After juveniles fledged from artificial nests, each cowbird was housed in a single cage with one heterospecific nestmate from its original nest. For the comparative study, fledglings of two close relatives of cowbirds, the Red-winged Blackbird and Common Grackle, were also housed with age-matched heterospecific nestling songbirds so rearing conditions were similar for cowbirds, blackbirds, and grackles. Permits for our research were granted by all appropriate institutional, state, and federal agencies.

Recordings and Analysis of Food Begging Calls

Begging calls were recorded from individuals from the early nestling stage (5 dph) to the late fledgling stage (30 dph). We defined a begging call as a vocalization produced by an individual when food was presented within 10 cm of its bill during laboratory feeding trials. Begging calls of fledgling Brown-headed Cowbirds and other non-parasitic songbirds were recorded two to three times per day until they reached independence or when they were sacrificed, with at least one recording session in the morning between 06:00 and 08:00 h before the first feeding of the day, and one in the late afternoon between16:00 and 18:00 h. Recordings of begging calls were made using an Audio Technica AT803 Lavalier microphone (Audio-Technica U.S., Inc. Stow, OH) placed in the top center of cages. The microphone was connected to an M-audio Audio-Buddy pre-amp (Avid Technology, Irwindale, CA) and an M-audio Delta 44 sound card, with calls being recorded with Raven Pro 1.3 recording software (Cornell Laboratory of Ornithology, NY).

Quantitative analysis was performed using Avisoft SAS-Lab Pro (Berlin, Germany) and Sound Analysis Pro (SAP) programs. Each bird's begging calls were characterized by their duration, acoustic features (see below), and total number of calls produced per minute. A begging call note was defined as a continuous sound preceded and followed by silent intervals longer than 5 ms. For within-day comparisons, we selected the first 3 min of begging call recordings (at the point of maximum hunger). Quantification of the acoustic properties of begging calls, and host *versus* parasite comparisons, were conducted using the similarity measurement asymmetric pairwise comparison programs from the SAS-Lab Pro and SAP. In SAP, the frequency range setting is adjusted to 11,800 Hz and this program calculates the Euclidean distance between all interval pairs from two notes over the course of the begging calls. The time window ("interval") used for comparisons was 9.27 ms long. Six acoustic features that characterize begging calls were measured: duration, pitch, frequency modulation (FM), Wiener entropy, mean frequency, and pitch goodness (PG) (Tchernichovski and Mitra, 2002).

Behavioral Manipulation of Host-Parasite

We experimentally tested whether cowbirds matched host young vocalizations by moving cowbirds between groups

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Figure 1 (A) Schematic representation of the songbird song system in sagittal view. Vocal learning requires a forebrain song system (left panel) consisting of the anterior forebrain circuit for acquisition of learned song (red) and the vocal-motor pathway for acquisition and production of learned song (green), both of which converge on nucleus HVC. The begging call of a fledgling brown-headed cowbird is associated with the forebrain's HVC_RA vocal-motor pathway (middle panel). In contrast, a midbrain/brainstem vocal pathway is sufficient for the production of unlearned sounds in non-learners (right panel). (B) Begging in brown-headed cowbird fledglings (but not nestlings) induced the expression of three immediate early genes (*c-fos Arc,* and *BDNF)* in the forebrain song nuclei HVC and RA (green), but not in the anterior forebrain circuit nuclei lMAN and AreaX (red). Begging induced forebrain IEG expression was not detected in the Red-winged Blackbird, one of the cowbird's close relatives, or the Blue-winged Warbler, a representative cowbird host. Repeated measures ANOVA testing differences among three *IEGs*: $p < 0.01$; unpaired *t*-test for each brain region relative to silent controls for HVC: for *BDNF*, $F = 4.49$; $p < 0.01$, for *Arc*, $F = 6.57$; $p < 0.01$, for *c-fos*, $F = 9.43$; $p < 0.001$, Tukey *post hoc* test. Scale bar = 1.5 mm. (C) Begging calls did not induce *c-fos, BDNF*, and *Arc* expression in song nucleus HVC in two closely related nonparasitic family members (Red-winged Blackbird and Common Grackle) or more distantly related host species. Repeated measures ANOVA, $F = 18.3$; $p > 0.05$ (mean ± SEM). (D) Begging-induced *Arc* protein expression in song nuclei HVC and RA of fledgling cowbirds (25 dph); (E) retrograde neuronal labeling of HVC after injecting retrograde tracer, flouro-gold at nucleus RA of fledgling cowbird at age of 20 dph; also, anterograde projecting labeling of nucleus RA after injecting tracer DiI (C12) at nucleus HVC, which indicates the connection between HVC and RA is well established by age of 20 dph. Scale bar = 0.5 mm. Anatomical abbreviations: nXIIts, tracheosyringeal hypoglossal nucleus; LMAN, lateral magnocellular nucleus of the anterior nidopallium; RA, robust nucleus of the arcopalium; DLM, nucleus dorsolateralis anterior thalamis; DM, dorsomedial nucleus of the intercollicular complex, Scale bar = 1 mm.

that contained different hosts young during their development. At the time of fledging, cowbirds were moved to a new cage that contained a different species of host young at similar age ($\pm$0–4 days) from which it was raised. We put newly created cowbird-host pairs in separate, acoustically-isolated rooms to prevent the cowbird from further hearing its natal nestmates. The new host species was selected so that it had a different peak frequency of calls relative to the host young with which the cowbird was raised in the natal nest. The host young used for these experiments included host species that have a lower call frequency <7.5 kHz, than previous nestmates (i.e. Eastern Phoebe = 6573.5 $\pm$ 119.1 Hz, mean- $\pm$ SEM, and Canary = 7276.4 $\pm$ 338.2 Hz) and host that have a higher call frequency, >8 kHz, than previous nest mates (i.e., Blue-winged Warblers = 8233.6 $\pm$ 321.5 Hz and Ovenbirds = 8057.4 $\pm$ 292.8 Hz). The begging calls of cowbirds and new host young were recorded as mentioned previously (Materials and Methods section). A total of eight cowbirds were raised in original nests and then either transferred to cages with new host parents.

Gene Expression of Food Begging Calls

Experimental Groups. Cowbirds were each randomly assigned to two experimental groups for the study of gene expression: (1) Begging group: a fledgling cowbird and its host sibling(s) were housed together in a single cage. After lights went on in the early morning (06:15–06:30), begging calls were recorded for 15 to 20 min. During these protracted recording period, we encouraged juveniles to beg for 15 to 20 min, with intervals of 2 to 5 min of begging followed by 3 to 5 min of silence. These birds were then sacrificed by decapitation approximately 45 to 50 min after the production of the first begging call and brain tissues stored in a $-80°$C freezer. (2) Hearing and nonbegging group: these fledglings heard begging calls of other young birds but did not beg themselves overnight (over 10 h) and they had no visual contact with the experimenter conducting hand feeding. These birds were sacrificed 40 to 45 min after lights went on in the same manner as individuals in the begging group.

In Situ *Hybridization.* Three immediate early genes (*IEGs*) *C-fos, Arc* (activity-regulated cytoskeleton-associated protein), and *BDNF* (brain derived neurotrophic factor) are used in this study to identify the begging induced gene expression pattern in the forebrain. These three genes are neural activity-dependent *IEGs*, functionally associated with learning, memory (Fleischmann et al., 2003, Tzingounis and Nicoll, 2006; Bramham et al., 2008), or neuronal survival (Korte et al., 1996). In songbirds, the expression of these *IEGs* is induced in the forebrain song nuclei by singing of learned song, particularly during the sensitive period of vocal learning (Kimpo and Doupe, 1997; Li et al., 2000; Wada et al., 2006).

Brains of collected birds were sectioned in a cryostat. *In situ* hybridization was then used to reveal the mRNA of chosen *IEGs*, following protocols previously described

using plasmids of *c-fos, Arc*, *BDNF*, and androgen receptor (Wada, personal communication). In brief, frozen brain sections (12 μm) were hybridized with ^{33}P-labeled antisense riboprobes and exposed to X-ray films for 2 days. Gene expression level in the specialized forebrain song nuclei region was then quantified following a previously described procedure with modification. The brain image on the exposed film was placed was scanned at 5000 dpi (Epson, Perfection V700, Long Beach, CA). Images were then exported to Adobe Photoshop CS2 (Adobe, San Jose, CA) and converted to 8-bit gray scale. The song nuclei and surrounding areas (lateral and medial parts) were outlined, and the average pixel density was calculated using the Photoshop histogram function. To quantify and compare the relative amount of *Arc*, *c-fos*, and *BDNF* expression in song nuclei of individuals in the two treatment groups we normalized the amount of *IEG* expression in each song nucleus to the average amount in silent controls.

Immunohistochemistry. Anesthetized cowbirds ($n = 6$ birds, three calling birds and three silent birds, see above) at age of 24 to 27 dph were perfused with 4% paraformaldehyde and 1$\times$ PBS, soaked in 40% sucrose overnight. Sagittal sections (40 μm), were cut on a freezing microtome. The brain sections were incubated in a blocking solution containing rabbit anti-*Arc* polyclonal antibody (1:500; Arc antibody H-300, Santa Cruz Biotech) overnight at 4°C. After washing three times with 1$\times$ PBS and 0.3% tritonX, the sections were incubated with secondary antibodies for anti-rabbit (1:1000; Invitrogen). After washing three times with 1$\times$ PBS, the sections were incubated in ABC for 45 min, washed and the DAB reaction.

Tract-Tracing

We used retrograde tract tracers, flouro-gold (4%; Fluorochrome Inc., Denver, CO), injected into the song nucleus RA at the arcopallium of young cowbirds at nestling and fledgling stages (10 and 20 dph; three birds per group); and we injected DiI (C12) into the nucleus HVC in young cowbirds ($n = 3$ birds). Anesthesia was first induced with intramuscular injection of Nembutal (1:5) and maintained by 1 to 1.5% isofluorane. The scalp was then retracted and a small craniotomy made over the injection site. Injections of flouro-gold and DiI (D12) were made through a glass micropipette. For each nucleus, four 70 to 100 nL injections were made with 50 s apart. The birds were then sacrificed 4 to 5 days after injection and the brain was sectioned with cryostat at 20 μm. Injection sites and track-tracing areas were examined with microscopy.

Statistics

We used Tukey *post hoc* test correction to test for the significance of differences for each of the acoustic features in food begging calls. We used one-way ANOVA and Mann-Whitney two-tailed *U* tests to determine if the gene expression ratio in the song nuclei of begging birds was
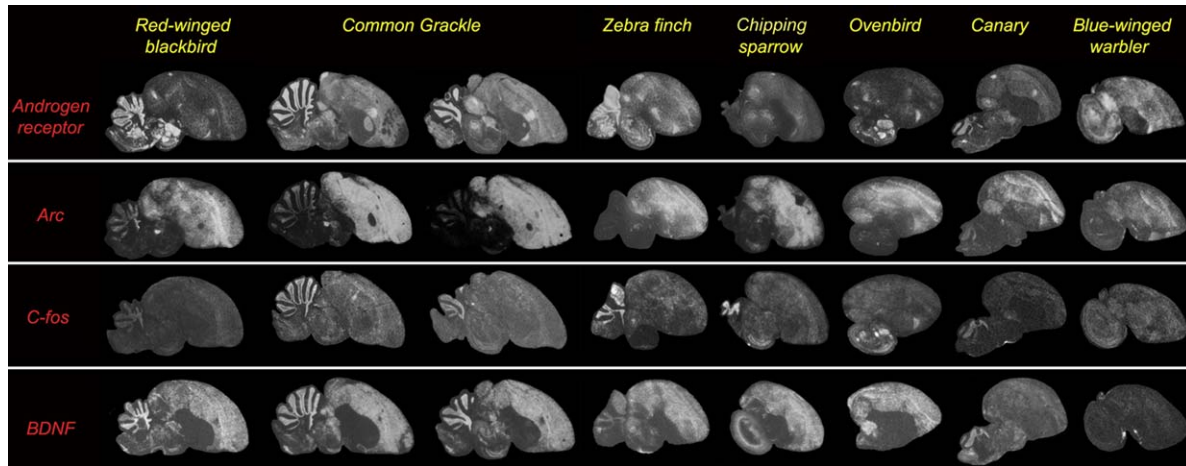
Figure 2 Begging calls in fledglings of seven nonparasitic songbird species did not induce the expression of immediate early genes (*IEGs*), *Arc*, *c-fos*, and *BDNF*, in the forebrain song nucleus HVC or the anterior forebrain circuit nuclei lMAN and Area X, including the closely related but non-parasitic Icterids: red-winged blackbirds and common grackles. Androgen receptor (labeled as the top row) was used here to identify the forebrain song nuclei in each of these songbird species. Scale bar = 2 mm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

significantly different from that of nonbegging individuals (PASW 18). One-way ANOVA was used for testing overall differences between silent controls and calling individuals for each of three genes we tested, followed by unpaired *t*-test among groups. To examine the amount of begging as a variable, we performed a regression analysis on total time spent (in seconds) for each animal of the 60 min begging group versus the amount of IEG expression in each nucleus.

RESULTS

Begging Induced IEG Expression in Forebrain Vocal Pathway

Production of begging calls in fledgling cowbirds (*n* = 7 birds, five males and two females; 20–25 dph) induced mRNA expression of three IEGs in two forebrain premotor song nuclei: HVC and RA, but not in the song nuclei of anterior pathway: lMAN and Area X [Fig. 1(B)]. No *IEG* expression was detected in control cowbirds that did not vocalize (*n* = 4 birds; Fig. 1). In addition, food begging did not induce forebrain *IEG* expression in younger, nestling cowbirds regardless of whether they produced begging calls (i.e., 6–13 dph, begging *vs*. nonbegging; *n* = 4 birds per group: repeated measures ANOVA, *c-fos*, $F = 4.61$; *Arc*, $F = 4.61$, $P > 0.05$).

Production of begging calls also induced protein expression of *Arc* in both HVC and RA of the fledgling cowbirds, and this was not detected in the silent controls or in younger nestlings that called or were silent (Fig. 1, *n* = 3 males, Mann-Whitney *U* test,

$Z = -2.15$; $p < 0.01$), indicating begging associated neural activity in forebrain nuclei HVC and RA. To determine whether these two song nuclei are connected, a retrograde tracer, fluorogold, was injected in song nucleus RA of fledgling cowbirds (19–21 days, *n* = 3 males) and retrogradely labeled nucleus HVC. Similarly, injection of lipophilic fluorescent dye, DiI (C12), in nucleus HVC anterogradely projects to nucleus RA, suggesting the connections between these two song nuclei was established by the fledgling stage (Fig. 1). In contrast, we found no begging-induced *IEG* expression in song nucleus HVC in non-parasitic songbirds at both the nestling (four host species at 8–13 days, Fig. 1) and fledgling stages (six host species at 19–27 days; Fig. 2), regardless of the number of begging calls produced or if they were silent controls. Importantly, we failed to detect forebrain *IEG* expression of begging fledglings in either of the two nonparasitic, close relatives of the cowbird we tested (i.e., the Red-winged Blackbird and Common Grackle). In nucleus RA, begging-associated *IEG* expression was more variable across species, as some species we examined (i.e., Oven-birds, Chipping Sparrows, and Zebra Finches). The IEG expression in RA of these host young is not directly associated with food begging behavior (Figs. 1 and 2).

The begging-induced *IEG* expression and early innervation in the HVC-RA vocal-motor pathway in parasitic cowbirds suggests an early establishment of connections between HVC, RA and brainstem vocal nuclei in young parasitic cowbirds, compared with the zebra finch (Konishi and Akutagawa, 1985;
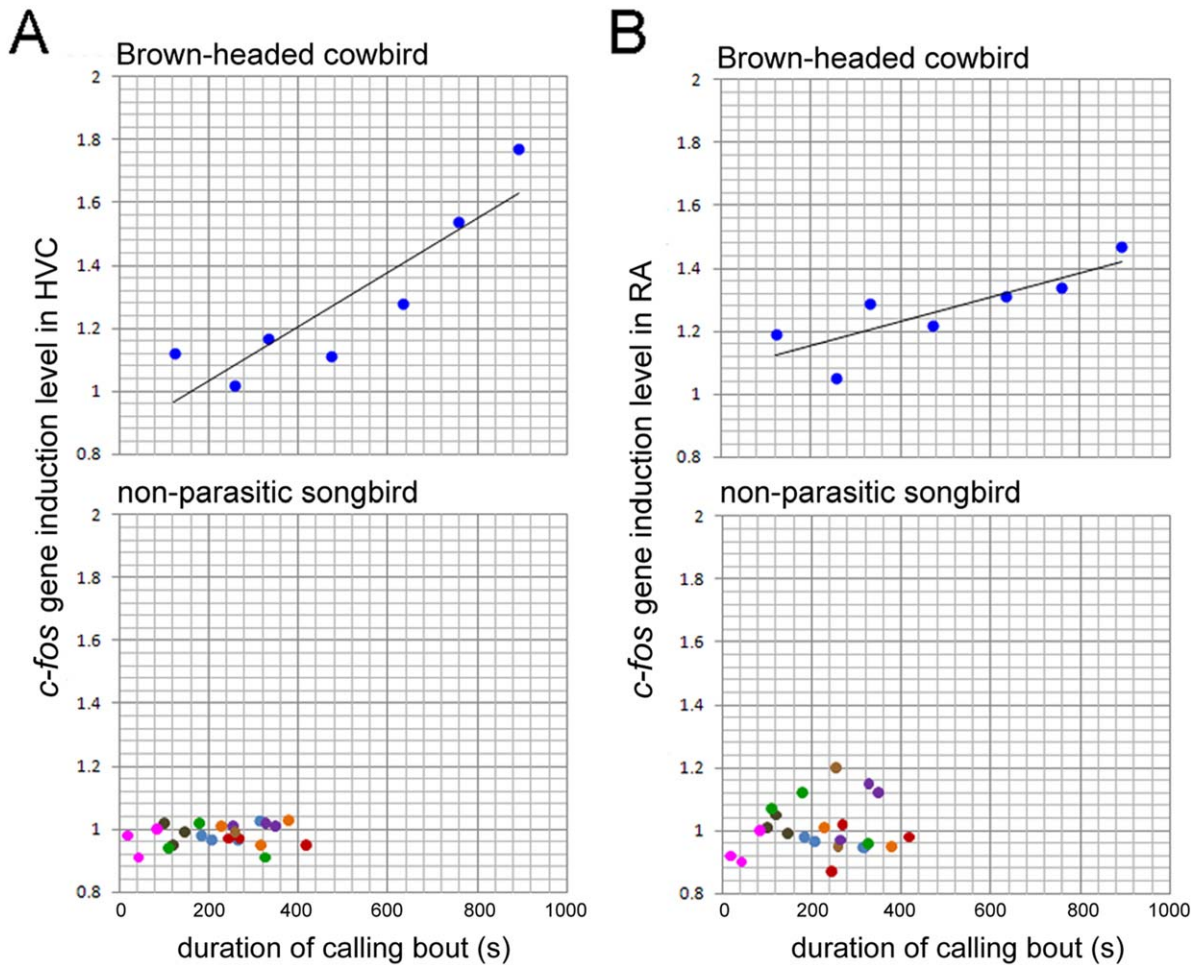
Figure 3 The duration of calling bout in fledgling cowbirds was positively linked to levels of *c-fos* expression in nucleus HVC, $R^2 = 0.911$, $p < 0.001$ (see A); and RA, $R^2 = 0.738$, $p < 0.01$ (B), but this relationship was not present in any of seven nonparasitic species ($n = 23$ birds), including two closely-related family members. The duration of calling bout was defined as the sum in seconds of the duration of all begging calls produced during the first hour of begging after sunrise Each color dot represents an individual species: Blue: Brown-headed Cowbird; Pink: Red-winged blackbird; Red: Zebra Finch; Light green: Blue-winged Warbler; Dark green: Common Grackle; Light blue: Canary; Purple: Chipping Sparrow; Orange: Ovenbird.

Mooney and Rao, 1994). These early innervations do not lead to early development of song learning, as the onset of subsong (the earliest known stage of song learning) does not occur earlier in cowbirds than in other songbird species (the earliest subsong between cowbirds and four other non-parasitic songbird species, $n = 5$ and 9 birds; mean $\pm$ SEM $= 36.1 \pm 2.5$ *vs.* 39.2 ± 2.4 dph, Mann-Whitney *U* test, $U = 35$, $Z = 1.45$, $p = 0.173$; see also Tchernichovski et al., 2001). Moreover, in non-parasitic songbirds, only subsong induced *IEGs* expression in HVC→RA vocal-motor pathway, but not begging calls (Wada et al., 2006).

The *IEG* expression in HVC→RA motor pathway was significantly and positively correlated with the number of begging calls produced by individual cowbirds [correlation coefficient = 0.827, $p < 0.01$; Fig.

3(A)]. However, the number of begging calls was not related to *IEG* expression in non-parasitic songbirds, as none of the *IEGs* (*Arc* and *c-fos*) were expressed in nucleus HVC regardless of the amount of calling in any non-cowbird species [Fig. 3(B)]. For instance, fledgling zebra finches produce higher intensity of begging calls yet there was no identifiable IEG expression in HVC [Fig. 3(B)]; this is in contrast to our finding that fledgling cowbirds still had *IEG* expression in HVC despite some of them (three out of seven) have a lower or similar calling intensity than their hosts or other non-parasites [Fig. 3(A); three cowbirds with low call intensity *vs.* seven hosts; 233 ± 97.3 *vs.* 297.6 ± 114.6 s; mean $\pm$ SD]. Furthermore, although nestling and fledgling cowbirds produced comparable intensity of begging calls (Mann-
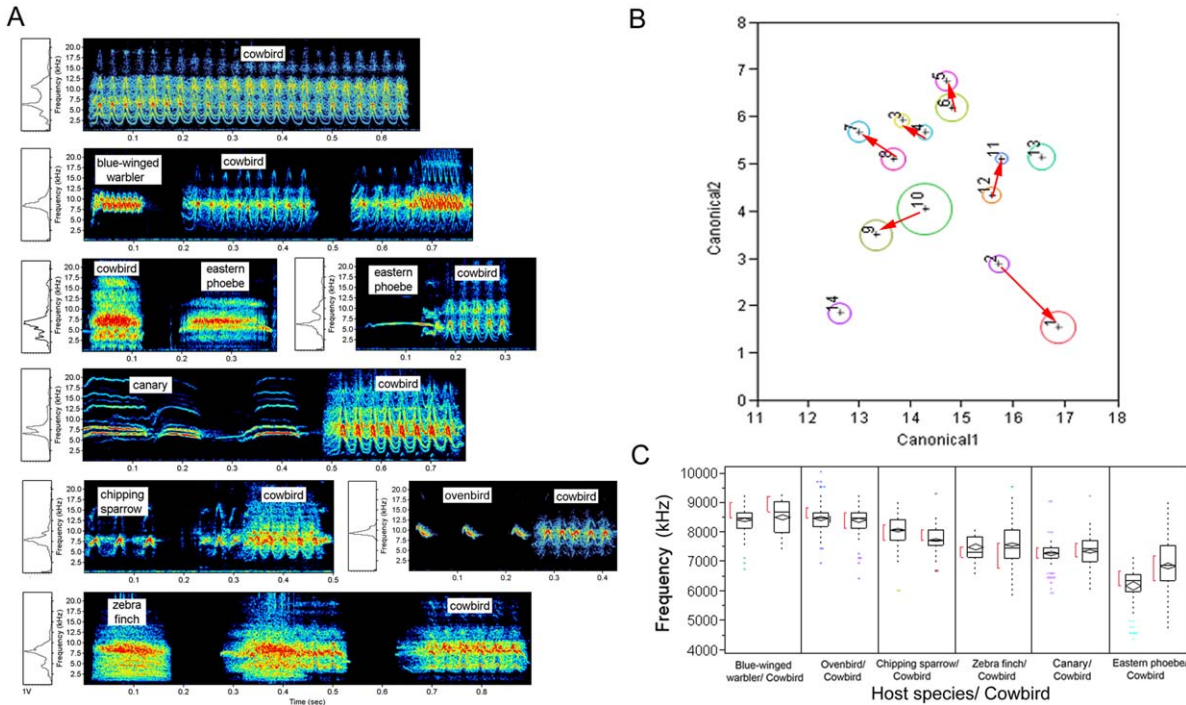
Figure 4 Apparent vocal matching in the begging calls of fledgling cowbirds (*n* = 7 birds). (A) A representative example of a begging call produced by a cowbird fledgling at 20 days after hatching. Cowbirds calls generally cover a wide frequency range and sometimes have a long duration of call rendition. Fledgling cowbirds appear to shift the peak frequency of their begging calls to match the calls produced by host young. The rendition of a begging call produced by a cowbird fledgling, matching the peak frequency of its foster host young (from the top to the bottom): the blue-winged warbler, eastern phoebe; canary; chipping sparrow; ovenbird, and zebra finch. The left panel shows the frequency distribution of the begging calls of cowbirds and the host siblings with which they were raised. Note the cowbird begging calls seem to match more to the contact call (right) than the begging call (left) of the phoebes. (B) Discriminant function analysis using three call features (peak frequency, amplitude, and Wiener entropy) to identify the vocal matching of begging calls among six parasite-host pairs (red arrow represents how each cowbird pairs with its host). 1←2: phoebe←cowbird; 3←4: blue←winged warbler←cowbird; 5←6: ovenbird←cowbird; 7←8: chipping sparrow←cowbird; 9←10: zebra finch←cowbird; 11←12: canary←cowbird; 13←14 canary←red-winged blackbird. (C) Potential matching of begging call peak frequency between cowbird fledglings and their host nestmates. Each pair of box-plot represents the call frequency of a host (left) and its parasitic cowbird "sibling" (right); all data were obtained from fledglings of similar age (see Methods for details). In the phoebe-cowbird pair, although call frequency did not match precisely, the cowbird produced lower-frequency calls compared with those of other cowbirds, and its frequency range is closer to the phoebe call than that of any other host young.

Whitney *U* test, two tailed, *p* = 0.14), we did not see *IEG* expression in nucleus HVC of nestling cowbirds. The begging call-induced, intensity-dependent forebrain *IEG* expression appears to be identified only at the fledgling stage of parasitic cowbirds.

Vocal Matching of Food Begging Calls

The begging calls of fledgling cowbirds covered a wide frequency range [Fig. 4(A)], and young cowbirds seem capable of emphasizing the amplitude and peak frequency of calls in a way that appeared to match the vocalizations of nestmates with whom they were raised (*n* = 4 female, 5 male cowbirds; 17–25

dph). This vocal matching between cowbirds and their host young was statistically significant during the fledgling stage, yet we did not find consistent call matching in nestling cowbirds (Fig. 4). Discriminant function analysis, using three acoustic features of calls (i.e., amplitude, peak frequency, and Wiener entropy), suggested that each parasitic cowbird's call features have a closer acoustic resemblance to its natal nestmate than those of other hosts under examination [Fig. 4(B)]. It is worth noting that, although the fledgling cowbird significantly dropped its call frequency in the phoebe-cowbird pair [Fig. 4(C)], the cowbird's begging calls imprecisely match with phoebe begging calls (Fig. 4). However, the cowbird's begging calls have a closer match to the

contact call of young phoebes. One potential explanation for this finding is that we observed young phoebes produced many more "contact" calls than food begging calls (first to five calls) during each feeding trial (Fig. 4), and the cowbird's begging calls seem to better match the "contact" calls. Thus, cowbirds may match the vocalizations they encounter most often, which are typically food begging vocalizations in hosts other than the phoebe.

Cross-Fostering Experiments

Call matching by cowbirds did not occur if the source host sibling was replaced with a different host species around the time of fledging ($n = 5$ cowbirds hatched by the foster species in the wild, and three additional cowbirds hatched and cared for by lab-bred domesticated canaries). Host-siblings were switched at 11 to 14 dph; the mean peak frequency of cowbird begging calls did not closely match the new host sibling after cowbirds were moved from their natal nests ($n = 8$ birds; Wilcoxon sign-rank match-paired test; $p = 0.12$). Therefore, the call matching exhibited by cowbirds appear to develop before fledging occurred.

DISCUSSION

Cowbirds are nonevicting brood parasites and are typically raised alongside host offspring as nestlings, and this often leads cowbirds gaining a disproportionate share of the food (Dearborn, 1998; Lichtenstein and Sealy, 1998; Kilner and Davies, 1999; Kilner et al., 2004). This may provide cowbirds with an opportunity to hear and memorize the vocal features of host young during the early development and later, during the fledgling stage, vocally match host calls to procure foster parental care. Indeed, such a system is found in *Vidua* indigobirds which imprint on host begging calls and later incorporated them into song for mate attraction (Payne et al., 2000).

Alternatively, the vocal plasticity and wide frequency range of begging calls in fledgling cowbirds may allow them to couple feedback with subtle vocal modification until the host's peak frequency is reached, potentially increasing feeding by host parents. Such a reinforcement learning strategy could be especially useful for parasitism of small host species where cowbirds are almost always raised alone (Goguen, 1996; Parker, 1999) and therefore have no basis for learning vocalizations of host young. In this scenario, the begging behavior of the young cowbirds might be shaped by adult responses, much as the learned song of young male cowbirds is shaped by

subtle responses of adult females, whose guidance results in song crystallization of higher sex appeal (West and King, 1988; Davies et al., 2006). Given its absence in closely related non-parasitic species, vocal matching either by imitation of foster young or by reinforcement from foster-parent response appears restricted to cowbirds and therefore may serve as an adaptation for brood parasitism.

In this study we provide the first evidence that a forebrain vocal-motor pathway, HVC–RA, is involved in the production of food begging call. This pathway is thought to be innervated and activated during the production of subsong singing, the earliest stage of song development (Liu et al., 2009). The positive correlation between *IEG* expression in HVC and the amount of begging calls suggests that the HVC–RA pathway is associated with the degree of begging behavior, but the exact role of HVC on cowbird begging calls remains unclear. The nucleus HVC is known to play a role on flexible and subtle vocal or respiratory control during song production (Aronov et al., 2008; Andalman et al., 2011; Veit et al., 2011), HVC may thus play a similar role during intense begging bouts, and this forebrain involvement may allow vocal matching to occur. Alternatively, the call intensity-dependent neural activation may be the result of retrofitting of the developing vocal-motor circuit for song learning. It remains unclear, from our current study, whether the cortical-basal ganglia-thalamol circuit is involved in vocal matching or production of begging calls. Additional experiments, such as temporary silence of HVC or neurophysiological recordings of HVC neurons, are needed to determine the role of HVC on the production of food begging calls in cowbirds.

The vocal matching in the fledgling begging call of the generalist parasitic cowbird adds to a growing body of evidence showing that host-specific vocal mimicry of the nestling begging call has occurred in a number of specialist brood parasites (Davies et al., 2006; Langmore et al., 2008; Anderson et al., 2009; De Marsico et al., 2012), including non-vocal learners. For example, vocal matching of host begging calls has been observed in the nestlings of the parasitic cuckoos (Cuculiformes) that comprise a clade that lacks song learning. The mimetic begging calls in specialist parasites could be either genetically predisposed innate behavior or socially acquired reinforcement learning (Ortega, 1998). Additionally, the relatively simple and universal acoustic structures of begging calls observed in many songbird nestlings suggest a simpler neural mechanism might be sufficient to induce early vocal learning. Perhaps a finer control of expiration during vocalizations can modulate subtle vocal change of simple vocalizations

(Simpson and Vicario, 1990) and induce early vocal matching such as begging-call matching observed in a few nestling songbirds (Davies et al., 2006; Langmore et al., 2008; Berg et al., 2011; Colombelli-Negrel et al., 2012). Therefore, the simpler form of early vocal matching of begging calls may not require a forebrain vocal circuit; a midbrain or brainstem circuit would be sufficient to perform a fine adjustment of vocal modification for vocal matching.

However, if the midbrain/brainstem vocal pathway is sufficient for early vocal learning, then why is the forebrain vocal pathway involved in begging calls of fledglings in the generalist cowbird? The forebrain involvement may provide more vocal plasticity and is particular useful for generalist parasitic young as its host parents are less predictable. Although more research in is needed, several studies (Eastzer et al., 1980; Woodward, 1983; Sealy, 1999) suggest that cowbird fledglings may be more successful in procuring parental care than their host siblings or cowbird nestlings. Fledgling cowbirds still rely heavily on parental feeding after leaving the nest, and cowbirds may have evolved a combination of begging strategies to secure food from host parents by increasing begging intensity and vocal plasticity of food begging calls through accelerated development of a forebrain vocal-motor pathway that is later used for song learning (Hauber and Ramsey, 2003). These behavioral and circuit modification seem to provide this generalist brood parasite with a flexible strategy for procuring foster-care from a wide range of host species. Despite these possibilities, our understanding of cowbird fledgling ecology is quite limited and more research is needed in this area, including whether the pattern we observed in our laboratory study occurs under natural conditions.

REFERENCES

Andalman AS, Foerster JN, Fee MS. 2011. Control of vocal and respiratory patterns in birdsong: Dissection of forebrain and brainstem mechanisms using temperature. PLoS One 6:e25461.

Anderson MG, Ross H, Brunton DH, Hauber ME. 2009. Begging call matching between a specialist brood parasite and its host: A comparative approach to detect coevolution. Biol J Linnean Soc 98:208–216.

Aronov D, Andalman AS, Fee MS. 2008. A specialized forebrain circuit for vocal babbling in the juvenile songbird. Science 320:630–634.

Berg KS, Delgado S, Cortopassi KA, Beissinger SR, Bradbury JW. 2011. Vertical transmission of learned signatures in a wild parrot. Proc R Soc London Ser B Biol Sci 279:585–591.

Bramham CR, Worley PF, Moore MJ, Guzowski JF. 2008. The immediate early gene arc/arg3.1: Regulation, mechanisms, and function. J Neurosci 28:11760–11767.

Budden AE, Wright WJ. 2001. Begging in nestling birds. Curr Ornithol 16:83–118.

Colombelli-Negrel D, Hauber ME, Robertson J, Sulloway FJ, Hoi H, Griggio M, Kleindorfer S. 2012. Embryonic learning of vocal passwords in superb fairy-wrens reveals intruder cuckoo nestlings. Curr Biol 22:2155–2160.

Davies NB, Madden JR, Butchart SH, Rutila J. 2006. A host-race of the cuckoo *Cuculus canorus* with nestlings attuned to the parental alarm calls of the host species. Proc R Soc London Ser B Biol Sci 273:693–699.

De Marsico MC, Gantchoff MG, Reboreda JC. 2012. Host-parasite coevolution beyond the nestling stage? Mimicry of host fledglings by the specialist screaming cowbird. Proc R Soc London Ser B Biol Sci 279:3401–3408.

Dearborn DC. 1998. Begging behavior and food acquisition by brown-headed cowbird nestlings. Behav Ecol Sociobiol 43:259–270.

Eastzer D, Chu PR, King AP. 1980. The young cowbird - Average or optimal nestling. Condor 82:417–425.

Farries MA. 2001. The oscine song system considered in the context of the avian brain: Lessons learned from comparative neurobiology. Brain Behav Evol 58:80–100.

Fleischmann A, Hvalby O, Jensen V, Strekalova T, Zacher C, Layer LE, Kvello A, et al. 2003. Impaired long-term memory and NR2A-type NMDA receptor-dependent synaptic plasticity in mice lacking c-Fos in the CNS. J Neurosci 23:9116–9122.

Godfray HCJ. 1991. Singaling of need by offspring to their parents. Nature 352:328–330.

Godfray HCJ. 1995. Evolutionary theory of parent-offspring conflict. Nature 376:133–138.

Goguen CB. 1996. Nest desertion by blue-gray gnatcatchers in association with brown-headed cowbird parasitism. Anim Behav 52:613–619.

Hauber MEd, Ramsey CK. 2003. Honesty in host-parasite communication signals: The case for begging by fledgling brown-headed cowbirds *Molothrus ater*. J Avian Biol 34:339–344.

Katz PS. 2011. Neural mechanisms underlying the evolvability of behaviour. Philos Trans R Soc London Ser B Biol Sci 366:2086–2099.

Kilner R, Johnstone RA. 1997. Begging the question: Are offspring solicitation behaviours signals of need? Trends Ecol Evol 12:11–15.

Kilner RM, Davies NB. 1999. How selfish is a cuckoo chick? Anim Behav 58:797–808.

Kilner RM, Madden JR, Hauber ME. 2004. Brood parasitic cowbird nestlings use host young to procure resources. Science 305:877–879.

Kimpo RR, Doupe AJ. 1997. FOS is induced by singing in distinct neuronal populations in a motor network. Neuron 18:315–325.

Konishi M. 1965. The role of auditory feedback in the control of vocalization in the white-crowned sparrow. Z Tierpsychol 22:770–783.

Konishi M, Akutagawa E. 1985. Neuronal growth, atrophy and death in a sexually dimorphic song nucleus in the zebra finch brain. Nature 315:145–147.

Korte M, Staiger V, Griesbeck O, Thoenen H, Bonhoeffer T. 1996. The involvement of brain-derived neurotrophic factor in hippocampal long-term potentiation revealed by gene targeting experiments. J Physiol 90:157–164.

Langmore NE, Maurer G, Adcock GJ, Kilner RM. 2008. Socially acquired host-specific mimicry and the evolution of host races in Horsfield's bronze-cuckoo *Chalcites basalis*. Evolution 62:1689–1699.

Li XC, Jarvis ED, Alvarez-Borda B, Lim DA, Nottebohm F. 2000. A relationship between behavior, neurotrophin expression, and new neuron survival. Proc Natl Acad Sci USA 97:8584–8589.

Lichtenstein G, Sealy SG. 1998. Nestling competition, rather than supernormal stimulus, explains the success of parasitic brown-headed cowbird chicks in yellow warbler nests. Proc R Soc London Ser B Biol Sci 265:249–254.

Liu WC, Wada K, Nottebohm F. 2009. Variable food begging calls are harbingers of vocal learning. PLoS One 4:e5929.

Marler P. 1997. Three models of song learning: Evidence from behavior. J Neurobiol 33:501–516.

Mooney R, Rao M. 1994. Waiting periods versus early innervation: The development of axonal connections in the zebra finch song system. J Neurosci 14:6532–6543.

Ortega CP. 1998. Cowbirds and Other Brood Parasites. Tucson: University of Arizona Press.

Parker TH. 1999. Responses of Bell's vireos to brood parasitism by the brown-headed cowbird in Kansas. Wilson Bull 111:499–504.

Payne RB, Payne LL, Woods JL, Sorenson MD. 2000. Imprinting and the origin of parasite-host species associations in brood-parasitic indigobirds, *Vidua chalybeata*. Anim Behav 59:69–81.

Rivers JW, Jensen WE, Kosciuch KL, Rothstein SI. 2010. Community-level patterns of host use by the brown-headed cowbird (*Molothrus ater*), a generalist brood parasite. Auk 127:263–273.

Sealy SG. 1999. Cowbird parasitism on lark buntings: Frequency, acceptance, and fledging. J Field Ornithol 70:182–186.

Simpson HB, Vicario DS. 1990. Brain pathways for learned and unlearned vocalizations differ in zebra finches. J Neurosci 10:1541–1556.

Strausberger BM, Ashley MV. 2005. Host use strategies of individual female brown-headed cowbirds *Molothrus ater* in a diverse avian community. J Avian Biol 36:313–321.

Tchernichovski O, Mitra PP. 2002. Towards quantification of vocal imitation in the zebra finch. J Comp Physiol A 188:867–878.

Tchernichovski O, Mitra PP, Lints T, Nottebohm F. 2001. Dynamics of the vocal imitation process: How a zebra finch learns its song. Science 291:2564–2569.

Ter Maat A, Trost L, Sagunsky H, Seltmann S, Gahr M. 2014. Zebra finch mates use their forebrain song system in unlearned call communication. PLoS One 9:e109334.

Tzingounis AV, Nicoll RA. 2006. Arc/Arg3.1: Linking gene expression to synaptic plasticity and memory. Neuron 52:403–407.

Veit L, Aronov D, Fee MS. 2011. Learning to breathe and sing: Development of respiratory-vocal coordination in young songbirds. J Neurophysiol 106:1747–1765.

Wada K, Howard JT, McConnell P, Whitney O, Lints T, Rivas MV, Horita H, et al. 2006. A molecular neuroethological approach for identifying and characterizing a cascade of behaviorally regulated genes. Proc Natl Acad Sci USA 103:15212–15217.

West MJ, King AP. 1988. Female visual displays affect the development of male song in the cowbird. Nature 334:244–246.

Wild JM. 1997. Neural pathways for the control of birdsong production. J Neurobiol 33:653–670.

Woodward PW. 1983. Behavioral ecology of fledgling brown-headed cowbirds and their hosts. Condor 85:151–163.

Woolfenden BE, Gibbs HL, Sealy SG, McMaster DG. 2003. Host use and fecundity of individual female brown-headed cowbirds. Anim Behav 66:95–106.