Neurophysiological coordination of duet singing

Melissa J. Coleman[a,1], Nancy F. Day[b], Pamela Rivera-Parra[c], and Eric S. Fortune[d]

[a]W. M. Keck Science Department, Scripps College, Pitzer College, and Claremont McKenna College, Claremont, CA 91711; [b]Department of Psychology, Whitman College, Walla Walla, WA 99362; [c]Departamento de Biología, Escuela Politécnica Nacional, Quito, Ecuador; and [d]Department of Biological Sciences, New Jersey Institute of Technology, Newark, NJ 07102

Edited by Daniel Margoliash, University of Chicago, Chicago, IL, and accepted by Editorial Board Member J. Anthony Movshon April 23, 2021 (received for review August 28, 2020)

Coordination of behavior for cooperative performances often relies on linkages mediated by sensory cues exchanged between participants. How neurophysiological responses to sensory information affect motor programs to coordinate behavior between individuals is not known. We investigated how plain-tailed wrens (*Pheugopedius euophrys*) use acoustic feedback to coordinate extraordinary duet performances in which females and males rapidly take turns singing. We made simultaneous neurophysiological recordings in a song control area "HVC" in pairs of singing wrens at a field site in Ecuador. HVC is a premotor area that integrates auditory feedback and is necessary for song production. We found that spiking activity of HVC neurons in each sex increased for production of its own syllables. In contrast, hearing sensory feedback produced by the bird's partner decreased HVC activity during duet singing, potentially coordinating HVC premotor activity in each bird through inhibition. When birds sang alone, HVC neurons in females but not males were inhibited by hearing the partner bird. When birds were anesthetized with urethane, which antagonizes GABAergic (γ-aminobutyric acid) transmission, HVC neurons were excited rather than inhibited, suggesting a role for GABA in the coordination of duet singing. These data suggest that HVC integrates information across partners during duets and that rapid turn taking may be mediated, in part, by inhibition.

cooperation | closed-loop control | sensorimotor integration | reciprocal inhibition | turn taking

Animals routinely rely on sensory feedback for the control of their own behavior. In cooperative performances, such sensory feedback can include cues produced by other participants (1–8). For example, in interactive vocal communication, including human speech, individuals take turns vocalizing. This "turn taking" is a consequence of each participant responding to auditory cues from a partner (4–6, 9, 10). The role of such "heterogenous" (other-generated) feedback in the control of vocal turn taking and other cooperative performances is largely unknown.

Plain-tailed wrens (*Pheugopedius euophrys*) are neotropical songbirds that cooperate to produce extraordinary duet performances but also sing by themselves (Fig. 1*A*) (4, 10, 11). Singing in plain-tailed wrens is performed by both females and males and used for territorial defense and other functions, including mate guarding and attraction (1, 11–16). During duets, female and male plain-tailed wrens take turns, alternating syllables at a rate of between 2 and 5 Hz (Fig. 1*A*) (4, 11).

There is a categorical difference between solo and duet singing. In solo singing, the singing bird receives only autogenous (hearing its own vocalization) feedback (Fig. 1*B*). The partner may hear the solo song if it is nearby, a heterogenous (other-generated) cue. In duet singing, birds receive both heterogenous and autogenous feedback as they alternate syllable production (Fig. 1*C*). Participants use heterogenous feedback during duet singing for precise timing of syllable production (4, 11). For example, when a male temporarily stops participating in a duet, the duration of intersyllable intervals between female syllables increases (4), showing an effect of heterogenous feedback on the timing of syllable production.

How does the brain of each wren integrate heterogenous acoustic cues to coordinate the precise timing of syllable production between individuals during duet performances? To address this question, we examined neurophysiological activity in HVC, a nucleus in the nidopallium [an analogue of mammalian cortex (17, 18)]. HVC is necessary for song learning, production, and timing in species of songbirds that do not perform duets (19–24). Neurons in HVC are active during singing and respond to playback of the bird's own learned song (25–27). In addition, recent work has shown that HVC is also involved in vocal turn taking (19).

To examine the role of heterogenous feedback in the control of duet performances, we compared neurophysiological activity in HVC when female or male wrens sang solo syllables with syllables sung during duets. Neurophysiological recordings were made in awake and anesthetized pairs of wrens at the Yanayacu Biological Station and Center for Creative Studies on the slopes of the Antisana volcano in Ecuador. We found that heterogenous cues inhibited HVC activity during duet performances in both females and males, but inhibition was only observed in females during solo singing.

Results

We measured HVC activity in four pairs of wrens as they sang solo syllables and duet songs. Sensorimotor activity in HVC was

Significance

Cooperation, turn taking, and other social behaviors often depend on temporal coordination between individuals. How brains use sensory cues from participants to synchronize performances is not known. We examined the interactions between sensory cues and motor activity in the brains of female and male plain-tailed wrens that rapidly take turns to produce a duet that sounds as if a single bird is singing. We made simultaneous neurophysiological recordings from the brains of pairs of awake, duetting wrens. We discovered that inhibition driven by auditory feedback from the partner alternated with the premotor activity used by each individual to produce its own vocalizations. These data show how sensory feedback links the brains of cooperating animals through the modulation of motor circuits.

Author contributions: M.J.C. and E.S.F. designed research; M.J.C., N.F.D., P.R.-P., and E.S.F. performed research; E.S.F. contributed new reagents/analytic tools; M.J.C., N.F.D., and E.S.F. analyzed data; and M.J.C., N.F.D., P.R.-P., and E.S.F. wrote the paper.

The authors declare no competing interest.

This article is a PNAS Direct Submission. D.M. is a guest editor invited by the Editorial Board.

See online for related content such as Commentaries.

[1]To whom correspondence may be addressed. Email: mcoleman@kecksci.claremont.edu.

This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2018188118/-/DCSupplemental.

Published May 31, 2021.

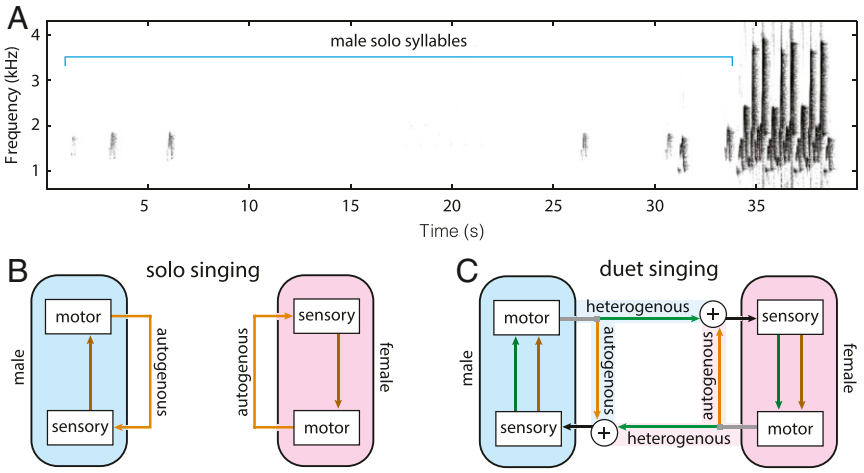

Fig. 1. Neural control of solo and duet singing in plain-tailed wrens. (*A*) Spectrogram of a singing bout that included male solo syllables (blue line, top) followed by a duet. Solo syllables for both sexes (only male solo syllables are shown here) are sung at lower amplitudes than syllables produced in duets. Note that the smeared appearance of wren syllables in spectrograms reflects the acoustic structure of plain-tailed wren singing. (*B* and *C*) Each bird has a motor system that is used to produce song and sensory systems that mediate feedback. (*B*) During solo singing, the bird hears its own song, which is known as autogenous feedback (orange). (*C*) During duet singing, each bird hears both its own singing and the singing of its partner, known as heterogenous feedback (green). The key difference between solo and duet singing is heterogenous feedback that couples the neural systems of the two birds. This coupling results in changes in syllable amplitude and timing in both birds.

recorded simultaneously in a female and male pair of wrens during singing bouts using a wireless neurophysiological system. Four nichrome/formvar electrodes were implanted unilaterally into HVC (either right or left side) in both the female and the male of each pair of wrens that were captured on their territory up to 5 d earlier. We recorded neurophysiological activity from several neurons near each electrode. We used principal component analysis to identify single units and analyzed the sum of all single units on each electrode (Fig. 2*A* and *Materials and Methods*).

In both females and males, HVC neurons increased firing during autogenous solo and duet syllable production (Fig. 2*A* and Movie S1). This premotor activity increased prior to the onset and decreased prior to the end of each autogenous syllable in both solo and duet singing (Fig. 2*A*). Premotor activity varied during the duration of each autogenous syllable, forming bursts of spiking activity. During duets, activity in HVC alternated across the two birds coincident with the production of autogenous syllables (Fig. 2*A* and Movie S1). In contrast, HVC neurons reduced firing during heterogenous syllables—while each bird was hearing its partner's vocalizations (Fig. 2*A*).

We characterized HVC activity during autogenous syllable production and while hearing heterogenous cues by averaging "response strength" (RS) of HVC neurons. RS was calculated by subtracting the spontaneous firing rate (spikes per second) from the firing rate during syllables. The spontaneous firing rate in HVC did not differ between females and males (median spikes per second: female = 4.4, $n = 8$ singing bouts; male = 2.8, $n = 8$ singing bouts; Mann–Whitney U, $P = 0.0830$, $U = 15$).

To visualize the average time-varying RS of HVC neurons during autogenous and heterogenous activity, we aligned simultaneously recorded neurophysiological data from females and males with respect to the onsets of each syllable across singing bouts and pairs of wrens. For example, we aligned all 13 male solo syllables (from four singing bouts produced by three pairs of birds) to their onsets and plotted the RS (mean ± SD) in both male HVC (autogenous activity) and female HVC (heterogenous activity) (Fig. 3*A*). The HVC RS averages include responses across syllable types, which differ in amplitude and time-varying frequency. The data across syllables are perfectly aligned at time 0, with variations in syllable structure leading to decreases in alignment with increasing time before and after syllable onset. Fig. 3 *A*–*D* shows ±250 ms around the onset of syllables. The duration of both female and male syllables was longer than this window (female: median 281 ms, $n = 53$; male: median 303 ms, $n = 45$).

During solo syllables, spiking rates increased in male HVC around 50 ms prior to the onset of its solo syllables (Fig. 3*A*). In contrast, HVC activity recorded in females as they heard these male solo syllables remained at or below baseline (Fig. 3*A*). Similarly, spiking rates in female HVC also increased about 50 ms prior to the onset of the production of their own solo syllables (Fig. 3*B*). Activity in male HVC remained near baseline while hearing these female solo syllables (Fig. 3*B*).

During duets, HVC activity in both females and males increased while producing autogenous syllables and decreased while hearing heterogenous syllables, creating an alternation of HVC activity in each bird (Fig. 3 *C* and *D*). For example, when female and male HVC activity was aligned to the onset of male duet syllables (Fig. 3*C*), there was an increase in male HVC activity approximately 50 ms prior to the onset. At the same time, HVC activity in females was high during production of her syllable, then decreased prior to the start of the male syllable, and eventually dropped below baseline. The decrease in RS in female HVC below baseline occurs while hearing the male syllable. For female duet syllables, activity patterns in female and male HVC are reversed (Fig. 3*D*). The decrease in RS below zero (below baseline) indicates that HVC activity is inhibited while each bird hears its partner.

To make statistical comparisons of HVC activity during autogenous and heterogenous syllables, we averaged the RS over the entire duration of each syllable (Fig. 3 *E* and *F*). As premotor HVC activity increased about 50 ms prior to syllable onset, we shifted the starts and ends of analysis windows by −50 ms (earlier) for HVC activity during production of autogenous syllables. Similarly, as auditory responses in HVC are delayed by about 30 to 50 ms (28), we shifted the starts and ends of analysis windows by +50 ms (later) for HVC activity responding to heterogenous syllables. These shifts did not affect the results, which were robust to shifts of 0 to over ±50 ms.

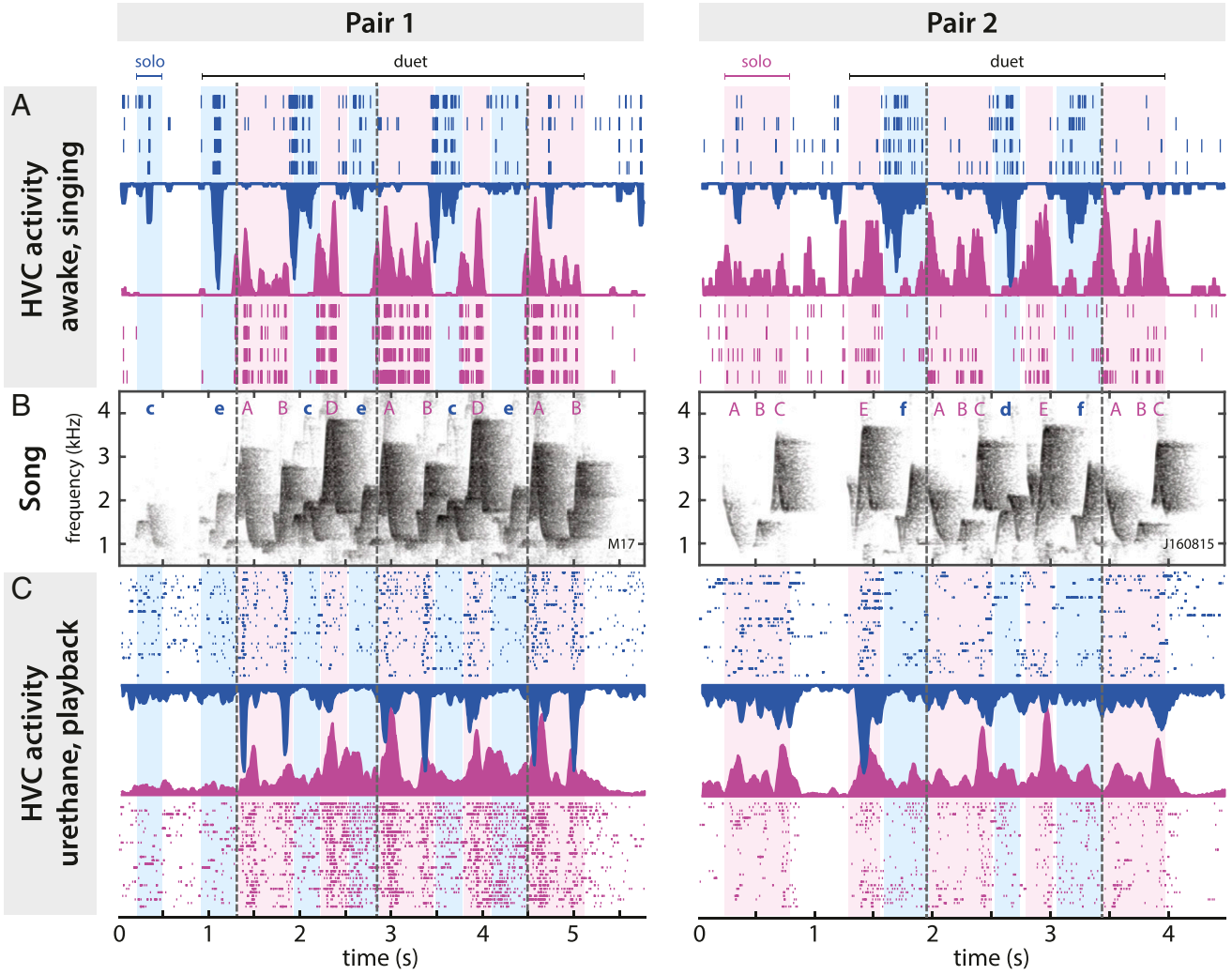

Fig. 2. HVC neurophysiology in two pairs of wrens. In pair 1, the duet was immediately preceded by a male solo syllable. In pair 2, the duet was immediately preceded by female solo syllables. Background shading indicates which bird sang each syllable: light blue for male and light magenta for the female. Dotted lines highlight repetitions of duet motifs (repeated sequences of syllables). (*A*) Neural activity in awake, singing wrens. Each row of raster marks indicates the time of action potentials from each of the four electrodes implanted in each bird (blue for male, magenta for female). Between the raster plots are normalized histograms of spiking activity. The histogram for activity in the male has been inverted to highlight the temporal relations in HVC activity between the two birds. (*B*) Spectrogram of solo syllables and duets produced by the pairs of wrens. Lowercase blue letters are for male syllables, and uppercase magenta letters are for female syllables. The motif for pair 1 was ABcDe, and that for pair 2 was ABCdEf. (*C*) Neural responses to playback of the song (*B*) after the wrens were anesthetized with urethane. Each row in the raster plots shows spike times from a playback. (For pair 1, rasters are shown for 30/77 song playbacks to male and 30/52 playbacks to female; for pair 2, rasters are shown for 30/53 for male and 30/63 for female.) Between the raster plots are normalized peristimulus time histograms (PSTHs).

As expected, HVC activity increased significantly during both solo and duet autogenous syllables in both females and males (median RS, Wilcoxon signed-rank test different from zero [baseline RS]: female solo = 10.09, $n = 7$, $P = 0.0156$; female duet = 17.19, $n = 40$, $P \ll 0.0001$; male solo = 38.32, $n = 13$, $P = 0.0024$; male duet = 39.73, $n = 20$, $P \ll 0.0001$) (Fig. 3*E*). Interestingly, we did not find differences in HVC activity for solo vs. duet syllable production (median RS; females, Mann–Whitney $U = 85$, $P = 0.1044$; males, $U = 102$, $P = 0.3157$). This finding is interesting because there was a significant difference in the amplitudes of solo and duet syllables; male solo syllables were 41.9 dB lower in amplitude than male duet syllables (median root mean square [rms]; solo = 0.0075, $n = 12$; duet = 0.0860, $n = 20$; Mann–Whitney $U = 7$, $P \ll 0.0001$), and female solo syllables were 15.2 dB lower in amplitude than duet syllables (median rms; solo = 0.0693, $n = 7$; duet = 0.1451, $n = 40$; $U = 32$, $P = 0.0005$).

In one male (blue squares in the two right columns in Fig. 3*E*), RS to autogenous syllables was higher than in all other birds, male or female. This difference could be due to the position of the electrodes in HVC or to individual differences between birds. Importantly, this difference did not affect the main result; we repeated the analysis using normalized RS, which reduces the effect of individual differences between birds. Normalized HVC activity, calculated by dividing RS by the spontaneous firing rate, was significantly above zero (baseline) for male HVC activity during autogenous syllable production (median activity, Wilcoxon signed-rank test: male solo = 19.46, $P = 0.0024$; male duet = 13.71, $P \ll 0.0001$).

RSs during heterogeneous syllables in duets were below zero (Fig. 3 *C* and *D*), indicating inhibition of HVC activity when birds heard their partners. To determine if HVC was inhibited, we calculated the RS over the duration of each heterogenous syllable. The RSs of HVC neurons in female wrens were significantly

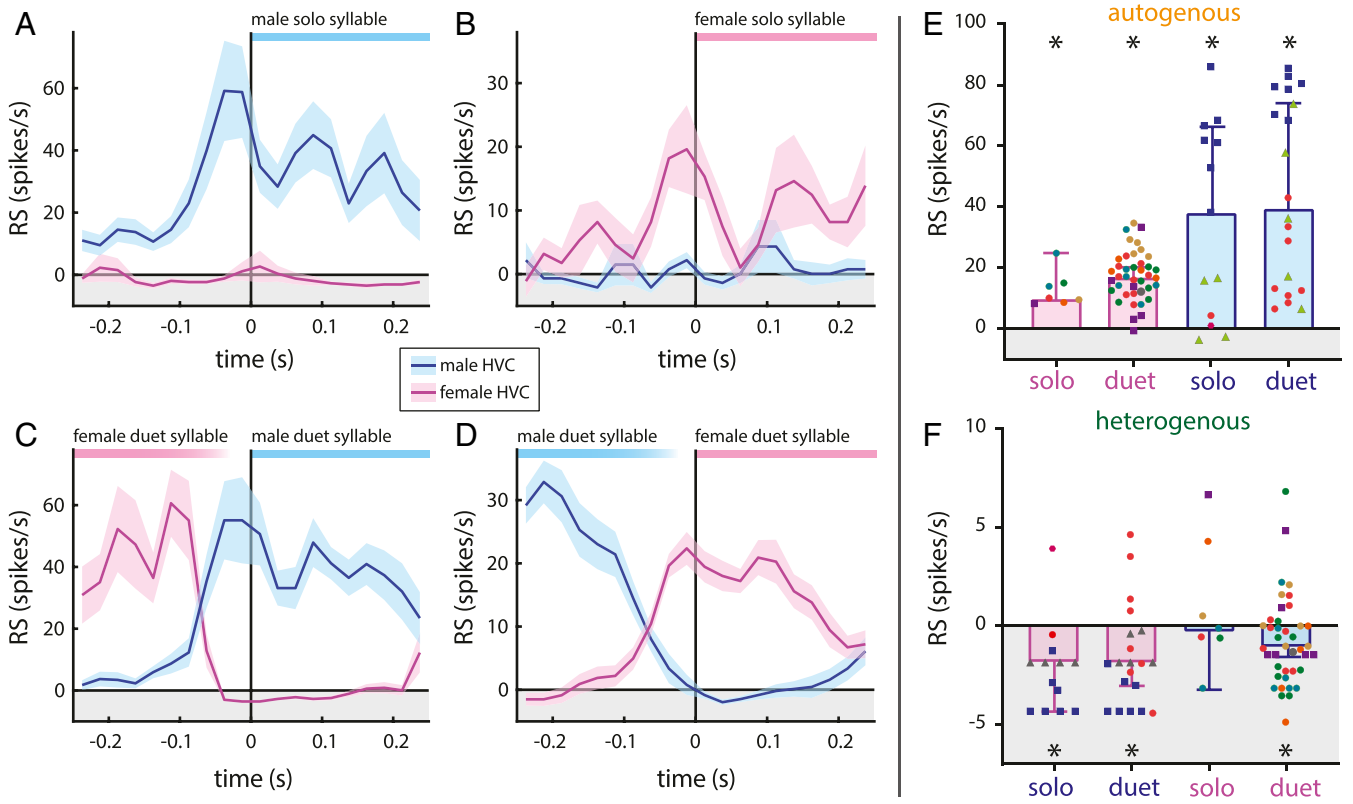

Fig. 3. HVC activity during solo and duet singing. Negative RSs (i.e., inhibition) are highlighted in gray. *A–D* show mean and SD of HVC RS (firing rate during syllables minus spontaneous firing rate) across syllables before and after (±250 ms) syllable onsets. (*A*) RS of male HVC (blue) and of female HVC (magenta) aligned to the beginning of male solo syllables. (*B*) Same as *A* but for HVC RS aligned to the beginning of female solo syllables. (*C*) RS of male and female HVC aligned to the onset of male duet syllables (female syllable precedes male syllable). (*D*) Same as *C* but for RS aligned to the onset of female duet syllables. (*E*) Median (+95% CI) RS of HVC during autogenous syllables in females (left two bars) and males (right two bars). Different symbols represent different pairs of wrens, and different colors represent different duets. The color of *x*-axis labels indicates the sex of the bird producing the syllables. (F) Same as *E* but median RS (−95% CI) in HVC during heterogenous syllables. *Significant difference from zero ($P < 0.05$, Wilcoxon signed-rank test).

below zero while hearing both male solo and duet syllables (median RS, Wilcoxon signed-rank test; female response to male solo syllables, median $= -1.875$, $n = 13$, $P = 0.0078$; female response to male duet syllables, median $= -1.903$, $n = 20$, $P = 0.0166$). RSs in male HVC neurons were significantly below zero while hearing female duet syllables (median $= -1.114$, $n = 40$, $P = 0.006$) but not female solo syllables (median $= -0.1529$, $n = 7$, $P = 0.9375$) (Fig. 3*F*). Because wrens use heterogenous information for the coordination of duet syllables (4, 10) and because neurons in HVC contribute to timing of syllable production in other songbird species (19, 24), we hypothesize that this inhibition is important for the coordination of duet singing.

To test whether HVC responded to acoustic cues while birds were not engaged in duet singing, we presented recordings of their own duets to two pairs of awake wrens. There were no increases or decreases in HVC activity during playback compared with baseline in either females (paired *t* test, $P = 0.2068$, $t = 1.341$, degrees of freedom [df] $= 11$) or males ($P = 0.0939$, $t = 1.797$, df $= 14$). This lack of auditory responses to playback is similar to results obtained in other species of songbirds (27, 29). In plain-tailed wrens, however, there seems to be context-dependent modulation of auditory responses of HVC neurons in awake birds. HVC neurons were inhibited while hearing heterogenous syllables during bouts of singing but not playback of recorded wren songs.

HVC Responses to Playback under Urethane Anesthesia. At the level of behavior, heterogenous input is critical for the timing of motor activity in cooperative behaviors. As an initial test of whether inhibition of HVC activity during heterogenous feedback was related to hearing the partner's syllables, we anesthetized each wren with urethane, which antagonizes GABAeric (γ-aminobutyric acid) transmission (30), and made neurophysiological recordings in HVC. We presented vocalizations sung by each pair of wrens while measuring responses in HVC (Fig. 2*C*). These recordings were made with carbon fiber electrodes after the chronically implanted electrodes had been removed.

We found that HVC neurons in both females and males always responded to female syllables, but responses to male syllables seemed to depend on the presence of female syllables. In both females and males, HVC neurons did not respond to playback of male solo syllables (Fig. 4*A*) (median RS; female $= -0.602$, $n = 5$, $P = 0.8125$; male $= 0.9729$, $n = 12$; $P = 0.1641$). However, HVC neurons in both sexes did respond to male syllables when they occurred within duets (median RS; female $= 9.713$, $n = 13$, $P = 0.0002$; male $= 4.333$, $n = 20$, $P = 0.0037$) (Fig. 4*A*). That is, HVC neurons responded to male syllables only when the male syllable was preceded by a female syllable. In contrast, HVC neurons in females and males responded to female syllables both during playback of solo (median RS: female $= 8.754$, $n = 6$, $P = 0.0313$; male $= 5.635$, $n = 7$, $P = 0.0469$) and duet songs (female $= 12.83$, $n = 34$, $P \ll 0.0001$; male $= 9.10$, $n = 40$, $P \ll 0.0001$) (Fig. 4*B*). These data are similar to our previous report in which we showed that HVC neurons in both female and male anesthetized wrens responded more strongly to playback of female duet syllables than to male duet syllables (4).

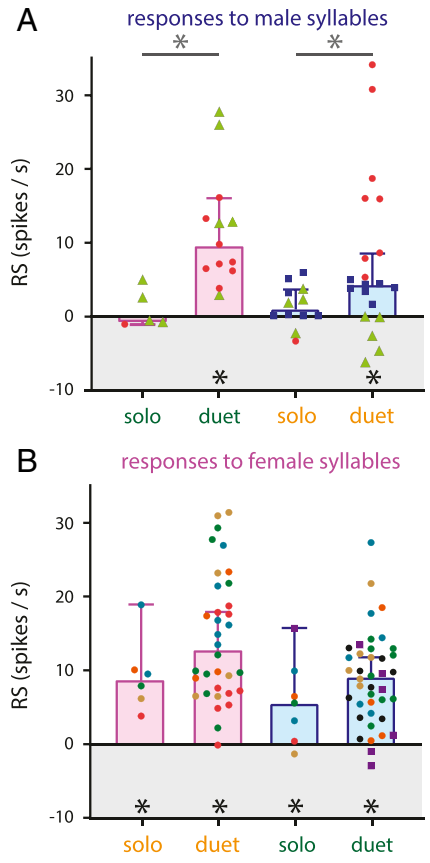

Fig. 4. HVC activity in response to playback of solo and duet syllables in urethane-anesthetized wrens. Negative RSs are highlighted in gray. Magenta bars (left two bars) represent median RS + 95% CI in females, and blue bars (right two bars) represent it in males. Orange labels indicate playback of autogenous syllables, and green labels indicate playback of heterogenous syllables. Black asterisks indicate significant difference from zero ($P < 0.05$, Wilcoxon signed-rank test). (*A*) RS in female and male HVC in response to playback of male solo and duet syllables. Dark gray asterisks at the top indicate significant differences between responses to solo and duet syllables ($P < 0.05$, Mann–Whitney *U*). Symbols are the same as in Fig. 3. (*B*) RS in female and male HVC in response to playback of female solo and duet syllables.

Discussion

We recorded neurophysiologically from HVC neurons in wild-caught plain-tailed wrens in Ecuador. We found an increase in premotor HVC activity in awake wrens during solo and duet syllable production, which resulted in an alternation in HVC activity during duets, as occurs in white-browed sparrow-weavers (*Plocepasser mahali*) (5). This alternation may result, in part, from the inhibition of HVC neurons when wrens hear heterogenous feedback from their partner.

HVC activity in female wrens was also inhibited when they heard male solo syllables. However, in male wrens, HVC activity did not change in response to hearing female solo syllables. In anesthetized wrens, HVC neurons in both females and males were excited by playback of female syllables and male syllables in a duet but not to male solo syllables. This sex difference may reflect the leading role of females in duet performances (4). Taken together, these data show that HVC circuits integrate information from sequences of autogenous and heterogenous cues during duet performances. We suggest the integration of heterogenous feedback alters the timing of autogenous syllable production by activating inhibitory circuits in HVC.

Cooperative performances, like vocal turn taking, require participants to modulate the timing of an animal's own vocalizations with its partner by integrating heterogenous feedback. Turn taking must manifest in alternating premotor activity in the brains of the participants. For example, HVC activity in white-browed sparrow-weavers alternated between females and males in conjunction with their own vocal performances (5). This alternating activity is mediated by heterogenous feedback that links motor activity across the brains of participants (Fig. 1*C*). This linkage can manifest within milliseconds; when male wrens drop a syllable during a duet, females show a delay in the production of the next syllable (4).

Role of Inhibition for Coordination of Motor Programs. Inhibition is a common mechanism for modulating pattern-generating circuits (19, 31, 32). In plain-tailed wrens, we found that heterogenous feedback may contribute to the synchronization of bouts of premotor activity in HVC via inhibition. In zebra finches (*Taeniopygia guttata*), GABAergic inhibition in HVC has been shown to mediate turn taking of innate calls (33). Infusion of GABAergic antagonists, muscimol or GABAzine, into HVC impaired the coordination of call production in these birds (19). We hypothesize that heterogenous acoustic feedback from partner wrens activates GABAeric circuits in HVC that alter the timing within the pattern-generating circuit (23, 24). Based on our results, we predict that blocking GABAergic activity will result in a disruption of temporal coordination between wrens, resulting in overlapping syllables during duet singing.

Such changes in temporal coordination sometimes occur during duet singing, demonstrating the potential influence of inhibition. For example, when male plain-tailed wrens omitted syllables during duets, the subsequent female syllables were delayed (4). Our hypothesis is that the lack of postinhibitory rebound resulted in delays in premotor activity and syllable production. Interestingly, in zebra finches, HVC interneurons and HVC_X neurons (HVC neurons that project to area X) have an H current that is activated upon hyperpolarization (34), a cellular mechanism for postinhibitory rebound. However, premotor neurons in HVC (HVC_{RA} neurons [HVC neurons that project to the robust nucleus of the arcopallium, RA]) do not have an H current. It may be that HVC_{RA} neurons in plain-tailed wrens differ from zebra finches and have an H current that is an adaptation for turn taking in duet singing.

When the birds were anesthetized with urethane, instead of inhibitory responses to heterogenous cues, we observed excitatory responses to playback of duet syllables. Urethane has been shown to act as an antagonist for GABAergic transmission (30). Urethane anesthesia, therefore, may reveal excitatory input to HVC that is gated by GABAergic circuitry when the wrens are awake.

Solo Vs. Duet Performances. We found that solo syllables in plain-tailed wrens are lower in amplitude than syllables produced in duets (4). This may reflect differences in the function of solo singing. In indigo buntings (*Passerina cyanea*), low-amplitude singing is a mechanism used in the development of new syllables (35). Low-amplitude singing may also be a form of aggressive signaling—a quiet threat (36). Data from other duetting species indicate that females use solo song to defend territories (12, 14).

The role of solo singing has not been directly investigated in plain-tailed wrens. We believe that there are two forms of solo syllables—low-amplitude syllables like those reported in this study and higher-amplitude solo syllables. We have heard the higher-amplitude solo syllables in response to playback of conspecific songs at home territories, suggesting that a single nearby wren may engage in territorial defense. Lower-amplitude solo syllables are produced by either sex preceding duet performances, as seen here, but they are also produced by lone males in the field. These low-amplitude syllables may be related to mate attraction and the process of initiating duets.

The change in amplitude between solo and duet singing likely reflect changes in function. Duet singing is believed to be used for mate guarding and territorial defense (1). Interestingly, plain-tailed wrens routinely sing in choruses of over four individuals (11). Chorusing may be a mechanism for further enhancing territorial defense (11) but may also contribute to learning duet performances among related wrens.

Cooperative Performances across Species. Cooperative performances are found across species and are used in behaviors that range from social displays (3, 37, 38) to prey capture (2). We envision cooperative performances as being mediated by feedback control systems that span individuals. These feedback control systems may be innate or learned.

In songbirds, duet singing requires two categories of learning. First, duetting songbirds must learn their own vocalizations, as is seen in all other species of oscine passeriform birds. Second, duetting songbirds learn to coordinate with partners. In canebrake wrens (*Cantorchilus zeledoni*), duet performances improve with practice, developing novel phrases with increased stereotopy (39). We have anecdotally observed the same phenomenon in plain-tailed wrens, as newly formed pairs produce shorter, seemingly less-coordinated duet performances than long-standing pairs.

Many behaviors that are described as having "senders" and "receivers" can be considered cooperative insofar as the behaviors of both sender and receiver are linked through heterogenous feedback (9, 40). Indeed, signaling often is not unidirectional, but rather, the roles of sender and receiver alternate between individuals. The rapid alternation of sending and receiving seen in plain-tailed wrens is controlled by an emergent feedback loop that spans individuals. The timing of this turn taking in wrens appears to be regulated within this feedback loop by inhibition.

Materials and Methods

All animal experiments were conducted according to guidelines established by the National Research Council, and all procedures were evaluated and approved by the animal care and use committee of Rutgers University/New Jersey Institute of Technology. Animal collection was made under the permit 01-16-1C-FAU-DPAN/MA issued by the Ministerio del Ambiente of Ecuador. All of the specimens were deposited at Museo de Zoología (QCAZ), Pontificia Universidad Católica del Ecuador (Quito), under mobilization permit 58/-08-2016-DPAN-MA. Additional collection permits, 14-2013-1214-IC-FAU-FLO-DPAI/MA, 02-2014-IC-FAU-DPAP-MA, and 40-IC-FAU-DPAN/MA (Ministerio del Ambiente, Napo Province, Ecuador), were also obtained for the conduct of this research.

Neurophysiology in Awake Animals. Four pairs of plain-tailed wrens, *P. euophrys*, were caught in mist nets at the Yanayacu Biological Research Station and Center for Creative Studies in Ecuador. Individuals were identified with leg bands and maintained either separately or in a single cage throughout the duration of the experiment. Birds were fed live crickets and mealworms (Wikiri) throughout the day and provided with water ad libitum. Overnight, birdcages were covered with blankets, and heat was provided by a hot-bead sterilizer (Germinator 500).

Wrens were housed up to 5 d prior to neurophysiological recordings. For implantation of electrodes, pairs of birds were anesthetized with sevoflurane (0.7 to 0.9 L/min oxygen) and placed in a custom stereotaxic apparatus. Vibration isolation was achieved using a heavy aluminum plate supported by tennis balls. The system was grounded using a 2-m copper stake driven into the soil adjacent to the rig.

Topical and/or subcutaneous lidocaine (1 to 2%) was used on the scalp prior to incision. A small craniotomy was made over HVC (3.0 mm lateral, 0 mm anterior to the bifurcation of the sagittal sinus). The location of HVC was confirmed by recording its characteristic neurophysiological activity using a carbon fiber electrode (41).

Four single-wire recording electrodes (50-μm nichrome–formvar electrodes; 700 kΩ to 1.5 MΩ) were implanted in either the right or the left HVC using micromanipulators (Narishige and Siskiyou). A 50-μm nichrome–formvar electrode was implanted adjacent to HVC as a reference. A 75- or 50-μm silver wire ground was implanted beneath the skull rostrally. Some electrodes were plated (nanoZ; White Matter, LLC) with gold (Neuralynx, Inc.) prior to implantation.

All wires were attached to a connector (single row, Nano-Miniature 0.025"/0.64 mm; Omnetics) that was cemented onto the bird's head using dental cement and cyanoacrylate glue. Birds recovered from anesthesia in a cage equipped with a heating pad. Birds were monitored every 30 min for the first day after surgery. Food and water were available ad libitum.

For recording sessions, wireless digital transmitters (model MCS-2100; MultiChannel Systems GmbH) that amplify and digitize (sample rates 10 to 25 kHz) neurophysiological signals were attached to each bird. A battery for each transmitter was attached with Velcro to a small backpack that was placed on the bird after surgery. Neural recordings were collected via MultiChannel Systems GmbH software (MC Rack). Vocalizations were recorded using microphones placed adjacent to the birds (Sennheiser ME66/KP6 or ATR-55; Saul Mineroff Electronics). Acoustic signals were digitized using a Micro1401 or Power1401 (Cambridge Electronic Design) at rates of 10 to 25 kHz, which matched the sampling rates for neural recordings. Neural and acoustic data were synchronized across recording systems using a custom transistor–transistor logic (TTL) pattern generator.

For each recording session, transmitters and batteries were placed on a pair of wrens. Recording sessions lasted up to 2 h. Birds were provided food and water throughout the session. After each recording session, the transmitters and batteries were removed from the animals.

Neurophysiology in Urethane-Anesthetized Animals. After the awake neurophysiological recordings, birds were prepared for neurophysiological recordings under urethane anesthesia. Each bird was given 80 to 100 μL of 20% urethane in water every hour for 2 h. These procedures for anesthesia and recordings are identical to those used in ref. 4.

After the bird was anesthetized, the nichrome–formvar electrodes used for the prior recordings were removed. Birds were then placed in the custom stereotaxic apparatus. Micromanipulators were used to position Carbostar-1 electrodes (Kation Scientific) in HVC.

Neural activity was amplified and filtered (300 to 5,000 Hz) using a Model 1700 differential amplifier (A-M Systems). Data were collected using Spike2 software to control a Micro1401 or Power1401 data acquisition system (CED). Playback of songs and other sounds were delivered through external speakers. We presented 20 to 40 repetitions of each acoustic stimulus (amplitudes 65- to 80-dB sound pressure level [SPL]; Radio Shack Sound Level Meter, 33 to 2,055) in a randomized order with 10 to 20 s between each stimulus presentation. Stimuli included the duets sung during earlier neurophysiological recordings in the awake wrens, solo male and female syllables, and conspecific duets. We did not experimentally manipulate the order of syllables or other acoustic features, as has been done in previous studies of HVC (4, 42, 43). We recorded up to five sites in each wren.

After recordings, animals were euthanized with an overdose of sevoflurane and perfused with 0.9% saline and 4% formaldehyde. These specimens are stored at the Museo de Zoología, Pontificia Universidad Católica del Ecuador (QCAZ).

Analysis. Data from neurophysiological recordings were imported into Spike2 software and filtered (high pass at 240 to 600 Hz and low pass at 5 kHz). For recordings in awake and anesthetized wrens, one to five neurons (electrical "units") were isolated at each recording site based on waveform using principal component analysis, as implemented in Spike2 software. Since the reliability of single-unit isolation varied between different units and different recording sites over recordings, we chose to report summed single units, making a "multiple single-unit" list of spike times on which all analyses were performed. For chronic recordings, each channel was analyzed independently (Fig. 2) as the electrode tips were spread over about 1 mm within HVC. We did not identify HVC neuron types; antidromic stimulation is necessary to differentiate projection neurons from interneurons. If the types of HVC neurons in plain-tailed wrens are similar to those in zebra finches, we expect that most spikes reported here were produced by interneurons.

RS was calculated as the firing rate (spikes per second) during production or hearing of syllables minus the spontaneous firing rate. Calculated this way, negative RSs indicate firing rates below baseline (i.e., inhibition). The window for calculating spontaneous rate was typically a few seconds prior to the playback stimulus or birds singing.

The plots in Fig. 3 *A–D* highlight HVC activity at syllable transitions. To average across syllable transitions, we synchronized activity in relation to the start of either solo or duet autogenous syllables. Because the types and durations of syllables differ, the temporal alignment of activity degrades the further away activity is from the start of the syllable,

both prior to and after syllable onset. Using this approach, the data are aligned at the start of the autogenous syllable, but because each performance is different, the further in time away from that synchronization point, the greater the desynchronization. This alignment is sufficient to show changes in activity near syllable onsets, the moment when turn taking occurs.

The windows for calculating RS (Fig. 3 *E* and *F*) during singing were shifted relative to the start and end times of syllables. The time windows for quantifying premotor activity were shifted by −50 ms to capture the increase in firing that preceded the onsets of autogenous syllables (Figs. 2 and 3 *A*–*D*). The time windows for responses to heterogenous syllables were shifted +50 ms, reflecting neural delays in ascending auditory pathways (28, 42). The windows for heterogenous responses during duets were truncated by 125 ms to avoid overlap with premotor increases in spiking activity. Calculation of RS in urethane-anesthetized birds was not shifted relative to syllable onset or offset; this allows a more direct comparison with previous data (4).

For recordings in urethane-anesthetized birds, we summed responses to each repetition of a stimulus across recording sites—up to 100 repetitions of the stimulus across five recording sites. RSs were calculated over the duration of each syllable. The time window for calculating spontaneous firing rate was identical to that used for the recordings in awake birds.

Spectrograms were rendered in Matlab (MathWorks) using the spectrogram function (95% overlap, either 512- or 1,024-point window, sample rates of 10 or 25 kHz). The amplitude mapping of the spectrograms was shifted to highlight low-amplitude solo syllables. Note that the "smeared" appearance of these spectrograms reflects the acoustic structure of the songs; similar smearing is seen in recordings taken in the field in previous publications (4, 11, 44). Demarcation of the starts and ends of syllables was based on visual inspection of the spectrograms.

Amplitudes were calculated as rms values of the audio recording over the duration of each syllable (Matlab "rms" function). Differences in solo and duet syllable amplitudes were not due to the placement of the microphone, which was adjacent to each cage.

All statistical calculations were performed in Matlab or Prism (GraphPad; version 8.0.2). Actual *P* values are reported, unless they were less than 0.0001, which are shown as "$P \ll 0.0001$."

Data Availability. Matlab code and data have been deposited in Dryad (https://doi.org/10.5061/dryad.q2bvq83hp).

ACKNOWLEDGMENTS. Ignacio Moore, Stephanie White, Santiago Burneo, and Alejandra Camacho provided critical logistical help and support. We thank Harold Greeney and José Simbaña for support of our efforts at Yanayacu. This work was supported by NSF Grants IOS-1146792 (to M.J.C.) and IOS-1146855 (to E.S.F.).

1. M. L. Hall, "A review of vocal duetting in birds" in *Advances in the Study of Behavior: Vocal Communication in Birds and Mammals*, M. Naguib, V. M. Janik, K. Zuberbuhler, N. S. Clayton, Eds. (Academic Press, Amsterdam, the Netherlands, 2009), vol. 40, pp. 67–121.
2. B. L. Moore, R. C. Connor, S. J. Allen, M. Krützen, S. L. King, Acoustic coordination by allied male dolphins in a cooperative context. *Proc. Biol. Sci.* **287**, 20192944 (2020).
3. D. E. Okobi, A. Banerjee, A. M. M. Matheson, S. M. Phelps, M. A. Long, Motor cortical control of vocal interaction in neotropical singing mice. *Science* **363**, 983–988 (2019).
4. E. S. Fortune, C. Rodriguez, D. Li, G. F. Ball, M. J. Coleman, Neural mechanisms for the coordination of duet singing in wrens. *Science* **334**, 666–670 (2011).
5. S. Hoffmann *et al.*, Duets recorded in the wild reveal that interindividually coordinated motor control enables cooperative behavior. *Nat. Commun.* **10**, 2577 (2019).
6. K. D. Rivera-Caceres, Plain wrens *Cantorchilus modestus zeledoni* adjust their singing tempo based on self and partner's cues to perform precisely coordinated duets. *J. Avian Biol.* **46**, 361–368 (2015).
7. M. Wirthlin *et al.*, A modular approach to vocal learning: Disentangling the diversity of a complex behavioral trait. *Neuron* **104**, 87–99 (2019).
8. S. C. Levinson, Turn-taking in human communication—origins and implications for language processing. *Trends Cognit. Sci.* **20**, 6–14 (2016).
9. J. E. Elie *et al.*, From perception to action: The role of auditory input in shaping vocal communication and social behaviors in birds. *Brain Behav. Evol.* **94**, 51–60 (2019).
10. M. J. Coleman, E. S. Fortune, Duet singing in plain-tailed wrens. *Curr. Biol.* **28**, 643–645 (2018).
11. N. I. Mann, K. A. Dingess, P. J. B. Slater, Antiphonal four-part synchronized chorusing in a neotropical wren. *Biol. Lett.* **2**, 1–4 (2006).
12. C. N. Templeton, A. A. Ríos-Chelén, E. Quirós-Guerrero, N. I. Mann, P. J. B. Slater, Female happy wrens select songs to cooperate with their mates rather than confront intruders. *Biol. Lett.* **9**, 20120863 (2013).
13. K. D. Rivera-Cáceres, C. N. Templeton, A duetting perspective on avian song learning. *Behav. Process.* **163**, 71–80 (2019).
14. R. N. Levin, Song behaviour and reproductive strategies in a duetting wren, *Thryothorus nigricapillus*. I. Removal experiments. *Anim. Behav.* **52**, 1093–1106 (1996).
15. R. N. Levin, Song behaviour and reproductive strategies in a duetting wren, *Thryothorus nigricapillus*. II. Playback experiments. *Anim. Behav.* **52**, 1107–1117 (1996).
16. N. Langmore, Functions of duet and solo songs of female birds. *Trends Ecol. Evol.* **13**, 136–140 (1998).
17. B. M. Colquitt, D. P. Merullo, G. Konopka, T. F. Roberts, M. S. Brainard, Cellular transcriptomics reveals evolutionary identities of songbird vocal circuits. *Science* **371**, eabd9704 (2021).
18. A. Reiner *et al.*, Revised nomenclature for avian telencephalon and some related brainstem nuclei. *J. Comp. Neurol.* **473**, 377–414 (2004).
19. J. I. Benichov, D. Vallentin, Inhibition within a premotor circuit controls the timing of vocal turn-taking in zebra finches. *Nat. Commun.* **11**, 221 (2020).
20. F. Nottebohm, T. M. Stokes, C. M. Leonard, Central control of song in the canary, *Serinus canarius*. *J. Comp. Neurol.* **165**, 457–486 (1976).
21. R. H. R. Hahnloser, A. A. Kozhevnikov, M. S. Fee, An ultra-sparse code underliesthe generation of neural sequences in a songbird. *Nature* **419**, 65 (2002).
22. W. Zhao, F. Garcia-Oscos, D. Dinh, T. F. Roberts, Inception of memories that guide vocal learning in the songbird. *Science* **366**, 83–89 (2019).
23. M. A. Long, M. S. Fee, Using temperature to analyse temporal dynamics in the songbird motor pathway. *Nature* **456**, 189–194 (2008).
24. E. T. Vu, M. E. Mazurek, Y. C. Kuo, Identification of a forebrain motor programming network for the learned song of zebra finches. *J. Neurosci.* **14**, 6924–6934 (1994).
25. J. F. Prather, S. Peters, S. Nowicki, R. Mooney, Precise auditory-vocal mirroring in neurons for learned vocal communication. *Nature* **451**, 305–310 (2008).
26. R. Mooney, W. Hoese, S. Nowicki, Auditory representation of the vocal repertoire in a songbird with multiple song types. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 12778–12783 (2001).
27. K. Murphy, L. S. James, J. T. Sakata, J. F. Prather, Advantages of comparative studies in songbirds to understand the neural basis of sensorimotor integration. *J. Neurophysiol.* **118**, 800–816 (2017).
28. M. J. Coleman, Synaptic transformations underlying highly selective auditory representations of learned birdsong. *J. Neurosci.* **24**, 7251–7265 (2004).
29. M. F. Schmidt, M. Konishi, Gating of auditory responses in the vocal control system of awake songbirds. *Nat. Neurosci.* **1**, 513–518 (1998).
30. D. Accorsi-Mendonça, R. M. Leão, J. F. Aguiar, W. A. Varanda, B. H. Machado, Urethane inhibits the GABAergic neurotransmission in the nucleus of the solitary tract of rat brain stem slices. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **292**, R396–R402 (2007).
31. R. L. Calabrese, Oscillation in motor pattern-generating networks. *Curr. Opin. Neurobiol.* **5**, 816–823 (1995).
32. C. J. Edwards, T. B. Alder, G. J. Rose, Auditory midbrain neurons that count. *Nat. Neurosci.* **5**, 934–936 (2002).
33. J. I. Benichov *et al.*, The forebrain song system mediates predictive call timing in female and male zebra finches. *Curr. Biol.* **26**, 309–318 (2016).
34. A. Daou, M. T. Ross, F. Johnson, R. L. Hyson, R. Bertram, Electrophysiological characterization and computational models of HVC neurons in the zebra finch. *J. Neurophysiol.* **110**, 1227–1245 (2013).
35. D. Margoliash, C. A. Staicer, S. A. Inoue, Stereotyped and plastic song in adult indigo buntings, *Passerina cyanea*. *Anim. Behav.* **42**, 367–388 (1991).
36. Ç. Akçay, R. C. Anderson, S. Nowicki, M. D. Beecher, W. A. Searcy, Quiet threats: Soft song as an aggressive signal in birds. *Anim. Behav.* **105**, 267–274 (2015).
37. G. L. Patricelli, A. H. Krakauer, Tactical allocation of effort among multiple signals in sage grouse: An experiment with a robotic female. *Behav. Ecol.* **21**, 97–106 (2010).
38. K. S. Bostwick, Display behaviors, mechanical sounds, and evolutionary relationships of the club-winged manakin (*Machaeropterus deliciosus*). *Auk* **117**, 465–478 (2000).
39. K. D. Rivera-Cáceres, E. Quirós-Guerrero, M. Araya-Salas, W. A. Searcy, Neotropical wrens learn new duet rules as adults. *Proc. Biol. Sci.* **283**, 20161819 (2016).
40. R. Laje, G. B. Mindlin, Highly structured duets in the song of the South American hornero. *Phys. Rev. Lett.* **91** (2003).
41. N. F. Day, K. L. Terleski, D. Q. Nykamp, T. A. Nick, Directed functional connectivity matures with motor learning in a cortical pattern generator. *J. Neurophysiol.* **109**, 913–923 (2013).
42. D. Margoliash, Acoustic parameters underlying the responses of song-specific neurons in the white-crowned sparrow. *J. Neurosci.* **3**, 1039–1057 (1983).
43. D. Margoliash, E. Fortune, Temporal and harmonic combination-sensitive neurons in the zebra finch HVC. *J. Neurosci.* **12**, 4309–4326 (1992).
44. N. I. Mann, K. A. Dingess, F. K. Barker, J. A. Graves, P. J. Slater, A comparative study of song form and duetting in neotropical *Thryothorus* wrens. *Behaviour* **146**, 1–43 (2009).