2023

Neural and Behavioral Evolution in an Eavesdropper with a Rapidly Evolving Host

Aaron W. Wikle
University of Denver

Neural and Behavioral Evolution in an Eavesdropper with a Rapidly Evolving Host

Abstract

The diversification of animal communication systems is driven by many interacting factors. Unintended receivers play an important part in this process, yet little is known about their role in signal evolution. Flies of the genus *Ormia* are parasitoids of crickets and rely on acoustic cues to locate hosts. In Hawaii, selection imposed by *Ormia ochracea* has led to recent and rapid diversification in the songs of their host. Here, we compare neural and behavioral responses of Hawaiian flies to those of an ancestral population to understand the role of parasitoid sensory and behavioral variation in the evolution of host songs. We demonstrate evolved differences in the auditory tuning and behavioral responses of Hawaiian flies that are likely facilitating the detection of the novel songs. This work heeds the recent call for better integration of the sensory and cognitive mechanisms of receivers into our understanding of the evolution of animal communication.

Document Type
Thesis

Degree Name
M.S.

Department
Biological Sciences

First Advisor
Robin M. Tinghitella

Second Advisor
Norman Lee

Third Advisor
Jonathan P. Velotta

Keywords
Behavior, Natural selection, Neurophysiology, Ormia ochracea, Rapid evolution, Teleogryllus oceanicus

Subject Categories
Developmental Neuroscience | Ecology and Evolutionary Biology | Evolution | Life Sciences | Neuroscience and Neurobiology

Publication Statement

Neural and Behavioral Evolution in an Eavesdropper with a Rapidly Evolving Host

_____

A Thesis

Presented to

the Faculty of the College of Natural Sciences and Mathematics

University of Denver

_____

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

_____

by

Aaron W. Wikle

June 2023

Advisor: Dr. Robin M. Tinghitella

Author: Aaron W. Wikle
Title: Neural and Behavioral Evolution in an Eavesdropper with a Rapidly Evolving Host Advisor:
Advisor: Dr. Robin M. Tinghitella
Degree Date: June 2023

ABSTRACT

The diversification of animal communication systems is driven by many interacting factors. Unintended receivers play an important part in this process, yet little is known about their role in signal evolution. Flies of the genus *Ormia* are parasitoids of crickets and rely on acoustic cues to locate hosts. In Hawaii, selection imposed by *Ormia ochracea* has led to recent and rapid diversification in the songs of their host. Here, we compare neural and behavioral responses of Hawaiian flies to those of an ancestral population to understand the role of parasitoid sensory and behavioral variation in the evolution of host songs. We demonstrate evolved differences in the auditory tuning and behavioral responses of Hawaiian flies that are likely facilitating the detection of the novel songs. This work heeds the recent call for better integration of the sensory and cognitive mechanisms of receivers into our understanding of the evolution of animal communication.

ACKNOWLEDGMENTS

TABLE OF CONTENTS

CHAPTER ONE: NEURAL AND BEHAVIORAL EVOLUTION IN AN EAVESDROPPER WITH

A RAPIDLY EVOLVING HOST

Introduction

Communication is fundamental to survival and reproduction, and the evolution of communication systems drives diversification (Bradbury and Vehrencamp, 1998). During communication, senders generate signals that are perceived and either ignored or acted on by receivers (Endler 1993; Bradbury and Vehrencamp, 1998; Breed 1999). Critical to the successful transfer of information between sender and receiver is congruence between a signal's properties and the sensory capabilities of the receiver – if a signal cannot be detected, it cannot convey information or change receiver behavior (Ryan 1988; Mason et al. 1999). Consequently, animal sensory systems evolve to be most sensitive to sensory stimuli that are critical for survival and reproduction (Gerhardt and Huber 2002; matched filter hypothesis: von der Emde and Warrant 2016; Römer 2016). This tuning enhances the reception of signals generated in the presence of seemingly infinite sources of sensory interference (e.g., conspecific, heterospecific, and abiotic stimuli; Ryan et al. 2007, 2013); for instance, the narrow tuning of receiver sensory systems to conspecific signals allows female frogs to identify the mating calls of conspecific males (Bee 2015) over the din of competing environmental noise (Brumm and & Slabbekoorn 2005). However, if the tuning of sensory systems to conspecific signals is very narrow, it may limit the ability of organisms to respond to signals and cues outside of that range, constraining the evolution of signals and receiver features, thus shaping evolutionary trajectories. In this paper, we capitalize on the highly unusual evolution of novel signals to capture how receiver sensory systems and behavior both respond to – and potentially shape – signal features.

While we often study how signal evolution is shaped by conspecific intended receivers (e.g., mates, competitors), diversification of communication systems is driven by a wide variety of interacting factors that co-occur within complex environments, including selection imposed by unintended receivers (Zuk and Kolluru 1998; Bernal and Page 2022), which is particularly underappreciated. Unintended receivers, or eavesdroppers, intercept signals intended for reception by other individuals (Peake 2005), usually to complete a critical life function such as locating a host or specialized prey item. Like intended receivers, eavesdroppers face a set of challenges - they must detect and perceive signals, extract information from them, and act upon that information (Zuk and Kolluru 1998). While generalist eavesdropping predators may not possess sensory systems that are narrowly tuned to the features of prey signals, some eavesdroppers are specialists on particular hosts or prey, at least locally, and risk forgoing any fitness if they fail to locate a host or prey (Thompson 1994; Zuk and Kolluru 1998), resulting in strong selection on sensory systems of such eavesdroppers. Consequently, intended and unintended receivers' sensory systems and preferences often converge (Robert et al. 1992; Wagner 1996), leading to opposing selective pressures that result in net stabilizing selection on host signal features (Lande 1981; Endler 1992). To maintain communication between signalers and receivers, signals should be relatively stable over evolutionary time, yet signals commonly diversify within species and play an important role in generating biodiversity (Price 1998, Seehausen 2000, Masta and Maddison 2002). 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 (Svensson and Gosden 2007; Svensson 2019).

Many have called for better integration of the underlying sensory and cognitive mechanisms of unintended receivers into our understanding of the evolution of animal communication (e.g., Rosenthal 2017; Romer 2020), as the sensory system defines the limits of behavioral responses that ultimately act on signals (selection), thus shaping intra- and interspecific communication (Zuk and Kolluru 1998). Whether the sensory system of an eavesdropper or predator evolves with changes in hosts and/or host signals has not been tested.

2

Here, we use complementary behavioral and neural-sensory experiments to ask how an acoustic eavesdropping parasitoid fly, *Ormia ochracea*, fares when faced with a change in host species as well as the rapid evolution of that hosts' signal (song).

Across its geographic range in North America, *O. ochracea* parasitize numerous cricket species (Lehmann 2003; Gray et al. 2019). As *O. ochracea* are obligate parasitoids of living crickets, locating a singing host is paramount for their survival (Edgecomb et al. 1995). While the host recognition template of *O. ochracea* appears remarkably flexible with respect to some spectral and temporal features of host song, a single defining parameter appears universally required for recognition of cricket song: a dominant frequency of 3–6 kHz (Gray 2007; Lakes-Harlan 2014). Indeed, neurophysiological recordings have also demonstrated that the auditory system of the fly is most sensitive to this narrow frequency range, although this has only been tested in Florida, where the fly is native (Robert et al. 1992). Sometime prior to 1989 (Eldridge et al. 2003), the fly was introduced to Hawaii, where none of its known continental U.S. hosts are found. 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 "ancestral"; Bennet-Cark 1989, 2003). Intriguingly, these eavesdroppers are thought to be responsible for the recent and remarkable evolution and spread of several wing and song-altering mutations in Hawaiian *T. oceanicus*. A silent morph (discovered in the early 2000s, Zuk et al. 2006) and several additional novel male morphs that produce unique, attenuated songs were recently discovered (Tinghitella et al. 2018; Rayner et al. 2019; Gallagher et al. 2022). Hawaiian populations of *T. oceanicus* are now highly dynamic, with some novel morphs increasing in abundance within populations and some spreading across the archipelago (Gallagher et al. in prep). Two of these morphs, purring and rattling, appeared as recently as seven years ago and produce songs with spectral characteristics (e.g., frequency, amplitude, bandwidth) that differ significantly from those of the ancestral song; for example, the average dominant frequency of purring song is nearly twice as high as that of the ancestral song (9.2 kHz vs 4.8 kHz; Gallagher et al. 2022). Despite this, in field studies *O. ochracea* can locate both novel songs, though at

3

much lower rates than ancestral song (Tinghitella et al. 2021; Gallagher et al. in prep). How does *O. ochracea* accomplish this feat? It is possible that the sensory system and/or behavior of *O. ochracea* has evolved in Hawaiian flies; the neural auditory sensitivity of the fly has only been examined in flies from Florida, and behavioral work with Hawaiian flies has only been conducted in the field (e.g., Gray et al. 2007; Tinghitella et al. 2021; Broder et al. 2022), where we cannot rule out plasticity (Paur and Gray 2011). Common garden approaches will allow us to establish whether responses observed in the field are genetically based evolutionary changes.

In this work, we use a complementary set of lab and field approaches to compare the neural and behavioral responses (important components of the 'receiver psychology' framework; Guilford and Dawkins 1993) of derived Hawaiian and ancestral Floridian *O. ochracea* to understand the role of parasitoid sensory and behavioral variation in host switching and the evolution of novel host songs. This experimental approach provides the opportunity to link the evolution of sensory systems, signals, and behavior, which together shape animal communication (Endler 1992). Further, while comparisons of sensory tuning and sensitivity are often performed across species (e.g., Hoke et al. 2022), comparing these characteristics *within* species (i.e., across populations; e.g., Capranica et al. 1973; Ryan and Wilczynski 1988) can provide a more refined understanding of the rate at which sensory systems can evolve and the selective pressures likely responsible. Hawaiian *O. ochracea* face two related dilemmas: introduction to Hawaii forced them to use a new cricket host and the new hosts' signal is now rapidly evolving. We hypothesized that neural and/or behavioral change has helped Hawaiian flies address these challenges and tested this by comparing responses of ancestral (mainland) and derived (Hawaiian) fly populations to playback of pure-tone pulses over a broad range of frequencies. We then compared behavioral responses of these same fly populations to the ancestral Hawaiian *T. oceanicus* song and two novel songs (purring and rattling) that arose within the past ~5-10 years. While dominant frequency appears to be the most essential host-recognition feature for *O. ochracea* across their range (Gray 2007; Lakes-Harlan 2014), the novel *T. oceanicus* songs vary along numerous spectral axes in addition to frequency (e.g. bandwidth, amplitude); therefore, we

4

use playback of recorded natural purring and rattling song in the lab under common garden and in the field where crickets and flies naturally occur to learn how an eavesdropping natural enemy fares when host songs evolve rapidly. We found that Hawaiian flies have evolved differences in their sensory tuning relative to flies in the ancestral Floridian range, particularly at frequencies important to host location. 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 these responses 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.

Methods

We compared the neural and behavioral response thresholds of lab reared *Ormia ochracea* derived from two populations: a Hawaiian population collected from Laie, Hawaii in November 2020 and a Floridian population collected from Gainesville, Florida in 2019. We chose these populations because they contain a high density of flies and are well studied (Broder et al. 2022). Additionally, the repertoire of host songs to which each population is exposed in nature also differs; Floridian *O.ochracea* use *Gryllus rubens* as a host (Walker 1986; Walker & Wineriter 1991; Gray et al. 2007), while Hawaiian *O. ochracea* use *T. oceanicus* as a host and encounter the ancestral pure-tone song as well as derived novel songs across Hawaii (Tinghitella et al. 2021; Gallagher et al. 2022). We maintained all flies at St Olaf University in an environmental chamber (Power Scientific, Inc, Model DROS52503, Pipersville, PA, USA) at 25° C and 75% humidity, on a 12:12 reverse light: dark cycle. We fed the flies butter nectar solution (The Birding Company, Yarmouth, MA, USA) *ad libitum*.

<u>Determining Neural Frequency Tuning Curves</u>

We generated all synthetic stimuli in MATLAB (R2021b). To determine neural frequency tuning curves of Hawaiian and Floridian *O. ochracea*, we used playback of single 10 ms trapezoidal pure tone sound pulses with a rise-fall ramp of 1 ms (see Oshinsky and Hoy 2002).

5

We varied the carrier frequency of the sound pulses from 2 - 44 kHz in ⅓ octave steps and the sound intensity from 20 - 90 dB SPL in 5 dB increments, resulting in 406 frequency-by-intensity pure tone stimuli (14 frequencies each played at 29 intensities). To confirm the neural activity in each recording was auditory-evoked, we also included 14 repetitions of silence for comparison. We converted all digital acoustic stimuli to analog signals using a data acquisition (DAQ) device (NI USB-6251, National Instruments, Austin, TX, USA).

We assessed overall peripheral auditory responsiveness to synthetic pure tone stimuli using extracellular multi-unit neural recordings from the frontal auditory nerve. To access the frontal auditory nerve, we used a dorsal dissection approach following Robert et al. (1992) and Lee and Mason (2017). We first immobilized subjects by placing them on ice for 5 minutes, then removed the subject's legs and mounted it on a custom holder using low-melting point wax. We then removed the subject's scutum, flight muscles, and gut. To ensure the flys' nervous system remained functional throughout the neurophysiological recordings, we bathed the thoracic ganglion in saline solution. We recorded auditory-evoked neural responses using a tungsten electrode (A-M Systems, 10 MΩ) inserted where the left and right frontal (auditory) nerve converges onto the thoracic ganglion. A silver wire that served as the reference electrode was inserted into the posterior tip of the subject's abdomen.

We collected all neural recordings at ambient temperature in a sound-isolated chamber. Due to the short window of time in which neural recordings could be collected (before desiccation) and speaker limitations, we collected neural responses to lower-end pure tone pulses (2 - 10 kHz; N = 11 flies per population) and higher-end pure tone pulses (12 - 40.3 kHz; N = 13 flies per population) separately using different speakers and animals. For playback of lower end pure tone pulses, we used a 1-⅛ Dayton Audio Classic Series DC28FS-8 silk-dome speaker, and for playback of higher-end pure tone pulses, we used an Avisoft Bioacoustics Ultrasound Dynamic Vifa speaker (part #60108). For both higher and lower-end stimuli, we situated the speaker 25 cm to the left (-80° azimuth relative to the subject's midline axis) of the subject. We attenuated all stimuli using a programmable attenuator (Tucker Davis Technologies System 3

PA5, USA) and amplified them using an audio amplifier (Crown XLS1002 Drive Core 2, USA). To ensure stimuli were presented at accurate intensities, we recorded the sound levels of all stimuli prior to initiating trials using a sound level meter (LZFmax function; B&K Type 2250, Denmark) connected to probe microphone (B&K Type 4182, Denmark) placed at the location of the subject's head. If sound intensities were not within 0.1 dB SPL of the desired RMS intensity, we adjusted them accordingly. To achieve synchronous sound presentation and data acquisition, we used a custom MATLAB script (StimProg V6: github link) that interfaced with the digital acquisition device (DAQ) and speakers. We amplified (100x gain) all neural recordings with an A-M Systems AC amplifier (Model 1800, place), digitized them with the DAQ, and saved them using custom software written in MATLAB (StimProg_V6).

We determined extracellular neural frequency tuning curves of Hawaiian and Floridian *O. ochracea* using a visual detection approach (e.g., Cone-Wesson et al. 1997; Gall et al. 2011; Lohr et al. 2013; Buerkle et al. 2014; Schrode et al. 2014). For each frequency, we used a custom MATLAB script to plot all frequency-by-sound intensity combinations in descending order. We established a timeframe of 0.05 - 0.1 s after the stimulus presentation as the window in which responses were visually assessed (there is an inherent delay in neural responses). We defined *neural threshold* as the lowest intensity at which an auditory-evoked waveform was visually detected (See Figure S1 for an example visual plot).

<u>Behavioral Responses to Synthetic Host Songs</u>

We determined behavioral frequency tuning curves of Hawaiian and Floridian *O. ochracea* using playback of pure-tone synthetic songs modeled after each population's primary host's calling song. To generate the synthetic *T. oceanicus* calling song for playback to Hawaiian flies, we used the SynSing (Tanner et al 2020) code in MATLAB. We modeled the temporal features of the synthetic *T. oceanicus* calling song after the mean temporal features of Hawaiian *T. oceanicus* calling songs reported in Tanner et al. (2019): song period = 1.144 s; duty cycle = 54%; proportion long chirp = 0.216 s; long chirp pulse duration = 34; short chirp pulse duration: 29 ms. We modeled the synthetic *G. rubens* calling song for playback to Floridian flies after the

natural calling song features reported in Walker (1998); 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. As was done with the pure-tone stimuli used to obtain neural frequency curves, we varied the carrier frequency of the synthetic calling songs from 2 - 44 kHz in ⅓ octave steps, resulting in 14 song frequencies. For each frequency, we repeated the synthetic songs with a 0.5 s inter-song-interval of silence to create a 6 s long bout of song.

To collect behavioral responses of Hawaiian and Floridian *O. ochracea* to the synthetic host calling songs, we conducted walking phonotaxis trials in an acoustically isolated chamber using a high-resolution spherical treadmill system (Lott et al. 2007). We performed all trials at ambient temperatures and in light conditions meant to mimic the flies' crepuscular hunting conditions. To ensure adequate visualization of the subjects, we further illuminated the trackball system with an infrared light (IR) and monitored the subject using an IR-capable camera (Chronos 1.3 high-speed camera, Krontech). As was done to collect neural recordings, we collected behavioral responses to lower and higher-end frequencies separately, using different speakers and animals. We used two 1-⅛ Dayton Audio Classic Series DC28FS-8 silk-dome speakers to broadcast the lower-end (2 - 10 kHz; N =17 Hawaiian flies; N =18 Floridian flies) synthetic calling songs and two Avisoft Bioacoustics Ultrasound Dynamic Vifa speakers (part #60108) for playback of higher-end and ultrasound (12 - 40.3 kHz; N = 19 Hawaiian flies; N = 20 Floridian flies) synthetic calling songs. To achieve synchronous sound presentation and data acquisition, we used a custom MATLAB script (StimProg V6: github link) that interfaced with the digital acquisition device (DAQ), speakers, and treadmill system.

For each behavioral trial, we first chilled the fly on ice for five minutes, attached it to a tether mounted to a micromanipulator (Narashige BC-4) using low-melting point wax, then positioned it on the treadmill in a natural walking posture (following Mason et al. 2001). To confirm the flies were placed and responding appropriately on the spherical treadmill, we began each trial by presenting the 5 kHz synthetic calling song (*T. oceanicus* pattern for Hawaiian flies, *G. rubens* pattern for Floridian flies) from the left speaker, followed by the right speaker; if the fly

responded positively to the control (walked at least 1 cm accurately towards the speaker broadcasting the control), we proceeded. To determine behavioral response thresholds to the synthetic calling songs, we used an adaptive tracking approach modeled after Lee et al. (2017); if a valid behavioral response (walking distance greater than 1 cm in any direction or flying behavior exhibited during stimulus playback) to the stimulus was observed, we decreased the intensity of the stimulus in 5 dB increments until no response was observed, after which we proceeded to the next stimulus; if a positive phonotactic response was not observed, we increased the intensity of the stimulus in 5 dB increments until either a response was observed or the upper playback intensity of 90 dB was reached, after which we proceeded to the next stimulus. We gave each fly a 1-minute recovery period between each stimulus, and we ensured the subject had stopped moving prior to stimulus presentation.

<u>Behavioral Responses to Natural Host Song Variants</u>

To measure behavioral responses of Hawaiian and Floridian *O. ochracea* to natural recordings of purring, rattling, and ancestral Hawaiian *Teleogryllus oceanicus* calling songs, we used playback of high-quality calling song recordings collected in previous work; purring songs were recorded in a recording studio at the University of Denver (see Tinghitella et al. 2021), and rattling songs were recorded at a field station in Hawaii (see Gallagher et al. 2022). We used a principal components analysis (PCA) approach to select calling song recordings that reflect the extreme spectral variation present in purring and rattling *Teleogryllus oceanicus* song, following Tinghitella et al. (2021). Briefly, song characteristics representing the many dimensions of variation in purring and rattling song (e.g., comprising frequency, bandwidth, and amplitude; see Table S1 and S2 for list 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 using slightly different recording techniques and equipment and differ in their spectral characteristics, we analyzed purring and rattling songs 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 purring

9

and rattling, PC1 largely captured frequency-related characteristics, whereas PC2 largely captured characteristics pertaining to broadbandedness (Table S1 and S2; Figure S2 and S3). 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 S3 and S4 for PC coordinates associated with the chosen exemplars). Because ancestral *T. oceanicus* song is preferred by flies over purring (Tinghitella et al. 2020) and rattling (Gallagher et al. 2022) song, we also included a representative ancestral Hawaiian *T. oceanicus* song we have used in previous work (Tinghitella et al. 2021; Broder et al. 2022). 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 (Tinghitella et al. 2021; Broder et al. 2022), we adjusted the RMS value of each song to standardize the amplitudes across all 11 natural songs.

To collect behavioral responses of Hawaiian and Floridian *O. ochracea* to the natural *Teleogryllus oceanicus* calling songs (N = 17 flies per population), we conducted walking phonotaxis trials identically to the synthetic calling song behavioral trials described above, except that only one set of speakers was used; because the majority of power in the natural *T. oceanicus* calling songs was present in lower-end frequencies (2 - 15 kHz), we presented all natural song stimuli using the 1-⅛ Dayton Audio Classic Series DC28FS-8 silk-dome speakers.

Responses to T. oceanicus Song Variants in the Field

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 = 8 trapping nights) using the same population of Hawaiian flies used in the neural and treadmill experiments (located at the Brigham Young University (Hawaii) campus in Laie, HI). We used circular funnel trap arrays deployed during the flies' active period (~1 h before and after sunset). Each replicate trapping array comprised 12 funnel traps (constructed from 2-L plastic bottles following Walker, 1989) placed 10 ft apart in a circle 120 ft in diameter. We placed a single speaker (BERENNIS A30-202201 MP3 player with an internal speaker) inside each trap,

from which we broadcast 1 of 13 stimuli (location determined using a random number generator); in addition to the 11 *T. oceanicus* song variants used in the treadmill experiments (an ancestral *T. oceanicus* calling song positive control and the 5 purring and 5 rattling exemplars), we also included a negative white noise control and a negative silent control. To ensure the stimuli were broadcast at realistic amplitudes (Tinghitella et al. 2021), we placed a sound level meter 1 m above each trap and adjusted the amplitude levels of each stimulus accordingly (53 dBA for the purring exemplars and white noise control; 60 dBA for the rattling exemplars; 70 dBA for the ancestral control).

<u>Statistical Analysis</u>

All statistical analyses were performed in RStudio (RStudio Team (2020); R version 4.2.2). To test for differences in the neural frequency tuning curves of Hawaiian and Floridian flies (N = 48), we first conducted a linear mixed model (LMM; package *lme4*; Bates et al. 2015). 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*; Lenth 2019).

To compare the behavioral response thresholds of Hawaiian and Floridian flies to pure-tone synthetic songs (N = 74), 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

11

used pairwise estimated marginal means with an FDR multiplicity adjustment to test for population-level differences in behavioral response thresholds within each frequency.

To examine differences in the proportion of Florida and Hawaii flies that responded behaviorally to each of the purring and rattling exemplars at biologically realistic amplitudes, we subset the data to only include observations made at 45 dB SPL for purring exemplars, 55 dB SPL for rattling exemplars, and 65 dB for the ancestral song. To test for overall differences in responsiveness to each of the three song types, we pooled the exemplars into their respective morph type then fit a generalized linear model with response (yes/no) as the response variable and an interaction between morph (purring, rattling, ancestral) and population. To test for differences in responses to each of the purring and rattling exemplars (i.e., unpooled), we then fit two separate bias-reduced generalized linear models (family = binomial), one for each morph, with response (yes/ no) as the predictor variable and exemplar (categorical) as the predictor variable in the package *brglm2* (Kosmidis et al. 2020). We subsequently used pairwise estimated marginal means with an FDR multiplicity adjustment to compare outcomes in behavioral response thresholds among predictors for each model.

We compared overall differences in behavioral response thresholds of Hawaiian and Floridian flies to each of the three song types by pooling exemplars within their respective morph and fitting a LMM with behavioral response threshold as the response variable, an interaction between song type (purring, rattling, ancestral) and population as the predictor variables, and a random effect of individual.

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. Therefore, we fit two separate LMMs, one for each morph, with behavioral response threshold (in dB SPL) as the response variable, an interaction between population (Florida, Hawaii) and the coordinates of each exemplar along the first two PCA axes (PC1, PC2; Tables S3 and S4) as predictor variables, and a random effect of individual.

12

To compare the responses of Hawaiian flies to the purring, rattling, and ancestral song types in the field (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 (5 purring exemplars, 5 rattling exemplars, ancestral positive control, white noise negative control, silence) as the predictor variable in the package *brglm2.*

Results

Neural Frequency Tuning Curves

To test for differences in the overall peripheral auditory responsiveness of Hawaiian and Floridian *O. ochracea*, we recorded extracellular multi-unit neural responses to a wide range of pure-tone frequencies (2 - 40.3 kHz) from the frontal auditory nerve. 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 =1413.63, *df* = 3, *p* < 0.0001; Figure 1a). Averaged across frequencies, the neural response thresholds of Hawaiian and Floridian flies did not differ (population: x^2 =1.084, *df* = 1, *p* = 0.2978); however, polynomial models revealed that the shape of each population's neural frequency tuning curve differed (frequency x population: x^2 =12.73, *df* = 3, *p* = 0.0053), indicating that the neural thresholds of Hawaiian and Floridian flies have evolved to differ at certain frequencies. Indeed, when we compared the neural thresholds of Hawaiian and Floridian flies at each of the 14 tested frequencies we found that the neural response thresholds of Hawaiian and Floridian flies differed at certain frequencies (Type III Wald chi-square; frequency x population: x^2 = 2850.38, *df* = 13, *p* < 0.001; see Table S5 for pairwise comparisons). We were particularly interested in knowing whether the neural response thresholds differed at frequencies relevant to the Hawaiian *T. oceanicus* morphs. We found that the neural response thresholds of Hawaiian flies were significantly lower (i.e., flies were more responsive) at the frequencies nearest the average dominant frequency of ancestral (dominant frequency: ~4.8 kHz) and purring *T. oceanicus* (dominant frequency: ~9.2 kHz); the neural response thresholds of Hawaiian flies were on average 6.5 dB SPL lower to the 5 kHz tone (mean ± SE; Hawaii: 29.77 ± 0.79; Florida: 36.59 dB SPL ± 1.08) and 6.8 dB SPL lower to 10.1 kHz tone (Hawaii: 36.82 ± 1.31; Florida:

13

43.64 ± 2.22) 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). Intriguingly, Floridian flies possess lower response thresholds at the two highest frequencies, 32.0 kHz and 40.3 kHz (see Table S5 for full list of contrasts).

<u>Behavioral Responses to Synthetic Host Songs</u>

To test if the behavioral response thresholds of Hawaiian and Floridian *O. ochracea* differ across frequencies, we collected behavioral responses to pure-tone synthetic host calling songs using a spherical treadmill. 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 = 1034.14, df = 1, p < 0.001; Figure 1b). Averaged across frequencies, the behavioral response thresholds of Hawaiian and Floridian flies did not differ (population: x^2 = 2.2, df = 1, p = 0.1378), however, the shape of the behavioral response tuning curves did differ for the two populations (frequency x population: x^2 = 33.82, df = 3, p < 0.001). There were particular frequencies at which the Hawaiian and Floridian flies differed in their neural response thresholds (frequency x population: x^2 = 55.78, df = 13, p < 0.001); the behavioral response thresholds of Hawaiian flies were on average 7.5 dB lower to the 4 kHz tone (mean ± SE; Hawaii: 39.41 ± 1.65 vs Florida: 46.94 dB ± 1.52) and 7.2 dB lower to the 5 kHz tone (Hawaii: 30.59 ± 2.26 vs Florida: 37.78 dB ± 1.47) than those of Floridian flies (Figure 1b; Table S6). As with the neural response thresholds, Floridian flies exhibited lower behavioral response thresholds than Hawaiian flies at some of the much higher-end frequencies (16.0, 20.2 kHz; see Table S6 for full list of contrasts).
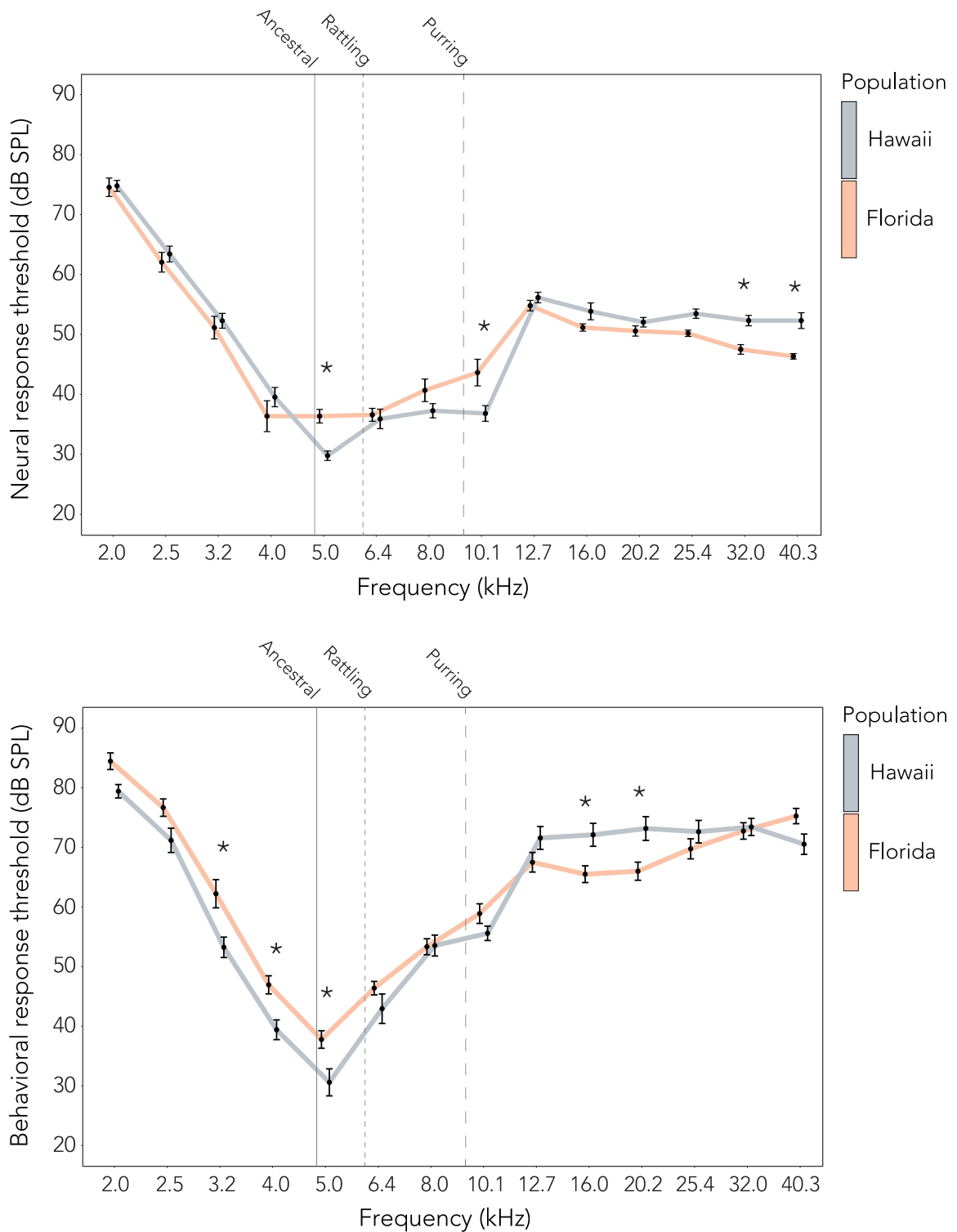
14

Figure 1: 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 (N = 26) were lower than those of ancestral Floridian flies (N = 26) at 5 kHz and 10.1 kHz, the frequencies nearest the average dominant frequency of ancestral (4.8 kHz: solid gray vertical

line) and purring (9.2 kHz: far right dashed vertical line) *T. oceanicus.* Floridian flies possess lower neural thresholds than Hawaiian flies at the two highest ultrasound frequencies (32 and 40.3 kHz). **b.** Behavioral response thresholds of Hawaiian flies (N = 36) were lower than those of Floridian flies (n = 38) at 5.0 kHz and several other lower-end frequencies (3.2 and 4.0 kHz), whereas Floridian flies possess lower response thresholds at several higher-end frequencies (16 and 20.2 kHz). Points and whiskers represent means ± SE and are jittered for easier comparison between populations.

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, we collected behavioral responses to representative naturally recorded purring, rattling, and ancestral *T. oceanicus* exemplars. We first examined bulk responses to the three morphs by pooling behavioral responses to exemplars within their respective morphs and limiting our observations to those made at realistic amplitudes (purring: 45 dB SPL; rattling: 55 dB SPL; Ancestral: 65 dB SPL at 25 cm). Overall, a much greater proportion of Hawaiian flies responded to the three song types when presented at realistic amplitudes than did Floridian flies (Fig. 2a), and Hawaiian flies also had substantially lower behavioral response thresholds to each song type than Floridian flies (Fig. 2b). The proportion of flies that responded differed by both song type and population (Type III Wald chi-square: population x song type x^2 = 21.55, *df* = 2, *p* < 0.0001); a greater proportion of Hawaiian flies than Floridian flies responded to each song type (Figure 2a). While the proportion of Hawaiian flies that responded to the ancestral (100%) and rattling songs (74%) did not differ, this is likely due to the smaller sample sizes associated with ancestral song (1 ancestral song played to each fly vs 5 rattling exemplars pooled within rattling) and complete separation in the model (100% of Hawaiian flies responded to the ancestral song). Interestingly, the proportion of Floridian flies that responded to the purring ancestral song types were nearly identical (see Table S7 for full list of pairwise contrasts). We found that the behavioral response thresholds of Hawaiian and Floridian flies also differed by song type (population x song type x^2 = 48.46, *df* = 2, *p* < 0.0001); the mean behavioral response threshold of Hawaiian flies was 19.5 db SPL (31%) lower to purring song, 31.5 dB SPL (43%) lower to rattling song, and 51 dB SPL lower to the ancestral song (Figure 2b) relative to Floridian flies. Interestingly, Floridian flies possess a lower

16

behavioral response threshold to purring song (63.4 dB SPL) than rattling (73.6 dB SPL) or

ancestral song (77.1 dB SPL; see Table S8 for full list of pairwise contrasts).
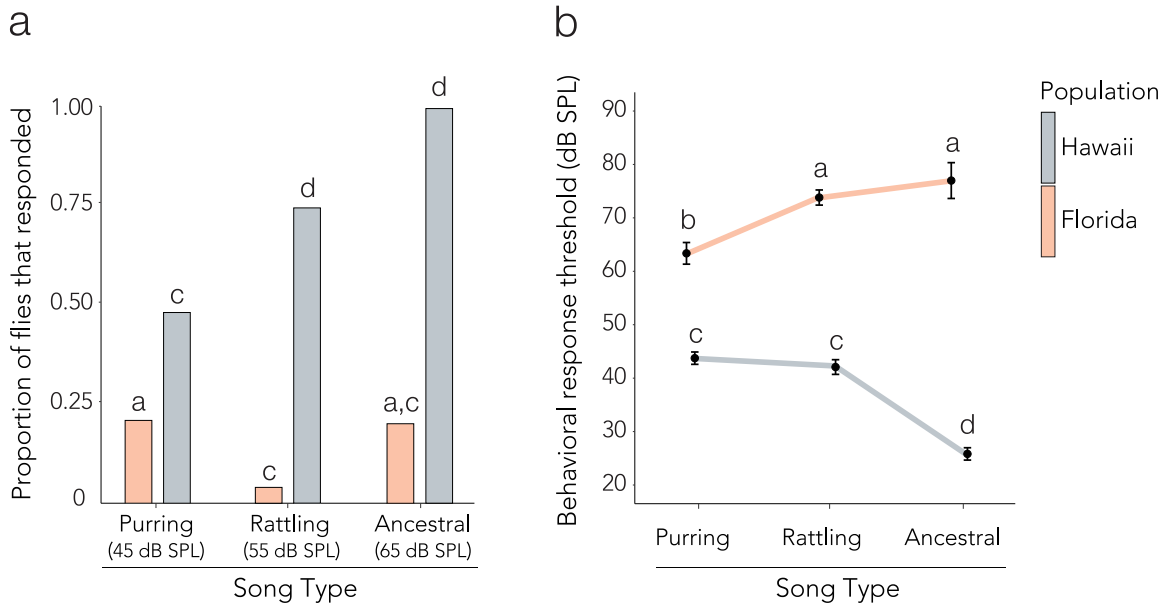
Figure 2: **a.** A much greater proportion of Hawaiian flies (N = 17) responded to each of the three Hawaiian *T. oceanicus* song types than did Floridian flies (N = 17). Here, we are interested in overall differences in responsiveness to each of the morph types, therefore exemplars are pooled within their respective morphs. Bars not sharing letters are significantly different from each other in post-hoc FDR tests. **b.** Hawaiian flies also possess significantly lower behavioral response thresholds to each of the three Hawaiian *T. oceanicus* song types. Again, exemplars are pooled within their respective morph types and points not sharing letters are significantly different from each other in post-hoc FDR tests. Points and whiskers represent mean ± SE.

Next, we interrogated whether flies exert selection differently on particular purring or

rattling songs, which is important for understanding how these songs will evolve in response to

natural enemies. In comparing the proportion flies that responded to the 5 purring exemplars at

realistic amplitudes, we found differences in *overall* responsivity between the two populations

(Type III Wald chi-square: population x^2 = 10.89, *df* = 1, *p* < 0.001); Hawaiian flies were overall

more responsive than Floridian flies to purring exemplars. We did not find a significant interaction

between population and exemplar (population x purring exemplar x^2 = 2.91, *df* = 4, *p* = 0.5728),

though there are some clear differences in response rates (Fig. 3a) that led us to believe this test

may be underpowered (see also fitness surfaces below). When we investigated the proportion

flies that responded to the 5 rattling exemplars at realistic amplitudes, we again found differences

in *overall* responsivity between the two populations (population x^2 = 43.09, *df* = 1, *p* < 0.0001), but again no significant interaction between population and ratting exemplar (population x rattling exemplar x^2 = 3.09, *df* = 1, *p* = 0.543; Fig. 3c).

We then 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 PCs for each song type; this approach, which uses behavioral responses thresholds, was a more sensitive metric of differences in fly responses across exemplars. We found that Hawaiian flies not only possess lower behavioral response thresholds to nearly all purring song exemplars (reflected in the height in the of the surfaces; Fig. 3b), but are also less sensitive to variation in purring song characteristics than Floridian flies (reflected in the relatively flat shape of Hawaiian purring fitness surface; Fig. 3b). Behavioral response thresholds to the purring exemplars were strongly predicted by their underlying PC1 and PC2 values (N = 34; Type III Wald chi-square: PC1 x^2 = 34.11, *df* = 1, *p* < 0.001; PC2 x^2 = 91.57, *df* = 1, *p* < 0.001); for both populations, as values of PC1 (largely frequency-related characteristics; Table S1) and PC2 (largely broadbandedness-related characteristics; Table S1) decreased, behavioral responses decreased (Fig. 3b), indicating that both Hawaiian and Floridian flies prefer lower frequency, less broadband purring songs. However, while both fly populations prefer lower PC1 and PC2 values, Floridian flies were over two times more sensitive to changes in PC1 values (PC1 x population: x^2 = 4.42, *df,* = 1 *p* = 0.0335; Hawaii estimate (slope) = 1.15, Florida estimate = 2.47) and over four times more sensitive to changes in PC2 values (PC2 x population: x^2 = 38.48, *df* = 1, *p* < 0.001; Hawaii estimate = 1.12, Florida estimate = 5.26) than Hawaiian flies. When we examined responses to rattling song exemplars, we found nearly the opposite; Hawaiian flies were far more sensitive to variation in rattling song characteristics along PC1 (PC1 x population x^2 = 8.23.11, *df* = 1 *p* = 0.0041) and PC2 (PC2 x population x^2 = 9.45, *df* = 1 *p* = 0.0021) than Floridian flies (reflected in surface slopes/shapes); while Floridian exhibited nearly flat responses to variation in PC1 (largely frequency-related characteristics; Table S2; Florida estimate = 0.62) and PC2 (largely broadbandedness-related characteristics; Table S2; Florida

estimate = -0.24), Hawaiian flies exhibited lower behavioral response thresholds to rattling

exemplars with higher PC1 (Hawaii estimate = -1.6) and PC2 (Hawaii estimate = -2.85) values

(Figure 3d). Interestingly, while Hawaiian flies exhibited lower behavioral response thresholds to

purring songs with lower frequency and broadbandedness content, they exhibited the opposite

trend for rattling songs, preferring rattling songs with higher frequency (higher PC1 values) and

broadbandedness (high PC2 values) content.
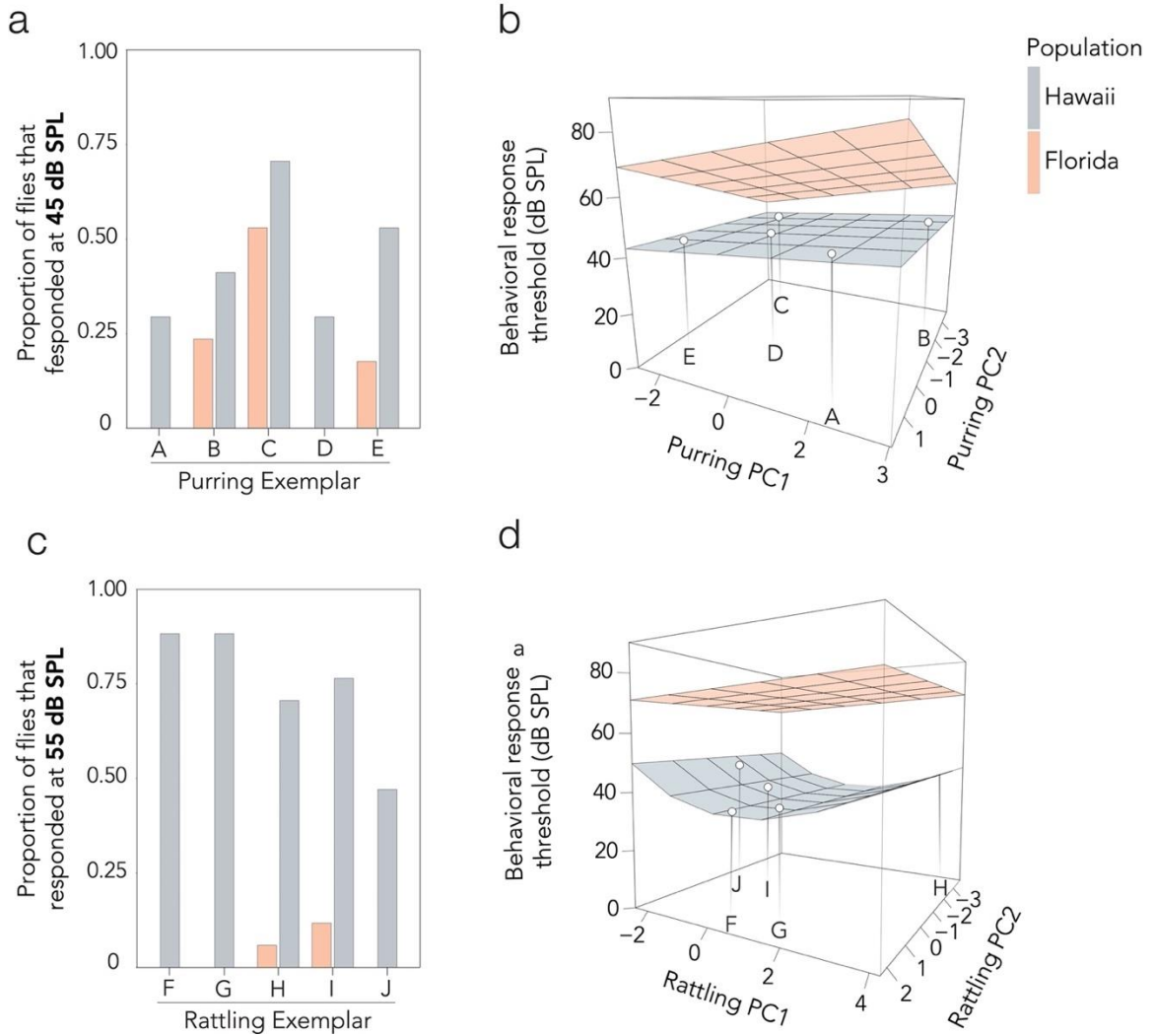
Figure 3: Hawaiian (N = 17) and Floridian (N = 17) flies exhibited clear differences in their behavioral preferences for certain purring and rattling song variants (exemplars). **a.** When broadcast at realistic amplitudes, a greater proportion of Hawaiian flies responded to the 5 purring exemplars (labeled A – E). **b.** Hawaiian flies possess lower behavioral response thresholds to purring song variants than Floridian flies. Both Hawaiian and Floridian flies exhibited lower response thresholds to purring exemplars with lower PC1 and PC2 values (e.g., behavioral response thresholds of both populations were lowest to exemplar C), but Hawaiian flies were far

less sensitive to changes in PC values (reflected in the slope of the surfaces). Letters on the bottom of the surface (A – E) show PC coordinates associated with each purring exemplar (see Table S1 for list of characteristics used in PCA, and Table S3 for PC coordinates associated with each exemplar). Vertical lines extending from each letter depict where each exemplar falls on the lower surface. **c.** When broadcast at realistic amplitudes, a greater proportion of Hawaiian than Floridian flies responded to the 5 rattling exemplars (labeled F – J). **d.** Hawaiian flies exhibited much lower behavioral response thresholds to the rattling variants than Floridian flies. While the behavioral thresholds of Floridian flies did not vary among the rattling exemplars (flat surface), the behavioral response thresholds of Hawaiian were lower to rattling exemplars with higher PC1 and PC2 values. Letters on the bottom of the surface (F – J) show PC coordinates associated with each rattling exemplar (see Table S2 for list of characteristics used in PCA, and Table S4 for PC coordinates associated with each exemplar). Vertical lines extending from each letter depict where each exemplar falls on the lower surface.

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 at one of our well-monitored field sites in Laie, Hawaii. The attraction of Hawaiian flies to the 13 stimuli 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 3). Of the 37 flies we caught, 30 (81%) were caught to the ancestral song, 2 (5%) were caught to purring songs, and 5 (14%) were caught to rattling songs (Benjamin-Hochberg contrasts of estimated marginal means: ancestral vs all 12 other stimuli: all p < 0.03). Similar to prior work in this system, this finding suggests that the flies only use these novel songs to find hosts at relatively close distances, which likely reflects the attenuated (quieter, more broadband) nature of the songs. We found no differences in the number of flies caught to any of the purring exemplars, rattling exemplars, silent, or white noise stimuli (Benjamin-Hochberg contrasts of estimated marginal means: all p > 0.5).

20
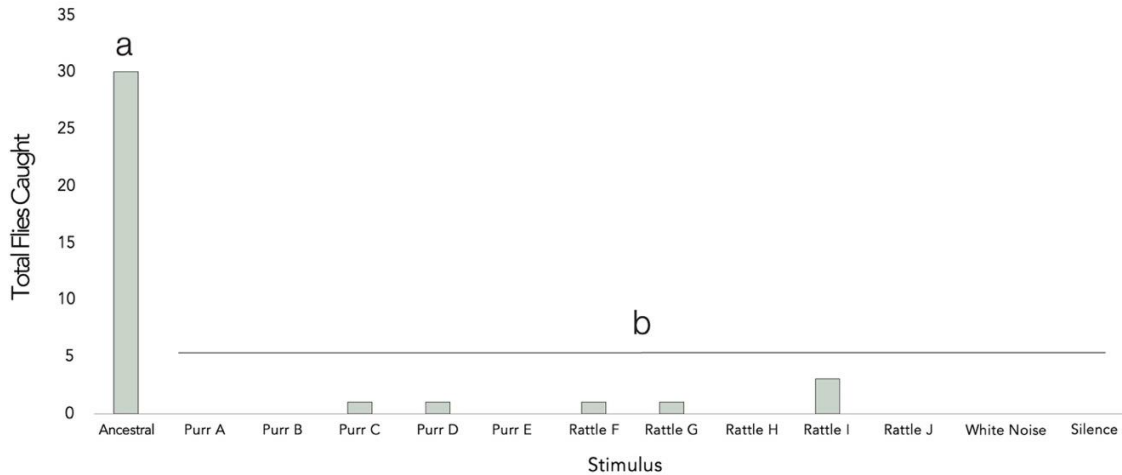
Figure 4: In long-distance field playbacks of song where crickets and flies naturally co-occur in Laie, Oahu (N = 8 trapping nights), Hawaiian *O. ochracea* were far more attracted to ancestral *T. oceanicus* song than purring and rattling song, and the latter two did not differ in attractiveness from white noise and silent controls.

Discussion

Opportunities to observe the evolution of novel animal communication signals in nature are exceedingly rare, and even rarer are studies investigating how the evolution of signals impacts interactions with eavesdropping natural enemies. Here, we used a series of complementary neural and behavioral experimental approaches in ancestral and derived populations of an eavesdropping natural enemy, *Ormia ochracea,* to learn how this parasitoid fly has evolved in the context of facing a novel host in Hawaii and ongoing rapid evolution of that host's sexual signal (Tinghitella et al. 2018; Tinghitella et al. 2021). Collectively, we demonstrate that Hawaiian flies have evolved sensory and behavioral differences that are likely facilitating the detection of novel host signals.

We first documented genetically based differences in the neural tuning of ancestral and derived populations of the fly to pure tone synthetic sounds (Fig. 1a). To our knowledge, this is the first evidence for rapid evolution in the sensory tuning of an eavesdropper to correspond with a rapidly evolving host. Previous work has demonstrated coevolution of sensory systems and signals within a species (i.e., between populations that differ in signals), for instance by shaping

in the tuning of female sensory systems to local male mating calls (Ryan and Wilczynski 1998 and Capranica et al. 1973). Interestingly, some intraspecific studies find that sensory tuning of intended receivers (females) does not match the spectral features of the signal (Mason et al. 1999; Zhao et al. 2017; Hoke et al. 2022). Such a mismatch may be explained by selection from unintended receivers, which is often ignored, but could shift signal features away from optimal sensory sensitivity of the intended receiver. Because signalers coevolve with both intended and unintended receivers (e.g., Wagner 1996; Zuk and Kolluru 1998), and many eavesdroppers must locate hosts using their signals in order to survive and/or reproduce, it is likely underappreciated just how common evolution in the sensory systems of eavesdroppers is; understanding such selective pressures is critical because eavesdroppers consequently shape interspecific communication and behavior (Zuk and Kolluru 1998; Bernal and Page 2022). The host switch and more recent evolution of novel host signals in our system provided the rare opportunity to test whether the sensory system and behavioral responses of a derived population (Hawaii) of parasitoid flies has evolved compared to an ancestral population (Florida). We show that Hawaiian flies possess significantly lower neural detection thresholds to frequencies relevant for detection of the ancestral (mean dominant frequency = 4.8 kHz) and purring (mean dominant frequency = 9.2 kHz) *T. oceanicus* morphs; this finding demonstrates coupling (i.e., coevolution) between host signal features and receiver sensory tuning in an eavesdropping predator. In contrast, Floridian flies possess lower neural response thresholds to much higher ultrasound frequencies (Figure 1a), which we discuss below.

While sensory systems define what 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 lab-reared Hawaiian and Florida flies differed in their behavioral responses to pure-tone synthetic host songs presented across a wide frequency range (Figure 2b); Hawaiian flies possessed lower behavioral response thresholds than Floridian flies to lower-end frequencies (3.2, 4.0, 5.0 kHz), which, importantly, correspond to the dominant frequency of some novel purring songs as well as the ancestral *T. oceanicus* song (Tinghitella et

22

al. 2018). As there is unusually high inter-individual variation in the characteristics of purring songs, including dominant frequency (Tinghitella et al. 2018; Gallagher et al. 2022), it may be advantageous for Hawaiian flies to possess lower response thresholds across a wider range of frequencies (relative to the Floridian flies that need only respond positively to the less variable song of their local host, *G. rubens*). However, there is not a perfect match between the neural and behavioral responses (e.g., populations differed in their behavioral thresholds at 4.0 kHz but not in their neural thresholds). While we are unaware of adaptive explanations for this slight mismatch, it stands to reason that animals might detect, but not attend to certain cues that do not resemble salient signals; after all animals are capable of detecting much more than the signals of their conspecifics and hosts (Ryan et al. 2007, 2013). Interestingly, as with the neural response thresholds, Floridian flies exhibited lower behavioral response thresholds to several higher-end frequency stimuli (Fig. 1b). One possible explanation for this finding is that Floridian flies are exposed to bat predation while flying and therefore benefit from exhibiting avoidance behavior (acoustic startle response) in response to higher, ultrasound frequencies (Rosen and Hoy 2009). Hawaiian flies, however, are not believed to be exposed to such bat predation, likely leading to relaxed selection to respond to such high-end frequencies in Hawaiian populations.

Selection exerted by *O. ochracea* on the song of its primary local cricket host in Hawaii, *T. oceanicus*, has facilitated the recent emergence and spread of several novel male morphs that produce song with spectral characteristics (e.g., bandwidth, amplitude) dramatically different from those of the typical ancestral song. Thus, our second major goal was to test whether Hawaiian flies have evolved to be more responsive to the purring, rattling, and ancestral *T. oceanicus* song types. Indeed, we found that Hawaiian flies were not only more likely than Floridian flies to respond to each song type when broadcast at realistic amplitudes (Fig. 2a), but also possess drastically lower behavioral response thresholds (i.e., are more responsive) to all three songs (Fig. 2b). When we compared responses to variants (exemplars) of the purring and rattling songs, we found that Floridian and Hawaiian flies have different preferences for, and sensitivity to, purring and rattling song characteristics (Fig 3b,d). While Hawaiian and Floridian flies both

23

exhibited preferences for lower frequency and less broadband purring song characteristics, Hawaiian flies were far less sensitive to changes in these features (and additional features; See Table S1) (Fig. 3b); in other words, Hawaiian flies responded more positively to a broader set of purring song variants than did Floridian flies. Furthermore, Floridian flies exhibited completely flat (no) preferences for rattling song variants, while Hawaiian flies exhibited preferences for rattling songs with higher frequency and higher broadbandedness content (Fig. 3d). Lower response thresholds in Hawaiian flies may reflect the extreme inter-individual variation in the acoustic characteristics underlying purring and rattling song (Tinghitella et al. 2018; Gallagher et al. 2022); possessing lower response thresholds across a broad range of acoustic characteristics likely improve their chances of finding a host cricket overall. While it would be informative to know whether the differences in sensory tuning and behavior between fly populations stems primarily from evolution associated with the change in host cricket or the evolution of novel T. oceanicus songs, that would require sampling flies before and after each event. We can gain some insight by sampling across additional N. American populations, but there are no appropriate fly neural and behavior samples from Hawaii that pre-date the origin of the novel songs.

What have we learned about how eavesdropper sensory tuning and host location behavior shape communication systems? The psychology of receivers plays an important role in shaping the evolution of signals (Guilford and Dawkins 1993), but the role of unintended receivers like eavesdroppers is less well understood (Bernal and Page 2022). Expanding our understanding of the fly's receiver psychology to Hawaiian populations where songs are evolving rapidly allows us to consider how signal evolution itself is impacted by evolution in eavesdroppers. One overarching theme in our work is that flies exert much stronger selection against ancestral *T. oceanicus* than novel morphs. Multiple lines of evidence support this pattern, including peak neural sensitivity and behavioral response thresholds at ~5kHz (Figure 1a,b), high behavioral response rates and low thresholds to recorded natural *T. oceanicus* songs (Figure 2a,b), and high fly trapping rates to ancestral song in the field (Figure 4). Given that, we might expect for novel males that are protected from the fly to increase in commonality, since some

24

female crickets are willing to accept novel male morphs as mates (Tinghitella et al. 2021, Fitzgerald et al. 2022, Gallagher et al. in prep). Indeed, repeated sampling efforts over the past 10 years reveal that Hawaiian populations of *T. oceanicus* are highly dynamic, with some novel morphs becoming more abundant within populations, likely, in part, due to selection imposed by *O. ochracea* (Gallagher et al. in prep). If and when males of novel morphs are chosen by flies as hosts, the specifics of Hawaiian fly receiver psychology we uncovered here will shape how novel signals evolve. Our playback of naturally recorded purring and rattling song exemplars allowed us to test for specific underlying acoustic characteristics favored by Hawaiian *O. ochracea*. Hawaiian flies exhibited preferences for purring songs with lower frequency and broadbandedness content (Figure 3b) and rattling songs with higher frequency and broadbandedness content (Figure 3d). The within-song-type preferences are particularly important to the evolution of purring songs in populations where only the highly protected purring morph is found (Tinghitella et al. 2018; Tinghitella et al. 2021). Flies are still found at these purring-exclusive sites, suggesting that purring males are successful hosts (Tinghitella et al. 2021).

We demonstrated evolved differences in the neural tuning and behavioral responses of Hawaiian and Floridian (ancestral) *O. ochracea* that are likely facilitating the detection of recently evolved novel host signals in Hawaii. This study, while illuminating, lays the foundation for several intriguing areas of interest that will be addressed in future and ongoing work. First, while the Hawaiian population of flies examined in this study co-occurs with purring and ancestral *T. oceanicus,* other Hawaiian populations of *T. oceanicus* currently only contain the highly protected purring morph; despite this, flies are still found at these purring-exclusive sites. Future studies should compare the neural thresholds of flies from populations that differ in cricket morph composition. Second, because plasticity likely shapes fly responses, future quantitative genetics studies should partition variance in neural thresholds and behavior into genetic and environmental components for each of these populations and address the role of developmental plasticity and learning in host song recognition and preference. Third, while the pure-tone stimuli used to obtain neural recordings in this study were well-suited for our primarily frequency-related

25

questions, there is a large amount of inter-individual variation in broadbandedness and other acoustic characteristics in addition to frequency in the novel purring and rattling songs. Thus, future work should attempt to collect neural responses to natural songs as well as a more comprehensive set of synthetic stimuli that capture the many additional underlying dimensions of natural cricket song (e.g, broadbandedness); expanding the array of characteristics to more accurately reflect natural songs would allow more accurate predictions to be made in regard to how selection by the flies may shape the characteristics of these novel songs in the future. Fourth, while a dominant frequency of 3–6 kHz is one of the most essential features of host detection in *O. ochracea* (Gray 2007; Lakes-Harlan 2014), preferences for particular temporal features have been shown (e.g, Floridian flies prefer pulse rates between 40-70 pulses/sec; Lee et al. 2019) and even vary geographically (Gray et al. 2007). Fifth, while natural selection imposed by *O. ochracea* is a major selective force shaping the features of these songs, it is not the only one; sexual selection by female crickets is simultaneously acting to shape their acoustic characteristics, yet the neuro-sensory tuning of Hawaiian crickets has not been tested. Future work should examine the neural auditory tuning of female crickets from Hawaii, for contrasting it with the neural auditory tuning of Hawaiian *O. ochracea* determined in this work will reveal how the receiver psychology of intended and unintended receivers jointly shape animal communication.

BIBLIOGRAPHY

Bates, D., Mächler, M., Bolker, B. M. & Walker, S. C (2015). Fitting linear mixed-effects models
using lme4. *Journal of Statistical Software*, https://doi.org/10.18637/jss.v067.i01.

Bee, M. A. (2015). Treefrogs as animal models for research on auditory scene analysis and the
cocktail party problem. *International Journal of Psychophysiology*, *95*(2), 216-237.

Bennet-Clark, H. C. *Songs and the physics of sound production in Cricket Behavior and
Neurobiology* (eds Huber, F., Moore, T. E. & Werner, L.) 227–261 (Cornell
University Press, New York, 1989).

Bennet-Clark, H. C. Wing resonances in the Australian field cricket *Teleogryllus oceanicus*.
Journal of Experimental Biology, 206, 1479–1496 (2003).

Bernal, X. E., & Page, R. A. (2022). How enemies shape communication systems: sensory
strategies of prey to avoid eavesdropping preda-tors and parasites. *Frontiers in Ecology
and Evolution*, 10, 989763.

Bradbury, J. W., & Vehrencamp, S. L. (1998). *Principles of animal communication*.

Breed, M. D. (1999). How do animals communicate?

Broder, E. D., Gallagher, J. H., Wikle, A. W., Venable, C. P., Zonana, D. M., Ingley, S. J., ... &
Tinghitella, R. M. (2022). Behavioral responses of a parasitoid fly to rapidly evolving
host signals. *Ecology and Evolution*, *12*(8), e9193.

Brumm, H., & Slabbekoorn, H. (2005). Acoustic communication in noise. *Advances in the
Study of Behavior*, *35*, 151-209.

Buerkle, N. P., Schrode, K. M., & Bee, M. A. (2014). Assessing stimulus and subject
influences on auditory evoked potentials and their relation to peripheral physiology in
green treefrogs (*Hyla cinerea*). *Comparative Biochemistry and Physiology Part A:
Molecular & Integrative Physiology*, *178*, 68-81.

Capranica, R. R., Frishkopf, L. S., & Nevo, E. (1973). Encoding of geographic dialects in the
auditory system of the cricket frog. *Science*, *182*(4118), 1272-1275.

Cone-Wesson, B. K., Hill, K. G., & Liu, G. B. (1997). Auditory brainstem response in tammar wallaby (*Macropus eugenii*). *Hearing Research*, *105*(1-2), 119-129.

Edgecomb, R. S., Robert, D., Read, M. P., & Hoy, R. R. (1995). The tympanal hearing organ of a fly: phylogenetic analysis of its morphological origins. *Cell and tissue research*, *282*(2), 251-268.

Eldredge, L. G., & Evenhuis, N. L. (2003). Hawaii's biodiversity: a detailed assessment of the numbers of species in the Hawaiian Islands. *Bishop Museum Press*.

Endler, J. A. (1992). Signals, signal conditions, and the direction of evolution. *The American Naturalist*, *139*, S125-S153.

Endler, J. A. (1993). Some general comments on the evolution and design of animal communication systems. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences*, *340*(1292), 215-225.

Gall, M. D., Brierley, L. E., & Lucas, J. R. (2011). Species and sex effects on auditory processing in brown-headed cowbirds and red-winged blackbirds. *Animal Behaviour*, *81*(5), 973-982

Gallagher, J. H., Zonana, D. M., Broder, E. D., Herner, B. K., & Tinghitella, R. M. (2022). Decoupling of sexual signals and their underlying morphology facilitates rapid phenotypic diversification. *Evolution Letters*, *6*(6), 474-489.

Gerhardt, H. C., & Huber, F. (2003). Acoustic communication in insects and anurans: common problems and diverse solutions.

Gray, D. A., Banuelos, C., Walker, S. E., Cade, W. H., & Zuk, M. (2007). Behavioural specialization among populations of the acoustically orienting parasitoid fly *Ormia ochracea* utilizing different cricket species as hosts. *Animal Behaviour*, *73*(1), 99-104.

Gray, D. A., Kunerth, H. D., Zuk, M., Cade, W. H., & Balenger, S. L. (2019). Molecular biogeography and host relations of a parasitoid fly. *Ecology and Evolution*, *9*(19), 11476-11493.

Kosmidis, I., Kenne Pagui, E. C., & Sartori, N. (2020). Mean and median bias reduction in generalized linear models. *Statistics and Computing*, *30*(1), 43-59.

Lakes-Harlan, R., & Lehmann, G. U. (2015). Parasitoid flies exploiting acoustic communication of insects—comparative aspects of independent functional adaptations. *Journal of Comparative Physiology A*, *201*, 123-132.

Lande, R. (1981). Models of speciation by sexual selection on polygenic traits. *Proceedings of the National Academy of Sciences*, *78*(6), 3721-3725.

Lee, N., & Mason, A. C. (2017). How spatial release from masking may fail to function in a highly directional auditory system. *ELife*, *6*, e20731.

Lee, N., Kirtley, A. T., Pressman, I. S., Jirik, K. J., Koucoulas, D., & Mason, A. C. (2019). Developing a phonotaxis performance index to uncover signal selectivity in walking phonotaxis. *Frontiers in Ecology and Evolution*, *7*, 334.

Lehmann, G. U. (2003). Review of biogeography, host range and evolution of acoustic hunting in Ormiini (Insecta, Diptera, Tachinidae), parasitoids of night-calling bushcrickets and crickets (Insecta, Orthoptera, Ensifera). *Zoologischer Anzeiger-A Journal of Comparative Zoology*, *242*(2), 107-120.

Lenth, R.V. (2021). Estimated marginal means, aka least-squares means [R Package Emmeans Version 1.6. 0]. Comprehensive R Archive Network (CRAN).

Lohr, B., Brittan-Powell, E. F., & Dooling, R. J. (2013). Auditory brainstem responses and auditory thresholds in woodpeckers. *The Journal of the Acoustical Society of America*, *133*(1), 337-342.

Lott, G. K., Rosen, M. J., & Hoy, R. R. (2007). An inexpensive sub-millisecond system for walking measurements of small animals based on optical computer mouse technology. *Journal of neuroscience methods*, *161*(1), 55-61.

Mason, A. C., Morris, G. K., & Hoy, R. R. (1999). Peripheral frequency mis-match in the primitive ensiferan *Cyphoderris monstrosa* (Orthoptera: Haglidae). *Journal of Comparative Physiology A*, *184*, 543-551.

Masta, S. E., & Maddison, W. P. (2002). Sexual selection driving diversification in jumping

  spiders. *Proceedings of the National Academy of Sciences*, *99*(7), 4442- 4447.

Oshinsky, M. L., & Hoy, R. R. (2002). Physiology of the auditory afferents in an acoustic

  parasitoid fly. *Journal of Neuroscience*, *22*(16), 7254-7263.

Paur, J., & Gray, D. A. (2011). Individual consistency, learning and memory in a parasitoid fly,

  Ormia ochracea. *Animal behaviour*, *82*(4), 825-830.

Peake, T. O. M. M. (2005). Eavesdropping in communication. *Animal communication*

  networks, 13-37.

Price, T. (1998). Sexual selection and natural selection in bird speciation. *Philosophical*

  Transactions of the Royal Society of London. Series B: Biological Sciences,

  353(1366), 251-260.

Rayner, J. G., Aldridge, S., Montealegre-Z, F. & Bailey, N. W. A silent orchestra: convergent

  song loss in Hawaiian crickets is repeated, morphologically varied, and widespread.

  Ecology, 100, https://doi.org/10.1002/ ecy.2694 (2019).

Robert, D., Amoroso, J., & Hoy, R. R. (1992). The evolutionary convergence of hearing in a

  parasitoid fly and its cricket host. *Science*, *258*(5085), 1135-1137.

Römer, H. (2016). Matched filters in insect audition: tuning curves and beyond. *The Ecology of*

  Animal Senses: Matched Filters for Economical Sensing, 83-109.

Römer, H. (2020). Insect acoustic communication: the role of transmission channel and the

  sensory system and brain of receivers. *Functional Ecology*, *34*(2), 310-321.

Rosen, M. J., Levin, E. C., & Hoy, R. R. (2009). The cost of assuming the life history of a host:

  acoustic startle in the parasitoid fly *Ormia ochracea*. *Journal of Experimental Biology*,

  212(24), 4056-4064.

Rosenthal, G. G. (2017). *Mate choice: the evolution of sexual decision making from*

  microbes to humans. Princeton University Press.

Ryan, M. J. (1988). Coevolution of sender and receiver: effect on local mate preference in

  cricket frogs. *Science*, *240*(4860), 1786-1786.

Ryan, M. J., & Cummings, M. E. (2013). Perceptual biases and mate choice. *Annual review of ecology, evolution, and systematics*, *44*, 437-459.

Ryan, M. J., Akre, K. L., & Kirkpatrick, M. (2007). Mate choice. *Current Biology*, *17*(9), R313-R316.

Schrode, K. M., Buerkle, N. P., Brittan-Powell, E. F., & Bee, M. A. (2014). Auditory brainstem responses in Cope's gray treefrog (*Hyla chrysoscelis*): effects of frequency, level, sex and size. *Journal of Comparative Physiology A*, *200*, 221-238.

Seehausen, O. (2000). Explosive speciation rates and unusual species richness in haplochromine cichlid fishes: effects of sexual selection. *Advances in ecological research*, *31*, 237-274.

Svensson, E. I. (2019). Eco-evolutionary dynamics of sexual selection and sexual conflict. *Functional Ecology*, *33*(1), 60-72.

Svensson, E. I., & Gosden, T. P. (2007). Contemporary evolution of secondary sexual traits in the wild. *Functional Ecology*, *21*(3), 422-433.

Tanner, J. C., Garbe, L. M., & Zuk, M. (2019). When virginity matters: age and mating status affect female responsiveness in crickets. *Animal Behaviour*, *147*, 83-90.

Tanner, J. C., Justison, J., & Bee, M. A. (2020). SynSing: open-source MATLAB code for generating synthetic signals in studies of animal acoustic communication. *Bioacoustics*, *29*(6), 731-752.

Thompson, J. N. (1994). *The coevolutionary process*. University of Chicago press.

Tinghitella, R. M., Broder, E. D., Gallagher, J. H., Wikle, A. W., & Zonana, D. M. (2021). Responses of intended and unintended receivers to a novel sexual signal suggest clandestine communication. *Nature Communications*, *12*(1), 797.

Tinghitella, R. M., Broder, E. D., Gurule-Small, G. A., Hallagan, C. J., & Wilson, J. D. (2018). Purring crickets: the evolution of a novel sexual signal. *The American Naturalist*, *192*(6), 773-782.

Von der Emde, G., & Warrant, E. (Eds.). (2015). *The ecology of animal senses: matched filters for economical sensing.* Springer.

Wagner Jr, W. E. (1996). Convergent song preferences between female field crickets and acoustically orienting parasitoid flies. *Behavioral Ecology*, *7*(3), 279-285.

Walker, T. J. (1986). Monitoring the flights of field crickets (Gryllus spp.) and a tachinid fly (*Euphasiopteryx ochracea*) in north Florida. *Florida Entomologist*, 678-685.

Walker, T. J. (1998). Trilling field crickets in a zone of overlap (Orthopter: Gryllidae: *Gryllus*). *Annals of the Entomological Society of America,* 91, 175–184. doi: 10.1093/aesa/91.2.175

Walker, T. J., & Wineriter, S. A. (1991). Hosts of a phonotactic parasitoid and levels of parasitism (Diptera: Tachinidae: *Ormia ochracea*). *Florida Entomologist*, 554-559.

Zhao, L., Wang, J., Yang, Y., Zhu, B., Brauth, S. E., Tang, Y., & Cui, J. (2017). An exception to the matched filter hypothesis: A mismatch of male call frequency and female best hearing frequency in a torrent frog. *Ecology and Evolution*, *7*(1), 419-428.

Zuk, M., & Kolluru, G. R. (1998). Exploitation of sexual signals by predators and parasitoids. *The Quarterly Review of Biology*, *73*(4), 415-438.

Zuk, M., Rotenberry, J. T., & Tinghitella, R. M. (2006). Silent night: adaptive disappearance of a sexual signal in a parasitized population of field crickets. *Biology letters*, *2*(4), 521-5

APPENDICES

Appendix A: Example plot demonstrating how neural thresholds were visually determined (note that in this shortened example there are just 5 amplitude levels, whereas there were 29 amplitudes per frequency used in the full data set). As amplitude of the stimulus increases, neural activity increases (larger, denser spikes). In these example recordings, there is clear neural activity during the established window in which neural activity was assessed (0.05 - 0.1 s; see methods) at each amplitude level except 20 dB, which contains the same level of neural activity as the silent control; thus, the neural threshold at this representative frequency would have been recorded as 20 dB SPL.
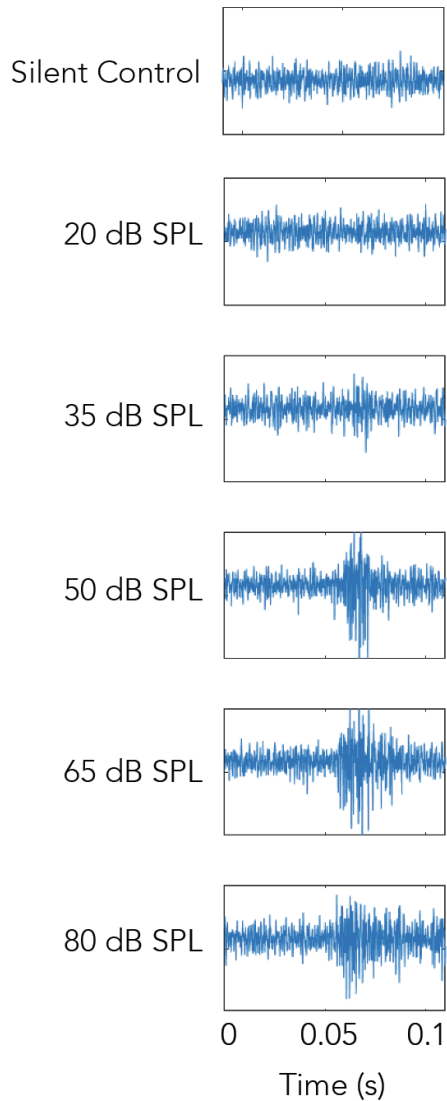
Appendix B: Characteristics used in the principal components analysis of purring *T. oceanicus* song (N = 46 males) and their associated PC1 and PC2 eigenvectors.

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

Appendix C: Characteristics used in the principal components analysis of rattling *T. oceanicus* song (N = 15) and their associated PC1 and PC2 eigenvectors.

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

Appendix D: PC coordinates of the 5 purring exemplars.

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

Appendix E: PC coordinates of the 5 rattling exemplars.

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

Appendix F: PCA loadings of the 11 purring song characteristics extracted from calling song recordings of 46 purring males.

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
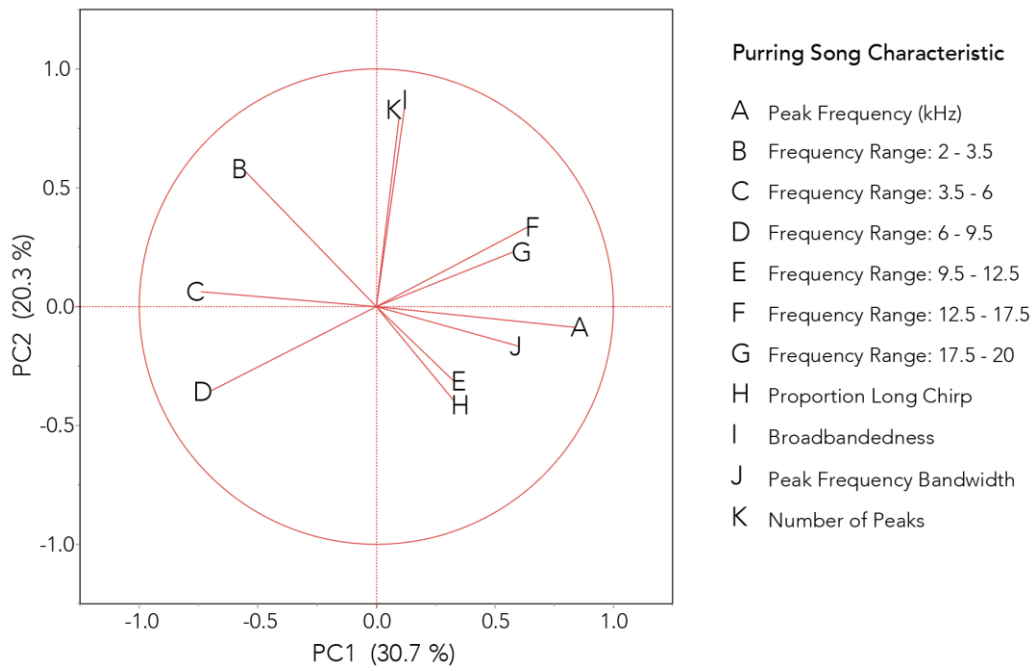
Appendix G: PCA loadings of the 9 rattling song characteristics extracted from calling song recordings of 16 rattling males.

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
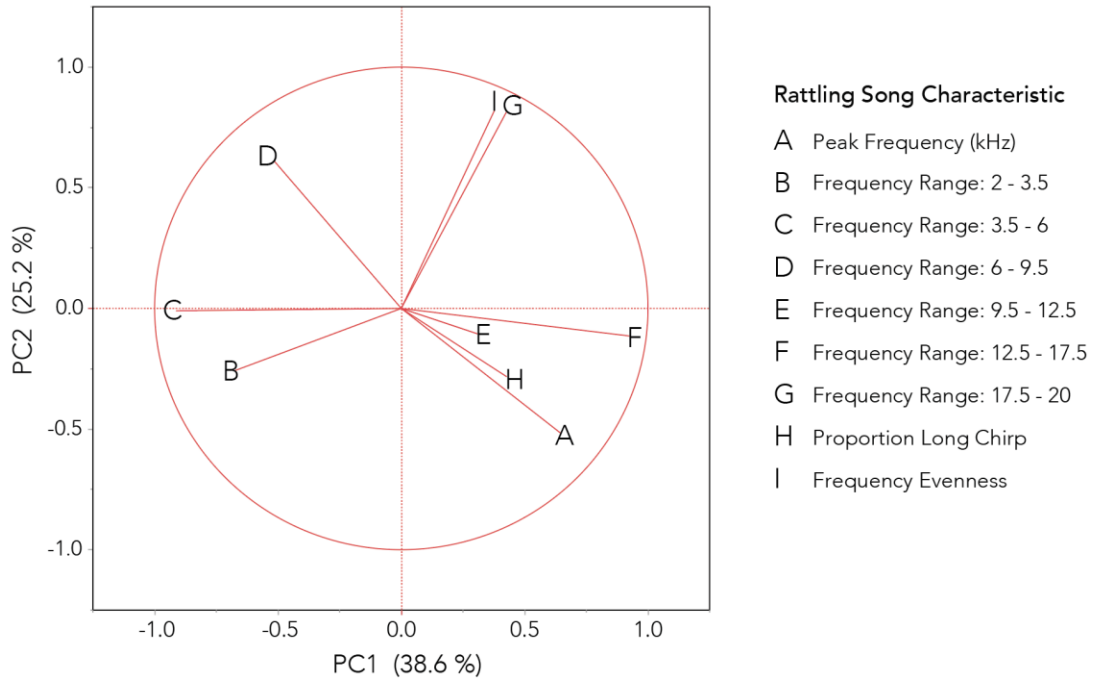
Appendix H: Pairwise contrasts of estimated marginal means of neural response thresholds with a false discovery rate (FDR) correction at each of the 14 frequencies for Hawaiian and Floridian *Ormia ochracea* (N = 48).

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	**2.45e-03**
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	**2.45e-03**
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	**2.45e-03**

Appendix I: Pairwise contrasts of estimated marginal means of behavioral response thresholds to pure-tone songs with a false discovery rate (FDR) correction at each of the 14 frequencies for Hawaiian and Floridian *Ormia ochracea* (N = 74).

Frequency (kHz)	estimate	se	df	lower.cl	upper.cl	t ratio	p value
2.0	5.03	2.44	393.53	-2.12	12.18	2.06	0.07
2.5	5.49	2.44	393.53	-1.66	12.64	2.25	0.06
3.2	8.99	2.44	393.53	1.84	16.14	3.68	**3.66e-03**
4.0	7.53	2.44	393.53	0.38	14.68	3.09	**0.01**
5.0	7.19	2.44	393.53	0.04	14.34	2.95	**0.01**
6.4	3.45	2.44	393.53	-3.70	10.60	1.41	0.22
8.0	-0.20	2.44	393.53	-7.35	6.95	-0.08	0.94
10.1	3.30	2.44	393.53	-3.85	10.45	1.35	0.22
12.7	-4.08	2.31	393.55	-10.85	2.69	-1.77	0.12
16.0	-6.61	2.31	393.55	-13.38	0.17	-2.86	**0.01**
20.2	-7.16	2.31	393.55	-13.93	-0.39	-3.10	**0.01**
25.4	-2.88	2.31	393.55	-9.65	3.89	-1.25	0.25
32.0	-0.67	2.31	393.55	-7.44	6.10	-0.29	0.83
40.3	4.72	2.31	393.55	-2.05	11.50	2.04	0.07

Appendix J: Pairwise contrasts of estimated marginal means of the proportion of flies that responded to each of the three song types (ancestral, purring, rattling) with a false discovery rate (FDR) correction (N = 34).

Contrast	estimate	se	asymp.lcl	asymp.ucl	z ratio	p value
FL ancestral - HI ancestral	-4.98	1.60	-9.67	-0.29	-3.11	**3.07e-03**
FL ancestral - FL purring	0.02	0.67	-1.96	1.99	0.02	0.98
FL ancestral - HI purring	-1.21	0.65	-3.12	0.70	-1.86	0.07
FL ancestral - FL rattling	1.74	0.82	-0.68	4.15	2.11	**0.04**
FL ancestral - HI rattling	-2.46	0.66	-4.40	-0.52	-3.72	**4.94e-04**
HI ancestral - FL purring	4.99	1.50	0.59	9.40	3.33	**1.65e-03**
HI ancestral – HI purring	3.77	1.49	-0.61	8.14	2.52	**0.02**
HI ancestral - FL rattling	6.72	1.57	2.09	11.34	4.26	**7.51e-05**
HI ancestral – HI rattling	2.52	1.50	-1.87	6.91	1.68	0.10
FL purring - HI purring	-1.23	0.35	-2.26	-0.20	-3.49	**1.02e-03**
FL purring - FL rattling	1.72	0.61	-0.08	3.52	2.80	**7.57e-03**
FL purring – HI rattling	-2.48	0.37	-3.56	-1.39	-6.69	**1.63e-10**
HI purring - FL rattling	2.95	0.59	1.22	4.68	4.99	**2.96e-06**
HI purring - HI rattling	-1.25	0.33	-2.21	-0.28	-3.79	**4.56e-04**
FL rattling - HI rattling	-4.20	0.60	-5.96	-2.43	-6.97	**4.61e-11**

Appendix K: Pairwise contrasts of estimated marginal means of the behavioral response thresholds of Hawaiian and Floridian flies to the three song types (ancestral, purring, rattling) with a false discovery rate (FDR) correction (N = 34).

Contrast	estimate	se	df	lower.cl	upper.cl	t ratio	p value
FL ancestral - HI ancestral	1.18	4.75	350.00	37.15	65.20	10.78	**1.00e-22**
FL ancestral – FL purring	3.65	3.47	344.05	3.38	23.91	3.93	**1.19e-04**
FL ancestral - HI purring	3.12	3.81	269.33	21.85	44.39	8.70	**7.05e-16**
FL ancestral - FL rattling	.47	3.47	344.05	-6.80	13.74	1.00	0.34
FL ancestral - HI rattling	5.00	3.81	269.33	23.73	46.27	9.20	**2.60e-17**
HI ancestral - FL purring	37.53	3.81	269.33	-48.80	-26.26	-9.86	**3.26e-19**
HI ancestral - HI purring	18.06	3.47	344.05	-28.33	-7.79	-5.20	**5.16e-07**
HI ancestral - FL rattling	47.71	3.81	269.33	-58.98	-36.44	-12.54	**1.56e-27**
HI ancestral - HI rattling	16.18	3.47	344.05	-26.44	-5.91	-4.66	**5.73e-06**
FL purring - HI purring	9.47	2.54	83.38	11.80	27.14	7.67	**4.72e-11**
FL purring - FL rattling	10.18	2.01	344.05	-16.10	-4.25	-5.07	**8.68e-07**
FL purring - HI rattling	1.35	2.54	83.38	13.68	29.02	8.42	**1.76e-12**
HI purring - FL rattling	29.65	2.54	83.38	-37.32	-21.98	-11.68	**9.12e-19**
HI purring - HI rattling	.88	2.01	344.05	-4.05	7.81	0.94	0.35
FL rattling - HI rattling	1.53	2.54	83.38	23.86	39.20	12.43	**5.71e-20**