Neural Mechanisms for the Coordination of Duet Singing in Wrens

Eric S. Fortune, *et al.*

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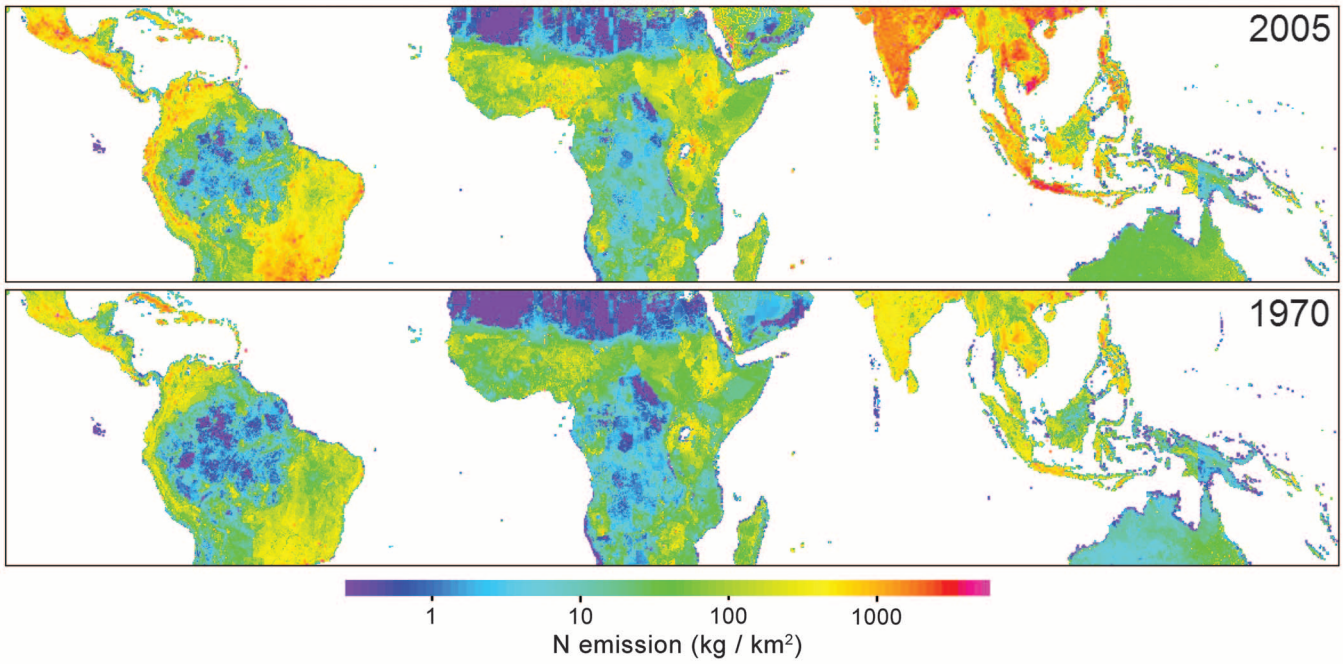

Fig. 3. Anthropogenic N emissions in 2005 and 1970. NO_x and NH_3 emissions per 0.1° grid cell were obtained from European Commission–Joint Research Centre/Netherlands Environmental Assessment Agency, EDGAR version 4.1, (http://edgar.jrc.ec.europa.eu/) 2010, and were converted to N emissions per surface area.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/334/6056/664/DC1
Materials and Methods
Figs. S1 to S6
Table S1
References (*27*–*33*)

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Neural Mechanisms for the Coordination of Duet Singing in Wrens

Eric S. Fortune,[1,2*] Carlos Rodríguez,[2] David Li,[1] Gregory F. Ball,[1] Melissa J. Coleman[3]

Plain-tailed wrens (*Pheugopedius euophrys*) cooperate to produce a duet song in which males and females rapidly alternate singing syllables. We examined how sensory information from each wren is used to coordinate singing between individuals for the production of this cooperative behavior. Previous findings in nonduetting songbird species suggest that premotor circuits should encode each bird's own contribution to the duet. In contrast, we find that both male and female wrens encode the combined cooperative output of the pair of birds. Further, behavior and neurophysiology show that both sexes coordinate the timing of their singing based on feedback from the partner and suggest that females may lead the duet.

Cooperative behaviors are found across taxa and can be critical for survival and reproduction (*1*–*6*). To achieve cooperative performances, brain circuits in each individual must integrate information both from the animal's own self-generated sensory feedback and from sensory cues produced by the partner or partners. We examined how sensory information from these two sources, "autogenous" and "heterogenous" respectively, is integrated in cortical (i.e., pallial) circuits. We used a model system, plain-tailed wrens (*Pheugopedius euophrys*) (*7*), a species of neotropical birds that sing duets in which females and males rapidly alternate syllable production, sounding as if a single bird sang it (see movies S1 and S2) (*8*, *9*).

[1]Department of Psychological and Brain Sciences, Department of Neuroscience, Johns Hopkins University, 3400 North Charles Street, Baltimore, MD 21218, USA. [2]Museo de Zoología, Pontificia Universidad Católica del Ecuador, Edificio de Ciencias Biológicas, 12 de Octubre 1076 y Roca, Quito, Ecuador. [3]W. M. Keck Science Department of Claremont McKenna College, Pitzer College, and Scripps College, 925 North Mills Avenue, Claremont, CA 91711, USA.

*To whom correspondence should be addressed. E-mail: eric.fortune@gmail.com

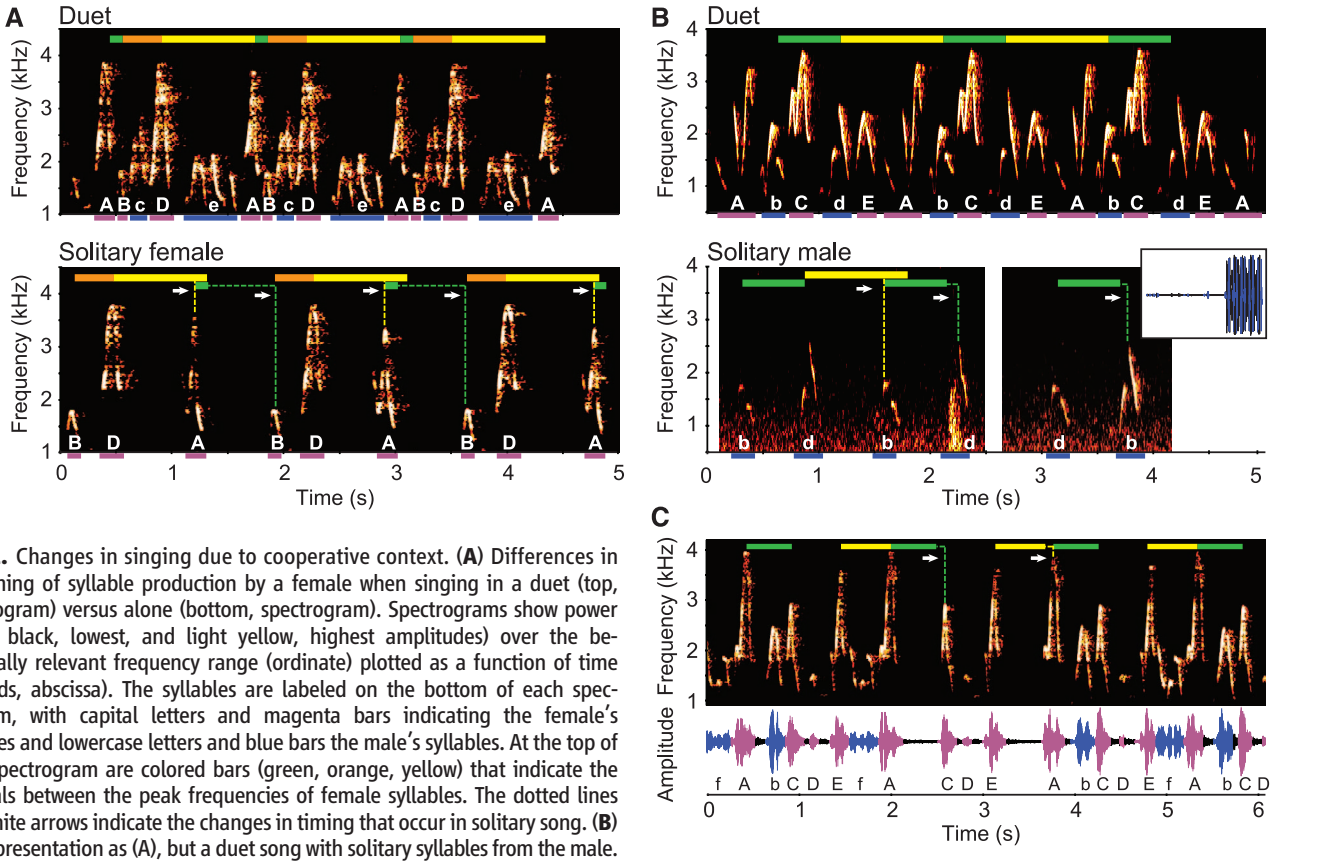

Fig. 1. Changes in singing due to cooperative context. (**A**) Differences in the timing of syllable production by a female when singing in a duet (top, spectrogram) versus alone (bottom, spectrogram). Spectrograms show power (color: black, lowest, and light yellow, highest amplitudes) over the behaviorally relevant frequency range (ordinate) plotted as a function of time (seconds, abscissa). The syllables are labeled on the bottom of each spectrogram, with capital letters and magenta bars indicating the female's syllables and lowercase letters and blue bars the male's syllables. At the top of each spectrogram are colored bars (green, orange, yellow) that indicate the intervals between the peak frequencies of female syllables. The dotted lines and white arrows indicate the changes in timing that occur in solitary song. (**B**) Same presentation as (A), but a duet song with solitary syllables from the male. In this example, the bars at the top indicate the timings of the male syllables. Inset oscillogram shows the change in amplitudes from male solitary singing to subsequent duetting; male syllables are marked in blue. (**C**) Timing changes were also seen during bouts of duetting when the male failed to produce its syllables during a motif of duet song. Spectrogram (top) has the same format used in (A); the corresponding oscillogram below indicates female syllables in magenta and male syllables in blue. Audio files are available in the supporting online materials.

What are the mechanisms that plain-tailed wrens use to cooperate for the production of their complex, learned duet songs? At one extreme, it is possible that the wrens each sing their sequence of syllables as a fixed action pattern (*10*–*14*), following a common cue that initiates singing in both individuals. Alternatively, the wrens could respond on a syllable-by-syllable basis to the singing of the partner throughout the duration of the duet (*15*). These mechanisms will be reflected in both the behavioral performances of the wrens and in the neurophysiological activity that mediates the behavior.

To assess the behavioral parameters that the wrens use for duetting, we monitored singing behavior of a population of plain-tailed wrens on the slopes of the Antisana volcano in Ecuador at the Yanayacu Biological Station and Center for Creative Studies (00.36°S, 77.53°W, altitude 2700 m) (fig. S1) between October 2009 and January 2011. We examined more than 1000 wren vocalizations captured in over 150 hours of acoustic recordings (see Materials and Methods) that were made in the *Chusquea* bamboo thickets where the wrens maintain territories. Duet singing is likely used in territorial defense (*9*, *15*), although a complete description of the functional roles of duetting has not been achieved.

We observed that wrens commonly produced duet songs, but both females and males also sang alone. In general, the acoustic structure and sequence of syllables produced by each individual were identical both in duet and solitary singing (Fig. 1, A and B). Solitary songs are easily recognized by long intersyllable intervals (range 0.34 s to 1.6 s) that occur when the partner would normally sing its syllables during a duet. The presence of these intersyllable intervals in solitary songs suggests that the motor pattern generator for singing in the brain includes the appropriate timing of syllable production during the duet.

Nevertheless, when either female or male wrens sing alone, they increase the durations of intersyllable intervals within each motif. These within-motif increases were on the order of tens of milliseconds (mode increase in duration from duet to solitary = 58 ms, n = 115 song samples; female intersyllable interval duration during a duet = 489 ± 69 ms and alone = 524 ± 99 ms; males during duet = 763 ± 23 ms and alone 885 ± 586 ms; mean ± standard deviation). Also, the durations of intersyllable intervals were significantly more variable in solitary songs than in duet songs (F test, $P < 0.05$, df = 66). These changes can be seen in the examples of female and male wren singing shown in Fig. 1, A and B. In Fig. 1A, the mean intersyllable intervals between the female syllables sung during its duets are indicated by the colored bars at the top of each spectrogram. Green bars indicate the (A) to (B) transition, orange (B) to (D), and yellow (D) to (A). The within-motif intervals, indicated by the green and orange bars, were either identical or longer in duration in the solitary song. In Fig. 1B, the mean intersyllable intervals between male syllables during duets are indicated with green bars, (b) to (d), and yellow bars (d) to (b). As in females, within-motif intervals, indicated by the green bars, are longer during solitary singing.

These results suggest that heterogenous acoustic cues modulate the motor program for singing on a syllable-by-syllable basis (*15*) and that these sensory cues affect at least the duration and variability of intersyllable intervals. These behavioral data therefore also suggest that the nervous system is not using a fixed-action pattern to generate duet song but relies on a unique combination of sensory feedback from both autogenous and heterogenous sources.

We also found more variability in male singing than in female singing. We commonly recorded solitary songs produced by females but infrequently recorded those by males. Solitary males produced low-amplitude songs, and therefore, the field recordings may have failed to capture many male songs because of lower amplitudes. The amplitude of male syllables, however, increased significantly by about 14.5 dB

from solitary to duet singing (Fig. 1B, inset) [*t* test, $P < 0.01$, df = 20; solitary syllable amplitude 17.4 ± 11.7 (arbitrary units) and duet amplitude 534.75 ± 52.85]. In contrast, females did not change syllable amplitudes [amplitude increase 0.13 dB, *t* test, $P = 0.86$, df = 20; solitary amplitude 565.9 ± 203.3 (arbitrary units) and duet amplitude 582.1 ± 219.1]. During duets, male syllable amplitudes were not significantly different from female syllables (*t* test, $P = 0.46$, df = 20), as can be seen in oscillograms in Figs. 1C and 2.

Male variability was also evident in failures of the male wren to produce its syllables within motifs in the middle of longer duet sequences (Fig. 1C). During these omissions, females continued singing and lengthened intersyllable intervals (mode increase 61 ms, $n = 44$ song samples). These rapid modulations of singing in the middle of duets provide additional evidence that the birds are not relying on fixed-action patterns in the brain to generate duet song. Also, the sex differences observed in singing indicate that females and males share similar, but not identical, mechanisms for cooperative production of duets. Further, these data suggest an interesting hypothesis, that female plain-tailed wrens may provide the "leading" cues for duet singing (*16*, *17*). Neurophysiological evidence may support this idea (below), but future experiments with manipulations of acoustic cues in playbacks are needed (*17*). Nevertheless, these data demonstrate moment-to-moment coordination of feedback arising from both partners during duet singing in plain-tailed wrens.

To examine how cooperative duet singing is encoded in cortical circuits, we captured birds and performed neurophysiological experiments. We made extracellular recordings in HVC, a song control nucleus (*18*, *19*), for up to 30 hours each in three female and three male wrens. Before capture, we recorded duets from each individual used in neurophysiological experiments, as HVC activity has been shown to be selective for the acoustic parameters of autogenous song elements (*20*–*24*). We isolated 75 "units" from 42 recording sites (see Materials and Methods for details). Stimuli included duets and a series of manipulations of duet motifs, including isolated autogenous and heterogenous syllables from the duet, time reversals of the entire duet and subsets of syllables, a duet in which the syllables were presented in reverse order, and other ad hoc temporal manipulations.

The majority of units in both females and males responded best to the duet song over all stimuli tested [85 and 87% for *Z* score and response strength measures (*25*), respectively, $n = 61$] (Fig. 2). The mean *Z* score for duets in females was 2.3 ± 0.83 (range 0.8 to 3.9, $n = 21$) and in males 1.3 ± 0.64 (range 0.2 to 3.0, $n = 40$). The distribution of response strengths (range 0.9 to 15.1 spikes/s) to duet songs for both females and males can be seen in Fig. 3A. The responses to duet stimuli were not simply a sum of responses to female and male syllables, because, in the majority of neurons, response strengths elicited by duet stimuli (7.9 ± 6.4 spikes/s in females, $n = 21$; 6.1 ± 3.7 in males, $n = 40$) were significantly greater (paired *t* tests: females, $P < 0.01$, $n = 21$; males, $P < 0.01$, $n = 40$) than the sum of the response strengths to female and male syllables presented alone (6.3 ± 4.7 spikes/s in females, $n = 21$; and 4.4 ± 2.8 males, $n = 40$) (Fig. 3, A and B).

Further, we calculated d' values (*26*) for each unit, comparing responses to duet stimuli with (i) the sum of the responses to the male and female syllables presented alone, (ii) the response to female syllables, and (iii) the responses to male syllables alone. The d' values express the ability to discriminate between two stimuli, and in the comparisons shown here, d' values greater than 0.5 indicate the selectivity of a neuron for the duet song over the other stimulus (*26*). Data for each unit in Fig. 3A are plotted with a symbol that represents the d' measures that are greater than 0.5 for multiple comparisons. Most units (36 out of 61) responded selectively to the duet song when compared with the sum of the responses to the male and female syllables presented alone (filled circles), whereas only one unit did not respond preferentially to the duet song relative to each of the other stimuli (open circle). Eleven units preferred duet songs over male syllables only (horizontal bar), 3 units preferred duet over female syllables only (vertical bar), and 10 units preferred duet over both male and female syllables alone (cross) but not the sum (female + male).

As female and male syllables were extracted unaltered from the duet, these stimuli had identical acoustic content, and therefore, it is the rapid alternation of female and male syllables in the duet that led to facilitated responses. In sum, both response strength and d' measures indicate that a majority of HVC units exhibited facilitated responses to the combined duet performance, rather than responding best to each individual's own contribution to duets.

We nevertheless found that both female and male syllables often elicited responses from HVC

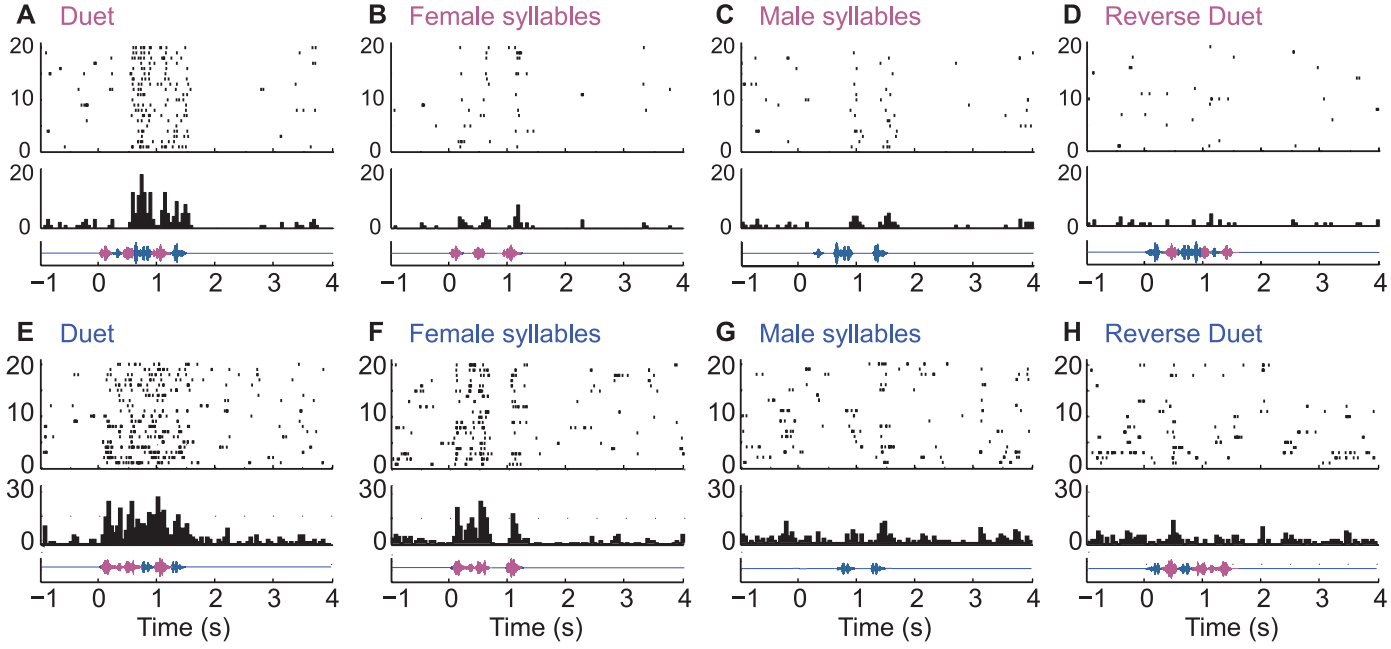

Fig. 2. Example responses in HVC to song stimuli. Responses recorded in a female (**A** to **D**) and a male (**E** to **H**) wren. Bottom in each panel shows the stimulus oscillogram, top are raster plots showing the times of spikes for 20 stimulus repetitions, and middle is a histogram (50-ms bins) of the activity. Magenta indicates syllables produced by the female, blue by the male. Stimuli were (A) and (E) duet song, (B) and (F) female syllables, (C) and (G) male syllables, and (D) and (H) reverse duet song. The female recording shown here had a strong preference for duet song over all other stimuli tested. The male recording shown here responded best to the duet song but, nonetheless, had a particularly strong response to the female syllables.

units in both female and male wrens. If these responses were related to the autogenous motor output, we would expect that female syllables would elicit stronger responses in female HVC than male syllables and vice versa for neurons in males. It is interesting that, in both females and males, the female syllables elicited significantly stronger responses than did the male syllables (Fig. 3) (paired t tests, females $P < 0.01$, $n = 21$ and males $P < 0.01$, $n = 40$). This is particularly surprising in males, because these responses are contrary to previous results obtained in several songbird species (*22*, *23*, *27*). Stronger responses to the conspecific partner in male wrens may be an adaptation for duet singing in these birds. The preference for female syllables in both female and male partners may be a neurophysiological correlate of the possible role of females in leading duet singing.

That HVC neurons respond consistently to partner syllables is itself a surprising result, as previous studies showed that conspecific vocalizations generally elicit weak responses in HVC (*20*, *21*). Indeed, such responses do not fit with current models of HVC function developed for nonduetting species, in which selective responses to autogenous stimuli in HVC are used to modulate motor programs (*27*, *28*). The responses in both sexes to the partner syllables support behavioral observations that both sexes adhere to "duet codes" that are influenced by the partner's previous syllables (*15*).

The question remains, how are temporal interactions between females and males for the cooperative production of duets encoded? To examine this issue, we altered the timings between syllables. For example, we removed the intersyllable intervals in stimuli composed of only female or only male syllable sequences. Even though the syllables are identical in these stimuli, sequences with naturally occurring intersyllable intervals elicited significantly stronger responses than stimuli without these intervals (Fig. 4) (paired t tests: females, $P < 0.01$, $n = 21$; males, $P < 0.01$, $n = 40$). This finding differs from previous results in which temporal combination–sensitive HVC neurons in nonduetting species were not sensitive to durations of intersyllable intervals (*29*). This sensitivity to intersyllable intervals is likely another adaptation of the song circuit for the coordination of duet singing.

The sensitivity to intersyllable intervals requires long-term, on the order of hundreds of milliseconds, integration of information. If these responses are produced by sensory information alone, a possible cellular strategy could use a combination of slow inhibition coupled with rapid excitation, as has been seen in "counting neurons" in amphibians (*30*). If these responses reflect underlying motor programs (*10*), it would suggest that sensory stimuli are activating and/or modulating a form of central pattern generator (CPG) (*11*, *13*). Indeed, the activation of a CPG could explain the structure of solitary songs, in which appropriate syllable sequences and intervals are retained.

The combination of mechanisms that we observed in plain-tailed wrens may be used to control cooperative behaviors across taxa. Consider, for example, two people cooperating to dance a tango. Certainly each person knows his or her own part of the dance and possibly the partner's contribution, but these data suggest that premotor circuits in both individuals preferentially encode the combined cooperative behavior. Further,

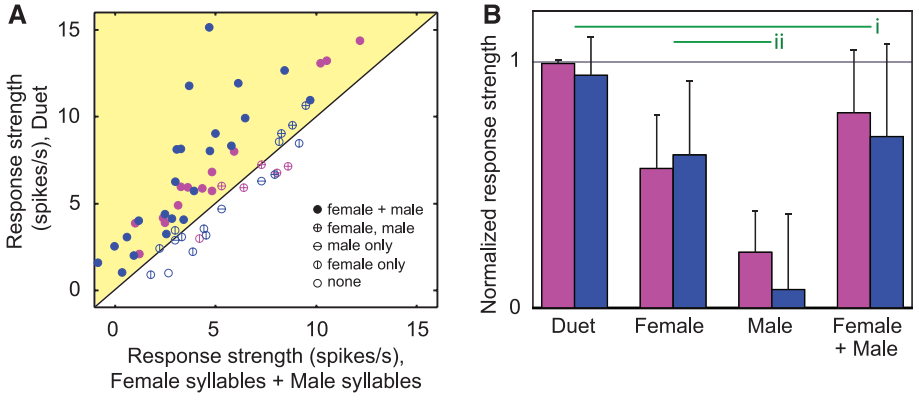

Fig. 3. HVC neurons in both females and males exhibit facilitated responses to the duet song. (**A**) Response strength of 20 female units (magenta) and 40 male units (blue) to the duet song (ordinate) plotted against the sum of response strengths to the separate female and male components of the duet song (abscissa). Data above the identity line, in the yellow region, indicate superlinear responses to the duet than the sum of responses to female and male syllables presented alone. Filled circles (●) indicate that the d' value (see text) for the comparison between the duet and the sum of the responses to the male and female syllables was > 0.5. Horizontal bar (⊖) indicates d' values > 0.5 for the comparison between responses to the duet and male syllables and vertical bar (⊕) for female syllables. A cross (⊕) indicates d' > 0.5 for both female and male syllables, but not female + male. Open circles (○) indicate d' values of < 0.5 in all comparisons of the duet to other stimuli. (**B**) Responses for female and male units normalized to the strongest response from each unit. Duet songs elicited responses that were significantly stronger (paired t tests, $P < 0.01$; females, $n = 21$; males, $n = 40$) than responses to female, male, and summed (female + male) responses (the significant difference between duet and female + male responses are indicated by the green line labeled "i"). In addition, in both female and male wrens, the female syllables elicited significantly stronger responses (paired t tests, $P < 0.01$; females, $n = 21$; males, $n = 40$) than male syllables (indicated by the green line labeled "ii"). Bars indicate means, error bars indicate standard deviations.

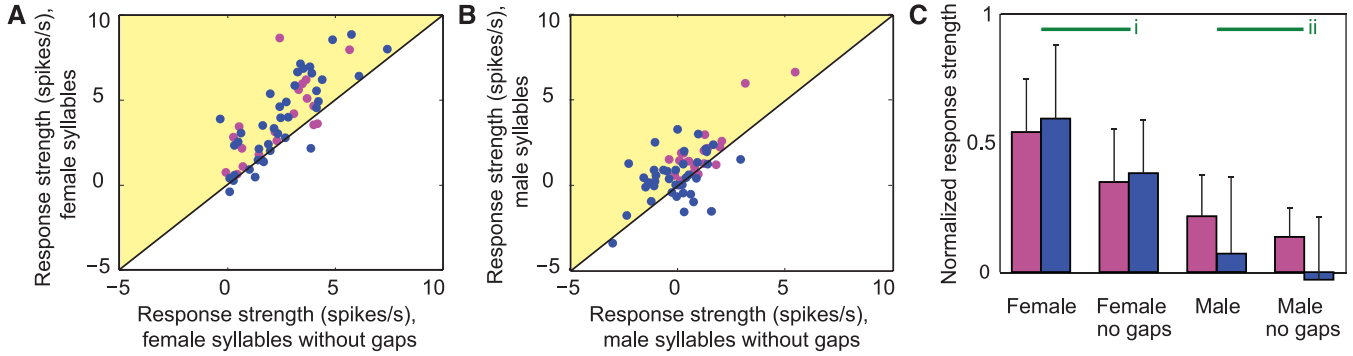

Fig. 4. Neurons in HVC require appropriate intersyllable intervals. (**A**) Response strengths (spikes/s) to unaltered female syllables (ordinate) versus response strengths to the same syllables, but with the intersyllable intervals removed (abscissa). Blue dots are units recorded in males ($n = 40$) and magenta in females ($n = 21$). Data points in the yellow shaded region indicate that the unaltered syllable sequence elicited a greater response than the same syllables without intersyllable intervals. (**B**) Same as (A) but for male syllables. (**C**) Normalized responses as in Fig. 2B. Removal of intersyllable intervals from either female or male syllable sequences resulted in significantly reduced response strengths in both male and female units (green lines, paired t tests, $P < 0.01$; female syllables, green line "i"; male syllables, "ii"). Bars indicate means, error bars indicate standard deviations.

information from the leader may be preferentially represented in the brains of both individuals. Finally, coordination of timing during cooperation is likely mediated by interactions between CPGs and both autogenous and heterogenous sensory information.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/334/6056/666/DC1
Materials and Methods
Fig. S1
References
Audio Clips S1 to S5
Movies S1 and S2

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Drosophila Microbiome Modulates Host Developmental and Metabolic Homeostasis via Insulin Signaling

Seung Chul Shin,[1,3]*† Sung-Hee Kim,[1]† Hyejin You,[1,2] Boram Kim,[1,2] Aeri C. Kim,[1,2] Kyung-Ah Lee,[1] Joo-Heon Yoon,[3] Ji-Hwan Ryu,[3] Won-Jae Lee[1]‡

The symbiotic microbiota profoundly affect many aspects of host physiology; however, the molecular mechanisms underlying host-microbe cross-talk are largely unknown. Here, we show that the pyrroloquinoline quinone–dependent alcohol dehydrogenase (PQQ-ADH) activity of a commensal bacterium, *Acetobacter pomorum*, modulates insulin/insulin-like growth factor signaling (IIS) in *Drosophila* to regulate host homeostatic programs controlling developmental rate, body size, energy metabolism, and intestinal stem cell activity. Germ-free animals monoassociated with *PQQ-ADH* mutant bacteria displayed severe deregulation of developmental and metabolic homeostasis. Importantly, these defects were reversed by enhancing host IIS or by supplementing the diet with acetic acid, the metabolic product of PQQ-ADH.

All metazoans harbor substantial numbers of commensal microorganisms in the gut. It has been well established that commensal bacteria have positive impacts across a wide range of host physiology, including regulation of immunity and metabolism (*1*–*3*). Recent progress toward understanding gut-microbe interactions using *Drosophila* revealed that a fine-tuned regulation of gut immunity is required for the preservation of a healthy commensal community structure to promote host fitness and ensure normal host survival rates (*4*). Furthermore, the indigenous gut microbiota also controls the normal turnover rate of gut epithelial cells by regulating intestinal stem cell activity (*5*).

Recently, it has been shown that the normal microflora is deeply involved in the energy balance and metabolic homeostasis of host animals (*6*–*9*). However, our current understanding of the impact of gut microbiota on host physiology is descriptive, due in part to technical difficulties associated with in-depth integrated genetic analysis of both the microbes and the host. To overcome these limitations, we used the combination of *Drosophila* and its commensal *Acetobacter* as a model of host-microbe interaction to enable us to perform a simultaneous genetic analysis of both host and microbe in vivo.

To observe the systemic effects of the symbiotic commensal community on the host, we first examined host growth rate and body size in the presence and absence of the commensal microflora by generating conventionally reared and germ-free animals (*10*). In conventionally reared fly larvae, the time to develop into a puparium was <7 days; it lengthened to ~9 days in germ-free larvae when they fed on the axenic standard cornmeal diet (Fig. 1A). Interestingly, the effect of commensal bacteria on host development was more pronounced when the amount of yeast in the diet was reduced (Fig. 1A and fig. S1). Most notably, conventionally reared larvae developed into puparia in ~9 days, whereas germ-free larvae died at first instar if fed a diet containing <0.1% yeast or if yeast was substituted by casamino acids (Fig. 1A and fig. S1). Casamino acids were found to be essential nutrients for host growth in the absence of yeast. Under these conditions, germ-free larvae had a body size <10% of corresponding conventionally reared larvae 120 hours after egg laying (Fig. 1A and fig. S1). At this time point, the effect of the microbiota on host growth was most pronounced. These results indicate that commensal microbiota is able to influence the systemic development of *Drosophila* by affecting both growth rate and body size.

All metazoan guts harbor complex commensal communities: hundreds of species are present in humans (*11*). In *Drosophila*, the adult midgut is typically in stable contact with a symbiotic commensal community composed of 5 to 20 different microbial species that consist primarily of members of the *Acetobacter* and *Lactobacillus* genera (*12*–*14*). We found that the midgut of laboratory-reared *Drosophila* harbors five major commensal bacterial species, *Commensalibacter intestini*, *Acetobacter pomorum*, *Gluconobacter morbifer*, *Lactobacillus plantarum*, and *Lactobacillus brevis* (*12*, *15*). Taking advantage of

[1]School of Biological Science, Seoul National University and National Creative Research Initiative Center for Symbiosystem, Seoul 151-742, South Korea. [2]Department of Bioinspired Science and Division of Life and Pharmaceutical Science, Ewha Woman's University, Seoul 120-750, South Korea. [3]Research Center for Human Natural Defense System, Yonsei University College of Medicine, CPO Box 8044 Seoul, South Korea.

*Present address: Korea Polar Research Institute, Incheon 406-840, South Korea.
†These authors contributed equally to this work.
‡To whom correspondence should be addressed. E-mail: lwj@snu.ac.kr