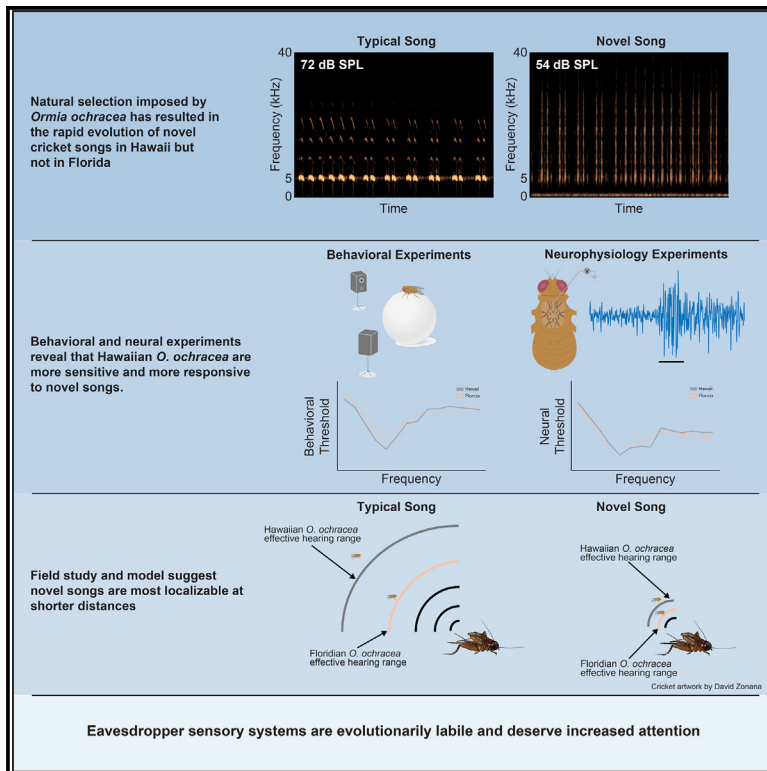# Current Biology

Neural and behavioral evolution in an eavesdropper with a rapidly evolving host

Graphical abstract

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In brief

Selection by the eavesdropping parasitoid *Ormia ochracea* has driven the rapid evolution of host cricket songs in Hawaii. Wikle et al. show that Hawaiian flies have evolved to better detect and respond to novel song types than flies from elsewhere, highlighting the evolutionary lability of eavesdroppers and their influence on animal communication.

Highlights

- Parasitism by *Ormia ochracea* favors cryptic novel cricket songs in Hawaii (HI)

- HI flies evolved more sensitive neural and behavioral responses to novel host songs

- Field study and model suggest novel songs are most localizable at shorter distances

- Eavesdropper sensory systems are evolutionarily labile and deserve attention

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Article

Neural and behavioral evolution in an eavesdropper with a rapidly evolving host

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SUMMARY

The diversification of animal communication systems is driven by the interacting effects of signalers, signal receivers, and the environment. Yet, the critical role of unintended receivers, like eavesdropping enemies, has been underappreciated. Furthermore, contemporary evolution of animal signals is rare, making it difficult to directly observe this process. Ormiine parasitoid flies rely exclusively on acoustic cues to locate singing male orthopteran hosts. In Hawaii, selection imposed by *Ormia ochracea* has led to recent and rapid diversification of their local host crickets' song. We use complementary lab and field experiments to understand how receiver psychology (sensory and cognitive mechanisms) evolves to accommodate a new host and the evolution of that host's signal. Receiver psychology is critical to our understanding of host-parasite coevolution and animal communication, as the sensory system establishes the limits of behavioral responses that exert selection on signals. We demonstrate that the neural auditory tuning and behavior of *O. ochracea* have evolved in Hawaii, and these differences likely facilitate the detection of novel host songs. Further, the recently evolved songs are highly variable among males, and flies prefer novel songs with particular spectral characteristics, enabling us to predict how eavesdroppers may shape host song evolution. To our knowledge, this is the first evidence for rapid evolution in the sensory tuning of an eavesdropper. Our work links the evolution of sensory systems, signals, and behavior, heeding the recent call for better integration of sensory and cognitive mechanisms of receivers into our understanding of the evolution of animal communication.

INTRODUCTION

Communication is fundamental to survival and reproduction, and the evolution of communication systems often plays a key role in diversification.[1–4] While studies frequently consider how signal evolution is shaped by conspecific intended receivers (e.g., mates and competitors), selection imposed by unintended receivers is also critical but often overlooked.[5–7] Unintended receivers, or eavesdroppers, intercept signals intended for other individuals,[8] co-opting them to locate hosts and prey across a diverse set of taxonomic groups and modalities of communication (e.g., Zuk and Kolluru,[5] Bernal and Page,[6] and Reichert et al.[7]). It is crucial to integrate the perspective of eavesdroppers into our understanding of the evolution of animal communication, as they are ubiquitous and often kill the unsuspecting signalers they locate, thus exerting strong selection on animal

signals.[9–11] And, like intended receivers, they must detect and perceive signals, extract information from them, and act upon that information, leading to congruence between a signal's properties and the sensory capabilities of the receiver (e.g., matched filter hypothesis).[5,12–16]

In this paper, we capture how the receiver psychology[17] of unintended receivers influences animal communication and host-parasite interactions by capitalizing on a near-ideal study system in which an eavesdropping natural enemy has adopted a new host species that is now evolving in response. Many have called for such integration of the underlying sensory and cognitive mechanisms of receivers into our understanding of the evolution of animal communication (e.g., Rosenthal[18] and Römer[19]), as the sensory system defines the limits of behavioral responses that ultimately act on signals (selection), thus shaping intra- and inter-specific communication.[5,20] The influential fields of receiver

psychology,[21] sensory drive,[22] and sensory bias[23,24] tend to assume that the sensory mechanisms of receivers are rather conserved, either because of the nervous system or environmental constraints. If receiver sensory systems are instead highly evolutionarily labile, this could change dynamics of coevolution between signalers and receivers, altering our predictions and even our research approaches. Pioneering work linking mechanisms to the evolution of animal communication has revealed much about how animal signal evolution depends on receiver sensitivities (e.g., Capranica et al.,[25] Boughman,[26] and Lee et al.[27]), but the overwhelming majority of that work considers intended receivers. Almost nothing is known about the role of unintended receivers when signals change, as opportunities to directly observe the contemporary evolution of animal signals are rare.[28,29] Here, we use complementary behavioral and neural experiments to ask how an acoustic eavesdropping parasitoid fly, *Ormia ochracea*, fares when invading a new habitat where they must adopt a new host species with rapidly evolving signals.

Female *O. ochracea* are obligate parasitoids that rely exclusively on acoustic cues to locate singing male cricket hosts.[30–32] Across their geographic range, *O. ochracea* parasitize numerous cricket species.[33–35] Host species recognition begins with the ability of the auditory system to detect the frequency content of an auditory input.[36] After signal detection, similar to crickets,[37] these flies use song temporal pattern recognition to identify a preferred host.[34,38–41] The auditory system of the fly is most sensitive to sound frequencies between 3–6 kHz, aligning with the dominant frequency of most cricket songs[41,42]; notably, though, neural audiograms have only been established in Floridian *O. ochracea*.[43,44]

O. ochracea was introduced to Hawaii sometime prior to 1989, where none of its known continental US hosts are found.[45] Despite that, the fly was able to persist within Hawaii by adopting a new host, the Pacific field cricket (*Teleogryllus oceanicus*), which sings a loud, nearly pure tone ~4.8 kHz calling song (hereafter "typical song," Bennet-Clark[46] and Bennet-Clark[47]). Intriguingly, selection imposed by the eavesdropping parasitoid fly is thought to be responsible for the recent and remarkable evolution of several cricket morphs that produce unique, attenuated songs (hereafter novel songs; Tinghitella et al.,[48] Rayner et al.,[49] and Gallagher et al.[50]), and such morphs are increasing in abundance within populations and rapidly spreading across the Hawaiian archipelago.[51,52] Critically, the novel songs have different acoustic properties than those of all known *O. ochracea* hosts (e.g., frequency, amplitude, and bandwidth; Figure 1), which should render the new male morphs more protected from the parasitoid—unless the parasitoid responds in kind.

Perhaps surprisingly, we previously found that Hawaiian *O. ochracea* can locate novel songs in the field, albeit at much lower rates than typical song.[50,51,54] How does *O. ochracea* accomplish this feat? Has the receiver psychology (sensory capabilities and behavior) evolved in Hawaiian flies (Figure 1)?

We used a complementary set of lab and field approaches to compare the neural and behavioral responses of derived Hawaiian and ancestral Floridian *O. ochracea* in order to understand how the flies have overcome the dual dilemmas of using a new host species and that host's signal evolving rapidly. Two novel

songs in particular, purring and rattling, arose within the past ~5–10 years and have dominant frequencies that fall well outside the range of that of North American hosts (purring = 9.2 kHz, rattling = 5.8 kHz; Gallagher et al.[50]). While dominant frequency is an essential host-recognition feature for *O. ochracea* across their range,[41,42] the novel *T. oceanicus* songs also vary along numerous other spectral axes (e.g., bandwidth and amplitude; Figure 1B) that should impact host-parasite interactions by making it more difficult for flies to locate host crickets. We therefore coupled pure-tone frequency playback experiments with playback of natural songs to capture responses to the many important axes of spectral variation in novel songs. First, to investigate auditory sensitivity (frequency tuning), we obtained auditory-evoked multi-unit neural recordings from the central nervous system in response to pure tones that vary in frequency and sound intensity. We then used a high-speed treadmill system coupled with a psychoacoustic adaptive tracking technique (modeled after Lee and Mason[38] and Bee and Schwartz[55]) to estimate behavioral response thresholds to pure tones with varied frequencies. Finally, we used playback of recorded natural purring and rattling songs in the lab and in the field where crickets and flies naturally co-occur. Our experimental approach links the evolution of sensory systems, signals, and behavior, which together shape animal communication.[22]

Multiple lines of evidence support our primary result that there are rapidly evolved differences in the neural auditory tuning and behavioral responses of Hawaiian flies that are likely facilitating the detection of the novel host songs. Hawaiian flies have evolved differences in their sensory tuning relative to flies in the ancestral Floridian range, particularly at frequencies important to host localization. Hawaiian flies were far more responsive across the board to novel host songs (purring, rattling) than the ancestral mainland fly population and expressed preferences for particular purring and rattling variants. Because we used a common garden rearing design, we demonstrate that both of these findings are genetically based. Collectively, we show the first evidence for intraspecific changes in the sensory system and behavior of an eavesdropper associated with changes in host species and the evolution of novel signals.

RESULTS

Neural audiograms (neural response thresholds)
To test for evolved differences in the overall peripheral auditory sensitivity of Hawaiian and Floridian *O. ochracea*, we recorded extracellular multi-unit neural activity from the neck connective of flies reared in a common garden. The neck connective contains auditory neurons that relay auditory input to the brain. We tested responses to pure tones that varied in frequency (2–40.3 kHz) and sound intensity (20–90 dB SPL re. 20 μPa). As expected, the frequency content of auditory stimuli strongly affected the neural response thresholds of *O. ochracea* (N = 48; type III Wald chi-square; frequency: x^2 = 1,300.65, df = 3, p < 0.0001; Figure 2A; Table S1). In models testing neural and behavioral outcomes, finding main effects of population or the interaction of population and frequency would support our central hypothesis that there are evolved differences in the auditory tuning and phonotaxis behavior of Hawaiian flies relative to
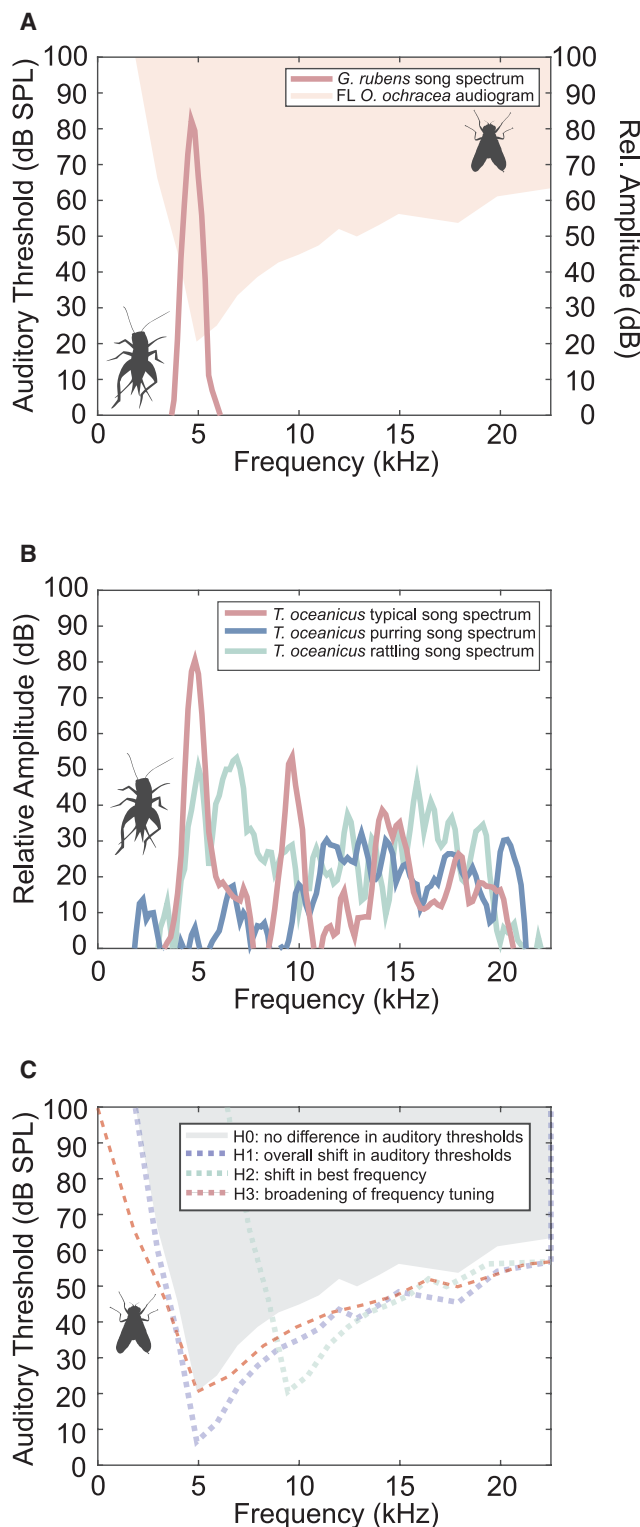
Figure 1. *Ormia ochracea* hearing and the evolution of novel host songs

(A) Our understanding of *O. ochracea* hearing comes from populations in Florida where flies parasitize *Gryllus rubens*, which produces a simple song with dominant frequency of 5 kHz (red line and corresponding y axis on the right, Robert et al.[43]). The filled light-pink audiogram shows the amplitudes at

ancestral Floridian flies. A population effect indicates that overall responsiveness has evolved (Figure 1C H1), while an interaction indicates that the shape of the function has evolved (Figure 1C H2 and H3). The shape of the fly's neural frequency tuning curves differed across populations (frequency x population: $x^2 = 14.55$, $df = 3$, $p = 0.0022$), but the responsiveness of Hawaiian and Floridian flies did not differ (population: $x^2 = 1.05$, $df = 1$, $p = 0.3057$; Table S1; Figure 2A).

We then identified the particular frequencies at which Hawaiian and Floridian flies differed in neural response thresholds (using a second linear mixed model [LMM] with the 14 frequencies treated categorically), finding that they diverge primarily at frequencies relevant to the host in Hawaii (type III Wald chi-square; frequency x population: $x^2 = 63.14$, $df = 13$, $p < 0.0001$; Table S1; also see Table S1 for full list of contrasts). The neural response thresholds of Hawaiian flies were on average 6.5 dB lower to the 5 kHz tone (mean ± SE; Hawaii: 29.77 ± 0.79 dB SPL; Florida: 36.59 ± 1.08 dB SPL) and 6.8 dB lower to the 10.1 kHz tone (Hawaii: 36.82 ± 1.31 dB SPL; Florida: 43.64 ± 2.22 dB SPL) than the thresholds of Florida flies (Benjamin-Hochberg contrasts of estimated marginal means; 5.0 kHz: estimate = 6.59, t-ratio = 3.57, $p = 0.0024$; 10.1 kHz: estimate = 6.82, t-ratio = 3.7, $p = 0.0024$; Table S1). These frequencies align with typical (~4.8 kHz) and purring (~9.2 kHz) *T. oceanicus* songs, respectively. Intriguingly, Floridian flies possess lower response thresholds at the two highest ultrasonic frequencies, 32.0 and 40.3 kHz (Table S1).
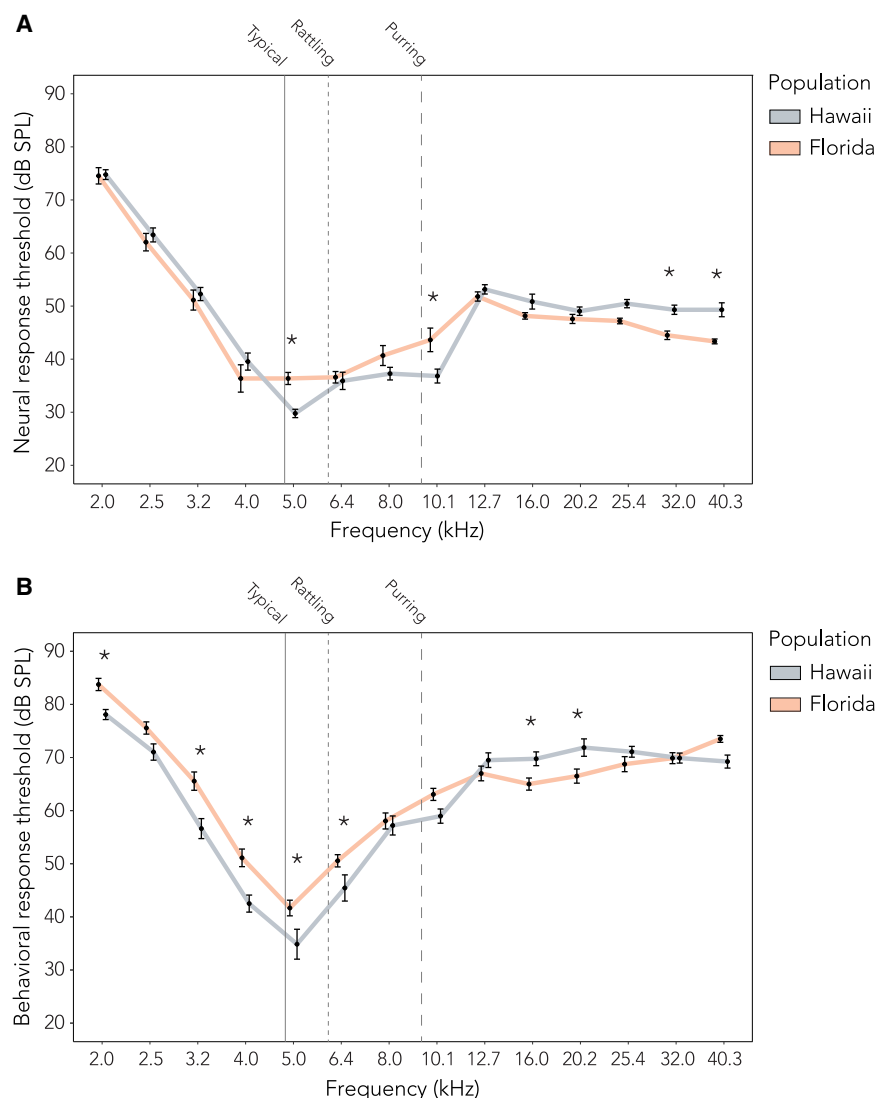
Behavioral audiograms (behavioral response thresholds)

To test for evolved differences in the behavioral response thresholds of Hawaiian and Floridian *O. ochracea* to different frequencies, we collected walking phonotactic responses using a spherical treadmill system[56] in the lab under common garden. Behavioral audiograms to pure-tone synthetic host calling songs were estimated using an adaptive tracking psychoacoustic approach.[38,55] As with the neural response thresholds, the behavioral response thresholds of *O. ochracea* were strongly affected by the frequency content of the stimuli (type III Wald chi-square; frequency: $x^2 = 924.99$, $df = 3$, $p < 0.0001$; Figure 2B; Table S2). The shape of the behavioral audiograms differed between populations (frequency x population: $x^2 = 31.82$, $df = 3$, $p < 0.0001$), and the Hawaiian

which flies in Florida can detect each frequency on the x axis (neural response thresholds; Robert et al.[43]). Auditory tuning of Florida *O. ochracea* peaks at 5 kHz, matching *G. rubens* song.

(B) In Hawaii, *O. ochracea* must locate a new host song (red line = typical *T. oceanicus* song) and are also exposed to the recently evolved purring (blue) and rattling (green) *T. oceanicus* host songs. Purring and rattling songs are highly variable among individuals but, on average, are higher in dominant frequency, more broadband, and quieter than typical *T. oceanicus* song.[48,50]

(C) When we measure receiver psychology (neural auditory and behavioral response thresholds) in Hawaiian *O. ochracea*, there are numerous possible outcomes (hypothetical auditory response thresholds shown here). There may be no evolved differences between populations (H0), responsiveness (elevation of the threshold line; Kilmer et al.[53]) could differ between populations (H1 shows one possible shift to increased responsiveness), peak auditory tuning may shift (H2 reflects a shift to the right toward higher peak frequency), and/or the tolerance (width of the preference function at a given elevation; Kilmer et al.[53]) could shift (H3 shows increased tolerance). Behavioral thresholds may evolve independently of neural thresholds.

A

B

Figure 2. Neural and behavioral response thresholds of Hawaiian and Floridian *O. ochracea* differ at frequencies relevant for host detection

(A) Neural response thresholds of Hawaiian flies were lower than those of ancestral Floridian flies at 5 and 10.1 kHz, the frequencies nearest the average dominant frequency of typical (4.8 kHz: solid gray vertical line) and purring (9.2 kHz: far right dashed vertical line) *T. oceanicus* songs. Floridian flies possess lower neural thresholds than Hawaiian flies at the two highest ultrasound frequencies.

(B) Behavioral response thresholds of Hawaiian flies were lower than those of Floridian flies at five lower-end frequencies, whereas Floridian flies possess lower response thresholds at two higher-end frequencies. Points and whiskers represent means ± SE and are jittered. Significant differences indicated by *.

See also Figure S2 and Tables S1 and S2.

Behavioral responses to novel host songs

To characterize responses of Hawaiian and Floridian *O. ochracea* to the continuous variation in acoustic features underlying the novel purring and rattling calling songs (beyond frequency), we collected behavioral responses from *O. ochracea* performing tethered walking phonotaxis on the spherical treadmill system to representative naturally occurring purring, rattling, and typical *T. oceanicus* exemplars. We first examined whether flies respond differently to typical, rattling, and purring exemplars by pooling behavioral responses to five exemplars of each song type. We used two approaches: first, we limited our observations to those

population was more responsive overall than the Florida population (population: $x^2 = 5.99$, $df = 1$, $p = 0.0144$). We then identified the particular frequencies at which the Hawaiian and Floridian flies differed in a second model with frequencies treated categorically (frequency x population: $x^2 = 56.71$, $df = 13$, $p < 0.0001$; Table S2; also see Table S2 for full list of contrasts), and the behavioral response thresholds of Hawaiian flies were on average 5.6 dB lower to the 2 kHz tone (mean ± SE; Hawaii: 78.09 ± 0.95 dB SPL vs. Florida: 83.75 ± 1.15 dB SPL), 8.6 dB lower to the 4 kHz tone (Hawaii: 42.5 ± 1.6 dB SPL vs. Florida: 51.11 ± 1.66 dB SPL), 6.8 dB lower to the 5 kHz tone (Hawaii: 34.85 ± 2.82 dB SPL vs. Florida: 41.67 ± 1.47 dB SPL), and 5.16 dB lower to the 6.4 kHz tone (Hawaii: 45.44 ± 2.47 dB SPL vs. Florida: 50.56 ± 1.15 dB SPL) than those of Floridian flies (Figure 2B; Table S2). Intriguingly, Hawaiian flies were more behaviorally responsive to frequencies aligning with typical and rattling *T. oceanicus* songs. As with the neural response thresholds, Floridian flies exhibited lower behavioral response thresholds than Hawaiian flies to some of the higher frequencies (16.0, 20.2 kHz; Table S2C).

made at realistic amplitudes (purring: 45 dB SPL; rattling: 65 dB SPL; typical: 85 dB SPL at 25 cm). Overall, a much greater proportion of Hawaiian flies responded to all three song types than did Floridian flies (Figure 3A; Table S3; type III Wald chi-square: population $x^2 = 23.46$, $df = 1$, $p < 0.0001$), particularly for the novel song types (population x song type $x^2 = 21.93$, $df = 2$, $p < 0.0001$). See Table S3 for a full list of pairwise contrasts. Second, we examined behavioral response thresholds to identify the amplitude at which flies positively respond to each morph. Hawaiian flies had substantially lower behavioral response thresholds to each natural *T. oceanicus* song type than Floridian flies (Figure 3B; Table S4; population $x^2 = 205.74$, $df = 1$, $p < 0.0001$; population x song type $x^2 = 47.68$, $df = 2$, $p < 0.0001$). The mean behavioral response threshold of Hawaiian flies was 18.6 dB lower to purring song, 30.2 dB lower to rattling song, and 48.5 dB lower to the typical song (Figure 3B) relative to Floridian flies. Interestingly, Floridian flies possess a lower behavioral response threshold to purring song (65.6 dB SPL) than rattling (75.1 dB SPL) or typical song (77.2 dB SPL; also see Table S4 for full list of pairwise contrasts).
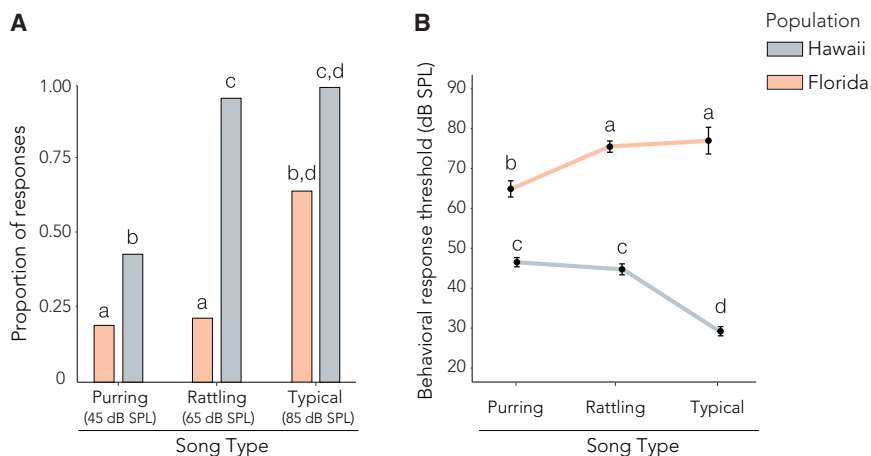
Figure 3. Hawaiian *O. ochracea* were more responsive to all three natural *T. oceanicus* song types than were Floridian *O. ochracea*

(A) A greater proportion of Hawaiian flies ($n = 17$) responded to each novel *T. oceanicus* song type than did Floridian flies ($n = 17$) when played at realistic amplitudes.

(B) Hawaiian flies also possess significantly lower behavioral response thresholds to each of the *T. oceanicus* song types.

In (A) and (B), exemplars are pooled within their respective morphs, and bars not sharing letters are significantly different from each other in post-hoc false discovery rate (FDR) tests. Points and whiskers in (B) represent mean ± SE.

See also Tables S3 and S4.

Next, we investigated whether flies exert selection differently on particular purring or rattling songs by examining variation in fly responses to the purring and rattling exemplars (not pooled within morph), which is important for understanding how novel song types will evolve in response to natural enemies. Again, we use two approaches, first looking at the proportion of flies that responded to each exemplar when played at realistic amplitudes and second identifying behavioral response thresholds. Purring exemplars differed in their attractiveness (type III Wald chi-square: exemplar $x^2 = 16.99$, $df = 4$, $p = 0.0019$), and a greater proportion of Hawaiian flies responded to purring songs played at realistic amplitudes than did Floridian flies (population $x^2 = 8.71$, $df = 1$, $p = 0.0032$; Figure 4A; Table S5). The populations did not differ in their relative preferences for purring songs (population x purring exemplar $x^2 = 2.85$, $df = 4$, $p = 0.5831$; Figure 4A; Table S5). Ratting exemplars did not differ from one another in their attractiveness when broadcast at biologically realistic amplitudes (type III Wald chi-square: exemplar $x^2 = 0.96$, $df = 4$, $p = 0.9160$); however, a much greater proportion of Hawaiian flies responded to rattling songs than did Floridian flies (population: $x^2 = 48.27$, $df = 1$, $p < 0.0001$; Table S5; Figure 4C). Again, the populations did not differ in their relative preferences for rattling songs (population × rattling exemplar $x^2 = 5.99$, $df = 1$, $p = 0.1996$; Figure 4C; Table S5). Note that there were some visible differences in response rates across purring and rattling exemplars, which may indicate these tests were underpowered, prompting further investigation with a more sensitive approach that used continuous behavioral threshold responses to purring and rattling song variation (described below).

We characterized behavioral responses to the continuous acoustic characteristics underlying the purring and rattling exemplars by generating fitness surfaces for purring and rattling with respect to the first two principal components (PCs) from a principal-component analysis (PCA) on numerous measured characteristics for each song type (Table S6; Figures 4 and S1). We found that Hawaiian flies not only possess lower behavioral response thresholds to nearly all purring song exemplars (reflected in the relative height in the of the surfaces; type III Wald chi-square: population $x^2 = 47.73$, $df = 1$, $p < 0.0001$; Figure 4B; Table S5) but are also less sensitive to variation in purring song characteristics than Floridian flies. This is reflected in the relatively flat shape of the Hawaiian purring fitness surface, as

compared with the Floridian purring fitness surface that has a clear peak (Figure 4B). Behavioral response thresholds did vary with their underlying PC1 and PC2 values (PC1 $x^2 = 29.46$, $df = 1$, $p < 0.0001$; PC2 $x^2 = 85.79$, $df = 1$, $p < 0.0001$; Table S5). For both populations, as values of PC1 (largely frequency-related characteristics; Table S6) and PC2 (largely bandwidth-related characteristics; Table S6) decreased, behavioral response thresholds generally decreased (Figure 4B), indicating that both Hawaiian and Floridian flies prefer lower frequency, less broadband purring songs (see Table S7 for PC coordinates associated with each purring exemplar). Both Hawaiian and Floridian flies exhibited the lowest behavioral response thresholds to the purring exemplar that had a dominant frequency of 8.80 kHz (exemplar C; Figure 4B). However, while both fly populations prefer lower PC1 and PC2 values, Floridian flies were over four times more sensitive to changes in PC2 values (PC2 × population: $x^2 = 32.16$, $df = 1$, $p < 0.0001$; Table S5; Hawaii estimate = 1.2, Florida estimate = 4.97) than Hawaiian flies.

When we examined the fitness surfaces for rattling song exemplars, we found nearly the opposite. While Hawaiian flies were again more responsive overall to rattling songs (type III Wald chi-square: population $x^2 = 150.51$, $df = 1$, $p < 0.0001$), they were also far more sensitive to variation in rattling song characteristics along PC1 (PC1 × population $x^2 = 8.36$, $df = 1$, $p = 0.0038$) and PC2 (PC2 × population $x^2 = 11.07$, $df = 1$, $p = 0.0009$) than Floridian flies (reflected in surface slopes/shapes; Table S5). Floridian flies exhibited nearly flat responses to variation in PC1 (largely frequency-related characteristics; Figure S1; Florida estimate = 0.63) and PC2 (largely bandwidth-related characteristics; Figure S1; Florida estimate = −0.12). Hawaiian flies exhibited lower behavioral response thresholds to rattling exemplars that had dominant frequencies (exemplars I = 4.76 kHz, F = 4.70 kHz) closest to typical *T. oceanicus* song (~4.8 kHz) (Figure 4D; Table S7).

Responses to *T. oceanicus* song variants in the field

Finally, we examined how wild Hawaiian *O. ochracea* respond at much longer distances to the purring, rattling, and ancestral *T. oceanicus* songs using fly trapping experiments in the field at one of our well-monitored field sites in Laie, Hawaii. The attraction of Hawaiian flies to 13 stimuli (the typical *T. oceanicus* song, five purring exemplars, five rattling
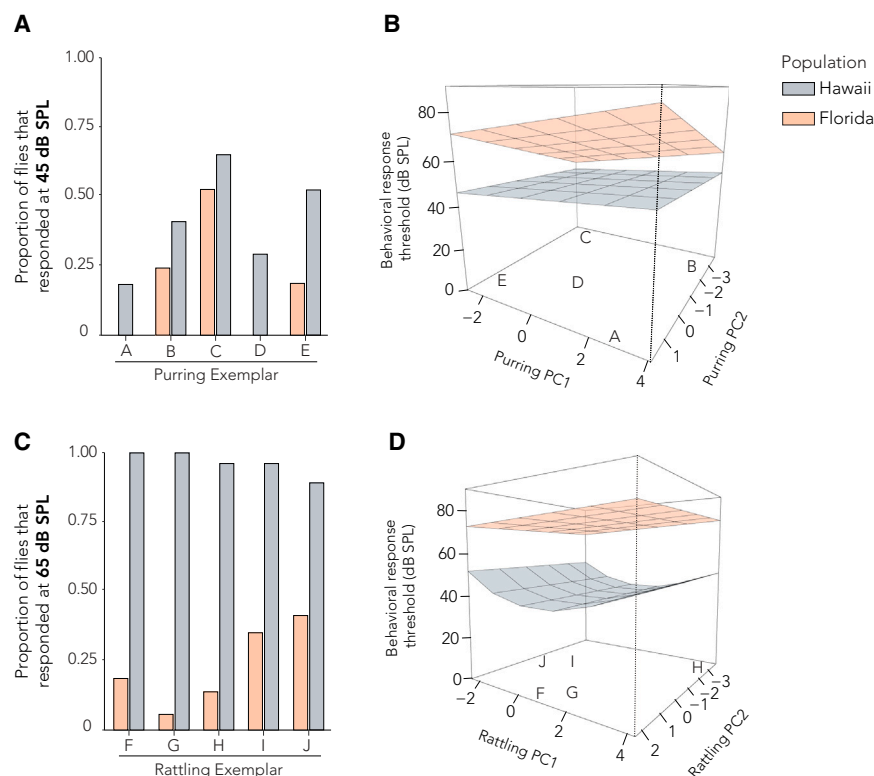
Figure 4. Hawaiian (_n_ = 17) and Floridian (_n_ = 17) flies exhibited clear differences in their behavioral preferences for purring and rattling song variants (exemplars)

(A) When broadcast at realistic amplitudes, a greater proportion of Hawaiian flies responded to the five purring exemplars (labeled A–E).

(B) Hawaiian flies also possess lower behavioral response thresholds to purring song variants than Floridian flies.

(C) When broadcast at realistic amplitudes, a much greater proportion of Hawaiian than Floridian flies responded to the five rattling exemplars (labeled F–J).

(D) Hawaiian flies also exhibited much lower behavioral response thresholds to the rattling variants than Floridian flies.

Letters on the bottom of the fitness surfaces (A–E in B and F–J in d) show PC coordinates associated with each purring and rattling exemplar, respectively.

See also Figure S1 and Tables S5–S7.

exemplars, and white noise and silent negative controls) differed dramatically when flies hunted for hosts from afar in the field (type II Wald chi-square: stimulus: x^2 = 93.13, df = 12, p = < 0.001; Figure 5). Remarkably, the novel _T. oceanicus_ songs, which evolved less than 10 years ago and differ dramatically in spectral content from the typical host song, were sufficiently detectable to some flies that they could locate simulated novel hosts from long distances in nature. Of the 37 flies we caught, 30 (81%) were caught at the typical song, 2 (5%) were caught at purring songs, and 5 (14%) were caught at rattling songs (Benjamin-Hochberg contrasts of estimated marginal means: ancestral vs. all 12 other stimuli: all p < 0.03). We found no differences in the number of flies caught at any of the purring exemplars, rattling exemplars, silent, or white noise stimuli (Benjamin-Hochberg contrasts of estimated marginal means: all p > 0.5).

To determine how auditory sensitivity translates to differences in the localizability of various song types, we developed a computational model that allowed us to estimate the distances at which Hawaiian and Floridian flies should be able to hear and thus exert selection on _T. oceanicus_ songs. This model accounts for non-frequency-dependent damping of sound with distance (following the inverse distance 1/r law), frequency-dependent attenuation due to atmospheric absorption,[57,58] data from our neural audiograms, and the absolute sound intensity levels and peak frequencies of calling songs, in order to estimate effective hearing distances for different song types. Because of the incredible variation in novel songs across many acoustic dimensions (Figure 1B), we model variation in both amplitude and peak frequency for the three song types,

estimating effective hearing distance for nine different combinations of frequency and amplitude for each song (Table S8). When mean peak frequencies and amplitudes were modeled (Figure 5B), we found significant differences in effective hearing distances across populations ($F_{(1, 60)}$ = 19.30, p < 0.001, η^2_p = 0.243), song types ($F_{(2, 60)}$ = 218.02, p < 0.001, η^2_p = 0.879), and we found that the effective hearing distances for each morph depended on fly population ($F_{(2, 60)}$ = 14.94, p < 0.001, η^2_p = 0.332). Consistent with lower capture rates among purring and rattling cricket songs in nature, estimated hearing distances for novel songs were substantially shorter than those for the typical calling song (Figure 5B).

DISCUSSION

To successfully elicit a response, animal signals need to be detected and distinguished from other sensory stimuli, making receiver psychology central to our understanding of how animal communication evolves. Without integrating the receiver psychology of unintended receivers (like eavesdropping natural enemies) into the study of animal communication, our understanding of how signals evolve is incomplete. This is because natural enemies can both act directly on signals and indirectly through impacts on intended receivers' sensory systems and signal processing, potentially generating coevolutionary relationships between signalers and unintended receivers. Here we provide multiple lines of evidence in support of our central finding that there are evolved differences in the neural auditory tuning and behavioral responses of Hawaiian flies that are likely facilitating the detection of the recently evolved, novel host songs. Rather than finding that eavesdropper sensory systems are constrained, such that pre-existing eavesdropper peak sensitivities shape novel animal signals, we find instead that eavesdropper sensory systems are highly evolutionarily labile, generating the opportunity for more complex reciprocal coevolutionary responses.
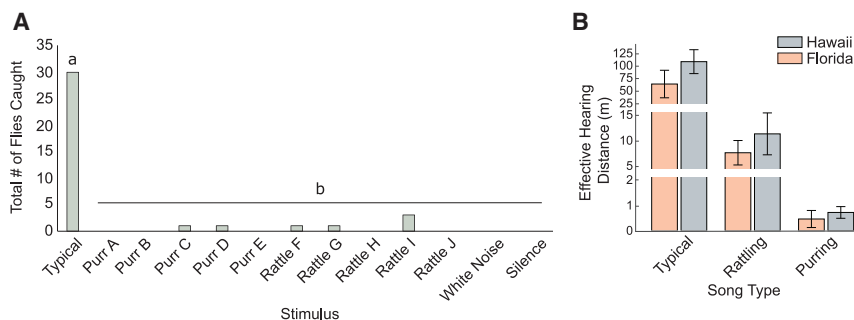
Figure 5. Field study and model suggest novel songs are localizable only at shorter distances

(A) In long-distance field playbacks of song where crickets and flies naturally co-occur in fields in Laie, Oahu (*n* = 16 replicates), Hawaiian *O. ochracea* were far more attracted to typical *T. oceanicus* song than purring and rattling song, and the latter two did not differ in attractiveness from white noise and silent controls. Letters indicate significant differences. Yet, some flies were able to detect and positively respond to traps broadcasting novel song exemplars.

(B) Estimated effective hearing distance (mean ± SD) of Floridian and Hawaiian flies for songs of the three morphs (mean peak frequencies and amplitudes are modeled for all morphs; see details in STAR Methods and Table S8). Note that the y axis on (B) is broken to facilitate ease of viewing the dramatic differences between morphs. See also Table S8.

We first documented genetically based evolved differences in the neural tuning of ancestral and derived populations of the fly (removing plasticity and learning as factors; Figure 2A). While the sensory systems of intended receivers are known to evolve with conspecific signals (e.g., female tuning to detect male signals; Capranica et al.[25] and Ryan[59]), this marks the first observation of evolution of the neural underpinnings of unintended receivers to detect hosts. Hawaiian flies possess significantly lower neural thresholds to frequencies relevant for detection of Hawaiian host songs (Figure 2A), suggesting that host signal features and receiver auditory tuning may be coevolving or that Hawaiian flies had a pre-existing bias that facilitated responding to these novel signals. Such a bias could result, for instance, from genetic drift associated with founding of Hawaiian populations; however, drift alone would be unlikely to produce the matching neural thresholds and novel song dominant frequencies we detected. Interestingly, Floridian flies exhibited lower behavioral response thresholds to several ultrasonic stimuli (Figure 2B), which could be due to coevolution with predatory bats that call in this range to detect prey in Florida[60] but are not found in Hawaii. One other compelling difference between Floridian and Hawaiian flies occurs at 5 kHz, the frequency closest to that of the Floridian host and ancestral *T. oceanicus*. Interestingly, here Hawaiian flies have a substantially lower threshold. We hypothesize that similar to the Kaneshiro effect[61,62] which proposes selection for increased responsiveness to a broad range of potential mating signals in small island populations, selection may also favor parasitoids that are more responsive to a broad range of host signals. Such selection should favor individuals that have overall higher responsiveness to a range of host song frequencies and temporal patterns.

While sensory systems define which stimuli eavesdroppers are capable of detecting and responding to, behavior (in this case, to choose or not choose a particular host) ultimately exerts selection on host signals. We found that Hawaiian flies have lower behavioral thresholds (are more responsive) to host songs with dominant frequencies that span the parasitoid's hearing range than Floridian flies (Figure 2B). Notably, Hawaiian flies were particularly phonotactic to songs played in the 3–6 kHz range, which aligns with the dominant frequencies of typical and rattling Hawaiian host crickets. There is unusually high inter-individual variation in the spectral characteristics of the novel purring and rattling songs, including dominant frequency;

purring song dominant frequencies range from ∼2–26 kHz and rattling from ∼4–8.6 kHz.[48,50] It may thus be adaptive for Hawaiian flies to be more behaviorally responsive across a wider range of frequencies than Floridian flies, whose main host, *G. rubens*, has a less variable song.

Armed with a mechanistic understanding of behavioral and neural response differences across populations, we then asked how the flies respond to their new host in Hawaii, *T. oceanicus*, and specifically to the recently evolved host songs there; this allowed us to assess the selection imposed by *O. ochracea* on novel signals. In playbacks of naturally recorded novel cricket songs, Hawaiian flies were far more responsive to the novel songs than were Floridian flies (Figure 3), supporting the idea that differences in neural auditory tuning and behavior facilitate localization of novel hosts. We then compared responses to variants (exemplars) of the purring and rattling songs, finding that Hawaiian flies prefer novel song variants with certain characteristics. Perhaps surprisingly, the shape of selection acting on purring and rattling depends on rather subtle characteristics of those signals, despite broad similarities between purring and rattling songs.[48,50] Hawaiian flies preferred rattling songs with dominant frequencies closest to that of typical *T. oceanicus* song, while they preferred purring songs with intermediate frequencies (Figure 4). Intriguingly, the shape of fitness surfaces differs between Hawaiian and Floridian flies, suggesting that they would exert very different selection on these novel signals (Figure 4). Further, Hawaiian flies responded more positively to a broader set of purring song variants than did Floridian flies, consistent with the idea above that possessing lower response thresholds across a broad range of acoustic characteristics likely improves their chances of finding a host with variable song.

We next asked if any of these evolved neural and behavioral changes in Hawaiian flies are reflected in the behavior of wild flies. Our field study revealed that despite maintaining a preference for the ancestral, typical *T. oceanicus* song over the recently evolved ones, some wild Hawaiian flies are indeed able to locate novel cricket songs, so they may exert selection on novel songs in nature. Similar to prior work in this system,[35,54,63] this finding, and its contrast with findings at short distances (Figure 4), suggests that the flies can only use these novel songs, especially purring, to find hosts at relatively close distances. Consistent with this, our model revealed that Hawaiian flies should be able to hear an average amplitude-typical song

at over 100 meters away, compared with just over 11 m for the average rattling song and ∼1 m for the average purring song (Figure 5B). This model illustrates that changes in spectral qualities (e.g., frequency, amplitude, and bandwidth) of the two novel songs result in functionally dramatic differences in how the signals are received by unintended receivers. In fact, these differences are likely even greater than we show because there is a large amount of inter-individual variation in the amplitude and frequency of males' songs within each novel morph. In Figure 5B, we show responses to average amplitude songs with average peak frequencies, but in many cases, flies would have to be much closer to purring and rattling individuals to detect them (Table S8). Further, while some Floridian flies respond positively to novel *T. oceanicus* songs (Figures 3 and 4), they would need to be ∼40 m closer than Hawaiian flies to locate a typical song, 3 m closer to locate a rattling male, and only 0.25 m closer to locate a purring song (Figure 5B). Finally, when flies did locate purring and rattling traps in the field study (Figure 5A), their song preferences aligned largely with those expressed in the lab experiments (Figures 4B and 4D), suggesting that preferences revealed in our laboratory experiments are likely to translate into selection on novel songs in nature.

Receiver psychology plays an important role in signal evolution,[21] but unintended receivers like eavesdroppers have received little attention in this context and are consequently much less well understood.[6] Our study sheds light on how signal evolution itself is impacted by evolution in eavesdroppers. Multiple lines of evidence support the idea that flies exert much stronger selection against typical than novel *T. oceanicus* morphs (Figures 2, 3, 4, and 5). Given that, we might expect for novel males that are protected from the fly to increase in commonality, since some female crickets do accept novel morphs as mates.[50,54,64,65] Indeed, repeated sampling efforts over the past ten years reveal that Hawaiian populations of *T. oceanicus* are highly dynamic, with some novel morphs becoming more abundant within populations.[51,52] The specifics of Hawaiian fly receiver psychology we uncovered here will shape how novel signals evolve within male morphs as well, especially in populations where typical *T. oceanicus* are absent. For instance, Hawaiian flies exhibited preferences for purring songs with lower frequency and bandwidth content (Figure 4B), and so this may favor individuals with higher frequency, more broadband purring songs.

Our work lays the foundation for ongoing and future work in several areas. First, Hawaiian flies co-occur with cricket populations that vary in morph composition, and some populations contain only the highly protected purring and flat-wing morphs.[48,52] Future studies should compare the neural thresholds of flies from Hawaiian populations that differ in cricket morph composition. Relatedly, similar auditory tuning work conducted in other ancestral fly populations (e.g., California; Gray et al.[34] and Dobbs et al.[66]) would reveal the extent to which the differences we find between Floridian and Hawaiian flies stem from adaptation to the acoustic host environment in Hawaii specifically, and there is likely variation in the fly's sensory system across North America, as they parasitize numerous Orthopterans therein and experienced serial bottlenecking events as their range expanded through western North America.[34] Next, we examined neural and behavioral variation in common garden,

eliminating the influences of plasticity and learning, yet fly responses are likely influenced by both. In future work, we will address experiential influences on host song recognition and preference. While dominant frequency is one of the most essential features of host detection in *O. ochracea*,[41,42] there are also strong, geographically variable preferences for particular temporal features (e.g., Gray et al.,[34] Lee et al.,[39] and Gray et al.[41]) that we plan to investigate in Hawaiian flies. Finally, while natural selection imposed by *O. ochracea* is a major selective force shaping cricket song in Hawaii, sexual selection by female crickets acts simultaneously to shape their acoustic characteristics. In ongoing work, we are testing the neuro-sensory tuning of Hawaiian crickets, as contrasting it with the neural auditory tuning of Hawaiian *O. ochracea* will help reveal how the receiver psychology of intended and unintended receivers jointly shapes animal communication.

Rapid evolution likely facilitates timely responses to global environmental change, particularly for organisms like insects with relatively short generation times (e.g., Garnas[67]), and animal communication is one of many traits greatly impacted by environmental change.[68,69] Because eavesdroppers co-opt host and prey signals and often kill unsuspecting signalers they locate, signalers coevolve with both intended receivers and eavesdroppers (e.g., Zuk and Kolluru[5] and Wagner[70]). It is thus crucial that we integrate the perspective of unintended receivers into our understanding of the evolution of animal communication.[7] This work strongly suggests that animal sensory systems may evolve at similarly rapid rates, for instance, in response to environmental change, generating the opportunity for complex reciprocal coevolutionary responses. It is likely that we do not appreciate just how common the coevolution of signals and the sensory periphery and perceptual processing of eavesdroppers really is. We hope this work illustrates the value of interrogating receiver psychology and catalyzes future work in this area.

AUTHOR CONTRIBUTIONS

A.W.W.: conceptualization (lead), methodology (lead), investigation (lead), formal analysis (lead), data curation (lead), writing – original draft (lead), writing – review & editing (equal), visualization (lead), project administration (equal), and funding acquisition (equal). E.D.B.: conceptualization (equal), investigation (equal), writing – original draft (lead), and writing – review & editing (equal). J.H.G.: conceptualization (equal), methodology (equal), writing – original draft (supporting), and writing – review & editing (equal). J.D.: investigation (equal) and writing – review & editing (supporting). M.C.: investigation (supporting) and writing – review & editing (supporting). Q.V.: investigation (supporting) and writing – review & editing (supporting). R.M.T.: conceptualization (lead), methodology (lead), formal analysis (equal), data curation (supporting), funding acquisition (lead), writing – original draft (lead), writing – review & editing (lead), visualization (equal), project administration (lead), and correspondence (lead). N.L.: conceptualization (lead), methodology (lead), software (lead), investigation (equal), formal analysis (equal), data curation (supporting), funding acquisition (equal), project administration (equal), writing – original draft (supporting), writing – review & editing (equal), and visualization (equal).

DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR★METHODS

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

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SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
NaCl	Sigma-Aldrich	S9888; CAS: 7647-14-5
KCl	Sigma-Aldrich	P9541; CAS: 7447-40-7
Trehalose	Sigma-Aldrich	T0167; CAS: 6138-23-4
TES	Sigma-Aldrich	T1375; CAS: 7365-44-8
Calcium chloride dihydrate	Sigma-Aldrich	223506: CAS: 10035-04-8
Sodium bicarbonate	Sigma-Aldrich	S8875; CAS: 144-55-8
Deposited data		
Raw and analyzed data	This paper	Mendeley Data: https://doi.org/10.17632/kg74mpjzfv.1.
Experimental models: Organisms/strains		
Female *Ormia ochracea*	Laboratory strains derived from Florida and Laie, HI	N/A
Female *Ormia ochracea*	Wild caught	N/A
Software and algorithms		
Statistical analysis R script	This paper	DOI: https://doi.org/10.17632/kg74mpjzfv.1.
Effective hearing distance computational model MATLAB script	This paper	DOI: https://doi.org/10.17632/kg74mpjzfv.1.
StimProg behavioral and neural data collection software	Norman Lee	Version 6 https://github.com/Ormia/Stimprog
Other		
Highspeed spherical treadmill system	Lott et al.[56]	N/A
National Instruments data acquisition system	National Instruments	NI USB-6363
Silk-dome tweeters (speakers)	Dayton Audio Classic	DC28FS-8
Ultrasonic speakers	Avisoft Bioacoustic	Vifa part #60108
Audio amplifier	Crown	XLS1002 Drive Core 2
Audio amplifier	Applied Research and Technology	SLA1
Programmable attenuators	Tucker-Davis Technologies	System 3 PA5
Highspeed camera	Chronos	1.3
Neural amplifier	A-M Systems	Model 1800
Audiomonitor	A-M Systems	Model 3300
Tungsten electrodes	A-M Systems	Catalog # 573220
Micromanipulator	Leica	N/A
Bluetooth MP3 player	BERENNIS	A30-202201
Sound level meter	Hottinger Brüel & Kjær	Type 2250
Probe microphone	Hottinger Brüel & Kjær	Type 4182
1/8-inch microphone	Hottinger Brüel & Kjær	Type 4138
Microphone preamplifier	Hottinger Brüel & Kjær	Type 2669
Microphone conditioning amplifier	Hottinger Brüel & Kjær	Type 1708

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Animals

Lab experiments were conducted from June 2021 to December 2022 on lab reared *Ormia ochracea* derived from two populations exposed to different host songs in nature: a Hawaiian population collected from Laie, Hawaii in November 2020 and a Floridian population collected from Gainesville, Florida in 2019. Both populations contain a high density of flies and are well studied.[63,71] Floridian *O. ochracea* use *Gryllus rubens* as their preferred host,[41,72,73] while Hawaiian *O. ochracea* use *T. oceanicus* as their host; Hawaiian flies encounter the typical ancestral pure-tone song as well as derived novel songs across Hawaii.[50,54] Flies for lab-based experiments were maintained at St. Olaf College in a temperature-, humidity- and light-controlled environmental chamber (Power Scientific, Inc., model DROS52503, Pipersville, PA) set to a 12 hr photoperiod, 25°C, at 75% humidity, and provided butterfly nectar solution (The Birding Company, Yarmouth, MA, USA) *ad libitum*. Field-based soundtrap experiments were conducted in Hawaii with wild gravid female *O. ochracea*.

METHOD DETAILS

Neural auditory threshold measurements (neural audiograms)
Acoustic stimuli

We first created acoustic stimuli to present to flies to produce neural audiograms. We used a custom MATLAB app (StimProg V6 - FTC Module) to create pure tone sound pulses (10 msec duration, 1 msec on/off cosine squared ramps). We varied the carrier frequency of sound pulses within both calling songs from 2 - 44 kHz in $\frac{1}{3}$ octave steps, resulting in 14 song frequencies (sample rate of 44100 samples/sec). Sound intensity varied from 20 - 90 dB SPL in 5 dB intervals, resulting in 406 frequency-by-intensity pure tone stimuli (14 frequencies each played at 29 intensities).

Speakers are limited in their ability to present sounds at the same intensity across all frequencies (e.g., to produce flat frequency response profiles without introducing harmonic distortions). Thus, in this study, we selected two speakers that have excellent performance in the lower frequency range and in the ultrasound range, respectively. We tested sound frequencies below 12 kHz using a 1-$\frac{1}{8}$" Dayton silk-dome speakers (Audio Classic Series DC28FS-8) with analog signals amplified by a Crown audio amplifier (XLS1002 Drive Core 2, amplifier limited to sound frequencies <21 kHz). Sound frequencies above 12 kHz were tested using Avisoft Bioacoustic ultrasonic speakers (Vlfa, part #60108) connected to an Applied Research and Technology (SLA1) audio amplifier (amplifier limited to sound frequencies <60 kHz). Calibration of sound intensities within the two frequency ranges also necessitated the use of two different Hottinger Brüel & Kjær microphones and calibration approaches. For sound frequencies <12 kHz, we used a Type 4182 probe microphone connected to a calibrated Hottinger Brüel & Kjær sound level meter (Type 2250, LZFmax measurements). Sound intensity values were read from this meter and the signals were digitally adjusted to achieve appropriate sound pressure levels relative to 20 μPa. For sound frequencies >12 kHz, we calibrated sound intensities using a 1/8-inch pressure-field microphone (Hottinger Brüel & Kjær, Type 4138) connected to a microphone preamplifier (Hottinger Brüel & Kjær, Type 2669) that was powered by a microphone conditioning amplifier (Hottinger Brüel & Kjær, Type 1708). Sound intensity values were calculated based on RMS measurements of the voltage output from the microphone conditioning amplifier while factoring in the calibrated sensitivity of the microphone. The amplitude of the digital sound files was adjusted to achieve target sound pressure levels.

Experimental Setup

Neurophysiology measurements were conducted in a dark, acoustically-dampened sound chamber (Wenger Soundlok, USA). Stimulus presentation was achieved with a single speaker (Dayton silk-dome speakers for experiments with frequencies <12 kHz or Avisoft Bioacoustics ultrasonic speakers for experiments with frequencies > 12 kHz) positioned at -90° azimuth (to the left) relative to the test fly's midline body axis. We used a programmable attenuator (Tucker Davis Technologies, System 3 PA5, USA) to rapidly adjust sound pressure levels in real-time. This allowed for quick randomized testing of stimuli at different frequency and intensity combinations within the most optimal recording time frame (first 30 mins) from a dissected fly.

To aid in positioning the recording electrode in the cervical neck connective of the test fly (see below), we used a Leica micromanipulator that was situated at +90° azimuth to the fly. This manipulator, and the area surrounding the test fly, was covered with acoustic attenuating foam to eliminate echoes. We obtained auditory evoked extracellular multi-unit recordings using a tungsten electrode (1-5 MOhms, A-M Systems) and a silver wire as the reference electrode. Both electrodes were connected to the headstage of an AC microelectrode amplifier (A-M Systems Model 1800, USA). The analog output of this neurophysiology amplifier was connected to an audiomonitor (A-M Systems Model 3300, USA) and was digitized (44100 samples/sec) with the National Instruments data acquisition system. Neurophysiology measurements were synchronized with sound presentation using StimProg that interfaced with the National Instruments data acquisition system.

Preparing test subjects

Only gravid female *Ormia ochracea* were tested in neurophysiology experiments. A total of 11 flies from each of the two populations (Hawaii and Florida) were tested in the lower frequency range (2-10 kHz) and 13 flies from each of the two populations were tested in the high frequency range (12 - 40 kHz). Flies were immobilized by anesthetization on ice for 5 mins. After anesthetization, we prepared them with a live dorsal dissection by removing the fly's legs, mounting the fly on a custom holder using low temperature melting wax,

and removing the dorsum, flight muscle, and gut to expose the thoracic ganglion.[38,43] The thoracic cavity was bathed with a saline solution throughout neurophysiology measurements.

Experimental Protocol

We recorded auditory evoked potentials from prepared flies using a tungsten electrode inserted into the cervical neck connective. The neck connective contains auditory neurons that presumably receive auditory input from the frontal (auditory nerve) or from local auditory interneurons within the thoracic ganglion, and project to the brain.[44] To maximize the signal-to-noise ratio (evoked auditory response relative to background neural activity) of recordings, we advanced the tungsten electrode into the fly's neck connective using the Leica micromanipulator (see above) while monitoring evoked neural activity in response to a 5 kHz search tone that was repeated at a rate of 1 Hz. We monitored activity both visually via the Stimprog app interface and aurally via headphones connected to the audiomonitor. Once an optimal signal-to-noise ratio was achieved, we began the experiment by presenting different frequency tone pulses at different intensities in randomized order. Each frequency/amplitude combination (e.g., 6.4kHz at 70dB) was presented for 10 msec and was preceded and followed by 50 msec of silence (110 msec total). Such data collection epochs were separated by 1-second intervals, resulting in a total data collection time of approximately 7 minutes per repetition of the stimulus sequence. Recording quality was most optimal during the first 30 mins of a freshly dissected fly, thus limiting recordings to 3-4 repetitions per individual.

Data Processing

We identified neural response thresholds with a widely used visual detection approach (e.g.,[74–77]; (see Figure S2 for an example). At each test frequency, we plotted a panel of auditory evoked responses arranged in the order of increasing stimulus intensity and established the lower and upper bounds based on the presence or absence of detectable neural responses during and after the stimulus epoch (see Figure S2). The lower bound is the lowest sound pressure level that we were able to visually detect an evoked response while the upper bound is the next intensity (5 dB lower) where we were not able to detect an evoked response. From these lower and upper bounds, we estimated the neural response thresholds using a calculation described in.[55]

$$\text{Estimated neural response thresholds} = 10 * \log_{10}[(10(UB/10) + 10(LB/10))/2]$$

Tethered walking phonotaxis experiments - behavioral audiograms

Acoustic stimuli

We created synthetic cricket songs to use as stimuli in this experiment using a MATLAB custom script for testing Floridian flies, and MATLAB based SynSing (v1.0)[78] for testing Hawaiian flies; this allowed for fine control of the carrier frequency and temporal characteristics of sound pulses that comprise the cricket songs. Unlike in the neural experiment where regular sound pulses elicit neural responses, behavioral responses can only be elicited when sound pulses are in the temporal pattern of host cricket song. Thus, we created stimuli for testing Floridian flies modeled after the calling songs of *Gyllus rubens* following natural song features reported in Walker.[79] The song consisted of a trill of 10 ms sound pulses (cosine squared ramps of 1 ms on/off) separated by an interpulse interval of 10 ms. We tested Hawaiian flies with stimuli modeled after the calling songs of typical Hawaiian *Teleogryllus oceanicus*. Temporal features were derived from those reported in Tanner et al.[78] Each long chirp is composed of 7 sound pulses with pulse durations = 34 ms, pulse periods = 51 ms (interval between sound pulses within long chirps = 17 ms) for a total long chirp duration of 340 ms. The period between long chirps = 1213 ms, which is separated by a series of 6 short chirps composed of 2 sound pulses with pulse durations = 29 ms and a pulse period = 43 ms (interval between sound pulses within short chirps = 14 ms) for a total short chirp duration = 72 ms, and the series of 6 chirps duration = 747 ms. We varied the carrier frequency of sound pulses within both calling songs from 2 - 44 kHz in $^1/_3$ octave steps, resulting in 14 song frequencies. Song stimuli were repeated with a 0.5 s inter-song-interval of silence to create a 6 s long bout of song. The resultant digital sound files were 16 bit with a sample rate of 44100 samples per second. We converted digital sound files to analog signals using a National Instruments data acquisition system (NI USB-6363).

Preparing test subjects

Only gravid female *Ormia ochracea* were tested in tethered walking phonotaxis experiments. A total of 17 Hawaiian flies and 18 Floridian flies were tested at the lower frequency range (2-10 kHz), and 19 Hawaiian flies and 20 Floridian flies were tested at the higher frequency range (12-40.3 kHz). Flies were immobilized by anesthetization on ice for 5 mins and then attached to a tether with low melting point wax. This tether was held by a Narishige micromanipulator (BC-4, Japan) attached to a magnetic base, which allowed for fine adjustments of the fly's standing posture on top of a high-speed spherical treadmill system (see below).

Experimental Setup

Tethered walking phonotaxis experiments were conducted in the dark, within a custom-made wooden box lined with acoustic attenuating foam. We used the same two types of speakers for broadcasting acoustic stimuli as in the neural physiology measurement (Dayton silk-dome speakers for experiments with frequencies <12 kHz or Avisoft Bioacoustics ultrasonic speakers for experiments with frequencies > 12 kHz). Two speakers of the same type (either Dayton silk-dome or Avisoft Bioacoustics ultrasonic speakers) were installed in this box at ±45° azimuth relative to the fly's midline body axis and at 25 cm away from the location of a fly that was tethered and held on top of the spherical treadmill system.[39] To ensure adequate visualization of the tethered fly in the dark, we illuminated the area surrounding the treadmill system with an infrared light (IR) and we monitored the fly via the digital display of an IR-capable camera (Chronos 1.3 high-speed camera, Krontech). The treadmill system consists of a lightweight spherical table tennis ball levitated by a constant airstream delivered through a perforated custom ball holder.[56] Walking phonotactic responses result in rotations of the ball, which actuates a modified optical mouse sensor below the ball. Locomotory responses in the left

and right directions are transduced as changes in x pixel values (positive values to the right), and forward and backward responses are transduced as changes in y pixel values (positive values in the forward direction). Synchronization of data capture via the treadmill system and stimulus presentation was achieved with a custom MATLAB app (StimProg V6, https://github.com/Ormia/Stimprog) that interfaced with a National Instruments data acquisition system. Each acoustic stimulus had a playback duration of 6 seconds, so we captured treadmill data for those 6 seconds plus one additional second beyond stimulus broadcast.

Experimental Protocol

As ambient temperature can affect song preferences,[40] we conducted tethered walking phonotaxis experiments at a constant ambient temperature of 21°C. Following the tethering procedure and after a 10 minute period of acclimation, we first verified that each experimental animal was phonotactic by presenting the 5 kHz synthetic calling song (*T. oceanicus* temporal pattern for Hawaiian flies, *G. rubens* temporal pattern for Floridian flies) at 70 dB SPL from the left speaker, followed by the right speaker. If the fly was responsive/phonotactic, we proceeded with the adaptive tracking procedure with that individual while unresponsive flies were excluded. To determine behavioral response thresholds, we used an adaptive tracking approach modeled after.[38,55] For a particular carrier frequency, we started with a playback intensity of 70 dB SPL. If a valid behavioral response to the stimulus was observed, the stimulus was decreased by 5 dB intervals until no response was observed. After a non-response, we increased the stimulus intensity by a half step (2.5 dB). If the fly responded at this intensity, then that intensity was specified as the upper bound of the behavioral response threshold and the lower bound was specified as the last intensity for which a non-response occurred. For example, if a fly responded to a frequency of 6.4kHz at 70 dB, then 65 dB, then 60 dB, then 55 dB, but did not respond to 50dB, we would then play the 6.4kHz stimulus at 52.5dB. If the fly responds to 52.5 dB, then we conclude that the fly's response threshold is between 50 and 52.5 dB. Thus, we name the upper bound (UB) as 52.5 dB and the lower bound (LB) as 50dB, and we use these values (see below) to calculate the response threshold. If the fly did not respond to the half step intensity (e.g., 52.5 dB), then the current intensity (e.g., 50 dB) was specified as the lower bound, and the last intensity for which a response occurred (e.g., 55 dB) was specified as the upper bound. Once the upper and lower bounds of the behavioral response threshold at a particular carrier frequency were established, we proceeded to the next frequency in random order. To reduce carryover effects from a prior stimulus, we allowed a 30 s silent period between stimuli, and we ensured the subject had stopped moving prior to stimulus presentation.

Data processing

Following[38] and,[55] the upper (UB) and lower bounds (LB) of the behavioral response thresholds were used in the following formula to calculate the estimated behavioral response thresholds:

$$\text{Estimated behavioral response thresholds } = 10 * \log_{10}[(10(\text{UB}/10) + 10(\text{LB}/10))/2]$$

Tethered walking phonotaxis measurements - behavioral responses to natural songs

Preparing test subjects

Gravid female flies (N = 17 Hawaiian flies, N = 17 Floridian flies) were prepared for behavioral experiments using the same approach as described in the tethered walking phonotaxis experiments - behavioral audiograms experiments.

Acoustic stimuli

To capture behavioral responses of flies to natural purring and rattling songs that vary in numerous spectral characteristics in addition to frequency, we used natural recordings of purring, rattling, and typical Hawaiian *Teleogryllus oceanicus* calling songs that were recorded as part of previous studies.[50,54] Purring songs were recorded in a recording studio at the University of Denver using a Sennheiser MKH800 microphone (Sennheiser, Wedemark, Germany) connected to a Millennia HV-3D preamplifier (Millennia, Diamond Springs, California) with gain set to 48 dB and recorded inputs through an Avid HD analog to digital converter (Avid, Burlington, Massachusetts) with sampling rate of 192 kHz at 24 bit depth. Rattling songs were recorded at a field station in Hawaii using a digital recorder (Marantz PMD620 MKII; Sound United LLC, Carlsbad, CA USA) connected to a RØDE NTG2 Multi-powered Condenser Shotgun microphone (RØDE Microphones LLC, Long Beach, CA USA) with a sampling rate of 192 kHz at 24 bit depth. Acoustic recordings were saved as WAV files. We used a principal components analysis (PCA) approach to select calling song recordings that reflect the extreme spectral variation present in purring and rattling *T. oceanicus* songs, following Tinghitella et al.[54] Briefly, song characteristics representing the many dimensions of variation in purring and rattling songs (e.g., comprising frequency, bandwidth, and amplitude; see Table S6 for lists of measured characteristics) were extracted from the first complete uninterrupted bout of calling song of each male's calling song recording. As purring (N = 46) and rattling songs (N = 15) were recorded under different conditions using slightly different recording techniques and equipment and differ in their spectral characteristics, we analyzed them and chose songs for playback separately. We visualized song variation along the first two PCA axes, which captured 51% of variation for purring song and 64% of variation for rattling song. For both song types, PC1 largely captured frequency-related characteristics, whereas PC2 largely captured characteristics pertaining to bandwidth (Table S6; Figure S1). We selected five purring and five rattling exemplar songs that spanned the acoustic space of song characteristics by choosing the four phenotypic extremes, as well as the most central song (see Table S7 for PC coordinates associated with the chosen exemplars). Because typical *T. oceanicus* song is preferred by flies over purring[54] and rattling[50] song, we also included a representative typical Hawaiian *T. oceanicus* song we have used in previous work.[50,54] For each of the 11 natural songs, we created standardized 6 s stimuli by repeating the songs and inserting a 0.5 s inter-song-interval of silence. As was done in previous work,[54,63] we adjusted the RMS value of each song to standardize the amplitudes across all 11 recorded natural songs for playback.

Digital sound files were converted to analog signals using a National Instruments data acquisition system (NI USB-6363), amplified with a Crown audio amplifier (XLS1002 Drive Core 2) and broadcast through 1-$\frac{1}{8}$ Dayton silk-dome speakers (Audio Classic Series DC28FS-8). Sound intensities were adjusted digitally by modifying the amplitude of the digital file and calibrated with a probe microphone (Hottinger Brüel & Kjær, Type 4182) connected to a sound level meter (LZeq measurements using Hottinger Brüel & Kjær, Type 2250).

Experimental Setup
We used the same experimental setup as described in the tethered walking phonotaxis - behavioral audiograms experiment.

Experimental Protocol
We again conducted trials at an ambient temperature of 21°C. Similar to the behavioral experiment described above, testing began with the presentation of the "preferred" synthetic cricket song (song modeled after the *Gryllus rubens* calling song for testing FL flies, or *Teleogryllus oceanicus* for testing HI flies) from the left speaker, followed by the right speaker to ensure that each fly was phonotactic and gravid. We then waited 30 seconds before playing all 11 natural songs to each test fly in a random order (again separated by 30 seconds of silence between stimuli). We chose a 30 second interval because that time period is substantially longer than demonstrated priming effects we are aware of Poulet and Hedwig.[80] We collected walking phonotaxis data using the same treadmill system described above.

Field sound trap experiments in response to *T. oceanicus* song variants
To examine responses of Hawaiian *O. ochracea* to the *T. oceanicus* calling song variants under natural conditions, we performed fly trapping choice tests in the field in June 2022 and November 2022 (N = 16 replicates across 8 trapping nights) using the same population of Hawaiian flies used in the laboratory experiments described above (located at the Brigham Young University (Hawaii) campus in Laie, HI).

Acoustic stimuli
We used the same 11 acoustic stimuli described in the laboratory-based natural songs experiment as well as two controls: silence and white noise.

Experimental Setup
We conducted fly sound trapping experiments with funnel traps constructed from 2 L plastic bottles as described in Walker.[81] We placed a single speaker (BERENNIS A30-202201 MP3 player with an internal speaker) at the bottom of each funnel trap from which we broadcast natural song variants to lure naturally occurring flies. We established a circular funnel trap array by positioning 13 funnel traps 3m apart in a circle (position within the circular array determined using a random number generator). Of the 13 stimuli, 11 were the *T. oceanicus* song variants used in the laboratory-based natural song experiments (a typical *T. oceanicus* calling song positive control and the 5 purring and 5 rattling recordings), 1 was a white noise negative control, and 1 was a silent negative control. We broadcast the stimuli at realistic natural amplitudes ([54]; 53 dBA for the purring exemplars and white noise control; 60 dBA for the rattling exemplars; 70 dBA for the typical control) for a minimum of two hours beginning one hour before sunset, which is the peak activity time for *O. ochracea*.[82] When we collected traps after the sampling period, we recorded whether and how many *O. ochracea* were present inside each trap. We held all captured flies until the experiment was complete to avoid resampling on subsequent nights and then released them at the collection site. We deployed two arrays per night (>50 meters from the closest edges of the arrays) for eight nights resulting in 16 total replicates.

Model of effective hearing distance
Model inputs
To approximate the selection that flies from each population could impose on these signals, we modeled effective hearing distances of *O. ochracea* by integrating our population-specific neural audiogram data (Figure 2A) with previously published bioacoustic data on the frequency characteristics and sound pressure levels of different song types. There is substantial inter-individual variation amplitude, dominant frequency, and bandwidth for both novel songs[48,50,52] (Figure 1), which would affect effective hearing distance. We are not able to incorporate bandwidth into our model, but do explore how variation in amplitude and dominant frequency impact effective hearing distances. We model effective hearing distances for the mean +/- 1 SD for both amplitude and peak frequency for the typical, purring, and rattling songs. We note that there are limitations to models such as this, that future work should explore, but we still find this model nevertheless valuable because it places our findings in a biological context for two important axes of variation (amplitude and frequency) that differ among songs. We incorporated previously published[50] dominant frequencies and variation (standard deviation) in dominant frequency (mean peak frequency for typical = 4877 Hz, purring = 9205 Hz, ratling = 5806 Hz; see Table S8 for all values modeled). We also used previously published measures of amplitude and variation therein (standard deviation) to parameterize the model. For typical song, we incorporated the morph's mean amplitude value, taken from the literature (typical = 92 dB SPL at 10 cm[83,84]). We then modeled the amplitudes for the novel morphs (rattling and purring from Gallagher et al.[50]) by calculating them relative to the published typical amplitude (mean purring amplitude = 53.71 dB SPL, rattling = 73.77 dB SPL at 10cm distance; Table S8).

Calculating attenuation over distance
To account for the non-frequency dependent damping of sound pressure with distance, we calculated the amount of sound attenuation following the 1/r inverse distance law[85]). In addition to distance related attenuation, sound pressure levels are also affected by atmospheric absorption, which depends on sound frequency, temperature, and humidity. Sound dissipation due to atmospheric

absorption was estimated using the "Volpe Method", which analyzes wideband sounds with $^1/_3$ octave band filters.[57] First, we calculated mid-band attenuation coefficients ($\alpha_{fm,i}$, expressed as dB/m).[58] Second, to calculate the mid-band attenuation values ($\Delta t_{fm,i}$), mid-band coefficients were multiplied by the path length (s).

$$\Delta t_{fm,i} = \alpha_{fm,i} \times s$$

Third, we used the Volpe Method equations to calculate mid-band attenuations at different nominal frequencies (fi) centered on the $^1/_3$ octave band filters.

For mid-band attenuation levels <150 dB

$$B_{fi} = \Delta t_{fm,i} \times \left(A + \left(B \big/ C - D \times \Delta t^E_{fm,i} \right) \big/ 1 + B \times \Delta t^E_{fm,i} \right)$$

where the following Volpe Method constants were applied:

$$A = 0.867942$$

$$B = 0.111761$$

$$C = 0.95824$$

$$D = 0.008191$$

$$E = 1.6$$

For mid-band attenuation levels >150 dB

$$B_{fi} = F + G \times \Delta t_{fm,i}$$

Where the following Volpe Method constants were applied:

$$F = 9.2$$

$$G = 0.765$$

Attenuation across a broadband sound was calculated by summing B_{fi} across all frequency bands. This attenuation was added to the distance related attenuation for the combined total attenuation due to distance and due to atmospheric dissipation.

Integrating cricket song sound pressure levels with neural audiograms to estimate effective hearing distance

Our neural audiogram data is based on sampling at discrete frequencies in $^1/_3$ octave band steps, so we used linear interpolation between frequency sampling points to estimate neural response thresholds at specific peak frequencies. Thresholds for song peak frequencies (see Table S8) were determined from the interpolated individual neural audiograms (low-frequency range: n=11 flies per population, high-frequency range: n=13 per population, Figure 2A). As these thresholds describe the minimum song sound pressure level that elicits neural responses, we calculated the attenuation over distance, starting at peak amplitude for each song type (see Table S8), and determined the distance at which sound pressure levels reached the threshold required to elicit a neural response. These estimated hearing distances were calculated across all neural audiograms for each song type and are plotted as mean±SD in Figure 5B.

QUANTIFICATION AND STATISTICAL ANALYSIS

All statistical analyses were performed in RStudio (RStudio Team (2020); R version 4.2.2). To test for differences in the neural response thresholds of Hawaiian and Floridian flies, we first conducted a linear mixed model (LMM; package *lme4*;[86]) with threshold (in dB SPL) as the response variable, an interaction between population (Hawaii, Florida) and frequency (in kHz; treated as continuous) as the predictor variable and a random effect of individual; as neural response thresholds were clearly non-linear, we also included orthogonal quadratic (frequency[2]) and cubic (frequency[3]) frequency terms in this model. To test for differences in the neural response thresholds of Florida and Hawaii at each of the 14 frequencies, we performed a separate LMM with threshold (in dB SPL) as the response variable, an interaction between population (Hawaii, Florida) and frequency (with each of the 14 frequencies treated as categorical, rather than continuous) as the predictor variables, and a random effect of individual. We subsequently tested for population-level differences using pairwise estimated marginal means with an FDR multiplicity adjustment (package *emmeans*;[87]).

Next, to compare the behavioral response thresholds of Hawaiian and Floridian flies to pure-tone synthetic songs, we used identical models as above (two separate linear models: one with frequency as a continuous predictor and the other with frequency as categorical) except with behavioral response threshold (in dB SPL) as the response variable. Again, we subsequently used pairwise estimated marginal means with an FDR multiplicity adjustment to test for population-level differences in behavioral response thresholds within each frequency.

We then considered how flies respond to novel songs evolving in Hawaii using playback of naturally recorded purring, rattling, and typical males. Here, we were interested in overall differences in responses to each of the song types; therefore, exemplars were pooled within their respective morphs for the following two models. First, to examine differences in the attraction of Floridian and Hawaiian flies to purring, rattling, and typical *T. oceanicus* songs at biologically realistic amplitudes, we subset the data to only include observations made at 45 dB SPL for purring exemplars, 65 dB SPL for rattling exemplars, and 85 dB for the typical song at 25 cm (realistic amplitudes determined from recordings). Note that these amplitudes match amplitudes used in the computational model of effective hearing distance; specifically the mean amplitudes for each morph type at 10 cm in Table S8 were adjusted to 25 cm distance, as this was the distance that the speaker was placed from the fly in these playback experiments. To test for overall differences in the proportion of flies responding to each of the three song types across populations we fit a generalized linear model with response (yes/no) as the response variable, and song type (purring, rattling, typical), population, and their interaction as effects. We next compared overall differences in behavioral response thresholds (the intensity at which flies responded to each exemplar) of Hawaiian and Floridian flies to each of the three song types. We fit a Linear Mixed Model (LMM) with behavioral response threshold as the continuous response variable, and song type (purring, rattling, typical), population, and an interaction between song type and population as the predictor variables, plus a random effect of individual.

Next, we examined responses to each of the purring and rattling exemplars (unpooled) using a parallel approach where we first filtered to examine responses only at realistic amplitudes and then examined continuous behavioral response thresholds. First, we again subset the data to only include observations made at 45 dB SPL for purring exemplars, 65 dB SPL for rattling exemplars (as above). To test for differences in responses to each of the purring and rattling exemplars, we fit two separate bias-reduced generalized linear models (family = binomial), one for purring and one for rattling, in the package *brglm2*.[88] In each, whether the fly responded (yes/ no) was the response variable and predictor variables included exemplar (categorical), population, and their interaction. Then, to compare differences in continuous behavioral response thresholds (the intensity at which flies responded to each exemplar) of Hawaiian and Floridian flies to each of the purring and rattling exemplars, we used a principal component approach. As the characteristics measured and incorporated into the principal components analyses of the purring and rattling song recordings differed slightly, we could not directly compare behavioral response thresholds between the purring and rattling exemplars. We then used two separate LMMs, one for purring and one for rattling, to examine variation in the shape of fitness surfaces with behavioral response threshold (in dB SPL) as the response variable and the following predictor variables: the coordinates of each exemplar along the first two PCA axes (PC1, PC2; Tables S6 and S7), population (Hawaii, Florida), and interactions between PC1 and population and between PC2 and population. Individual was included as a random effect.

To compare the responses of Hawaiian flies to the purring, rattling, and typical song types in the field trapping experiment, we ran a bias-reduced generalized linear model (family = Poisson) with total number of flies caught as the response variable and stimulus type (five purring exemplars, five rattling exemplars, typical positive control, white noise negative control, silence) as the predictor variable in the package *brglm2*.[88]

Finally, we analyzed effective hearing distance data using a two-way analysis of variance (ANOVA) to evaluate the effects of fly population (Hawaiian and Floridian), song type (typical, rattling, and purring), and their interaction on effective hearing distances. Effect sizes were reported as partial eta squared (η^2) to quantify the proportion of variance explained by each factor.

Supplemental Information

Neural and behavioral evolution in an eavesdropper

with a rapidly evolving host

Aaron W. Wikle, E. Dale Broder, James H. Gallagher, Jimena Dominguez, Mikayla Carlson, Quang Vu, Robin M. Tinghitella, and Norman Lee

A
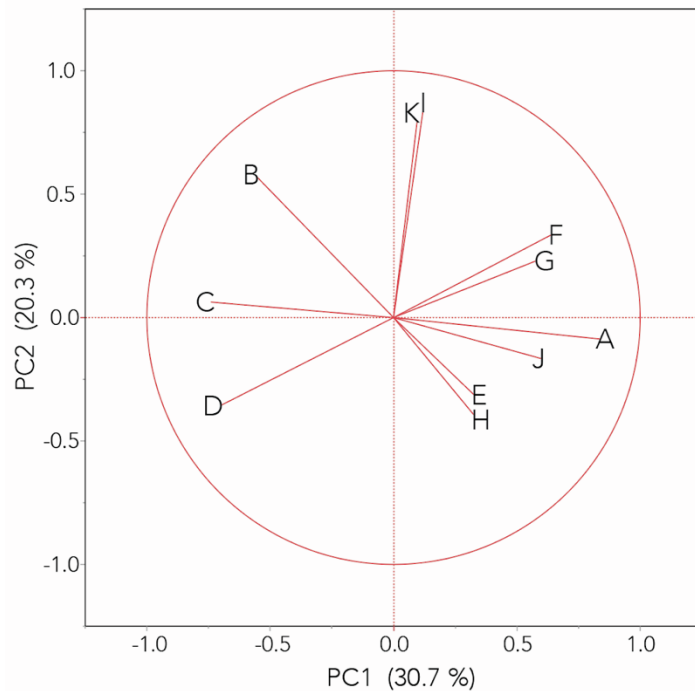
Purring Song Characteristic

A   Peak Frequency (kHz)
B   Frequency Range: 2 - 3.5
C   Frequency Range: 3.5 - 6
D   Frequency Range: 6 - 9.5
E   Frequency Range: 9.5 - 12.5
F   Frequency Range: 12.5 - 17.5
G   Frequency Range: 17.5 - 20
H   Proportion Long Chirp
I   Broadbandedness
J   Peak Frequency Bandwidth
K   Number of Peaks

B
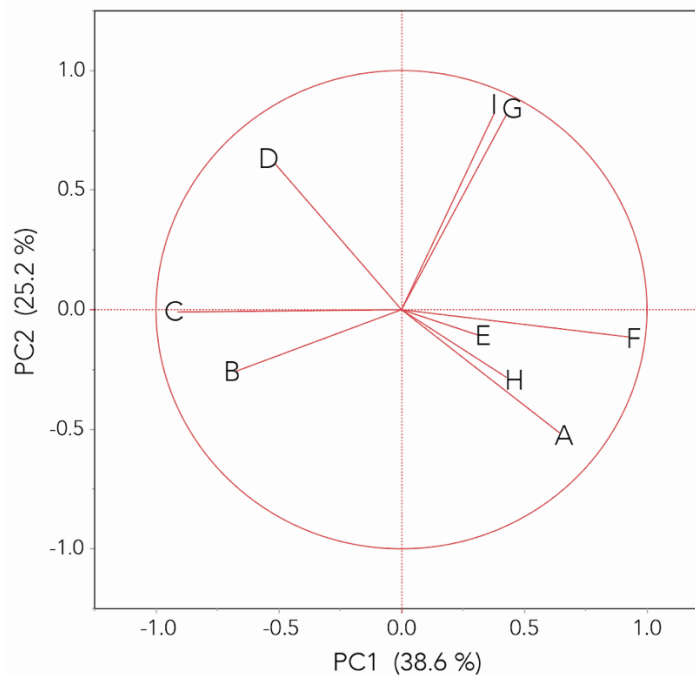
Rattling Song Characteristic

A   Peak Frequency (kHz)
B   Frequency Range: 2 - 3.5
C   Frequency Range: 3.5 - 6
D   Frequency Range: 6 - 9.5
E   Frequency Range: 9.5 - 12.5
F   Frequency Range: 12.5 - 17.5
G   Frequency Range: 17.5 - 20
H   Proportion Long Chirp
I   Frequency Evenness

Figure S1. PCA loadings of the characteristics extracted from purring (a) and rattling (b) calling songs. Related to Figure 4.
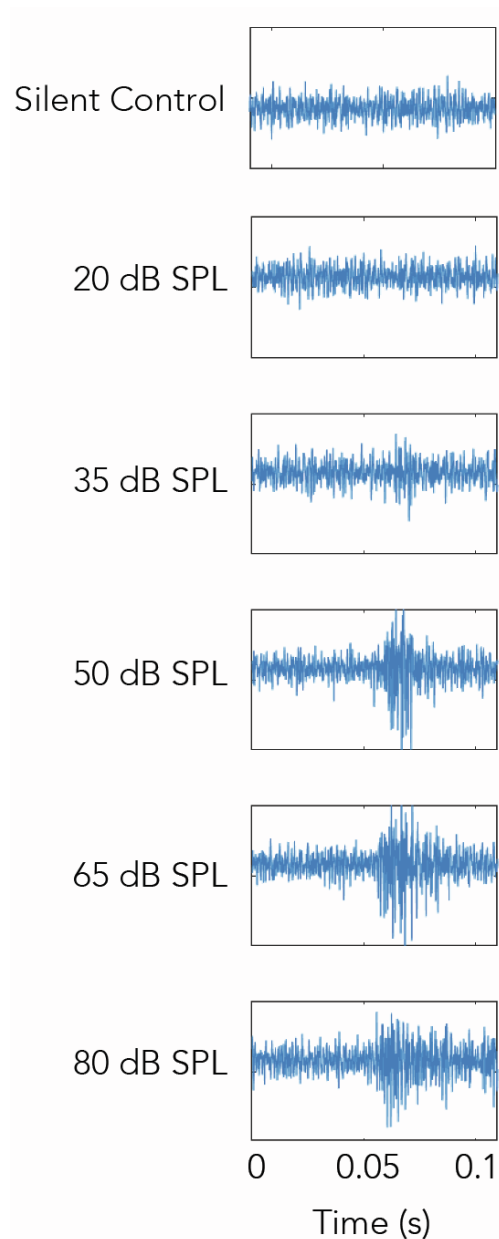
Figure S2. Example plot demonstrating how neural thresholds were visually determined from audiograms collected across intensities for each frequency. Related to Figure 2. Note that in this example there are just five intensities (20, 35, 50, 65, 80 dB SPL), whereas there were 29 intensities in our dataset. As amplitude of the stimulus increases, neural activity increases (larger, denser spikes). In these example recordings, 20 dB SPL and the silent control have the same level of neural activity, whereas there is enhanced neural activity at all higher sound intensities. In this example the upper bound of neural response is 20 dB SPL and the lower bound is 35 dB SPL. These upper and lower bound values were used to calculate the neural response thresholds.

Polynomial model

Model effect	Type III Wald Chi-square	df	p value
Polynomial frequency	1300.65	3	**<0.0001**
Population	1.049	1	0.3057
Polynomial frequency*Population	14.55	3	**0.0022**

Categorical model

Model effect	Type III Wald Chi-square	df	p value
Frequency	2815.09	13	**<0.0001**
Population	0.56	1	0.4545
Frequency*Population	63.14	13	**<0.0001**

Pairwise contrasts

Frequency (kHz)	estimate	se	df	lower.cl	upper.cl	t-ratio	p value
2.0	-0.23	1.84	243.99	-5.65	5.20	-0.12	0.90
2.5	-1.36	1.84	243.99	-6.79	4.06	-0.74	0.59
3.2	-1.14	1.84	243.99	-6.56	4.29	-0.62	0.63
4.0	-3.18	1.84	243.99	-8.61	2.24	-1.73	0.17
5.0	6.59	1.84	243.99	1.17	12.02	3.57	**0.0025**
6.4	0.68	1.84	243.99	-4.74	6.11	0.37	0.77
8.0	3.41	1.84	243.99	-2.02	8.83	1.85	0.15
10.1	6.82	1.84	243.99	1.39	12.24	3.70	**0.0025**
12.7	-1.35	1.70	244.02	-6.34	3.64	-0.79	0.59
16.0	-2.69	1.70	244.02	-7.68	2.30	-1.59	0.20
20.2	-1.46	1.70	244.02	-6.45	3.53	-0.86	0.59
25.4	-3.27	1.70	244.02	-8.26	1.72	-1.93	0.15
32.0	-4.81	1.70	244.02	-9.80	0.18	-2.83	**0.02**
40.3	-5.96	1.70	244.02	-10.95	-0.97	-3.51	**0.0025**

Table S1. Polynomial and categorical models comparing neural audiograms of Hawaiian and Floridian *Ormia ochracea* across sound frequencies. Related to Figure 2. Pairwise contrasts of estimated marginal means of neural response thresholds are provided with a false discovery rate (FDR) correction at each of the 14 frequencies (N = 48).

Polynomial model

Model effect	Type III Wald Chi-square	df	p value
Polynomial frequency	924.99	3	**<0.0001**
Population	5.99	1	**0.0144**
Polynomial frequency*Population	31.82	3	**<0.0001**

Categorical model

Model effect	Type III Wald Chi-square	df	p value
Frequency	2036.72	13	**<0.0001**
Population	7.32	1	**0.0068**
Frequency*Population	56.71	13	**<0.0001**

Pairwise contrasts

Frequency (kHz)	estimate	se	df	lower.cl	upper.cl	t ratio	p value
2.0	5.66	2.11	361.32	-0.52	11.84	2.69	**0.02**
2.5	4.53	2.11	361.32	-1.65	10.70	2.15	0.05
3.2	8.94	2.11	361.32	2.76	15.11	4.24	**0.004**
4.0	8.61	2.11	361.32	2.43	14.79	4.09	**0.004**
5.0	6.81	2.11	361.32	0.64	12.99	3.24	**0.006**
6.4	5.11	2.11	361.32	-1.06	11.29	2.43	**0.03**
8.0	0.85	2.11	361.32	-5.33	7.03	0.40	0.74
10.1	4.08	2.11	361.32	-2.09	10.26	1.94	0.07
12.7	-2.50	1.99	361.32	-8.35	3.35	-1.25	0.27
16.0	-4.76	1.99	361.32	-10.61	1.09	-2.39	0.03
20.2	-5.37	1.99	361.32	-11.22	0.48	-2.69	**0.02**
25.4	-2.33	1.99	361.32	-8.18	3.32	-1.17	0.28
32.0	-0.02	1.99	361.32	-5.87	5.83	-0.01	0.99
40.3	4.26	1.99	361.32	-1.59	10.11	2.14	0.05

Table S2. Polynomial and categorical models comparing behavioral audiograms of Hawaiian and Floridian *Ormia ochracea* across song frequencies. Related to Figure 2. Pairwise contrasts of estimated marginal means of behavioral response thresholds to pure-tone songs are provided with a false discovery rate (FDR) correction at each of the 14 frequencies comparing Hawaiian and Floridian *Ormia ochracea* (N = 74).

Proportion of flies responding to novel host songs (exemplars pooled within song types)

Model effect	Type III Wald Chi-square	df	p value
Population	23.46	1	**<0.0001**
Song Type	37.49	2	**<0.0001**
Population*Song Type	21.93	2	**<0.0001**

Pairwise contrasts of estimated marginal means

Contrast	estimate	se	asymp.lcl	asymp.ucl	z ratio	p value
FL typical - HI typical	-2.99	1.56	-7.56	1.59	-1.91	0.0696
FL typical - FL purring	2.01	0.58	0.32	3.70	3.50	**0.0018**
FL typical - HI purring	0.92	0.55	-0.69	2.54	1.68	0.1083
FL typical - FL rattling	1.80	0.57	0.13	3.46	3.17	**0.0029**
FL typical - HI rattling	-2.33	0.70	-4.39	-0.27	-3.32	**0.0023**
HI typical - FL purring	4.99	1.50	0.59	9.40	3.33	**0.0023**
HI typical – HI purring	3.91	1.49	-0.47	8.29	2.62	**0.0132**
HI typical - FL rattling	4.78	1.50	0.38	9.18	3.20	**0.0029**
HI typical – HI rattling	0.66	1.55	-3.90	5.22	0.42	0.6716
FL purring - HI purring	-1.09	0.35	-2.12	-0.05	-3.08	**0.0035**
FL purring - FL rattling	-0.21	0.38	-1.32	0.90	-0.56	0.6177
FL purring – HI rattling	-4.33	0.56	-5.98	-2.69	-7.75	**<0.0001**
HI purring - FL rattling	0.87	0.34	-0.12	1.87	2.57	**0.0138**
HI purring - HI rattling	-3.25	0.54	-4.82	-1.68	-6.08	**<0.0001**
FL rattling - HI rattling	-4.12	0.55	-5.74	-2.50	-7.47	**<0.0001**

Table S3. Models addressing the proportion of flies responding to novel host songs and pairwise contrasts of estimated marginal means with a false discovery rate (FDR) correction (N = 34). Related to Figure 3. While the proportion of Hawaiian versus Florida flies that responded to the typical songs did not differ, this is likely due to the smaller sample sizes associated with typical song (1 typical song played to each fly vs 5 each for purring and rattling) and complete separation in the model (100% of Hawaiian flies responded to the typical song). The proportion of Floridian flies that responded to the purring and rattling song types were nearly identical.

Behavioral thresholds to novel song types (exemplars pooled within song types)

Model effect	Type III Wald Chi-square	df	p value
Population	205.74	1	**<0.0001**
Song Type	13.01	2	**0.0015**
Population*Song Type	46.68	2	**<0.0001**

Pairwise contrasts

Contrast	estimate	se	df	lower.cl	upper.cl	t ratio	p value
FL typical - HI typical	48.53	4.57	341.98	35.02	62.04	10.62	**<0.0001**
FL typical – FL purring	11.56	3.32	344.05	1.75	21.37	3.48	**0.0006**
FL typical - HI purring	30.18	3.68	255.97	19.27	41.08	8.20	**<0.0001**
FL typical - FL rattling	2.06	3.32	344.05	-7.75	11.87	0.62	0.54
FL typical - HI rattling	32.29	3.68	255.97	21.39	43.20	8.77	**<0.0001**
HI typical - FL purring	-36.97	3.68	255.97	-47.88	-26.06	-10.04	**<0.0001**
HI typical - HI purring	-18.35	3.32	344.05	-28.17	-8.54	-5.53	**<0.0001**
HI typical - FL rattling	-46.47	3.68	255.97	-57.38	-35.56	-12.63	**<0.0001**
HI typical - HI rattling	-16.24	3.32	344.05	-26.05	-6.42	-4.89	**<0.0001**
FL purring - HI purring	18.62	2.49	79.37	11.08	26.15	7.48	**<0.0001**
FL purring - FL rattling	-9.50	1.92	344.05	-15.17	-3.83	-4.96	**<0.0001**
FL purring - HI rattling	20.74	2.49	79.37	13.20	28.27	8.33	**<0.0001**
HI purring - FL rattling	-28.12	2.49	79.37	-35.65	-20.58	-11.29	**<0.0001**
HI purring - HI rattling	2.12	1.92	344.05	-3.55	7.78	1.10	0.29
FL rattling - HI rattling	30.24	2.49	79.37	22.70	37.77	12.14	**<0.0001**

Table S4. Models addressing the behavioral thresholds of Hawaiian and Floridian flies responding to typical, purring, rattling songs (exemplars pooled within song types) and pairwise contrasts of estimated marginal means with a false discovery rate (FDR) correction (N = 34). Related to Figure 3.

Proportion flies responding to purring song exemplars

Model effect	Type III Wald Chi-square	df	p value
Purring Exemplar	16.99	4	**0.0019**
Population	8.71	1	**0.0032**
Purring exemplar*Population	2.85	4	0.5831

Behavioral response thresholds to purring song continuous variation

Model effect	Type III Wald Chi-square	df	p value
Purring PC1	29.46	1	**<0.0001**
Purring PC2	85.79	1	**<0.0001**
Population	47.73	1	**<0.0001**
Purring PC1*Population	2.45	1	0.1177
Purring PC2*Population	32.16	1	**<0.0001**

Proportion flies responding to rattling song exemplars

Model effect	Type III Wald Chi-square	df	p value
Rattling Exemplar	0.99	4	0.9160
Population	48.27	1	**<0.0001**
Rattling Exemplar*Population	5.99	4	0.1996

Behavioral response thresholds to rattling song continuous variation

Model effect	Type III Wald Chi-square	df	p value
Rattling PC1	1.37	1	0.2417
Rattling PC2	13.16	1	**0.0003**
Population	150.51	1	**<0.0001**
Rattling PC1*Population	8.36	1	**0.0038**
Rattling PC2*Population	11.07	1	**0.0008**

Table S5. Models addressing the proportion of flies responding to purring song exemplars, the behavioral response thresholds to natural purring song continuous variation, the proportion responding to rattling song exemplars, and the behavioral response thresholds to natural rattling song continuous variation. Related to Figure 4.

Purring song PCA (N = 46 males)

Song Characteristic	PC1 Eigenvector	PC2 Eigenvector
Peak frequency kHz	0.455144	-0.05821
2-3.5 kHz relative amplitude adjusted for silence	-0.30373	0.384734
3.5-6 kHz relative amplitude adjusted for silence	-0.40232	0.042014
6-9.5 kHz relative amplitude adjusted for silence	-0.38075	-0.23811
9.5-12.5 kHz relative amplitude adjusted for silence	0.176747	-0.26318
12.5-17.5 kHz relative amplitude adjusted for silence	0.347432	0.224167
17.5-20 kHz relative amplitude adjusted for silence	0.312712	0.153115
Proportion long chirp	0.177031	-0.2088
Broadbandedness	0.064558	0.557383
Peak frequency bandwidth	0.325313	-0.11104
Number of peaks	0.051821	0.529686

Rattling song PCA (N = 15)

Song Characteristic	PC1 Eigenvector	PC2 Eigenvector
Peak frequency kHz	0.347022	-0.34368
2-3.5 kHz relative amplitude adjusted for silence	-0.3627	-0.17154
3.5-6 kHz relative amplitude adjusted for silence	-0.48958	-0.00652
6-9.5 kHz relative amplitude adjusted for silence	-0.27675	0.406105
9.5-12.5 kHz relative amplitude adjusted for silence	0.166124	-0.07005
12.5-17.5 kHz relative amplitude adjusted for silence	0.497128	-0.07599
17.5-20 kHz relative amplitude adjusted for silence	0.242028	0.568726
Proportion long chirp	0.22897	-0.18769
Frequency evenness	0.214498	0.564037

Table S6. Characteristics used in the principal components analyses of purring and rattling *T. oceanicus* song, respectively. Related to Figure 4.

Purring song PC coordinates

Exemplar	PC1 (30.7%)	PC2 (20.3%)
Purring A	2.26137414	1.80608466
Purring B	3.77025122	-2.8902445
Purring C	-1.443401	-3.6681889
Purring D	0.09061599	0.50282539
Purring E	-2.7068921	0.94963963

Rattling song PC coordinates

Exemplar	PC1 (38.6%)	PC2 (25.2%)
Rattling F	0.62163031	2.32367921
Rattling G	1.63120188	1.27227414
Rattling H	4.21220433	-3.8391841
Rattling I	-1.0998869	-0.5353704
Rattling J	-2.464672	-0.5642039

Table S7. PC coordinates of the five purring and rattling exemplars. Related to Figure 4.

Typical Effective hearing distance (m)		Amplitude in dB at 10 cm		
		-1 SD (82.82)	mean (92)	+1 SD (96.38)
Peak frequency (Hz)	-1 SD (4686)	FL: 22.86±9.53 HI: 29.98±3.72	FL: 64.42±26.87 HI: 84.49±10.48	FL: 102.10±42.59 HI: 133.91±16.61
	mean (4877)	FL: 22.56:±9.67 HI: 38.27±8.40	*FL: 63.56±27.26 HI: 107.85±23.67*	FL: 100.72±43.21 HI: 170.93±37.51
	+1 SD (5069)	FL: 22.63±9.62 HI: 45.25±14.29	FL: 63.77±27.10 HI: 127.53±40.27	FL:101.07±42.96 HI: 202.12±63.82

Rattling Effective hearing distance (m)		Amplitude in dB at 10 cm		
		-1 SD (69.29)	mean (73.77)	+1 SD (76.73)
Peak frequency (Hz)	-1 SD (3179)	FL: 0.97±0.66 HI: 0.74±0.27	FL: 1.73±1.16 HI: 1.32±0.48	FL: 2.44±1.65 HI: 1.86±0.67
	mean (5806)	FL: 4.26±1.34 HI: 6.33±2.30	*FL: 7.58±2.38 HI: 11.26±4.09*	FL:10.70±3.37 HI: 15.91±5.77
	+1 SD (7509)	FL: 3.36±1.85 HI: 4.21±1.33	FL: 5.97±3.28 HI: 7.49±2.37	FL: 8.42±4.64 HI: 10.57±3.35

Purring Effective hearing distance (m)		Amplitude in dB SPL at 10 cm		
		-1 SD (49.35)	mean (53.71)	+1 SD (56.62)
Peak frequency (Hz)	-1 SD (5263)	FL: 0.44±0.17 HI: 0.82±0.24	FL: 0.78±0.31 HI: 1.46±0.42	FL: 1.10±0.43 HI: 2.06±0.59
	mean (9205)	FL: 0.30:±0.19 HI: 0.41±0.13	*FL: 0.47±0.33 HI: 0.72±0.23*	FL: 0.66±0.47 HI: 1.02±0.32
	+1 SD (13147)	FL: 0.11±0.02 HI: 0.13±0	FL: 0.16±0.05 HI: 0.13±0.04	FL: 0.20±0.07 HI: 0.18±0.05

Table S8. Estimated effective hearing distance (mean ± SD) of Floridian (FL top) and Hawaiian (HI bottom) flies for songs of the three morphs: typical, rattling, purring. Related to Figure 5b. For each morph type, we modeled variation in amplitude at 10 cm distance and peak frequency using the mean value and +/- 1 standard deviation (SD; from Gallagher et al. 2022). See details in Methods. Note that the italicized cell in the center of each table (mean amplitude and mean peak frequency) shows the values that are plotted in Figure 5b.