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Surviving the Serenade: How Conflicting Natural and Sexual Selection Drove Rapid Diversification of Mating Signals in an Insect

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Abstract

Understanding how the early stages of sexual signal diversification proceed is of critical importance because the nature of these microevolutionary dynamics can directly shape species trajectories and macroevolutionary patterns. Unfortunately, studying signal diversification is challenging because signals involve complex interactions between behavioral, morphological, and physiological components, many of which can only be measured in real time. Here we had the opportunity to directly study this process with the recent increase in sexual signal variation in Hawaiian populations of the Pacific field cricket (*Teleogryllus oceanicus*). In Hawaii, male song attracts both female crickets and a deadly acoustically orienting parasitoid fly (*Ormia ochracea*). This interaction led to the emergence of a silent morph that evades costly parasitism, and, more recently, novel male morphs that produce attenuated songs and may balance the natural-sexual selection conflict differently. We capitalize on this case of rapid evolution to answer questions about the earliest stages of signal diversification by 1) characterizing novel signal diversity, 2) investigating mechanisms underlying the production of new signals, 3) tracking evolutionary change in the relative abundance of morphs, and 4) interrogating the selective landscape driving such rapid evolutionary change. We first characterize the signals of novel cricket morphs, finding that several differ in how their wings generate song. This work illustrates how the rewiring of wing-song relationships can facilitate phenotypic diversification. Evidence also points to ongoing rapid evolution within and among populations. Our repeated sampling over approximately 12 generations revealed that some novel morphs are increasing in commonality within populations, some are spreading across the archipelago, and at least one has already diverged across island populations. Finally, we investigate ultimate explanations for such rapid evolution by characterizing major selective pressures imposed by intended and unintended receivers. We find that each novel morph's sexual signal is a unique evolutionary solution to the challenge of attracting mates while evading parasitism. Overall, our findings demonstrate the rapid pace of evolution in island populations, provide insights into the origins and divergence of new sexual signals over time, and illustrate the utility of this emerging model system as a microcosm for answering fundamental questions in evolutionary biology.

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Surviving the serenade: how conflicting natural and sexual selection drove rapid diversification of mating signals in an insect

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by

James H. Gallagher

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Understanding how the early stages of sexual signal diversification proceed is of critical importance because the nature of these microevolutionary dynamics can directly shape species trajectories and macroevolutionary patterns. Unfortunately, studying signal diversification is challenging because signals involve complex interactions between behavioral, morphological, and physiological components, many of which can only be measured in real time. Here we had the opportunity to directly study this process with the recent increase in sexual signal variation in Hawaiian populations of the Pacific field cricket (*Teleogryllus oceanicus*). In Hawaii, male song attracts both female crickets and a deadly acoustically orienting parasitoid fly (*Ormia ochracea*). This interaction led to the emergence of a silent morph that evades costly parasitism, and, more recently, novel male morphs that produce attenuated songs and may balance the natural-sexual selection conflict differently. We capitalize on this case of rapid evolution to answer questions about the earliest stages of signal diversification by 1) characterizing novel signal diversity, 2) investigating mechanisms underlying the production of new signals, 3) tracking evolutionary change in the relative abundance of morphs, and 4) interrogating the selective landscape driving such rapid evolutionary change. We first characterize the signals of novel cricket morphs, finding that several differ in how their wings generate song. This work illustrates how the rewiring of wing-song relationships can facilitate phenotypic diversification. Evidence also points to ongoing rapid evolution within and among populations. Our repeated sampling over approximately 12 generations revealed that some novel morphs are increasing in commonality within populations, some are spreading across the archipelago, and at least one has already diverged across island populations. Finally, we investigate ultimate explanations for such rapid evolution by characterizing major selective pressures imposed by intended and unintended receivers. We find that each novel morph's sexual signal is a unique evolutionary solution to the challenge of attracting mates while evading parasitism. Overall, our findings demonstrate the rapid pace of evolution in island populations, provide insights into the origins and divergence of new sexual signals over time, and illustrate the utility of this emerging model system as a microcosm for answering fundamental questions in evolutionary biology.

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Chapter One

Decoupling of sexual signals and their underlying morphology facilitates rapid phenotypic diversification

(Chapter One is published in *Evolution Letters*, Volume 6, pp. 474-489.)

ABSTRACT

How novel phenotypes evolve is challenging to imagine because traits are often underlain by numerous integrated phenotypic components, and changes to any one form can disrupt the function of the entire module. Yet novel phenotypes do emerge, and research on adaptive phenotypic evolution suggests that complex traits can diverge while either maintaining existing form-function relationships or through innovations that alter form-function relationships. How these alternate routes contribute to sexual signal evolution is poorly understood, despite the role of sexual signals in generating biodiversity. In Hawaiian populations of the Pacific field cricket, male song attracts both female crickets and a deadly acoustically orienting parasitoid fly. In response to this conflict between natural and sexual selection, male crickets have evolved altered wing morphologies multiple times, resulting in loss and dramatic alteration of sexual signals. More recently, we and others have observed a radical increase in sexual signal variation and the underlying morphological structures that produce song. We conducted the first combined analysis of form (wing morphology), function (emergent signal), and receiver responses to characterize novel variation, test alternative hypotheses about form-function relationships (Form-Function Continuity vs. Form-Function Decoupling) and investigate underlying mechanistic changes and fitness consequences of novel signals. We identified three sound-producing male morphs (one previously undescribed, named “rattling”) and found that relationships between morphology and signals have been rewired (Form-Function Decoupling), rapidly and repeatedly, through the gain, loss, and alteration of morphological structures, facilitating the production of signals that exist in novel phenotypic space. By integrating across a hierarchy of phenotypes, we uncovered divergent morphs with unique solutions to the challenge of attracting mates while evading fatal parasitism.

INTRODUCTION

The origin of evolutionary novelty is one of the most perplexing yet fundamental processes in the generation of biodiversity. It is difficult to envision how novel traits arise, as many traits are complex and underlain by multiple morphological and physiological components (forms) that interact to dictate trait function (Wagner and Altenberg 1996). Because natural and sexual selection act upon trait function rather than the underlying forms themselves (Arnold 1983; Losos 2011) (e.g., selection acts on the bite force exerted by a jaw structure; (Alfaro et al. 2005), and the perceived color emitted from a pigmented wing spot; (Grether et al. 2004)), it is necessary to carefully consider the relationships between form and function in order to understand the diversification of complex traits. There are many uses of the term "function" in the study of ecology and evolution, but here we follow Bock (Bock 1980) and use the term to refer to all emergent "physical and chemical properties of a feature arising from its form" (a concept also sometimes referred to as functional "-consequences" or "-capabilities"; Losos 2011). Decades of research suggests that phenotypic evolution can be either hindered or facilitated when multiple forms contribute to trait function. On one hand, phenotypic components of complex traits may covary in their expression due to genetic linkage, pleiotropy, and developmental constraints (i.e., phenotypic integration; Lande and Arnold 1983; Cheverud 1996; Pigliucci 2003; Cooper et al. 2011), constraining potential evolutionary trajectories (Lande and Arnold 1983; Klingenberg 2008). But trait complexity may also provide the conditions for novelty to evolve (Navalón et al. 2020). Many-to-one mapping (Alfaro et al. 2005; Wainwright et al. 2005) allows multiple phenotypic combinations to reach equivalent functional outcomes (alternate relationships between form and function), and may facilitate the evolution of new paths to fitness peaks (Wainwright 2007).

Evolution that rewires form-function relationships has long been recognized as facilitating the colonization of new ecological spaces (Mayr 1960; Simpson 1984; Heard and Hauser 1995; Wainwright 2007), but its role in the diversification of sexual signals (i.e., emergent sensory characteristics that receivers experience) is less well understood (Eliason 2018; but see Clark et al. 2011; Maia et al. 2013; Eliason et al. 2015), despite the key role of sexual signal divergence in the generation and maintenance of biodiversity (West-Eberhard 1983; Pomiankowski and Iwasa 1998; Gray and Cade 2000; Panhuis et al. 2001; Mendelson and Shaw 2002; Niehuis et al. 2013; Servedio and Boughman 2017; Kopp et al. 2018). The diverse, and often conflicting, selective pressures acting on sexual signals (e.g., from intended and unintended receivers; Rosenthal 2017) make them a particularly interesting case in which to study the origins of novelty. Research on the role of sexual selection in signal evolution frequently focuses on the directional and incremental elaboration of ornaments (Coyne, Jerry A and Orr, H Allen 2004; Bradbury and Vehrencamp 2011). However, novel sexual signals (*sensu* Broder et al. 2021a) may also evolve through complex modifications to relationships between form and function, as they often include multiple sensory components (Elias et al. 2005; Hebets and Papaj 2005; Mullen et al. 2007), each of which is produced by underlying morphology (Hebets et al. 2016). It remains unclear whether sexual signal novelty is more often generated through the evolution of exaggerated forms that maintain ancestral form-function relationships (e.g., Møller 1988), or through morphological innovations that decouple structures from signal properties (Mhatre et al. 2012; Figure 1.1). However, testing how form-function relationships are maintained or altered during periods of signal divergence is challenging due to the difficulty of reconstructing the causes and consequences of evolutionary changes that took place long ago, and the extreme rarity of opportunities to directly observe signal divergence (Svensson and Gosden 2007; Svensson 2019).

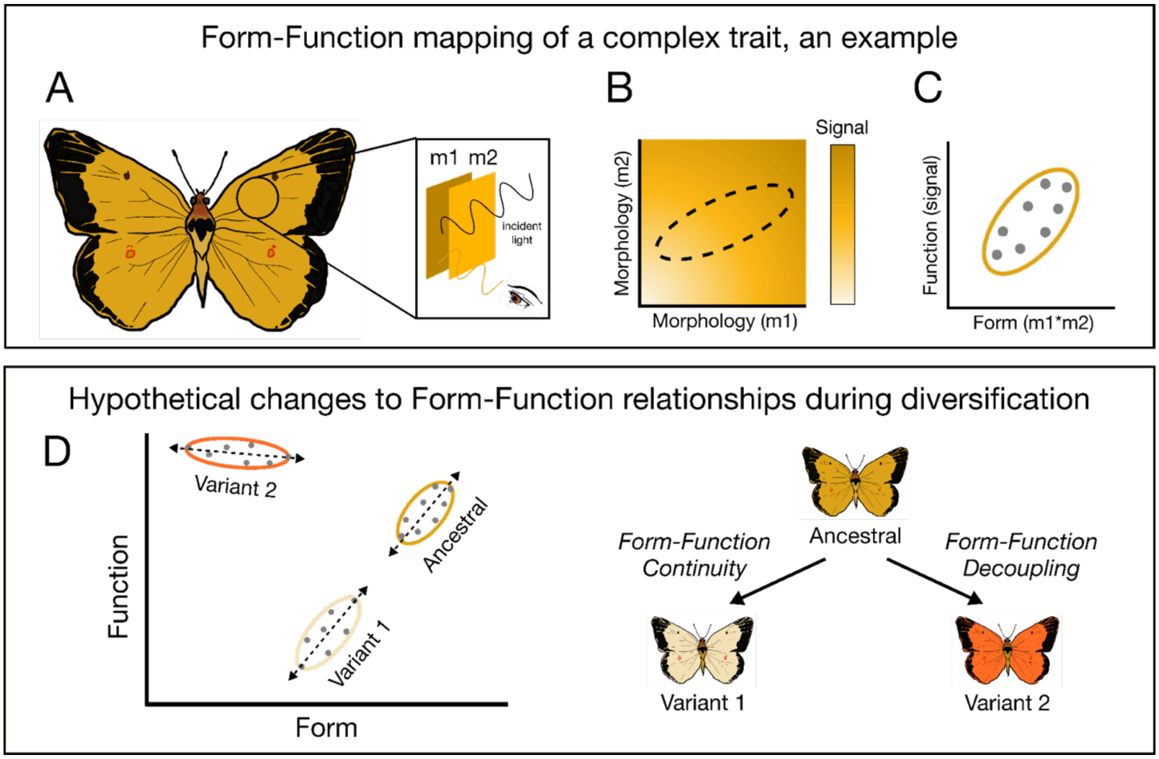

Figure 1.1. Phenotypic diversification of complex traits depends upon form-function relationships. A) A hypothetical scenario where a complex sexual signal (wing coloration; example inspired by Rutowski et al. 2005) is produced by two morphological components: separate cell layers containing different pigments (m1, m2). B) In the simplest case, two components map independently and additively to dictate function (signal value). There are multiple ways to achieve equivalent signal values within the resulting morphospace. However, functionally related traits are rarely fully independent, but rather are correlated in their expression (indicated by dashed oval), constraining the morphospace into which future phenotypes could evolve. C) We can similarly visualize form-function relationships (here, signal-by-morphology composite variable). D) The form-function plot from C is expanded to include novel variants 1 and 2. Diversification of complex traits can occur while maintaining the established, ancestral form-function relationships (Form-Function Continuity); variant 1 has the same form-function

relationship (slope) as the ancestral cluster. Alternatively, innovations that rewire form-function relationships can facilitate diversification by expanding available morphospace (Form-Function Decoupling); the variant 2 cluster has a novel slope, indicating that the previous form-function relationship has been changed, allowing the new orange color to evolve.

In this study we capitalize on the real-time, rapid evolution of acoustic sexual signals in Hawaiian populations of the Pacific field cricket, which provides a rich opportunity to characterize novel signal variation and test how form-function relationships are reconfigured during bursts of increased signal variation. Male crickets use song in two contexts associated with mating: they produce a long-distance calling song to attract females from afar and a courtship song to entice females to mount once they are in close proximity (Alexander 1962). These songs convey various information to receivers, with calling song primarily providing species, sex, and location-based information, and courtship song indicating fitness-related traits, such as immune function (Tregenza et al. 2006; Zuk et al. 2008; Simmons et al. 2010). Sound is generated when crickets drag the scraper of one wing across the file, a modified wing vein with a row of many continuous small teeth, on the other wing, resonating important veins and structures such as the harp and mirror to create sound (Ewing 1989, Bennet-Clark 1999a); changes to these structures can affect sound characteristics of the resulting songs (Bennet-Clark 1987, 2003; Koch et al. 1988; Desutter-Grandcolas 1998). However, in Hawaiian populations, male calling songs attract not only potential mates (female crickets) but also a recently introduced parasitoid fly, *Ormia ochracea* (Lehmann 2003). After locating a potential host by eavesdropping on their song, gravid female flies deposit their planidia (specialized larvae) on the male cricket (Adamo et al. 1995). These larvae develop inside the cricket's body cavity and after devouring the animal's insides, eat their way out in a dramatic scene that harkens childhood nightmares spurred on by the movie “Alien.” In response to this strong selective pressure from the fly (historically 27% of males parasitized; Zuk et al. 1993), separate populations of Hawaiian *T. oceanicus* independently lost sound-producing structures on their wings, rendering these males obligately silent (named “flatwing” or “silent” males; Zuk et al. 2006; Tinghitella 2008; Pascoal et al. 2014). Silent males are protected from parasitism, but their inability to sing makes mate attraction challenging (Zuk et al. 2006; Tinghitella 2008; Pascoal et al. 2014). Silent crickets do, however, retain ancestral wing movement patterns (stridulation; Rayner et al. 2020) and some vestigial wing structures, features which have been hypothesized to provide opportunity for the evolution of novel signal function (Bailey et al. 2019). Indeed, in 2017 a new male morph called “purring” was discovered that produces a novel song that attracts mates but evades the parasitoid fly (Tinghitella et al. 2018, 2021). Thus, purring appears to be a novel solution to the conflict between natural and sexual selection in this system; it has since become common across Hawaii (Tinghitella et al. 2021).

While the evolution of two novel morphs in two decades is itself remarkable, the story is far from complete. Since the discovery of purring, we and others have observed a radical increase in sexual signal variation and the underlying morphological structures that produce song (e.g., Rayner et al. 2019). Much of this variation has not been characterized, and the underlying mechanisms and fitness consequences of novel signals remain largely unknown. Here, we conduct the first combined analysis of detailed morphological, song, and fitness data from six Hawaiian populations of *T. oceanicus* to: 1) ask whether male signal diversification supports patterns of evolutionary change through Form-Function Continuity or Form-Function Decoupling (Figure 1.1), 2) characterize groups of males with shared morphology and signals (hereafter, morphs) in order to compare form-function relationships among morphs, and 3) investigate the fine-scale morphological mechanisms and fitness trade-offs underlying novel songs. We find that form-function relationships between morphology and emergent sexual signals have been rewired, rapidly and repeatedly, through the gain, loss, and alteration of morphological structures, demonstrating how innovations that decouple form and function can facilitate the evolution of novel phenotypes.

METHODS

Collection, Recording, and Photography

In June 2019, we collected 153 adult males and 172 adult females from six Hawaiian populations: Hilo, Kalaupapa, Manoa, La'ie, Wailua, and Kapa'a (see Appendix A and Supplemental Methods for sampling details). We housed animals with ad libitum rabbit food, damp cotton (for water), and an egg carton shelter; males were housed individually in 0.5 L plastic deli cups, and females were housed in groups, by site, in 15 L plastic containers. We recorded both calling and courtship songs of individual males 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) positioned 10 cm above the cricket. For courtship recordings we added an adult female to the male's container to encourage courtship stridulation. All recordings were conducted indoors during the animals' natural scotoperiod in rooms, lit with only red light. Each recording captured at least one complete bout of uninterrupted song. We took photographs of each male's right wing under natural daylight using a digital SLR camera (Pentax K-5, Hoya Corp., Tokyo, Japan; Tamron SP 90mm F/2.8 macro lens, Tamron USA Inc., Commack, NY) positioned 10 cm directly above the wing. We gently lifted forewings and pressed them flat on a piece of paper with a printed ruler to facilitate visualization of wing venation. After recording and photographing was complete, we returned all crickets to their collection sites.

Song Analysis

We analyzed the second cleanly recorded (without background noise) song from the first bout of continuous song from each male's calling and courtship recordings (see Supplemental Methods for more detail). We measured nine sound characteristics that capture variation in frequency, amplitude, and broadbandedness (Figure S1.1). We first determined each song's dominant frequency in Audacity (version 2.3.1, The Audacity Team) using the plot spectrum analysis function (settings: Hanning window, size=256, log frequency axis). All remaining song analyses were conducted in Logic Pro X (version 10.4.8, Apple Inc., Los Altos, CA USA). We determined the amplitude (RMS level) of each song using Logic Pro X's Level Meter, and then measured the amplitude of six different frequency ranges (Figure S1.1), chosen because they reflect natural clusters of auditory receptor fibers, and thus 'peaks' and 'valleys' in *T. oceanicus* hearing ability (Imaizumi and Pollack 1999; Tinghitella et al. 2021). We calculated the relative amplitude of each frequency range by dividing the range's amplitude by the sum of all frequency range amplitudes. We calculated frequency evenness as the additive inverse of the standard deviation of the relative amplitudes of all frequency ranges (Figure S1.1).

Some song characteristics were correlated with one another, so to understand how songs differed among morphs, we first used principal component analysis (PCA) as a variable reduction technique, collapsing characteristics into fewer axes that describe independent covariance. Because male crickets produce both a calling and courtship song, we conducted separate PCAs on recordings of these distinct signals (Calling Song: N=143; Courtship Song: N=112).

Wing Morphometrics

We chose fourteen landmarks (Figure S1.2, Table S1.2) based on previous morphometric work in this species (Pascoal et al. 2014, 2017) that capture variation in wing structures known to play a role in sound production (Huber et al. 1989,Bennet-Clark 1999b, 2003; Prestwich et al. 2000). We placed landmarks on photos of the right wing of each male using tpsDIG2 (v2.3.1; Rohlf 2006; see Supplemental Methods), and used the R package *geomorph* (Adams and Otárola Castillo 2013) to reduce the dimensionality of morphological data using a PCA (gm.prcomp function) that included xy coordinates of all fourteen wing landmarks (N=131). In addition to the composite morphological variables generated by the PCA, we extracted further information from photographs about specific wing structures by: 1) scoring the presence or absence of the scraper and the mirror, two potentially sound-altering structures that are sometimes absent in the recently evolved male morphs, 2) measuring the width of the harp (an important resonator in sound production; Bennet-Clark 1999b, 2003; Prestwich et al. 2000) by calculating the linear distance along the x-axis between landmarks 5 and 14 (Figure S1.2), and 3) measuring mirror size by subsetting landmark data to only include points marking the mirror's perimeter (landmarks 6-11, Figure S1.2), and extracting centroid sizes (gpagen function in *geomorph*).

Morphology and Performance of Novel Morphs

Because the above morphometric analyses used photographs of live animals (to avoid destructive sampling), we could not examine microstructures in these animals. In 2020 we collected an additional 48 males from the field and removed right wings (22 ancestral (Mo'orea, Hilo), 11 rattling (Hilo), and 15 purring (Manoa)). We used a VHX-7000 Digital Microscope (Keyence Corporation, Itasca, IL USA) to view and measure the spacing of teeth on the files of purring, rattling, and ancestral males (see Supplemental Methods).

In January 2020 we also collected female *T. oceanicus* and *O. ochracea* (see Walker 1989; Tinghitella et al. 2021) from Hilo for use in behavioral phonotaxis experiments. See Tinghitella et al. 2021 for detailed cricket and fly phonotaxis methods. Briefly, crickets were placed in an arena and played stimuli (purring, rattling, ancestral, and white noise control) in a random order for one minute each or until speaker contact (ancestral always played last). For each phonotaxis trial (N=30 females), we measured whether or not the female cricket exhibited positive phonotaxis and whether they contacted the speaker. Flies (N=8) were tested using the same set of stimuli during their active searching time (dusk) in a 40 x 40 x 61 cm mesh cage where they traveled down (flying and/or walking) towards a speaker broadcasting sound, and we recorded whether they contacted the speaker (yes/no).

To investigate how purring wings produce audible song despite lacking many of the same important sound-producing structures as silent males, we measured the presence/absence of the scraper on a set of wings from first-generation, lab-born Wailua males (N=27) that hatched from eggs collected in the field in 2015.

Statistical Analysis

We performed all statistical analysis using RStudio (RStudio Team 2020, R version 3.6.3; see supplemental R script and data). We first visualized form-function relationships by plotting features of wing morphology against a subset of calling song characteristics using all males in our sample. In order to identify major clusters of variation based on (dis)similarities in both song and wing phenotypes, we subset 59 individuals which had complete morphological and song (both calling and courtship) data (using all individual characteristics for calling and courtship song, plus all wing morphology variables from Table S1.3), and performed hierarchical clustering using the hclust function (Ward.D2 agglomeration method) in the *factoextra* package (Kassambara et al. 2017). The gap statistic calculated using the hcut and fviz_nbclust functions in *factoextra* identified k=3 as the best supported number of phenotypic clusters (morphs). We next used the phenotypic characteristics that defined morphs in the cluster analysis to manually classify a larger sample (N=105) of field-caught males for which we had both morphological data and recordings of at least one song type (but not necessarily both calling and courtship songs, as was required for inclusion in the initial clustering analysis). To further assess differences among morphs in song and wing variation using this larger data set, we conducted multivariate analysis of variance (MANOVAs) separately for calling song, courtship song, and wing morphology, using the first two composite axes of phenotypic variation (PC1-2) as response variables and morph as a predictor. We next calculated correlations amongst scaled wing and song traits within-morphs by calculating Pearson’s product moment correlations, and estimated statistical significance using asymptotic t approximations using the rcorr function in the package *Hmisc* (Harrell et al. 2008). A Welch’s two-sample t-test tested for differences in calling song mean dominant frequency between purring males with and without scrapers.

To test for differences in female cricket responses to song variants, we conducted generalized linear mixed models with binomial error structures in *lme4* (Bates et al. 2007), with the presence/absence of phonotactic behavior as the response variables, song variant as a fixed predictor variable and a female’s individual ID as a random effect (to account for individual-level variation in overall responsiveness). We compared outcomes between song stimuli using pairwise estimated marginal means in the package *emmeans* (Lenth 2021). Due to complete separation in our data when using contact with the playback speaker as a response (no crickets ever contacted a speaker broadcasting white noise), we performed a penalized logistic regression (Firth’s bias-reduced Logistic Regression) in the *logistf* package in R (Heinze et al. 2020).

RESULTS

Decoupling of form and function during phenotypic diversification

To test whether wing morphology and song characteristics covary in similar or different ways among male *T. oceanicus*, we comprehensively measured morphology and signals of male crickets across six populations on four Hawaiian islands. Form-function relationships were nonlinear across males; a range of wing morphologies produce similar signal values, while highly variable songs result from wings with similar morphology (Figure 1.2C). These patterns suggest that males produce different sexual signals via alternate pathways between wing and song (Form-Function Decoupling; Figure 1.1D, 1.2B). Notably, these nonlinear relationships were evident for all major signal characteristics we investigated (frequency, amplitude, broadbandedness, and a composite of all three, PC1; Figure S1.1), indicating broadscale decoupling of wing morphology and signal. Having found support for Form-Function decoupling, we next asked if decoupling occurred once or multiple times, which required us to first identify clusters of males with shared morphology and signals (morphs).

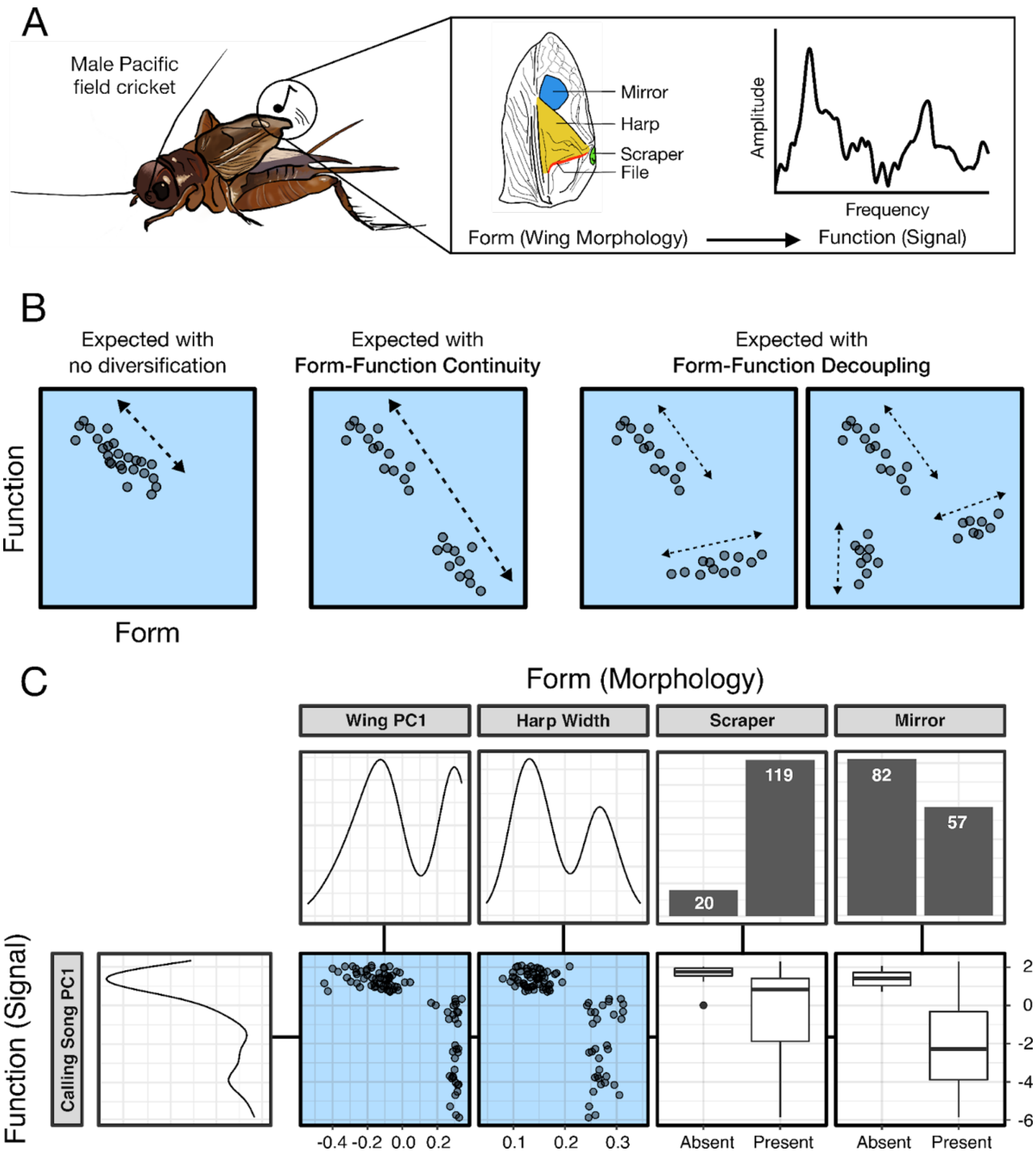

Figure 1.2. Rapid evolution of sexual signals in T. oceanicus provides a rare opportunity to test how complex traits diversify. A) Rapid evolution of sexual signals in T. oceanicus provides a rare opportunity to test how complex traits diversify. Morphological components of wings (mirror, harp, scraper, file) produce mating signals (songs) that vary in spectral characteristics (function, e.g. frequency and amplitude). B) As described in Figure 1.1, diversification of sexual signals may occur while maintaining or rewiring form-function relationships. Hypothetical data display patterns supporting no diversification, diversification with Form-Function Continuity, and diversification with Form-Function Decoupling. C) We investigate form-function relationships across male Hawaiian T. oceanicus, using form to describe wing morphology and function to describe

song characteristics (analogous to the use of, for instance, form to describe jaw morphology and function to describe bite force in Alfaro et al. 2005). Calling song recordings and morphometric analyses of field-caught males showed inconsistent relationships between morphological and signal components across Hawaii (blue boxes), matching patterns shown in panel B that are expected given Form-Function Decoupling. Form-function relationships differ among males across Hawaii, as points do not fall along a single axis of covariation. Two important wing structures (scraper, mirror) are present in some sound-producing males, but absent in others.

We performed hierarchical clustering using 33 measures of song and wing characteristics (Table S1.2 and Figures S1.1-S1.2) from field-caught male crickets for which we had complete data (N=59; calling and courtship song recordings, and wing morphometrics). We uncovered three distinct phenotypic clusters that we define as “ancestral,” “purring,” and a new “rattling" morph that we describe for the first time here (Figure 1.3A; gap statistic: k=3; see Table S1.3 for morph-level means and SDs of all traits). Ancestral males had traits characteristic of *T. oceanicus* from their ancestral range in Australia: wings with fully developed harps and mirrors, and loud, tonal songs, with a low dominant frequency (Bennet-Clark 1999b, 2003). Consistent with previous work (Tinghitella et al. 2018), purring males lacked mirrors altogether, had reduced harps (Figure 1.3B, S1.3; similar to silent males; Zuk et al. 2006), and produced detectable but dramatically quieter (low amplitude), more broadband songs (high frequency evenness) with variable dominant frequencies (Figure 1.3C, S1.3; as in (Tinghitella et al. 2018)). In contrast, the newly discovered rattling morph had categorically different songs from the other two morphs (more power in middle frequencies, intermediate amplitude and frequency evenness; Figure 1.3C, S1.3), and differed from ancestral males in song but not wing morphology (as measured by traditional landmarking; Figure 1.3B). Corroborating the discrete phenotypic groupings revealed by hierarchical clustering (Figure 1.3), MANOVAs of wing and song variation from a larger sample of field-caught males (see methods for criteria for inclusion; N = 105) showed dramatic differences among morphs (MANOVA, Calling Song: $F_{4,174}$=77.8, p<0.0001; Courtship Song: $F_{4,140}$=32.2, p<0.0001; Wing Morphology: $F_{4,204}$=48.8, p<0.0001; Figure S1.3). Morph-level clustering persisted in lab-reared animals after two generations in a common garden, suggesting that rearing conditions have little effect on these distinct phenotypes (Figure S1.4, Supplemental Methods; MANOVA; Morph: $F_{4,248}$=60.0, p<0.0001; Rearing Treatment: $F_{2,123}$=2.0, p=0.14; Morph x Rearing Treatment: $F_{4,248}$=0.58, p=0.68).

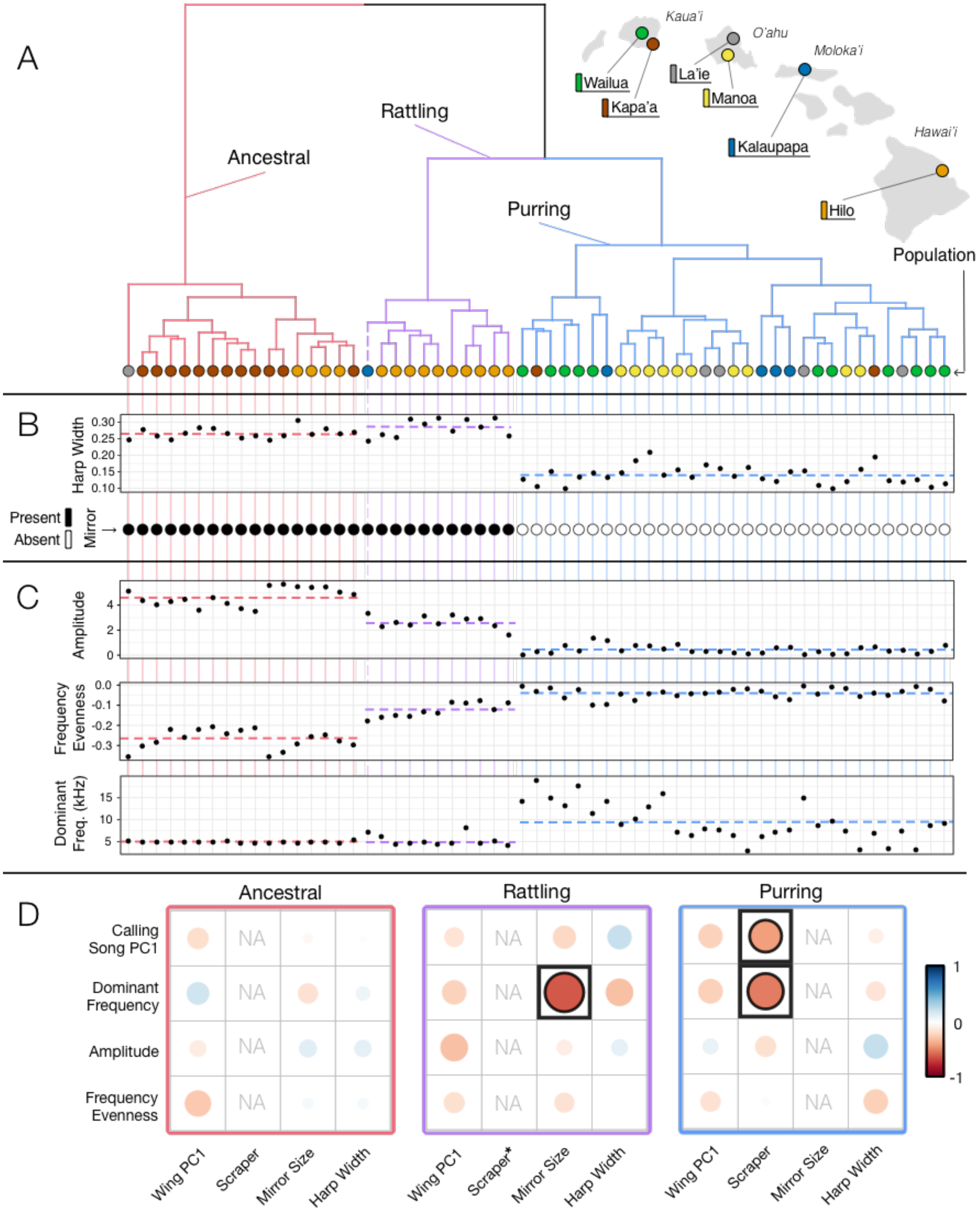

Figure 1.3. We identified three distinct morphs (ancestral=red, purring=blue, and previously undescribed rattling=purple) with different morphology-song relationships. A) Dendrogram of 59 males from across Hawaii, generated via hierarchical clustering based on phenotypic (dis)similarities (Gap statistic: k=3). Leaves of branches are colored by population. Rattling appears unique to the Hilo population; a single individual from

Kalaupapa (dashed branch) clustered with rattling, but this was due to uniquely abnormal harp venation, generating songs similar to rattling. B) Morphology: ancestral and rattling individuals have similar wing morphology, possessing mirrors and wide harps. In contrast, purring males lack mirrors and have reduced harps. C) Signal: all three morphs differ in amplitude, frequency evenness, and dominant frequency. D) Differences among morphs in the correlations between wing structures and calling song characteristics illustrate form-function decoupling (all continuous variables except scraper presence/absence). Bold cells highlight significant morphology-song relationships, which differ among morphs. The NAs represent cases where within-morph variation was insufficient for calculating meaningful correlations (e.g., all ancestral males possessed scrapers, while no purring males had mirrors). *Only a single rattling individual was missing a scraper (1/13), so it was impossible to calculate meaningful correlation coefficients between scraper and rattling song characteristics. Note that different morphological features in rattling and purring males (mirror size and scraper presence, respectively) were correlated with the same song component (Dominant Frequency).

To examine how form-function relationships differ among the three morphs we just described, we tested how song variation correlates with morphology within each morph. As expected for a trait that has historically been under strong stabilizing selection, we found significantly lower variation in the morphology of ancestral males (Levine's test: $F_{2,103}$=21.2, p<0.0001; Figure S1.5) resulting in weak correlations between morphology and song features (Figure 1.3D). But both of the derived morphs, purring and rattling, had unique sets of correlations between calling song and morphology components (Pearson's correlations Table S1.4; Figure 1.3D). For instance, dominant frequency varies with mirror size in rattling males, but with scraper presence in purring males. This is further evidence that form-function relationships have been decoupled across Hawaii, as different morphological changes correspond with novel variation in the same song characteristic.

Collectively, we see strong evidence for Form-Function Decoupling in this system (Figures 1.2 and 1.3). Novel broadband, attenuated songs are produced by two separate wing types (purring and rattling), and it appears that males with similar wing morphology can produce dramatically different songs (rattling and ancestral; Figure 1.3A-C). These findings raise additional questions about the mechanistic basis of morphology-signal novelty that has evolved over the last two decades. Mirror size does appear to influence the frequency of rattling calls to some degree (Figure 1.3D), yet there is much overlap in wing morphology (including mirror size) of ancestral and rattling males despite their categorically distinct songs (Figure S1.3C). This suggests mirror size cannot explain the dramatic differences between rattling and ancestral songs (Figure S1.4B). How can morphs that appear to overlap in wing morphology (ancestral and rattling) produce non-overlapping signals (Figure S1.3C)? How do purring wings produce audible song despite lacking many of the same important sound-producing structures as silent males (Zuk et al. 2006; Tinghitella et al. 2018)?

Morphology and Performance of Novel Morphs

To further understand the morphological mechanisms producing novel signals, and because our morphometrics above did not explain the discrete differences between rattling and ancestral songs, we used digital microscopy to compare microstructures on the underside of the wing that are not measured by common landmarking approaches (Pascoal et al. 2014, 2017). Crickets make sound by moving the scraper of one wing across the file (a modified vein containing continuous microscopic teeth) on the other wing (Ewing 1989, Bennet-Clark 1999b). All rattling wings, but no purring or ancestral wings, had distinct gaps where file tooth development was disrupted (Figure 1.4A). Spacing among individual teeth contributes to song differences among cricket species (Desutter-Grandcolas 1998; Montealegre-Z 2009; Montealegre-Z et al. 2011) and is typically invariant within species due to stabilizing selection from choosy females

(Duncan et al. 2021). However, the pattern of larger gaps between groups of teeth seen here in rattling males has not been documented in crickets before, to our knowledge. Importantly, gaps in the teeth of the file were immediately apparent upon eclosion to the adult stage in lab-reared rattling males, and the proportions of rattling males were remarkably consistent when comparing field-sampled (N=8/31 males; 26% rattling) and lab-born animals (N=13/48; 27% rattling), demonstrating that gaps are not likely caused by environmental differences or age-related wear. Detailed song analysis revealed categorically different courtship songs between groups of lab-born males that differed only in the presence of file tooth gaps, further implicating tooth gaps in the generation of the distinct rattling song (Figure S1.6; t-test: t=6.68, df=7.88, p=0.0002, n=10). It is possible that the wing movements of novel male morphs like rattling differ from that of ancestral males and that this could contribute to song differences. Note, however, that both purring and silent males retain the stridulatory patterning of ancestral males (Tinghitella et al. 2018; Rayner et al. 2020). Our discovery of gaps in the file likely explains why rattling males produce dramatically different songs from ancestral males, despite largely overlapping wing morphology (Figures 1.3B, S1.3).

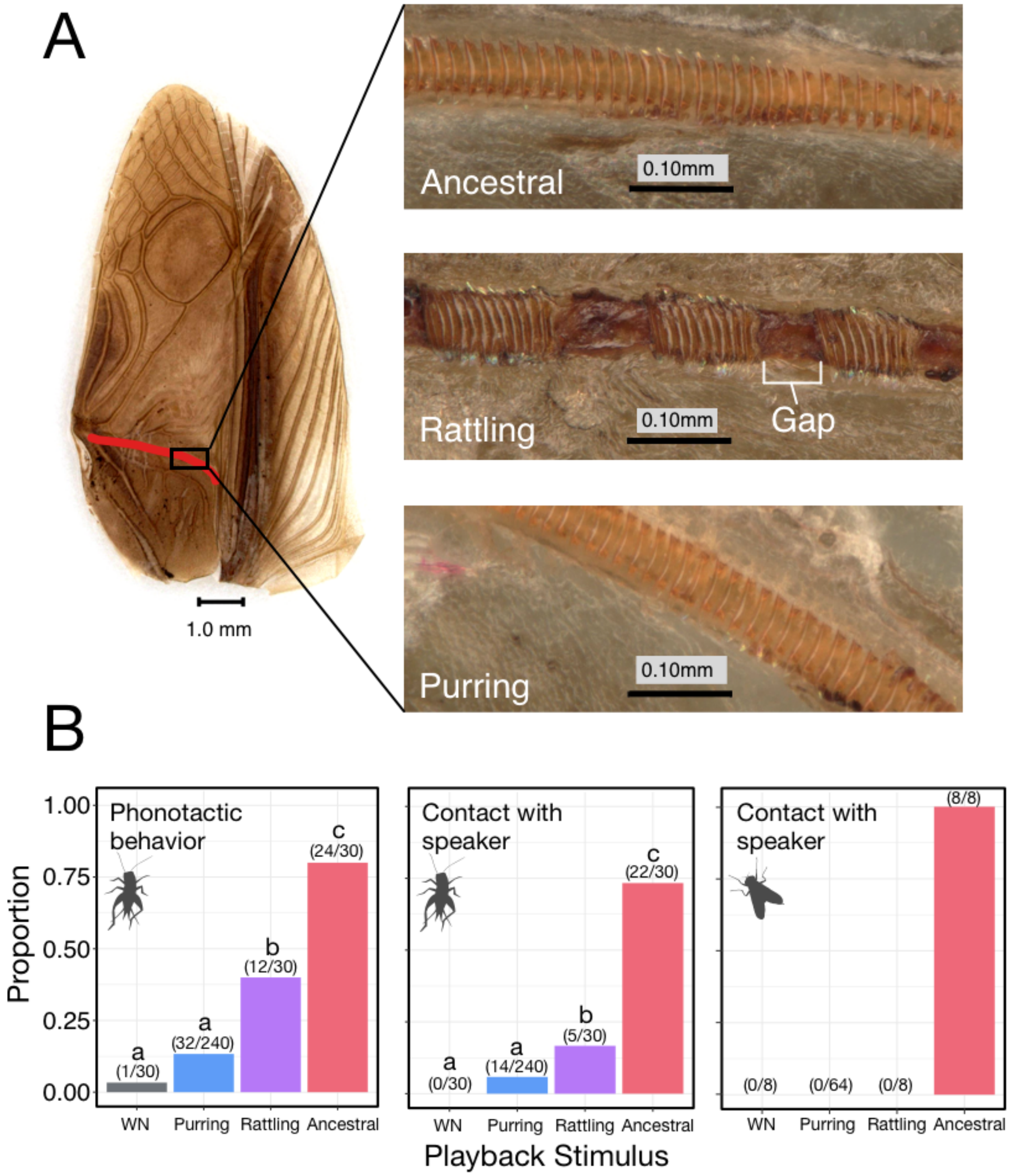

Figure 1.4. Rattling song is produced via discrete modification of an existing structure (the file), and provides a solution to the problem of attracting mates while avoiding parasitism. A) Unlike ancestral (N=0/22) and purring males (N=0/15), all rattling males (N=11/11) had distinct gaps between groups of teeth on the file (red line on example rattling wing; see Figure S1.3 for ancestral and purring example wings). B) In Hilo (where rattling exists but purring does not), rattling was more attractive to female crickets than purring and white noise (WN), but less attractive than ancestral calling song (attractiveness measured as phonotactic behavior and contact with speaker). Rattling was unattractive to parasitoid flies—they did not contact the speaker when rattling song was played, but did when ancestral song was played.

We next tested whether rattling song functions as a signal within a mating context, or as a cue to eavesdropping parasitoids, by measuring responses of female crickets and flies to playback stimuli (ancestral, rattling, purring, and white noise, following Tinghitella et al. 2021) in the population where rattling exists (Hilo). We found that female crickets, but not flies, are attracted to rattling songs (Figure 1.4B; Table S1.5), suggesting that rattling is a private mode of communication (with regard to the primary eavesdropper, *O. ochracea*), as has recently been shown for purring (Tinghitella et al. 2018, 2021). Because purring and rattling appear to be two alternative solutions to shared, conflicting natural and sexual selection pressures, selection may increase the frequencies of these morphs in the populations where they are found.

We then turned to the morphology that underlies the production of purring songs. The morphology of purring wings is very similar to that of silent male wings (Tinghitella et al. 2018). In our above analyses (Figure 1.3D), the scraper was the only wing feature significantly correlated with purring calling song variation and was specifically associated with overall variation (PC1) and dominant frequency (Figure 1.3D). Further analysis of frequency differences among purring males revealed that individuals with scrapers had calling songs with dramatically lower median dominant frequencies than scraperless males (scraper present: 7.6kHz, scraper absent: 13.6kHz; $t=-4.66$, $df=13.6$, $p=0.0004$; Figure 1.5A). Crickets are more sensitive to certain sound frequencies than others (Hoy et al. 1982), so frequency properties of a signal will affect its perceived loudness to the animal. Therefore, a shift in frequency, even without a change in overall amplitude, impacts the ability of a stimulus to elicit a behavioral response from females. The lower dominant frequencies of purring male songs with scrapers fall in a range to which female crickets are more sensitive (closer to ancestral song frequencies; Bennet-Clark 2003). Based on previously published behavioral response thresholds (Hoy et al. 1982), tones with dominant frequencies matching those of scraperless purring males would need to be approximately 19 dB louder to elicit a positive female response than those of males with scrapers (Figure 1.5A, adapted from Hoy et al. 1982; note that perceived differences by female crickets may be less extreme because purring songs are not pure tones, unlike stimuli used to generate response curves). Given the low overall amplitude of purring songs (many within 5 dB of background noise in the field), even subtle differences in detectability by females could determine which males' displays can operate as signals and which cannot (functionally silent). Identifying where exactly this sensory threshold lies will require additional neurophysiological and behavioral studies of receivers.

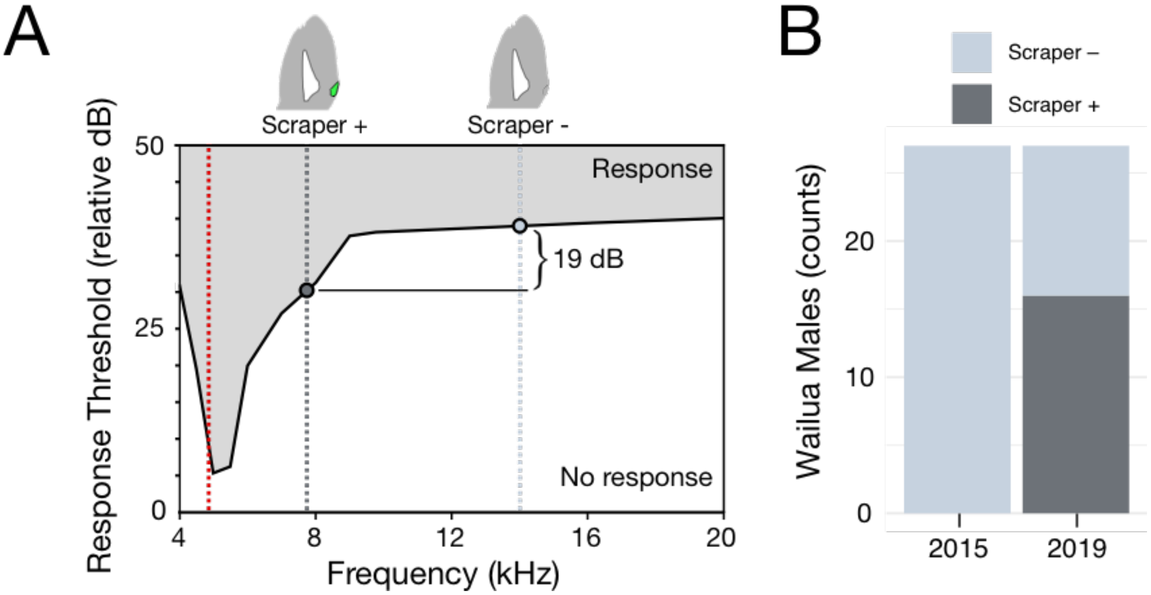

Figure 1.5. Purring males with scrapers produce lower frequency (closer to ancestral), and therefore more detectable, songs; this structure is rapidly increasing in prevalence in a population where it was previously absent. A) In this female response threshold figure

(adapted from Hoy et al. 1982), the shaded gray section shows the signal space that elicits female response. Because the amplitudes required to trigger female responses vary across frequencies, changes to mean frequency affect detectability. Dotted lines show the median calling song dominant frequency for purring males with and without scrapers (gray vertical lines), and for ancestral males (red vertical line). Purring males without scrapers would need to produce louder songs than those with scrapers to elicit female response. B) The proportion of Wailua males with scrapers increased from 0% (0/27) to ~60% (16/27) between 2015 and 2019.

Interestingly, the scraper has been implicated as a potential difference between silent and purring morphs (Tinghitella et al. 2018), which have historically been classified based on morphology (lack of many wing structures) and/or detectability by human observers. The effects of song frequency changes on perceived loudness that we describe above would not only influence intended female recipients, but also human researchers (Jackson et al. 1999; Gelfand 2001). Therefore, having identified the substantial effect of scraper on calling song characteristics, we assessed whether the increased abundance of purring males recently observed in some populations (Tinghitella et al. 2021) has coincided with an increase in scraper presence. We measured the presence or absence of scrapers on archived male wings from a population that was historically silent (10 years ago; Zuk et al. 2018) but was predominantly purring in our 2019 sample. In 2015, no sampled males (0/27) had scrapers, while ~60% (16/27) had scrapers in 2019 (Figure 1.5B). This reappearance of scrapers in Wailua—whether due to mutation, gene flow, or standing genetic variation—suggests that over only a four year period (~16 generations), the sound produced during male displays may have evolved to become more detectable, potentially restoring sexual signal function (purring; Tinghitella et al. 2018).

DISCUSSION

By integrating data across a hierarchy of phenotypes and resulting performance, we show that changes to multiple different wing structures have resulted in the evolution of novel acoustic signals (purring and rattling), each of which appears effective at attracting mates while avoiding fatal parasitism (Tinghitella et al. 2021). Divergent male morphs of the rapidly evolving Hawaiian populations of *T. oceanicus* achieve fitness through alternate relationships between morphology and signal, illustrating how the process of Form-Function Decoupling (Figure 1.1) may be important during the evolution of novel sexual signals, as is well documented for ecological traits (Mayr 1960; Simpson 1984; Heard and Hauser 1995; Wainwright 2007). The causes and consequences of evolution involving complex restructuring of relationships among phenotypic components may be mischaracterized by studies that do not jointly analyze form and function. We bridge previous work in this system on the functional genetics of wing morphology (Tinghitella 2008; Pascoal et al. 2014, 2020; Zhang et al. 2021) and the fitness consequences of signal variation (Zuk et al. 2006; Tinghitella et al. 2018, 2021), and we illustrate this in Figure 1.6. In the same way that independent mutations converged upon wings lacking sound-producing structures (silent; Pascoal et al. 2014), we show alternate routes from morphology to novel signals (non-parallel connections between Morphology and Signal in Figure 1.6). Many-to-one mapping is inherent in complex traits, allowing multiple routes from form to function (Wainwright et al. 2005); it may facilitate phenotypic innovation and expand the number of possible evolutionary trajectories (Thompson et al. 2017). Therefore, in this system, future directional selection for song characteristics that differ from ancestral song (and protect against fatal parasitism) may result in further morphological divergence among morphs (Lande 1980; Thompson et al. 2017) due to the fact that they produce signal variation through alternate morphological pathways. Finally, selection from receivers (Behavior level, Figure 1.6) is critically important in the diversification of sexual signals (Page et al. 2014; Rosenthal 2017,Broder et al. 2021a; Rosenthal and Ryan 2022), and strong natural selection against an ancestral signal (Tinghitella et al. 2021) coupled with relaxed sexual selection (Bailey and Zuk 2008; Tinghitella and Zuk 2009), as we see in Hawaiian *T. oceanicus*, may facilitate signal novelty.

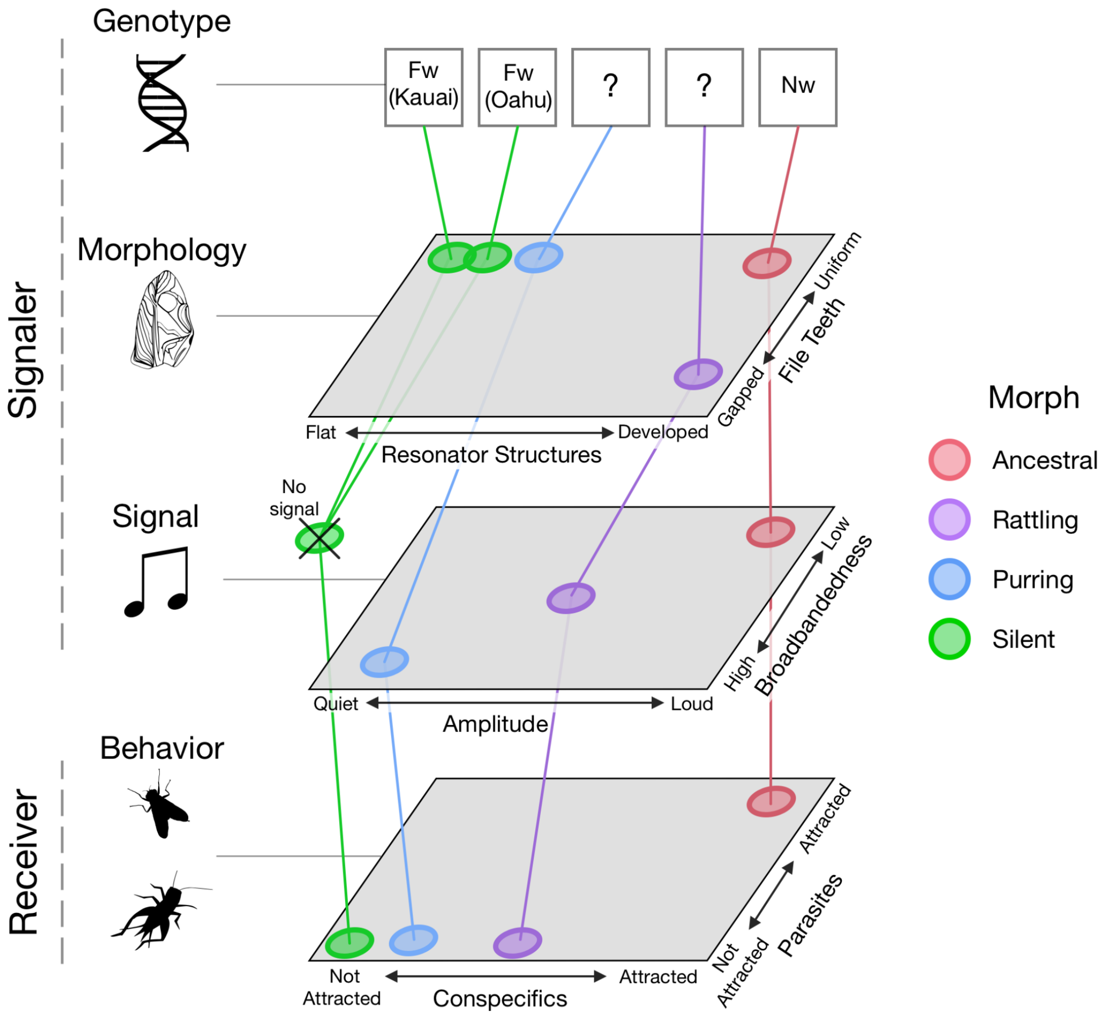

Figure 1.6. A schematic of the causes and consequences of sexual signal diversity in Hawaiian T. oceanicus showing how signals emerge through the interacting levels of genes, morphology, signal, and behavior (inspired by Figure 1.1 in (Eliason 2018)), necessitating an integrative research framework. We synthesize our findings from this paper and others (Bennet-Clark 2003; Zuk et al. 2006; Tinghitella 2008; Pascoal et al. 2014; Tinghitella et al. 2018, 2021) to place four prominent male morphs relative to each other on these levels (we simplify to two, out of many possible dimensions). Clearly, signals are generated through alternate paths across morphs, as indicated by non-parallel connections between levels. Genotype abbreviations: "Fw"=flatwing, "Nw"=normal wing, "?"=unknown.

The types of mechanistic changes that could theoretically result in form-function decoupling are finite; forms could either be gained, lost, or altered in ways that break ancestral relationships amongst phenotypic components, resulting in novelty (Broder et al. 2021a; Starrett et al. 2022). The well-documented evolution of silent *T. oceanicus* occurred through a major mutation that resulted in the loss of important resonator structures on male wings (mirror, scraper; (Tinghitella 2008; Pascoal et al. 2014). Bailey et al. (2019) demonstrated that signal loss in silent crickets has led to increased variation in vestigial wing structures, and proposed that this release of variation could facilitate novel signal values if and when song is restored. Males from the population of Wailua, where silent crickets were first discovered, are now producing novel, attenuated songs (purring; Figure 1.3), suggesting that Bailey's prediction may be coming to fruition. While we do not know the genetic architecture underlying purring, our data implicate the gain of the scraper, a previously lost structure, as a potential mechanism for signal restoration in this population (Figure 1.5). Additionally, our results on the morphological underpinnings of the newly discovered rattling morph (Figure 1.4A) points to a categorical alteration of an existing wing structure (the file) in the generation of a novel signal. Over very short timescales, phenotypic evolution in Hawaiian *T. oceanicus* appears to provide examples of gain, loss, and alteration of forms modifying signal function, however additional work is needed to fully resolve how these morphs relate to one another.

We found support for Form-Function Decoupling (Figures 1.1, 1.2) here, but sexual signal novelty can also evolve via Form-Function Continuity, as is likely in the cases of the diversification of the avian syrinx (Kingsley et al. 2018) and song in *Gryllus* field crickets (Caetano and Beaulieu 2020). Indeed, there may also be a case of signal novelty evolving via Continuity in *T. oceanicus*. The wing of the recently discovered "small-wing" morph (Rayner et al. 2019) appears to produce a new song but retain all of the structures on the ancestral wing (scaled down to a smaller size), though relationships between wings and songs of this morph should be analyzed in further detail. It would be interesting to explore the relative importance of Form-Function Continuity and Form-Function Decoupling in the evolution of sexual signal novelty using multi-species comparative studies.

Understanding the evolutionary processes that facilitate rapid phenotypic diversification may provide insight into the earliest stages of animal signal radiations, which remains somewhat of a black box in evolutionary biology despite much emphasis on the importance of sexual signal radiations in generating diversity (Seehausen et al. 1997; Coyne, Jerry A and Orr, H Allen 2004; Boake 2005; Mendelson and Shaw 2005; Wilkins et al. 2013). Changes to the many selective pressures acting upon a given trait can strongly influence the ability for variation to be generated and persist within populations. One well documented change in selective pressures is weakened selection on a previously optimal phenotype (relaxed selection; Lahti et al. 2009). Relaxed selection allows for the accumulation of genetic and phenotypic variation, which may provide the material that other selective pressures can act on (Lahti et al. 2009). Sexual selection appears to be weakened in Hawaiian *T. oceanicus*, as females there are more likely to mount non-ancestral males than are females from the crickets' native range in Australia (Tinghitella and Zuk 2009), a phenomenon that is well-documented in small populations where the initial costs of being choosy following colonization are heightened (McPhail 1969; Kaneshiro 1980; Shaw and Lugo 2001; Tinghitella and Zuk 2009). Indeed, the novel morphs that we describe here should have greater success if females are willing to accept a broad range of signal values (Figure 1.4B;

Tinghitella et al. 2021). At this early stage of diversification, we find that females do not systematically prefer particular purring songs with specific acoustic properties (Tinghitella et al. 2021). While the sensory capabilities of *T. oceanicus* from Australia have been studied (Hoy et al. 1982), it's possible that these capabilities have changed in Hawaii, broadening the range of acceptable signal values. Alternatively, selection pressures may become relaxed if the information content of a signal changes or becomes less relevant to receivers. Beyond their efficacy in the important task of mate location, we know little about if and how the information content of these novel signals differs from that of the ancestral songs. Future studies should test relationships between signal variation and male quality in these morphs.

Selective pressures may also be reversed, where a previously advantageous phenotype becomes strongly selected against (reversed selection; Rayner et al. 2022). In Hawaiian populations of *T. oceanicus*, the arrival and proliferation of the fly changed the selective landscape so that net selection on ancestral song was reversed; selection from flies against males producing ancestral song may have allowed for multiple successful new morphs to become quickly established, as nearly any deviation from the previously optimal ancestral signal may increase male fitness (Figure 1.4B; Tinghitella et al. 2021). Relaxed or reversed selection may be a broadly important precursor for the generation and success of novel variation in complex traits.

The novelty we discovered points to ongoing phenotypic diversification across Hawaii. Because we found significant differences in performance among signal variants (Figure 1.4B; Tinghitella et al. 2021), and gene flow is ongoing among islands (Zhang et al. 2021), we can now watch evolution in action. Real-time research on rapid evolution, as we present here, provides unique opportunities to test the immediate fitness consequences of novel forms within the very environments in which they first appear. Close observation of emerging phenotypic variation in Hawaiian *T. oceanicus* allows for a deeper understanding of which phenotypic innovations are successful, which are evolutionary dead ends (insights missed by retrospective approaches; Rabosky 2017), and whether novel phenotypes arise that rewire form-function relationships in even more successful and surprising ways.

Chapter Two

A novel cricket morph has diverged in song and wing morphology across island populations

ABSTRACT

Divergence of sexual signals between populations can lead to speciation, yet opportunities to study the immediate aftermath of novel signal evolution are rare. The recent emergence and spread of a new mating song, purring, in Hawaiian populations of the Pacific field cricket (*Teleogryllus oceanicus*) allows us to investigate population divergence soon after the origin of a new signal. Male crickets produce songs with specialized wing structures to attract mates from afar (calling) and entice them to mate when found (courtship). However, in Hawaii, these songs also attract an eavesdropping parasitoid fly (*Ormia ochracea*) that kills singing males. The novel purring song, produced with heavily modified wing morphology, attracts female crickets but not the parasitoid fly, acting as a solution to this conflict between natural and sexual selection. We've since observed increasing numbers of purring males across Hawaii. In this integrative field study, we investigated the distribution of purring and the proportion of purring males relative to other morphs in six populations on four islands, and compared a suite of phenotypic traits (wing morphology, calling song, and courtship song) that make up this novel signal across populations of purring males. We show that purring is found

in varying proportions across five, and is locally dominant in four, Hawaiian populations. We also show that calling songs, courtship songs, and wing morphology of purring males differ geographically. Our findings demonstrate the rapid pace of evolution in island populations and provide insights into the emergence and divergence of new sexual signals over time.

INTRODUCTION

Many closely related populations differ in their sexual signals, and this variation is thought to play a role in generating reproductive isolation and even speciation (West-Eberhard 1983; Panhuis et al. 2001; Mendelson and Shaw 2002; Wilkins et al. 2013; Servedio and Boughman 2017; Hund et al. 2020; Mendelson and Safran 2021). Even subtle differences in signals across populations can be important. For instance, swordtail cricket species of the genus *Laupala*, which are ecologically indistinguishable, have mating songs that differ in only one characteristic (pulse rate), yet coupling of mating preferences with divergent pulse rates has led to reproductive isolation (Mendelson and Shaw 2002) and likely speciation of *Laupala* across the Hawaiian islands (Mendelson and Shaw 2005). Despite the important role that signal novelty can play in initiating speciation, our understanding of how new sexual signals arise and diverge remains limited (West-Eberhard 1983; Panhuis et al. 2001; Broder, Elias, et al. 2021); evidence for speciation by sexual selection is nearly always retrospective because direct observation of divergence in sexually selected traits is incredibly rare (Svensson and Gosden 2007; Svensson 2019).

Understanding how signals come to differ across populations is critical to illuminating the path from signal divergence to speciation (Panhuis et al. 2001; Ritchie 2007; Servedio and Boughman 2017). Variation in sexual signals across populations might stem from local adaptation, founder effects, genetic drift (especially in small populations), or some combination thereof, and may be particularly common in island populations. Such circumstances led to the rapid adaptive radiation of Hawaiian drosophila from a single common introduced ancestor to hundreds of endemic species across the island chain (Templeton 1979; reviewed in Boake 2005). The recent appearance of a novel sexual signal in multiple Hawaiian populations of the Pacific field cricket (*Teleogryllus oceanicus*) provides an unusual opportunity to study the process of signal divergence in real time (Tinghitella et al. 2018, 2021).

Hawaiian populations of *T. oceanicus* have a propensity for rapid evolutionary change in their sexual signals. In response to strong natural selection pressure (Zuk et al. 1993) from a recently introduced deadly parasitoid fly (*Ormia ochracea*; Lehmann 2003) that hunts for hosts using their songs, male crickets in several Hawaiian populations independently lost sound-producing morphological structures on their wings (the instrument that males use to produce song), leaving them silent (Zuk et al. 2006; Tinghitella 2008; Pascoal et al. 2014). The inability to produce song protects silent males from the parasitoid fly, which allowed the silent morph to spread through populations rapidly (Zuk et al. 2006) despite strong sexual selection favoring males that can sing (Tanner et al. 2019). Even though the costs of stridulation (rubbing their wings together) are high (Mowles 2014), silent males continue to stridulate (Rayner et al. 2020). It’s been hypothesized that wing variation among silent males and retention of resonating properties may provide the fodder for novel acoustic signals to evolve if missing sound-producing structures were regained (Bailey et al. 2019).

In 2017, male *T. oceanicus* on the island of Moloka’i were discovered that produced a novel, detectable song (Tinghitella et al. 2018). The spectral characteristics of purring songs (both long-distance calling and short-distance courtship songs) differ dramatically from the ancestral song—purring songs have higher dominant frequency, lower amplitude, and are more broadband (Tinghitella et al. 2018; Gallagher et al. 2022). Purring wing morphology resembles that of silent male crickets, and may stem from the regain of a previously lost wing structure (the scraper; Gallagher et al. 2022), as predicted in Bailey et al. (2019). Since their discovery, purring males have arisen in or spread to several additional locations (Tinghitella et al. 2021) and anecdotally appear to be increasing in prevalence within populations as well. This is likely because of the way natural and sexual selection act on purring; the parasitoid flies greatly prefer ancestral over purring song, and the purr's low amplitude makes it fairly inconspicuous, protecting purring males from parasitism (Tinghitella et al. 2021; Broder et al. 2022). Unlike silent males, however, the purr is attractive to conspecific females (Tinghitella et al. 2018, 2021), suggesting that purring serves as a private mode of communication among crickets. Interestingly, other novel morphs have also arisen in various Hawaiian populations very recently (e.g. smallwing and curlywing, Rayner et al. 2019; rattling, Gallagher et al. 2022). We focus here on purring because it appears to have become locally abundant in multiple Hawaiian populations very recently. Determining if and how purring populations differ in their sexual signals may be important to understanding the early stages of the speciation process, as divergence in acoustic signals, in particular, is associated with species diversification across taxa (e.g., Mendelson and Shaw 2005; Seddon et al. 2008; Wilkins et al. 2013).

In this paper, we first report field-collected data showing that purring is found in different proportions across five Hawaiian populations. Because many of these sites are fairly geographically isolated from one another, population sizes are relatively small, and there exists a large amount of variation in purring songs and wings, it is possible that purring (like the mutation causing silence; Pascoal et al. 2014) may have evolved multiple times independently, and/or that selection or drift could shape populations of purring males in different ways over a very short period of time. Indeed, these populations are evolving independent of one another (Zhang et al. 2021) and our anecdotal observations suggest there is geographic variation in purring songs. Thus, we ask whether the characteristics of purring males differ across populations. To do this we took an integrative approach, interrogating a suite of phenotypic traits that make up sexual signal production (wing morphology, calling song, courtship song), as even a thorough analysis of one phenotypic level may not fully capture population differences and the mechanisms underlying them (Gallagher et al. 2022). Finding differentiation in purring across populations so soon after its origins will demonstrate the remarkable speed at which evolution can occur in island populations and yield insight into the process that initiates speciation by sexual selection.

METHODS

Male morph proportions across Hawaii

To determine the morph composition of Hawaiian *T. oceanicus* we collected up to 30 males from each of six populations from four islands (Kalaupapa, Moloka’i; Manoa, O’ahu; La’ie, O’ahu; Wailua, Kaua’i; Kapa’a, Kaua’i; Hilo, Hawai’i; total N=303) during three sampling trips (January 2019, June 2019, January 2020) using sampling methods that were unbiased with respect to morph (capturing them visually, rather than by song). We categorized each individual male as one of seven described *T. oceanicus* morphs (purring, ancestral, rattling, small-wing, curly-wing, silent, or undetermined) by comparing their wings and song characteristics to previously published descriptions of each morph (Zuk et al. 2006; Rayner et al. 2019; Gallagher et al. 2022).

Data Collection

To investigate whether and how purring songs and wings differ across populations, we capitalized on song recordings and wing photographs taken across the Hawaiian archipelago in June 2019 as part of a longitudinal study in the four populations (Kalaupapa, Manoa, La'ie, Wailua) where we determined purring to be prominent. We housed males individually in 0.5 L deli cups at ambient temperatures with rabbit food, wet cotton, and egg carton shelters. We recorded calling songs from males in empty 0.5 L deli cups using a RØDE NTG2 Multi-powered Condenser Shotgun microphone (RØDE Microphones LLC, Long Beach, CA USA) placed 10 cm above the male. We recorded courtship songs in the same way, but added a female cricket to the deli cup to initiate male courtship (any mating attempts were disrupted). We measured each male's pronotum width using calipers (to the nearest hundredth of a millimeter). After males were recorded, we photographed their right wing using a digital SLR camera (Pentax K-5, Hoya Corp., Tokyo, Japan) with a metric ruler included in each image for scale. Males were then returned to their respective collection sites.

Morphometric and acoustic analyses

Song production in crickets occurs when the scraper of one wing is dragged across the file of the other. As the male opens and closes his wings, this rapid and repeated rubbing of the scraper over the file (a modified vein containing a row of many small, tightly packed teeth located along the basal vein of the harp) sends vibrations through the wing, resonating other important veins and structures such as the harp and mirror to create sound (Ewing 1989; Bennet-Clark 1999, 2003).

To comprehensively describe differences in wing morphology among populations of purring males, we placed fourteen landmarks on each wing photograph (Table S1.2; Gallagher et al. 2022). We conducted morphometric analysis on these landmarking data using the *geomorph* R package (Adams and Otárola Castillo 2013). We collapsed wing morphometric data using a Principal Component Analysis that included xy coordinates of landmarks. We additionally measured the width of the harp (linear distance along the x-axis between landmarks 5 and 14; Table S1.2) and presence or absence of the scraper (Gallagher et al. 2022), the two individual sound-affecting structures on the wing (Bennet-Clark 2003).

We measured nine acoustic characteristics of calling and courtship songs (see Table S2.1 for descriptions; as in Gallagher et al. 2022). We selected one calling song and one courtship song from each individual by choosing the second cleanly recorded song from the first bout of continuous song, then analyzed calling and courtship songs independently using the same methods (Gallagher et al. 2022). We measured characteristics of the chirp and trill separately in courtship songs because their sound properties differ from one another (Hoy 1974; Bennet-Clark 2003). After selecting a song, we removed background frequencies below the range of detectable signal in Audacity by using a high-pass filter at 1500 Hz (version 2.3.1, The Audacity Team; roll-off: 48 dB per octave). We measured dominant frequency using the plot spectrum analysis function (settings: Hanning window, size=256, log frequency axis). We next measured the amplitude of songs using the Level Meter in Logic Pro X (version 10.4.8, Apple Inc., Los Altos, CA USA; Gallagher et al. 2022). In addition to overall amplitude, we also measured the relative amplitude of six frequency ranges, allowing us to capture details of each song's frequency distribution (Table S2.1). Lastly, we determined how broadband songs were by calculating frequency evenness (Table S2.1). After analyzing calling and courtship songs, we used a Principal Component Analysis (PCA) to collapse variables into fewer axes that captured variation in calling and courtship song characteristics, separately (see Table S2.2 for trait loadings).

Because we were only able to collect a smaller sample of crickets from Kalaupapa in 2019 (N=5, owing to small population size), and because we found large differences in courtship songs between Kalaupapa and the other sites (see results), we wanted to confirm that the differences in courtship songs of field-caught Kalaupapa males were not a result of small sample size. Thus, we also analyzed the courtship songs from Kalaupapa males that had been reared in common garden for at least two generations (N=20), chosen haphazardly from a large lab population of the animals (>200 adult animals) that had been reared in a walk-in growth chamber set to 26 degrees celsius with ad lib access to rabbit food, moist cotton for water, and egg carton for shelter. These males were recorded in 2018 inside of an acoustically isolated recording studio at XXX using a Sennheiser MKH800 microphone (Sennheiser, Wedemark, Germany) positioned 40 cm above the cricket. Because these lab recordings were conducted in a recording studio and therefore contain less ambient noise than field recordings, and because the distance between the microphone and the crickets was different, we did not measure amplitude-related characteristics in lab males that may be affected by different recording environments. Instead we measured dominant frequency (following the same methods described above for field-caught males), which is not dependent on successful calculation of amplitude and should be robust to differences in low-level background noise spread across a wide range of frequencies (Araya-Salas et al. 2019) and changes in microphone distance from the cricket.

Statistical Analysis

We conducted all statistical analysis using RStudio (RStudio Team 2022, R version 4.1.2). To test whether wing morphology, calling song, and courtship song differ across predominantly purring populations, we performed multivariate analysis of variance (MANOVA with Pillai's Trace test statistic); for each trait, we included population, body size, and their interaction as our independent variables and the first three composite axes of variation (PC1-3; see Table S2.2 for PC details) as our dependent variables. Body size (measured as pronotum width; Moradian and Walker 2008; Broder, et al. 2021; Tan 2022) can impact an individual's song and wing characteristics in some cricket species (Moradian and Walker 2008; Whitman 2008; Tan 2022), and since the purring is generated using an altered instrument with novel wing-song relationships (Gallagher et al. 2022), we wanted to be sure that any phenotypic differences we found among populations were not simply due to differences in body size. To determine whether there were morphological differences among populations in harp width we used a two-way analysis of variance (ANOVA) with population, pronotum width, and their interaction as independent variables. We addressed whether scraper presence differed across populations with a Pearson's chi-squared test (population = independent, scraper presence = dependent). To confirm that dominant frequency of courtship song did not differ between field- and lab-reared Kalaupapa males, we performed a two-way analysis of variance (ANOVA) with rearing environment as the independent variable and dominant frequency as the dependent variable.

RESULTS

Our sampling between January 2019 and January 2020 showed that purring was present on three different islands and in five of six regularly sampled populations (Figure 2.1). Purring is now the most prominent morph in four locations (Kalaupapa, Manoa, La'ie, and Wailua) across the Hawaiian archipelago. See Table S2.3 for the number of individual males per morph in each population.

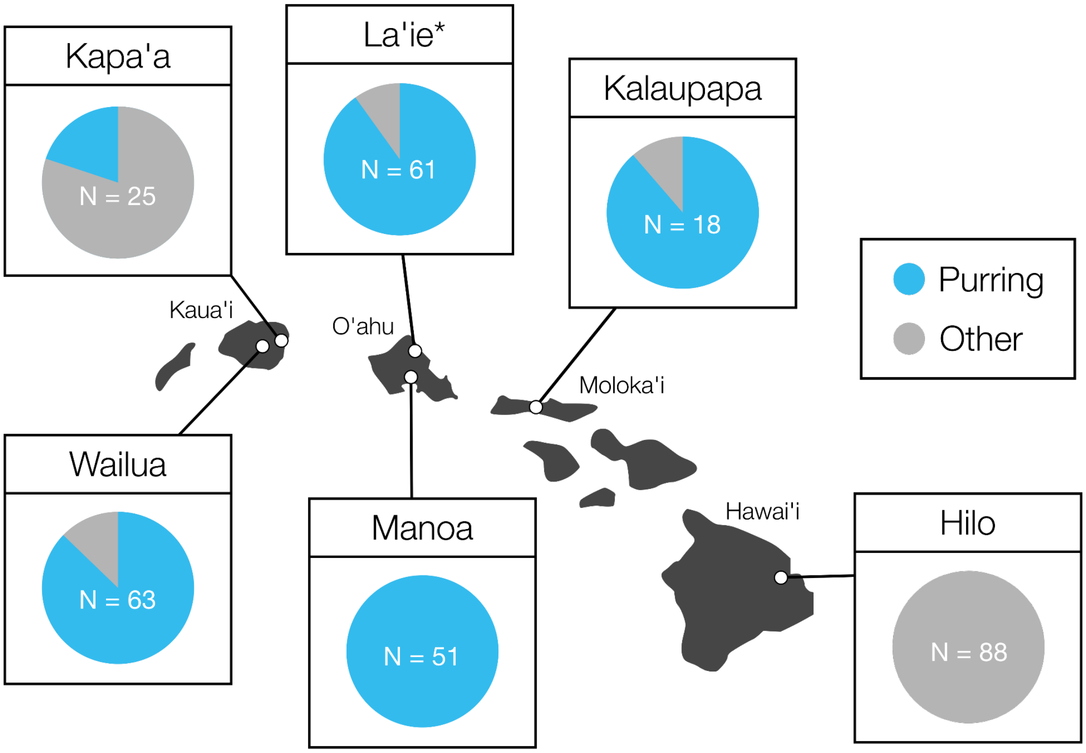

Figure 2.1. Purring is widespread, present at five and prominent at four of our six study sites across Hawaii. All non-purring males were placed into the "other" category. Total N across populations = 303 males. *At the La'ie site, ancestral males burrow, making it

difficult to collect them, which may cause underestimates of the proportion ancestral at that site. Based on the number of ancestral males we heard singing, we estimate that they made up 5% to 20% of the male population at La'ie during this sampling period.

Across Hawaii, wing morphology of purring males differed across the four sites where the morph is prominent (MANOVA: $F_{9,165}=7.23$, $N=67$, $p<0.0001$; Figure 2.2A). The presence and size of important sound-producing structures, the harp and scraper, differed among populations (Figure 2.2B, harp width, ANOVA: $F_{3,57}=8.90$, $N=77$, $p<0.0001$; Figure 2.2C, scraper presence, chi-square: $\chi^2=11.80$, $N=75$, $df=3$, $p=0.008$; see the drawings of representative wings in Figure 2.2A).

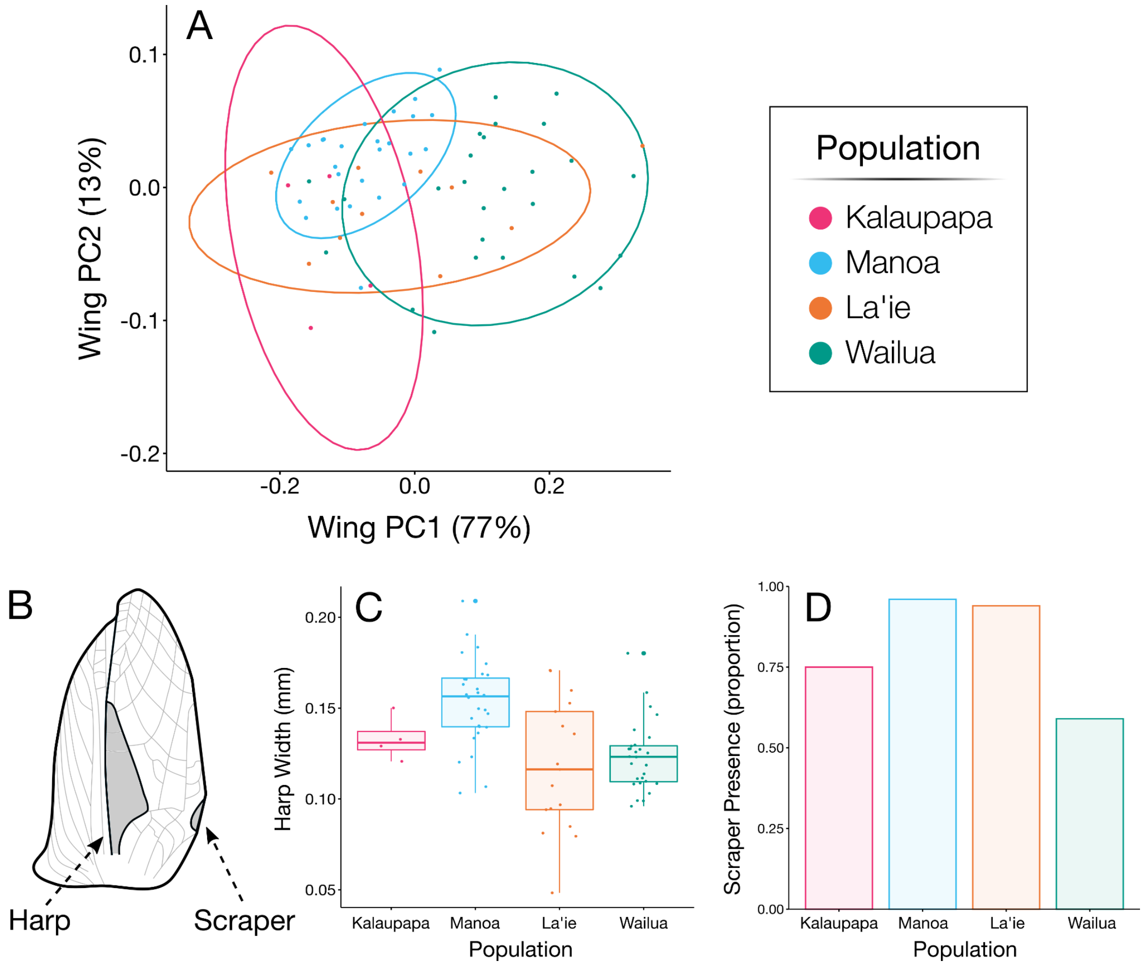

Figure 2.2. Purring males from different populations differed in overall wing morphology (A). Ellipses represent 90% data coverage for each population. Purring wings have two

important structures, the harp and scraper, that are known to vary in their size and presence among males (B). Males differed in harp width (C) and scraper presence (D) among populations.

The calling songs of purring males differed among populations (MANOVA: $F_{9,165}$=2.31, N=67, p=0.018; Figure 2.3A). Purring courtship songs also differed among populations (MANOVA: $F_{9,114}$=6.58, N=46, p<0.0001; Figure 2.3B). Certain populations, such as Wailua and in particular Kalaupapa, appeared to differ primarily from other populations by having louder and higher frequency courtship songs (see Figure 2.3C for representative songs from each population, and Table S2.4 for means and standard deviations of major song characteristics). Because Kalaupapa (the population that had the smallest sample size) differed most notably from other populations in courtship song characteristics (Figure 2.3B), we re-ran this model, excluding Kalaupapa from analysis. Even then, we found differences in purring courtship song among populations ($F_{6,68}$=5.34, p<0.001), suggesting that differences in courtship song were not entirely driven by Kalaupapa. When we compared the small sample size of field-recorded Kalaupapa males to a larger sample of lab-reared males from Kalaupapa (see methods), we found that the dominant frequency of lab-recorded Kalaupapa purring courtship songs was nearly identical to that of Kalaupapa field recordings (ANOVA: $F_{1,23}$ =0.045, p=0.834; Kalaupapa, lab: 11.02 kHz; Kalaupapa, field: 11.59 kHz; all other purring populations, field: 7.41 kHz), supporting the field-collected courtship song patterns and suggesting that these population-level differences were not environmentally induced. We never found that population and pronotum width (our proxy for body size) interacted to affect wing morphology or song characteristics (wing morphology, harp width, calling song, courtship song), indicating that differences in purring songs and wings across populations are not driven by body size differences.

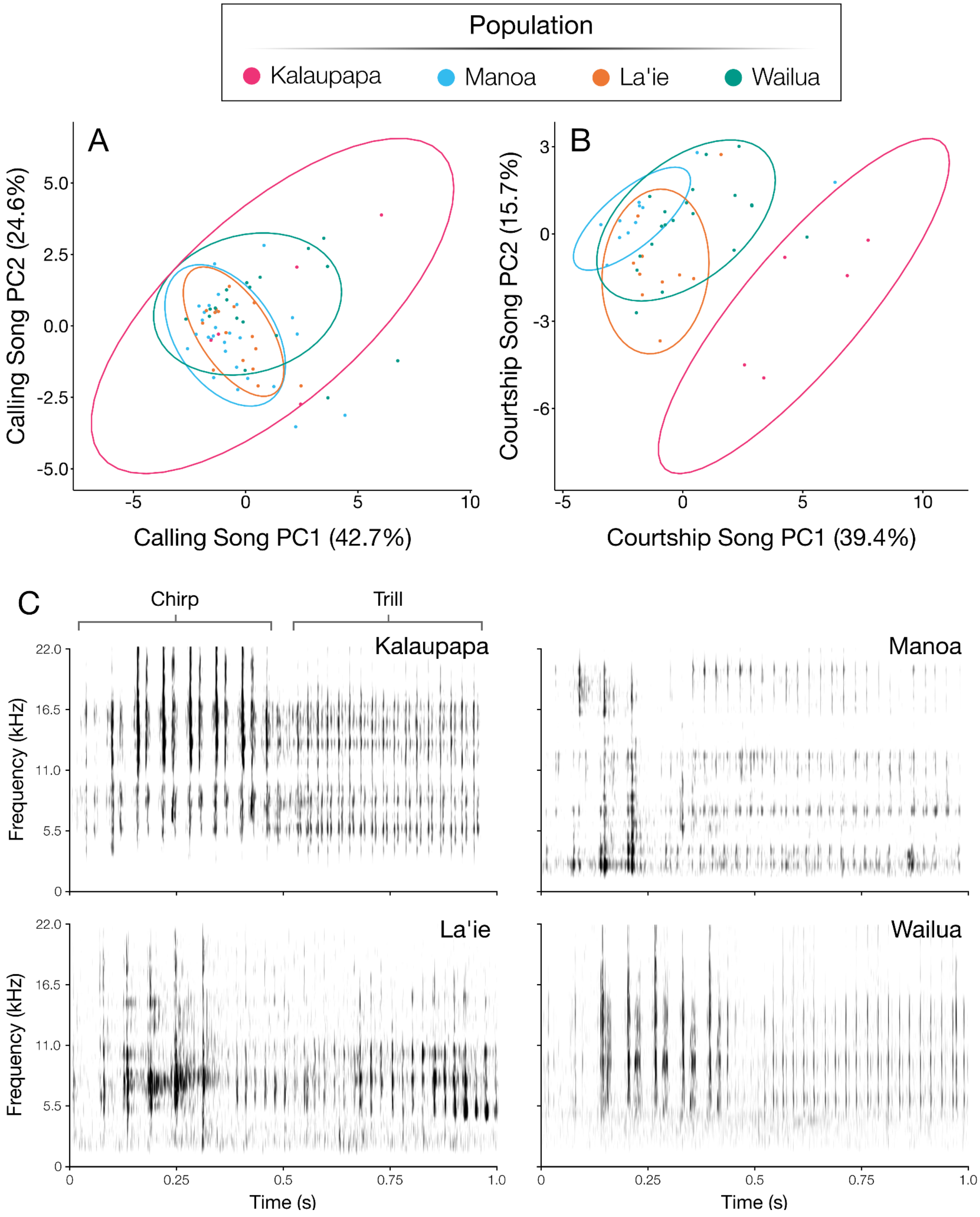

Figure 2.3. Purring males differed in calling song (A) and courtship song (B) characteristics among populations. Ellipses represent 90% data coverage for each

population. C) Spectrograms of representative purring courtship songs from the four purring populations. Courtship songs are composed of two sections: the chirp and the trill. Darker regions indicate greater relative acoustic power within the song.

DISCUSSION

We investigated whether a novel sexual signal (purring) has diverged among small, relatively isolated island populations of crickets. In field studies, we first found that purring is now present on three different islands and in five of six long-studied populations. In four of the six populations, purring has become the most common morph (Figure 2.1), despite arising recently in these locations (Tinghitella et al. 2021). We next characterized a suite of phenotypic traits relating to this new signal and its production (wing morphology, calling song, and courtship song) and found that island populations already differ in each major trait (Figures 2.2-2.3).

Here we document that purring is the most common morph at several sites across Hawaii. Our previous data show that purring attracts females but protects signaling males from the parasitoid fly (Tinghitella et al. 2021; Broder et al. 2022), suggesting that purring males achieve higher fitness than the other morphs in locations where purring has become prominent. This selective advantage could explain the prevalence of purring morphology that we document across Hawaii (Figure 2.1). In fact, purring has already gone to fixation in one location (Figure 2.1); Manoa consisted of more than 50% ancestral males as recently as 2012 (Pascoal et al. 2014; Zuk et al. 2018). This provides a powerful example of the speed at which evolution can occur. Hypothetically, if purring were to eventually go to fixation across Hawaii (or even on a single island), Hawaiian males would have a completely separate sexual signal than ancestral males in Australia.

It’s likely that Australian females discriminate against purring males (since they discriminate against silent males in courtship; Tinghitella and Zuk 2009), and signals and preferences could diverge in allopatry creating reproductive barriers should these groups come into secondary contact (reviewed in: West-Eberhard 1983; Servedio and Boughman 2017; e.g., Shaw and Lugo 2001). Future work should assess whether premating barriers already exist between this new morph and ancestral populations where it is absent, such as in long distance mate location.

How do we explain the phenotypic differentiation among populations that exists so soon after the emergence of purring? One possible driver is selection shaping purring differently in the various populations. Indeed, despite the presence of the parasitoid fly in all of these locations, the selective landscape is not identical across localities; populations have different morph compositions (Figure 2.1), abundances of parasitoids, and acoustic environments, for instance: variation in each of these factors could affect the strength of selection acting directly upon purring songs and underlying morphology. Furthermore, selection acting on other traits (e.g., life history) that may be genetically linked with wing morphology or singing behavior could indirectly lead to population-level differences in wings or songs. A second likely contributor to population differences is genetic drift. Purring is a novel sexual signal and there is quite a lot of variation in the songs produced by different purring males (Tinghitella et al. 2018). We previously demonstrated that population level preferences for existing natural variants of purring songs have not yet developed in female crickets or parasitoid flies (Tinghitella et al. 2021; Broder et al. 2022). While crickets and flies prefer the songs of certain morphs over others (Tinghitella et al. 2018, 2021; Gallagher et al. 2022), the absence of selection on within-purring variation could allow drift to shape purring differently across populations. Another explanation for the geographic variation we uncovered may lie in the genetic origins of purring. Purring may have arisen independently in multiple locations (as occurred with the silent morph; Tinghitella 2008; Pascoal et al. 2014), which could explain some of the consistent phenotypic differences among purring populations. Alternatively, purring could have emerged once, subsequently spread across Hawaii, and diverged quickly across these small island populations through some combination of selection, drift, and gene flow. We find either of these origin scenarios to be exciting because purring arose or arrived at some of these locations as recently as three years (approximately 12 cricket generations) before this study was conducted (Tinghitella et al. 2018). Future genetic work should attempt to tease apart these two alternative evolutionary histories for purring.

In addition to evolutionary mechanisms, plasticity may underlie population-level differences in purring wings and songs. While within- and among- morph differences persist for multiple generations when crickets are reared in common garden (Gallagher et al. 2022); Kalaupapa in this paper), some of the variation we uncovered could still be due to plastic responses to environmental differences. Importantly, plasticity could facilitate alternate evolutionary trajectories if it acts differently in different populations and is later incorporated into the genome via genetic accommodation (West-Eberhard 2003; Barrett and Schluter 2008; Pfennig et al. 2010; Broder, Elias, et al. 2021). As we continue to follow these populations over time, it will be important to consider plasticity's role in shaping variation in the new purring signal.

We show that the morphology and signals of purring males differ across populations, but previous work has shown no population-level differences in female preferences for variation in purring song (Tinghitella et al. 2021). This lack of preference for specific purring variants suggests that directional selection by female choice is unlikely responsible for the population-level differences in purring that we show here. In fact, our results may provide support for the "signal first" hypothesis of divergence in animal communication, where signals diverge first among groups, followed by the evolution of receiver preferences for these new signal characteristics and ultimately reproductive isolation (Bradbury and Vehrencamp 2011; Broder et al. 2021). If preferences for certain purring songs do subsequently develop in females at certain locations but not others, or differences in preferences evolve across populations, divergence among populations could accelerate. Currently there is no evidence of reproductive isolation among morphs within Hawaii (Fitzgerald et al. 2022), but it will be important to measure receiver preferences over time both between and within morphs as these populations evolve.

Finally, our finding that populations differ in the type and extent of phenotypic variation could impact evolutionary trajectories. For example, the presence or absence of a scraper on the wing dramatically affects the frequency of calling song in purring males (Gallagher et al. 2022), and we found variation in the proportion of individuals with scrapers among populations in this study (Figure 2.2C). If future selection were to favor purring songs with higher frequencies, evolution could be constrained in populations where scraperless males (who are capable of producing very high frequency calls) are absent or in low abundance. It will also be important to consider how existing variation in purring songs and morphology will be shaped by the sensory systems of intended and unintended receivers; neural responses of Hawaiian populations of crickets and flies have not been measured and just like receiver preferences, variation in receiver sensory systems could impact the success of different purring variants. Taking into consideration how existing variation in both signalers and receivers limits or promotes the evolvability of traits in these populations will be important as we monitor the evolutionary trajectory of purring.

Chapter Three

Surviving the serenade: how conflicting selection pressures shape the early stages of sexual signal diversification

ABSTRACT

Understanding how the early stages of sexual signal diversification proceed is critically important because such microevolutionary dynamics directly shape species trajectories and can impact macroevolutionary patterns. Unfortunately, studying signal diversification is particularly challenging because signals involve complex interactions between behavior, morphology, and physiology, much of which can only be measured in real time. In Hawaii, male Pacific field crickets (Teleogryllus oceanicus) attract both female crickets and a deadly acoustically orienting parasitoid fly (Ormia ochracea) when they sing. We and others have recently observed a radical increase in sexual signal variation in Hawaiian populations of these crickets, including novel male morphs with attenuated mating songs. We track the relative abundance of four morphs (three novel) in one population over time and interrogate the natural (parasitism) and sexual (mating) selection pressures driving this evolutionary change. We find dramatic fluctuation in morph proportions over three years, including the arrival and rapid increase of one novel morph to 40% prevalence. Each morph balances the natural-sexual selection conflict differently, with some more attractive to mates and others more protected from

parasitism. Collectively, we argue that of the two primary selection pressures that male crickets face in the wild, parasitism is driving recent rapid evolutionary change.

INTRODUCTION

Changes to the selective landscape can drive diversification of phenotypes (Schluter 2000; Nosil and Crespi 2006; Glor 2010; Losos 2010; Stroud and Losos 2016, 2020; Stephan 2021). In particular, dramatic shifts in the shape or direction of selection (i.e., reversed selection; Gimelfarb 1986; Rayner et al. 2022) can trigger rapid evolution of novel variants within a species (Zuk et al. 2006; Latta et al. 2007; Rosenblum et al. 2010). Such diversification is often driven by natural selection, sexual selection, or a combination of selective pressures (West-Eberhard 1983; Losey et al. 1997; Nosil and Crespi 2006; Rosenblum 2006; Meyer and Kassen 2007; Scordato et al. 2014; Servedio and Boughman 2017; Tinghitella et al. 2018*b*; Bush et al. 2019). One variant might have the highest fitness and outcompete others (Wright 1978; Daborn et al. 2002; Zuk et al. 2006; Cook et al. 2012; Hallgrímsson et al. 2012), leaving behind an evolutionary graveyard of experimental forms. Alternatively, multiple variants could have roughly equivalent fitness and be maintained long-term within populations (for instance through negative frequency dependence or by variants balancing selection pressures differently; Adamkewicz 1969; Sinervo and Lively 1996; Brooks 2002; McLean and Stuart-Fox 2014), though this process is poorly understood (Bataillon et al. 2022).

Understanding how within species evolutionary dynamics progress during the early stages of phenotypic diversification is of critical importance because the nature of these microevolutionary dynamics can directly shape species' trajectories and even macroevolutionary patterns (McLean and Stuart-Fox 2014; Aguilée et al. 2018; Li et al. 2018; Stroud and Losos 2020; Chaparro-Pedraza et al. 2022; Rhoda et al. 2023).

Unfortunately, researching the early stages of diversification is incredibly challenging because we often do not have the opportunity to interrogate the process as it is happening (Stroud and Losos 2020). Many studies attempt to make inferences about how diversification occurs by associating phenotypic traits with known phylogenetic relationships (Rabosky 2017; Stroud and Losos 2020). While this type of work is valuable, it necessitates making assumptions about the process that cannot be resolved after the fact (Stroud and Losos 2020). For instance, many traits that may be important during diversification can only be measured in living organisms (such as those related to behavior, physiology, communication, etc.; West-Eberhard 1983; Pomiankowski and Iwasa 1998; Gray and Cade 2000; Panhuis et al. 2001; Mendelson and Shaw 2002; Niehuis et al. 2013; Servedio and Boughman 2017; Kopp et al. 2018; Cooney et al. 2019; Zhang et al. 2020). In this study we capitalize on a rare opportunity to investigate the ongoing evolutionary dynamics in a population of crickets that has recently experienced diversification of sexual signals.

To produce their sexual signal, male Pacific field crickets (*Teleogryllus oceanicus*) use specialized structures on their wings that produce sound when rubbed together (Bennet-Clark 1999, 2003). They generate species-specific songs that attract potential mates in both long-distance (calling) and short-distance (courtship) contexts (Alexander 1962; Hoy et al. 1982). In their introduced range in Hawaii, male *T. oceanicus* song also attracts *Ormia ochracea*, a deadly parasitoid fly originally from North America (Lehmann 2003). The flies listen for the same long-distance calling songs as female crickets to locate (and eventually kill) their cricket hosts (Wagner 1996). They recognize a cricket's calling song, cruise towards a singing male from afar, approach the singing male more closely by flying in a downward spiral pattern, then walk to the sound source (Müller and Robert 2001; Mason et al. 2005). The introduction of the fly to Hawaii dramatically altered the selective landscape for crickets in ways that should favor males that can evade parasitism. Indeed, beginning approximately two decades ago, multiple independent mutations arose that eliminated most of the crickets' sound-producing wing structures, rendering them obligately silent in some populations (Zuk et al. 2006; Tinghitella 2008; Pascoal et al. 2014). These silencing mutations protected males from parasitism but prevented them from attracting mates using song. Despite being unable to attract females from afar (unlike the ancestral males that produce loud, tonal songs), silent males became increasingly common throughout Hawaii and, in one population, reached near fixation in fewer than 20 generations, highlighting how quickly evolution can occur in this species when faced with strong natural selection pressure from the fly (Zuk et al. 2006).

More recently, there has been remarkably rapid diversification of sexual signals across Hawaii (Figure 3.1). Four additional morphs, each with different modified wing morphology that generate attenuated songs, have been discovered: purring (Tinghitella et al. 2018*a*), rattling (Gallagher et al. 2022), smallwing (Rayner et al. 2019), and curlywing (Rayner et al. 2019). Purring and rattling males make acoustically distinct songs using unique morphological structures on their wings (Gallagher et al. 2022). These songs are much less attractive to the parasitoid fly than the ancestral song but are still attractive to female crickets (less so than ancestral but more so than no song; Tinghitella et al. 2018*a*, 2021; Gallagher et al. 2022). The song produced by curlywing males is also less attractive to flies than ancestral male song, though its ability to attract mates is unknown

(Rayner et al. 2019). The acoustic properties of smallwing song, whether it can be used to attract females, and the extent to which it protects against parasitism has yet to be tested (Rayner et al. 2019). Though the genetic underpinnings are not yet fully understood, there is much evidence suggesting that all of these novel morphs are heritable (pure breeding lines exist in several labs, morph is observable immediately upon reaching adulthood, and song and wing characteristics are persistent over multiple generations in common garden; Rayner et al. 2019; Gallagher et al. 2022; Gallagher et al. in review). The morphs are present in populations across the Hawaiian Islands and exist in different proportions in each (Gallagher et al. in review). However, in one population on the Big Island of Hawaii (hereafter, "Hilo"), three of the novel morphs (rattling, smallwing, and curlywing) are present on the very same lawns and are thus directly competing with each other and with the ancestral morph for mate acquisition and survival (Figure 3.1). Over time, polymorphism may be maintained in this population, or one morph may come to dominate.

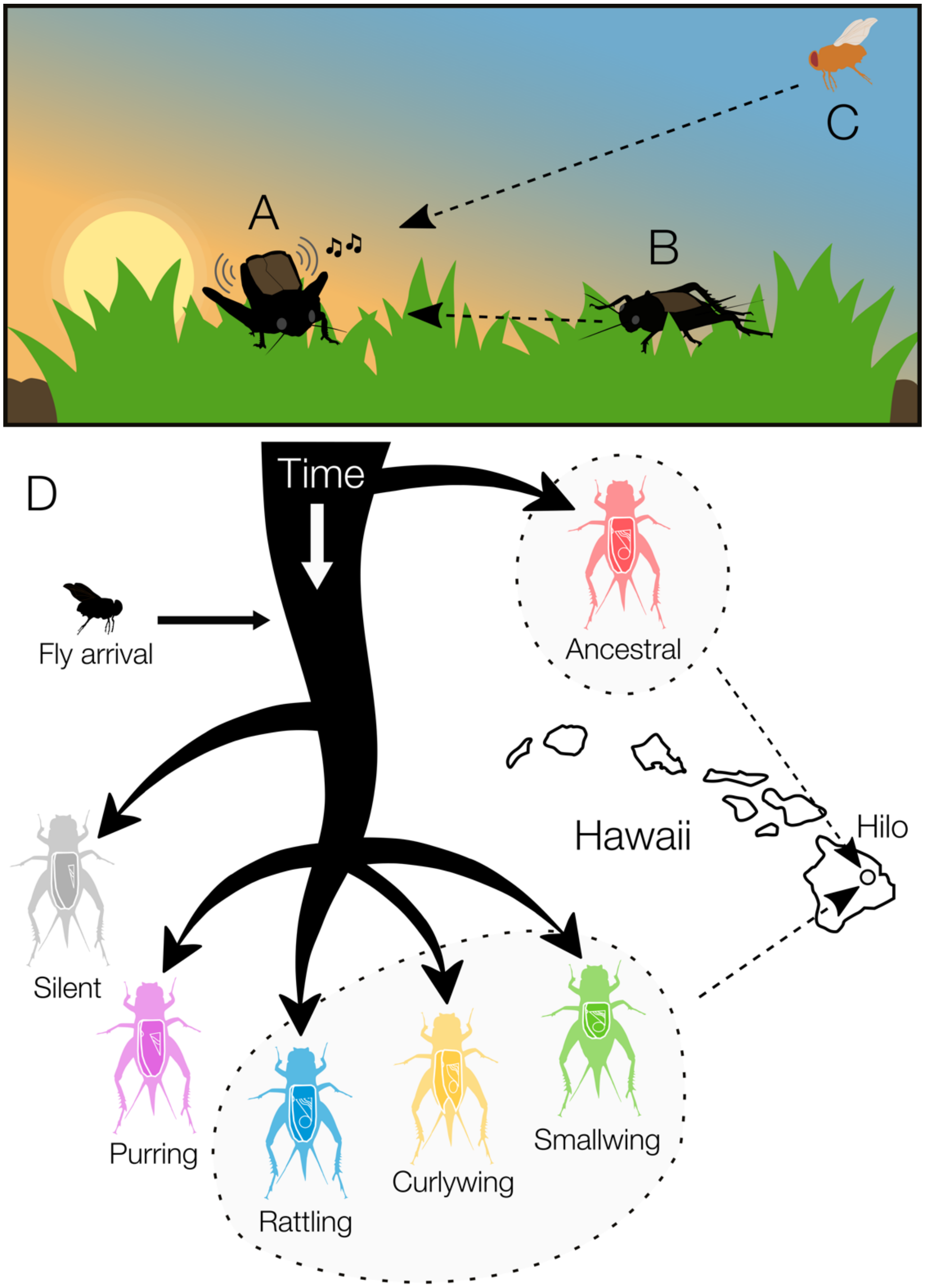

Figure 3.1. Conflicting natural and sexual selection pressures have resulted in rapid diversification of sexual signals in Hawaiian populations of the Pacific field cricket over the past two decades. Male crickets (A) sing by rubbing their wings together to attract females (B), but their song also attracts a deadly eavesdropping parasitoid fly (C). D) Stylized timeline of sexual signal diversification over the past two decades. The cricket and fly interaction led first to the complete loss of signal (silent males), and more recently

to the arrival of several new male types (morphs), each having unique wing morphology that generates attenuated songs. Note that four morphs (purring, rattling, smallwing, and curlywing) were all discovered in the past six years and the chronological order in which they originated and their evolutionary history and genetic underpinnings are unknown. Three of these novel morphs currently co-occur in a single location on the Big Island of Hawaii (Hilo) with ancestral males, and thus compete directly with one another for mate acquisition and survival.

The unusual circumstance of several novel morphs competing in a single population allows us to investigate the evolutionary dynamics soon after diversification of sexual signals, which cannot be adequately captured through retrospective approaches (Stroud and Losos 2020). Our study system is highly tractable, allowing a multifaceted approach where we combine careful field-based experiments measuring the natural and sexual selection acting on the four morphs, and map those results onto a time-series of morph proportions in the population where the morphs are naturally found. We first ask whether proportions of the four morphs are changing over time in the field using repeated sampling over three years (~12 cricket generations), tracking rapid evolution in real time. We then characterize the natural and sexual selection landscapes proposed to shape changes in morph proportions in this population by measuring both mate attraction and parasitoid attraction to the four different morphs in long- and short-distance song contexts. It was particularly important to investigate the selection acting on male morphs in both long- and short-distance contexts because female crickets assess mates during both long-distance phonotaxis and close-range courtship interactions (Alexander 1962), and parasitoid flies have multiple phases of host location, including long-distance flight and shorter distance walking phonotaxis (Oshinsky 1998; Mason et al. 2005). Finally, we remove the influence of the fly by comparing the morph proportions in the field to their first-generation lab-reared offspring, hypothesizing that if natural selection plays an important role in the relative prevalence of morphs, ancestral males should be more common among the lab-reared offspring than their wild parents. We predict that natural selection from the fly is the primary driver of changes in morph proportions for three reasons: first, theoretical and empirical work suggests that natural enemies are broadly important as drivers of diversification (Nosil and Crespi 2006; Meyer and Kassen 2007; Bush et al. 2019); second, our previous work in this system shows that sexual selection is relatively relaxed in Hawaiian populations of *T. oceanicus* (Bailey and Zuk 2008; Tinghitella and Zuk 2009; Fitzgerald et al. 2022); and third, fly parasitism facilitated the spread of silent and purring males in other populations (Zuk et al. 2006, 2018; Pascoal et al. 2014; Tinghitella et al. 2021; Gallagher et al. 2022). Placing our work in the context of a single actively evolving population enables us to discuss which of these selection pressures contribute most to the relative success of the competing alternative morphs because our study is not confounded by geographic variation (e.g., environmental differences across populations) in the selective landscape. Our work provides a rare window into the evolutionary mechanisms that shape the early stages of diversification, allows us to make predictions about future evolutionary change, and offers insights into how novelty is eroded or maintained in populations.

METHODS

Determining male morph proportions over time

To determine whether morph proportions of male *T. oceanicus* had changed over time, we collected >30 males per time point at the Hilo site (University of Hawai'i at Hilo) on the Big Island of Hawaii during five time points over three years (winter 2018, summer 2019, winter 2019, summer 2021, and winter 2021). At each time point, we collected crickets using a standardized visual sampling technique that does not bias collection of certain morphs (Tinghitella et al. 2021; Gallagher et al. 2022). After collection, we housed males in individual 0.5 L plastic deli cups with ad lib rabbit food, damp cotton for water, and egg cartons for shelter, and used a combination of song recordings and wing photographs to determine each male's morph. We recorded male calling and/or courtship song in their deli cups at night illuminated only by red light using a Rode NTG2 Multi-Powered Condenser Shotgun microphone (Rode Microphones LLC, Long Beach, CA USA) positioned 10 cm above the cricket and connected to a digital recorder (Marantz PMD620 MKII; following Tinghitella et al. 2021 and Gallagher et al. 2022). If a male did not readily produce calling song, we enticed him to sing by placing a female into the cup to elicit courtship song. Following recording, we took photographs of every male's right wing on a white background with a mm scale in the image using a digital SLR camera (Pentax K-5, Hoya Corp., Tokyo, Japan; following Gallagher et al. 2022). We determined a male's morph by comparing their song recordings and wing photographs to published acoustic and morphological morph descriptions (Zuk et al. 2006; Tinghitella et al. 2018*a*; Rayner et al. 2019; Gallagher et al. 2022). Note that the three novel morphs we observed in Hilo were rattling (as defined in Gallagher et al. 2022), and Nw curlywing and Nw smallwing (as defined in Rayner et al. 2019). There were no flatwing (silent) individuals found in this population over the course of the study. If a male's morph was unclear based on recording and/or photography, we clipped their right wing and investigated the male's file under a VHX-7000 Digital Microscope (Keyence Corporation, Itasca, IL USA) to confirm the male's morph (Gallagher et al.

2022). Any males that could still not be classified as a particular morph were excluded from subsequent work (N=13 total over the five sampling trips). We returned all males to the field from which they were collected following recording and wing photography. To determine whether morph proportions had changed over the three years of the study, we ran a Fisher's exact test to compare the frequency of morphs (ancestral, rattling, smallwing, curlywing) at the beginning of the study (winter 2018) and the end of the study (winter 2021). All statistical analyses were conducted in R Studio (RStudio Team 2023, R version 4.1.2).

Building playback loops

We first analyzed the calling song recordings of males of each novel morph to generate song loops for playback experiments. We used existing recordings from Gallagher et al. 2022 for rattling (n=16) and recorded smallwing and curlywing males from Hilo in summer 2021 (smallwing: n=5; curlywing: n=5). We identified the first cleanly recorded song from the first bout of calling song from each male using Audacity (version 2.3.1, The Audacity Team) and applied a high-pass filter at 1500 Hz (roll-off: 48 dB per octave) to remove unrelated background noise (as in Gallagher et al. 2022). We measured two important sound characteristics that receivers use to assess a male and are known to vary among the novel morphs previously analyzed (purring and rattling): dominant frequency and amplitude (Tinghitella et al. 2018*a*; Gallagher et al. 2022; Gallagher et al. in review). We measured dominant frequency of the song using the "plot spectrum analysis" function in Audacity (settings: Hanning window, size = 256, log frequency axis), and amplitude using Audacity's "measure RMS" tool. We then converted measurements from dB to amplitude ratio to compare them on a linear scale for principal component analysis (see below; as in Gallagher et al. 2022). See Table S3.1 for morph song characteristics.

To choose three representative calling songs of each morph for playback, we ran a principal component analysis for each novel morph using the standardized (z-score) measured characteristics (dominant frequency and amplitude). For each morph, using the first two PC axes generated, we chose the three most central songs to build a playback loop for the following phonotaxis experiments. We combined the three songs in succession in a random order, resulting in a single track with a length of 1.5 seconds for each morph. The ancestral loop was built for use in earlier studies (see Tinghitella et al. 2018*a*, 2021; Broder et al. 2022; Gallagher et al. 2022) using the same methods except that the songs were chosen at random from a set of previously recorded ancestral males, instead of using the PCA approach (Tinghitella et al. 2018*a*).

Long-distance mate attraction

In winter 2021, we collected adult female crickets for long-distance mate attraction (phonotaxis) trials using the same methods used to collect males (described above). Females were housed in 15 L plastic containers with rabbit food, damp cotton for water, and egg carton for shelter, and kept in a separate room from males to prevent them from hearing song at least 24 hours prior to trials. To test that ancestral calling song remains most attractive to females, as has been shown in prior work (Tinghitella et al. 2018*a*, 2021; Gallagher et al. 2022), we ran competitive phonotaxis trials in which we broadcast the looped songs of the four morphs simultaneously (ancestral, rattling, smallwing, curlywing; all mate attraction trials were conducted during the night at indoor field stations). We placed one speaker (AGPTEK A02 MP3 player) in each corner of a 113 cm x 113 cm arena, each broadcasting the calling song loop of one morph. We placed each test female in the center of the arena, 51 cm from each speaker, and randomized the corner from which each song was played before each trial (N=20 females/trials; Figure S3.1). Females were given five minutes from the onset of song to contact a speaker. Two trained observers (JG and EDB) watched and recorded which speaker the female first contacted. To test whether morphs differed in their relative attractiveness, we used a Fisher's exact test to compare the observed number of positive phonotaxis responses (speaker contact) per morph to the null expectation of 25% (no preference).

We investigated the relative long-distance attractiveness of the three novel morphs (rattling, smallwing, curlywing) using competitive phonotaxis tests in summer 2021. In these phonotaxis trials (hereafter "two-way phonotaxis trials"), each female (N=44) was presented with every pairwise combination of the four stimuli (rattling loop: 60 dBA from speaker to cricket, smallwing loop: 60 dBA, curlywing loop: 55 dBA, and a silent negative control) resulting in six trials per female (minimum one hour rest between trials). Two speakers were placed 30 cm from each end of a 50 cm x 195 cm phonotaxis arena, facing the center and each other. We then placed a female in the center of the arena under a deli cup, gave her 30 seconds to acclimate to the conditions while speakers broadcast song, and then released the female to make a choice. To prevent masking, stimuli from the two speakers were played in an alternating fashion so that songs did not overlap with each other (as in Hirtenlehner and Römer 2014; each song separated by 1.5

seconds of silence). We gave females up to two minutes to contact one of the two speakers. Three trained observers (JG, AW, HO) measured which speaker was contacted. Following completion of the phonotaxis experiment, we returned females to their original collection field.

We assessed the relative attractiveness of the novel morphs' songs in the two-way phonotaxis experiment by calculating attractiveness scores for each morph based on speaker contacts. Scores were calculated for each morph by taking the sum of the following equation solutions for each of the morph's competitive match-ups: the proportion of "wins" (speaker contacts) plus half the proportion of "ties" (no speaker contacted during the trial), divided by the total number of individual match-ups against each of the other morphs, minus 0.5 (following Andrews and David 1990). To compare the observed scores to the distributions of scores under the null model of equal attractiveness of all songs, we recalculated scores after permuting morph type from the observed data for each female. We calculated 5000 such sets of scores, then examined the frequency of scores in the permuted data that were at least as extreme as those observed. To make pairwise comparisons in attractiveness among morphs, we used a bootstrap approach that allows for somewhat different proportions of female behaviors between the observed and simulated data. We determined how robust the differences between the two scores for each pair-wise comparison were to changes in the sample by drawing 5000 bootstrap samples of female IDs, calculating scores for the morphs from the trial data for the sample of females, then calculating the difference in scores for each pair of songs in each bootstrap sample. We assessed these differences using basic and percentile bootstrap intervals (listed as "bootstrap-p" values in the results).

This two-way phonotaxis experimental design also allowed us to examine whether female preference for these songs was different when they were tested in a choice vs no-choice scenario (both speakers broadcasting song vs one speaker that was silent), as has been recently discussed in the literature (Dougherty and Shuker 2015). To compare whether females were more likely to contact one of the two speakers (make a decision) in choice vs no-choice trials, we ran a generalized linear mixed model in the lme4 package (family=binomial; Bates et al. 2015), with trial type (choice or no-choice) as the independent variable and decision made in the trial (yes/no) as the dependent variable, and female ID as a random effect. We took a model comparison approach (likelihood ratio test), comparing the full model with trial type as a fixed effect to a reduced model without trial type, allowing us to determine if trial type explained contact rates.

Short-distance mate attraction

In winter 2021, we collected and housed males (N=45) and females (N=28) using the methods described above to compare the relative attractiveness of morphs in a short-distance, courtship context. We paired females with males randomly in standardized no-choice courtship trials (following Tinghitella et al. 2018*a*; Broder et al. 2021; Fitzgerald et al. 2022) so that N≥15 trials for each male morph (ancestral, rattling, smallwing, curlywing). Each trial occurred in a 0.5 L deli cup lined with filter paper that was changed between trials to prevent accumulation of scent or debris that might bias female behavior, and we conducted all trials at night inside a field station in a quiet, acoustically isolated room lit with red light. After introducing a male and female to the deli cup, we gave males five minutes to begin courting (determined by him rubbing his wings together, known as stridulation), and if he did not, we ended the trial (and tried that male again after an hour). If the two crickets did not encounter each other within the first minute, we carefully moved them closer to each other using a pencil. After the male started courting, the trial lasted 10 minutes or until the female mounted the male. We recorded whether the female mounted and, if so, the time until mounting after the male started courting (stridulating). For trials where the male was mounted before stridulation, we assigned a time of 0 for "time until mount" (this only occurred once).

We compared mounting numbers among morphs using a generalized linear mixed model in the lme4 package (family=binomial; Bates et al. 2015), with morph as our independent variable and mount (yes/no) as our dependent variable. Females were used in 1-3 trials each so we included female ID in the model as a random effect. Because male morphs had uneven sample sizes (due to differences in how common each morph was in our field samples), some males were used in multiple trials, so we also included male ID as a random effect in the model. We compared models with and without morph type as a predictor (likelihood ratio test) to determine if morph explained mounting rates. We compared the time it took for females to mount among morphs using a linear mixed-effects model in the lme4 package (family=gaussian; Bates et al. 2015), with morph as our independent variable, time until mount as our dependent variable, and both male and female ID as random effects. We also used the same model comparison approach to determine the significance of male morph on time until mount.

Long-distance fly attraction

To test relative fly attraction to the songs of each morph in a competitive context, we conducted two different experiments in summer 2021, winter 2021, and summer

2022. First, to test that ancestral song is preferred by flies (as shown in Tinghitella et al. 2021, Broder et al. 2022, and Gallagher et al. 2022), we used sets of four traps that included the three novel morphs as well as ancestral song (hereafter, squares; 14 replicates, 56 traps). We then compared fly attraction to the novel morphs excluding ancestral (only three traps) in a second experiment (hereafter, triangles; 24 replicates, 72 traps; following Tinghitella et al. 2021; Broder et al. 2023). Each trap broadcasted calling songs of a different morph (same song loops played for crickets) at amplitudes that were as similar as possible to cricket long-distance phonotaxis trials. We built traps using clear 2-L plastic bottles with a speaker placed inside (AGPTEK A02 MP3 player), following established methods (Walker 1989; Tinghitella et al. 2021; Broder et al. 2022). We placed traps 10 m apart from each other in both experiments (squares and triangles) with the relative positions of the songs determined randomly, and we placed sets of traps at least 20 m apart from each other in the fields where we collected crickets and where flies have been successfully caught in recent studies (Tinghitella et al. 2021; Gallagher et al. 2022) for one hour each evening during sunset (when flies are actively searching for hosts; Wineriter and Walker 1990). At the end of each playback night, we recorded the number of flies caught in each trap. Then, to avoid pseudoreplication by catching the same flies the following night, we held the flies at a local field station for the duration of the experiment, then returned all flies to the fields where we caught them. We ran two generalized linear models (one for the square experiment and one for the triangle experiment using the bias reduction method, family=poisson) using the R package brglm2 (Kosmidis et al. 2020; Kosmidis and Firth 2021), with morph as our independent variable and flies caught per trap as our dependent variable. We used a model comparison approach to determine the significance of male morph on flies caught for squares and triangles, as used in short-distance cricket trials above, and conducted further pairwise comparisons when applicable using the emmeans package (adjust=”fdr”; Lenth 2021).

Short-distance fly attraction

In summer 2021, winter 2021, and winter 2022, we tested the attractiveness of songs of the different morphs to flies in a close-range, non-competitive context. First, we collected flies using funnel traps broadcasting ancestral song. We housed flies (N=26) in mesh butterfly cages indoors at ambient temperatures and under natural day and night light cycles for 24 hours before being used in the experiment. We ran each trial in a butterfly cage to which a measuring tape was attached so that we could measure vertical distance traveled. Trials began at sunset (fly active period) in a room lit by natural ambient light plus red light. After ensuring that the fly was at the top of the 40 x 40 x 61 cm cage, we began playback from an AOMAIS Sport II speaker placed face-up under the mesh cage, positioned in one of the four corners of the cage randomly for each stimulus. We played five stimuli (rattling, smallwing, curlywing, silence, and ancestral) to each fly at the same amplitudes we played to crickets in random order, except ancestral song which was always played last to avoid biasing the responses to the novel songs (as in: Tinghitella et al. 2021; Broder et al. 2022; Gallagher et al. 2022). During the trial, two trained observers measured the maximum vertical distance (cm) traveled by the fly toward the speaker and whether or not the fly contacted the speaker, two measures of positive phonotaxis (Tinghitella et al. 2021; Broder et al. 2022). After trials were completed, we returned flies to their collection fields. To compare the attractiveness of each morph’s song, we ran separate models for distance traveled and speaker contact. For distance traveled we ran a linear mixed-effects model (family=gaussian) using the R package lme4 (Bates et al. 2015), with stimulus (ancestral, rattling, smallwing, curlywing, and silence) as the independent variable, distance traveled as the dependent variable, and individual ID as a random effect. For speaker contact we ran a generalized linear mixed-effects model (family=binomial), with stimulus played (same as for distance but silence was excluded due to zero positive contacts) as the independent and contact (yes/no) as the dependent variables, and fly ID as a random effect. We used model comparison to determine the effect of male morph on fly response (both distance traveled and contact), and conducted pairwise comparisons among morphs using the emmeans package (adjust=”fdr”; Lenth 2021).

Comparing lab and field morph proportions

To test whether certain morphs were being disproportionately mated with (sexual selection) and/or surviving to adulthood in the wild (natural selection), we compared the morph proportions of lab- and field-reared animals from the same generation. We took eggs laid by females in the field, let them hatch in the lab, and reared them to adulthood, removing the natural selection pressure from the fly. Lab-reared crickets were housed in 15 L plastic containers at 26° Celsius inside a humidity-controlled room and given regular access to fresh food (Fluker’s cricket chow), water (moistened cotton), and shelter (egg carton). Upon reaching adulthood, we determined the morph of each male using the methods described above. We then compared the morph proportions of these lab-reared males and the proportions of field-reared males from the same time point to the proportions of their field-caught parents’ generation using a Fisher’s exact test to determine whether proportions changed in lab vs field rearing conditions.

Natural fly parasitism

During the winter 2021 sampling trip, we kept male crickets for up to seven days after collection. We took this opportunity to monitor males for fly parasitism by observing the emergence of fly pupae. Each day we checked each individual container for newly emerged fly pupae. We recorded the number of fly pupae that emerged per male and the male's morph to determine which morphs were being naturally parasitized in our sample (sample sizes per morph: ancestral=10, rattling=12, smallwing=3, curlywing=11).

RESULTS

Morph proportions over time

The relative abundance of morphs in the field changed over the three years of the study between winter 2018 and winter 2021 (Fisher's exact test: $p = 0.016$; Figure 3.2), consistent with ongoing evolution leading to changes in morph prominence; morph proportions do not appear to be at equilibrium. Curlywing, which was absent from sampling at the beginning of the study, arose or arrived in the population by time point 3 (winter 2019) and quickly increased in prevalence. Rattling, smallwing, and ancestral males decreased between the start and end of the study, although changes in abundance of rattling and ancestral males were dynamic among individual time points. Rattling was initially the most abundant and steadily increased in proportion over our first three sampling trips, but decreased overall by the last time point. By the end of the study, rattling, curlywing, and ancestral males were all found in similar proportions (0.33, 0.31, and 0.28, respectively), and smallwing was less common (0.08).

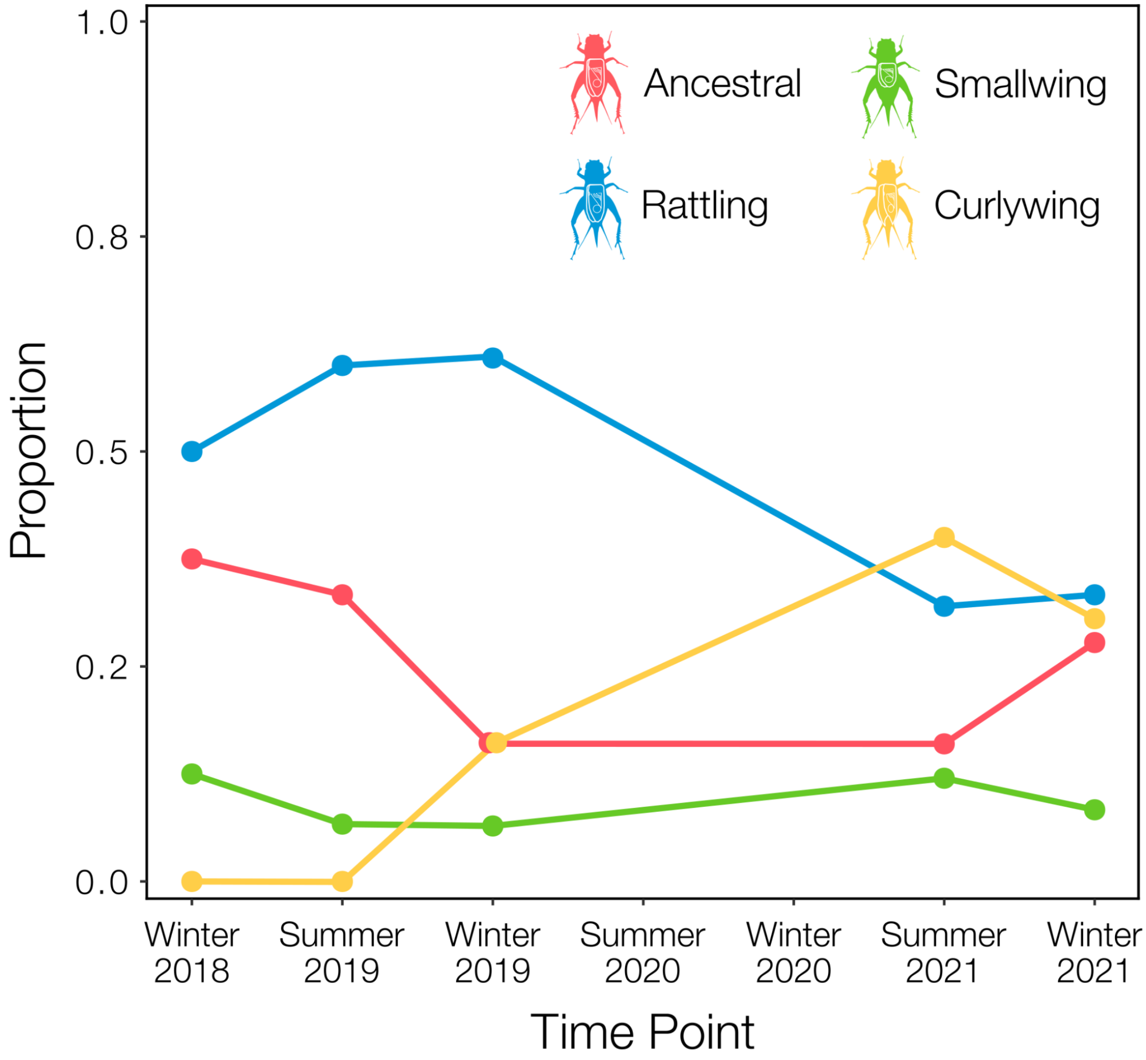

Figure 3.2. Morph proportions (relative abundance of each morph in the population) changed over the course of the three-year study, demonstrating rapid evolutionary change in the population. Notably, curlywing males were absent during the first two time points but arrived at some point before winter 2019, becoming increasingly common in the population soon after. As a result, there was a subsequent overall decrease in the proportion of all other morphs.

Long-distance mate attraction

In the four-choice phonotaxis trials, we found that speakers broadcasting some songs were contacted more often than others (Fisher's exact test: p = 0.018, N = 20), with ancestral being preferred over all other alternatives, confirming results from prior work (Figure S3.1A; Tinghitella et al. 2018*a*, 2021; Gallagher et al. 2022). Similarly, in no-choice trials (from the two-way phonotaxis experiment), speaker contact proportions of novel songs (rattling: 16%, smallwing: 18%, curlywing: 9%) were dramatically lower than contact proportions of ancestral song (73%) using data from a previous experiment (Figure S3.1B; Gallagher et al. 2022). The relative attractiveness of the novel morphs differed in two-choice phonotaxis trials (Figure 3.3A; N = 44 females, 262 trials). Smallwing and rattling did not differ in attractiveness from each other (boostrap-p = 0.395), but both were more attractive than curlywing (smallwing: bootstrap-p < 0.0001; rattling: bootstrap-p = 0.042) and silence (smallwing: bootstrap-p = 0.002; rattling: bootstrap-p = 0.031). Interestingly, curlywing was not more attractive to crickets than silence in long-distance phonotaxis trials (bootstrap-p = 0.781).

Previous work in the system has compared long-distance mate attraction among morphs in a no-choice format (Tinghitella et al. 2018*a*, 2021; Gallagher et al. 2022), whereas here we used both two- and no-choice designs. We found that females were more likely to make a decision (contact one of the two speakers) in competitive trials where two stimuli were being broadcast simultaneously (two-choice) than in non-competitive trials where one of the speakers was playing nothing (no-choice; Figure 3.3B; GLMM: z = -3.593, df = 1, p = 0.0003).

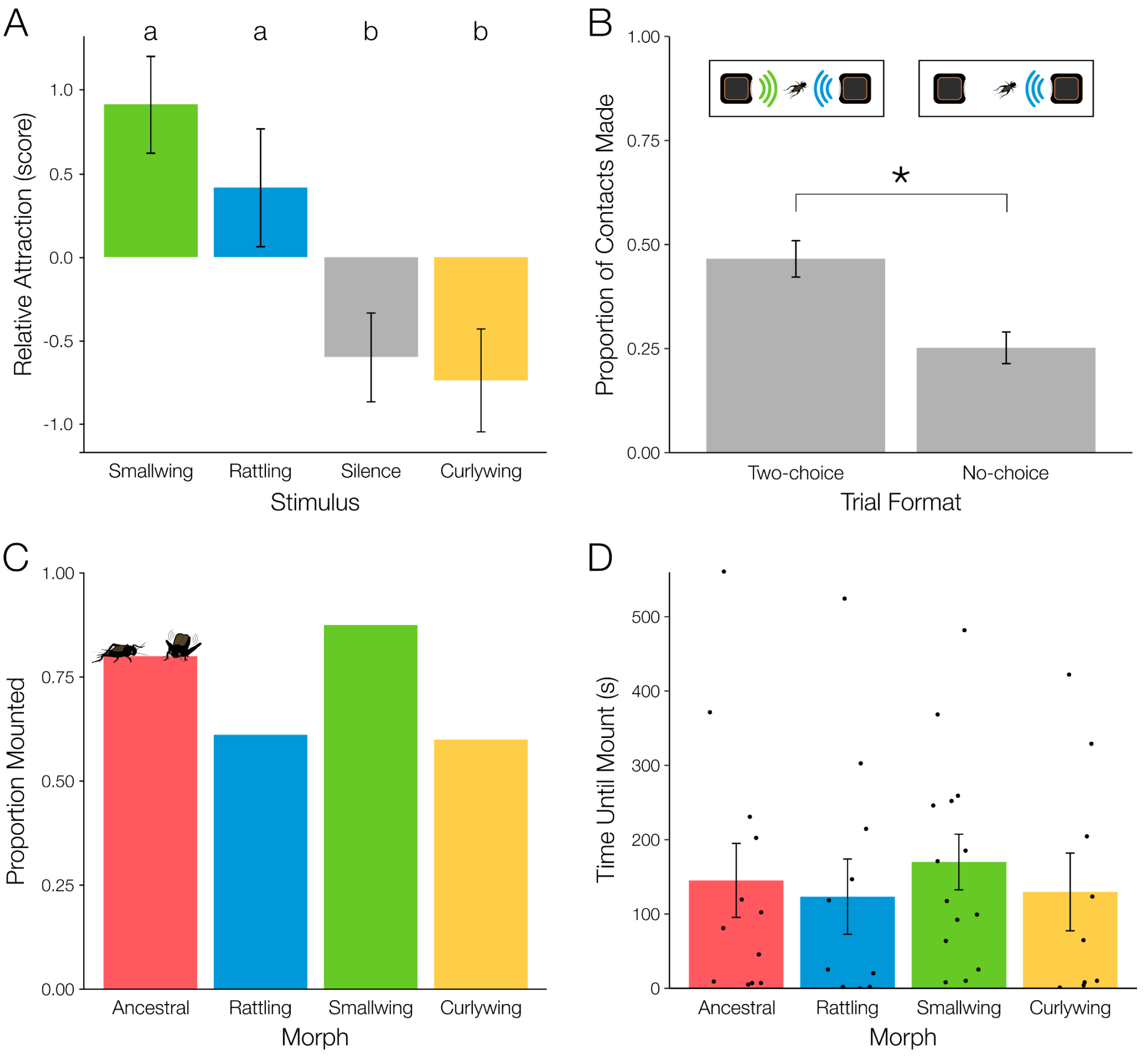

Figure 3.3. Morphs differed in their ability to attract mates in long-distance (A-B) but not short-distance contexts (C-D). A) In long-distance phonotaxis trials comparing the performance of novel morphs, smallwing and rattling calling songs were more attractive than curlywing (ancestral song remains more attractive than novel songs; see Figure S3.1). Relative attraction scores >0 indicate that the stimulus was more effective than expected if all stimuli were equally attractive, while scores <0 indicate the opposite. Curlywing did not differ in attractiveness from silence. B) Interestingly, females were more likely to make a decision (contact one of the two speakers) in two-choice trials than in no-choice trials, indicating that the relative attractiveness of songs may be heightened in a competitive context (as is likely common in nature). C-D) Morphs did not differ in attractiveness during close-range courtship encounters, both in the proportion of females that mounted (C) and the latency to mount (D).

Short-distance mate attraction

Unlike the long-distance phonotaxis context, mounting rates did not differ by male morph (likelihood ratio test: $X^2 = 2.781$, df = 3, N = 64, p = 0.427; Figure 3.3C), nor did the latency to mount after males began courting (likelihood ratio test: $X^2 = 0.478$, df = 3, N = 46, p = 0.924; Figure 3.3D).

Long-distance fly attraction

In long-distance field fly trapping squares, flies caught per trap differed by morph (likelihood ratio test: $X^2 = 61.690$, df = 3, $p < 0.0001$); flies preferred ancestral over all other morphs (Figure 3.4A). Flies caught per trap did not differ by morph in long-distance field fly trapping triangles that did not include a trap broadcasting ancestral song (likelihood ratio test: $X^2 = 1.551$, df = 2, p = 0.460; Figure 3.4A). In both trapping experiments, we never caught a fly to a trap broadcasting curlywing song.

Short-distance fly attraction

In short-distance fly phonotaxis trials (no-choice trials), distance traveled to the speaker differed by morph (likelihood ratio test: $X^2 = 578.525$, df = 4, N = 26, $p < 0.0001$), and speaker contact differed by morph (likelihood ratio test: $X^2 = 39.176$, df = 3, N = 26, p = < 0.0001). By both measures (distance and contact), ancestral was preferred by flies followed by smallwing and rattling with curly as the least preferred (Figure 3.4B).

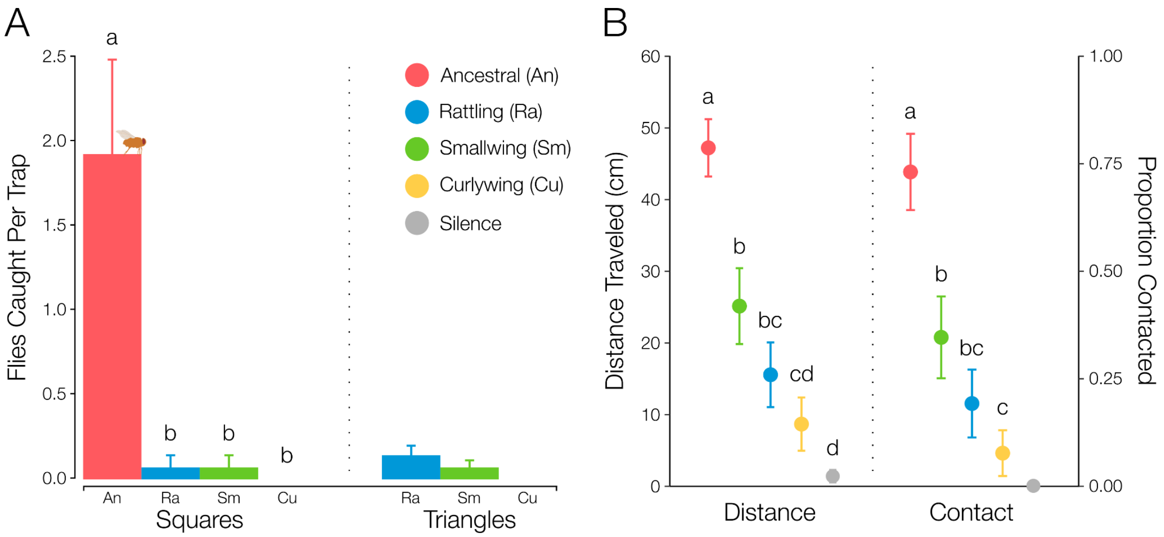

Figure 3.4. Fly attraction differed by morph in both long- (A) and short-distance (B) contexts. A) In long-distance fly trapping, we caught more flies in traps broadcasting ancestral song than the novel songs. In both the experiment with competing ancestral song (squares) and the experiment excluding ancestral (triangles), the number of flies caught did not differ among novel morphs. We never caught flies to speakers broadcasting curlywing song. B) In short-distance no-choice trials, attractiveness differed by morph in both our measures of positive phonotaxis: distance traveled and speaker contact. Like the long-distance experiment, ancestral song was the most attractive to flies. However, here we also found differences among the attractiveness of other songs; smallwing was more attractive than curlywing and silence, and rattling was more attractive than silence. Curlywing did not differ from silence in attractiveness. Overall, while ancestral song remains by far the most attractive to the fly, we found evidence that novel morphs differ among one another in their level of protection from parasitism.

Change in morph proportions between field and lab

We found that morph proportions changed between first-generation field-caught and second-generation lab-reared crickets (Fisher's exact test: p = 0.016), but not between first-generation field-caught and second-generation field-caught crickets (Fisher's exact test: p = 0.728; Figure 3.5).

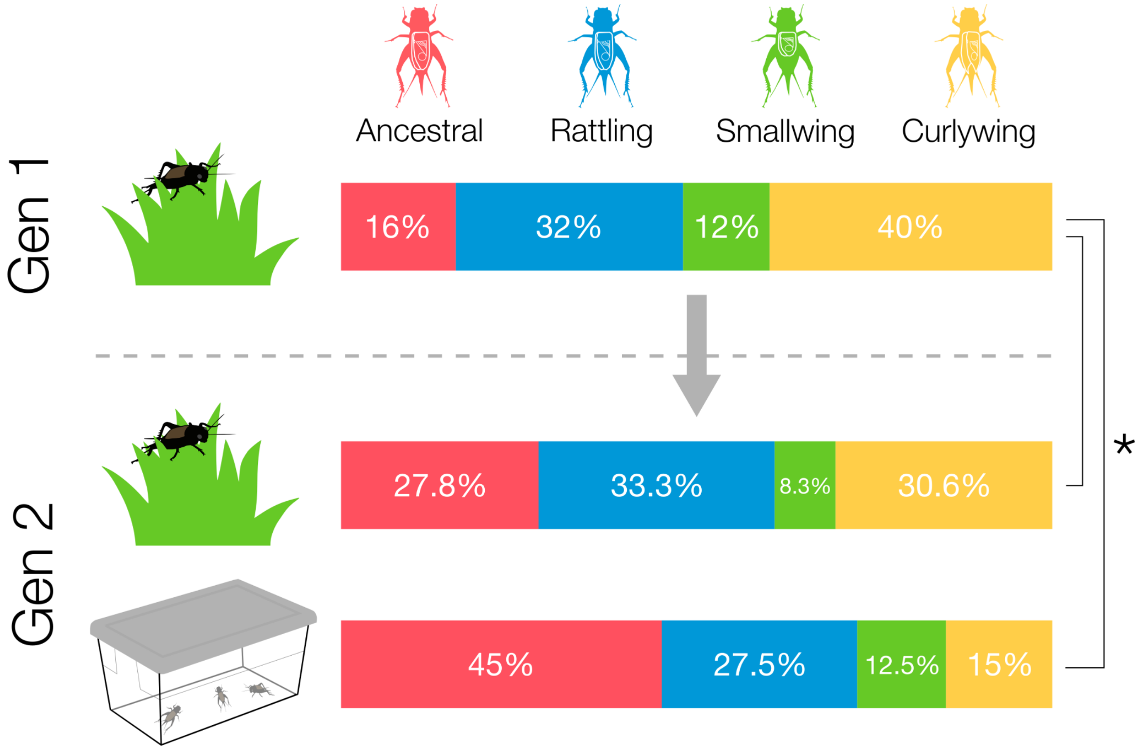

Figure 3.5. Morph proportions of second-generation males differed from first-generation males when reared in the lab but did not when sampled from the field (Gen 1 n=25, Gen 2 field n=36, Gen 2 lab n=80). Curlywing males were much more common in the field than in the lab, while ancestral males were much more common in the lab than in the field. Rattling males were slightly more common in the field, and smallwing males were slightly more common in the lab. This may indicate that ancestral males are being disproportionately mated with by females, but are not surviving in the wild as well as other morphs, possibly due to selection imposed by the fly; the opposite seems true for curlywing males—greater survival but fewer matings. Rattling and smallwing males, which are intermediate in mate attraction and parasitism avoidance, had very small changes between rearing conditions.

Observing natural fly parasitism

Of the 45 males of various morphs that we collected and monitored for evidence of parasitism, three had at least one pupa emerge from them. These parasitized males were all ancestral (30% of ancestral males we collected were parasitized), and each had multiple pupae emerge from them (three, four, and six).

DISCUSSION

We investigated the evolutionary mechanisms that shape the early stages of sexual signal diversification in nature to gain insight into how opposing natural and sexual selection pressures erode or maintain novel variation within populations. We first documented rapid evolutionary change in the morph composition of a single polymorphic population of crickets over three years (Figure 3.2) and then investigated the selective landscape contributing to those changes by characterizing the primary sexual and natural selection pressures that male crickets face in the field (Figure 3.3, 3.4). This combination of field-based approaches allows us to map dynamic evolutionary change that is happening in real time to the selection pressures most likely to drive that change. We found that four cricket morphs present in a single population (ancestral, rattling, smallwing, and curlywing) balance sexual and natural selection pressures differently. Overall, ancestral males are most attractive to mates but least protected from fly parasitism, while curlywing males are least attractive to mates but most protected from flies. Two other novel morphs, rattling and smallwing males, are intermediate to ancestral and curlywing in mate attraction and fly protection.

How then does the relative performance of each morph map onto the relative changes we observed in morph proportions in nature over time? This will allow us to make inferences about which selection pressures are driving evolutionary change in the population. Regarding sexual selection, the main effect we found was in long-distance mate attraction; as in previous work, ancestral males enjoy an advantage over all novel morphs in phonotaxis tests (Figure S3.1; Tinghitella et al. 2018*a*, 2021; Gallagher et al.

2022). We also detected a strong disadvantage for curlywing in long-distance mate attraction (Figure 3.3A). If sexual selection were driving patterns of morph changes in the wild, we would expect to see the ancestral morph increasing and curlywing decreasing. Instead, we found the opposite (Figure 3.2). When we investigated natural selection using short- and long-distance host location trials, we found that ancestral males were the least protected from parasitism, while curlywing was most protected (Figure 3.4). This aligns with the patterns of morph changes we observed in the field in that when curlywing males arrived or arose in the population sometime after summer 2019, they rapidly increased in prevalence over the following ~two years (Figure 3.2). Interestingly, rattling, which is more protected from flies than ancestral, was initially common, but became less frequent after the arrival and subsequent increase of curlywing (Figure 3.2), which appears more protected than rattling. Collectively, these patterns suggest that of the two major selection pressures that male crickets face, natural selection from parasitism has been the primary driver of rapid evolutionary change in the population (Figure 3.6). There are other interesting patterns in the field data we collected that beg explanation, such as the relatively constant but low proportion of smallwing in Hilo (Figure 3.2). We are very interested to interrogate the roles of drift, plasticity, underlying genetic architecture, gene flow, and other selection pressures that could contribute to the evolutionary dynamics of this population.

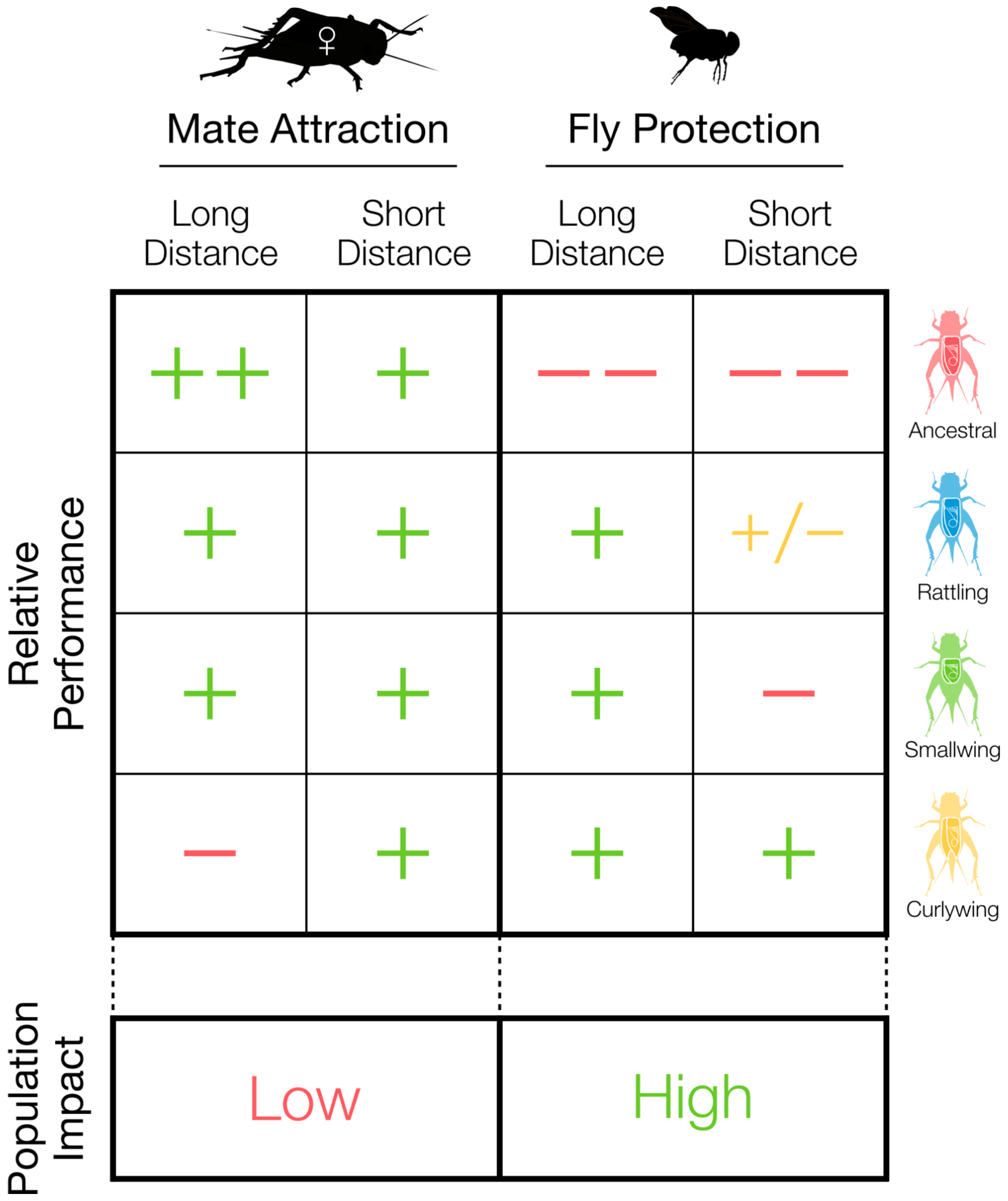

Figure 3.6. Mate attraction and fly protection, the primary sexual and natural selection pressures shaping male song morphs, differ among the four morphs in long- and short-distance contexts. Pluses and minuses indicate the relative performance among morphs in each of the four experiments conducted (represented by columns) in this study. For instance, all morphs are equally capable of attracting mates in short-distance courtship encounters, but curlywing song doesn't differ from silence in its ability to attract mates at long distance, unlike the other three morphs. Rattling was intermediate to (but did not differ from) smallwing and curlywing during the short-distance fly experiment and so was given +/- (Figure 3.4B). We mapped these performance results onto the changes in

morph proportions we observed over time (Figure 3.2) and found that the morphs that were most protected from parasitism increased in proportion over time (first rattling, then curlywing after its arrival), while morphs that were more attractive to mates did not increase (they mostly decreased). This suggests that natural selection from parasitism (High) has likely impacted morph proportions in the population more than sexual selection from mates (Low).

The lab-rearing experiment supports several findings above (Figure 3.5). When we removed natural selection from flies by rearing first generation offspring of field-caught females in the lab, we found that the proportion of ancestral males was greater in the lab-reared animals than we documented in the field. Conversely, the proportion of curlywing males decreased in the lab. Over that same time period, the morph proportions in the field did not change significantly. This may suggest that ancestral males are disproportionately mated with, but may have reduced survival relative to other morphs in the wild, and that curlywing males are mated with less frequently but have greater rates of survival. These patterns support our earlier results in that ancestral males are most preferred by potential mates and parasitoids, curlywing is least preferred by potential mates and parasitoids (Figure 3.3-3.4), and the four morphs balance natural and sexual selection pressures differently (see Figure 3.6 for a summary of those results). One possible alternative interpretation for this difference in lab-reared males is that developmental plasticity is affecting morph proportions. However, all previous work (Rayner et al. 2019; Gallagher et al. 2022) and personal observations strongly suggest that rearing conditions do not determine morph or affect morph-level morphological features. Of course other factors like genetic drift, gene flow, genetic architecture, and unmeasured selective pressures may influence our measures of morph proportions in the field, so we interpret this result with caution.

Understanding how selection has been shaping morph proportions allows us to make predictions about the future trajectory of the population immediately following sexual signal diversification. Will one morph ultimately reach fixation, or will polymorphism be maintained? If the same patterns continue, we might expect morphs that are best protected from parasitism to further increase in the population—in this case, curlywing would eventually outcompete the other morphs (Figure 3.6). It might seem unlikely that novel morphs would replace ancestral males, given that they are not preferred by female crickets (discussed below); yet novel morphs have indeed recently gone to fixation in two other long-studied populations in Hawaii (Tinghitella et al. 2018; Gallagher et al. 2022; Gallagher et al. in review). However, numerous other considerations instead suggest that multiple morphs may be maintained within the population. First, at our final sampled time point (winter 2021) morph proportions deviated slightly from the clear patterns observed throughout the rest of the three-year study, as curlywing males decreased and, surprisingly, ancestral males increased. At this time point, in fact, rattling, curlywing, and ancestral males were all in similar proportions, suggesting that the system is not at equilibrium and that perhaps polymorphism will be maintained long-term in the population. Such fluctuations can be due to negative frequency dependent selection among morphs, where the decrease in prevalence of a morph improves its relative fitness (Sinervo and Lively 1996; Brooks 2002), creating evolutionary cycles where phenotypic diversity is maintained over time. In particular, the density dependent aspect of predation (in this case, from the fly) can drive such evolutionary cycles and maintain polymorphism in a population (Lerch and Servedio

2023). Polymorphic populations may also be maintained through gene flow, which can bring new morphs into the population or counteract selection by introducing non-adaptive alleles. Indeed, there is some evidence of ongoing gene flow in Hawaiian *T. oceanicus* populations (Zhang et al. 2021), and curlywing's arrival in Hilo during this study may reflect ongoing gene flow from other island populations. Alternatively, we cannot rule out the possibility that curlywing evolved convergently across populations, as was the case for silent crickets (Pascoal et al. 2014). Given the recent propensity for the evolution of novelty in this system, it's also possible that yet another new morph could arise that balances selection pressures in new ways, affecting evolutionary dynamics in the population.

This leads to the final reason why our predictions may not play out as anticipated: the genetic underpinnings of these morphs are not fully resolved. During the last time point of this study and in our subsequent field surveys, the number of males that could not be assigned to one particular morph increased, and we've since collected males that express qualities of two different morphs (i.e., smallwing-curlywing males). If some of these wing characteristics can segregate independently (as is the case for flatwing; Rayner et al. 2019), there may be the potential for further novel signal values to evolve which could reach new fitness peaks (e.g., if smallwing-curlywing males produce a song that is more protected from flies and more attractive to mates). Even more recently, we discovered a male that had the distinct wing morphology of one morph on his right wing and a different morph on his left wing, and his song switched between morphs depending on which wing was on top during stridulation. We cannot at the moment fathom the genetic architecture underlying this type of "chimera" male, but we implore others to investigate the phenomenon further. These unusual male types did not begin appearing until the end of our study, so it will be important to continue monitoring the population over time for new phenotypic variation that may impact its evolutionary trajectory.

We found that curlywing did not differ from silence in long-distance mate attraction, suggesting that its calling song may not serve as a sexual signal that attracts mates (Figure 3.3A). Yet despite this, curlywing has increased in commonality in the population (Figure 3.2), likely due to its superior protection from parasitism (Figures 3.4, 3.6). If curlywing males do not attract mates from afar, how are they able to acquire mates at all? Silent males, which face a similar dilemma in other locations, exhibit satellite behavior where they are phonotactic to male ancestral song, consequentially increasing their likelihood of encountering a female that is also attracted to the song and thus in the same vicinity (Zuk et al. 2006). Because we found that all four morphs perform similarly well in close-range courtship encounters (Figure 3.3C-D)—suggesting weak (if any) sexual selection on male morph during courtship—a satellite behavioral strategy and/or increased ambulatory behavior (Balenger and Zuk 2015) could help less preferred morphs with attenuated song to find potential mates and offset their acoustic shortcomings. Another factor that could influence the ability of curlywing males (and other attenuated morphs) to locate mates is the local population density. Anecdotally, we note that populations with a large proportion of novel morphs have higher local population densities, perhaps as a consequence of human infrastructure (such as physical restriction to grass between parking lots and buildings on a university campus, as in this study). This type of geographical constraint may benefit males that are inferior in long-distance mate attraction by increasing the likelihood of them encountering a female.

Future work should explore whether novel morphs exhibit differing behavioral strategies and the role that population density plays in their relative successes.

Presence and composition of competing males may also impact the relative success of mate attraction. We found that female crickets were more likely to make a phonotaxis decision in the presence of two competing songs than when only one was broadcast and silence was the alternative (Figure 3.3B), suggesting that the overall success of males in attracting a female may increase when there are more males in close proximity, which is likely to occur in densely populated fields. This is contrary to other work in *T. oceanicus* where positive phonotaxis rates dropped upon increasing numbers of competing songs (Tanner and Simmons 2021). However, it is worth noting that this referenced study was conducted in Australia, where all males are ancestral, and females may assess male songs differently than in Hawaii. This finding supports other studies showing that trial format can significantly affect measures of attractiveness (Dougherty and Shuker 2015) and should be considered thoughtfully when designing mate choice experiments.

During this study, we also monitored males for parasitism (emergence of pupae). We found that only ancestral males were parasitized, consistent with our fly trapping and phonotaxis results that ancestral males are by far the most attractive to flies (Figure 3.4). We only observed males for up to seven days (some males as little as four days due to their later collection dates) and pupae can take nine days to develop inside a host (Wineriter and Walker 1990), so this rate is likely under-representing rates of parasitism. We were intrigued to find that the three parasitized males had three, four, and six pupae emerge (mean 4.3), which is more than previously published rates (average 1.8 for

Gryllus integer; Adamo et al. 1995) and more than rates measured 30 years ago in the very population of *T. oceanicus* we study here (where no more than two pupae ever developed inside an individual host; Zuk et al. 1993). The large number of pupae emerging from ancestral males in this study may stem from the rarity of easily detectable hosts leading to superparasitism (attacked by multiple flies; Salt 1934), or evolutionary or plastic increases in parasitoid clutch sizes. Future work should investigate in more depth the effects of changing morph proportions and thus findable host availability on the behavior and life history of the fly.

Our work provided an unusual opportunity to learn how evolution's playbook unfolds during the early stages of sexual signal diversification, and to follow along as it is written. Conducting this study within a single population allowed us to deeply interrogate the selective landscape driving evolutionary dynamics; we could eliminate many confounding environmental variables (all crickets and flies were from the same site with the same conditions), and accurately capture morph proportions by thoroughly sampling a small population. Our findings show how novel variants compete with each other through complex tradeoffs between selective pressures, and how these dynamics are shaped by a recently introduced parasitoid fly. Though experimental and modeling studies have supported the theory that predation can shape the early stages of diversification (Nosil and Crespi 2006; Meyer and Kassen 2007; Bush et al. 2019), our findings provide evidence of this in a natural, unmanipulated context. It will be useful to conduct this type of study in other locations across Hawaii with different combinations of morphs, and to use multi-generation mesocosm studies with varied socio-sexual and predation regimes to isolate the importance of specific selection pressures and learn whether or not selection shapes populations in a repeatable way. The tractability of this emerging model system makes it a powerful microcosm for answering fundamental questions in evolutionary biology.

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APPENDIX

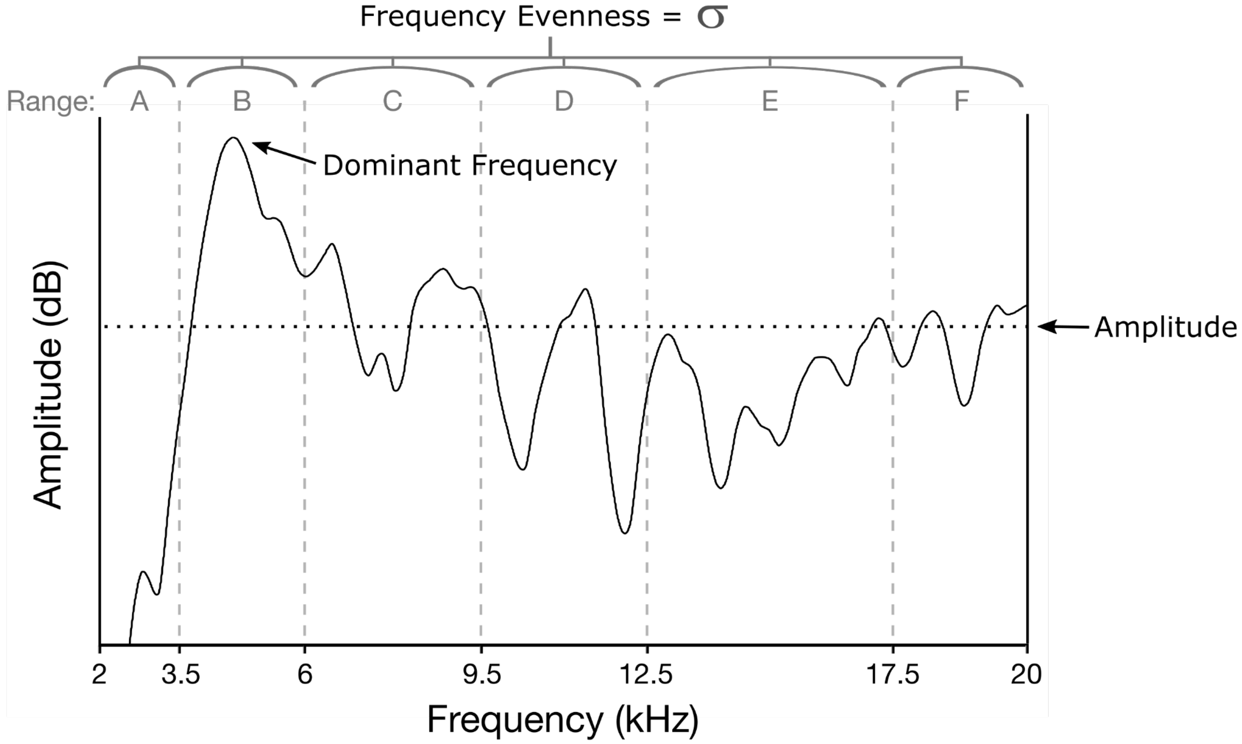

Figure S1.1. Annotated power spectrum of a single sample calling song displaying the nine sound characteristics measured in each calling and courtship song. “Dominant frequency” is the frequency with the greatest acoustic power. “Amplitude” is a measure of how loud the song is across all frequencies (using RMS level). Songs were spectrally divided into six frequency ranges (A-F), chosen because they represent natural clusters of auditory receptor fibers in *T. oceanicus*, indicating hearing sensitivity at different frequencies (Imaizumi and Pollack 1999). We divided the amplitude of each frequency range by the sum of all ranges’ amplitudes to determine the proportion of acoustic energy (“relative amplitude”) in each frequency range. We took the standard deviation of all relative amplitude ranges (A-F), multiplied by -1, as a measure of how evenly distributed the acoustic energy is across the song’s frequency spectrum (a measure of how broadband the sound is). We called this final characteristic “frequency evenness” (Formula: -(relative amplitude of ranges A,B,C,D,E,F)).

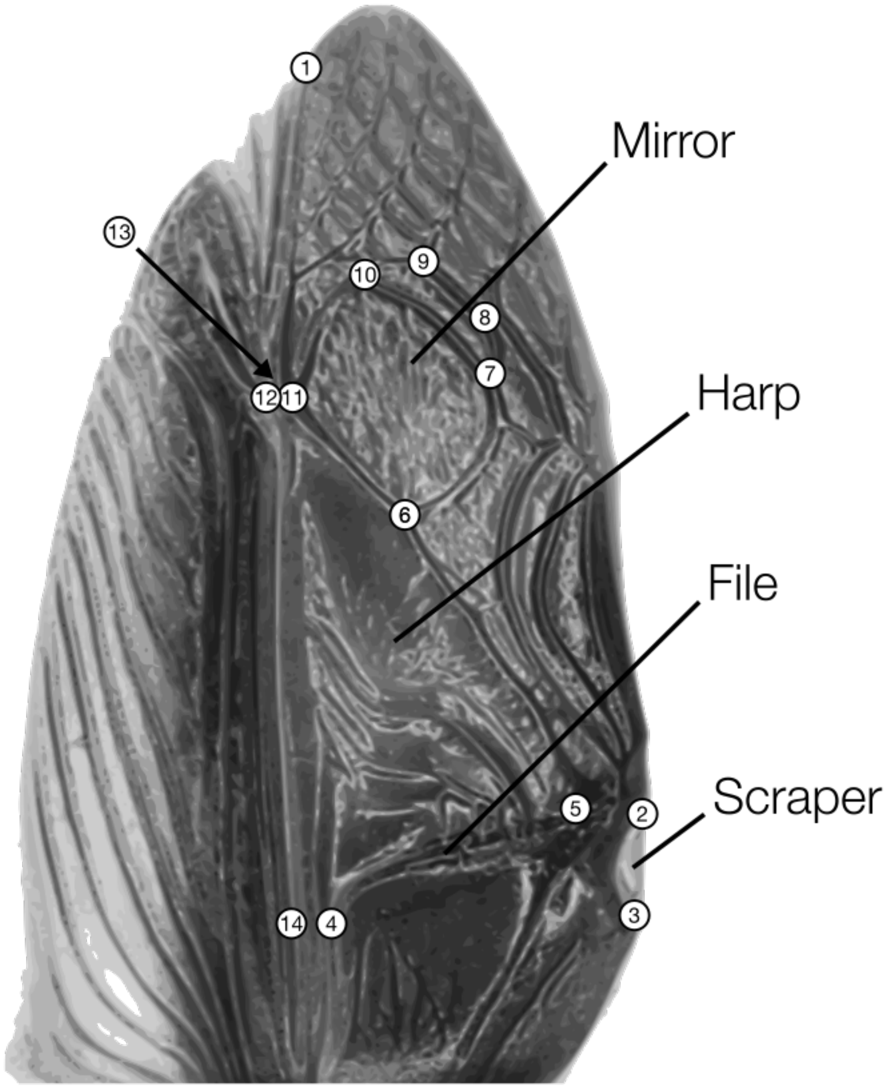

Figure S1.2. Example wing with landmarks placed at their respective locations. See Table S1 for location descriptions. Landmark locations adapted and modified from (Pascoal et al. 2014, 2017).

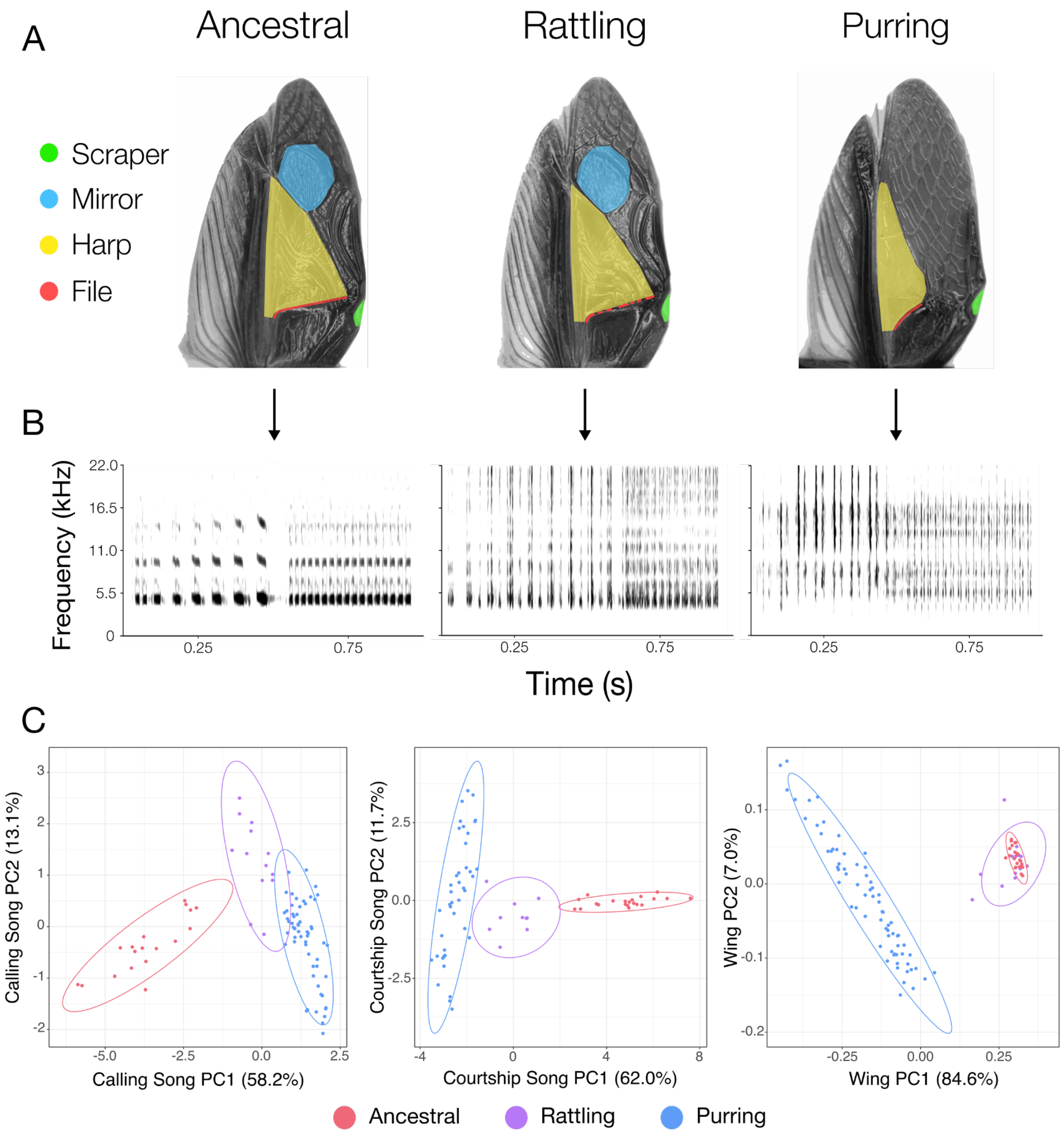

Figure S1.3. Example wings (A) and courtship songs (B) of ancestral, rattling, and purring males. Structures highlighted are important in sound production, and thus changes to them may alter (or even prevent) song (Desutter-Grandcolas 1998; Bennet-Clark 1999, 2003; Zuk et al. 2006; Montealegre-Z et al. 2009, 2011; Tinghitella et al. 2018; Duncan et al. 2021). Ancestral and rattling males both have fully intact harps, mirrors, and scrapers, while purring males have reduced harps and no mirrors. Rattling males have unique gaps between groups of teeth on the file (see Figure 4A). C) Principal component analyses for calling songs, courtship songs, and wing morphology of ancestral, rattling, and purring phenotypes using a sample of field-caught males manually classified to morph using diagnostic phenotypic characteristics from the cluster analysis (N=105). Songs differ among morphs for both calling (MANOVA: $F_{4,174}$=77.8, p<0.0001) and courtship songs (MANOVA: $F_{4,140}$=32.2, p<0.0001), as well as morphology (Wing Morphology: $F_{4,204}$=48.8, p<0.0001). Ellipses represent 90% confidence intervals. PC1 largely captures the extent to which a song is ancestral-like. Song characteristics of rattling are in many ways intermediate to those of ancestral and purring songs.

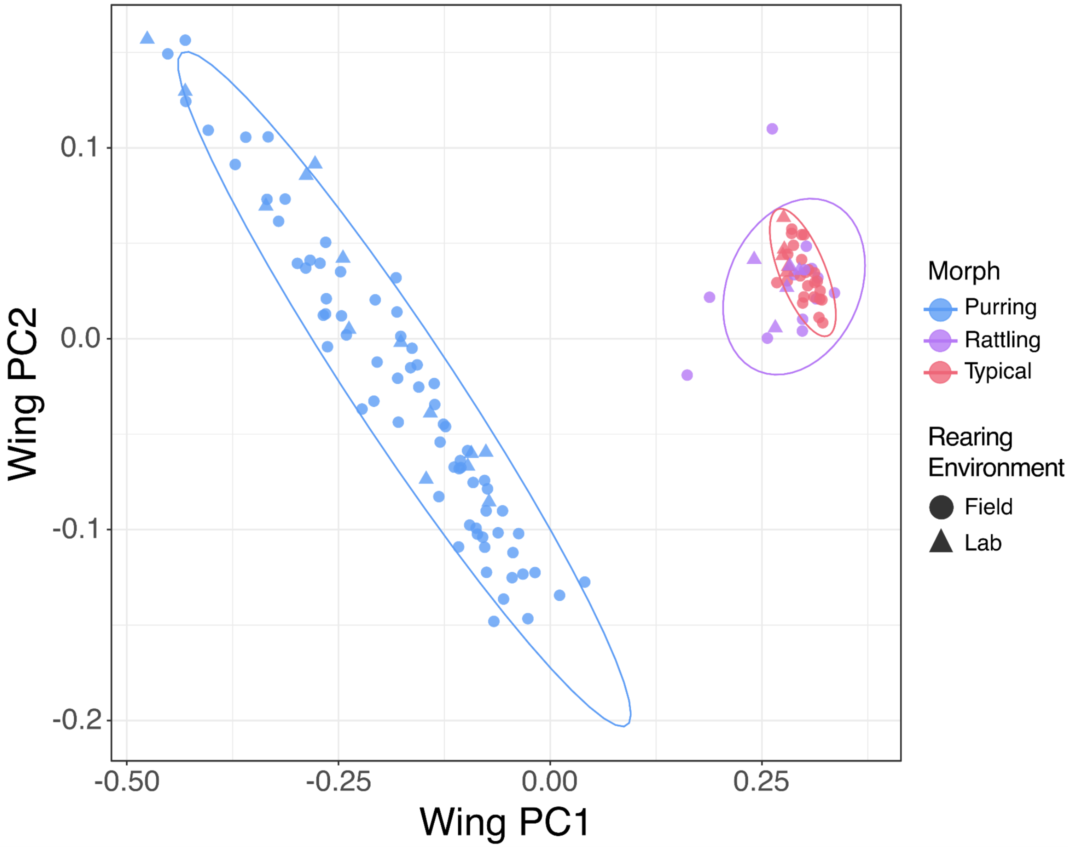

Figure S1.4. Morph-level differences in wing morphology (based on geometric morphometrics of 14 landmarks of the dorsal side of the wing; Figure S2, Table S2) were robust to differences in rearing environment. Morph was strongly predictive of morphological differences (MANOVA; Morph: $F_{4,248}$=60.0, p<0.0001) while rearing treatment and the interaction between morph and rearing treatment were not (MANOVA: Rearing Treatment: $F_{2,123}$=2.0, p=0.14; Morph x Rearing Treatment: $F_{4,248}$=0.58, p=0.68). The consistency of morphological differences across morphs within a common-garden, lab context suggests that differences have a genetic basis.

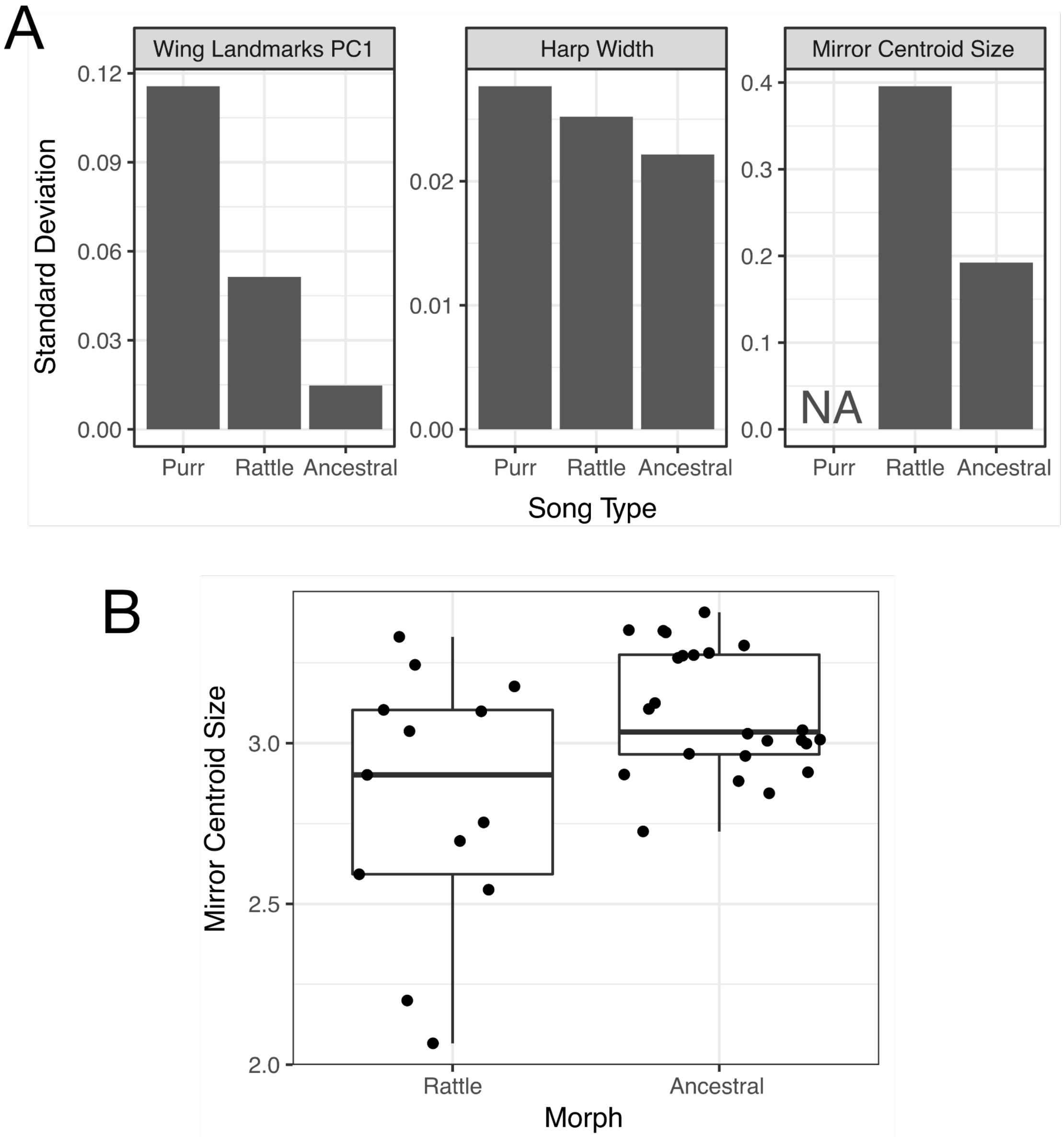

Figure S1.5. Differences in morphology among morphs. A) Standard deviations of wing morphometric variables show greater levels of morphological variation amongst alternate morphs (purring and rattling) compared to wings of ancestral males (Levine's test: $F_{2,103}$=21.2, p<0.0001). B) Though there is a statistically significant difference in mirror size between rattling and ancestral wings, it cannot explain the dramatic differences in song between morphs. There is much overlap in mirror size between morphs, and morph-level differences became non-significant when two outlier rattling males (with small mirrors) were removed from the dataset. Additionally, a larger resonator (mirror) should be associated with lower frequency sound (Bennet-Clark 1999), but we found the opposite pattern in rattling, but not ancestral, males—rattling songs have higher mean frequency than ancestral songs.

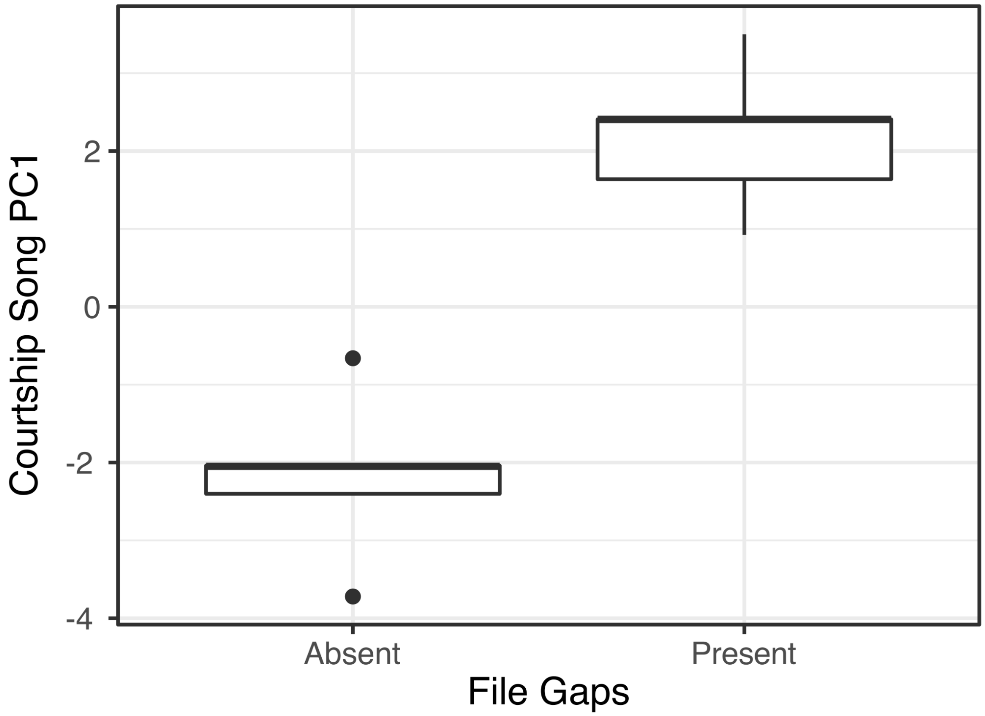

Figure S1.6. Courtship songs of second-generation, lab-reared males differed greatly depending on the presence of file tooth gaps, a diagnostic characteristic of the rattling morph. Males with tooth gaps (rattling males) had significantly greater courtship song PC1 values than males without gaps (ancestral males; T-test: t=6.68, df=7.88, p=0.0002, n=10). Courtship songs and wings were analyzed within two weeks of males eclosing to the adult stage in a common-garden, lab setting, removing most environmental/age effects on phenotypic differences.

Table S1.1. Sampling sites across Hawaii used in this study.

Site Name	Island	Males (N)	Females (N)	Location
Hilo	Hawai'i	35	30	University of Hawai'i at Hilo
Kalaupapa	Moloka'i	7	26	Kalaupapa National Historical Park
Manoa	O'ahu	31	30	University of Hawai'i at Manoa, Astronomy Center
La'ie	O'ahu	26	30	Brigham Young University – Hawaii
Wailua	Kaua'i	28	31	Kaua'i Research Station at the University of Hawai'i College of Tropical Agriculture
Kapa'a	Kaua'i	26	25	Kapa'a Pono Kai Resort

Table S1.2. Descriptions and notes for wing landmarks.

Landmark #	Description	Notes
1	Apical tip of Cu1	
2	Apical tip of scraper	If no scraper, directly below wing bend
3	Basal tip of scraper	If no scraper, on top of landmark 2
4	Basal point of Cu2 where the vein begins to curve away from Cu1	Usually proximal end of the file and basal point of the harp, where Cu1 and Cu2 are no longer parallel
5	Distal corner of the harp	In ancestral wings there is usually a junction of Cu2 and distal harp vein. In flatwings (wings with reduced harps and no mirrors), distal harp vein often appears to merge seamlessly with Cu2
6	Basal junction of mirror and distal harp vein	Placed on top of landmark 11 if mirror is absent
7	Basal junction of upper and lower mirror cells	Placed on top of landmark 11 if mirror is absent
8	Distal junction of apical accessory vein and upper mirror cell	Placed on top of landmark 11 if mirror is absent
9	Proximal junction of apical accessory vein and upper mirror cell	Placed on top of landmark 11 if mirror is absent
10	Apical junction of upper and lower mirror cells	Placed on top of landmark 11 if mirror is absent
11	Junction of lateral vein and distal harp vein	Lateral vein is the most basal connector between Cu1 and distal harp vein. In ancestral, this is at the proximal edge of the mirror. In flatwings, the lateral vein may not be at the apical tip of the harp
12	Junction of lateral vein and Cu1	Lateral vein is the most basal connector between Cu1 and distal harp vein. This will always be across from landmark 11
13	Apical tip of extended harp	May overlap with landmark 12 in many wings, including most wings with a mirror
14	Basal, proximal tip of harp on Cu1	Adjacent to landmark #4

Table S1.3. Means and standard deviations of trait values of ‘ancestral’, ‘rattling’, and ‘purring ’individuals from the dataset used in Figure S1.3.

		Ancestral (n = 24)	Rattling (n = 13)	Purring (n = 69)
Calling Song (composite variables)				
	PC1	-3.665 (1.101)	-0.216 (0.428)	1.384 (0.404)
	PC2	-0.386 (0.534)	1.292 (0.776)	-0.261 (0.784)
Calling Song (individual characteristics)				
	Amplitude	126.434 (82.566)	15.497 (6.263)	1.539 (0.609)
	Dominant Frequency	4877.105 (191.528)	5805.923 (1702.966)	9204.915 (3942.025)
	rangeA	0.014 (0.014)	0.030 (0.02)	0.154 (0.041)
	rangeB	0.713 (0.095)	0.300 (0.073)	0.152 (0.034)
	rangeC	0.083 (0.032)	0.282 (0.072)	0.193 (0.039)
	rangeD	0.100 (0.053)	0.137 (0.039)	0.173 (0.037)
	rangeE	0.059 (0.027)	0.160 (0.074)	0.170 (0.032)
	rangeF	0.031 (0.017)	0.091 (0.073)	0.157 (0.037)
	Freq. Evenness	-0.271 (0.043)	-0.121 (0.03)	-0.036 (0.024)
Courtship Song (composite variables)				
	PC1	4.841 (1.303)	-0.002 (0.771)	-2.448 (0.51)
	PC2	-0.055 (0.149)	-0.502 (0.586)	0.058 (1.903)
Courtship song (individual characteristics)				
	Amplitude (chirp)	172.234 (85.612)	8.030 (5.037)	2.850 (3.162)
	Dominant Frequency (chirp)	4868.318 (183.622)	4859.500 (477.827)	8051.233 (4186.844)

	rangeA (chirp)	0.006 (0.003)	0.019 (0.016)	0.129 (0.053)
	rangeB (chirp)	0.779 (0.084)	0.357 (0.071)	0.154 (0.041)
	rangeC (chirp)	0.073 (0.037)	0.228 (0.053)	0.183 (0.059)
	rangeD (chirp)	0.074 (0.037)	0.114 (0.033)	0.184 (0.044)
	rangeE (chirp)	0.041 (0.023)	0.138 (0.036)	0.189 (0.079)
	rangeF(chirp)	0.026 (0.015)	0.144 (0.043)	0.160 (0.053)
	Freq. Evenness (chirp)	-0.302 (0.039)	-0.121 (0.028)	-0.055 (0.036)
	Amplitude (trill)	113.409 (52.582)	7.971 (4.745)	1.795 (0.952)
	Dominant Frequency (trill)	4772.864 (168.234)	4909.200 (583.036)	7343.548 (3906.386)
	rangeA (trill)	0.009 (0.005)	0.026 (0.029)	0.142 (0.044)
	rangeB (trill)	0.716 (0.098)	0.348 (0.077)	0.170 (0.03)
	rangeC (trill)	0.104 (0.052)	0.242 (0.053)	0.190 (0.051)
	rangeD (trill)	0.097 (0.053)	0.120 (0.033)	0.182 (0.04)
	rangeE (trill)	0.039 (0.018)	0.138 (0.033)	0.170 (0.037)
	rangeF (trill)	0.035 (0.017)	0.127 (0.035)	0.145 (0.035)
	Freq. Evenness (trill)	-0.274 (0.041)	-0.118 (0.035)	-0.041 (0.024)
Wing Morphology				
	PC1	0.305 (0.015)	0.283 (0.051)	-0.170 (0.116)

	PC2	0.035 (0.014)	0.028 (0.032)	-0.021 (0.078)
	Scraper (proportion present)	24/24	12/13	55/68 *scraper presence undetermined for one male due to obscured photo
	Mirror (proportion present)	24/24	13/13	0/69
	Harp Width	0.271 (0.022)	0.281 (0.025)	0.138 (0.028)
	Mirror Size	3.098 (0.192)	2.826 (0.396)	NA

Table S1.4. Correlations (r) between song and wing traits within A) ancestral (N = 23), B) rattling (N = 13), and C) purring males (N = 69) from the dataset used in Figure S1.3. P-values shown in parentheses. All variables are continuous except scraper (presence/absence). Bold cells highlight significant morphology-song relationships. Note that we show correlations for scraper presence in rattling males, but these patterns are driven by a single rattling male that lacked a scraper and should be interpreted with caution.

A) Ancestral males

	Wing PC1	**Scraper**	**Mirror Size**	**Harp Width**
Calling Song PC1	-0.177 (0.483)	NA	-0.038 (0.881)	0.014 (0.957)
Peak Frequency	0.198 (0.432)	NA	-0.157 (0.534)	0.079 (0.757)
Amplitude	-0.107 (0.673)	NA	0.121 (0.632)	0.111 (0.660)
Frequency Evenness	-0.269 (0.280)	NA	0.046 (0.858)	0.047 (0.852)

B) Rattling males

	Wing PC1	**Scraper**	**Mirror Size**	**Harp Width**
Calling Song PC1	-0.148 (0.630)	-0.159 (0.604)	-0.206 (0.500)	0.227 (0.457)
Peak Frequency	-0.228 (0.453)	0.195 (0.524)	-0.611 (**0.027**)	-0.294 (0.329)
Amplitude	-0.298 (0.323)	-0.448 (0.125)	-0.093 (0.764)	0.107 (0.728)

Frequency Evenness	-0.169 (0.582)	-0.368 (0.217)	-0.151 (0.623)	0.001 (0.998)
C) Purring males				
	Wing PC1	**Scraper**	**Mirror Size**	**Harp Width**
Calling Song PC1	-0.227 (0.084)	-0.417 (**0.001**)	NA	-0.084 (0.528)
Peak Frequency	-0.234 (0.074)	-0.513 (**<0.0001**)	NA	-0.144 (0.275)
Amplitude	0.096 (0.465)	-0.170 (0.199)	NA	0.234 (0.072)
Frequency Evenness	-0.159 (0.225)	0.025 (0.851)	NA	-0.231 (0.076)

Table S1.5. Pairwise comparisons of the effects of song stimuli (purring, rattling, ancestral, and white noise (WN)) on A) female cricket phonotactic behavior and B) contact with playback speaker. Comparisons made with estimated marginal means, and contrasts from Firth's Penalized Logistic Regression for phonotactic behavior and contact with speaker models, respectively (N = 30 females from Hilo).

A) Phonotaxis				
Contrast	**estimate**	**SE**	**z-ratio**	**P**
Purr:Rattle	-1.68	0.463	-3.619	**0.0017**
Purr:Typical	-3.77	0.579	-6.512	**<0.0001**
Purr:WN	1.58	1.052	1.504	0.4353
Rattle:Typical	-2.09	0.648	-3.231	**0.0068**
Rattle:WN	3.26	1.119	2.912	**0.0188**
Typical:WN	5.35	1.177	4.547	**<0.0001**
B) Contact with Speaker				
Contrast	**estimate**	**SE**	**Chisq**	**P**
Purr:Rattle	1.215	0.550	4.294	**0.038**
Purr:Typical	3.722	0.491	Inf	**<0.0001**

Purr:WN	-1.362	1.475	1.352	0.245
Rattle:Typical	2.507	0.629	19.968	**<0.0001**
Rattle:WN	-2.577	1.526	5.323	**0.021**
Typical:WN	-5.084	1.506	40.237	**<0.0001**

Chapter One Supplementary Methods

Sampling

To collect crickets, we used an unbiased sweeping method in the fields at each location, capturing crickets visually. This method does not use sound to locate crickets and so allows us to collect all sexes and morphs equally (i.e., louder males are not "hunted down"), and has been used for many years in previous work (Tinghitella et al. 2018; Tinghitella et al. 2021).

Song Analysis

For courtship songs we selected and measured characteristics of the chirp and trill separately, as their sound properties differ from one another (Hoy 1974; Bennet-Clark 2003), and it's possible that selection on behavior could shape the two parts of the song in different ways. There is an incredible amount of variation in the length of a trill, and so analyzing the entire courtship song together may have heavily weighted results for one part over the other in our analyses. Before measuring song characteristics, we used Audacity to apply a high-pass filter at 1500 Hz (roll-off: 48 dB per octave) to remove background frequencies well below the hearing range of *T. oceanicus* (Hoy et al. 1982) and because we did not detect any song frequencies below this in our recordings. We measured the relative amplitude of six different frequency ranges in order to give us a much more detailed understanding of song frequency composition (as opposed to solely measuring dominant frequency), which was important because purring songs are quite broadband and frequency composition varies greatly among individual purring males (Tinghitella et al. 2018). For each frequency range, we applied a bandpass filter with a steep slope to eliminate frequencies outside the desired range (MBandPass 13.01, MeldaProduction, settings: Q=1.00, slope=96), then measured the amplitude of the sound. For our measure of how broadband songs were (frequency evenness), a greater value indicates greater frequency evenness within the song, meaning that acoustic energy is distributed more evenly across frequency ranges, resulting in a more broadband sound.

In order to measure amplitude from calling and courtship song recordings, we also measured the overall amplitude (and the amplitude of each frequency range) of ambient noise in the recording room each night to use as a 0 dB reference. Any males with overall song amplitudes at or below this threshold (undetectable over ambient noise) were deemed non-sound-producing and excluded from further analyses. Decibels are measured on a logarithmic scale, so we converted our amplitude measurements in dB to their amplitude ratio (a linear format) prior to subsequent analyses (Brown and Riede 2017), as is standard in bioacoustics work (e.g., Broder et al. 2021; Tinghitella et al. 2021). Courtship song PCAs used characteristics from both the chirp and trill.

Wing Morphometrics Repeatability

After a training and quality checking period with an experienced landmarker, two independent observers placed landmarks on the photos of the right wing of each male using tpsDIG2. For any wings where the Procrustes distance between the two observers was > 0.3, the wing was rescored by an expert observer (N=18 wings) and these new measurements were instead used in the final dataset. The expert observer then rescored a random subset of 10 wings. We compared each landmark from these 10 wings among observers—the initial observer's data whose measurements most closely matched those of the expert observer were used in the final dataset.

Morphology and Performance of Novel Morphs

When we examined wings using microscopy, we observed and needed to define a "gap." To determine a threshold for what was considered a gap between teeth, we measured the distance between teeth on five ancestral male wings. All ancestral teeth were spaced < 0.02 mm apart, so we conservatively defined gaps as any spacing between teeth > 0.03 mm. We measured the total file length, number of gaps, and proportion of the file that was made up of gaps (sum of length of gaps divided by total file length).

For the phonotaxis experiments using female crickets and flies from Hilo, we played purring, ancestral, and rattling calling songs. Using a PCA based on the same nine calling song characteristics described above (Figure S2), we selected and used the rattling song with the most central (closest to the origin) sound characteristics for use in the phonotaxis experiments.

Common Garden Rearing

In July 2021, we collected eggs from field-caught individuals from Hilo, Manoa, and Kapa'a and reared them for two generations in the lab to reduce the impact of plastic or transgenerational effects (Kawecki and Ebert 2004), following Pascoal et al. (2014). To ensure that morph differences were not strictly due to rearing conditions, we reared crickets for two generations in common garden and found that the same morph-level clustering exists in lab-reared animals (Figure S4). To test the possibility that rattling morphology is environmentally induced, we first compared the proportion of males expressing rattling morphology in the field (N=8/31 males; 26% rattling; identified using song and wing characteristics identified in clustering analysis, as well as file tooth gaps) to proportions from the first-generation lab reared males (N=13/48; 27% rattling). We next recorded courtship songs of second-generation lab–born rattling males and used a t-test to compare their courtship song PC1 values to those of rattling males recorded in the field. Finally, to further compare field and lab-reared animals across all morphs, we measured wing morphometrics (using landmarking methods described above) from newly-eclosed, lab-born males (rattling: N=5; ancestral: N=5; purring: N=14; all source populations had been in the lab for at least two generations), and ran a MANOVA, with the first two axes of morphometric PCA as the response variables, and morph, rearing treatment (lab vs. field-born), and their interaction as predictor variables. A significant interaction term in this model would suggest that morph-level differences in wing morphology are dependent upon environmental (i.e., rearing) differences. In all instances, we recorded male courtship song and excised right wings from lab-reared males within two weeks of their eclosion to the adult stage to remove phenotypic differences due to natural, age-related wear.

Table S2.1. Characteristics used in song analysis. Frequency ranges (A-F) were chosen because they represent natural clusters of auditory receptor fibers in *T. oceanicus* (Imaizumi and Pollack 1999; Gallagher et al. 2022).

Song Characteristic	**Description**
Peak frequency	Frequency with the greatest acoustic power
Amplitude	Overall amplitude (RMS level) ratio compared to background noise
Relative amplitude of frequency range A	The amplitude of 2-3.5 kHz, divided by the sum of all ranges' amplitudes
Relative amplitude of frequency range B	The amplitude of 3.5-6 kHz, divided by the sum of all ranges' amplitudes
Relative amplitude of frequency range C	The amplitude of 6-9.5 kHz, divided by the sum of all ranges' amplitudes
Relative amplitude of frequency range D	The amplitude of 9.5-12.5 kHz, divided by the sum of all ranges' amplitudes
Relative amplitude of frequency range E	The amplitude of 12.5-17.5 kHz, divided by the sum of all ranges' amplitudes
Relative amplitude of frequency range F	The amplitude of 17.5-20 kHz, divided by the sum of all ranges' amplitudes
Frequency evenness	Standard deviation of all relative amplitude ranges, multiplied by -1

Table S2.2. Trait loadings for calling and courtship song principal component analyses.

Eigenvector	**PC1** Calling: 42.7% Courtship: 39.4%	**PC2** Calling: 24.6% Courtship: 15.7%	**PC3** Calling: 11.3% Courtship: 11.4%
Calling Song			
Dominant frequency	0.25	0.26	0.58
Amplitude	0.44	-0.14	-0.33
Range A	-0.40	0.10	-0.23
Range B	-0.45	-0.08	0.03

Range C	0.11	-0.54	-0.09
Range D	0.25	-0.34	0.53
Range E	0.35	0.41	-0.01
Range F	0.17	0.53	-0.22
Frequency Evenness	-0.40	0.21	0.40
Courtship Song			
Dominant frequency - Chirp	0.24	0.16	0.18
Amplitude - Chirp	0.28	-0.13	0.20
Range A - Chirp	-0.33	0.06	-0.09
Range B - Chirp	-0.28	-0.13	-0.13
Range C - Chirp	0.08	-0.42	-0.18
Range D - Chirp	0.04	0.25	-0.44
Range E - Chirp	0.30	0.08	0.21
Range F - Chirp	-0.01	0.19	0.45
Frequency Evenness - Chirp	-0.28	0.15	-0.15
Dominant frequency - Trill	0.20	0.27	0.05
Amplitude - Trill	0.31	-0.130	-0.07
Range A - Trill	-0.32	-0.02	0.06
Range B - Trill	-0.14	-0.37	0.10
Range C - Trill	0.22	-0.37	-0.03
Range D - Trill	0.13	0.33	-0.46
Range E - Trill	0.26	0.30	-0.02
Range F - Trill	-0.20	0.20	0.39
Frequency Evenness - Trill	-0.27	0.18	0.13

Table S2.3. Number of males of each morph per population, based on field sampling in January 2019, June 2019, and January 2020. Differences in sample sizes are due to natural variation in population sizes among sites and because we did not go to all sites during every sampling trip.

Population	Sampling Dates	Purring	Ancestral	Rattling	Silent	Smallwing	Curlywing	NA
Manoa	June 2019, Jan 2020	51	0	0	0	0	0	0
La’ie	Jan 2019, June 2020, Jan 2020	55	2	0	4	0	0	0
Wailua	Jan 2019, June 2020, Jan 2020	55	0	0	8	0	0	0
Kalaupapa	Jan 2019, June 2020	16	0	0	1	0	0	1
Kapa’a	June 2020, Jan 2020	5	20	0	0	0	0	0
Hilo	Jan 2019, June 2020, Jan 2020	0	24	49	0	7	5	3

Table S2.4. Major individual song and wing characteristic means (and standard deviations) for each purring population. See Table S2 for descriptions of each song characteristic.

Characteristic	**Manoa**	**Laie**	**Wailua**	**Kalaupapa**
Calling Song				
Dominant Frequency (Hz)	8081 (3218)	8282 (3612)	10721 (4131)	8290 (3386)
Amplitude (ratio)	1.467 (0.50)	1.428 (0.43)	1.599 (0.76)	1.834 (0.83)
Frequency Evenness	-0.035 (0.02)	-0.027 (0.02)	-0.035 (0.03)	-0.056 (0.03)
Courtship Chirp				
Dominant Frequency (Hz)	3857 (2922)	6663 (2997)	9635 (3636)	11587 (3440)
Amplitude (ratio)	1.708 (0.64)	1.757 (0.49)	2.301 (1.35)	9.859 (5.97)
Frequency Evenness	-0.052 (0.03)	-0.038 (0.02)	-0.046 (0.02)	-0.124 (0.04)
Courtship Trill				
Dominant Frequency (Hz)	4119 (3449)	5693 (3475)	9320 (3504)	7244 (1376)
Amplitude (ratio)	1.560 (0.89)	1.538 (0.40)	1.629 (0.81)	3.803 (1.19)
Frequency Evenness	-0.035 (0.03)	-0.035 (0.02)	-0.037 (0.02)	-0.078 (0.02)
Wing Morphology				
Harp Width (mm)	0.153 (0.02)	0.118 (0.04)	0.123 (0.02)	0.133 (0.01)
Scraper Presence (proportion)	0.96	0.94	0.59	0.75

Table S3.1. Calling song trait means (and standard deviations) of the four morphs in this study. Ancestral and rattling data are adapted from Gallagher et al. 2022, while smallwing and curlywing were collected for this study.

Song Trait	**Ancestral (N=24)**	**Rattling (N=16)**	**Smallwing (N=5)**	**Curlywing (N=5)**
Dominant frequency (Hz)	4877 (192)	5805 (1703)	6854 (272)	8163 (5733)
Amplitude (dB above background noise)	42.04 (38.34)	23.81 (15.93)	18.60 (9.38)	11.98 (8.70)

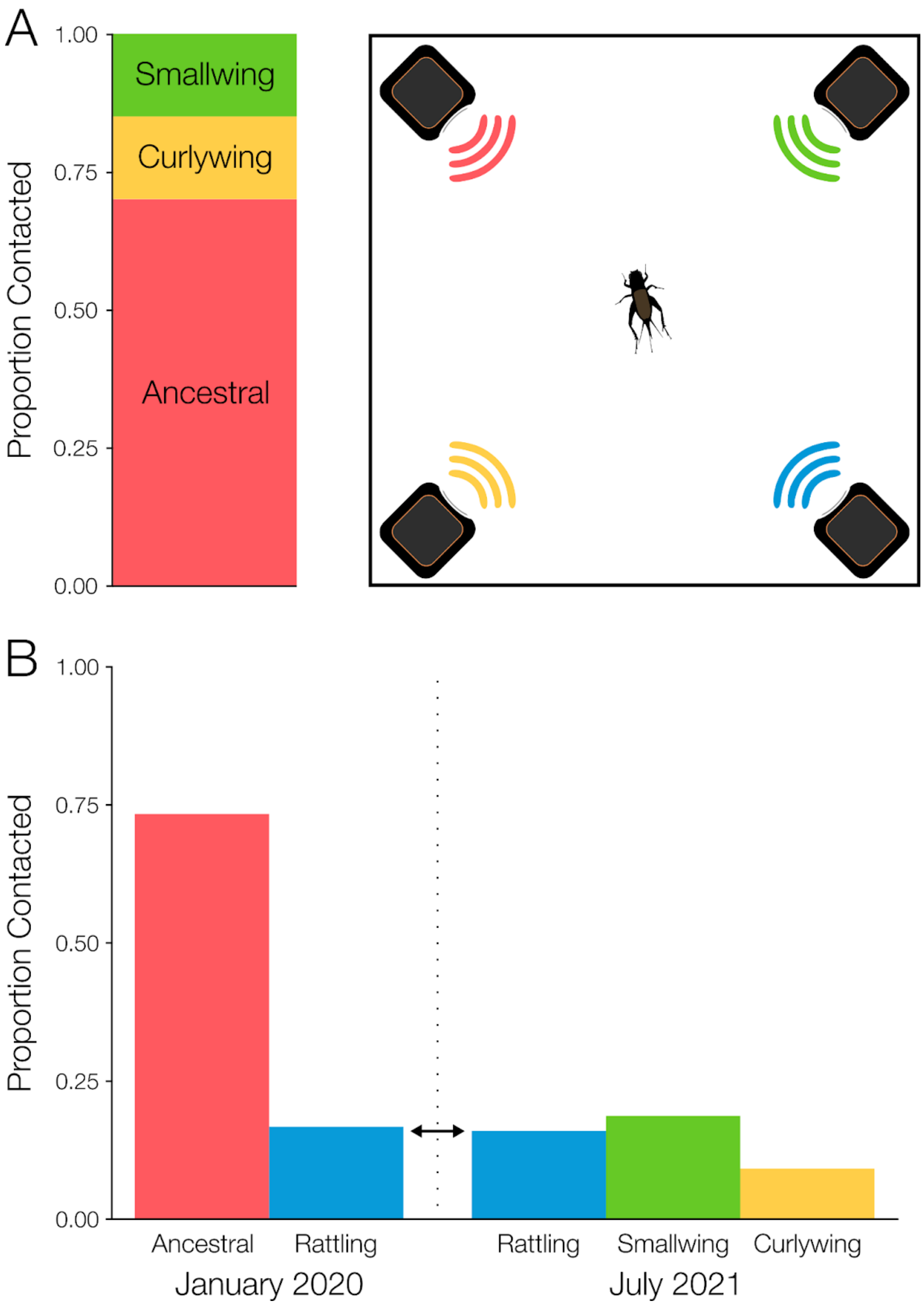

Figure S3.1. Ancestral song remains by far the most attractive to female crickets. A) Four-choice phonotaxis trials confirmed results from previous work showing that ancestral calling song is still more attractive (Tinghitella et al. 2018*a*, 2021; Gallagher et al. 2022), with 70% of females choosing ancestral over the songs of novel morphs. In these trials, 15% of females chose smallwing and curlywing songs, while no females chose rattling. Of the novel morphs, the frequencies of rattling song overlap most with ancestral frequencies (Table S3.1). Therefore, rattling song may be more masked than smallwing and curlywing when ancestral song is being broadcast, perhaps explaining why no females chose rattling in this particular experimental setup. B) Proportion of speakers contacted broadcasting various songs in no-choice trials across two different experiments shows the large difference between ancestral and novel morph calling song attraction. Data for January 2020 were collected as part of Gallagher et al. 2022, using very similar methods. In January 2020, the same arena, speakers, and conditions were used, but there was only one speaker placed in the arena (as opposed to two speakers with one not broadcasting song in July 2021), and the cricket was placed at the end of the arena with the speaker in the middle (January 2020) instead of the cricket in the middle with the speakers on either end (July 2021). Proportion contacted for rattling was nearly identical between the two experiments, justifying comparison of contact proportions between experiments.