ABSTRACT

Title of Document: PATTERNS OF SONG AND PREFERENCE VARIATION IN *LAUPALA CERASINA* AND THEIR EVOLUTIONARY IMPLICATIONS

Jaime Leigh Grace, Ph.D., 2010

Directed By: Professor Kerry L. Shaw, Department of Biology

Closely related species often differ conspicuously in secondary sexual characters, many of which may be shaped by sexual selection. Changes in these characters may directly influence speciation, since females use them to assess males and to choose a mate. I investigate the hypothesis that in closely related populations, divergence in preference for sexually selected characters may promote sexual isolation through assortative mating. Ultimately, if reproductive isolation is complete, this process of divergent sexual selection through preference divergence may result in the formation of new species. I have investigated the relationship between divergence in the pulse rate of male song and female preference for pulse rates in a group of Hawaiian crickets, *Laupala cerasina*. Within a population, males sing at a characteristic pulse rate, but males from different populations sing with significantly different pulse rates. The degree of population divergence is almost an order of magnitude less than that which distinguishes closely related species of *Laupala*. Using phonotaxis trials, I found that females show acoustic preferences for males whose pulse rates fall nearest

to the population mean. Across populations, male pulse rates and female preferences are significantly correlated, suggesting that they are coevolving. I have demonstrated that females discriminate among songs whose pulse rates vary within the range of pulse rates found among neighboring populations. Furthermore, in choice trials, females prefer the songs of males from the same population to those from neighboring populations. These females also exhibit significant assortative mating based on their acoustic preferences. Divergence in female acoustic preference among populations thus provides a mechanism for generating and maintaining biodiversity through divergence in sexually selected characters used in mate choice.

PATTERNS OF SONG AND PREFERENCE VARIATION IN *LAUPALA CERASINA* AND THEIR EVOLUTIONARY IMPLICATIONS

By

Jaime Leigh Grace

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Advisory Committee:
Professor Kerry L. Shaw, Chair
Professor Gerald S. Wilkinson
Professor Gerald Borgia
Professor David J. Hawthorne
Professor David D. Yager

Preface

This dissertation contains three chapters. Chapters I, II, and III are presented in manuscript form, with abstract, introduction, methods, results, and discussion, followed by tables and figures. A single bibliography section occurs at the end for references cited throughout the dissertation.

Acknowledgements

Table of Contents

List of Tables

List of Figures

Chapter I: Female preference covaries with male acoustic signal in the geographically widespread Hawaiian cricket, *Laupala cerasina*

ABSTRACT

Sexual selection is a powerful evolutionary force shaping phenotypes involved in mate choice, and can cause phenotypic shifts resulting in (or reinforcing) population divergence and speciation when such shifts reduce mating probabilities among divergent populations. In the Hawaiian cricket genus *Laupala*, pulse rate of male calling song is a conspicuous mating signal that differs among species, and may behave as a speciation phenotype. Females prefer pulse rates that roughly correspond to the male population mean, and signal and receiver values appear to be coupled across species. Populations of *L. cerasina* show subtle but significant differences in pulse rate across the species' range. This study documents the degree of differentiation among populations in three features of calling song: pulse rate, pulse duration, and carrier frequency. All three show significant population differentiation, with pulse rate showing the greatest heterogeneity; however, a Mantel test found no relationship between geographic distance and pulse rate divergence, indicating a simple model of greater divergence with increasing distance is insufficient to explain the observed pattern of differentiation. Female preference functions for pulse rate also show significant differentiation among populations. Furthermore, the

estimates of pulse rate preference are highly correlated with mean pulse rates across populations. This pattern of correlated divergence between sender and receiver suggests that sexual selection on song actively maintains population differentiation, and is consistent with the hypothesis that sexual selection may have promoted speciation in this genus. The correlated divergence in a sexually selected trait and preference among populations has the potential to reduce gene flow among divergent populations that are sexually isolated, which may ultimately result in speciation.

INTRODUCTION

Many closely related species differ conspicuously in secondary sexual signals and preferences of the mate recognition system (Darwin 1871; West-Eberhard 1983; Ritchie 2007). The processes by which these patterns arise are of intense interest because of their direct bearing on the origins of species. While the processes causing the evolution of such phenotypes occur within populations, the consequential divergence among populations can have significant repercussions for communication between populations (Lande 1981; Endler 1992). Evolved mating signals within a population may result in sexual isolation from other populations whose mating signals have remained the same or evolved differently (Kirkpatrick 1982; Lande 1982). If signals and preferences covary among populations, assortative mating could result in reduced mating probabilities and depressed gene flow between populations (Panhuis et al. 2001; Uyeda et al. 2009).

2

While interspecific differences in mate recognition phenotypes appear to make critical contributions to reproductive isolation among species, determining the origins and evolutionary dynamics of these differences must involve the study of intraspecific variation in signals and preferences (Härdling and Karlsson 2009; Uy et al. 2009). The functional interdependence of signal and preference suggests that a preference function (often expressed by females) can describe the selective pressure on signals (often expressed by males) due to female choice (Wagner 1998). The degree of overlap between signal and preference variation is an important factor in predicting the evolutionary potential and trajectory of signal-preference evolution (Ritchie 1996). For example, it is often hypothesized that a mate recognition system with strong stabilizing selection will tend toward extensive trait-preference matching within and covariance across populations (Butlin and Ritchie 1989). In addition, the genetic architecture of these traits can constrain, or promote, the tempo and coevolutionary precision of trait and preference that ultimately leads to interspecific differences. For example, theory suggests that details of the genetic architecture of diverging sexual traits can influence the likelihood and rate of speciation (Dieckmann and Doebeli 1999; Kondrashov and Kondrashov 1999; Verzijden et al. 2005; Hall and Kirkpatrick 2006; Hayashi et al. 2007).

Elucidation of the potential pathways by which interspecific differences in mate recognition evolve requires systems that exhibit intraspecific variation in these same traits. While numerous studies are beginning to examine geographic variation in mating signals and preferences (Simmons et al. 2001; Simmons 2004; Prohl et al. 2006; Prohl et al. 2007; Reynolds and Fitzpatrick 2007; Ryan et al. 2007) particularly

3

valuable would be studies that simultaneously document 1) variation in both signals and preferences for those signals among populations, 2) a genetic basis to covariation between trait and preference, and 3) interspecific variation in these same traits, enabling mechanistic and evolutionary studies of different stages of the speciation process (Panhuis et al. 2001; Boake 2002). Intraspecific variation in such systems can yield temporally fine-grained observations of trait differentiation, promising insights into 1) how interspecific differences accumulate from intraspecific differentiation and 2) the relationship between female preference functions and the degree to which signal and preference co-evolve or become decoupled during divergence.

The Hawaiian cricket genus *Laupala* has undergone rapid speciation (Mendelson and Shaw 2005), resulting in 38 closely related species that differ conspicuously in acoustic aspects of sexual communication, but that are otherwise cryptic (Otte 1994). Males of the different species of *Laupala* produce structurally similar songs that vary primarily in pulse rate, and species occurring in sympatry can always be distinguished by pulse rate (Shaw 1999; Parsons and Shaw 2001; Mendelson and Shaw 2002; Shaw 2002; Mendelson and Shaw 2005). Furthermore, females preferentially respond to the pulse rate produced by males from their own species (Shaw 2000b; Shaw and Herlihy 2000; Mendelson and Shaw 2002). Genetic studies of species differences in *Laupala* show that both pulse rate and preference variation is due to relatively small, additive allelic effects (Shaw et al. 2007; Shaw and Lesnick 2009). Furthermore, loci underlying both song and preference differences between species show genomic linkage (Shaw and Lesnick 2009; Wiley and Shaw 2010). These observations between species suggest the hypothesis that

4

divergent evolution of song and preference within species occurs through small, coordinated changes in signal and preference.

We examine this hypothesis in a study of *Laupala cerasina*, a widely distributed species endemic to the Big Island of Hawaii. Field recordings have suggested extensive pulse rate differentiation across the range of *L. cerasina* (Otte 1994), indicating that *L. cerasina* populations have diverged in the same character that varies conspicuously among species. The current study provides statistical support for the extensive pulse rate variation in *L. cerasina* and tests the hypothesis that male sexual signals and female preferences for those signals covary across the range of the species. The hypothesis that differences among populations in male song and female preference have a genetic basis is tested using a common garden experiment. The results are discussed with respect to the mode of change in both male and female sides of the acoustic communication system and in the context of geographic structure among populations.

METHODS

Cricket subjects and rearing conditions

Subadult *L. cerasina* crickets were collected from thirteen locations (Pololu Valley, Muliwai Plateau, Waimea Reservoir, Kalopa Park, Laupahoehoe, Akaka Falls, Eucalyptus Toe, Kaiwiki, Ola'a Flume, Glenwood, Wright Road, Naulu Trail, and Kaiholena), hereafter referred to as populations, from the Big Island of Hawaii in July 2004, 2005, and 2006 (Figure 1). In the laboratory at the University of Maryland, individuals were reared to maturity under a 12:12 light: dark cycle in a temperature-

controlled environment maintained at 20˚C, reflecting field conditions. Two to three

wild caught individuals of the same sex were reared together in medical specimen

cups, with a moistened kimwipe for water and humidity. Fresh cricket chow (Fluker's

Farms, LA, USA) was provided twice per week (2004, part of 2005) or cricket chow

treated with the anti-fungal agent methyl paraben (Tegosept, Fisher Scientific) once

per week (part of 2005, 2006). First laboratory generation crickets (G_1) were collected

as eggs from naturally inseminated females caught in the wild or paired with a wild

caught male in the laboratory. Second laboratory generation crickets (G_2) were

generated through random pairing of non-sibling males and females from the same

population.

Male song measurements

Single mature males were recorded with a Sony Professional Walkman (model WM-

D6) and Sony condenser microphone from a clear plastic cup fitted with a screen top

in a temperature-controlled room maintained at 20˚C. A female from the same

population was placed with males that failed to sing spontaneously (males produce

only one type of song in *Laupala*). Songs were digitized and analyzed using Raven

version 1.2.1 (Bioacoustics Research Program, Cornell University Lab of

Ornithology). From each song, five non-consecutive measurements were taken of

pulse period, pulse duration, and carrier frequency. Pulse period, measured as the

time between the beginning of one pulse and the beginning of the next pulse, was

transformed to calculate the pulse rate (the inverse of the pulse period). Pulse duration

is the time elapsed between the beginning and end of a single pulse. Finally, carrier frequency is calculated as the dominant frequency of the pulse.

Male song data were analyzed using SAS version 8.2 for Windows (SAS Institute, Cary, NC). The five measurements of pulse rate, pulse duration, and carrier frequency were averaged for each male to get a single measurement per individual. The coefficient of variation (CV) was calculated for each male by dividing the standard deviation by the mean and multiplying by 100 to get a percent. A low CV suggests that using mean values from individuals is appropriate for subsequent analysis. An analysis of variance was performed to test the hypothesis that song variables show significant heterogeneity among populations. Additionally, a correlation analysis was performed using pulse rate, pulse duration, carrier frequency, and temperature to test for independence of song characters. Each song value was adjusted to account for population differences by subtracting the value for each trait from the population mean for each individual, and using those values (the residuals) in the analysis.

To determine whether geographic variation in song has a genetic component, individuals from the 2005 collections at Glenwood Road, Kalopa Park, Laupahoehoe, Muliwai Plateau, Naulu Trail, and Waimea Reservoir were subjected to a common-garden study in the laboratory for two generations. Then the songs of the resulting progeny of outbred pairs were recorded under the same experimental conditions as the wild-caught generation. Measurements of pulse rate, pulse duration, and carrier frequency were conducted in the G_2 males using the same procedures as in the wild-caught males, and the results were compared across generations. The mean song

values measured from the G_2 progeny were used in a correlation analysis with the means from the parental (wild-caught) generation.

To test for a relationship between geographic distance and pulse rate divergence, a Mantel test was performed using XLSTAT in Microsoft Excel. Geographic distances were calculated using the ruler tool in Google Earth, with collecting locations estimated within one second of the recorded coordinates listed in Figure 1. Song divergence was measured as the absolute value of the difference between the mean male pulse rates for each population pair.

Female acoustic preference measurements

Phonotaxis methods were identical to those in Shaw and Herlihy (2000). Briefly, a circular test arena (47 cm radius) covered in fiberglass screen was housed in a temperature-controlled acoustic chamber (Acoustic Systems) for phonotaxis trials. Two 8.5 cm speakers (Radio Shack model 40-1218) placed 180˚ apart just outside the arena broadcast synthesized songs offered in pairs from a PC computer using custom software. From each speaker, a pulsed, sinusoidal tone was played back through a 16-bit digital/analogue converter (Tucker-Davis Technologies, Gainesville, FL). The pulse amplitude envelope had a rise time of 10 ms and a fall time of 30 ms and the acoustic output was filtered at 10 kHz using a Krohn-Hite filter (model 3322) to avoid aliasing. Sound pressure levels were equalized to 90 dB on a 4.0-pulses per second (pps) tone monitored with a Bruel and Kjaer sound pressure level meter (type 2230, fast-root-mean-square setting).

To conduct a trial, a female was placed beneath a plastic cup in the center of the arena. Pulse duration and carrier frequency were held constant at 40 ms and 5.0 kHz, respectively, while pulse rate varied depending on the trial. Each female was used in a series of six different trials (2.0 versus 2.5 pulses s^{-1}, 2.2 versus 2.7 pulses s^{-1}, 2.4 versus 2.9 pulses s^{-1}, 2.6 versus 3.1 pulses s^{-1}, 2.8 versus 3.3 pulses s^{-1}, and 3.0 versus 3.5 pulses s^{-1}) in randomized order, two per day, over the course of three days. These pulse rate values more than span the natural range of variation observed in male calling song across *L. cerasina* populations (Otte 1994). Paired songs were broadcast for five minutes before the cup was remotely raised, allowing the female access to the arena. If the female approached within 10 cm of one of the speakers within the trial period, the pulse rate choice was recorded and the trial ended. If the female did not approach either speaker after five minutes, the trial was ended and no choice was recorded. Only sexually mature, virgin females 20 days past the final molt were used in trials. In each trial, the speakers were randomly assigned a pulse rate.

In general, females responded to the faster pulse rate at the slow end of the trial series and to the slower pulse rate at the fast end of the trial series. We conducted an analysis to estimate the pulse rate corresponding to the inflection point, or point of indifference, where the proportion of females responding to the faster (or slower) pulse rate equaled 0.5 in a given population. Responses from a given population were analyzed simultaneously using logistic regression with the statistical software package R. In order to determine a standard error for these estimated mean preferences, the preference data sets (preference functions) of females within a given population were sampled with replacement to generate 1000 bootstrap replicates per

9

population, maintaining the original sample size for each replicate. For each bootstrap replicate, the pulse rate corresponding to the inflection point was calculated, providing a pulse rate preference estimate for that replicate sample. The mean preference estimate and its standard deviation (i.e. standard error) were calculated for each of the 1000 bootstrapped replicates.

To determine whether there is a genetic component to geographic variation in female acoustic preference, individuals from the 2005 collections at Glenwood Road and Naulu Trail were reared in a common garden study for two generations (as with males; see above). The resulting G_2 progeny were used in the same experimental design as the wild-caught individuals. Logistic regressions of phonotaxis response were compared across generations within populations (to test whether population preferences change across generations), and between populations within generations (to test whether differences persist across generations between populations) using Wald chi-square tests (Grace and Shaw 2004).

Song-preference comparisons

To test for population-level correlations between song and preference, a Spearman rank correlation test was performed using the mean pulse rate and preference estimates for each population for each year separately. We also performed a global correlation by combining all of the data for each population and performing a single correlational analysis across all 13 populations.

For the two populations (Glenwood Road and Naulu Trail) where song and preference data were available for wild-caught and G_2 generations, we tested the

10

hypothesis of no difference between pulse rate and pulse rate preference between populations in each generation.

RESULTS

Male song variation

Wild-caught generation: Temperature varied little throughout the course of the experiment (average recording temperature = 20.09 ± 0.17), and as expected, there was no relationship between temperature and any of the measured song characters (Pearson r = -0.01 to -0.05, p = 0.29-0.84). Analyses were conducted separately for each collection year, and results were compiled separately. In total, songs were recorded from 328 males. Pulse rates were significantly heterogeneous among populations of *L. cerasina* in all years sampled (2004: $F_{(5,103)}=24.07$, p<0.0001; 2005: $F_{(6,101)}=42.97$, p<0.0001; 2006: $F_{(6,104)}=32.13$, p<0.0001). In 2004, 7 out of 15 pairwise comparisons were significantly different (p<0.05) following Sidak correction for multiple comparisons; in 2005, 16 out of 21 pairwise comparisons were significantly different; and in 2006, 11 out of 21 were significantly different (Figure 2; Table 1). Carrier frequency was also significantly heterogeneous among populations in all years (2004: $F_{(5,103)}=7.02$, p<0.0001; 2005: $F_{(6,101)}=3.62$, p=0.0027; 2006: $F_{(6,104)}=4.27$, p=0.0007), but with fewer significant pairwise comparisons (5 out of 15 in 2004, 1 out of 21 in 2005, and 2 out of 21 in 2006; Figure 3; Table 1). Pulse duration was significantly heterogeneous among populations in 2004 ($F_{(5,103)}=6.12$, p<0.0001), but not in 2005 or 2006 (2005: $F_{(6,101)}=1.84$, p=0.099; 2006: $F_{(6,104)}=0.37$, p=0.89). In 2004, 4 out of 15 pairwise comparisons were significantly different

(Figure 4; Table 1). The pulse rates for Glenwood Road males sampled in 2004 were significantly slower than males sampled in 2005, as was true for Wright Road in these same years (Table 1; Figure 2). Wright Road males also had a significantly lower carrier frequency in 2004 than 2005 (Table 1; Figure 3). No other year effects were found within populations. A correlation analysis of pulse rate, pulse duration, carrier frequency and recording temperature found one significant negative correlation between carrier frequency and pulse duration, with all other relationships non-significant (Table 2). There was no support for a relationship between geographic distance and pulse rate divergence across populations (Mantel test: 50,000 permutations; Pearson r = -0.168, p = 0.346).

Laboratory generation: Songs were analyzed from 284 males in the G_2 generation. The six populations bred for two generations in the lab (G_2 males) were significantly heterogeneous for pulse rate ($F_{(5,278)}$=67.84, p<0.0001) and carrier frequency ($F_{(5,278)}$=3.67, p=0.0031), but not pulse duration $F_{(5,278)}$=1.69, p=0.136). Fewer pairwise comparisons were significant for pulse rate (9 out of 15), but all of the comparisons that were significant in the G_2 generation were also significant in the parental generation. For carrier frequency, 2 out of 15 pairwise comparisons were significant, including the pair that was different in the parental generation. Population means derived from wild-caught and G_2 males were significantly correlated across the six populations in pulse rate (Pearson r=0.972, p=0.0012) and carrier frequency (Pearson r=0.899, p=0.0149), but not pulse duration (Pearson r=0.699, p=0.51).

The pulse rate means of wild-caught males from Glenwood Road and Naulu Trail collected in 2005 were among those that differed significantly ($t_{(29)}$=6.19,

12

p<0.0001). While we found no significant differences in pulse rate between the parental and G_2 generations within each population (Glenwood $t_{(56)}=-0.93$, p=0.36; Naulu $t_{(51)}=-0.66$, p=0.51; Figure 5), the pulse rates of G_2 songs did differ between the two populations ($t_{(78)}=10.71$, p<0.0001; Figure 5). From the analysis of wild-caught males, the two populations also differed in carrier frequency ($t_{(29)}=-2.12$, p=0.043), but not pulse duration ($t_{(29)}=-0.30$, p=0.77). However, the difference in carrier frequency did not persist across generations between these populations (G_2 male analysis: $t_{(78)}=-1.30$, p=0.20).

Female preference variation

Wild-caught generation: In total, 269 wild-caught females were run in preference trials. Typically, females responded to the faster song of the pair at the slow end of the trial range, and to the slower song at the fast end of the trial range. Population means and standard errors within populations were estimated using the bootstrap analysis described above. After applying logistic regression to the bootstrap replicates, it became apparent that estimates of pulse rate preference scaled with pulse rate means from their respective populations (see below). However, preference estimates from slower singing populations were associated with substantially larger 95% confidence intervals when based on all six acoustic choice trials. This likely occurred because the trials at the fast end of the range were beyond the normal discrimination range of females from slower populations. To improve estimates, the means and standard errors were recalculated using the first four trials only, the first five trials only, and all six trials, for those populations where male pulse rates were

less than 2.5 pulses per second (pps), between 2.5 and 2.8 pps, and over 2.8 pps,

respectively. Of the 17 groups that had recalculated estimates, 14 showed a reduced

standard error. With this systematic adjustment, standard errors dropped by 35% and

were similar across populations (Table 3). Population preference means were

considered significantly different when the mean of one population was excluded

from the 95% confidence interval (=1.96SE) of a second population. Note that this

allows for three categories of relationship: when the means of both populations are

within the other's confidence intervals (NS), when one population's mean is excluded

from the other's confidence interval, but the other mean is included within the

confidence interval of the first (1-way), and when both means are reciprocally

excluded from each other's confidence intervals (2-way).

Populations were analyzed separately by year to mirror the analysis of song

variation, although no two-way significant year effects were detected in female

preference estimates (see below). As with song, statistically significant differences in

pulse rate preference among populations were detected in all three years of the study.

In 2004, 9 out of 15 pairwise comparisons were significantly different, with 2

comparisons showing one-way significance and 7 comparisons showing two-way

significance (Figure 6). In 2005, 15 out of 21 pairwise comparisons were significantly

different, with 5 comparisons showing one-way significance and 10 comparisons

showing two-way significance (Figure 6). In 2006, 16 out of 21 pairwise comparisons

were significantly different, with 2 comparisons showing one-way significance and

14 comparisons showing two-way significance (Figure 6). There was one significant

year effect on preference: at Ola'a Flume, our analysis revealed a one-way significant

difference between 2004 and 2006, with the 2004 estimate (2.61) falling just outside the 95% confidence interval of the 2006 estimate (2.62-2.76).

Laboratory generation: From the G_2 generation (derived from the 2005 wild-caught generation), a total of 16 females from Glenwood Road and 16 females from Naulu Trail were run in preference trials. Consistent with the hypothesis that pulse rate preferences breed true and thus have a genetic basis, a Wald chi-square test for logistic regression showed no significant difference between the parental and G_2 generations within the Naulu Trail or Glenwood Road populations, regardless of whether trials 1-4, 1-5, or all trials 1-6 were considered (Trials 1-4: Glenwood: Wald $\chi^2_{(1)}$=1.63, p=0.20; Naulu: Wald $\chi^2_{(1)}$=0.10, p=0.75). Analyses using 1-4 trial data revealed significant differences between Glenwood and Naulu populations in both the wild-caught and G_2 generations (wild-caught: Wald $\chi^2_{(1)}$=5.38, p=0.0204; G_2: Wald $\chi^2_{(1)}$=5.79, p=0.0161; Figure 5). Consistent with our observation that the fifth and sixth phonotaxis trials inflated the standard error estimates for populations with slow songs and preferences (like Naulu), we found no significant differences between these two populations when data from the fifth trial or all six trials were included.

The bootstrap method for estimating preference and 95% confidence intervals (above) revealed a two-way significant difference between Naulu and Glenwood in the wild-caught generation (Table 4). When applied to the G_2 generation, the analysis resulted in a one-way significant difference, with the Naulu estimated preference mean falling outside the 95% confidence interval of Glenwood, but the Glenwood estimated preference mean falling just inside the Naulu 95% confidence region (Table 4). This was true when data from trials 1-4 and 1-5 were used for Naulu and

Glenwood, respectively (consistent with our inclusion criteria outlined above) or when only trials 1-4 were used for both populations.

The 95% binomial confidence intervals were also calculated for wild-caught and G_2 generations for each pulse rate trial separately. Females from Naulu and Glenwood in both generations responded significantly differently only in trial 2 (trial midpoint pulse rate = 2.45 pps), corresponding to the pulse rate range (2.2 versus 2.7) that distinguishes males of these two populations (Figure 5). Collectively, these results suggest that the observed differences in phonotaxis preference among populations persist across generations, demonstrating a genetic basis to population variation.

Song-preference covariation

For 2005 and 2006, pulse rate and preference were significantly correlated (2005: n=7 populations, Spearman r=0.964, p=0.0005; 2006: n=7 populations, Spearman r=0.929, p=0.0025; Figure 7). Although there was a qualitatively similar trend, there was no significant relationship between song and preference in 2004 (n=6, Spearman r=0.148, p=0.67; Figure 7). A global analysis combining data across years for each population found a significant correlation between the mean estimated female preference and the mean male pulse rate across all thirteen populations (n=13, Spearman r=0.934, p<0.0001; Figure 8). Due to significant year effects, only 2004 data (yielding the largest sample) were used from Glenwood Road and Wright Road populations in the global analysis.

DISCUSSION

Recent, rapid radiations present ideal opportunities to study species that have diverged in relatively few characters, facilitating focus on evolutionary processes relevant to speciation. If such radiations harbor species that exhibit intraspecific differentiation as well, this provides the opportunity to examine the multiple temporal stages of differentiation that Mayr (1963) viewed as essential to understanding the mechanisms of speciation. When the divergent characters in question are involved in sexual communication and mate recognition, the populations are predisposed toward the evolution of sexual isolation, likely the earliest form of reproductive isolation to evolve in many taxa (Lande 1981; West-Eberhard 1983). In the genus *Laupala*, the pulse rate of the male calling song is a conspicuous character that varies among otherwise cryptic species, suggesting that this trait may be a speciation phenotype, i.e., a trait whose divergence reduces gene flow among incipient species (Shaw 2001; Shaw and Danley 2003). If this is true, then populations of *L. cerasina* that have diverged in pulse rate may be incipient species, provided that pulse rate preference has evolved in a correlated fashion with pulse rate.

Male song variation

We found that the mean pulse rates across the range of *L. cerasina* are heterogeneous to a statistically significant degree. Whereas Otte (1994) presented qualitatively similar results from field-recorded *L. cerasina*, the variation demonstrated here is observed among populations of wild-caught crickets brought to the laboratory where temperature conditions were monitored and controlled. Because temporal components

of song in orthopterans are notoriously sensitive to temperature variation (Gerhardt and Huber 2002; Grace and Shaw 2004), any demonstration that pulse rates are statistically different among populations requires measurement at constant temperatures. Two populations, Glenwood Road and Wright Road, showed a significant year effect on pulse rate between 2004 and 2005. Because pulse rate can be measured very precisely, it is possible that these differences are due to subtle temperature differences at the field sites prior to their collection. The environmental temperature experienced during development can have a lasting effect on adult song phenotypes (Grace and Shaw 2004), so any temperature differences experienced at those locations between 2004 and 2005 could have contributed to the differences in pulse rate observed under laboratory conditions.

Following two generations of laboratory rearing (G_2 generation), we showed that the pulse rate heterogeneity among populations persisted, and that the pulse rate means of wild-caught and G_2 lab-reared males were highly correlated. The mean pulse rate difference between wild-caught males from Naulu Trail and Glenwood Road, the two populations examined for preference variation in the G_2 generation, were among those that remained significantly different in the G_2 generation. These second lab generation observations allow us to rule out the possibility that a maternal by environment effects account for the difference between populations. Instead, these findings implicate a genetic contribution to the pulse rate variation among natural populations of *L. cerasina*.

To a lesser degree, significant variation was observed in carrier frequency and pulse duration among wild-caught *L. cerasina* populations, although only carrier

frequency differences remained significantly heterogeneous in the G_2 generation. However, fewer pairwise significant differences in carrier frequency were found among populations, and the significant difference in carrier frequency between Naulu and Glenwood wild-caught males did not breed true in the G_2 generation. Thus, male condition, natal environment and/or maternal effects may play a larger role in both pulse duration and carrier frequency variation. In a previous study, Shaw and Herlihy (2000) found that pulse rate and carrier frequency showed relatively low levels of intra-male variability, whereas pulse duration exhibited relatively high intra-male variability. Nevertheless, a negative correlation was found between carrier frequency and pulse duration, suggesting a constraint to the independent evolution of each. Previous work showed that females express unimodal preference functions for carrier frequency but open-ended preferences for pulse duration (Shaw and Herlihy 2000). These patterns match those found in frogs (Gerhardt 1991; Gerhardt and Huber 2002) and are consistent with the suggestion that variation is influenced by environment or male condition (although this hypothesis was not explicitly tested). Both low carrier frequency and long pulse duration have been associated with large body size in other crickets (Brown et al. 1996; Simmons and Ritchie 1996).

Female preference variation

The phonotaxis data indicate that females across the range of *L. cerasina* express unimodal preference functions for pulse rate, but females differed by population as to which pulse rates they are most attracted. Unlike the pulse rate estimates for male song, there was no effect of year on estimates of female pulse rate preference. In

general, variability in the standard errors associated with mean pulse rate preference estimates were higher than for mean pulse rates, likely due to either the nature of the measurement or expression of preference. Nonetheless, we found statistically significant differences in pulse rate preference among populations of wild-caught females as measured under controlled temperature conditions in the laboratory. Furthermore, the significant difference in mean pulse rate preference between wild-caught Naulu and Glenwood females persisted in the G_2 generation. As with pulse rate, these findings demonstrate a genetic contribution to the variation in preference for pulse rate among natural populations of *L. cerasina*.

Song-preference covariation

Pulse rate variation was strongly predicted by pulse rate preference across the range of *L. cerasina* (Figures 7, 8). This finding provides support for the hypothesis that the divergent evolution of song and preference among populations is characterized by small, parallel changes to both trait and preference characters. Additionally, we found a high degree of synchrony between trait and preference means across populations (Figure 8). Phenotypic correlations such as these could be explained by genetic correlations (through linkage disequilibrium or genetic coupling), pleiotropy (where a shared genetic element similarly affects both song and preference to the same degree), or environmentally based divergence, including genetic x environment interactions. The persistence of song-preference covariation across generations in a common garden environment indicates that environmental differences among populations is not sufficient to explain variation, and suggests that there is a genetic

20

basis to divergence. Although the genetic architecture of song and preference variation has not been examined in *L. cerasina*, these findings are consistent with a genetic correlation between trait and preference within populations underlying the correlated phenotypic divergence of these characters among populations. In a closely related pair of congeners, *L. kohalensis* and *L. paranigra*, song and preference variation colocalized to a common QTL (Shaw and Lesnick 2009), and multiple QTL that were associated with song variation also predicted preference variation (Wiley and Shaw 2010), suggesting that linkage disequilibrium and/or genetic coupling may keep song and preference characters matched during population divergence.

There was one deviation from this pattern of correlated divergence. At Kalopa Park, females appear to prefer songs that are faster than the population average. This is likely a statistical anomaly produced from the experimental design. At the faster end of the range of preference trials, the choices of pulse rates presented to females (2.8 vs 3.3 pps; 3.0 vs 3.5 pps) lie far outside the range of male song variation (2.19-2.47 pps). Females were less responsive, and exhibited no significant preference for either song. When the final trial was removed, the mean preference decreased from 2.61 to 2.55, and the standard error was cut in half. When the final two trials were removed, preference dropped to 2.51 and the standard error decreased another 25%. While this estimate is still faster than the average male song at Kalopa Park, it is likely a more accurate representation of the true preference. Kalopa Park males sang more slowly than preliminary research had suggested, and a better experimental design for these females would have included slower song choices. Despite the coarseness of the method for estimating female preference across a wide spectrum of

variation, the results are remarkably consistent overall, suggesting that the striking

pattern of song and preference covariation is a real biological phenomenon, and

strong enough to overcome the limitations of the methods for measuring it.

Causes of differentiation

While this study was not designed to address the causes of divergence, it provides

data that can be considered with regard to evolutionary causes. Divergent sexual

selection and reproductive character displacement have both been proposed as

hypotheses to explain the diversification of *Laupala* (Otte 1989; Otte 1994;

Mendelson and Shaw 2005).

     If reproductive character displacement influences population divergence in

song, there should be evidence that males have shifted their pulse rates to maximize

the difference from sympatric congeners (Hoskin and Higgie 2010). The congeners

that live sympatrically with *L. cerasina* are part of a distinct morphological and

genetic lineage of the *Laupala* radiation (the *pacifica* group) and are not close

relatives (Otte 1994; Shaw 2002), despite extensive sharing of mitochondrial DNA

haplotypes in zones of sympatry, indicating hybridization may have occurred in the

past (Shaw 2002). However, if reproductive character displacement has occurred, it is

not readily apparent. For example, the population at Pololu Valley is known to co-

occur with a fast-singing species (*L. kohalensis*, pulse rate ~4.0 pps) (Shaw 1996), but

the pulse rate of *L. cerasina* males here is intermediate (~2.6 pps) and nearby males at

Waimea Reservoir, where apparently no congeners occur, have a somewhat slower

song (~2.5 pps). In addition, while much of the range of *L. cerasina* is shared with *L.*

pruna (~2.0 pps) (Shaw 1999), this includes not only the populations where the fastest *L. cerasina* songs are documented (Eucalyptus Toe, Akaka Falls, Kaiwiki), but also populations where songs are intermediate (Ola'a Flume, Laupahoehoe, Glenwood Road) and slow (Naulu). However, the pulse rates of *L. pruna* are also geographically heterogeneous (Otte 1994), and may shift in concert with *L. cerasina* across their collective ranges. The question of whether reproductive character displacement influences diversification within *L. cerasina* deserves further attention.

Sexual selection could influence population divergence in song through either genetic correlations between trait and preference (Lande 1981), sensory drive using pre-existing biases in female perception (Boughman 2002), or sexual conflict (Gavrilets and Hayashi 2005). If sexual selection influences population divergence through genetic correlations, then variation in pulse rate should be correlated with variation in female preference for pulse rate, and this correlation should be found at every stage of the speciation process (Panhuis et al. 2001). In contrast, a model based on sensory drive or sexual conflict may not consistently find correlations between trait and preference across disparate populations, due to the inherent mismatch between trait and preference that drives evolution in those models. This study found that song and preference are significantly correlated across thirteen geographically disparate populations of *L. cerasina*, representing nearly the entire range of the species. However, these data represent a current snapshot in time and in the continuum of population divergence, and can only support the conclusion that sexual selection on song likely maintains the divergence that currently exists. Divergence of sexual traits among populations could be driven by a multitude of sexual selection or

reinforcement processes. For example, while the Fisher process (Lande 1981), good genes (Iwasa and Pomiankowski 1994), sensory drive (Boughman 2002), or sexual conflict (Gavrilets and Hayashi 2005) models suggest powerful mechanisms for the evolution of signals and preferences, each can operate with multiple preference functions, varying degrees of matching, and alternative genetic architectures of trait and preference. For these reasons, and because signal and preference are often expressed in multiple sensory modalities (Candolin 2003; Papke et al. 2007), the mate recognition system is a complex trait (Boake 2002; Chenoweth and Blows 2006; Mackay and Anholt 2007). Yet, even within a single sensory modality, we have an insufficient understanding of how and why signals and preferences diverge, obscuring our view of the speciation process. While the finding of a significant correlation among trait and preference suggests that sexual selection is involved in maintaining population differences, it would be difficult to disentangle the ultimate cause of the initial divergence from the consequential effect of that divergence among isolated populations.

Conclusions

In *L. cerasina*, there is statistically significant variation among populations in male acoustic traits, variation among populations in female acoustic preferences, and evidence for a genetic basis of the variation among populations. The evidence presented here supports the hypothesis that acoustic preference covaries with song. Taken together, these results indicate the potential for a direct role for these traits in the evolution of reproductive isolation.

These experiments have characterized a pattern of song differentiation in a species that is part of a rapidly diversifying clade of insects. This pattern of divergence within species is similar to the pattern of divergence among species, although to a lesser degree, which makes an investigation of the consequences of population divergence potentially useful for understanding the mechanisms that may influence speciation in the group. This study finds evidence for divergence in female preference for pulse rate among populations and shows that females prefer songs similar to those produced by males from the same population. The correlated divergence of song and preference among populations has the potential to reduce gene flow across the species and could promote speciation, if the differences among populations in song and preference are sufficient to cause sexual isolation through assortative mating.

TABLES

Table 1. Geographic variation in male song. Mean male pulse rate (PR), pulse duration (PD), and carrier frequency (CF) with one standard error (SE) for all 13 populations, for each year sampled. Populations are ordered from north to south. Coefficient of variation (CV) shown for each measurement is averaged across all individuals.

Population	Year	N	PR (SE)	PD (SE)	CF (SE)
Pololu Valley	2005	5	2.58 (0.037)	39.1 (2.65)	4780 (47)
Muliwai Plateau	2004	11	2.55 (0.026)	40.6 (1.65)	4840 (62)
Muliwai Plateau	2005	20	2.62 (0.019)	42.7 (0.90)	4840 (40)
Muliwai Plateau	2006	28	2.59 (0.020)	41.0 (1.38)	4980 (40)
Waimea Reservoir	2004	23	2.49 (0.017)	44.5 (0.88)	4530 (44)
Kalopa Park	2004	24	2.33 (0.015)	46.0 (0.68)	4600 (33)
Laupahoehoe	2005	18	2.74 (0.026)	44.3 (1.64)	4730 (36)
Laupahoehoe	2006	16	2.65 (0.023)	41.3 (1.78)	4730 (61)
Akaka Falls	2006	21	2.95 (0.024)	39.2 (1.16)	4910 (40)
Eucalyptus Toe	2005	19	2.91 (0.020)	40.3 (1.17)	4900 (47)
Eucalyptus Toe	2006	12	2.91 (0.025)	39.6 (1.51)	4870 (57)
Kaiwiki	2006	5	2.80 (0.046)	40.4 (1.54)	4700 (112)
Ola'a Flume	2004	9	2.61 (0.035)	40.9 (1.65)	4690 (102)
Ola'a Flume	2006	14	2.70 (0.022)	38.9 (1.37)	4750 (64)
Glenwood Road	2004	25	2.53 (0.024)	40.0 (0.97)	4590 (28)
Glenwood Road	2005	18	2.63 (0.020)	39.7 (1.16)	4740 (48)
Naulu Trail	2005	13	2.44 (0.023)	40.2 (1.28)	4880 (36)
Wright Road	2004	17	2.61 (0.022)	40.0 (1.60)	4420 (45)
Wright Road	2005	15	2.75 (0.019)	40.3 (1.69)	4680 (44)
Kaiholena	2006	15	2.73 (0.033)	40.3 (1.71)	4780 (32)
Coefficient of Variation (CV)			0.69%	4.35%	0.55%

Table 2. Correlations among song characters in wild-caught males. The correlation coefficients from a Pearson correlation analysis using pulse rate, pulse duration, carrier frequency, and recording temperature are shown below, adjusted to account for population differences. Following a Bonferroni correction for multiple comparisons, only one correlation, between pulse duration and carrier frequency, showed a significant (negative) relationship (in bold), with a p<0.0001. All other relationships were non-significant (p>0.05).

	Pulse Rate	Pulse Duration	Carrier Frequency
Pulse Rate	---		
Pulse Duration	0.053	---	
Carrier Frequency	0.119	**-0.213**	---
Temperature	0.074	-0.054	0.013

Table 3. Geographic variation in female preference for pulse rate. Mean female preference plus one standard error (SE) for all 13 populations, for each year sampled. The trials used in calculating the estimate are also shown for each population and year (see text). Populations are ordered from north to south. Average male pulse rates (PR) are reported here again for reference.

Population	Year	N	PR preference (SE)	Trials used	Male PR
Pololu Valley	2005	10	2.48 (0.040)	1-5	2.58
Muliwai Plateau	2004	16	2.56 (0.050)	1-5	2.55
Muliwai Plateau	2005	6	2.52 (0.070)	1-5	2.62
Muliwai Plateau	2006	20	2.58 (0.047)	1-5	2.59
Waimea Reservoir	2004	19	2.56 (0.063)	1-4	2.49
Kalopa Park	2004	18	2.51 (0.033)	1-4	2.33
Laupahoehoe	2005	8	2.77 (0.081)	1-5	2.74
Laupahoehoe	2006	25	2.73 (0.044)	1-5	2.65
Akaka Falls	2006	11	2.88 (0.069)	1-6	2.95
Eucalyptus Toe	2005	9	2.91 (0.105)	1-6	2.91
Eucalyptus Toe	2006	14	2.90 (0.060)	1-6	2.91
Kaiwiki	2006	9	2.85 (0.040)	1-5	2.80
Ola'a Flume	2004	10	2.61 (0.044)	1-5	2.61
Ola'a Flume	2006	8	2.69 (0.035)	1-5	2.70
Glenwood Road	2004	21	2.63 (0.029)	1-5	2.53
Glenwood Road	2005	7	2.64 (0.030)	1-5	2.63
Naulu Trail	2005	11	2.40 (0.063)	1-4	2.44
Wright Road	2004	17	2.75 (0.029)	1-5	2.61
Wright Road	2005	12	2.77 (0.157)	1-5	2.75
Kaiholena	2006	18	2.78 (0.035)	1-5	2.73

Table 4. Wild-caught and G_2 comparisons. Female mean pulse rate preference (PR pref) estimates and male mean pulse rates, both measured in pulses per second (pps) are compared across generations for two populations of *L. cerasina*.

Population	Generation	PR pref (pps) Mean (SE, N)	95% CI (pps)	Pulse Rate Mean (SE, N)
Naulu Trail	2005 (wild-caught)	2.40 (0.063, 11)	2.28-2.52	2.44 (0.023, 13)
Glenwood Road	2005 (wild-caught)	2.64 (0.030, 7)	2.58-2.70	2.63 (0.020, 18)
Naulu Trail	G_2 (lab-reared)	2.36 (0.112, 16)	2.14-2.58	2.43 (0.011, 40)
Glenwood Road	G_2 (lab-reared)	2.56 (0.054, 16)	2.45-2.67	2.61 (0.013, 40)

FIGURE LEGENDS

Figure 1. Topographic map of the Big Island of Hawaii, showing the approximate locations of the thirteen collecting sites where *Laupala cerasina* was sampled, along with their estimated coordinates.

Figure 2. Geographic variation in the pulse rate of male song, by year. Mean male pulse rates (plus one standard error) are reported for each population, arranged geographically from north to south, with each collecting year reported separately.

Figure 3. Geographic variation in the carrier frequency of male song, by year. Mean male carrier frequencies (plus one standard error) are reported for each population, arranged geographically from north to south, with each collecting year reported separately.

Figure 4. Geographic variation in the pulse duration of male song, by year. Mean male pulse durations (plus one standard error) are reported for each population, arranged geographically from north to south, with each collecting year reported separately.

Figure 5. Comparison of male pulse rate and female preference for pulse rate across generations. The histograms on the left show male pulse rate from second-generation (G_2) laboratory-reared individuals (bottom) and their wild-caught ancestors (top)

from Naulu and Glenwood. The figures on the right show female phonotaxis results from G_2 individuals (bottom) and their wild-caught ancestors (top) from Naulu and Glenwood. The y-axis shows the proportion of females responding to the faster pulse rate in a two-choice trial, along with the 95% confidence intervals. The dashed line at 0.5 indicates no preference for a given trial; populations whose confidence intervals exclude 0.5 show significant preference for one of the two pulse rates in the trial. Trials in which the confidence intervals of one population exclude the mean preference of the other have significantly different pulse rate preferences for that trial.

Figure 6. Geographic variation in female pulse rate preference, by year. Mean female pulse rate preferences (plus one standard error) are reported for each population, arranged geographically from north to south, with each collecting year reported separately.

Figure 7. Correlation between female preference and male song, by year. Female pulse rate preference means were plotted against male pulse rate means for all populations of *L. cerasina*, across all years sampled. Lines show the slope of the linear regression (trend line) for each year.

Figure 8. Covariation between female preference for pulse rate and male pulse rate across all populations of L. cerasina. Preference and pulse rate means were averaged across all years for which there were data. The error bars shown are the 95% confidence intervals for each estimate.

FIGURES

Figure 1.
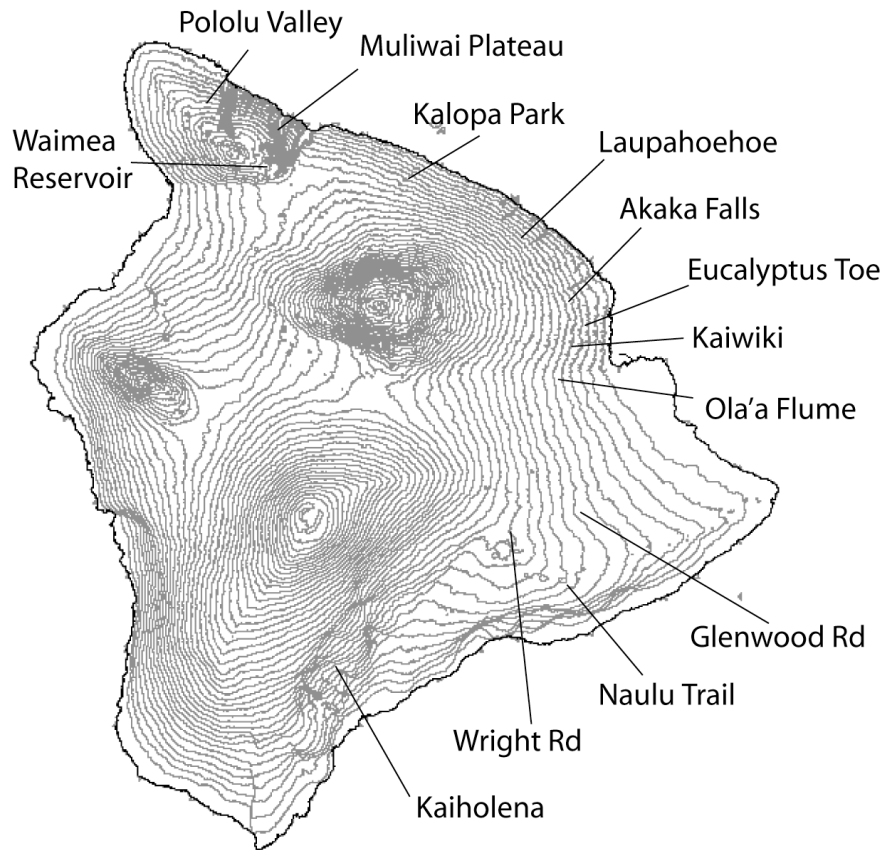
Population	Latitude (N)	Longitude (W)
Pololu Valley	20 deg 10'	155 deg 46'
Muliwai Plateau	20 deg 7'	155 deg 37'
Waimea Reservoir	20 deg 3'	155 deg 38'
Kalopa Park	20 deg 2'	155 deg 27'
Laupahoehoe	19 deg 57'	155 deg 16'
Akaka Falls	19 deg 51'	155 deg 10'
Eucalyptus Toe	19 deg 48'	155 deg 9'
Kaiwiki	19 deg 46'	155 deg 10'
Ola'a Flume	19 deg 42'	155 deg 11'
Glenwood Rd	19 deg 28'	155 deg 8'
Wright Rd	19 deg 26'45"	155 deg 14'30"
Naulu Trail	19 deg 21'30"	155 deg 10'
Kaiholena	19 deg 10'30"	155 deg 35'

Figure 2.

Pulse Rate Variation
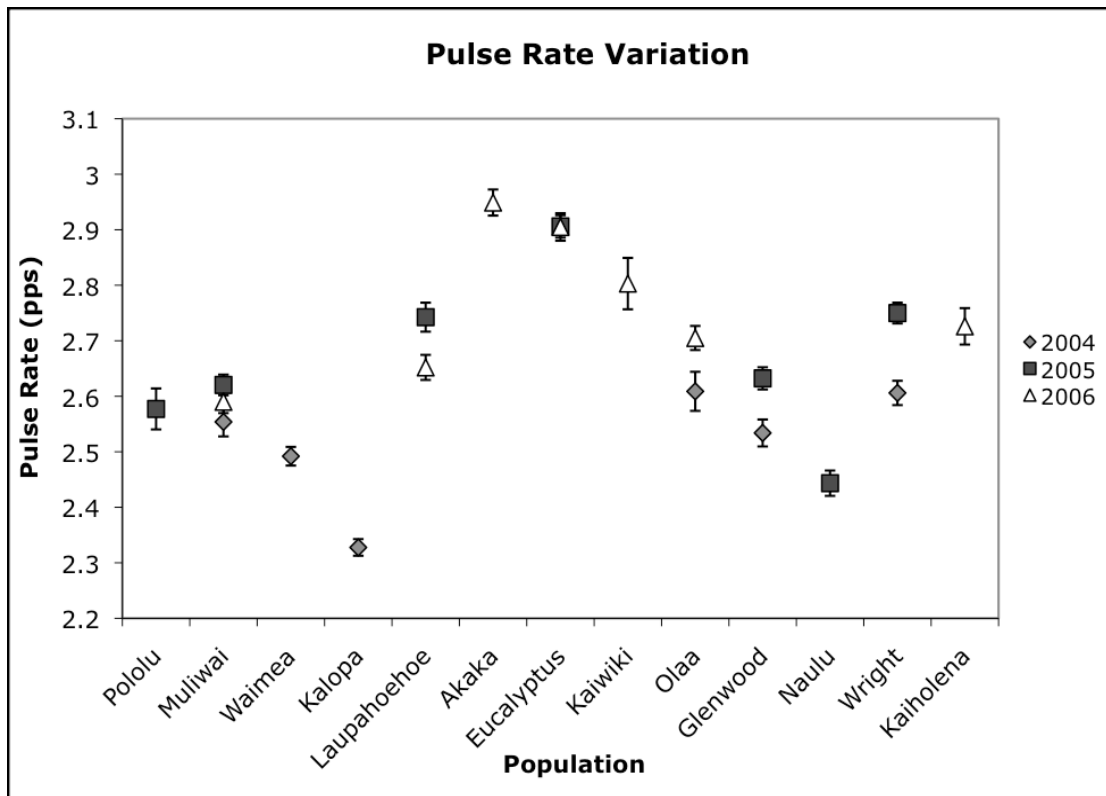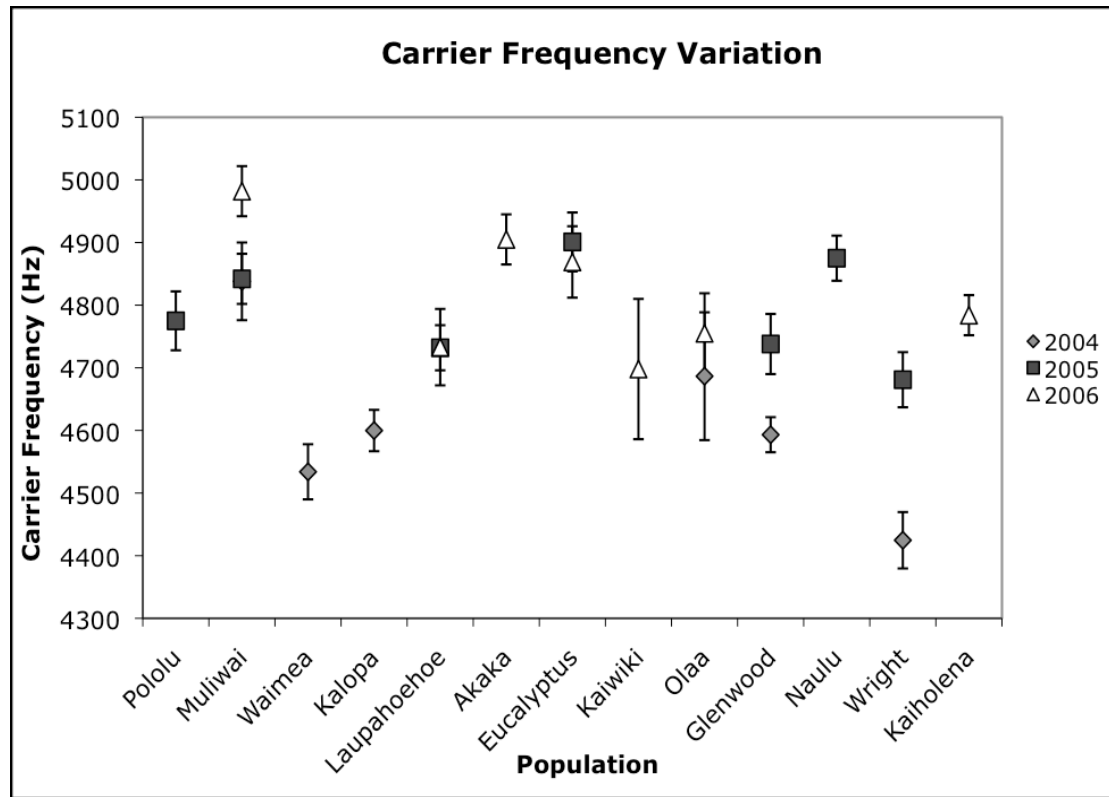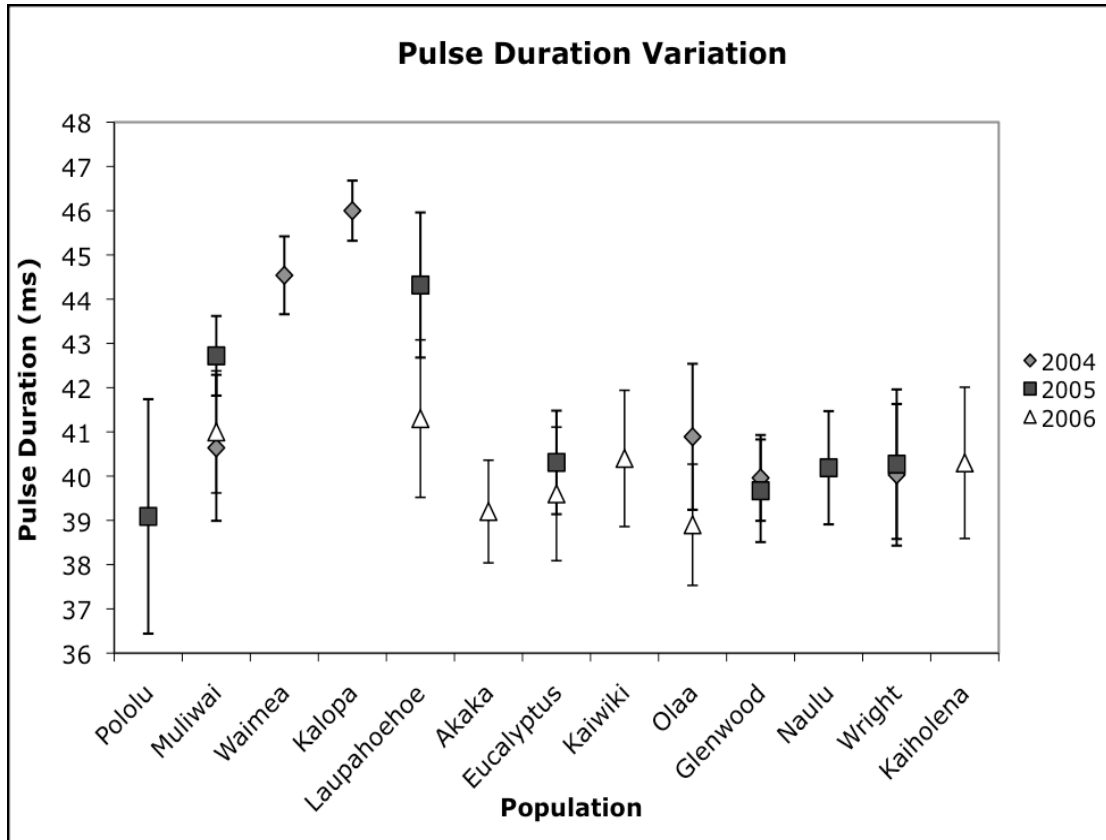
Figure 3.

Figure 4.

Figure 5.
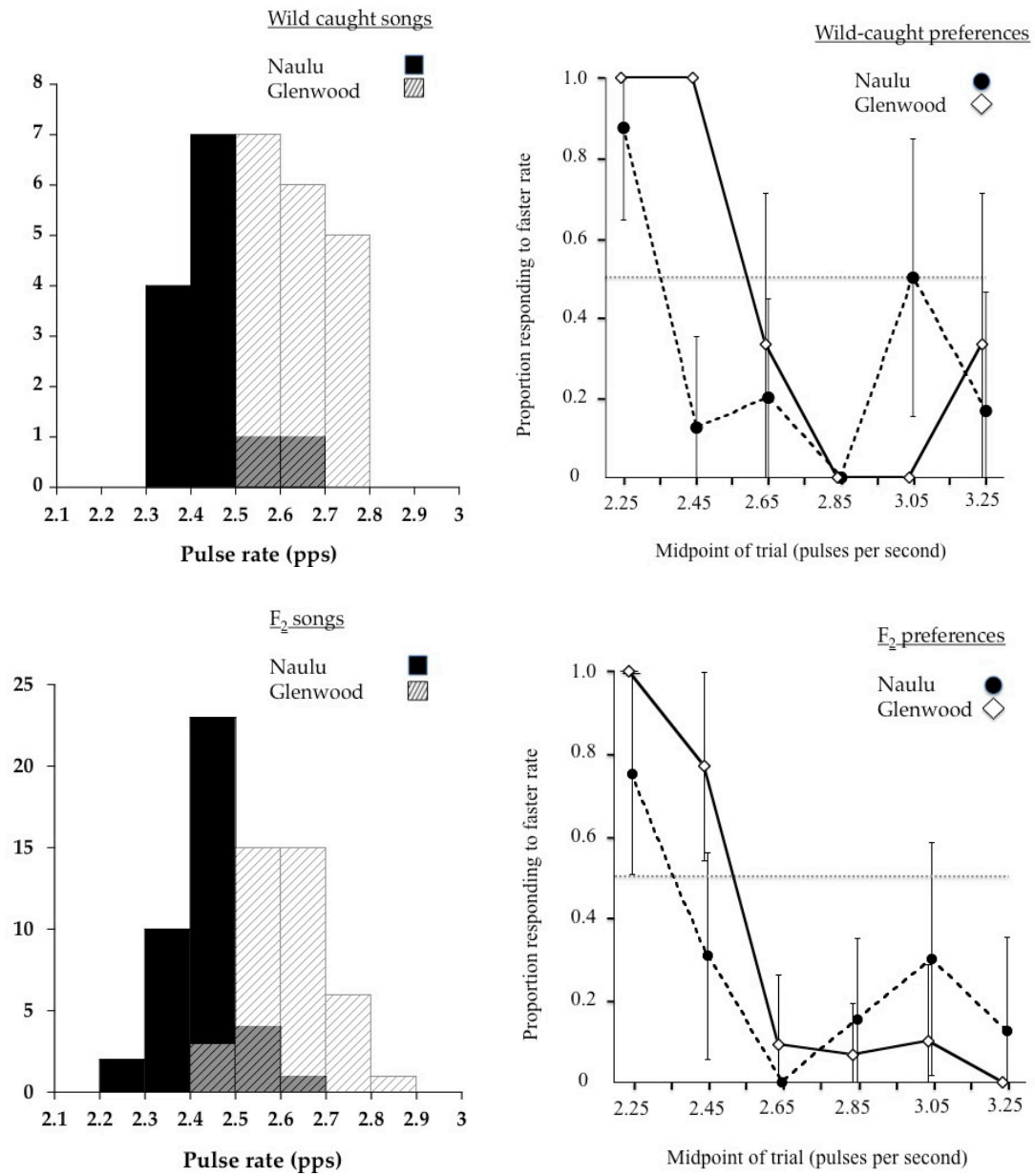
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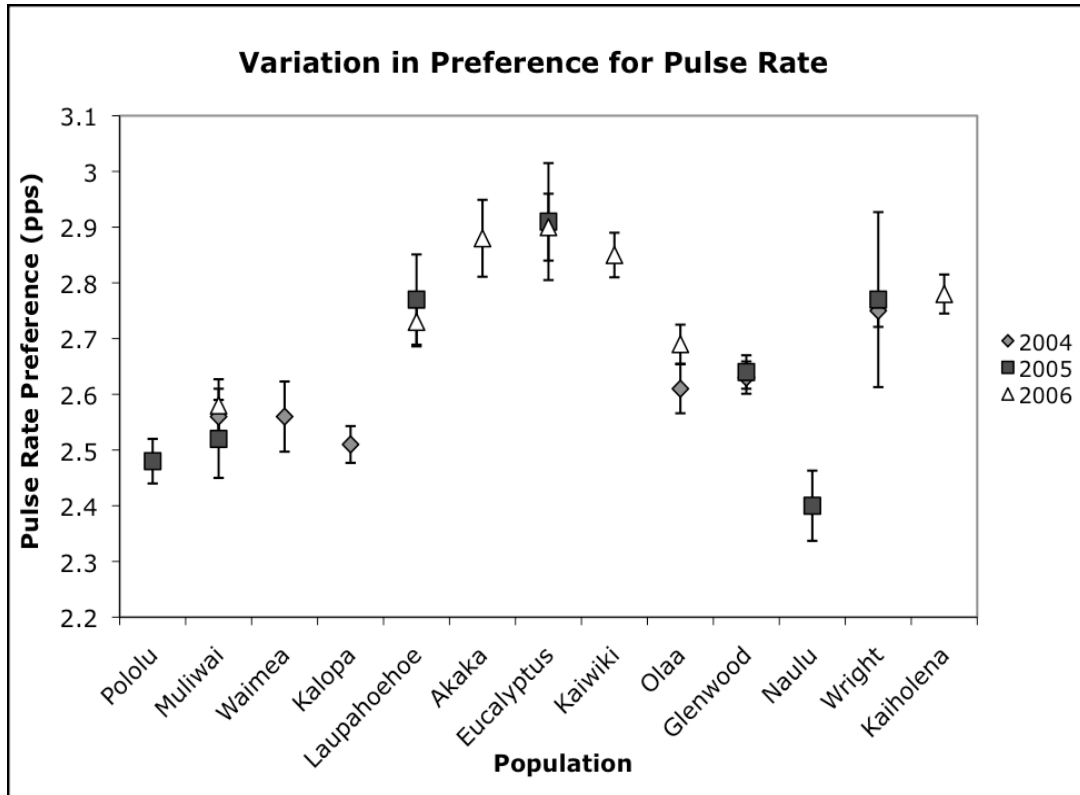
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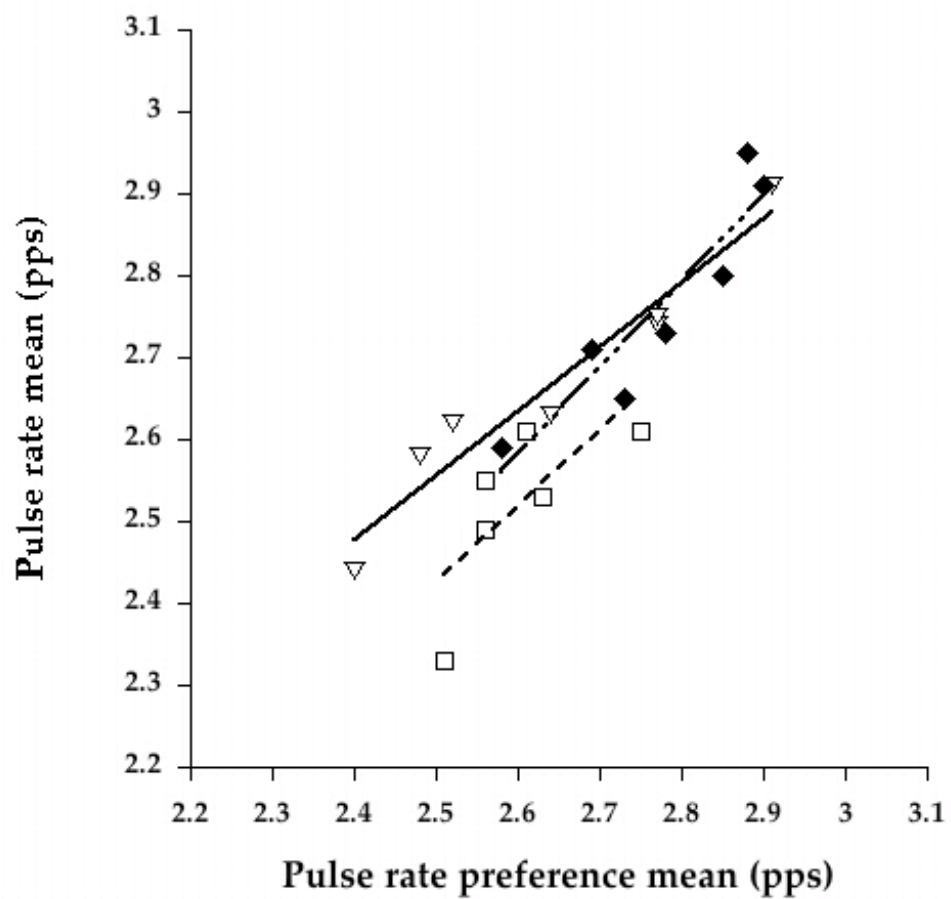
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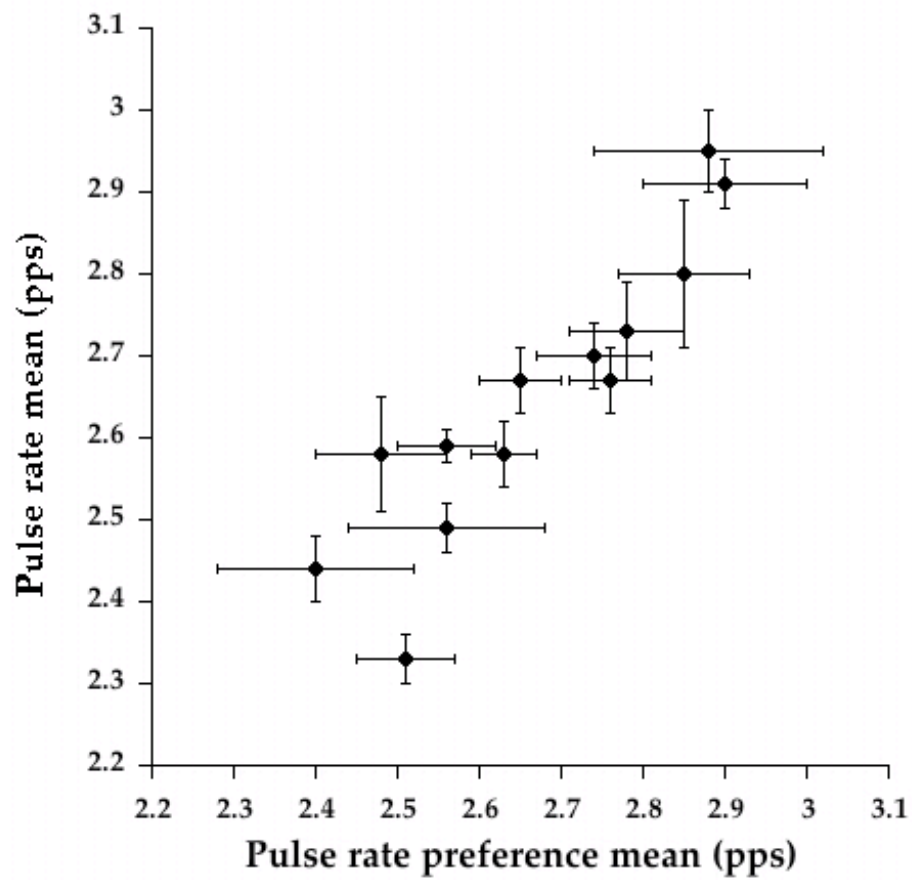
Chapter II: Rapid evolution of divergent female acoustic preferences facilitates sexual isolation in a Hawaiian cricket, *Laupala cerasina*

ABSTRACT

Sexual selection may cause the rapid evolution of sexually preferred characters within populations, since the most attractive individuals experience a direct increase in fitness through increased mating success. Consequently, isolated populations that experience divergence in their preferences for sexually selected traits may experience rapid evolution of those traits, resulting in population differentiation in a sexually selected character. In order for speciation by sexual selection to occur, females in diverging populations must discriminate among sexual signals that are in the process of divergence, and show preference for sexual signals representative of their own population over others. Previous research has documented that populations of the Hawaiian cricket *Laupala cerasina* show significant differentiation in the pulse rate of male song, a sexually selected character. However, nearby populations, whose songs tend to be less differentiated, have greater opportunities for migration and gene exchange, and this gene flow is reduced if migrants from sexually diverged populations experience reduced mating success. The current study investigates whether females can discriminate between songs whose differences are typical of those separating neighboring populations. Females from eight populations of *L. cerasina* were given the opportunity to choose between pairs of songs that

differed by only 0.2 pulses per second (pps), consistent with the divergence typical of adjacent populations. Not only did females discriminate among songs differing by 0.2 pps, but mean preferences also closely matched the mean pulse rates measured for males from the same population. Divergence in preference was observed only between populations that also differed in song. The correlated divergence of song and preference could be a mechanism that promotes assortative mating among populations, thereby reducing gene flow, and may contribute to the speciation process in *Laupala*.

INTRODUCTION

More than a century ago, Darwin (1871) first observed that secondary sexual characters diverge rapidly among closely related species, suggesting that sexual selection is an important mechanism influencing evolution within species. Much more recently, divergent sexual selection among populations has been proposed as a mechanism that could drive speciation (West-Eberhard 1983; Panhuis et al. 2001; Coyne and Orr 2004; Ritchie 2007). Since sexual selection within species is widely reported (Andersson 1994), its action within isolated populations may lead to population divergence across a broader geographic range (Lande 1982; Endler 1992). Indeed, because isolated populations experience no selection pressure to maintain reproductive compatibility, the divergence of geographically isolated populations is possibly inevitable (Mayr 1963; West-Eberhard 1983; Turelli et al. 2001).

While divergence through natural selection (Schluter 2009; Schluter and Conte 2009; van Doorn et al. 2009) or the evolution of genetic incompatibilities

(Pryke and Griffith 2009) as by-products may collaborate, there are several reasons to suspect that sexual selection actively facilitates speciation. First, reproductive isolation is more likely to result from a divergence in traits directly involved in mate choice, such as sexually selected characters (West-Eberhard 1983; Panhuis et al. 2001). Second, in several taxonomic groups, behavioral (and especially sexual) isolation appears to evolve at a faster rate than hybrid incompatibilities (Coyne and Orr 1997; Mendelson 2003; Alexander and Breden 2004). Third, comparative studies using various proxies for the intensity of sexual selection have indicated that phylogenetic clades with higher levels of sexual selection (and sexual conflict) tend to be species-rich (Barraclough et al. 1995; Mitra et al. 1996; Arnqvist et al. 2000). Lastly, the rapid evolution of sexual signaling is a key feature associated with diversification in two systems demonstrating accelerated rates of speciation (cichlids, Danley and Kocher 2001; crickets, Mendelson and Shaw 2005).

Conclusive demonstration of sexual selection-driven speciation requires detailed studies of specific populations in the process of divergence. Many species exhibit minor inter-population differentiation in male sexual signals (Andersson 1994; Gerhardt and Huber 2002); however, geographic variation in sexual signals may not always reflect a correlated divergence in female preference for those signals (Hill 1994; Zuk et al. 2006; van der Sluijs et al. 2010). Under most circumstances, coevolution of signal and preference are necessary to bring about the assortative mating that can close the reproductive boundary between incipient species (Lande 1981; Kirkpatrick 1982). Furthermore, the significance of population differences in male sexual signals must be assessed relative to whether or not females discriminate

differences in these male signals, a necessary prerequisite to skewed male mating success (Schluter and Price 1993). Several studies of various frog species have found evidence that divergence in male acoustic characters among populations may have been the result of divergent sexual selection (Prohl et al. 2006; Boul et al. 2007); however, these studies have not addressed differences in the shape of female preference functions in populations in the process of divergence. In contrast, many studies of divergent sexual selection in crickets do indicate divergent preference functions (Gray and Cade 2000; Shaw 2000b); however, these studies document differences at the interspecific level. There seems to be a lack of information about the shape of female preference functions among populations in the early stages of divergence, when these differences have the greatest potential to either accelerate or hinder the rate of population divergence by impacting the degree of gene flow.

The Hawaiian cricket genus *Laupala* makes an ideal candidate for studying questions of sexual selection and speciation. The 38 constituent species evolved recently and are morphologically cryptic (Otte 1994; Shaw 2000a; Mendelson and Shaw 2005), yet distinctive in pulse rate of the male calling song, a sexually selected trait (Shaw and Herlihy 2000; Mendelson and Shaw 2002). Male song is easily quantified and synthesized (Otte 1994; Shaw 1996) and females readily respond to these synthesized songs, exhibiting preferences for songs with pulse rates at or near the male population mean (Shaw 2000b; Shaw and Herlihy 2000). Both pulse rate and preference variation have a genetic basis (Shaw et al. 2007; Shaw and Lesnick 2009; Wiley and Shaw 2010; Oh et al. in prep). *L. cerasina* show differentiation in pulse rate (Otte 1994) and preference across its geographic range (Chapter 1), providing an

opportunity to investigate the importance of this variation female mating behavior. This study examines whether females can discriminate among slight differences in the songs that characterize adjacent populations (~ 0.2 pps). If female preference divergence influences male song evolution, females from neighboring populations are expected to exhibit biased phonotactic responses to songs that differ by this order of magnitude. Furthermore, when neighboring populations are compared, females should express divergent preferences, thus favoring songs representative of homotypic over heterotypic males.

METHODS

Overview

Laupala cerasina were collected from eight locations on the Big Island of Hawaii in July 2005 (Figure 1), hereafter referred to as populations. The collecting sites cover the majority of the range of *L. cerasina*, both geographically and phenotypically, and included the following paired comparisons: A. Muliwai Plateau (MP) vs. Waimea Reservoir (WM) (7.55 km apart), B. Kalopa Park (KP) vs. Laupahoehoe (LP) (21.26 km apart), C. Eucalyptus Toe (ET) vs. Kaiwiki (KW) (4.08 km apart), and D. Glenwood Road (GR) vs. Naulu Trail (NT) (12.46 km apart). In a previous study of wild-caught individuals, two of these population pairs showed significant divergence in song (B and D) and two did not (A and C) (Chapter 1).

Rearing conditions

In the laboratory at the University of Maryland, individuals were reared under a 12:12 light/dark cycle in a temperature-controlled room maintained at 20°C. Small nymphs were reared in 1-quart glass jars with no more than 20 individuals per jar, and larger nymphs and adults were kept in plastic specimen cups, with no more than three per cup, separated by sex. Individuals were provided with moistened kimwipes for moisture and fresh cricket chow (Fluker Farms, Louisiana) treated with methyl paraben (Tegosept, Fisher Scientific) once per week. Females collected from Hawaii as adults were housed individually to allow collection of eggs produced from natural matings prior to capture. All other individuals were reared to maturity and then randomly paired with a mate from the same population to generate offspring. Eggs collected from wild-inseminated adult females and through laboratory matings were reared to maturity under the same conditions as the parents. These lab-reared offspring were used in the experiments described below.

Quantifying song differentiation

To record songs, males were placed beneath clear plastic cups fitted with mesh covers in a temperature-controlled room maintained at 20°C. Upon singing, males were recorded onto cassette tapes using a Sony Professional Walkman (Model WM-D6) and Sony condenser microphone. A female from the same population was introduced if the male did not sing spontaneously. The songs of 260 males were recorded and digitized using Raven 1.2 (Cornell University, Ithaca, NY). Five non-consecutive measurements per male were made for pulse period, pulse duration, and carrier

frequency. These were averaged to obtain a single value for each male for each character. Pulse period, measured as the time between the beginning of one pulse and the beginning of the next pulse, is transformed to calculate pulse rate (the inverse of the pulse period), which is the primary song feature that differs among species of *Laupala*. Pulse duration is measured as the time between the beginning and end of a single pulse. Carrier frequency is measured as the dominant frequency of a single pulse. These are the same measurements that have previously been reported for wild-caught *L. cerasina* individuals (Chapter 1). Planned comparisons were conducted between the songs of males from neighboring populations (population pairs A-D) using t-tests.

Quantifying female discrimination

Virgin females approximately 20-25 days past the final molt were used in phonotaxis trials. Females of this age show a high level of responsiveness to synthesized cricket songs (Chapter 1). Since several adjacent populations of *L. cerasina* express a song divergence of around 0.2 pulses per second, our experimental design tested for this level of discrimination. To test for female discrimination behavior, six trials were conducted which differed in the pulse rate choices presented to the female: 2.0 vs. 2.2, 2.2 vs. 2.4, 2.4 vs. 2.6, 2.6 vs. 2.8, 2.8 vs. 3.0, and 3.0 vs. 3.2 pps. These trials span the natural range of variation for *L. cerasina* songs and song preferences (Chapter 1). All parameters other than pulse rate of the synthetic songs were held constant at values that approximate the natural parameters found in *Laupala* species:

pulse duration, 40 ms and carrier frequency, 5 kHz (Shaw and Herlihy 2000). A total of 233 females were used, once in each trial (six times total per female).

Phonotaxis trials were conducted in a circular arena (radius 47 cm) housed in a temperature-regulated (20°C), acoustically buffered sound chamber (Acoustic systems) illuminated by red gel-filtered lights. Two speakers (Radio Shack 40-1218) broadcast programmable, digitally synthesized, pulsed sinusoidal tones created using custom-designed software (T.G. Forrest, Univ. of North Carolina, Asheville) from opposite ends of the arena. A 16-bit digital/analog converter (Tucker-Davis Technologies) and 10 kHz filter (Krohn-Hite 3322) processed the acoustic output. A digital attenuator (Bruel and Kjaer 4155) was used to monitor and equalize sound pressure levels between speakers.

For each trial, playbacks occurred for an acclimation period of five minutes, during which time a female was restrained beneath a plastic cup in the center of the arena. Once the cup was raised, she received an additional five minutes to respond to the acoustic stimuli. A 10-cm score zone was designated around each speaker (Figure 2). Once a female entered the score zone associated with a speaker, the trial was ended. She received a score of one for approaching the faster song, or zero for the slower song. If the female did not approach either score zone after five minutes, no score was recorded for that trial. A random number generator was used to determine the order of presentation and the speaker assigned to play the faster/slower song. Females were used in two trials per day over three consecutive days to complete the series of six trials.

To determine whether females discriminate between songs differing by 0.2 pulses per second, the proportion of females that approached the faster song of a given pair was compared to the null expectation under a model of random choice, where the probability of choosing either song is 0.5. The 95% confidence intervals for each proportion were calculated from the binomial distribution. If these confidence intervals excluded 0.5, the population showed a significant response bias towards either the fast or the slow song of a given trial.

To determine whether neighboring populations differ in their song preferences, female preference data were further analyzed using the statistical program R. To estimate the mean female preference for a population, the entire set of female phonotaxis data is analyzed simultaneously using logistic regression. The point at which females are equally likely to choose either the fast or the slow song (probability of 0.5) was determined for each population, providing an estimate of the mean pulse rate preference (Shaw and Herlihy 2000; Grace and Shaw 2004; Chapter 1). Standard errors for this estimate were calculated for each population by bootstrap analysis, which randomly resampled the data with replacement, while maintaining the original sample size, for 1000 replicates (Chapter 1). For each replicate, the mean pulse rate preference was estimated, allowing a calculation of the standard deviation of the means (i.e., the standard error). Planned comparisons between neighboring population pairs (shown in Figure 1) were also performed using the Wald χ^2 test (Grace and Shaw 2004).

RESULTS

Male song variation

Populations that showed evidence of pulse rate differentiation in the wild-caught generation (KP-LP and GR-NT; Chapter 1) also showed significant pulse rate differentiation in this experiment (KP-LP: $t=-12.21$, $p<0.0001$; GR-NT $t=8.36$, $p<0.0001$; ET-KW: $t=1.68$, $p=0.095$; MP-WM: $t=0.10$, $p=0.92$; Figure 3; Table 1). Additionally, two pairs (MP-WM and KP-LP) showed significant differences in carrier frequency (MP-WM: $t=2.80$, $p=0.055$; KP-LP: $t=2.23$, $p=0.0267$; GR-NT: $t=-1.06$, $p=0.29$; ET-KW: $t=-0.31$, $p=0.76$; Figure 3; Table 1), but none were differentiated in pulse duration (GR-NT: $t=1.91$, $p=0.057$; MP-WM: $t=1.40$, $p=0.16$; ET-KW: $t=-0.57$, $p=0.57$; KP-LP: $t=-0.25$, $p=0.80$; Figure 3; Table 1).

Female discrimination

For each population, females showed biased responses (differing significantly from 0.5) in at least two of the six trials (average: 4; range: 2 to 6; Figure 4). At the low end of the series of trials, females respond preferentially to the faster song of the pair, and at the high end, they tend to respond to the slower song (Figure 4). For each population, this resulted in a single transition of preference from the faster to the slower song, allowing an estimation of the inflection point where preference for the faster song equals that of the slower song, which provides an estimation of the mean preference.

Female preference variation

Using the results from all six trials together allows estimation of the mean preference for each population. A significant logistic regression is possible only when sufficient signal exists in the data to allow a single transition from a preference for faster songs to a preference for slower songs across the whole range of phonotaxis trials, which appears as a sigmoidal pattern in the data (Figure 4). All of the populations showed this pattern, which allowed mean preferences to be estimated using logistic regression (Table 2). Standard errors were calculated using the bootstrapping procedure outlined above. When preferences were compared across populations, two of the four planned comparisons (KP-LP and GR-NT) showed significant differentiation in female preference functions (KP-LP: Wald χ^2=12.12, p=0.0005; GR-NT: Wald χ^2=11.49, p=0.0007; Figure 4; Table 2). These were the same populations that showed differentiation in pulse rate (Figure 3; Table 1). Populations that did not show significant pulse rate divergence also did not show divergence in preference (MP-WM: Wald χ^2=0.57, p=0.45; ET-KW: Wald χ^2=0.10, p=0.75; Figure 4; Table 2).

DISCUSSION

One of the biggest weaknesses in our understanding of the evolution of reproductive isolation occurs at the interface between population divergence and speciation. At some point along this continuum, divergent selection among populations results in reproductive isolation among species, but the factors that facilitate this transition are relatively poorly understood. If divergent sexual selection among populations drives speciation through sexual isolation and assortative mating, it must be shown that

females from the diverging populations have different preferences. If divergent preferences are to have an impact on gene flow, females must be able to discriminate between the signals of local males (homotypic) and those from divergent populations (heterotypic). Furthermore, females must exhibit clear preferences for homotypic male characteristics and discriminate against heterotypics even at these early stages of trait divergence.

In *Laupala cerasina*, when neighboring populations exhibit small but significant differences in pulse rate, females display preferential phonotaxis toward songs more similar to males from the same local population than males from adjacent populations. These data indicate that females across all populations could discriminate the pulse rate differences in the trials and, further, that they demonstrated a choice based on the perceived difference between them. This suggests both that females can discriminate between the songs and that populations exhibit significant preferences for one song over another in a given pair. This study reveals that even small differences in song and preference are biologically meaningful and may be sufficient to initiate a pattern of assortative mating preferences across populations whose songs and preferences are divergent. These results demonstrate how female acoustic preference within a population can result in sexual isolation between populations.

This study is the first to demonstrate the precision with which females are able to discriminate among divergent pulse rates in *Laupala*, and to provide an estimate as to what magnitude of change is sufficient to induce a pattern of acoustic preference divergence among populations. Divergent phonotaxis preferences were found in two

independent population pairs, KP-LP and GR-NT. These same pairs also differed in the pulse rate of the male song. Population pairs that did not show significant song divergence, MP-WM and ET-KW, likewise, did not show divergence in female preference. Based on these results, the degree of sexual isolation is expected to be greatest between populations having the greatest divergence in sexual communication traits, regardless of physical proximity. However, it is worth noting that the two population pairs that showed the least divergence in song and preference also happened to be separated by the shortest distances. Although there is no evidence for isolation by distance with respect to divergent pulse rates (Chapter 1), such a pattern may be more evident over the shorter distances involved in this study. The correlated divergence of trait and preference among populations (Chapter 1) is a potential signature of speciation by sexual selection (Lande 1981), but not necessarily predicted by other models of speciation, such as ecological speciation (Hill 1994; Zuk et al. 2006).

A QTL (quantitative trait locus) study in the closely related species pair *Laupala paranigra* and *L. kohalensis* has provided evidence that some of the same loci or closely linked loci may be involved in phenotypic divergence of both song and preference (Shaw and Lesnick 2009). Furthermore, the average effect size of QTL alleles that differ between species underlying male song was found to be around 0.2 pps (Shaw et al. 2007), which is the same magnitude of differentiation used to examine female preference functions and discrimination. If the effect sizes of the QTL differences identified are due to single mutations, our results here suggest that females are able to discriminate the effects of such mutations to single QTL.

However, we have yet to determine whether common QTL underlie song divergence in *L. cerasina*. A similar genetic architecture in *L. cerasina* would suggest that physical linkage and/or pleiotropy is responsible for maintaining the behavioral coupling of song and preference that we have observed. If song and preference were genetically coupled, this would provide a powerful mechanism for explaining their correlated divergence among populations (as shown here and in Chapter 1). Alternatively, genetic correlations could be maintained through the joint inheritance of song and preference alleles that occurs when females with strong preferences choose males with preferred traits, passing on the genes for both trait and preference to the next generation, as described by Fisher (1930) and modeled by Lande (1981). Since both song and preference are likely polygenic traits (Shaw 1996, 2000b; Wiley and Shaw 2010), both physical linkage and selection-generated linkage disequilibrium may contribute to the observed pattern of covariation.

In other organisms where sexual selection acts on traits used in sexual communication, few studies have been able to demonstrate female preference for local signals over foreign ones. In the Amazonian frog *Physalaemus petersi*, females prefer the songs of local males to those of foreign males, and song divergence is correlated with reduced gene flow (Boul et al. 2007). However, a separate study in the closely related congener *P. pustulosus* found that females prefer the calls of local males over those of foreign conspecifics only one-third of the time in choice trials (Pfennig and Ryan 2007). All of these studies were conducted under field conditions in the natural environment, so the additive genetic component of these phenotypic differences may be difficult to disentangle from environmental and developmental

differences. The use of laboratory-born individuals in this experiment provides additional evidence that the observed differences among populations of *Laupala* have a genetic basis. The environmental variation that might contribute to total phenotypic variation is reduced, since these individuals were born and raised entirely under laboratory conditions, and individuals experienced the same common garden environment throughout their entire lives.

Comparisons between different populations of *L. cerasina* showed that divergence in the pulse rate of male song predicts a correlated divergence in female preference, consistent with previous analyses using wild-caught individuals (Chapter 1). This study provides further evidence of a genetic basis to covariation, since individuals were bred and reared in a common garden environment. Furthermore, females could resolve differences in pulse rate approximating the differences between neighboring populations, setting the stage for assortative mating. This sexual isolation has the potential to reduce gene flow, since rare male migrants to a new population may have difficulty attracting a mate. This preference for local mates is astonishing, given the relatively minor divergence in pulse rates between neighboring populations (~0.2 pps), compared to that observed between *L. cerasina* and its sister species (~1.0 to 1.5 pps). In all other characters that have been measured, *L. cerasina* populations are considered to be a single species, with little population-specific variation (Otte 1994; Mendelson et al. 2004; Mendelson and Shaw 2005). The fact that populations could be reproductively isolated based on divergence in a single character would provide evidence that acoustic preference for song contributes to the divergence among populations. However, phonotaxis preference is only the first opportunity for

sexual selection to influence mate choice, and further studies are needed to specifically address the hypothesis that divergent phonotaxis preference predicts assortative mating. In addition, the influence of variation in pulse rate relative to other male song characters (e.g., pulse duration, carrier frequency) has not been assessed in *Laupala*, but could have major consequences for female preference evolution (Gerhardt 2008; Gerhardt and Brooks 2009). The finding that females can discriminate among songs so precisely goes a long way towards demonstrating that these subtle differences in song are maintained by sexual selection, and ultimately, that sexual selection may be driving speciation in this group.

TABLES

Table 1. Population variation in mean pulse rate (in pulses per second), pulse duration (in milliseconds), and carrier frequency (in Hertz) of male songs, plus one standard error (SE).

Population	N	Pulse Rate (SE)	Pulse Duration (SE)	Carrier Frequency (SE)
MP	33	2.604 (0.019)	40.45 (1.03)	4834 (27)
WM	33	2.602 (0.016)	38.19 (1.07)	4725 (29)
KP	46	2.421 (0.014)	40.67 (0.96)	4835 (26)
LP	33	2.695 (0.015)	41.04 (1.23)	4755 (26)
ET	35	2.903 (0.016)	38.47 (0.99)	4846 (19)
KW	15	2.852 (0.030)	39.63 (1.34)	4861 (48)
GR	33	2.683 (0.018)	36.42 (1.21)	4755 (26)
NT	32	2.479 (0.017)	33.30 (1.39)	4797 (30)

Table 2. Population variation in estimated mean female preference for pulse rate (in

pulses per second) plus one standard error (SE).

Population	N	Preference (SE)
MP	30	2.439 (0.123)
WM	30	2.541 (0.054)
KP	28	2.325 (0.097)
LP	32	2.693 (0.091)
ET	28	2.830 (0.057)
KW	25	2.851 (0.096)
GR	30	2.673 (0.065)
NT	30	2.354 (0.099)

FIGURE LEGENDS

Figure 1. Topographic map of the Big Island of Hawaii showing collecting locations of eight populations of *L. cerasina*. Boxes indicate pairwise planned comparisons between neighboring populations.

Figure 2. Schematic of arena used in phonotaxis trials. An acoustic preference is recorded if the female enters the score zone immediately adjacent to one of the two speakers.

Figure 3. a) Geographic variation in pulse rate of male song, organized from north to south. Mean pulse rate in pulses per second (pps) plus one standard error are plotted for each population. b) Geographic variation in carrier frequency of male song, organized from north to south. Mean carrier frequency in Hertz (Hz) plus one standard error are plotted for each population. c) Geographic variation in pulse duration, organized from north to south. Mean pulse duration of male song in milliseconds (ms) plus one standard error are plotted for each population.

Figure 4. Geographic variation in female phonotaxis responses. The y-axis shows the proportion of females responding to the faster pulse rate in a two-choice trial, along with the 95% confidence intervals. The dashed line at 0.5 indicates no preference for a given trial; populations whose confidence intervals for a given trial exclude 0.5 show significant preference for one of the two pulse rates in the trial. Trials in which

the confidence intervals of one population exclude the mean preference of the other population have significantly different preferences for that trial. Plots are organized according to planned population comparisons: a) Muliwai Plateau and Waimea Reservoir b) Kalopa Park and Laupahoehoe c) Eucalyptus Toe and Kaiwiki d) Glenwood Road and Naulu Trail

Figure 5. Geographic variation in mean female pulse rate preference, organized from north to south. Mean pulse rate preferences in pulses per second (pps) plus 1 standard error are plotted for each population.
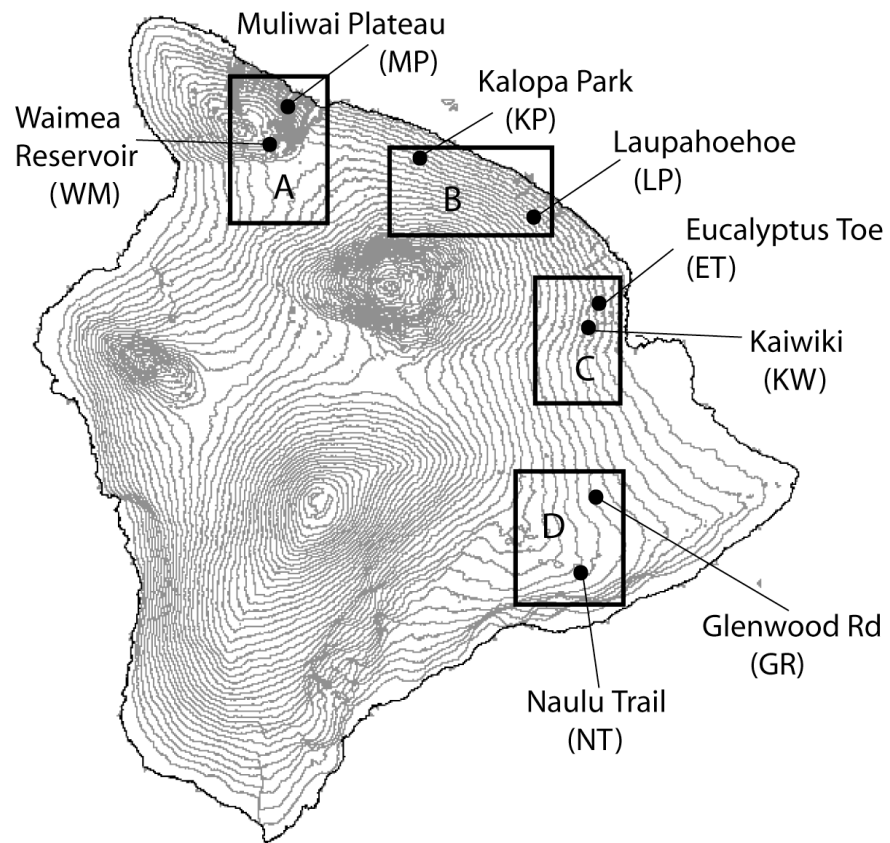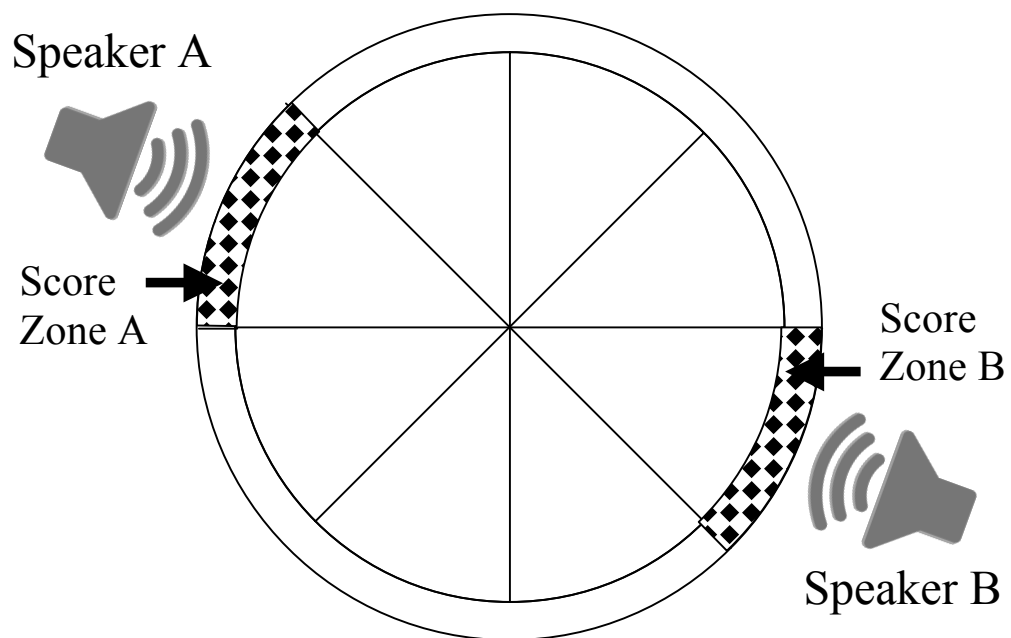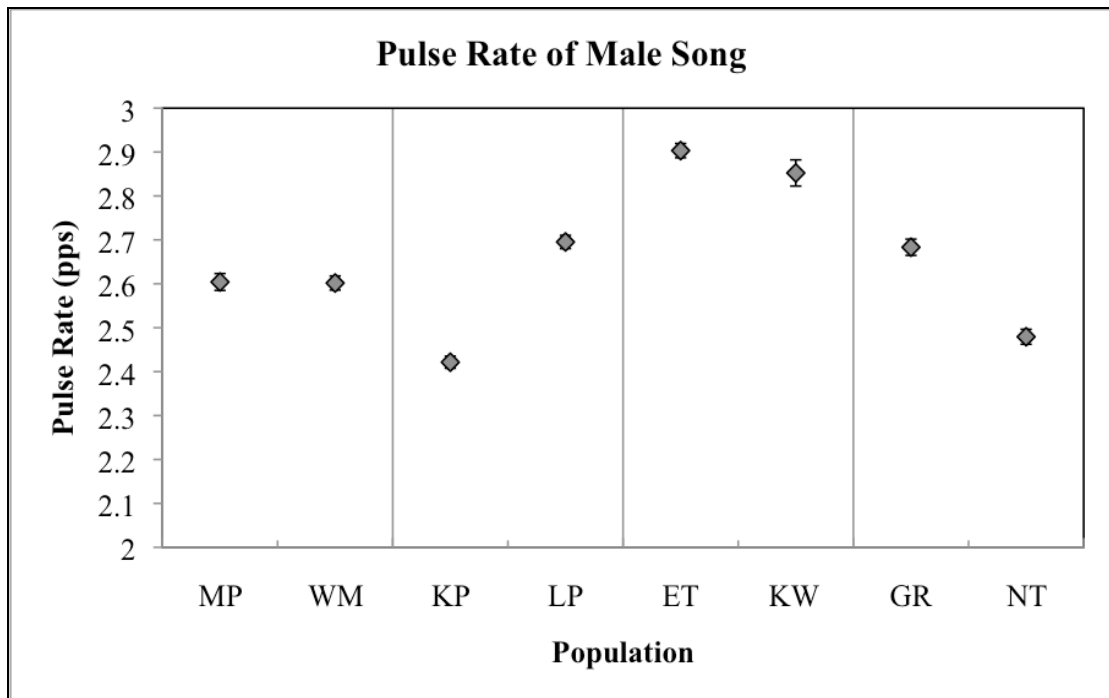
FIGURES

Figure 1.

Figure 2.

Speaker A

Score Zone A

Score Zone B

Speaker B

Figure 3.

a)

b)

c)

Pulse Duration of Male Song
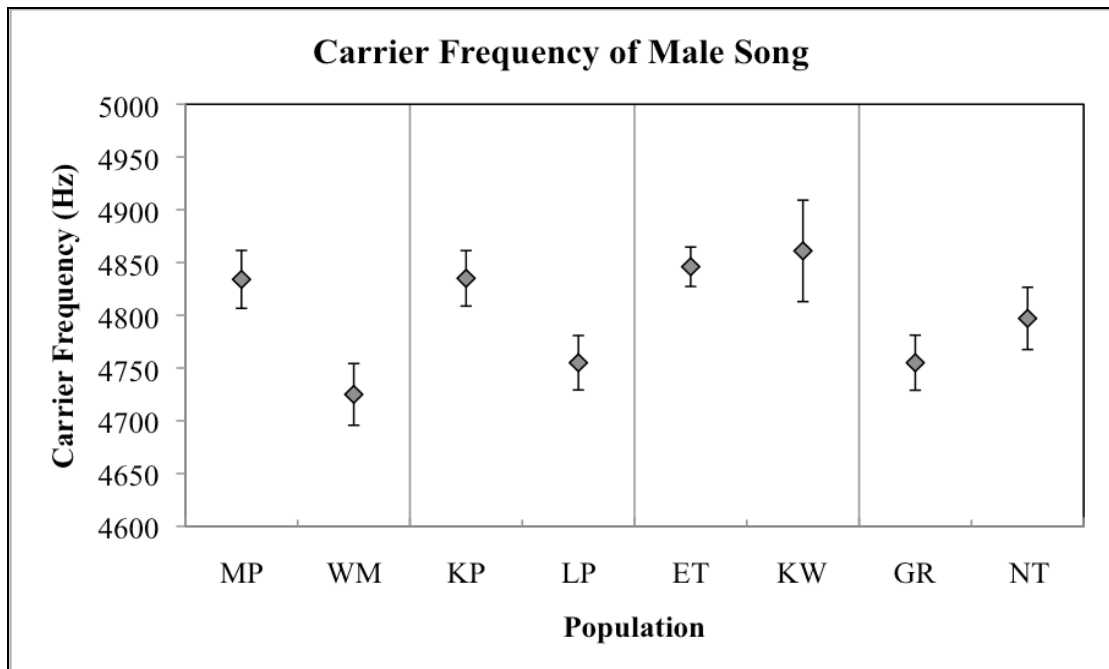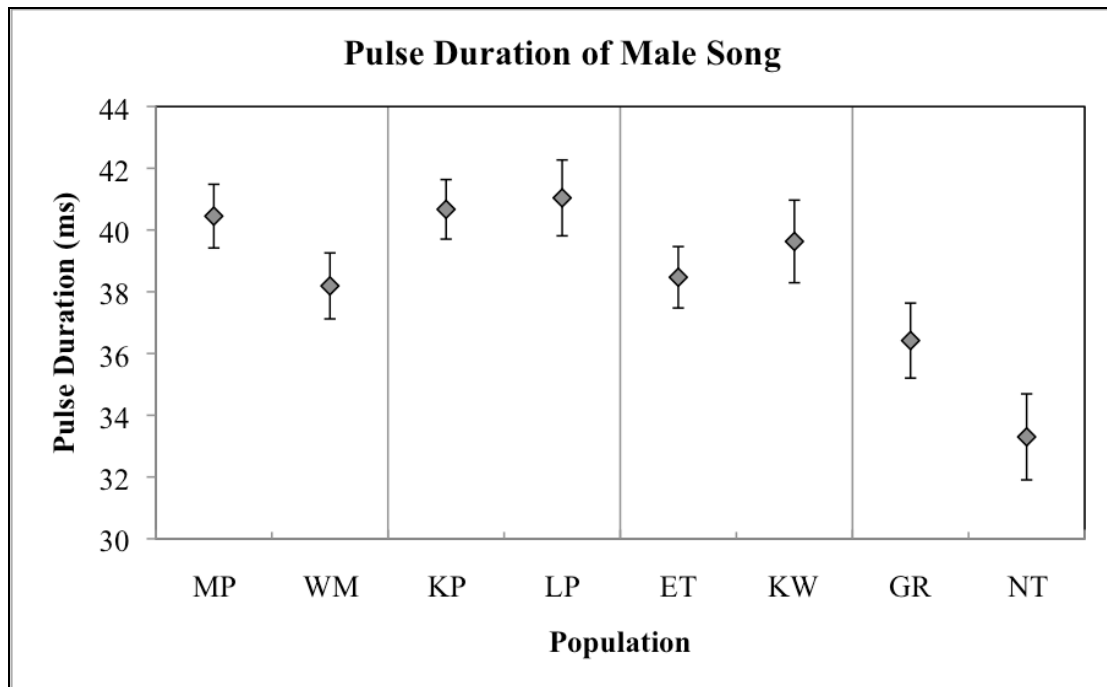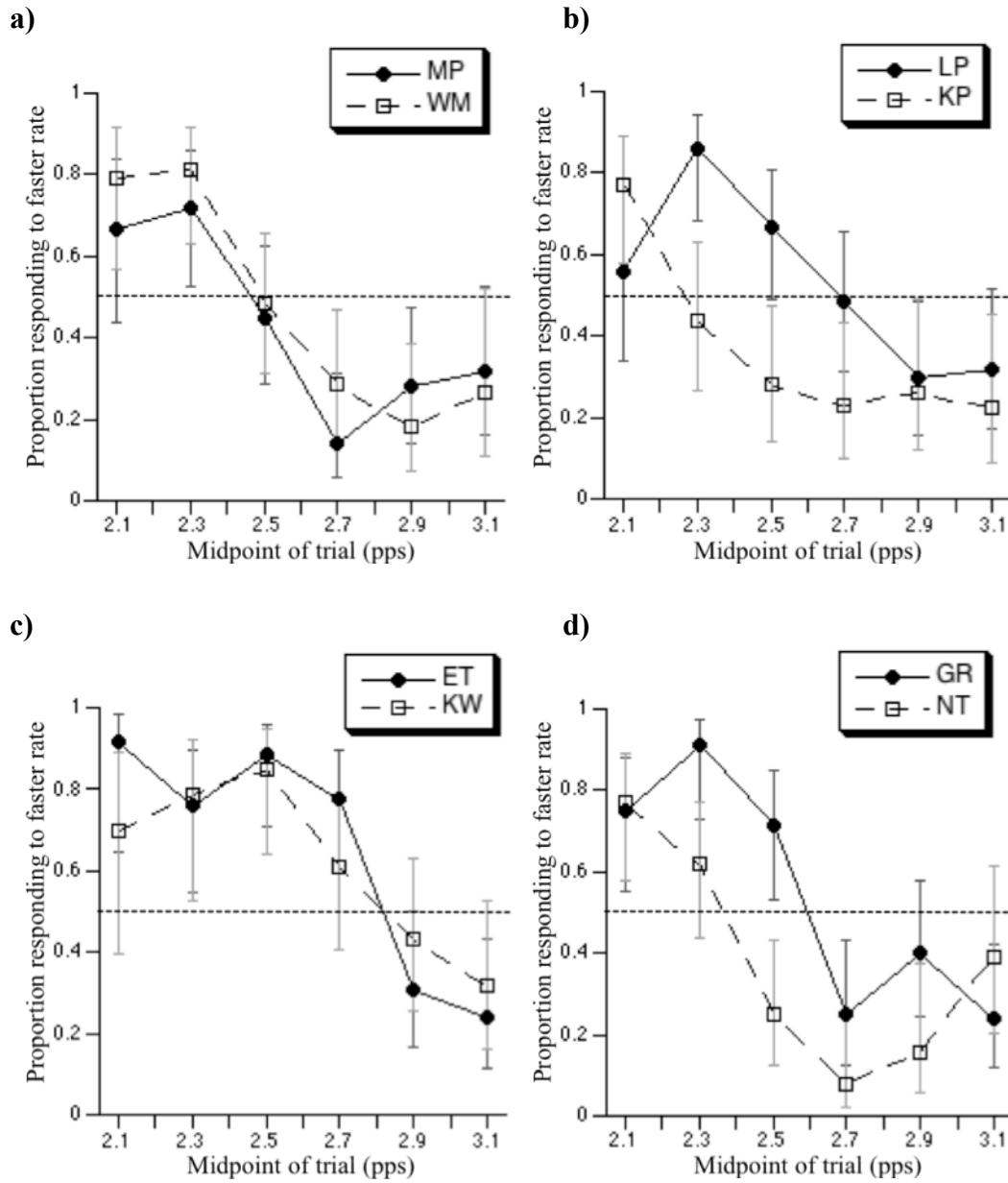
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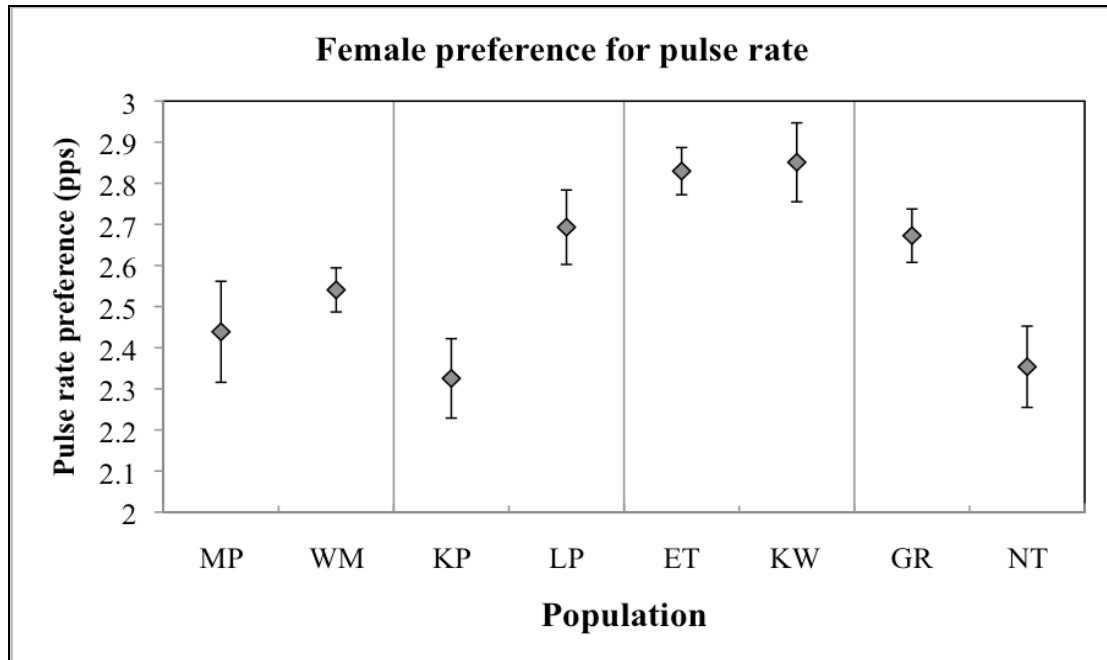
CHAPTER III: Divergent female acoustic preference facilitates sexual isolation through assortative mating in the Hawaiian cricket *Laupala*

ABSTRACT

Closely related species often differ in secondary sexual characters, prompting the hypothesis that sexual selection may promote speciation; however, strong evidence supporting this hypothesis remains elusive. Crickets in the genus *Laupala* are morphologically and ecologically cryptic, but have diverged in a secondary sexual character, pulse rate of male song. In phonotaxis trials, females prefer the pulse rates of conspecific males. It is hypothesized that divergent phonotaxis preferences promote sexual isolation between closely related species, and that divergent sexual selection may also promote isolation among populations within a species. The mate choice preferences of two closely related sister species, *L. cerasina* and *L. eukolea*, were examined, as well as the mate choice preferences of neighboring populations of *L. cerasina*. Females were presented the songs of two males: one from the same population and species (homotypic) and one from the contrasted population or species (heterotypic). Following phonotaxis, females were presented with either the male whose song they preferred or the alternate male. When females selected males on the basis of their phonotaxis preference, they showed significant assortative mate preference in intraspecific trials and complete assortative mate preference in interspecific trials. Females usually completed a 4-7 hour courtship culminating in

insemination by the male with whom they were presented, whether it was the male they selected on the basis of song or the male that they did not select. The number of offspring produced did not differ among the different groups, although the rate of failure to produce any offspring was higher in the heterospecific pairs. These results support the hypothesis that divergent phonotaxis preference contributes to the sexual isolation observed among populations and species of *Laupala*.

INTRODUCTION

Sexual selection is invoked to explain the evolution of a wide variety of phenotypes associated with mate choice throughout the animal kingdom and beyond (Darwin 1871; Andersson 1994; Andersson and Simmons 2006); however, its role in generating reproductive isolation is not fully understood (Ritchie 2007). Because sexual selection directly affects traits involved in sexual communication and mate recognition, population divergence in sexually selected traits and preferences has the potential to generate sexual isolation through assortative mate choice (Questiau 1999; Panhuis et al. 2001). This may occur when sexual selection within populations results in the strengthening of a correlation between preference and trait (Lande 1981, 1982), or when preferences have diverged among populations (Boughman 2002). Either of these processes would contribute to a reduction in gene flow among populations, since assortative mating would cause migrants to be less successful in obtaining mates from populations with divergent sexual signaling. Sexual selection, ultimately, is a process that occurs within populations, so any consideration of its potential to

maintain population divergence must consider both the action of sexual selection within populations and the consequences of sexual selection across populations (Boughman 2001). Thus, it is imperative to address this question in populations that are still in the process of divergence, rather than those in which speciation is already complete.

In order to demonstrate a causative role of sexual selection on species divergence, several requirements must be met (Panhuis et al. 2001). First, the trait (or traits) that differs among species should be sexually selected, and females must mate assortatively due to variation in trait preference. Assortative mating will establish a genetic correlation between trait and preference (Lande 1981), making interbreeding and gene flow increasingly unlikely as populations continue to diverge. Second, for a response to selection and evolutionary divergence to occur, the trait must have a genetic basis. The hypothesis that sexual selection is the primary driver of speciation would be bolstered by documenting that the traits involved in population divergence are the same as those underlying species differences (Boake 2002). Finally, little or no divergence in traits unrelated to mate choice provide ancillary evidence of the role of sexual selection, as divergence in other characters may suggest that other processes, such as natural selection, are the cause of divergence (Schluter 2001, 2009). For example, an absence of postzygotic reproductive isolation would rule out the process of reinforcement (Marshall et al. 2002; Servedio and Noor 2003).

Studies of sexual selection often rely on the assumption that female preference for a trait predicts mate choice, but this assumption is not always subjected to rigorous analysis (Walling et al. 2010). Particularly in phonotaxis trials, it is rarely

tested whether positive phonotaxis predicts mating success. In *Laupala*, this assumption is not trivial, because a single mating bout will take several hours to complete, and sperm is transferred only at the end of this extended courtship (Shaw and Khine 2004; deCarvalho and Shaw 2005, 2010). Thus, there are many opportunities for additional factors to influence mating decisions beyond phonotaxis behavior. This would be true for all courtships involving long-range attraction (Leonard and Hedrick 2010), but particularly when the duration of courtship - following initial attraction but prior to insemination - is so prolonged. This makes it particularly important to test the underlying assumption that a phonotaxis preference is indicative of a mate choice preference.

Several recent studies have begun to explicitly test the hypothesis that sexual selection drives speciation (Ritchie 2007); however, most of these studies do not meet the necessary standards to demonstrate an exclusive role of sexual selection in driving divergence. While collectively they make a compelling argument that sexual selection likely contributes to population divergence and reproductive isolation, individual studies rarely meet all the conditions necessary to demonstrate conclusively that sexual selection is the driving force of speciation (Panhuis et al. 2001). One notable exception are studies involving Amazonian frogs from the genus *Physalaemus* (Prohl et al. 2006; Boul et al. 2007; Ryan et al. 2007; Guerra and Ron 2008); however, even in these cases, more recent evidence indicates more ecological divergence than previously thought (Prohl et al. 2010). It is worth noting that very few closely related species groups differ only in sexually selected traits, and many examples of sexual selection driving speciation also involve ecological adaptations (Endler and Houde

1995; Boughman et al. 2005; Kronforst et al. 2006; Cocroft et al. 2010). This is likely because once speciation by sexual selection has occurred, the newly formed, reproductively isolated species will acquire new adaptations post-speciation that obscure the signature of sexual selection.

Speciation in the Hawaiian cricket genus *Laupala* may have occurred by the action of divergent sexual selection. The 38 described species are morphologically and ecologically cryptic, and are primarily differentiated using the pulse rate of male calling song (Otte 1994; Shaw 2000a; Mendelson and Shaw 2005), a sexually selected trait (Shaw 2000b; Shaw and Herlihy 2000; Mendelson and Shaw 2002). Previous studies of *L. cerasina*, a species showing intraspecific variability in pulse rate, showed significant differentiation in pulse rate and pulse rate preference (Chapter 1). Additional studies of phonotactic behavior in several populations of *L. cerasina* document that females from neighboring populations with slightly, but significantly differentiated songs are capable of distinguishing these small, interpopulational differences in pulse rate (Chapter 2), and furthermore, show an acoustic preference for pulse rates characteristic of their own population (Chapter 1; Chapter 2). Here, we investigate the hypothesis that females of *L. cerasina* from acoustically distinct populations mate assortatively based on their phonotactic preferences. If sexual selection on song maintains or accelerates population divergence, then female phonotactic preference across variant populations should be assortative. If phonotactic behavior is predictive of mate choice, then following phonotaxis, female mating success should be high. Song acts as a long-distance signal and may have less influence on mate choice at close range (Mendelson and Shaw

70

2006), even though males use the same calling song during both long-distance and close-range courtship. If close-range signals have also diverged among populations or species, such as cuticular hydrocarbons (Mullen et al. 2007; Mullen et al. 2008), phonotactic behavior may not be predictive of mate choice once potential partners are in physical contact. In addition, postzygotic isolation among populations or species would indicate a need for further investigation of other potential divergence processes in *Laupala* speciation. We further provide a comparative context for the analysis of mate choice in *L. cerasina* through similar studies of its sister species, *L. eukolea*, which shows much greater divergence both genetically and phenotypically from *L. cerasina* than populations within *L. cerasina* (Mendelson et al. 2004).

METHODS

Overview

Tests of assortative mating based on phonotaxis preference and staged encounters were performed between neighboring populations of *Laupala cerasina* (intraspecific) and between *L. cerasina* and its allopatric sister species, *L. eukolea* (interspecific). For the intraspecific comparisons, neighboring populations of *L. cerasina* were chosen, since they likely interact through chance migration events. Assortative mating in *L. cerasina* was assessed between two pairs of populations: (1) Kalopa Park (KP) and Laupahoehoe (LP) (distance: 21.3 km), and (2) Glenwood Road (GR) and Naulu Trail (NT) (distance: 12.5 km) (Chapter 2; Figure 1). Each population pair shows significant divergence in female pulse rate preference that is consistent with the divergence in male pulse rate: KP-LP mean pulse rates are 2.42 and 2.70 pulses per

second (pps), respectively, with estimated preferences of 2.33 and 2.69 pps; GR-NT mean pulse rates are 2.68 and 2.48 pps, respectively, with estimated preferences of 2.67 and 2.35 pps (Chapter 2). The same experimental design (see below) was used to quantify sexual isolation between *L. cerasina*, endemic to the Big Island of Hawaii, and the Maui endemic *L. eukolea*, its sister species. For this interspecific comparison, populations of *L. cerasina* from Muliwai Plateau (MP) and Waimea Reservoir (WM) were contrasted with the Ginger Camp (GC) population of *L. eukolea* (Figure 1). These two populations of *L. cerasina* have similar song and preference means: MP and WM mean pulse rates are both 2.60 pps and estimated preferences are 2.43 and 2.54 pps, respectively (Chapter 2). These populations occur 7.5 km apart along the region of the Big Island nearest to Maui, where *L. eukolea* occurs (Figure 1). *L. eukolea* from Ginger Camp sing much faster than *L. cerasina* males, with pulse rate and estimated preference around 3.9-4.0 pps (Oh et al. in prep).

Rearing conditions

Crickets from the six populations of *L. cerasina* and one population of *L. eukolea* were collected in July 2005 and brought to the laboratory at the University of Maryland, College Park. Females collected as adults were housed individually to allow collection of eggs produced through natural inseminations prior to capture. All other individuals were reared to maturity and paired randomly with a mate from the same population to generate offspring. These lab-reared offspring were reared to adulthood and then randomly paired with a mate from the same population but

different family (i.e., no inbreeding) to produce a second generation of laboratory-reared offspring for experimental trials.

All crickets were maintained on a 12:12 light: dark cycle in a temperature-controlled room maintained at 20˚C. Inseminated females were provided with moistened kimwipes as a substrate for oviposition. These kimwipes were collected, transferred to plastic specimen cups lined with additional moistened kimwipes, and monitored for hatching nymphs. Small nymphs were reared in one-quart glass jars with 15-20 individuals per jar, while larger nymphs and adults were housed in plastic specimen cups, no more than three crickets per cup, separated by gender and grouped according to approximate age. All crickets were provided moist kimwipes for water and humidity and fed weekly with cricket chow (Fluker Farms, LA) treated with methyl paraben (Tegosept, Fisher Scientific) to reduce mold. Crickets were checked twice weekly to determine approximate date of adult eclosion, and individuals were considered sexually mature approximately 20-25 days post-eclosion.

Pre-trial song analysis

To generate playback parameters for mating trials (see below), songs were recorded from the experimental males 1-7 days prior to the experiment using a Sony Professional Walkman (Model WM-D6) and Sony condenser microphone. To record a song, an individual male was placed beneath a clear plastic cup with screen top in a temperature-controlled room maintained at 20˚C. Males that did not sing spontaneously were presented with a female from the same population and species until singing commenced (*Laupala* males produce only one type of song, regardless

of social context). Approximately one minute of song was recorded from each male, after which males were returned to an individual cup with fresh water and food. If a female was introduced into the recording cup, she was removed before any mating activity occurred. Song recordings were digitized using Raven 1.2 (Cornell University, Ithaca, NY). For each male, five measurements were made of pulse period, pulse duration, and carrier frequency, using non-consecutive pulses (as in Chapter 1). Pulse period is measured as the time between the beginning of one pulse and the beginning of the next. Pulse duration is measured as the time between the beginning and end of a single pulse (duration of wing closure). Carrier frequency is the dominant frequency of a pulse. All three values were used, since all are known to be variable among males (Shaw and Herlihy 2000; Chapter 1). Only pulse rate has diverged among populations and among species, but pulse duration and carrier frequency differ among males to varying degrees. The average values for each song parameter were determined for each male and used in the experiment as described below. The two males used in each trial were paired randomly while matching for age similarity prior to song analysis, and each male was used only once.

Trial conditions

Song playback*:* Custom software (Tucker-Davis hardware/software system) was used to generate synthetic song that reflected the mean song characteristics of a given experimental male. The intensity of the speakers (Radio Shack model 40-1218) was calibrated to 80 dB on a 4.0 pulse/second, 5 kHz tone using a Bruel and Kjaer digital attenuator (type 4155) approximately once per week throughout the experiment to

ensure speaker equality. Each trial was randomized using a binomial probability generator to determine which male was assigned to each speaker.

Phase one (phonotaxis): Playback experiments were performed in a rectangular phonotaxis arena (dimensions: 3 feet x 1 ft X 1 ft) constructed of a balsa wood frame enclosed on all sides by dark mesh window screening (Figure 2) and located within an Acoustic Systems acoustically buffered sound chamber. Detachable mesh tubes were placed at either end of the arena, adjacent to each speaker. During a trial, the female was placed beneath a cup in the center of the arena. Synthetic songs from one male of each population (two total) were simultaneously broadcast from the two speakers. After a five-minute acclimation period, the cup was raised, allowing full access to the arena. If the female entered the mesh tube adjacent to one of the speakers, we regarded this as her acoustic preference, and she entered the next phase of the experiment. The duration of the phonotaxis period was recorded. If the female did not enter either mesh tube after 15 minutes, the trial was ended, and a second female was tested using the same parameters. However, once a female showed acoustic preference for one of the two songs, that pair of songs (and therefore, those two males) was never used again. Experimental males were kept outside the phonotaxis chamber during this portion of the trial, to prevent acoustical interference with the synthesized playbacks.

Phase two (mate choice): In *Laupala*, courtship lasts for 4-7 hours and consists of the production and transfer of numerous spermatophores throughout the day (Shaw and Khine 2004). Once a female entered the mesh tube adjacent to a broadcasting speaker, that tube was carefully detached from the rest of the arena with

the female inside. The male whose song was chosen, housed in a specimen cup, was introduced at one end of the tube, and the lid of a specimen cup closed the other end of the tube, forming the mating chamber. The male and female were observed for the entire day and the following courtship and mating behaviors were recorded: microspermatophore (micro) production, micro transfer, macrospermatophore (macro) production, macro transfer, and macro attachment duration. Only the macro contains sperm (Shaw and Khine 2004); thus, the successful transfer of a macro is used to define mating success.

Control manipulation: In a subset of trials, the female was presented with the male whose song she did not choose. The male and female were observed and the same behavioral data listed above were collected. This allowed a comparison of the mating behaviors and reproductive output of females who were paired with their preferred male and those who were paired with the non-preferred male.

Post-trial conditions: Following mating, females were kept in isolation and provided with clean, moistened kimwipes as substrate for oviposition. Eggs were collected from all mated females until their natural death. The number of live offspring hatched from each mating was recorded for each female as a measure of fitness. Starting about four weeks following successful mating, egg cups were monitored for hatching nymphs. Every two to three days all nymphs were collected from each egg cup. When at least one month had passed with no new nymphs hatching, the egg cup was discarded. If no nymphs hatched from an egg cup, the cup continued to be monitored for at least three months or until at least four weeks following the death of the female, whichever was longer, and then discarded.

RESULTS

Song analysis

The songs of males used in the choice trials were typical of their populations (Table 1). Additionally, mean male song characters for a given population did not differ between the two female populations being tested in each pairwise set (p>0.05 for all within-population comparisons for pulse rate, pulse duration, and carrier frequency). For each female population tested, paired t-tests showed that the pulse rates from the two contrasted populations (or species) being offered were significantly different (Table 2). Conversely, pulse duration and carrier frequency did not differ significantly between the contrasted populations (or species), with one exception: in trials where *L. eukolea* (GC) females were tested with males from MP (*L. cerasina*) or GC (*L. eukolea*), carrier frequency was significantly higher for MP males (Table 2).

Phonotaxis preference

A total of 162 phonotaxis trials ended with females expressing biased phonotaxis behavior. A global χ^2 test indicated that females showed no preference for one side of the arena over another ($\chi^2_{(df=1)}$=1.58, p=0.2). In intraspecific trials, when populations are considered in pairs, females significantly preferred males from the same population (Fisher's exact test: KP/LP, p=0.0011 (n=50); GR/NT, p=0.0031 (n=40). However, when females from each population are considered separately, only two of the four populations, KP (n=23) and NT (n=20) showed assortative mating preferences, where the 95% confidence interval of the proportion showing assortative

preference excludes 50% (random preference), while the other two populations, LP (n=27) and GR (n=20) did not (Figure 3). In interspecific trials, both *L. cerasina* (n=38) and *L. eukolea* (n=34) females showed complete assortative phonotaxis preference, meaning females always preferred the songs of conspecifics over those of heterospecifics (Figure 3).

In intraspecific trials, there was no significant heterogeneity among female populations in the phonotaxis response time, nor did it differ between homotypic and heterotypic responses. There was also no significant difference in the phonotaxis response time between *L. cerasina* and *L. eukolea* in interspecific trials. However, females responded significantly faster in interspecific trials when compared to intraspecific trials (average duration of phonotaxis: interspecific, 289 seconds; intraspecific, 382 seconds; t=2.9, p=0.0042).

Mating success

Mating success, defined by the successful transfer of a macrospermatophore, was high across all trial types (Figure 4). Across all 90 intraspecific trials, mating success was 82% for homotypic matings (n=67) and 87% for heterotypic matings (n=23). A G-test of heterogeneity indicated no difference in mating success between homotypic and heterotypic pairings, whether populations were considered separately ($\chi^2_{(df=7)}$=2.108, p=0.95) or combined ($\chi^2_{(df=1)}$=0.306, p=0.58). In 38 interspecific trials involving *L. cerasina* females, mating success was 86% for conspecific trials (n=28) and 80% for heterospecific trials (n=10). For the 34 *L. eukolea* females, mating success was 96% for conspecific trials (n=24) and 60% for heterospecific trials

78

(n=10). We did not find evidence of mate discrimination; a G-test of heterogeneity failed to reject the null hypothesis of equal mating success among all four interspecific trial types ($\chi^2_{(df=3)}$=6.811, p=0.078).

Hatching success

Of the 74 intraspecific trials where a male transferred a macrospermatophore, 14 (19%) produced no offspring. A G-test of heterogeneity indicated that these failures were randomly distributed across populations and across homotypic and heterotypic pairings ($\chi^2_{(df=7)}$=5.322, p=0.62). Considering only those matings where offspring were produced, there was no significant difference in offspring production between homotypic (mean=17.9, SD=12.1) and heterotypic (mean=19.8, SD=13.4) trials ($\chi^2_{(df=1)}$=0.1227, p=0.73), or between trials where females received the chosen (mean=21.5, SD=15.1) or the non-chosen (mean=18.0, SD=12.1) male ($\chi^2_{(df=1)}$=0.3957, p=0.53).

Of the 61 interspecific trials where a male transferred a macrospermatophore, 14 (23%) produced no offspring. Of those 14 trials failing to produce offspring, seven (50%) were male-manipulated trials where a female was paired with the male she did not choose (i.e., a heterospecific). A G-test of heterogeneity indicated that failure to produce offspring was significantly higher in heterospecific pairings ($\chi^2_{(df=3)}$=8.156, p=0.0429). Considering only those matings in which offspring were produced, a Kruskal-Wallis test found that the number of offspring produced from heterospecific (mean=28.3, SD=9.7) pairings did not differ from conspecific (mean=39.0, SD=16.2) pairings ($\chi^2_{(df=1)}$=2.90, p=0.0883).

DISCUSSION

If sexual selection can create and maintain population differentiation in traits associated with mate choice, then it has the power to cause reproductive isolation among populations that differ in these traits (West-Eberhard 1983; Panhuis et al. 2001). However, in order to demonstrate that populations are sexually isolated, it must be shown that females mate assortatively on the basis of these divergent, sexually selected traits (Lande 1981; Walling et al. 2010).

While previous research in *Laupala* has indicated that average preference functions have significantly diverged among neighboring populations, this is insufficient to demonstrate that sexual isolation has evolved. The current experiment used a combination of phonotaxis and staged mating encounters to demonstrate that females preferentially respond to songs produced by homotypic males. Furthermore, females exhibiting phonotaxis behavior have a high propensity to mate, as evidenced by the high percentage of successful courtships. While phonotaxis preferences were largely assortative, following phonotaxis, females generally engaged in courtship behavior with the male they were given, regardless of whether it was the same male they had initially selected based on song preference. This suggests that phonotaxis behavior is extremely important in establishing mating pairs in *Laupala*; thus, divergence in this phenotype will likely result in reduced gene flow across the divergent types.

Consistent with the hypothesis that sexual selection on song within populations may cause sexual isolation among divergent populations, female phonotaxis preference favored homotypic males in both intraspecific trials, where the

overall pattern was significantly assortative, and in interspecific trials, where preference was completely assortative. In intraspecific trials, females from two of the four populations deviated significantly from random choice; and when pairs were analyzed jointly, phonotaxis preference was significantly assortative. These results are consistent with the pattern expected under a model of sexual selection driving sexual isolation, since population divergence occurs in the same trait (pulse rate of male song) as species divergence. Also consistent with this model, the degree of sexual isolation is stronger between species than within species, where between-species trait divergence almost an order of magnitude greater than within-species divergence.

Populations of *L. cerasina* used in this experiment inhabit nearby locations, making their interaction more probable. This experiment used songs based on measurements made from individual males, which provides a more realistic assessment of the likelihood of assortative mating in their natural habitat. The average song of a population does not provide a complete picture of the full range of variation, and in fact, the population pairs being contrasted showed a small degree of overlap in pulse rate measurements, with the fastest males from the slower population KP (NT) overlapping with the slowest males from the faster population LP (GR). Likewise, individual variation in pulse duration and carrier frequency was also incorporated into the experimental design, allowing the relative roles of these characters in determining mate choice to be evaluated. The use of multiple cues in mate assessment has been observed in several other systems (Candolin 2003; van Doorn and Weissing 2004; Leonard and Hedrick 2009), and it has been demonstrated

in *L. cerasina* that females have acoustic preferences for long pulse duration and intermediate carrier frequency (Shaw and Herlihy 2000); however the interaction of multiple variable acoustic characters had been previously unknown. In KP and NT (the two slower populations), female phonotaxis preference departed significantly from random choice, with females favoring songs with the slower pulse rate, which corresponded to the homotypic male. However, in LP and GR (the two faster populations), the binomial confidence intervals for preference based on pulse rate did not exclude random choice (50%). In these cases, the proportion of females choosing males with the longer pulse duration in a given trial did deviate significantly from random choice, indicating that on balance, while females still more often chose the homotypic males, they also apparently also favored males whose songs had longer pulse durations. For LP females choosing KP males, in 8 out of 11 trials the KP males had longer pulse durations (8.4 ms longer on average); for GR females choosing NT males, in 4 out of 7 trials the NT males had longer pulse durations (4.75 ms longer on average). Additionally, for 10 out of 16 LP/LP trials and 12 out of 13 GR/GR trials, the females choosing homotypic males were also choosing the male with the longer pulse duration. This apparent preference for longer pulse duration was not seen in KP or NT females. However, since the components of song are not independent, it is difficult to conclude that pulse duration is more important than pulse rate in determining mate choice in these groups, and further work is needed to disentangle the multiple signals that females may receive from any given song.

Mating success was high throughout the experiment, demonstrating that females that express phonotaxis behavior are likely to engage in courtship and

copulation with the male they encounter. Given that the duration of courtship in *Laupala* is so prolonged, and that there are many opportunities for mutual mate assessment following initial contact but prior to sperm transfer, it is somewhat surprising that the initial female phonotaxis response resulted in such high rates of mating success. In control trials, where the male given to the female was the opposite male than the one she chose, no significant difference in mating success was observed, indicating that additional assessments made during the course of courtship had relatively little impact on the final outcome of the encounter. Even in interspecific trials, where all control trials were heterospecific since females never preferred heterospecific male song, females typically completed courtship and accepted a macrospermatophore from the heterospecific male. While this indicates that females are extremely selective when it comes to song preferences, it also indicates that once a female has selected a male based on song, she is unlikely to deviate from her initial choice. This suggests that female acoustic preferences are extremely important in determining male mating success, indicating sexual selection may be likely to influence song evolution through differential male mating success.

In intraspecific crosses, there were no significant differences in offspring production among any group. This indicates that females are just as likely to produce offspring when they are mated with the preferred male or another male, and when they are mated with a homotypic or heterotypic male. There is no indication of any kind of postzygotic incompatibility among populations; however, offspring fertility was not assessed. In interspecific trials, a greater number of the heterotypic crosses failed to produce offspring compared to conspecific crosses. However, these results

are confounded by the fact that the female was both being paired with the non-preferred male and the heterotypic male. Of the heterospecific pairs that did produce offspring, the numbers were similar to those produced in conspecific crosses. This suggests that the failure to produce offspring in a high proportion of trials is likely not due to postzygotic reproductive incompatibility, which would be expected to reduce the number of hybrids in all heterospecific crosses. While a failure rate of 50% is rather high, the sample size of 14 heterospecific crosses is still too low to make definitive conclusions about postzygotic isolation in this species pair, and the reasons for failure to produce offspring (fertilization failure, oviposition failure, egg inviability, etc.) are unknown.

The process of speciation is likely ongoing in *L. cerasina*. Populations that have diverged in the same character (pulse rate) that is divergent among species show assortative mating on the basis of this divergence. This suggests that population divergence of female acoustic preference (and correlated divergence in male song) may reduce gene flow among neighboring populations through sexual isolation. These patterns indicate that population divergence in song and preference may initiate processes that lead to reproductive isolation through assortative mating, and that the initial barriers to reproduction are likely behavioral.

TABLES

Table 1. Average song values (and standard errors) for males used in phonotaxis trials. Pulse rate in pulses per second (pps), pulse duration in milliseconds (ms), and carrier frequency in Hertz (Hz) are averaged across all female trials for each male population. The "c" denotes *L. cerasina* populations and the "e" denotes *L. eukolea*. For each male population, there was no difference between the songs given to the homotypic vs. heterotypic female population of each pair (p>0.05 all comparisons).

Male population/sp	Pulse Rate (pps)	Pulse Duration (ms)	Carrier Frequency (Hz)
Kalopa Park/c	2.391 (0.014)	41.6 (0.70)	4811 (19.9)
Laupahoehoe/c	2.620 (0.010)	40.9 (0.79)	4764 (16.0)
Naulu/c	2.429 (0.011)	38.2 (0.78)	4856 (23.1)
Glenwood/c	2.612 (0.013)	40.3 (0.95)	4816 (24.6)
Muliwai/c	2.597 (0.011)	40.3 (0.93)	4809 (27.2)
Ginger Camp/e	3.931 (0.021)	39.6 (0.72)	4707 (29.4)
Waimea/c	2.460 (0.013)	40.8 (0.78)	4744 (23.5)
Ginger Camp/e	3.955 (0.019)	39.9 (0.75)	4715 (28.9)

Table 2. Comparison of mean male pulse rate (PR), pulse duration (PD), and carrier frequency (CF) between contrasted populations/species. Populations designated "c" are members of *L. cerasina*, while those designated "e" are *L. eukolea*. All contrasted populations were significantly differentiated in pulse rate, but the other song characters rarely showed significant differences between populations.

Female pop/sp	Comparison	PR difference	PD difference	CF difference
Kalopa Park/c	KP vs LP	***	NS	NS
Laupahoehoe/c	KP vs LP	***	NS	NS
Naulu Trail/c	NT vs GR	***	NS	NS
Glenwood Road/c	NT vs GR	***	NS	NS
Muliwai Plateau/c	MP vs GC	***	NS	NS
Ginger Camp/e	MP vs GC	***	NS	*
Waimea Reservoir/c	WM vs GC	***	NS	NS
Ginger Camp/e	WM vs GC	***	NS	NS

$*p<0.01$
$**p<0.001$
$***p<0.0001$
NS: $p>0.05$

FIGURE LEGENDS

Figure 1. Map of Hawaii, showing the populations used for intraspecific and interspecific mate choice trials. All Big Island populations are *L. cerasina*, and the Maui population (Ginger Camp) is *L. eukolea*.

Figure 2. Phonotaxis arena. The mesh tubes on either end of the linear arena were removable, and could be detached from the rest of the setup. Once a female entered one of the tubes, she proceeded to the mating phase of the trial.

Figure 3. Female phonotaxis preference. Error bars indicate the 95% confidence intervals for the proportion based on the binomial distribution. The light gray bars indicate preference for the homotypic male, and the dark gray indicates preference for the heterotypic male. a) Intraspecific trials of *L. cerasina*, with KP-LP comparison on the left and GR-NT comparison on the right. b) Interspecific trials between *L. cerasina* and *L. eukolea*, with MP-GC comparison on the left and WM-GC comparison on the right.

Figure 4. Mating success. The proportion of trials ending in a macrospermatophore transfer is indicated for each population. The light gray bar indicates homotypic pairings, while the dark gray bar shows heterotypic pairings. a) Intraspecific trials of *L. cerasina*. The proportion of successful matings are contrasted between KP and LP populations and between GR and NT populations, plus a final column provides a

summary across all populations b) Interspecific trials between *L. cerasina* and *L. eukolea*. The proportion of successful matings from MP and WM populations of *L. cerasina* are each contrasted with the GC population of *L. eukolea*.
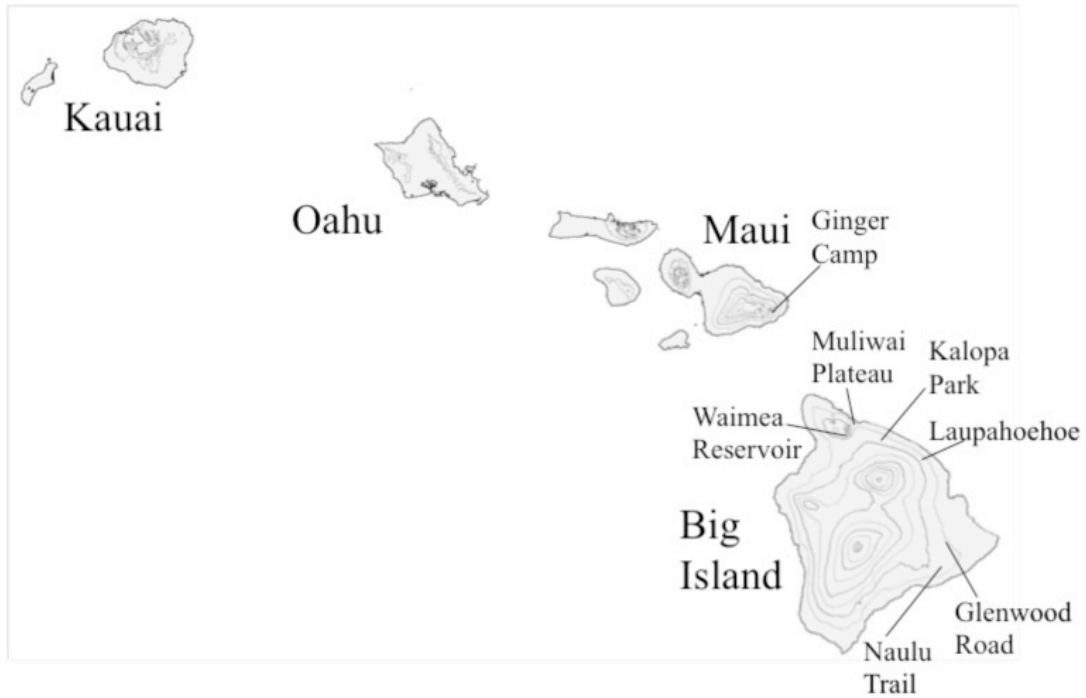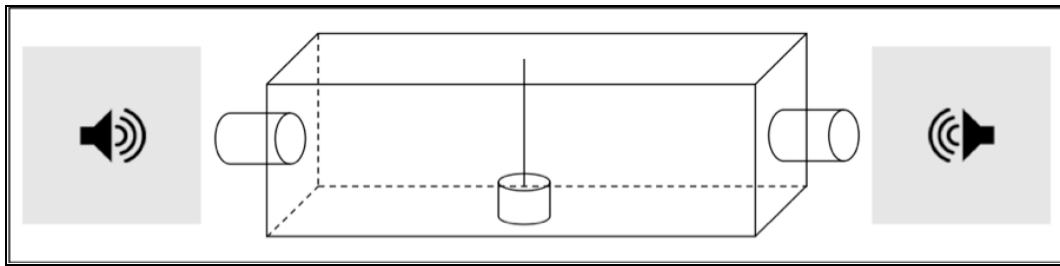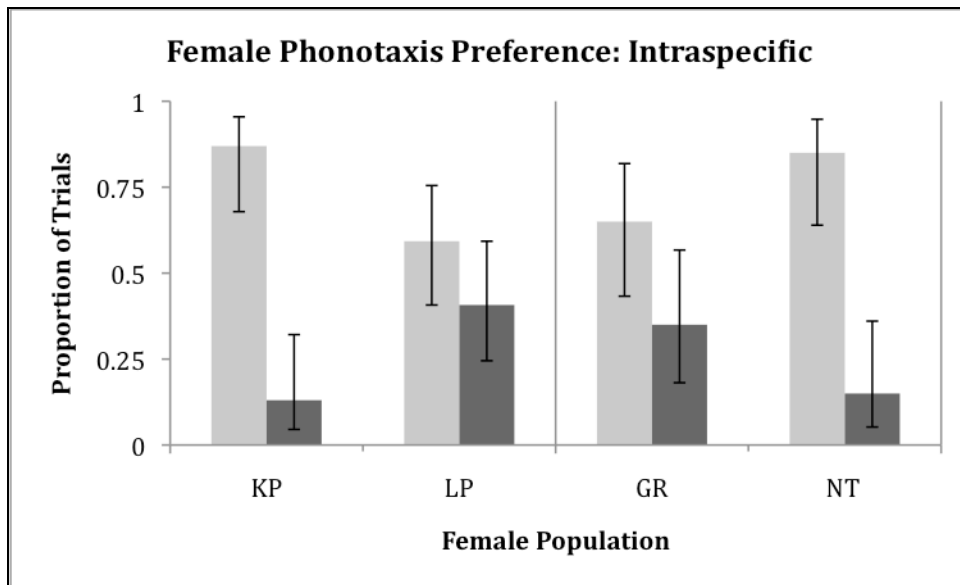
FIGURES

Figure 1.

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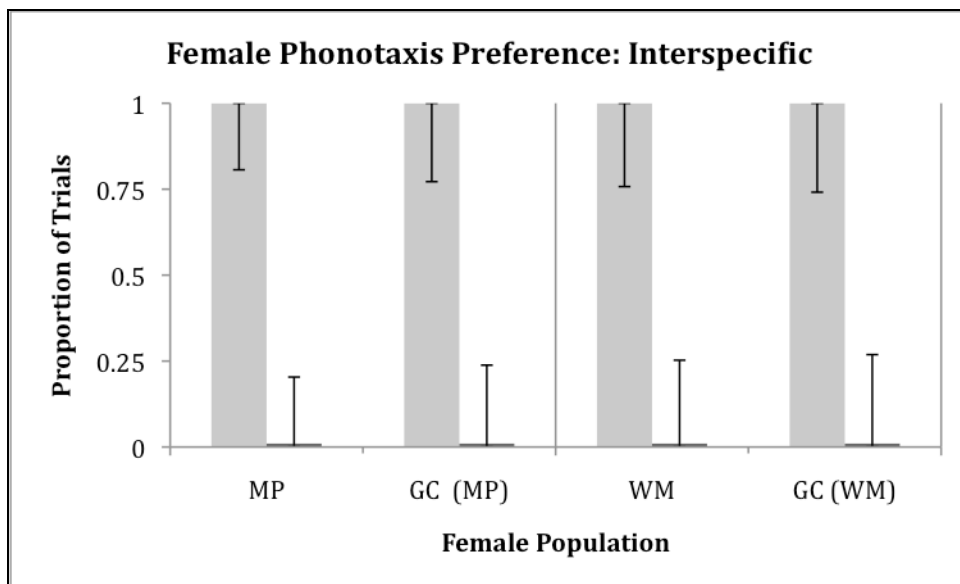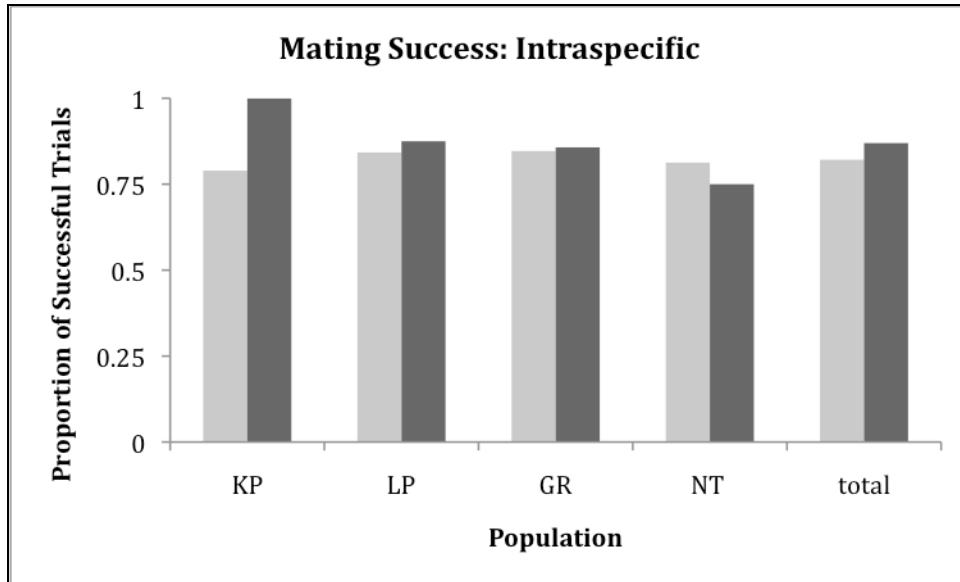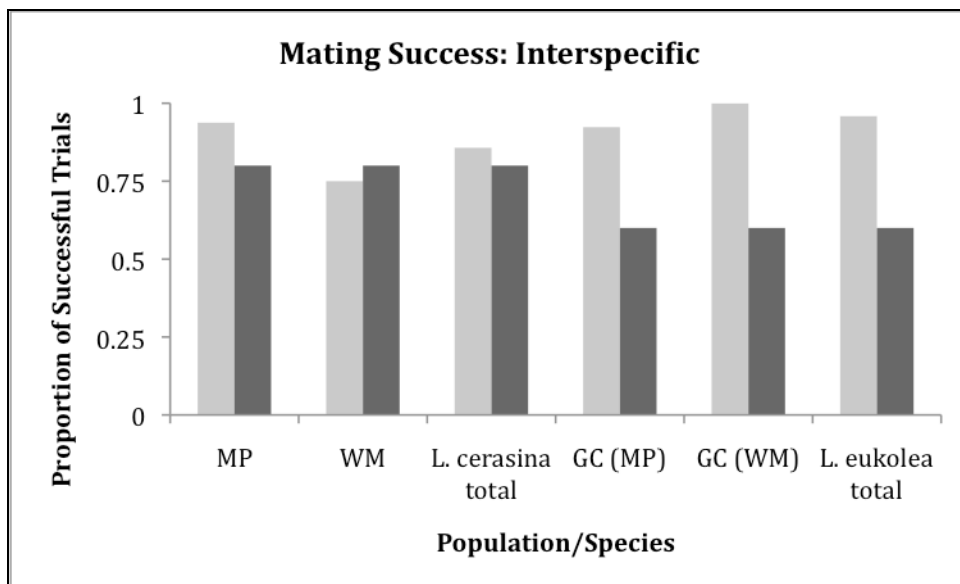
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