CHEMICAL COMMUNICATION AND SPECIATION IN HAWAIIAN CRICKETS

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The main objective of this doctoral dissertation is to explore the role chemical communication may play in the diversification of the Hawaiian swordtail crickets (genus *Laupala*). *Laupala* are known for their diversification in male acoustic signaling and associated female preferences. They also possess a complex courtship which includes extensive antennal interaction and during which the male gives nuptial gifts. Despite historically being seen as indiscriminate, males are predicted to make mating decisions under certain circumstances, such as when males are limited in the resources they are able to invest in females. Given that females are silent, males are likely using some other signaling modality, such as through contact pheromones on the antennae. Here, I tested the male use of chemical cues in initiating mating decisions, the impact of these cues on species boundaries, and how pheromones may be evolving in relation to song. Using both gas chromatographic analysis and novel behavioral assays, I examined the hypothesis that male *L. pruna* are using chemical signals to distinguish between males and females. I found that males and females differed quantitatively in their expression of shared peaks. Further, access to the antennae alone was sufficient cause aggressive behavior or courtship behavior towards males and females, respectively. I tested the hypothesis that males are using chemical information to distinguish between species. I used *L. pruna* and a closely related species, *L. kohalensis*, and found low interspecific mating success. Chemical analysis also determined that these species differed in their pheromone profiles. Males initiated

courtship in the presence of conspecific, but not heterospecific female antennae. I explored the hypothesis that male song and CHC expression are evolving together. I found significant heterogeneity among populations in both signals. The distribution of these signals follows the nonlinear ages of the volcano, versus a simple isolation-by-distance model. Together, these experiments establish chemical communication in male mate choice, demonstrate that differences in chemical expression matter for species boundaries, and suggest that acoustic and chemical signals may be coevolving early in the speciation of *Laupala*.

BIOGRAPHICAL SKETCH

Glenn Francis Stamps was born August 19, 1985 to Wayne and Joan Stamps in Royal Oak, MI. He was the middle child between his older brother, David, and his younger sister Holly. Shy, but intelligent, Glenn always enjoyed being out in nature and exploring, especially when the family would go on their yearly vacations. He would often wander ahead of the family to "scout" and would enjoy the flora and fauna he would find along the way. One particularly memorable summer vacation was when they spent a week in the Florida Keys. The dock near the cabin where they were staying allowed Glenn to view a wide variety of aquatic wildlife including nerve sharks, barracudas, and clown fish. Most memorably he was able to catch a puffer fish and a baby stingray in a hand net to observe them further. The pinnacle of the experience was having a wild manatee swim by as he explored the water.

At Shrine Catholic High School, Glenn developed an interest in music and in musical theater in particular. He performed in the performing vocal group Goliards as well the musicals *Into the Woods*, *Godspell*, *Anything Goes*, and *Bye Bye Birdie*, and *Oklahoma!*. In addition, he assisted with the theater program at the Academy of the Sacred Heart in Bloomfield Hills, MI and appeared in *Pride and Prejudice* as Mr. Darcy and *Arsenic and Old Lace* as Mortimer. His success in the performing arts, but mediocre success in chemistry and physics deluded him into thinking the sciences were not for him.

Glenn then did his undergraduate education at Central Michigan University in Mount Pleasant, MI. He pursued theater and then vocal music education initially, but quickly discovered that this was not the path for him. His love for biology was reawaken, specifically in the form of herpetology and animal behavior in Dr. James Gillingham's classes. He then joined the Applied Technology in Conservation Genetics lab of Dr. Bradley Swanson where he pursued polyploidy and its effects on

developmental mortality in mole salamanders (*Ambystoma* sp.) as his Honors thesis. Glenn also developed an interest in mate choice and species boundaries. As a senior, Glenn also took Dr. Swanson's Conservation Genetics course which he received a failing grade for. This caused Glenn to cry his eyes out, as he was applying to graduate school at the time. In reality, there had been a mistake and Glenn actually earned an "A" in the course. There was much rejoicing.

Glenn had more success in the sciences as an undergraduate than in high school, including in chemistry and physics. In fact, he was so taken with the logic and order of his organic chemistry courses that he momentarily considered pursuing chemistry in graduate school. Momentarily. It would, however, be a foreshadowing of Glenn's trajectory in graduate school. Glenn was accepted to Cornell University as an advisee of Prof. Kerry Shaw. He quickly centered on chemical communication and its role in mate choice and diversification.

During this time, Glenn also began dating his future wife, Jennifer Pizzie. She and Glenn both shared a love of animals and nature. They were married January 1, 2010 in Royal Oak MI. They made the trek through the snow back to Ithaca, NY to start their new life together. They bought a house in Trumansburg, NY in July 2011, shortly before Hurricane Irene would flood their basement. They then had their first child, Alyssa Thérèse Stamps on December 18, 2012. They enjoyed exposing Alyssa to nature and animals, to the point where Alyssa (five years old at the time of this writing) became the self-proclaimed "Queen and Protector of Nature."

This dissertation is dedicated to my amazing wife, Jennifer, my daughter, Alyssa, and any future children we may be blessed with. It would have been impossible without them.

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Like my time in theater, this doctoral dissertation has been a production. While the final performance has my dissertation and me at center stage, it would have been impossible without the blood, sweat and tears, supporting cast, and production team. The first thank you goes to Kerry Shaw for taking a chance on me and letting me join her lab. I am grateful for her wisdom, support, and healthy skepticism that have shaped me into the scientist I have become. I am also grateful for her stellar record of choosing lab members who have been essential to my development as well. Thank yous go out to the post docs Chris Wiley, Chris Ellison, Kevin Oh, Nick Ratterman, Mingzi Xu, and Thomas Blankers and the graduate students Dan Fergus, Biz Turnell, Jon Lambert, Qionghua Gao, and Hayden Waller. Their ideas and support were crucial to the progression of my ideas. Next, I thank Robert Raguso for his service on my committee, inspiration, effusive passion about chemical ecology, and hard work to get a GC-FID and then a new one when the old one crapped out. Thanks also to members of his lab, especially Geoffrey Broadhead and Kata Boroczky for their help in training me and troubleshooting the chemical analyses. Thank you to Tom Seeley for his service on my committee, especially for the wisdom, experience, and grounding in natural history he brought that have helped me to think about a cricket's sensory world better. I would also like to thank the other graduate students who participated in "Lunch Bunch" and "Research Jams" as well as Francoise Vermeylen and Erika Mudrak from the Cornell Statistical counseling Unit. Big thank yous also go out to the Department of Neurobiology and Behavior, Cornell Sigma Xi, Cornell Graduate School, and the National Sigma Xi for funding two expeditions to Hawaii and allowing me to buy the equipment I needed to do behavioral trials. I am also grateful for the many semesters of teaching experience I have gained over the years, which have helped me to be a better teacher, student, and public speaker. I think my time

trying to be creative in the classroom have definitely affected the breadth of my thinking and helped me to escape my myopic world and to see how amazing and interconnected all of biology is. Lastly, and most importantly, I am eternally grateful to family and friends for their unwavering support. Brad Swanson told me as I prepared to leave for graduate school, “Ninety-five percent of a PhD is determination and the other 5% is luck.” Having a good support network helped me keep my determination, especially when things were darkest. Thank you to my parents, Wayne and Joan Stamps, and my siblings, David and Holly, for helping to foster the seeds of passion for biology within me and to continue doing so, even when I didn’t see them growing. Many thanks also to my “extended family”, Rob, Annette, Sarah, and Catherine Pizzie who have been patient as they waited for me to grow up and start taking care of their daughter! Thank you to other forms of support, such as Leigh Ann Smith-Gary, Patrick Cayouette, and Tony Bordenkircher. Finally, my eternal gratitude to Jenny and Alyssa for keeping me grounded and reminding me that my life and my worth are more than my dissertation. I wouldn’t be here without you. I love you more that words could ever express.

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CHAPTER 1

MALE USE OF CHEMICAL SIGNALS IN SEX DISCRIMINATION OF *LAUPALA PRUNA*

INTRODUCTION

Much of the mate choice and mate recognition literature has focused on signal perception from the female perspective (Cummings 2015, Kelly 2018, Morina 2018). However, under certain conditions males are expected to make mating investment decisions as well, such as when males contribute more resources towards offspring than females, females vary in their fecundity, there is high sperm competition, or there females show variability in quality (Trivers 1972, Bonduriansky 2001). Part of understanding male mating decisions therefore requires determining what information males might be using to make these decisions. This information may activate courtship and suppress aggressive behavior (Rence and Loher 1977, Sakura and Aonuma 2013, Sakai et al. 2017), coordinate the timing of courtship initiation (Connolly et al. 1969, Wood and Ringo 1980, Markow 1981, Otte 1992, Clowney et al. 2015), or provide a basis for how much a male should invest in a given mating event (Gage and Barnard 1996, Gage 1998, Simmons et al. 2007, Thomas and Simmons 2009, Crean et al. 2016).

Crickets have been studied extensively for male use of acoustic signals (Snedden and Sakaluk 1992, Desutter-Grandcolas 2003, Bailey et al. 2017), and female preferences for male song (Simmons 1986, Brown et al. 1996, Shaw and Herlihy 2000, Grace and Shaw 2011, Orci et al. 2017). In addition, crickets have also served

as models for male choosiness and sexual role reversal, particularly in Mormon crickets (*Anabrus simplex*) (Gwynne 1981, Smith et al 2017). However, as females do not sing, information about sexual identity, receptivity, or mate quality is not available to males via acoustic means. One alternative mode of communication that may provide males with this information is through chemical signaling, specifically with low volatility cuticular hydrocarbons (CHCs) (Whitman 1990, Thomas and Simmons 2009, Blomquist and Bagneres 2010, Steiger et al 2013, Rapkin et al. 2017). CHCs have been found to be important in the social interactions of eusocial insects, such as determining reproductive status (Monnin 2006), and facilitating colony functioning (Richard and Hunt 2013).

Distinct CHC variation has been found to correspond with species boundaries within crickets (Mullen et al. 2007, Moran 2017) and other insects (Kather and Martin 2012, Guillem et al. 2016) as well as impact mating decisions affecting sexual isolation (Zhang et al. 2014, Shahandeh et al. 2018). Moreover, many insect species have been shown to use CHCs in mating decisions (Howard and Blomquist 2005, Blomquist and Bagneres 2010, Guillem et al. 2016). For example, removal and reapplication of all or some of the CHCs affected male courtship initiation in rustic borers (Ginzel et al. 2003). In crickets, males have been shown to exert selective pressure on female CHC composition (Thomas and Simmons 2010) and may influence male mate choice (Maroja et al. 2014). Males use information from female CHCs to alter their nuptial investment based on perceived sperm competition (Thomas and Simmons 2007, 2009, Assis et al. 2017). Males will also alter their CHC profiles shortly after losing fights to other males to designate subordinate status (Thomas and

Simmons 2011, Loranger and Bertram 2016). Further, a gene-by-environment study of CHC composition in *Gryllodes sigillatus* found that environmental effects on female CHCs is independent of genotype, while male CHCs varied in quantity and content (Weddle et al. 2012). Weddle et al. (2012) suggest that female CHCs might be consistent to express identity while males show greater variability and condition dependence. While we are beginning to understand the potential information that CHCs may contain, it is still unclear whether their early divergence plays a role in the diversification of species.

Crickets of the genus *Laupala* are endemic to the Hawaiian islands and are known for their rapid diversification in male song and associated female preferences despite being morphologically and ecologically cryptic species (Mendelson and Shaw 2005, Grace and Shaw 2011, Wiley et al. 2011). Like most crickets, males sing via wing stridulation and females exhibit phonotaxis toward males until antennal contact is made. After antennal contact occurs, a complex interaction unfolds that includes pumping, additional singing, and the production and transfer of a series of spermless microspermatophores (micros) by the male to the female over four to six hours before transfer of the sperm-containing macrospermatophore (macro) (Shaw and Khine 2004, Fig. 1.1). Thus, courtship presents at a minimum the cost of time and energy for courting males, including the cost of producing multiple spermatophores. Males who produced micros one day were found to be less likely to initiate courtship or produced fewer micros the following day, suggesting that courtship and the production micros may indeed be costly (Jadin PhD thesis), supporting the idea of costliness of courtship and protracted spermatophore production.

Several indirect lines of evidence suggest *Laupala* may be using CHCs as part of courtship and mating decisions. First, there are extended periods of antennal interaction during courtship (Shaw and Lugo 2001, Shaw and Khine 2004, Fig. 1.1). Cuticular hydrocarbons have low volatility and their perception requires close to direct contact, and the extensive, close physical contact during *Laupala* courtship provides the behavioral context for CHCs to be assessed. Second, while females may identify males by song, *Laupala* females do not sing. Thus, males might rely on chemical signals or cues to gain information about sex identity. Of course, visual and tactile information may also be present, although crickets are not known for use of visual signals during courtship. Yamawaki and Ishibashi (2014) found that house crickets (*Acheta domisticus*) orient their antennae towards approaching objects, presumably to better discriminate the identity of an object through antennal stimulation. Lastly, as noted above, Mullen et al. (2007) demonstrated that species of *Laupala* differ both qualitatively and quantitatively in their CHC profiles. Moreover, Mullen et al. (2007) provided preliminary evidence for sexual dimorphism in CHC composition. While these studies suggest that *Laupala* use chemical communication in mating decisions, this hypothesis has not been formally tested with behavioral assays.

Here, I tested whether chemical communication is used in sex recognition by male *L. pruna*. I hypothesized that chemical information is being used to determine sexual identity and predicted that CHCs should be differentially expressed by males and females within a species. Further, I predicted differential composition should be detectable by males and thereby evoke differential behavioral responses to males versus females. I tested this hypothesis by 1) quantifying CHC composition in males

and females using gas chromatography, 2) using behavioral assays to measure courtship and aggressive responses of focal males to the antennae of other males or females, and 3) observing behavioral responses of focal males to filter paper exposed to living males or females.

MATERIALS AND METHODS

Rearing

I used a combination of offspring of *L. pruna* collected from the Big Island of Hawaii at Alili Springs (19° 13' 33.29" N, 155° 31' 19.49" W) in June 2014 and wild caught individuals collected August 2016. Nymphs were raised in 1 qt glass jars with moist Kimwipe (Kimberly Clark Kimtech Science, Irving, Texas, USA) and Fluker's Hi Calcium Cricket (Fluker's Cricket Farm, Port Allen, LA, USA) diet twice a week. F1 nymphs were reared collectively in 1qt jars and larger nymphs (~5 months post hatching) were moved to same-sex specimen cups with no more than 3 individuals per cup and fed Purina organic cat chow (Nestle Purina Petcare, St. Louis, MO, USA) twice per week. Upon maturation, crickets were moved to individual specimen cups with a moist Kimwipe. All individuals were kept in a temperature controlled room set to 20 ±1˚C and a 12:12h light/dark cycle. All focal and treatment individuals were between 30 and 45 days post final molt.

Cuticular hydrocarbon preparation

Mullen et al (2008) found that CHCs expressed in *Laupala* ranged from 22 to 33 carbons. Therefore, I dissolved a small amount (0.034g) of nonadecane (C19) and

pentatriacontane (C35) in the extraction hexane and used these peaks as boundaries to determine with peaks to quantify. All glassware and forceps were cleaned with two washes of clean HPLC-grade hexane before use. Wild caught individuals were collected from Alili Springs in 2014 (males n=14, females n=13) for full body analysis. Individuals were anesthetized with CO2 before being submerged in 300 µl of HPLC grade hexane with C19 and C35 for 5 minutes. Vials were lightly swirled at the beginning and end of extraction and individuals were then removed. All samples collected above were blown down under a gentle nitrogen stream and then re-suspended in 60 µl of heptane. Samples were then pipetted into 2ml autosampler vials with 100µl glass inserts.

CHC analysis

A Shimadzu AOC-20i autoinjector was used to inject 1µl samples into a Shimadzu GC-2014 gas chromatograph with flame ionization detection (GC-FID) equipped with an HP-5 capillary GC column (20m, 0.180mm diameter, and a film thickness of 0.18um). Runs consisted of a starting temperature of 60˚C held 1 min, a ramp 20˚C/min to 200˚C. The temperature was then ramped 5˚C/min to 320˚C and held for 15 min, yielding a total run time of 47 min. The FID was set to 340˚C and a sampling rate of 40 msec. The injection port was 300˚C with a pressure of 144.2 kPa. The total flow rate of gasses was 10ml/min with column flow of 0.80 ml/min, and purge flow of 3.0ml/min, yielding a linear velocity of 32.5 cm/sec. The split ratio was calculated to be 7.7 given the other parameters.

Peaks were selected by first using the Shimadzu, Inc. “Labsolution” software’s

autointegrate function set at a slope of 18,000μV/s for full body extracts and 3,000 μV for antennal extracts, respectively. The threshold of autointegration was lower for antennal samples due to the smaller overall sample volume. Using similar parameters for full body and antennal trials led to a loss of available peaks for analysis. Peak retention times shared by at least half of the individuals in each sex (i.e. males and females separately) were selected and missing values were manually integrated. Values were log transformed using the formula $Z_{i,j} = \log(X_{i,j}/g(X_j))$, (Aitchison 1986, Mullen 2007). The high dimensionality of the chromatograms was reduced with a principal components analysis (PCA) carried out in R (R Development Core Team 208). Principal components with eigenvalues greater than 1 were input into a linear discriminant analysis (LDA) to determine if males and females could be assigned to their sex based on their chemical profiles. Similar analyses were performed independently for both whole body extracts and antennal-only extracts.

Antennal trials

To test whether *Laupala* males could distinguish between male and female chemical signals, I first conducted behavioral trials where ("focal") males were given access to antennae of males or females ("treatment"). Access to the antennae alone has been found in other studies on crickets to be sufficient to evoke behavioral responses (Rence and Loher 1977). Thirty minutes before trials, cohorts of three focal males were placed individually into petri dishes (9 cm diameter x 4 cm height) and allowed to acclimate. A small opening (size) was cut into the side of the petri dish to allow exposure of antennae of treatment individuals to the focal male. Immediately before

the start of trials, the treatment individual's antennae (see description below) were inserted, giving the focal male access to the treatment individuals' antennae (Fig. 1.2). Petri dishes were cleaned between trials using a fresh Kimwipe soaked in 70% ethanol.

Treatment males and females were cold anesthetized by placing them in a -20˚C freezer for 3 minutes on the day of trials. If the individual was still partially active after 3 minutes, it was placed in the freezer for an additional minute. Individuals were placed head first into a 0.5 ml eppendorf tube (Eppendorf North America, Hauppauge, NY, USA) with the tip removed. Curved tweezers were used to thread the antennae of the cricket through the hole at the tip. The back of the tube was plugged with Kimwipe to prevent the individuals from backing out. Individuals constrained in these tubes were able to wave their antennae, but were otherwise not exposed to the focal individuals (Fig. 1.2). After 15 minutes to warm up, the treatment individual was introduced into a trial arena and a Canon HD VIXIA HFR600 camera began recording. For each trial, contact time, aggressive and reproductive behaviors were measured (see below under video analysis). Each trial lasted 45 minutes. After trials and while still in the eppendorf tubes, the antennae of treatment individuals were immediately immersed into a 2ml autosampler vial filled with hexane for 5 minutes. Extracts were not available for 6 trials because most or all of the antennae had been bitten off during the trial.

Filter paper trials

Petri dishes (9cm diameter x 2cm depth) were lined with 8 pieces of filter

paper (Whatman 1, 42.5mm diameter, GE Healthcare, UK), four on each surface. Five males or five females of *L. pruna* from Alili Springs were placed in the petri dishes (Fisher Scientific, Waltham, MA, USA) simultaneously and allowed to walk around the dish for 48 hours. A moist Kimwipe was placed along the side of the dish vertically to prevent crickets from desiccating. The petri dish was flipped after 24 hours to control for any differential use of the top or the bottom of the petri dish. This method allows for the passive transfer of cuticular hydrocarbons and likely other chemical cues from the individuals to the filter paper (Borges et al. 2003). After 48 hours, the individuals were removed and returned to cups. Each piece of filter paper was cut in half and one half of a piece of filter paper was used for each trial. Therefore, each iteration of this setup yielded chemical-dosed filter paper sufficient for 16 behavioral trials. Filter paper halves were used in only one trial and were then discarded.

Filter paper trials consisted of placing a focal male in a petri dish with two pieces of filter paper of equal size from different sources, and recording the male for 45 minutes with a Canon HD VIXIA HFR600 camera. Three different filter paper trials were conducted: 1) a blank vs. a filter paper exposed to females; 2) a blank vs. a filter paper exposed to males; and 3) a filter paper exposed to males vs. a filter paper exposed to females. Each focal male was only used once across all experimental trials. These filter paper trials were conducted to test if chemical information is being used by males to initiate courtship independent of other kinds of information they may be detecting from the antennae (e.g. antennal structure or movement patterns). I predicted that males should spend more time investigating and also be more likely to exhibit

courtship behaviors near filter paper with female chemicals than male chemicals or blank filter paper.

Video Analysis

I analyzed 30 minutes of each trial video for behavior displayed (Table 1). In antennal trials, analysis began once the focal male made antennal contact with the treatment individual. Antennal contact was defined by 1) the onset of rapid antennal movement by one or both individuals or 2) conspicuous movement of the treatment individual's antennae (i.e. bending of part of the antenna). I noted 1) the duration (minutes) that the focal individual was in contact with the antennae of the treatment individual (when the antennae first touched until the focal male left a 30mm radius zone around the treatment individual's antennae; Fig. 1.2) the duration of each singing bout; and 3) the total duration of biting by the focal male (min) of the treatment individual's antennae (operationally defined as when the mouthparts of the focal male were over or grabbing the experimental individual's antennae with its mouthparts). I also counted the number of pumps a male performed in a trial and whether or not he produced a micro (no male produced more than one micro during the trial period).

For filter paper trials, analysis began after the male made contact with one side of filter paper. I noted 1) the time in contact with each piece of filter paper (total time when at least one leg was in contact with the paper), 2) the time spent singing on each side, 3) the number of pumps on each side, and 4) rubbing behavior when on each side of the filter petri dish (whereby a male arches his dorsal thorax and rubs it against the substrate; Fig. 1.3). Trials were excluded from analysis if 1) a male did not make

contact with the second piece of filter paper within 30 minutes or 2) a male did not make contact with either piece of filter paper within the first 15 minutes of recording (Table 1).

RESULTS

Whole-Body Extract Analysis

Fourteen wild caught males and 13 wild caught females were extracted and analyzed (Fig. 1.4). Males and females shared 34 peaks, making PCA analysis appropriate. They did not differ in the uncorrected total abundance of CHCs on their bodies for these peaks (males: n=14; 7642972.5 ± 1924346.59μV, females n=13; 6616251.385 ± 2791056.485 μV, Welch two sample t-test, t= -1.105, df=21.131, p=0.282). The principal components analysis reduced the dimensionality of the data into seven canonical variates explaining 81.7% of the variance between males and females. Using all seven PCs, LDA was able to correctly assign all 14 males and 10 of 13 females. A MANOVA test on the first 7 PCs indicate that males and females significantly differ from one another in their CHCs (Wilks λ= 0.3194, F=5.7839, NumDF= 7 DenDF=19, p=1.063×10^{-3}, Fig. 1.5).

Antennal Extract Analysis

Twenty male and 31 female antennal samples were extracted and analyzed (Fig. 1.6). Peak areas were manually integrated and retention times were aligned by sight yielding 49 peaks. Males and females did not differ in the total untransformed total abundance of CHC expressed on their antennae (females= 475142 ± 787685 μV,

males= 455730 ± 1101958 μV, Welch two sample t-test, t = 0.068321, df = 31.428, p-value = 0.946). A PCA resulted in seven canonical variates with an eigenvalue greater than 1, explaining 79.19% of the variation. The LDA correctly identified 25 of 31 females and 10 of 20 males and a MANOVA test on the first seven PCs determined that males and females are marginally different in the CHCs on their antennae (df=1, Wilks λ= 0.81843, approx. F= 2.5514, num df= 4, df=46, p= 0.05, Fig. 1.7).

Antennal Trials

Of the 57 antennal trials conducted, 52 were used for analysis. In the other 5 trials, focal males did not make contact with the antennae of the treatment individual within the first 15 minutes of the trial and were therefore removed from analysis because a 30-minute video sample was not possible. Males spent more time with female than male antennae (female: n=28, 22.61 ± 9.38 min; male: n=24, 18.25 ± 7.85 min; Wilcoxon rank sum test, W = 464, p-value = 0.0190, Fig. 1.8A). Males also spent more time singing after exposure to female antennae than to male antennae (female: n=28, 9.40 ± 10.64 min; male n=24, 4.26 ± 7.37 min; Wilcoxon rank sum test W = 446.5, p-value = 0.0384, Fig. 1.8B). Focal males performed about eight times more pumps across each trial, on average, in the presence of female than male antennae (female: n=28, 30.93 ± 35.89 pumps; male: n=24, 3.71 ± 5.89 pumps; Wilcoxon rank sum test, W = 502, p-value = 0.00191, Fig. 1.8C). Moreover, focal males also spent nearly three times more time biting male antennae versus female antennae (female: n=28, 0.39 ± 0.77 min; male: n=24, 1.10 ± 1.28 min, Wilcoxon rank sum test, W = 168.5, p = W = 209, p-value = 0.0181, Fig. 1.8D). Overall, micro

production was an uncommon occurrence in the antennal trials (9/52 trials). However, males did not differ in their likelihood of producing a microspermatophore in the presence of male versus female antennae (females: 7 of 28 trials, males: 2 of 24 trials, Barnard's Unconditional Test, p=0.068, Fig. 1.8E).

Filter Paper Trials

A total of 119 filter paper trials were conducted, of which 14 were removed because the focal male did not 1) make contact with at least one piece of filter paper before 15 minutes of recording or 2) make contact with both pieces of filter paper within the 30 minutes of data collection. In trials where males interacted with female-exposed versus a blank piece of filter paper, focal males spent more time in contact with the female-exposed than the blank filter paper (n=22, mean with female 63.38%, one sample t-test, t = 2.7368, df = 21, p = 0.0124). Focal males also sang more (n=7, blank: 1.04 ± 2.74 min, female: 10.64 ± 7.10 min, Wilcoxon rank sum test, W = 3, p = 5.22×10^{-3}, Fig. 1.9A) and pumped more near the female-exposed than the blank filter paper (n=6, blank: 4.17 ± 10.21 pumps, female: 22.00 ± 38.73 pumps; Wilcoxon rank sum test, W =5, p-value = 0.037, Fig. 1.9C). However, focal males did not preferentially rub their dorsal thorax on or near either filter paper (n=9, blank: 1.33 ± 1.12 rubs, female: 0.89 ± 0.60 rubs, Wilcoxon rank sum test, W=45, p= 0.46, Fig. 1.9B).

Given a choice between filter paper exposed to males and blank filter paper, males showed no difference in contact time (n=43, mean with male filter paper=55.48%, one sample t-test, t = -1.7322, df = 42, p = 0.091). They also did not

differ on which side they sang, although the number of trials in which males sang was very small (n=3, blank: 0.717 ± 1.24 min, male: 1.13 ± 0.96 min, Wilcoxon rank sign test, W= 2, p = 0.3758). Males also did not pump more on one side versus the other (n=27 males, blank: 2.33 ± 2.51 pumps, male: 3.59 ± 3.054 pumps, Wilcoxon rank sign test, W = 273, p = 0.1107) or rubbed by (n=16 males, blank: 4.19 ± 5.15 rubs, male: 4.06 ± 3.42 rubs, Wilcoxon rank sign test, W=120, p= 0.7749).

When focal males were given a choice between male or female pheromones, males did not preferentially spend more time with either paper (n= 43, mean with female 45.41%, one sample t-test, t = -1.6082, df = 39, p = 0.116). Focal males did spend more time singing near male-exposed filter paper (n=19, male: 7.11 ± 6.13 min, female: 2.70 ± 3.58 min, Wilcoxon rank sign test V= 9, p-value = 0.03223, Fig. 1.10), but did not differ in their amount of pumping (n=11, male: 15.91 ± 17.63 pumps, female: 16.45 ± 23.05 pumps, Wilcoxon rank sign test with continuity correction, V=25, p = 0.5045) or rubbing behavior (n=9, male: 1.22 ± 1.92 rubs, female 2.00 ± 2.83 rubs, Wilcoxon sign rank test with continuity correction, V = 8, p = 0.99).

DISCUSSION

Chemical signals are a ubiquitous source of information that many organisms use to make decisions impacting fitness. Male crickets produce acoustic information that females and other males can use to locate and assess a potential mate or rival. Males, however, receive no such information from females. It follows that information must be used by males to identify potential mates. While information in other modalities may also be available (e.g. visual, tactile), chemical communication is

known to be a mode of information transfer for crickets facilitated in part by their long, filiform antennae (Chapman 1998, Kostromytska et al. 2015, Sharma et al. 2015). Male and female crickets also make antennal contact for extended periods of time generally during courtship, suggesting that close range signaling is important for mate recognition. Multiple lines of evidence suggest the use of chemical communication in *Laupala* (Shaw and Khine 2004; Mendelson and Shaw 2006; Mullen et al. 2007, 2008), but the experiments here are the first to empirically show that male-female differences in chemical cues are meaningful to intrasexual and intersexual interactions. Further, these results open up the possibility of further exploring CHCs as a speciation phenotype in *Laupala* (Shaw and Mullen 2011, Mullen and Shaw 2014) along with acoustic signaling. Exploring these signaling modalities together in *Laupala* can help to elucidate how complex courtship and its components change over time and lead to eventual sexual isolation between incipient species.

Chemical differentiation

Males and females express similar compounds in different relative ratios, rather than differences in unique compounds, both in overall body extracts (Fig. 1.4) and in antennal-only extracts (Fig 1.6). I found a strong signal of overall differences in CHCs between males and females in the whole-body extracts, but a marginally significant ($p=0.052$) difference in antennal CHCs. This apparent lack of difference may be due to a low signal to noise ratio, given the smaller amounts of CHCs present on the antennae. Given the small amount of sample available from the antennae, finding a

strong signal may be more difficult given background noise. The individual compounds or combinations of compounds that males are assessing remains to be determined. Mullen et al. (2007, 2008) found little notable qualitative variation between males and females across *Laupala* crickets, despite significant quantitative variation in CHCs between species. However, a notable exception was that *L. makaio* males expressed significantly more C_{31} tetraene and differed in their abundance of C_{27} alkenes (Mullen et al. 2008). Their analysis included sampling of *L. pruna* from Glenwood Rd, (19° 26' 49.45" N , 155° 7' 20.85" W) whereas here I analyzed *L. pruna* from Alili Springs, a population 45.33 km apart. While *L. pruna* from Glenwood express heavier compounds as seen in the retention times (not shown), Alili Springs crickets do not express these compounds, demonstrating that marked qualitative differences in CHCs exist not only between species, but within species of *Laupala* as well (see Chapter 3).

Behavioral Discrimination

Males exposed to female antennae were significantly more likely to exhibit courtship behaviors and relatively reduced aggression compared with male antennae. *Laupala pruna* males would pump and sing more after contact with female antennae, whereas they spent more time biting male antennae. In these experiments, there was only one trial where a male sang spontaneously before making contact with antennae. However, this trial was one of the trials not included in the analysis because both the spontaneous singing bout and the antennal contact occurred after the 15-minute cutoff. Males never pumped or produced micros spontaneously, and only exhibited courtship

or aggressive behaviors after physical contact with the antennae of a treatment individual. These statistically different, and biologically relevant, behavioral responses to the antennae of female or males suggest that differences in behavior are due to a sex-specific contact cue or signal, rather than an acoustic or chemically volatile one. These results are consistent with studies in other cricket species, in which males displayed different behavioral responses to male vs female antennae in *Gryllus bimaculatus* (Haberkern and Hedwig 2016). Rence and Loher (1977) exposed blinded male *Teleogryllus commodus* to male or female antennae mounted on a stick and found that they showed higher levels or aggressive or courtship behavior, respectively. Other studies found that chemical ablation of the antennal contact chemoreceptors of crickets led to reduced mating behavior or an increase in courtship latency (Balakrishnan and Pollack 1997 (*Teleogryllus oceanicus)*, Ryan and Sakaluk 2009, *Gryllodes sigillatus),* further demonstrating the importance of contact chemical communication in cricket mating behavior.

The filter paper trials further support the hypothesis that some chemical substance derived from females elicits male courtship behavior more often than male-derived chemical substances. Males were more likely to exhibit singing and pumping in the presence of filter paper exposed to females compared with untreated filter paper (Fig 1.9), but showed no bias toward male-exposed filter paper, compared to untreated filter paper. Filter papers for these trials were treated by cohorts of males or females walking over them for two days, which likely caused a passive transfer of a stable chemical cue. This information appears to trigger behavioral responses of males, such as singing or pumping (Fig. 1.8-1.9). Given the low volatility of CHCs (Anthony and

Jallon 1982, Stinziano et al. 2015, Yew and Chung 2015), CHCs transferred from the tarsi are a likely candidate for this cue.

The results from trials where focal males interacted with both male and female filter paper simultaneously are less clear. There was no difference in contact time, rubbing, or pumping toward male versus female filter paper, although song was significantly biased towards the male side (Fig. 1.10). One possibility for these results is that access to conspecific CHCs, whether male or female, is sufficient to activate long distance courtship behaviors and rubbing. This scenario would suggest that the statistical difference in singing is a false positive and there is no true bias in singing. Another possibility is that *Laupala* males respond more strongly to the presence of potential competitors than they do to females being in the area. The difference in contact time, though not statistically significant, was slightly biased towards the male filter paper, rather than the female. If this bias is a false negative due to a modest sample size, these results suggest that males take a greater interest in assessing the level of competition they are likely to experience and therefore respond more strongly to it. Given how closely aggressive and courtship neural circuits can be (Koganezawa et al. 2016), it is possible that detection of male chemical signal is sufficient to activate aggressive singing and repress courtship behavior. Lastly, the lack of difference might be due to the constrained size of the petri dish where there was not a large enough area for males to accurately demonstrate their choice, so the expression of their preference for one piece of filter paper extended past the arbitrary divide of the petri dish. However, given the ability for males to exhibit behavioral preferences in trials with only male or female filter paper present make this third scenario seem the least likely.

Males differed strongly in the probability of producing micros between the antennal and filter paper trials. About 17% of males overall produced micros in the antennal trials whereas no males produced micros in the filter paper trials. This result suggests either 1) antennal and tarsal CHCs are different, where tarsal CHCs can evoke a calling response, but antennal female CHCs are needed to produce micros, a step further along in the courtship sequence (Shaw and Khine 2004); 2) that males need additional information from females (such as stimulation from the movement of female antennae); or 3) female CHCs can be dynamic where she secretes additional CHCs during antennal contact that encourage the male to proceed with courtship.

Despite quantitative differences in CHCs and differences in courtship behavior between males and females, a moderately high number of focal males nonetheless initiated courtship behavior when given access to the antennae of other males. The reasons for this responsiveness to male antennae, especially when costly reproductive behaviors like singing, pumping, and micro production are concerned, is unclear. However, evidence of same-sex sexual behavior in other crickets (Bailey and French 2012, Boutin et al. 2016) suggests that it might be a case of mistaken identity. Prior to trials, males were isolated or in small male-only groups before their final molt and therefore had not yet encountered females as adults. Consequently, their ability to discriminate between male and female signals might be compromised in some way. I found that male and female CHCs, while statistically different, was qualitatively similar overall. Moreover, the LDA did not produce perfect assignment of individuals to their correct sex category (Fig. 1.5, Fig. 1.7), suggesting that that there may be a continuum between male-like and female-like CHC profiles. Alternatively,

unrestrained males that are paired together usually separate shortly after contact because of aggressive interactions, as opposed to females, which stay relatively close to males (Stamps, personal observation). Restrained males in this experiment are unable to move away from focal males or can provide very limited behavioral feedback. The continued interaction with the antennae of a conspecific may therefore be sufficient to elicit courtship behavior. Further studies examining the chemical discriminatory abilities of naïve and mated males would further clarify if discrimination were physiological, developmental, or ecological.

Overall, the results of this chapter demonstrate that male and female *L. pruna* differ in their CHCs, both in whole body extracts and in antennae-only extracts. Further, these quantifiable chemical differences elicit differential behavioral responses across different trial setups. While females have access to male acoustic communication, males do not have similar information available to them when making courtship and mating decisions. Males, however, have access to chemical information from females, though the degree to which males discriminate among females due to differences in CHCs have yet to be determined. Conversely, females may well be using male CHCs to assess male quality, though females lack easily observable behaviors, such as pumping, singing, and micro production by which to monitor such assessments. Nevertheless, this chapter confirms the importance of chemical communication in initiating male courtship in *Laupala* and opens up the possibility of further exploring CHCs as a speciation phenotype in *Laupala* (Shaw and Mullen 2011, Mullen and Shaw 2014) along with acoustic signaling. Exploring these signaling modalities together in *Laupala* can help to elucidate how complex courtship and its

components change over time and lead to eventual sexual isolation between incipient species.

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FIGURES AND CHARTS

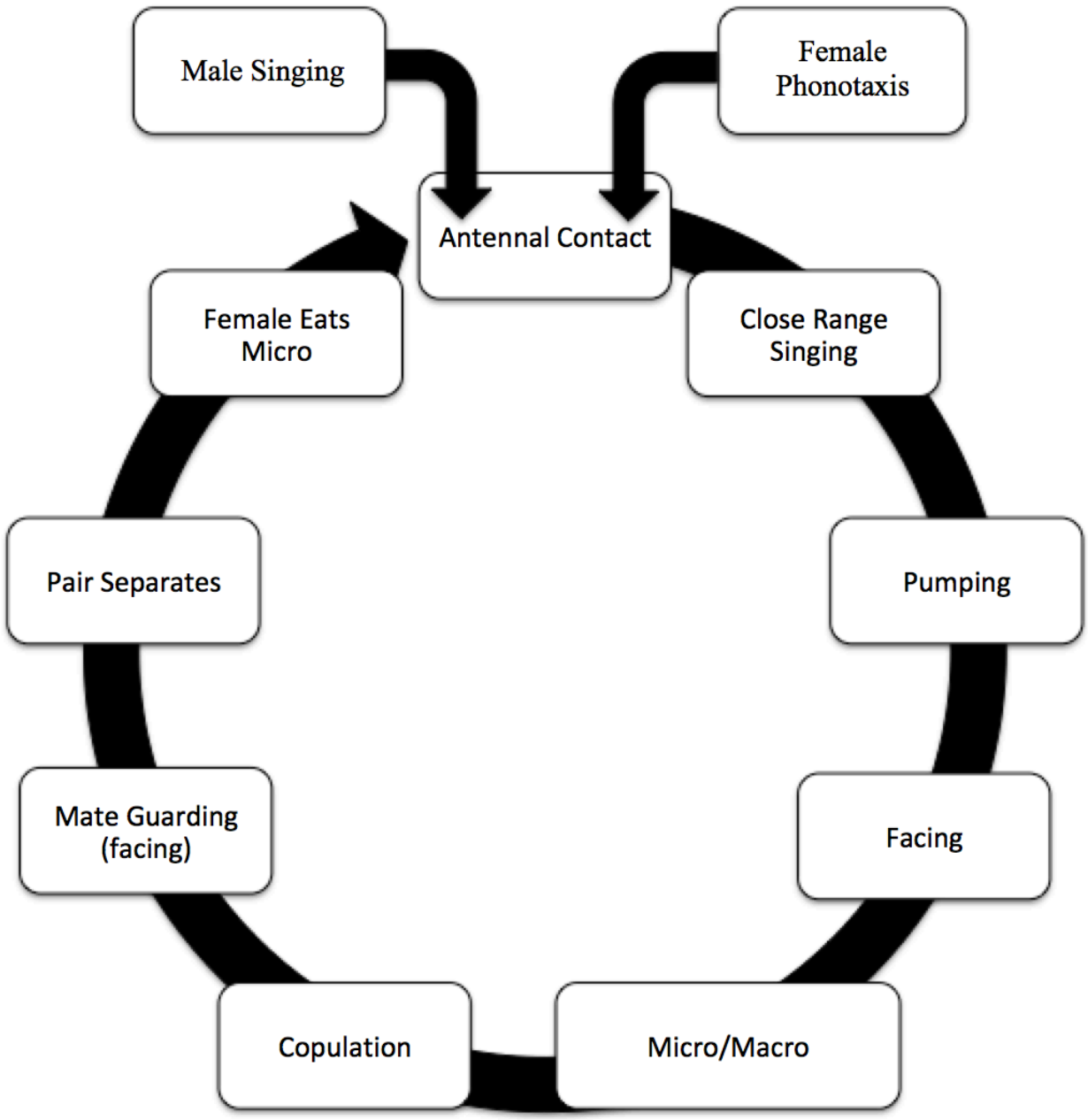

Figure 1.1: General progression of *Laupala* courtship. Events where chemical communication is likely occurring are marked with an asterisk. Adapted from Shaw and Khine 2004.

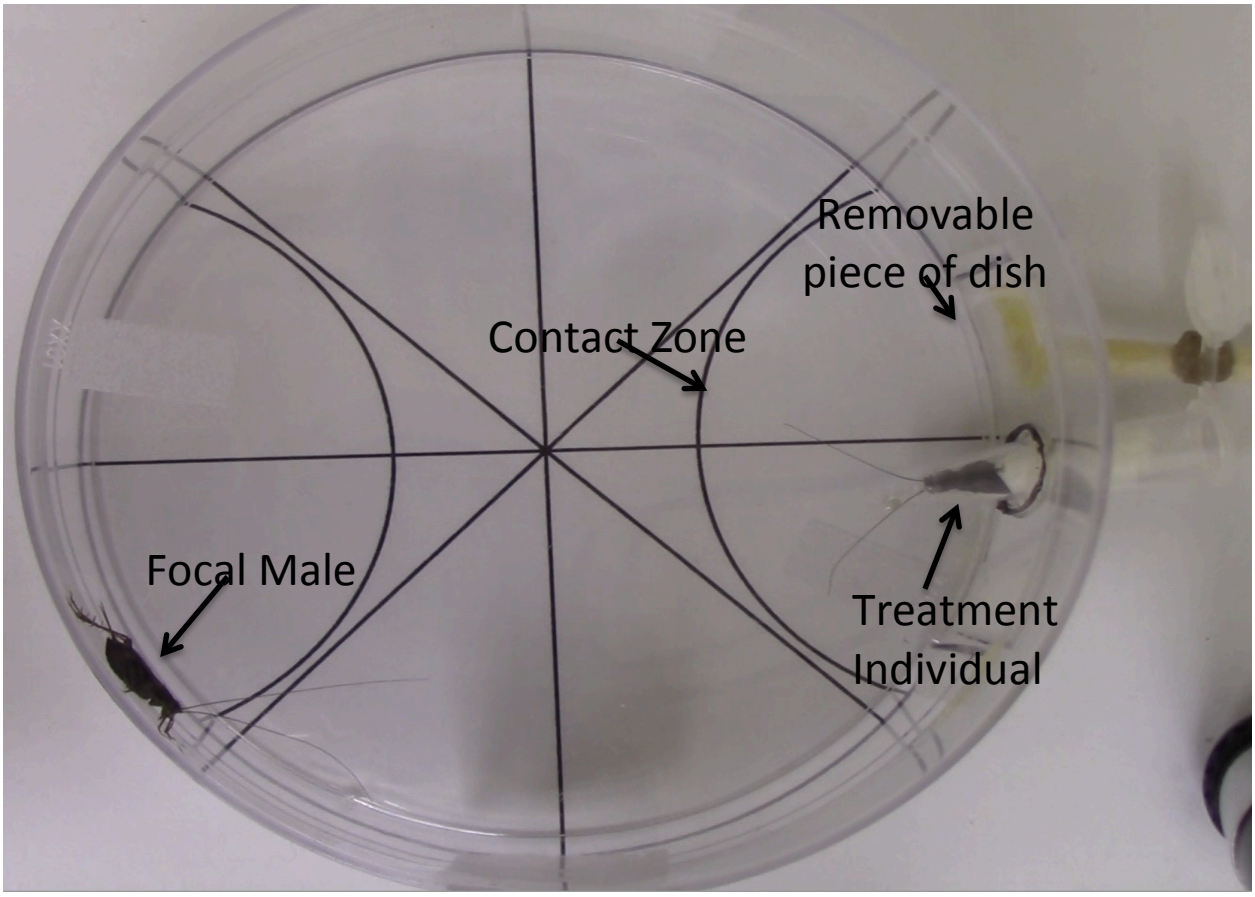

Figure 1.2: Experimental setup for antennal trials. The Experimental individual's antennae were inserted into the petri dish via a removable piece of a dish attached to a wooden dowel.

Figure 72: Male exhibiting "rubbing behavior" where male twists his back and presses it against the substrate

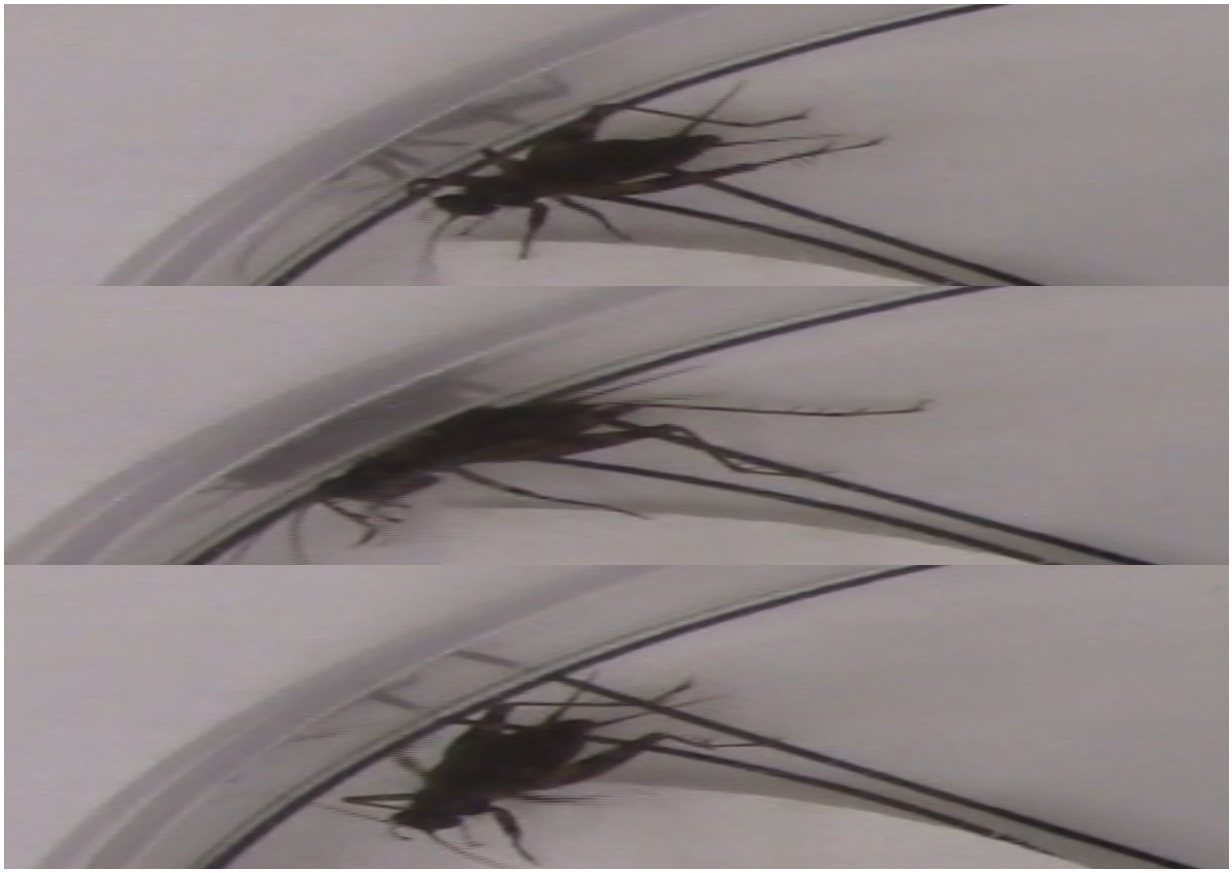

Figure 1.3: Male exhibiting “rubbing behavior” where male twists his back and presses it against the substrate

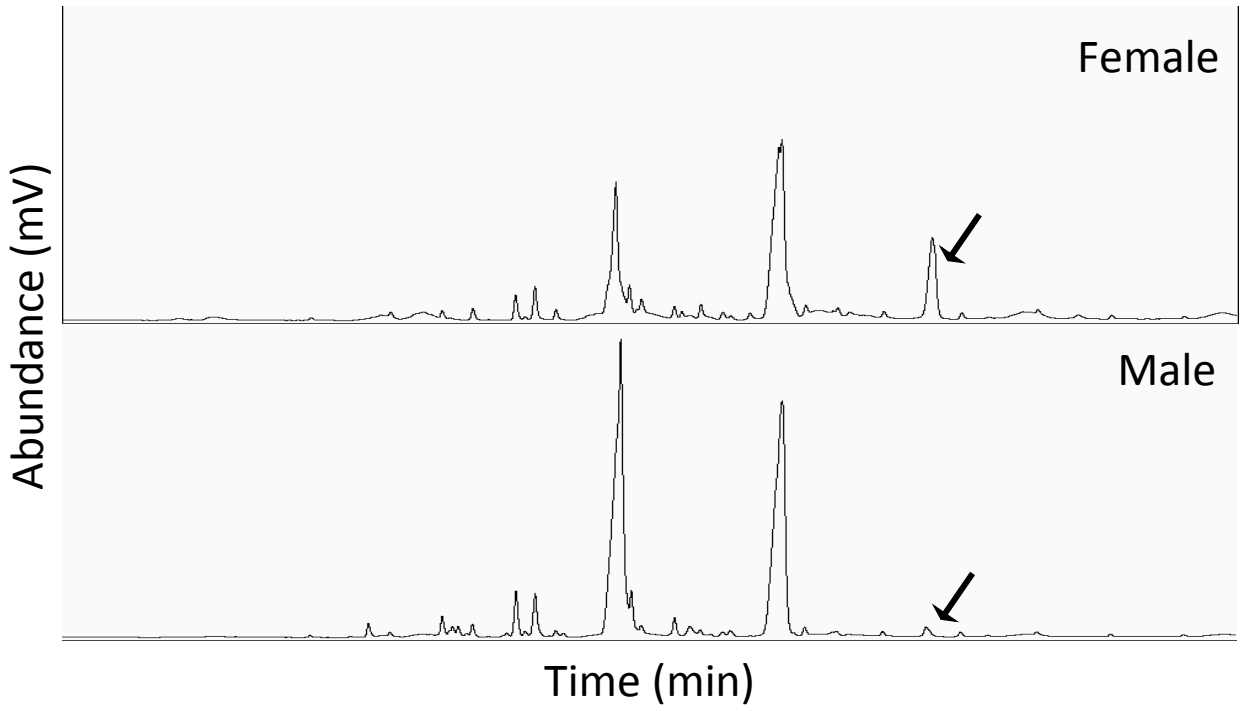

Figure 1.4: Chromatograms of male and female whole body CHC extracts. Peaks show individual compounds present in an individual. Arrows designate one peak that is quantitatively different between males and females

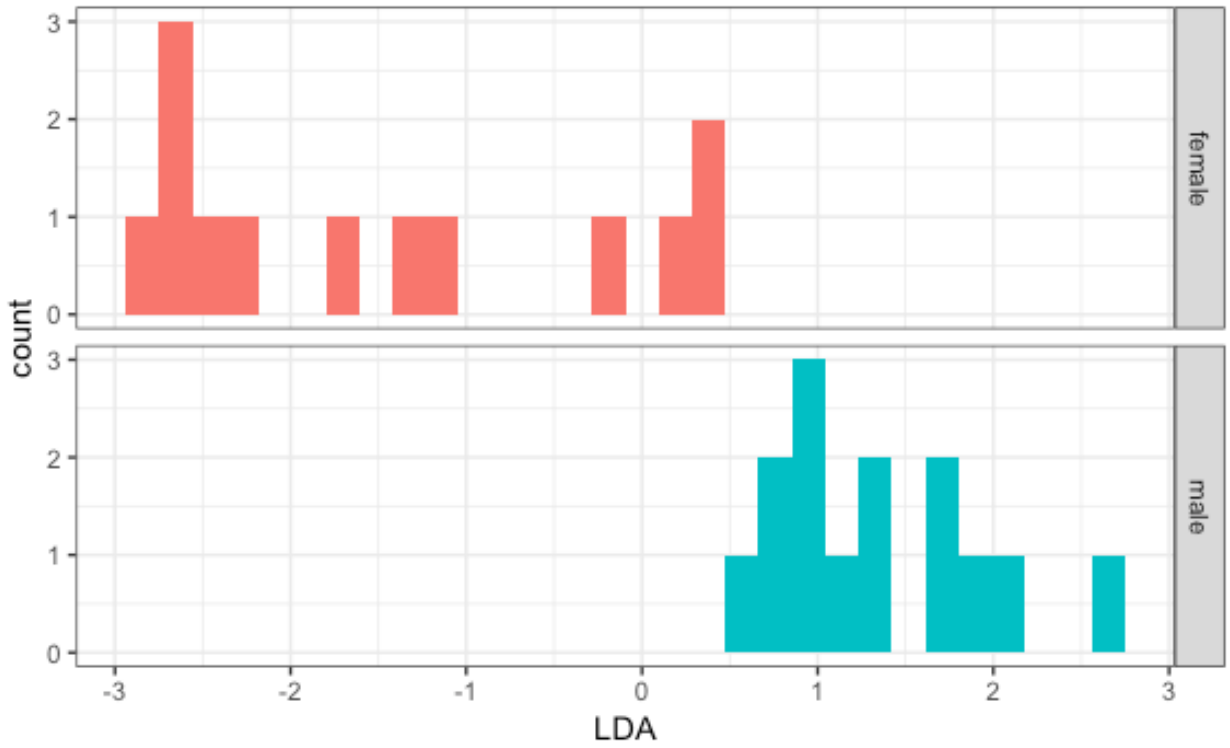

Figure 1.5: A LDA comparing males (n=14) and females (n=13) whole body extracts from 7 canonical variates, Males had more positive canonical scores while females had more negative canonical scores

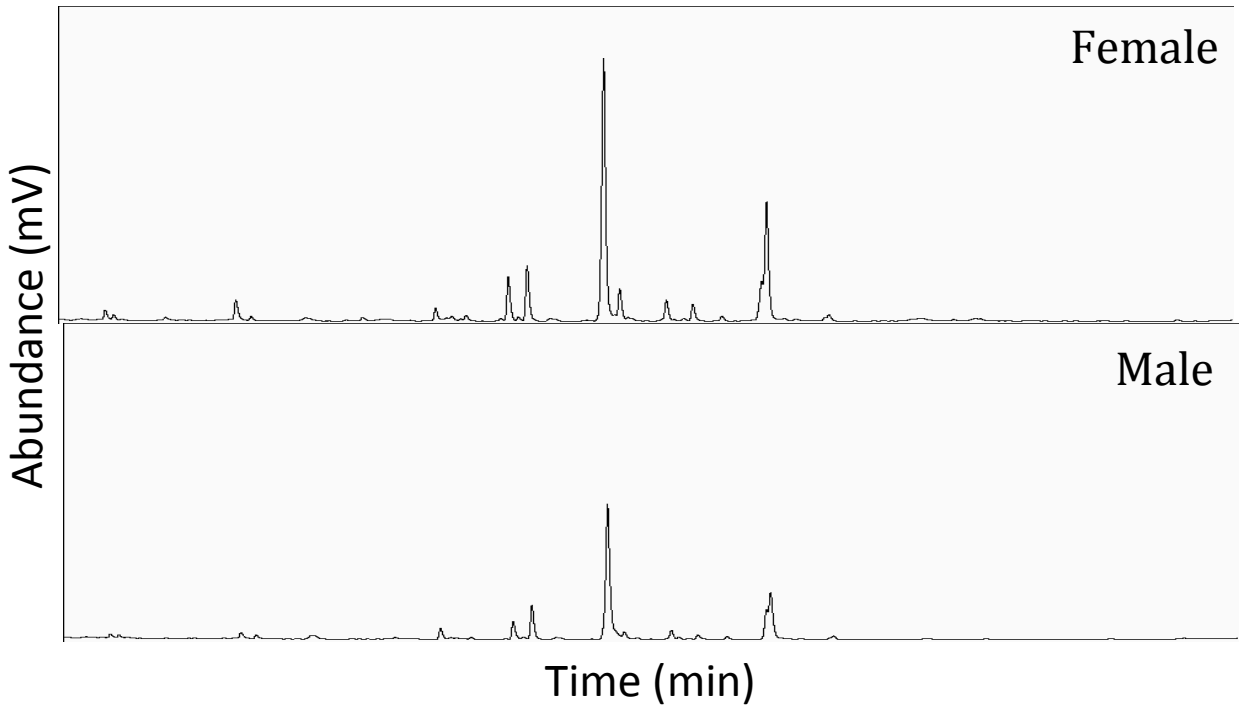

Figure 1.6: Chromatograms of male and female antenna-only CHC extracts. Peaks show individual compounds expressed by an individual.

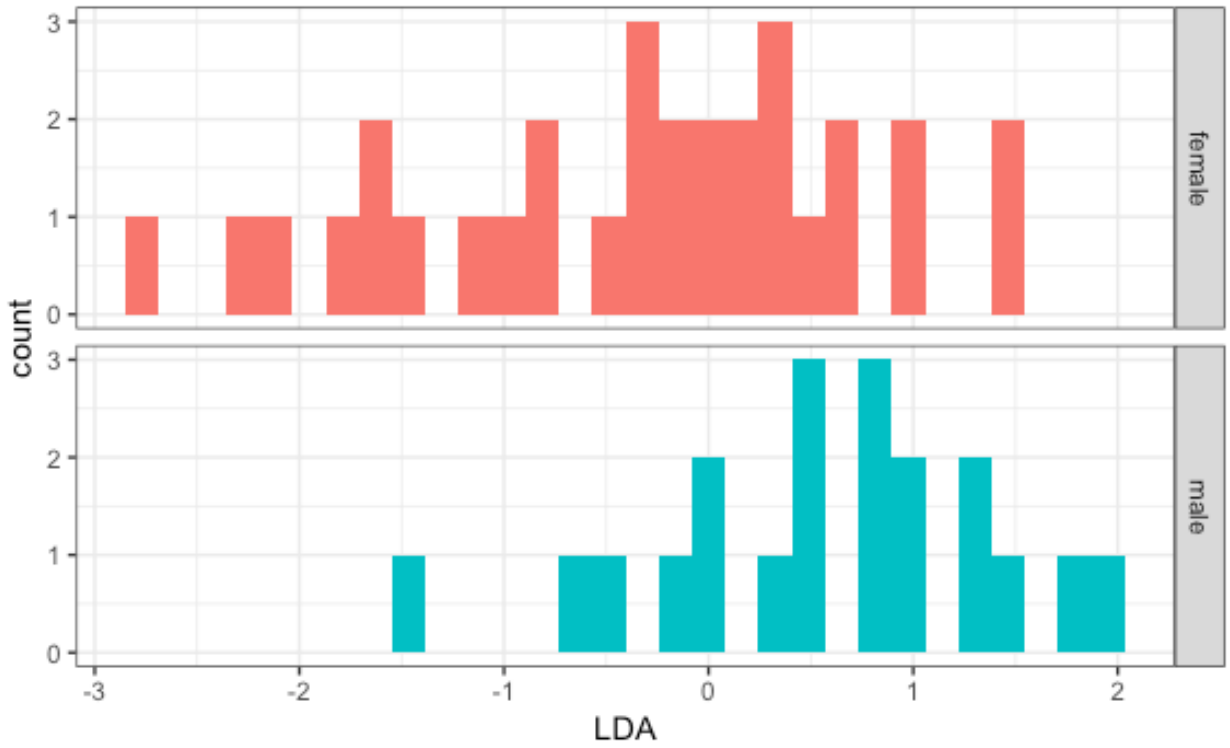

Figure 1.7: A LDA comparing males (n=20) and females (n=31) antennal extracts based canonical variates. Males had more positive canonical scores while females had more negative canonical scores

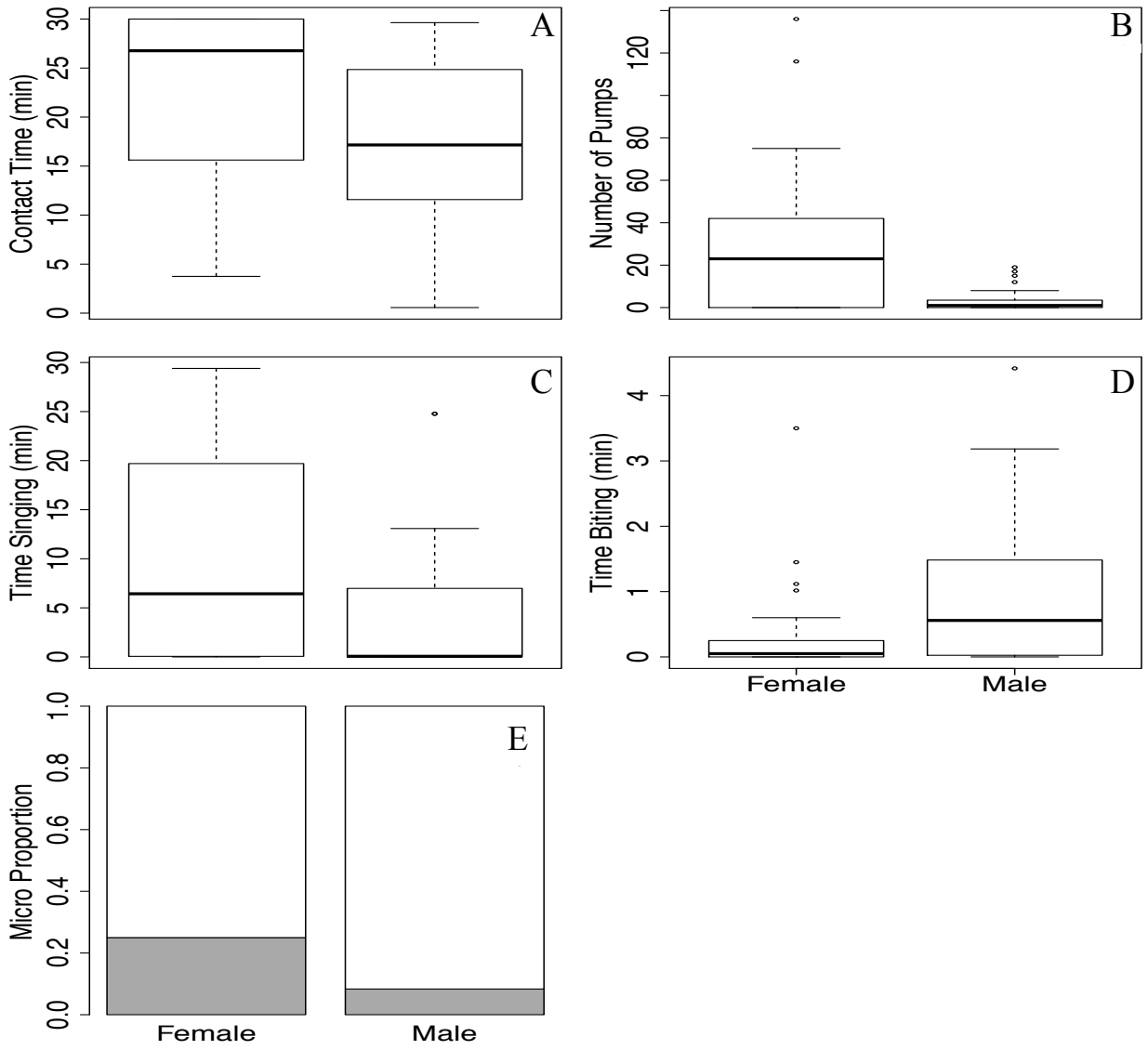

Figure 1.8: Behavioral results of antennal trials. A) Total time (min) focal males spent in contact with female versus male antennae B) Number of pumps focal males performed in presence of female versus male antennae C) Total time (min) focal males spent singing in presence of female versus male antennae D) Time (min) spent biting female versus male antennae E) proportion of males producing a microspermatophore (micro) in the presence of female versus male antennae)

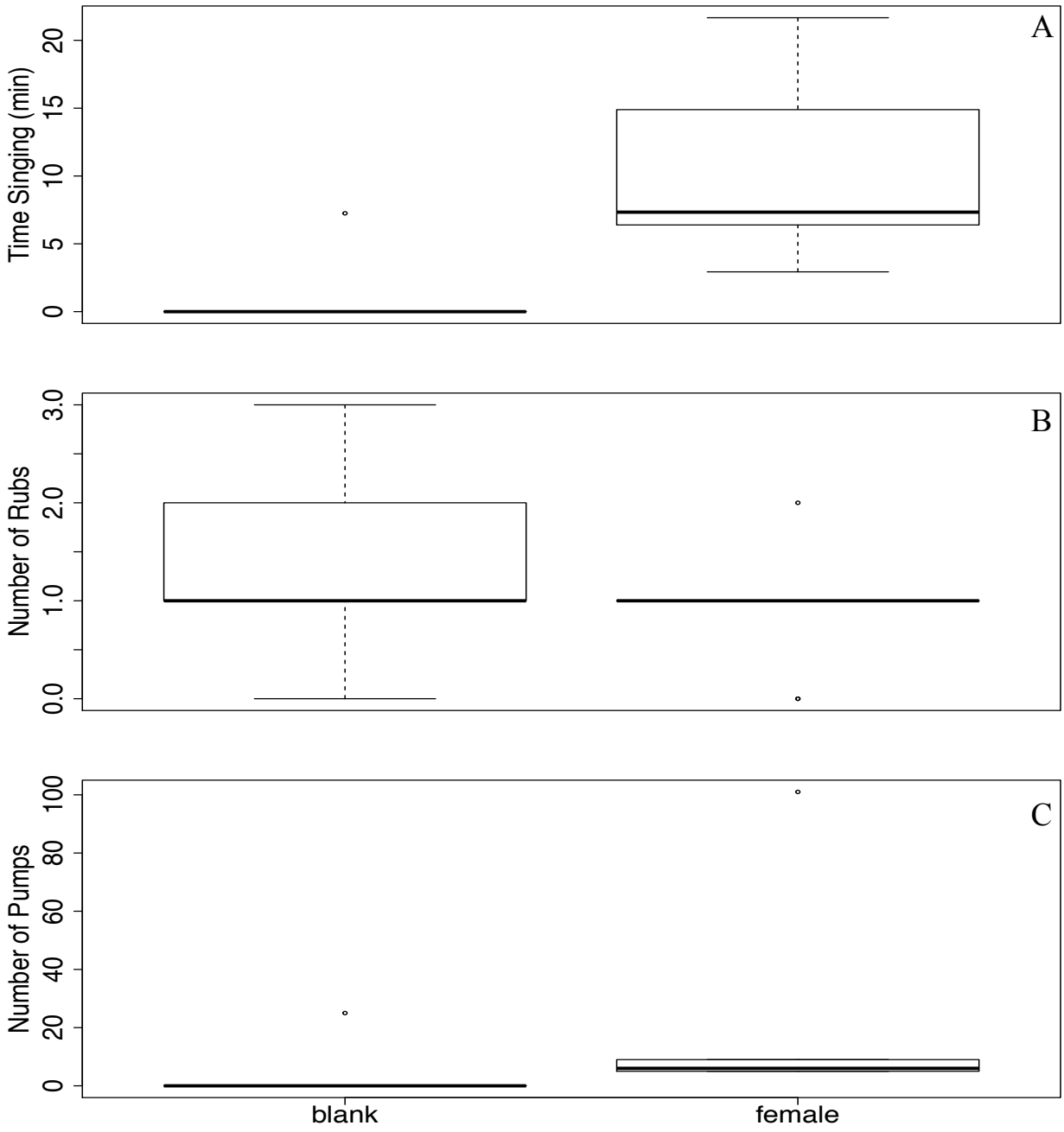

Figure 1.9: Filter paper trials where a focal male interacted with blank (control) and filter paper walked on by multiple females. A) Time (min) males spent singing on each side B) number of rubs males performed on each side C) number of pumps performed on each side

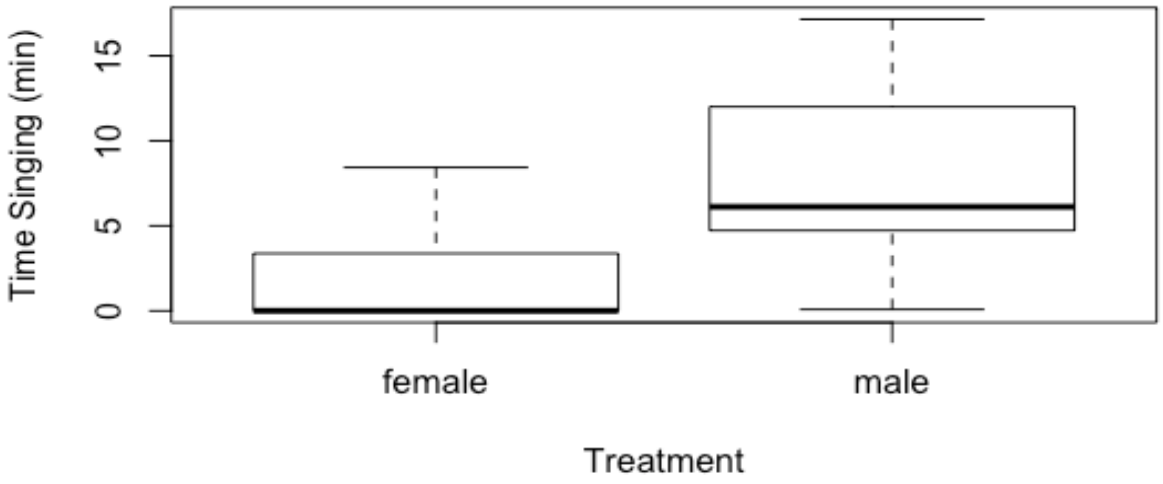

Figure 1.10: Time a focal male spent singing (min) near filter paper walked on by multiple females versus filter paper walked on by multiple males.

Table 1.1: Description of behaviors and scoring method for antennal trials and filter paper trial

Behavior	Description	Scoring Method (antennal trials)	Scoring Method (filter paper trials)
Contact	Focal male's antennae touch antennae of experimental individual	Contact begins when the experimental individual's antennae are touched and observably displaced by the focal individual. Contact ends when the male leaves the circle around the experimental individual	Begins when male places more than one foot on filter paper until male is completely off filter paper
Song	Male raises wings ~45 to 90˚from body and stridulates them against each other	The beginning (wing raising) and end (wings down) of song bouts, as long as the wings opened and closed at least once (i.e. one pulse).	Same as antennal trials, but assigned to the half of the petri dish male began singing on
Pumping	Either 1) Male thrusts his abdomen up a single time (silent) or vibrates slightly (while singing)	Individual pumps were counted when singing or silent and combined together	Same as antennal trials, but pumps were assigned to the side on which they occurred or if on line, the side the male was on last.
Rubbing	Male twists his body ~90˚ and drags his body across some surface of the arena	Rubbing behavior was not tallied during antennal trials	Single count with each drag against the petri dish or filter paper. Assigned to side male was on or last on if on the line.
Micro	Male everts genitalia and fills a spermatophore	Presence or absence of micros based on when genitalia eversion was noted	Males did not produce micros during filter paper trials

CHAPTER 2

CHEMICALLY MEDIATED ISOLATION BETWEEN TWO CLOSELY RELATED *LAUPALA* SPECIES

INTRODUCTION

Historically, the study of speciation has been synonymous with the study of reproductive isolation between groups of organisms (Mayr 1963, Coyne and Orr 2004, Merot et al. 2017). However, as many have pointed out, the major problem of this approach is that reproductive isolation is a consequence, rather than a cause of speciation (Patterson 1985, Harrison 1998, Shaw 2001, Via and West 2008, Mallet 2010, Shaw and Mullen 2011). Shaw and Mullen (2011) outlined a research paradigm to shift focus from reproductive isolation to phenotypes that are diverging early in the speciation process. Focusing on phenotypes that lead to assortative mating early in divergence allows us to determine which phenotypes are "drivers" of reproductive isolation between groups, rather than exploring phenotypes that may have evolved secondarily and maintain isolation. Identifying candidate "speciation phenotypes" can then lead to an exploration of the selective forces that cause divergence in these traits (Shaw and Mullen 2011, Mullen and Shaw 2014). Given the complexity of an organisms' natural history, it is unlikely that a single phenotype is responsible for differentiation (Dobzhansky 1972, Shaw and Mullen 2011), especially since organisms often use multiple signaling modalities to communicate (Hebets and Papaj 2005, Partan and Marler 2005, Uetz et al. 2017).

Chemical communication is an ancient form of signaling that is widespread

across taxa (Ache and Young 2005, Wyatt 2014, Brunetti et al. 2018). For insects in particular, chemical communication is widely used in mate choice, kin recognition, dominance hierarchies, aggregation pheromones, and in defense (Wyatt 2003, Eisner et al. 2005, Pelosi et al 2014, Smith and Liebig 2017.). Chemical signals may be particularly informative signals, as they are often metabolic byproducts and can be shaped by natural and sexual selection (Akino et al. 1999, Guillem et al. 2014). Given that half of all described species are insects (Mayhew 2007), and that chemical communication is not only widespread but plays a central role in social communication, it is highly plausible that chemical communication influences the divergence and maintenance of boundaries between incipient species (Smadja and Butlin 2009, Wyatt 2014).

Among chemical signals, cuticular hydrocarbons (CHCs) in particular play several important roles in the lives of insects. In social insects, they can indicate reproductive status (Monnin 2006) and facilitate intracolony activities (Richard and Hunt 2006). CHCs are thought to have evolved primarily to aid in desiccation resistance and were secondarily co-opted for use in chemical signaling (Howard and Blomquist 2005, Chung and Carroll 2015, Ginzel and Blomquist 2016). They may therefore be "magic traits", that is, traits under divergent natural selection, but also contributing to assortative mating between groups (Servedio 2011, Chung et al. 2014, Chung and Carroll 2015). Moreover, CHC variation is heritable, and the evolution of CHCs may be influenced by sexual selection at various points in the process of divergence. CHC's are used to discriminate against closely related individuals or family groups in Australian field crickets (*Teleogryllus oceanicus*) (Thomas and

Simmons 2008). Steiger et al. (2015) found that female choice for male CHC profiles was not influenced by similarity to her own profile in decorated crickets (*Gryllus sigillatus*). Ginzel and Hanks (2003) demonstrated that the presence of CHCs was a necessary condition for several different species of male longhorn beetles to recognize conspecific females while Zhang et al. (2014) found that differences in CHC expression mediated mate choice in conspecifics and reduced courtship behavior towards heterospecifics in leaf beetles. Shahandeh et al. (2018) found that CHCs cause male mediated reproductive isolation in *Drosophila*, while Higgie and Blows (2007) found that female *D. birchii* and *D. serrata* preferred the CHCs of allopatric males over sympatric males from hybrid zones. These patterns in part justify the use of cuticular hydrocarbons in taxonomy to hypothesize species boundaries (Kather and Martin 2012) and may be especially useful for distinguishing cryptic or closely related species (Sakolsy et al. 1999; Mullen et al. 2007, 2008, Vaníčková et al. 2014, Fox et al. 2017). Investigation of what role CHCs may have in speciation requires looking at taxa that are in the early stages of divergence and determining whether they are involved in mate discrimination among populations or closely related species.

While females have been traditionally viewed as the gatekeepers who determine whether or not hybridization occurs, a growing literature testifies to a role for males in mating decisions when those decisions affect male fitness (Zhang et al. 2014, Roberts and Mendelson 2017, Shahandeh et al. 2018). Males are predicted to be more selective in mate choice in species where they invest costly resources into mating, such as nuptial gifts (Trivers 1972, Simmons 1990, Wedell 1993, Macedo-Rego et al. 2016) or defense of a resource that females need for reproduction (Otsfeld

1985, White and Rundle 2015). Males may also express mating preferences when there is significant variation in female quality that they can detect (Bonduriansky 2001, Servedio and Lande 2006, Edward and Chapman 2011). We might expect males to discriminate against heterospecific females as well, when there is direct or indirect selection against mating with heterospecific females. While female use of cuticular hydrocarbons in mate choice and sexual isolation has been studied (e.g. Higgie and Blows 2007, Tyler et al. 2015), the role of chemically mediated male choice as a reproductive barrier is less explored (but see Zhang et al. 2014, Shahandeh et al. 2018).

Hawaiian swordtail crickets (genus *Laupala*) are a rapidly speciating group of 38 species found across the Hawaiian archipelago (Otte 1994, Shaw 2000; Mendelson and Shaw 2005). Evidence suggests that the evolution of male calling song and concurrent female acoustic preference has contributed to this diversification (Mendelson and Shaw 2005, Grace and Shaw 2011, Wiley et al. 2012). Acoustic behavior enables mate location and may coincidentally discourage hybridization with sympatric heterospecifics. However, courtship in *Laupala* is complex, occurring over several hours and is highlighted by the transfer of several spermless microspermatophores (micros) from the male to the female before the transfer of the sperm-bearing macrospermatophore (macro) (Shaw and Khine 2004). Once antennal contact is made, most of the courtship occurs in the absence of male song, but in the presence of extensive antennal interaction (Shaw and Khine 2004). Like song, all *Laupala* species studied to date have been shown to vary in CHCs (Mullen et al. 2007, 2008). However, whether these differences impact probabilities of interbreeding early

in *Laupala* divergence is unknown.

Here, I explore whether CHCs are a speciation phenotype, the differentiation of which can restrict gene flow early in divergence. Specifically, I focused on the role of male behavior in interspecific reproductive interactions between *L. pruna* (Lp) and *L. kohalensis* (Lk), closely related, allopatric species (Mendelson and Shaw 2005) endemic to the Island of Hawaii (Otte 1994). Prior work has shown that, despite their close relationship, Lp and Lk differ greatly in pulse rate of the male mating song (Otte 1994, Shaw et al. 2007), a long distance, sex-limited sexual signal. These two species also have distinct hydrocarbon expression which may present a barrier to close range reproductive interactions. If chemical communication has the potential to act as a barrier to gene flow between species of *Laupala*, I predict that males should be more likely to initiate courtship when exposed to conspecific versus heterospecifc chemical signals. I tested this hypothesis by 1) comparing CHC expression between Lp and Lk, 2) conducting behavioral trials where males were exposed to the antennae of conspecific or heterospecifc females, and 3) conducting filter paper trials where males interact with filter paper exposed to living conspecific and heterospecifc females. Results show that differences in CHC expression mediated by antennal interactions reduce reproductive interactions between these closely related *Laupala* species.

MATERIALS AND METHODS

Rearing

I used offspring of *Laupala pruna* (Lp) collected from Glenwood Road (19° 26' 49.45" N, 155° 7' 20.85" W) in July 2012, and Halema'uma'u (19°24' 36.10" N,

155°16'44.60" W) in 2012, and *L. kohalensis* (Lk) collected from Pololu Valley (20° 12' 22.81" N, 155° 44' 0.98" W) in 2007 on the island of Hawaii for behavioral trials. Nymphs were raised in 1 qt glass jars with moist Kimwipe and fed Fluker's Cricket diet twice a week. F1 nymphs were reared collectively in 1qt jars and larger nymphs (~5 months post hatching) were subsequently moved to same-sex specimen cups with no more than 3 individuals per cup. Upon maturation, crickets were moved to individual specimen cups with a moist Kimwipe. All individuals were kept in a temperature controlled room set to 20 ±1˚C and a 12:12h light/dark cycle.

Cuticular hydrocarbon preparation

All glassware and forceps were cleaned with two washes of clean HPLC-grade hexane before use. Wild caught *L. pruna* from Glenwood Road in 2012 (males n=5, females n=4) for and offspring of *L. kohalensis* collected in 2007 from Pololu Valley (males n=15, females=12) were used for full body extractions Individuals were anesthetized with CO2 before being submerged in 300 µl of HPLC grade hexane for 5 minutes. Vials were lightly swirled at the beginning and end of extraction and individuals were then removed with foreceps cleaned with hexane between samples. All samples collected above were blown down under a gentle nitrogen stream and then re-suspended in 60 µl of heptane. Samples were then pipetted into 2ml autosampler vials with 100µl glass inserts.

CHC analysis

A Shimadzu AOC-20i autoinjector was used to inject 1µl samples into a Shimadzu GC-2014 gas chromatograph with flame ionization detection (GC-FID)

equipped with a HP-5 column (20m, 0.180mm diameter, and a film thickness of 0.18um. Runs consisted of a starting temperature of 60˚C held 1 min, a ramp 20˚C/min to 200˚C. The temperature was then ramped *5˚C*/min to 320˚C and held for 15 min, yielding a total run time of 47 min. The FID was set to 340˚C and a sampling rate of 40 msec. The injection port was 300˚C with a pressure of 144.2 kPa. The total flow rate of gasses was 10ml/min with column flow of 0.80 ml/min, and purge flow of 3.0ml/min, yielding a linear velocity of 32.5 cm/sec. The split ratio was calculated to be 7.7 given the other parameters.

Peaks were selected by first using Labsolution's autointegrate function set at 18,000μV and a width of 2ms for full body extracts and 3,000 μV and a width of 3ms for antennal extracts, respectively. The threshold of autointegration was lower for antennal samples due to the smaller overall sample volume. Using similar parameters for full body and antennal trials led to a loss of available peaks for analysis. Retention times that were shared by at least half of the individuals in each sex and species were selected and missing values were manually integrated. Therefore, in initial peak binning within each species, there were peaks that were selected in one species, but not the other (i.e. peaks unique to each species). However, in order to meet the assumptions of PCA, manual integration of any charge above baseline at that binned retention time was included so that all retention time bins had a value. These abundances were log transformed using the formula $Z_{i,j} = \log(X_{i,j}/g(X_j))$, (Aitchison 1986, Mullen 2007). The retention times that differed between sexes were determined by performing t-tests on the log-transformed values and using a Bonferroni correction for multiple comparisons. The high dimensionality of the chromatograms was reduced

with a principal components analysis carried out in R (R Development Core Team 208). Principal components with eigenvalues greater than 1 were input into a linear discriminant analysis (LDA) to determine if males and females could be assigned to their sex and species in independent analyses based on their chemical profiles. Analyses were performed independently for both whole body extracts and antennal-only extracts. In addition, since the initial retention time selection was done independently for both species, there are retention times that are shared by both species and some that are unique to each species. I performed two analyses, examining "shared" and "all peaks" between Lp and Lk to determine on what levels discrimination between these species can occur. All samples from Lp were from the Glenwood Rd. population.

Mating Trials

To determine baseline levels of behavioral isolation between Lp and Lk, I conducted mating trials of Lp from the Halema'umau Trail and Lk from Pololu Valley. Males and females of these species were paired in a full factorial design: 1) Lk male x Lk female, 2) Lk male x Lp female, 3) Lp male x Lk female, 4) Lp male x Lp female. Pairs were placed in 9cm diameter x 2cm depth petri dishes from 0900h to 1000h and observed until 1800h. During these trials, I recorded 1) the time of initial antennal contact 2) the time of the first singing bout 3) the time of the first micro, 4) the number of micros successfully transferred, and 5) whether or not a macro was successfully transferred. I then calculated 1) the latency from first antennal contact to first singing bout and 2) the latency from first antennal contact to first micro produced. I measured

both of these latencies because not all males sang before producing micros or might sing, but not produce a micro. Both of these measures quantify a male's reproductive response to a female (Krstic et al. 2009).

Antennal Trials

To test whether *Laupala* males could distinguish between chemical signals of Lp (Glenwood Rd) versus Lk, I conducted behavioral trials where males were given access to antennae of either a conspecific or heterospecific female for forty-five minutes. Thirty minutes before trials, cohorts of three focal males were placed individually into petri dishes (9 cm diameter x 4 cm height) and allowed to acclimate. These petri dishes were modified to have a removable piece with tape on the sides to prevent the males from escaping. A wooden dowel was glued to the removable piece as a handle and to give stability. Immediately before the start of trials, the removable piece of the petri dish was replaced with one with a hole in it through which the treatment individual's antennae (see description below) were inserted, giving the focal male access to the treatment individuals' antennae. Petri dishes and removable pieces were cleaned between trials using a new Kimwipe soaked in 70% ethanol.

Treatment Lp or Lk females were cold anesthetized by placing them in a -4˚C freezer for 3 minutes on the day of trials. If the individual was still partially active, it was placed in the freezer for an additional minute. Each treatment female was placed in a modified 0.5 ml eppendorf tube and curved forceps were used to thread the antennae of the cricket through a hole at the tip. The back of the tube was plugged with Kimwipe to prevent the individuals from backing out. Individuals constrained in

these tubes were able to move their antennae normally, but were otherwise inaccessible to the focal individuals. After 15 minutes to warm up, a treatment individual was immediately introduced into the trial arena and a Canon HD VIXIA HFR600 camera began recording. A focal male of each species was given access to the antennae of either conspecific or heterospecific treatment female. Each focal male and treatment female was used only once.

Trials lasted 45 minutes after which the focal male was returned to his cup and the antennae of the treatment female was immediately immersed for five minutes into a 2ml autosampler vial filled with hexane. While still in the eppendorf tube, antennae of treatment individuals were immediately immersed into a 2ml autosampler vial filled with hexane for 5 minutes. Extracts were not available for 7/32 Lp and 7/27 Lk because most or all of the antennae were bitten off during trials and were therefore unavailable. All Lp antennal trials were conducted between 1000h and 1400h.

Filter paper trials

To test whether males could distinguish species-specific chemical signals independent of the antennae, I allowed males to interact with filter paper exposed to females from Lp (Glenwood Rd) or Lk for 48 hours prior to trials. Petri dishes (9cm diameter x 2cm depth) were lined with 8 pieces of filter paper (Whatman 1, 42.5mm diameter, GE Healthcare, UK), four on each surface. Five Lp females from Glenwood Rd or Lk from Pololu Valley were placed in the petri dishes simultaneously and allowed to walk around the dish for 48 hours. A moist Kimwipe was placed along the side of the dish vertically to prevent females from desiccating. The petri dish was

flipped after 24 hours to control for any differential use of the top or the bottom of the petri dish. This method allows for the passive transfer of cuticular hydrocarbons and likely other chemical compounds from the individuals to the filter paper (Borges et al. 2003). After 48 hours, the individuals were removed and returned to cups. Each piece of filter paper was cut in half and one half of a piece of filter paper was used for each trial. Therefore, each replication of the procedure described above yielded filter paper for 16 trials. Filter paper halves were used in only one trial and then discarded.

Filter paper trials consisted of placing a focal male in a petri dish with two pieces of filter paper of equal size from different sources, and recorded for 45 minutes with a Canon HD VIXIA HFR600 camera. Only *L. pruna* were used for the focal males of these trials because mature, sexually receptive *L. kohalensis* males were unavailable at the time of these trials. Three different filter paper trials were conducted: 1) a blank piece of filter paper vs. a filter paper exposed to *L. pruna* females 2) a blank vs. a filter paper exposed to *L. kohalensis* females; and 3) filter paper exposed to *L. pruna* females versus paper exposed to *L. kohalensis* females. Each focal male was only used in experimental trials.

Video Analysis

I analyzed 30 minutes of the videos for each set of trials. During antennal trials, observations began once the focal male made initial antennal contact with the treatment female. Antennal contact was usually accompanied by rapid antennal movement by one or both focal and treatment individuals or conspicuous displacement of the treatment individual's antennae (i.e. bending of part of the antenna). I then

noted the amount of time a male spent in contact with the treatment individual's antenna, the time spent singing, the number of pumps, whether or not a micro was produced, and how much time the male spent biting the antennae of the treatment individual. In a given contact episode, males were considered to be in contact with a female from the time that the antennae first touched until the male left a 30 mm radius zone around the treatment individual's antennae (Table 2.1).

In each filter paper trial, observations began after the male made contact with one side of filter paper. I noted the time in contact with each piece of filter paper, the amount of time spent singing, number of pumps, and a putative marking behavior. During this behavior, males would contort their backs and drag them along the surface of the petri dish or the filter paper. Contact was defined as a male beginning to walk on one piece of filter paper until all legs were off the filter paper. Trials where the male did not make antennal contact (antennal trials) or contact with one or both pieces of filter paper (filter paper trials) within the first 15 minutes were excluded from further analysis.

RESULTS

Gas Chromatography Analysis

Whole-Body Extract Analysis

The cuticular lipids of five wild caught *L. pruna* males and four wild caught females were analyzed along with 15 *L. kohalensis* males and 12 females (collected in 2007). Forty-three peaks were identified from *L. pruna* and 47 from *L. kohalensis,* yielding a total of 56 peaks. Among these peaks, 26 were shared by both species after

initial binning. There was no difference in the total amount of uncorrected CHC between the two species (i.e. the total abundance of all peaks used in analysis) (Lp= $9.44x10^6 \pm 3.66x10^5$ μV, Lk= $8.96 x10^6 \pm 1.18 x10^6$ μV, Welch two sample t-test, t = -0.38, df = 8.56, p-value = 0.71). However, *L. pruna* had a significantly higher total CHC abundance than *L. kohalensis* with respect to only shared compounds (*L. pruna*= $7.42x10^6 \pm 2.00 x 10^6$ μV, *L. kohalensis*= $5.25x10^6 \pm 7.91x10^5$ μV, Welch two sample t-test, t = -3.18, df = 8.85, p-value = 0.011).

Lp males and females did not differ in the total uncorrected expression of CHCs (females: n=4, $7.46x10^6$ μV, male: n=5, $8.96 x10^6$ μV, t = -1.1499, df = 4.7806, p-value = 0.3044). A principal components analysis revealed 8 PCs with eigenvalues greater than 1. However, including all 8PCs caused the cumulative variation explained to approximate 100%. Therefore, only the first 5PCs were used, explaining 88.72% of the variation. The first 5 PCs were chosen based on the % of variation explained, which approximates that of other PC analyses. Including all 8 PCs would cause an overfitting of the model to these data. A MANOVA test on the identified PCs indicated that male and female Lp are significantly different in their CHC expression (Wilks λ= 0.020722, F= 28.354, NumDF = 5, DenDF 3, $p=9.94x10^{-3}$, Fig. 2.1). The LDA was correctly able to identify all Lp individuals to the correct sex. After a Bonferroni correction for multiple comparisons ($\alpha=0.05/42=1.19x10^{-3}$), three peaks (representing the raw data) were found to be significantly different (Table 2.2).

Lk males and females did not differ in the total uncorrected expression of CHCs (female: n=12, $9.10x10^6 \pm 975 x10^5$ μV, male: $8.92 x10^6 \pm 1.21x10^6$ μV, t = 0.42454, df = 24.991, p-value = 0.6748). A principal components analysis revealed 11

PCs explaining 91.12% of the variation. A MANOVA test on the identified PCs indicated that male and female Lk are significantly different in their CHC expression (Wilks λ= 0.10968, F=11.069, NumDF =11, DenDF 15 p=2.612×10^{-5}). Using all 11 PCs, the LDA was correctly able to identify all Lk individuals to the correct sex (Fig. 2.2). After a Bonferroni correction for multiple comparisons (α=0.05/47=1.064×10^{-3}), sixteen peaks were still found to be significantly different (Table 2.3).

To compare Lp and Lk CHC differences, I performed two multivariate analyses: one examining shared peaks and another using all peaks. For shared peaks, principal components analysis reduced the dimensionality of the data into 5 PCs explaining 85.4% of the variation between species. Using all 5 PCs, the LDA was able to correctly assign all individuals to their nominal species. A MANOVA test on the identified PCs indicated that *L. pruna* and *L. kohalensis* CHC expression differs significantly (Wilks λ= 0.0461 F= 124.19, NumDF= 5 DenDF= 30, p=2.2×10^{-16}, Fig. 2.3). Using all peaks, including those qualitatively unique to each species, principal components analysis reduced the dimensionality of the data into 10 PCs explaining 85.9% of the variation. Using all 10 PCs, the LDA also correctly assigned all individuals to their nominal species. Likewise, A MANOVA test on the identified PCs indicates that the two species differ in their expression of CHCs (Wilks λ= 0.0138, F= 179.18, NumDF= 10 DenDF= 25, p< 2.2×10^{-16}, Fig. 2.4).

Antennal Extract Analysis

Antennal extracts were analyzed from 25 Lp and 20 Lk females. Females of the two species did not differ in the uncorrected total amount of CHC present on their antennae either when considering only shared compounds (Lp = 303293.4 ± 59639.32 μV, Lk = 30329.34 ± 36918.11 μV, Welch two sample t-test, t = -0.44189, df = 40.702, p-value = 0.661) or all compounds (Lp = 18174.85 ± 28917.27 μV, Lk= 12728.4 ± 16683.55 μV, Welch two sample t-test, t = -0.64608, df = 39.491, p-value = 0.522). After the Bonferroni correction for multiple comparisons (α=0.05/27=1.085x10^{-3}), nine peaks were still found to be significantly different (Table 2.3).

Using the antennal extracts, I again performed multivariate analyses using both shared peaks and all peaks. For shared peaks, principal components analysis reduced the dimensionality of the data into 3 PCs explaining 81.3% of the variation between species. Using all 3 PCs, the LDA was able to correctly assign 18/20 Lk and 24/25 Lp. A MANOVA test on these identified PCs indicates that these species differ in the expression of CHCs (Wilks λ= 0.812, F= 3.1612, NumDF= 3 DenDF= 41, p= 0.0346, Fig. 2.5). Using all peaks, including those unique to each species, principal components analysis reduced the dimensionality of the data into 6 PCs explaining 78.8% of the variation. Using all 6 PCs, the LDA was able to correctly assign 19/20 Lk and all 25 Lp. A MANOVA test on these identified PCs indicates that the species differ in the expression of CHCs (Wilks λ= 0.20062, F= 25.235, NumDF= 6 DenDF= 25, p< 7.651x10^{-12}, Fig. 2.6).

Mating Trials

Males of both *L. pruna* and *L. kohalensis* initiated and invested in courtship more readily with conspecifics than heterospecifics. Males of both species were significantly more likely to successfully transfer a macro to a conspecific than a heterospecific female (Lk_Lk: 19/23 males transferred a macro, Lk_Lp: 0/10 males transferred a macro, Lp_Lk: 0/9 males transferred a macro, Lp_Lp males: 10/15 males transferred a macro; Fisher's Exact Test for count data, $p=6.0 \times 10^{-8}$). Moreover, no macros were produced in heterospecific crosses. Even before the macro stage of the courtship sequence, differences were evident. The time from first antennal contact to first singing bout was shorter in the presence of conspecifics than heterospecifics for both *L. pruna* and *L. kohalensis* (Lk_Lk: n=17/23, 12.36 ± 12.92 min, Lk_Lp: n=8/10, 99.01 ± 84.37 min, Lp_Lk: n=8/9, 18.33 ± 15.64 min, Lp_Lp: n=13/15, 17.82 ± 38.26 min, ANOVA df=3, F= 9.148, $p= 8.87 \times 10^{-05}$, Fig. 2.7). While male *L. pruna* and *L. kohalensis* did not differ significantly in the time they took to produce their first micro in the presence of conspecific females (Lk_Lk: n=22/23, 62.33 ± 55.34 min, Lp_Lp: n=13/15, 37.48 ± 48.93 min, Welch's two sample t-test, t= 1.382, df=27.89, p=0.1779), only a single micro was produced in heterospecific crosses by a *L. kohalensis* male after 256.15 min. There was also no difference in the number of micros successfully transferred by males to conspecific females (Lk_Lk: n=21/23, 5.43 ± 2.23 micros, Lp_Lp: n=12/15, 4.40 ± 2.69 micros, two sample t-test, t = 1.0651, df = 25.177, p = 0.2969), but no micros were successfully transferred in heterospecific encounters (Lk_Lp: n=0/10, 0±0 micros, Lp_Lk: n=0/9, 0±0 micros).

Antennal Trials

A total of 71 trials were conducted but 10 were removed from analysis because they did not yield 30 minutes of data (i.e. contact did not occur within the first 15 minutes of video) and two males were removed because they escaped before the end of the trial. Both Lk and Lp males spent more time in contact with conspecific than heterospecific female antennae (Lk_Lk: n=16/18, 23.47 ± 7.75 min; Lk_Lp: n=13/18, 11.66 ± 6.86 min; Lp_Lk: n=11/13, 13.28 ± 3.89 min; Lp_Lp: n=19/20, 21.37 ± 9.80 min; Kruskal-Wallis chi-squared = 19.56, df = 3, p-value = 2.093×10^{-4}, Fig. 2.8A). Males of both species spent significantly more time singing in the presence of conspecific versus heterospecific female antennae (Lk_Lk: n=9/16, 5.44 ± 7.67 min; Lk_Lp: n=1/13, 0.01 ± 0.02 min; Lp_Lk: n=1/11, 0.04 ± 0.14min; Lp_Lp: n=12/19, 4.55 ± 8.01 min; Kruskal-Wallis chi-squared = 17.311, df = 3, p-value = 6.098×10^{-4}, Fig. 2.8B). Males of both species pumped more for conspecific females than for heterospecific female antennae, but Lk pumped the most towards conspecific female antennae overall (Lk_Lk: n=12/16, 30.28 ± 32.12 pumps: Lk_Lp: n=0/13, 0.0 ± 0 pumps; Lp_Lk: n=2/11, 0.18 ± 0.40 pumps; Lp_Lp: n=13/19, 10.05 ± 12.62 pumps; Kruskal-Wallis chi-squared = 26.049, df = 3, p-value = 9.314×10^{-6}, Fig. 2.8C). In trials included in further analysis, males of both species spent more time biting conspecific than heterospecific antennae, (Lk_Lk: n=13/16, 0.98 ± 1.60 min; Lk_Lp: n=5/13, 0.20 ± 0.49 min; Lp_Lk: n=11/11, 1.29 ± 1.43 min Lp_Lp: n=15/19, 2.72 ± 3.97 min; Kruskal-Wallis chi-squared = 12.469, df = 3, p-value = 5.94×10^{-3}, Fig. 2.8D). More than half of *L. pruna* males produced micros for conspecific antennae, but no males produced micros for heterospecific antennae, while *L. kohalensis* males

did not produce micros for either conspecific or heterospecific antennae. (Lk_Lk: n=0/16, 0.0 ± 0 micros; Lk_Lp: n=0/13, 0.0 ± 0 micros; Lp_Lk: n=0/11, 0.0 ± 0 micros; Lp_Lp: n=11/19, 0.58 ± 0.51 micros; Fisher's Exact Test for Count Data p-value = 8.656×10^{-7}, Fig. 2.8E).

Filter Paper Trials

Blank and *L. pruna*

All 10 trials where Lp males were exposed to blank filter paper versus Lp female exposed filter paper yielded usable data, though not all exhibited courtship behavior. Males did not spend significantly more time with one type of filter paper over another, (n= 10, LP %: 65.97 ± 25.78%, difference= 4.39 ± 11.71 min, one sample t-test, p-value = 0.082), though a highly significant difference emerges with the removal of one outlier data point (n=9, % LP: 72 ± 16.23%, difference =7.18 ± 8.15 min, t = 4.166, df = 8, p-value = 0.0031). Lp males did not differentially rub on one side (n=6, LP: 3.33 ± 5.85 rubs, blank 2.50 ± 2.59 rubs, difference: 0.83 ± 5.88 rubs, Wilcoxon-Mann-Whitney Test, Z = 0.54, p-value = 0.59) or pump (n=2, LP: 6 ± 7.07, blank: 0.0 ± 0, difference 3.33 ± 5.85, p-value = 0.59). A single male sang on the Lp filter paper side for 18.56 min and spent no time singing on the blank side.

Blank and *L. kohalensis*

Fifteen trials were conducted, fourteen of which were usable for further analysis. *L. pruna* males spent significantly more time with *L. kohalensis* filter paper than with blank filter paper (n=14, 64.86 ± 20.45% on LK filter paper, difference=

4.89 ± 7.48 min, one sample t-test, p-value = 0.018). Males did not differ on which side they exhibited rubbing (n=10, blank: 2.20 ± 2.78 rubs, LK: 2.40 ± 2.41 rubs, difference= 0.20 ± 2.57 rubs, Wilcoxon-Mann-Whitney Test, p-value = 0.66), in the amount of pumping they performed near each piece of filter paper (n=3, blank: 0.33 ± 0.58 pumps, LK: 0.67 ± 0.58 pumps, difference= 0.33 ± 1.15 pumps, Wilcoxon-Mann-Whitney Test, p-value = 0.56), nor in the amount of time they spent singing on each side (n=3, blank: 2.35 ± 4.04 min, LK 0.06 ± 0.10 min, difference=-2.29 ± 4.09 min, Wilcoxon-Mann-Whitney Test, p-value = 0.58).

L. pruna and *L. kohalensis*

Twenty-two trials were conducted with 17 producing usable data. Five were removed from analysis because the male failed to make contact with both pieces of filter paper. *L. pruna* males did not differentially spend more time with filter paper exposed to *L. pruna* females than with filter paper exposed to *L. kohalensis* females (n=17, % time on LP filter paper =46.91 ± 28.20%, difference =0.27 ± 6.60 min, One Sample t-test, p-value = 0.66). Males also did not differ where they rubbed their abdomens (n=8, LP: 2.63 ± 3.42 rubs, Lk: 3.75 ± 3.62 rubs, Wilcoxon-Mann-Whitney Test p-value = 0.27) or filter paper they associated with while singing (n=3, LP: 3.06 ± 4.65 min, LK: 0.31 ± 0.54 min, Wilcoxon-Mann-Whitney Test p-value = 0.58).

DISCUSSION

Male Mate choice and Species Boundaries

Historically, it has been assumed that males lack mate choice and would achieve maximal fitness by mating with any female given the chance (Andersson 1994, Janicke et al. 2016). Perhaps because of this, our understanding of the role males play in evolving or maintaining species boundaries has lagged behind our understanding of the role females play. Why males might be expected to discriminate against heterospecific females can be understood in at least two ways. First, a growing body of evidence suggests that males exhibit mating preferences under circumstances when choice would enhance fitness, such as in cases of role-reversed species (Petrie 1983, Flanagan et al 2016, Booksmythe et al. 2017), when females vary in fecundity and/or when male mating rate is limited by some resource (Bonduriansky 2001, Edward and Chapman 2011). If males can discriminate among conspecific females of varying quality in these circumstances, they should theoretically be able to discriminate between the cues or signals of conspecific and heterospecific females if such discrimination results in fitness benefits. Secondly, males may be secondarily selected to discriminate against heterospecific females if they experience a high rejection rate by heterospecific females (Roberts and Mendelson 2017). Males who initiate courtship with females only to be rejected after extensive energetic or resource expenditures may suffer lower lifetime reproductive success than males who discriminate between conspecific and heterospecific females and decide not to court heterospecifics. Therefore, when reproductive costs are high, we expect males to choose against heterospecific females. Note that this process does not result in the

phenomenon of "species recognition" *per se* (Mendelson and Shaw 2012), but simply, males that respond adaptively to mate choice decisions.

Gas Chromatography analysis

Here, I confirmed differences in whole body extracts between Lp and Lk found by Mullen et al. (2007, 2008). While Mullen et al. (2007, 2008) used a more stringent filter for investigating CHCs, I applied a more relaxed filter so that I could investigate additional compounds. While they examined 19 and 15 compounds in Lp and Lk, I found 43 and 46 compounds, respectively, and 56 compounds total between them. Further, I extracted CHCs from the antennae only and similarly found a difference in chemical signature between Lp (24 compounds) and Lk (13), for a total of 27 compounds. Given that males can discriminate between conspecific and heterospecific females based solely on access to the antennae, it is likely that males are using these differences in chemical signal to determine whether to initiate courtship.

Mating Trials

Lp and Lk are closely related species both found on the Big Island of Hawaii (Mendelson and Shaw 2005). When males were paired with conspecific females, I found that courtship proceeded at a high rate. However, when paired with a heterospecific female, courtship initiation was delayed or non-existent. Relative to conspecific pairings, males paired with heterospecific females took considerably longer to sing, and only one micro was successfully transferred (Fig. 2.7). Over longer-term exposure (of days to weeks), a low frequency of interspecific matings

have been observed in the lab and viable offspring were produced between these two species (Blankers et al. in preparation), reinforcing the hypothesis that premating barriers are responsible for the lack of courtship I observed. No macrospermatophores were produced during trials in the present study, however, and coupled with the long distance effect of an acoustic barrier to mate attraction (Shaw 2000), I conclude strong to near complete reproductive isolation between these two species despite their close phylogenetic relationship (Mendelson and Shaw 2005).

Although Lp and Lk differ greatly in pulse rate of the male mating song (both Glenwood and Halema'uma'u Lp sing at ~1.5 pps, Lk ~ 3.7 pps, Mendelson and Shaw 2005), the absence of heterospecific mating is likely influenced by the chemical modality as well. First, no males were observed to sing spontaneously before initial antennal contact was made during these trials. Therefore, rejection of males by heterospecific females due to differences in acoustic signaling only seems unlikely. Secondly, males paired with heterospecific females took longer to initiate courtship, both in latency to first song and first micro produced. If males showed no difference in the timing of courtship initiation towards conspecific and heterospecific females, it might suggest that males are indiscriminate while implicating female behavior as the cause of sexual isolation. However, my results show a courtship delay in heterospecific pairings, suggesting a behavioral barrier to courtship. Males cannot use acoustic cues to reject females because females are silent. However, males, through antennal contact with females, are likely using some signal associated with the antennae to accept or reject females. Overall, these results demonstrate a significant contribution to sexual isolation between Lp and Lk that is not attributable to acoustic

behavior, and that is likely attributable to male behavior.

Antennal Trials

To test if there was something specific about the antennae that allowed for discrimination among females, I gave males access to the antennae of either conspecific or heterospecific females. Access to conspecific and heterospecific antennae evoked distinct behaviors in males of both species. Males of both species spent more time in contact with conspecific compared to heterospecifc antennae (Fig. 2.8A), suggesting a greater interest in conspecific antennae. Further, males were much more likely to express courtship behavior and exhibited more intense courtship behavior toward conspecific females compared to heterospecific females (specifically, singing (Fig. 2.8B) and pumping (Fig. 2.8C)). Although Lp showed more aggression overall, Lp and Lk males differed in the time they spent biting conspecific versus heterospecific antennae and were more significantly more aggressive towards conspecific antennae (Fig. 2.8D). Higher aggression towards conspecific versus heterospecific antennae suggests that males are more likely to ignore heterospecific compared to conspecific females. In the bird literature, conspecific acoustic playback experiments are often used to test for reproductive isolation, wherein aggressive responses from resident males are interpreted as an indication of reproductive recognition (e.g. Ratcliff and Grant 1985), though this may also depend on whether habitat use overlaps (Reif et al. 2015). Elevated aggression toward conspecific antennae may indicate something similar in crickets. Lp and Lk males also differed in their likelihood to produce a micro during the trials, even towards conspecifics (Fig.

2.6). While males of neither species produced micros in the presence of heterospecific antennae, approximately 60% of Lp produced micros when exposed conspecific antennae while Lk males never produced micros during these antennal trials (Fig. 2.8E). This difference in probability of producing micros is puzzling, but several explanations are possible. First, some property of the antennae from Lk females may be sufficient to elicit initial courtship behaviors, such as singing and pumping, but more feedback from females is required to proceed to subsequent elements of the courtship, such as the production of micros. If female feedback is required for Lk males to produce micros, this additional information could not be conveyed because females were constrained into eppendorf tubes during these trials. These results suggest that access to the antennae is sufficient to influence the differential onset of courtship towards conspecific and heterospecific females and that some cue associated with the antennae, whether the movement, physical structure, or CHC blend, that allows males make this distinction.

The experiments of Rence and Loher (1977) found that stroking the antennae of *Teleogryllus commodus* crickets with the excised antenna of a male or female was sufficient to evoke aggressive or courtship behaviors, respectively, in blinded males. Further, they found if they stripped the chemical signal off the antennae, no differences in behavior were observed towards the antennae, suggesting there was a contact pheromone responsible for male discrimination (Rence and Loher 1977). Importantly, they found no differences in the antennal structure between male and females. Together, these results suggest that neither the structure of the antennae nor its pattern of movement are likely candidates to explain the behavioral differences

seen by Rence and Loher (1977). To my knowledge, there are no studies in the Orthoptera that find fine differences in antennal structure or antennal movement patterns between species. Therefore, while possible, it seems antennal movement and differences in fine structure of the antennae are less likely explanations for the differences I observed in this study.

Filter Paper Trials

When Lp males were allowed to choose between blank filter paper and filter paper exposed to Lp females, males spent more time in contact with filter paper exposed to conspecific females after an outlier was removed. However, very little courtship behavior was exhibited across trials, so drawing conclusions is tenuous. A similar response was seen when Lp males were exposed to blank versus Lk filter paper. Males preferred to investigate heterospecific cues over blank filter paper, but showed little courtship behavior. Finally, Lp males did not show a difference in contact time when exposed to Lp and Lk filter paper simultaneously. Again they showed little courtship behavior. These results are puzzling, as males seem to associate with cricket smells versus non-cricket smells, but the cue did not appear to be sufficient to consistently evoke early stages of male courtship like pumping and singing. Further, when comparing the difference in time Lp males spent interacting with Lp filter paper over blank filter paper (i.e. Lp contact minutes minus blank contact minutes) to the difference in time Lp males spent with Lk filter paper over blank filter paper (i.e. Lk contact mins minus blank contact mins), males did not show a difference in exploration time towards conspecific filter paper (blank vs. Lp

difference: 7.18 ± 8.15 min, blank vs. Lk difference: 4.89 ± 7.48 min, t = 0.69304, df = 21, p-value = 0.2479).

Other studies that have manipulated CHCs on filter paper have found mixed results. Paul (1976) found that males increased antennal rate and initiated calling song shortly after exposure to filter paper exposed to females in three species of *Allonemobius* and one species of *Pictonemobius* crickets. Wehi et al. (2017) found that male wetas discriminated between tree cavities lined with female-filter paper, but did not discriminate between male versus female filter paper or conspecific-heterospecific filter paper. Finck et al. (2016) found that male *Chorthippus* sp. grasshoppers discriminate between conspecific and heterospecific as well as male and female conspecific CHC extracts applied to filter paper. Therefore, the lack of behavioral discrimination of *L. pruna* males when directly choosing between Lp and Lk filter paper is not unusual.

CONCLUSIONS

This chapter explored reproductive isolation between *L. pruna* and *L. kohalensis* and how CHCs may contribute to sexual isolation from the male perspective. Compared with conspecific interactions, I found that Lp and Lk are far less likely to initiate or persist in courtship with heterospecific females. Trials that gave males access only to the antennae of conspecific or heterospecific females suggest that some cue associated with the antennae is sufficient to evoke courtship from conspecifics, but not heterospecifics. One possible signal is the differential expression of CHCs, both on the antennae and on the body as a whole, which is

known to play a role in social behaviors in insects (Blomquist and Bagneres 2010). Overall, these results highlight the importance of chemical signals in sexual isolation early in divergence and the roles males may play in reproductive incompatibility.

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FIGURES AND CHARTS

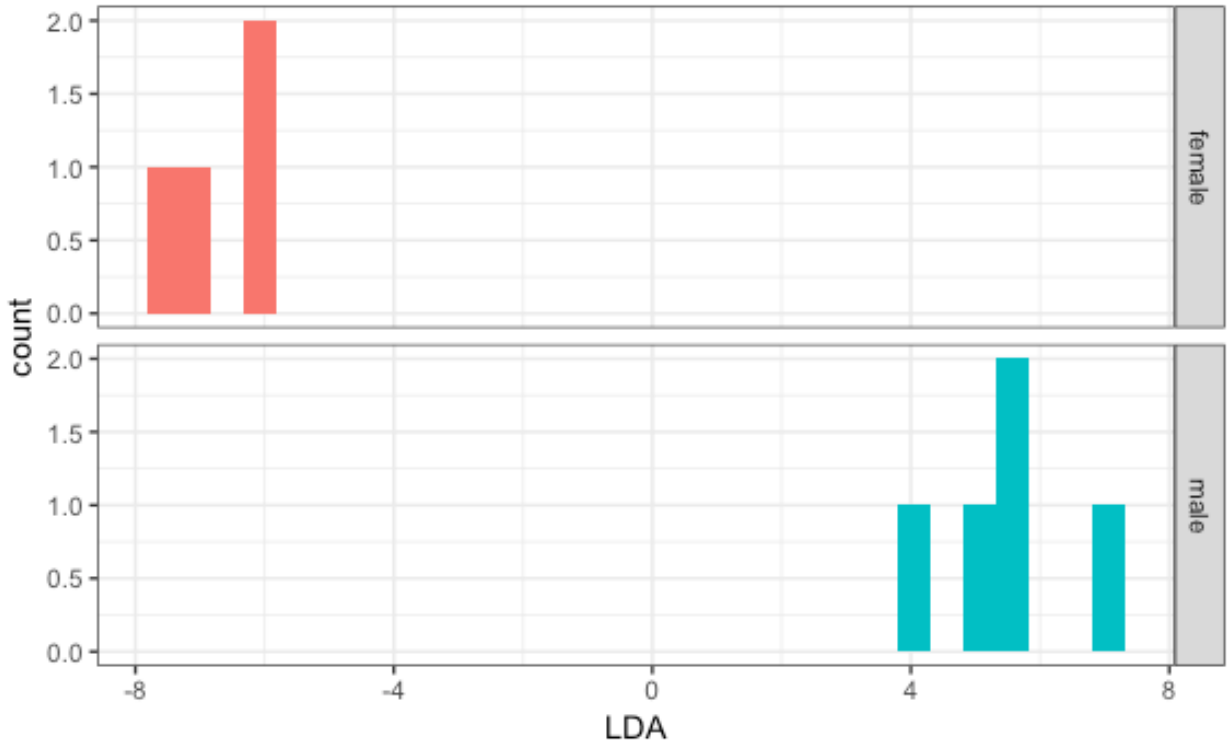

Figure 2.1: LDA discrimination between male and female *Laupala pruna* whole body extracts

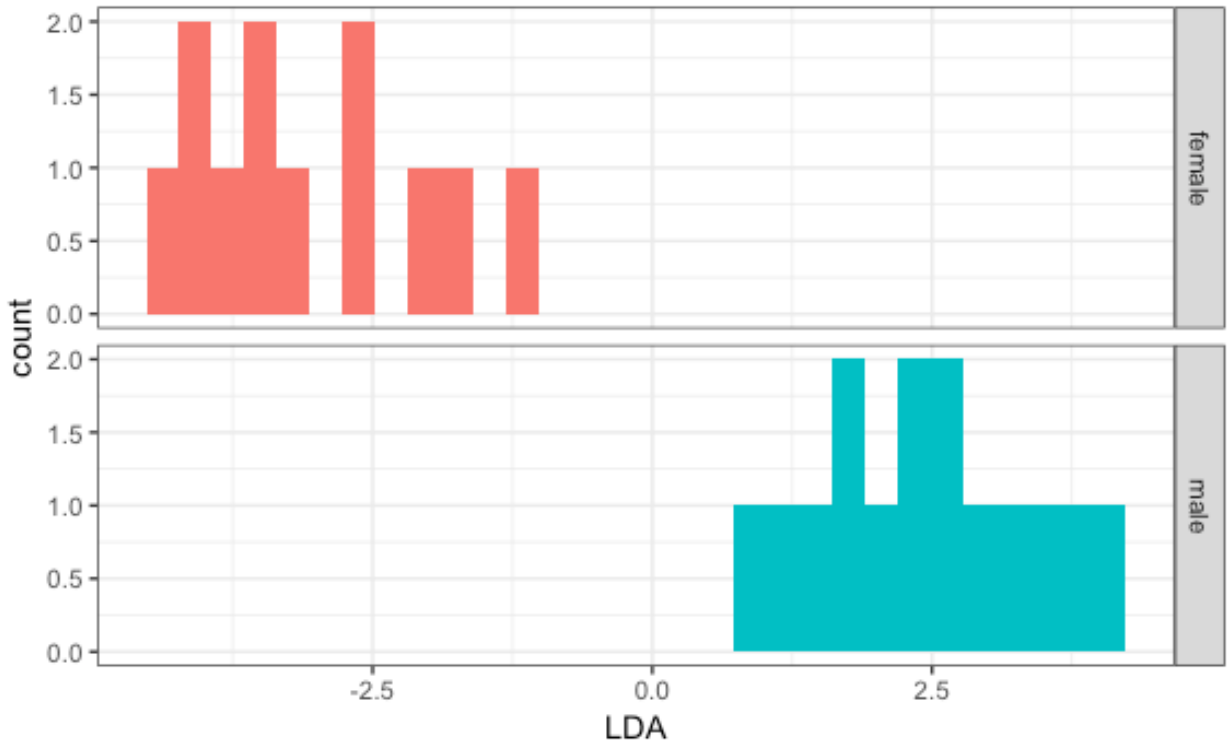

Figure 2.2: LDA discrimination between male and female *Laupala kohalensis* whole body extracts

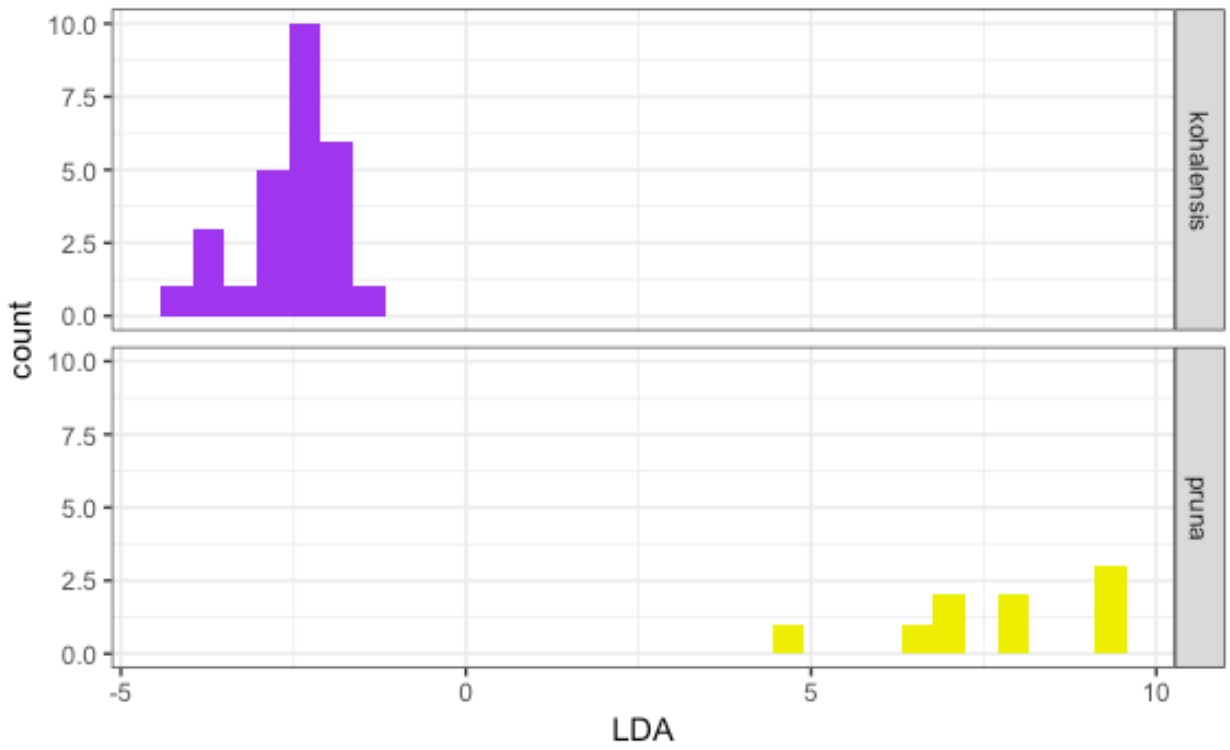

Figure 2.3: Comparison of LDA scores between *L. pruna* and *L. kohalensis* whole body extracts. Only peaks shared between both species were used

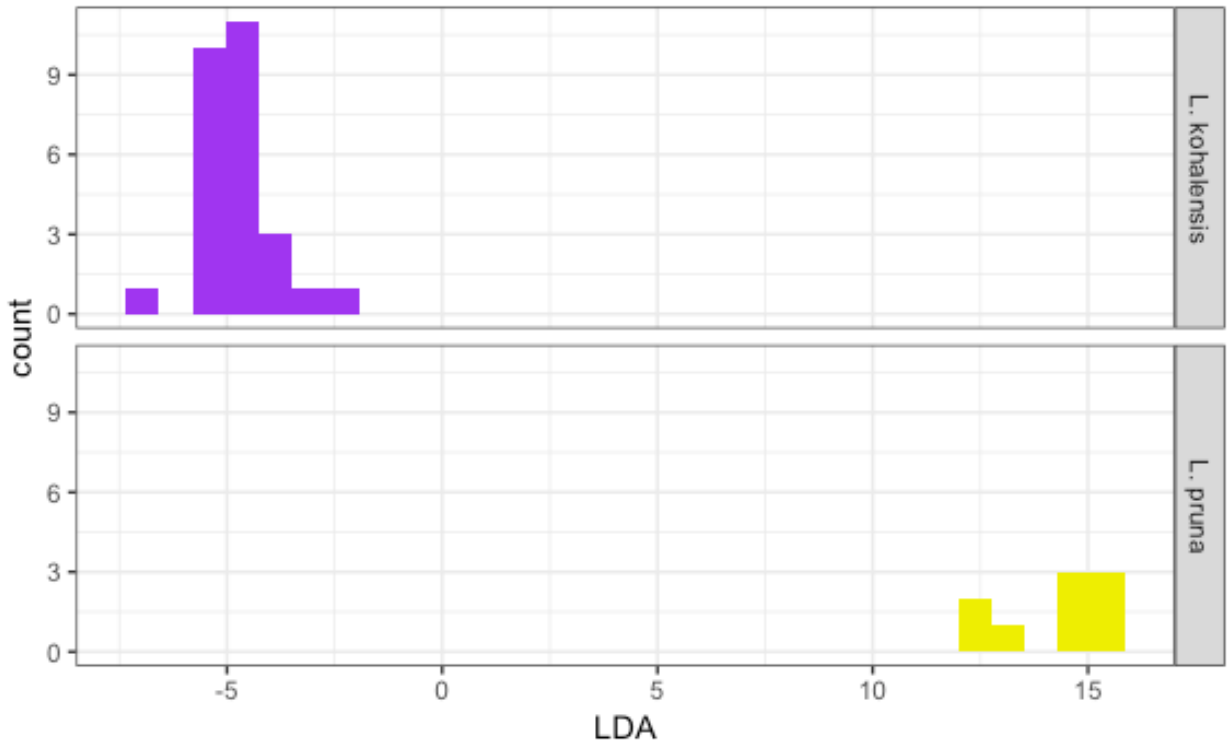

Figure 2.4: Comparison of LDA scores between *L. pruna* and *L. kohalensis* whole body extracts. All selected peaks, including those unique between each species were used

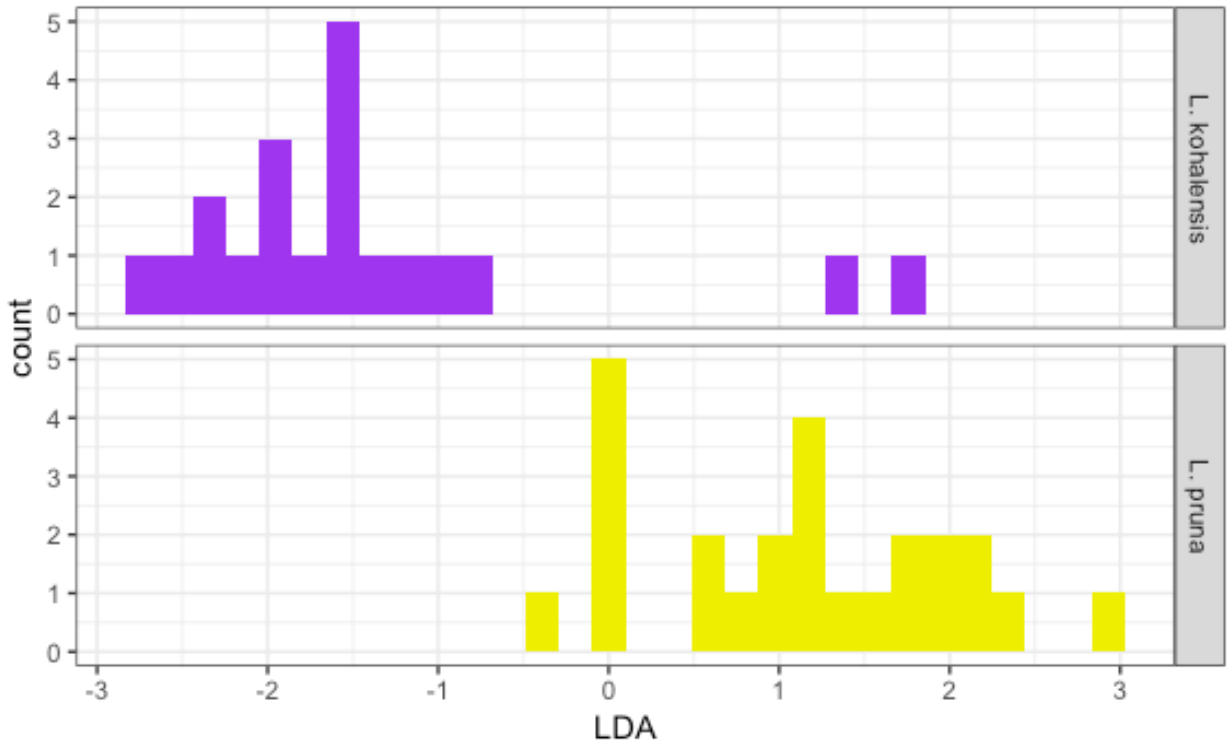

Figure 2.5: Comparison of LDA scores between *L. pruna* and *L. kohalensis* antennal extracts.

Only peaks shared between both species were used

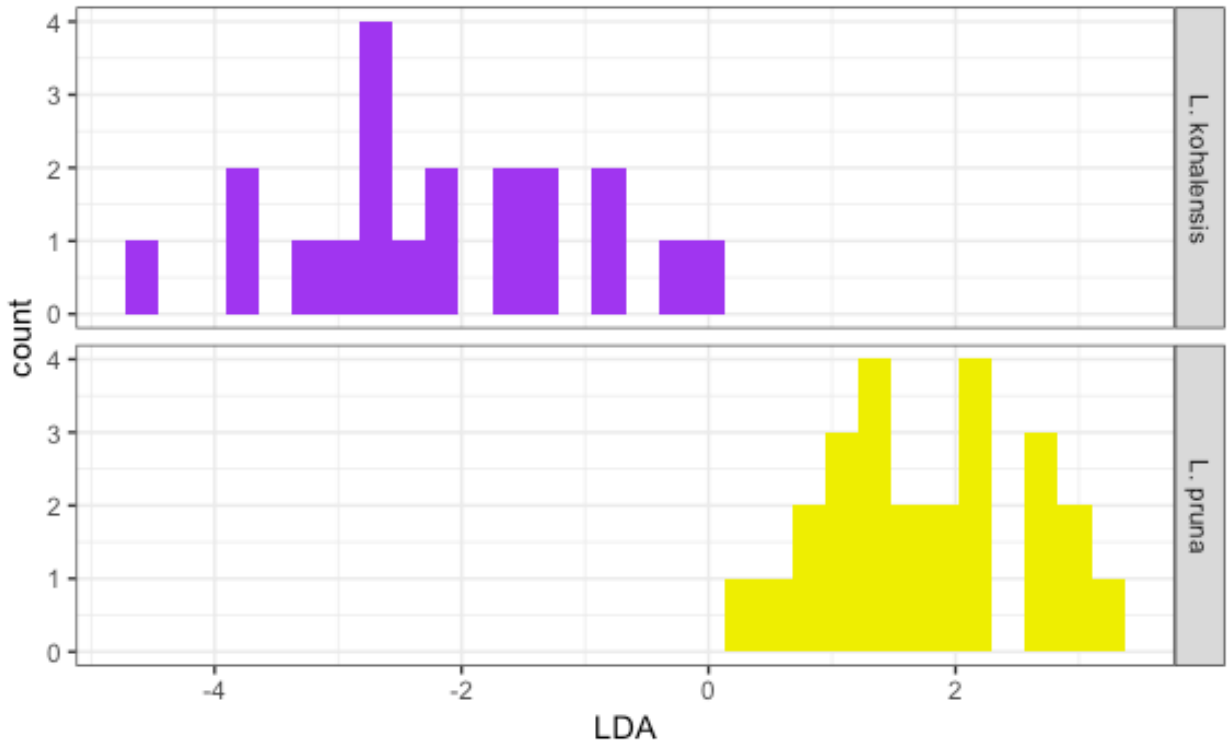

Figure 2.6: Comparison of LDA scores between *L. pruna* and *L. kohalensis* antennal extracts. All selected peaks, including those unique between each species were used

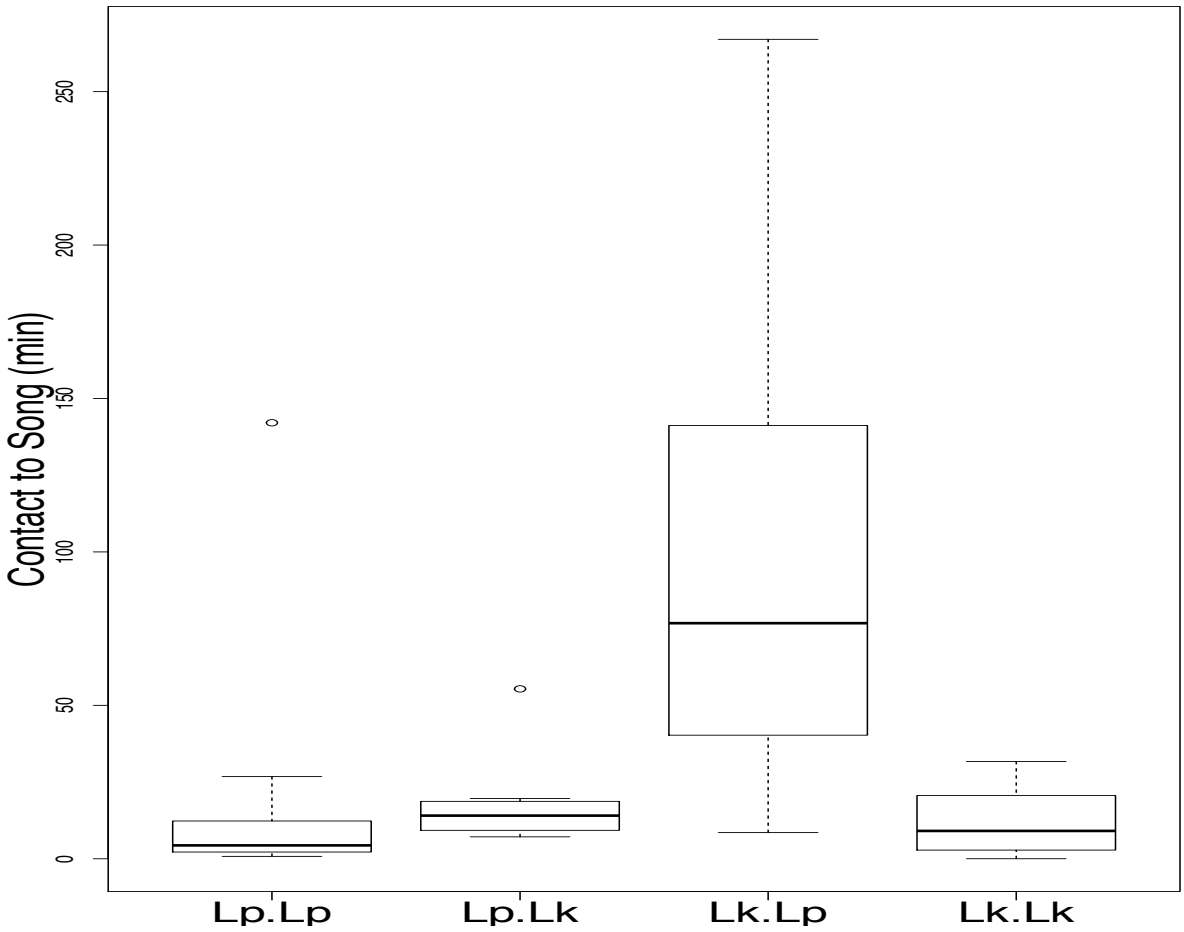

Figure 2.7: Mating trial experiments between *L. kohalensis* (Lk) and *L. pruna* (Lp). Time (min) from first antennal contact to first courtship song bout.

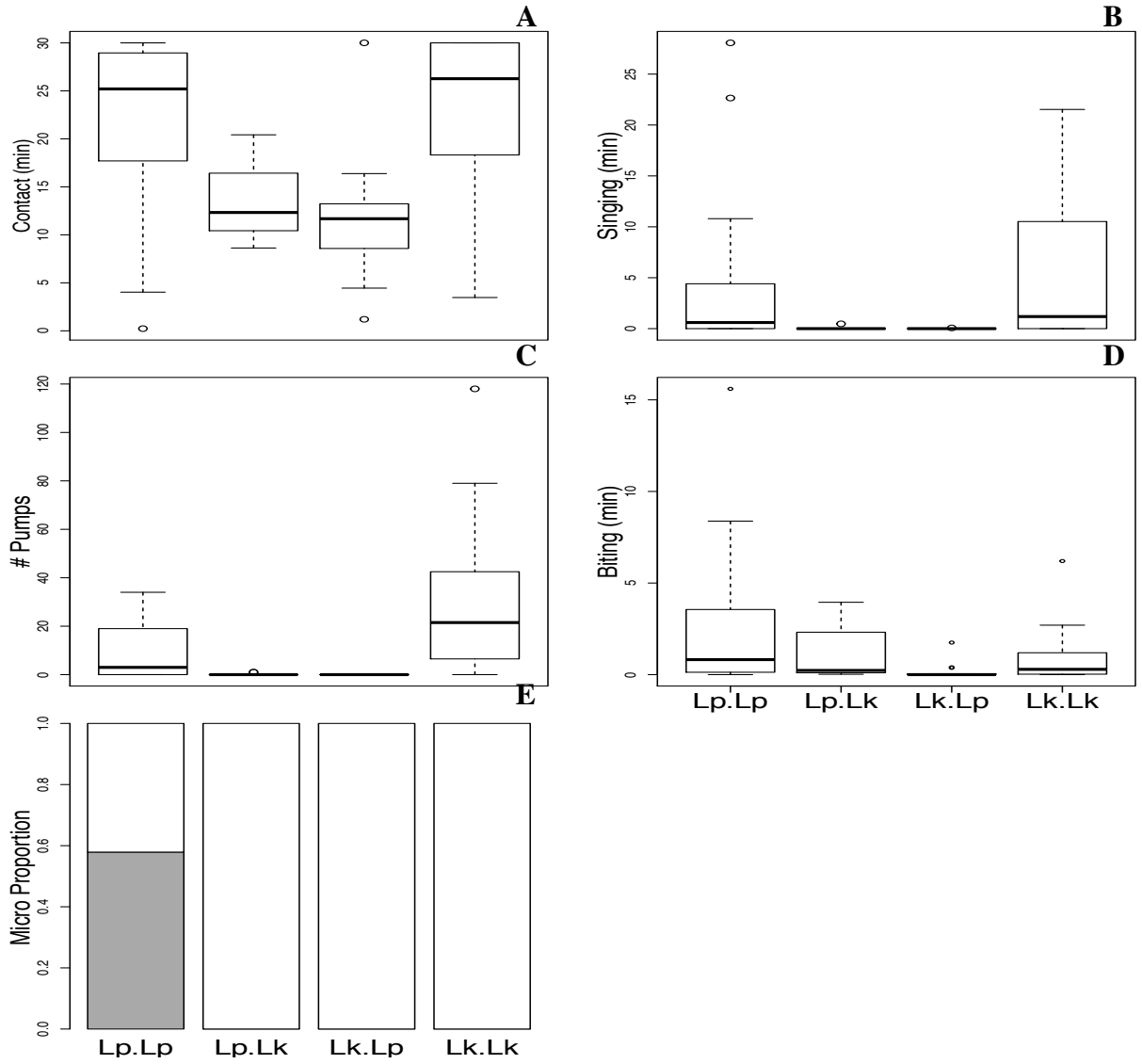

Figure 2.8: Results of antennal behavioral trials Pairs are male.female. Lp=Laupala pruna Lk=L. kohalensis A) The total time (min) males spent in contact with conspecific versus heterospecific female antennae B) Total time (min) males spent singing after contact with heterospecific versus heterospecific female antennae C) The total number of times males pumped near conspecific versus heterospecific female antennae D) The total time males spent biting conspecific and heterospecific female antennae E) The proportion of males producing a microspermatophore (micro) in the presence of conspecific versus heterospecific female antennae

Table 2.1: Description of behaviors and scoring method for antennal trials and filter paper trial

Behavior	Description	Scoring Method (antennal trials)	Scoring Method (filter paper trials)
Contact	Focal male's antennae touch antennae of experimental individual	Contact begins when the experimental individual's antennae are touched and observably displaced by the focal individual. Contact ends when the male leaves the circle around the experimental individual	Begins when male places more than one foot on filter paper until male is completely off filter paper
Song	Male raises wings ~45 to 90˚from body and stridulates them against each other	The beginning (wing raising) and end (wings down) of song bouts, as long as the wings opened and closed at least once (i.e. one pulse).	Same as antennal trials, but assigned to the half of the petri dish male began singing on
Pumping	Either 1) Male thrusts his abdomen up a single time (silent) or vibrates slightly (while singing)	Individual pumps were counted when singing or silent and combined together	Same as antennal trials, but pumps were assigned to the side on which they occurred or if on line, the side the male was on last.
Rubbing	Male twists his body ~90˚ and drags his body across some surface of the arena	Rubbing behavior was not tallied during antennal trials	Single count with each drag against the petri dish or filter paper. Assigned to side male was on or last on if on the line.
Micro	Male everts genitalia and fills a spermatophore	Presence or absence of micros based on when genitalia eversion was noted	Males did not produce micros during filter paper trials

Table 2.2: Retention times that differed between whole body extracts of male and female *Laupala pruna* CHCs after Bonferroni correction (α=0.05/42=1.19x10^{-3}) Df=7 for all.

Retention Time (min)	t-statistic	p-value
19.80	-6.8099	2.5 x10^{-4}
25.11	5.3255	1.1x10^{-3}
26.02	-7.7403	1.13x10^{-4}

Table 2.3: Retention times that differed between whole body extracts of male and female *Laupala kohalensis* CHCs after Bonferroni correction (α=0.05/47=1.064x10^{-3}) Df=25 for all.

Retention Time (min)	t-statistic	p-value
16.72	-5.328	1.599 x10-^{5}
18.24	-4.3181	2.18 x10^{-4}
19.29	-7.9942	2.381 x10^{-8}
21.29	-9.4437	1.005 x10^{-9}
22.05	-8.0296	2.197 x10^{-8}
23.68	4.6995	8.12 x10^{-5}
24.53	-4.3226	2.16 x10^{-4}
24.69	-5.0668	3.14 x10^{-5}
25.18	6.8432	3.579 x10^{-7}
25.32	4.043	4.44 x10^{-4}
25.59	-9.951	3.54 x10^{-10}
25.65	-7.0801	2.021x10^{-7}
25.99	-3.7334	9.79 x10^{-4}
26.04	-9.8867	4.033 x10^{-10}
29.47	-4.1488	3.38 x10^{-4}
29.80	6.311	1.324 x10^{-6}

Table 2.4: Retention times that differed between antennal extracts of female *L. pruna* and *L. kohalensis* CHCs after Bonferroni correction (α=0.05/27=1.085x10^{-3}) Df=43 for all.

Retention Time (min)	t-statistic	p-value
16.71	5.2169	4.961x10^{-6}
17.92	-5.8392	6.263 x10^{-7}
19.76	-4.5667	4.126 x10^{-5}
20.55	-3.5797	8.69 x10^{-4}
21.47	5.2268	4.801 x10^{-6}
22.38	-4.1133	1.73 x10^{-4}
23.25	-3.5019	1.091x10^{-3}
25.05	6.412	9.193 x10^{-8}
27.49	6.1882	1.946x10^{-7}

CHAPTER 3

GEOGRAPHIC VARIATION IN MULTIMODAL SIGNALS IN HAWAIIAN SWORDTAIL CRICKETS (GENUS *LAUPALA*)

Introduction

The processes by which new species are generated are mysterious (Darwin 1869), but recent changes in framing the problem are enabling progress. Speciation begins as a population-level process, where, for example, natural or sexual selection acts on a phenotype in one population, causing it to diverge from the phenotypes of other populations within a species. Although much of the speciation literature has focused on "isolating mechanisms" and their genetic bases (Coyne and Orr 2004, Moyle and Payseur 2009, Presgraves, 2010, Mack and Nachman 2017), several authors (e.g. Harrison 1998) have argued that these barriers are consequences, rather than causes, of the speciation process. Shaw and Mullen (2011) proposed an alternative approach that focuses on diverging phenotypes between populations, the evolutionary forces that cause their differentiation, and their putative roles as speciation phenotypes.

Groups of closely related species are compelling subjects for the study of speciation because they offer the opportunity to compare phenotypic variation in the context of recent evolutionary differentiation. This recent phylogenetic context can inspire hypotheses about the phenotypic causes of speciation and a hypothesized role for selection in bringing about diversification. Organisms are complex compositions of phenotypes that contribute to biological processes related to fitness such as securing food (White 1984, Lihoreau et al. 2015), avoiding predators (Festa-Bianchet 1988, Rito et al 2016), finding potential mates (Gibson and Langen 1996, Gibson and Cocroft 2018) and convincing a potential mate that they are worthy of reproductive

investment (Andersson 1994, Arnold et al. 2017). Divergent selection in any of these different phenotypes can propel geographically or genetically isolated populations to differentiate, and potentially contribute to the build up of reproductive barriers leading to speciation.

Courtship behaviors and associated morphological phenotypes often differ conspicuously among closely related species of animals, for example in color (Endler and Mappes 2017), sound (Snijders and Naguib 2017), and scent (Wyatt 2014). Sexual and/or ecological selection acting on such traits can lead to their divergence between populations in the absence of the homogenizing effect of gene flow, if such divergence results in assortative mating. It is then straightforward to hypothesize that changes in courtship signals among diverging populations can result in depressed gene flow and the genetic separation that characterizes speciation (Servedio and Boughman 2017). However, a challenge still remains in connecting divergence in courtship traits to speciation. Courtship signaling is often complex, integrating several sensory modalities (Candolin 2003, Greig and Webster 2013, Papaj and Hebets 2005, Partan and Marler 2005, Uy et al. 2009). Understanding how courtship repertoires evolve and contribute to reproductive isolation will require determining the contributions of each component to assortative mating of diverging populations early in the speciation process.

Showing that divergence in courtship signaling can cause speciation is further complicated by how long it takes to complete speciation. While interspecies comparisons can inspire hypotheses, speciation begins as an evolutionary process within species. Therefore, the best context for the study of speciation occurs when closely related species differ in the same traits that vary within and among intraspecific populations, suggesting that the same processes that caused speciation are still at play within existing species. Although directly observing the process of

speciation may be extremely rare (Lamichhaney et al. 2018), identifying variation in traits relevant to reproductive divergence within and across populations, and among close species relatives, will provide contexts from which a general understanding of speciation can emerge.

Hawaiian swordtail crickets (genus *Laupala*) are a group of 38 identified species found across the larger islands of Hawaii (Otte 1994, Shaw 2000). They are best known for their rapid speciation; thought to be fueled primarily by divergence in the pulse rate of male courtship song and female acoustic preference (Mendelson and Shaw 2005). Further, Mendelson and Shaw (2005) found evidence for two independent radiations across the islands, both going from older islands to younger islands. The consequence is the formation of the "cerasina" and "pacifica" clades. While song has been a primary focus in the diversification of this group, Mullen et al. (2007) demonstrated that some species in the pacifica clade of *Laupala* also differ markedly in their expression of cuticular hydrocarbons (CHCs), blends of long chain waxes known to aid is desiccation resistance, as well as chemical communication across insect species (Blomquist and Bagneres 2010). *Laupala* courtship has been studied in detail (Shaw and Khine 2004, de Carvalho and Shaw 2009) and involves sustained interactions between males and females where singing and antennation behaviors are expressed repeatedly. Moreover, recent evidence demonstrates the involvement of chemical communication in mating behavior and species boundaries in this genus (See chapters 1 and 2). As both acoustic and chemical communication are involved in courtship and differ markedly between closely related species, observable differences in these phenotypes should exist among populations within a species as well.

Laupala pruna is part of the pacifica clade on the Big Island, the youngest and most rapidly radiating part of the *Laupala* genus (Mendelson and Shaw 2005, Otte

1994). This species is distributed from north to south along the windward side of the Big Island of Hawaii (Fig. 3.1) (Otte 1994). Its range overlaps with much of the explored range of *L. cerasina* (Otte 1994, Mendelson and Shaw 2005, Grace and Shaw 2011), though *L. pruna* has not been as extensively studied as *L. cerasina*. One difference between these two clades is that *L. cerasina* is the only species on the island of Hawaii from the cerasina clade whereas several species in the pacifica clade inhabit Hawaii (Mendelson and Shaw 2005). Thus, diversification in the pacifica clade, may be ongoing and therefore should show evidence of incipient diversification. In order to explore this possibility, I investigated the diversification of song and chemical communication characteristics across the range of *L. pruna*. I quantified geographic variation in both male mating song and cuticular hydrocarbon (CHC) components, which then allowed me to test two main hypotheses. First, I tested whether these characteristics follow a simple isolation by distance model of phenotypic divergence (e.g. Rousset 1997), hypothesizing that CHC profiles and song characteristics should greater phenotypic differences with increased geographic distances. . Second, given the importance of both of these signals in *Laupala* courtship and their differentiation among species, I hypothesized that song and CHC variation diverge in tandem as part of a multicomponent behavioral repertoire (Hebets and Papaj 2005).

MATERIALS AND METHODS

Sample Collection

Laupala pruna were collected from the Big Island of Hawaii from 9 locations, hereafter referred to as populations. Sites were chosen that span different ages of

volcanic substrate and capture most of the range of *L. pruna* (Otte 1994) across Mauna Kea, Mauna Loa, and Kilauea (Fig. 3.1). Kaiwiki Rd (KW, 2014, 2016 collections) is near the most northern part of *L. pruna*'s range and is located on Mauna Kea. Glenwood Road (GW, 2012, 2014), Halema'uma'u, Trail (HM, 2012), and Naulu Trail (NT, 2016) are found on the southeast part of the island of Hawaii on Kilauea's flanks, Hawaii's youngest island volcano. Ola'a Flume (OF, 2014), Stainback Hwy (SB, 2016), Kea'iwa (KI, 2012), Alili Springs (AS, 2016), Kaiholena (KH, 2012) are found on Mauna Loa, which is of intermediate age to Mauna Kea and Kilauea. A mix of adults and nymphs were brought back to a laboratory setting in Ithaca, NY. Crickets were given at least two weeks to acclimate to lab conditions before song recordings or being frozen for later CHC analysis. Since *L. pruna* and *L. cerasina* are morphologically cryptic and co-occur, females can be harder to identify than males because they lack a distinguishable calling song. Males were identified on the basis of song (Otte 1994). Females were paired with males of known identity and were observed for initiation of male courtship. Females receptive to *L. cerasina* courtship were removed form analysis. Females of indeterminable identity were frozen along with identified *L. pruna* females. Nymphs were allowed to mature before song testing or being frozen for CHC analysis. All individuals were given at least two days in isolation after song recordings before being frozen to account for any physiological or mechanical transfer of CHCs that may come with interacting with a member of the opposite sex. In addition, males and females from a lab stock of *L. kohalensis* collected from Pololu Valley in 2007 were used as an out-group for CHC analysis. All crickets were kept in the lab on a 12:12 light: dark cycle in a temperature controlled

room held at 20˚C.

Song Recording and Analysis

Male song was recorded using an Olympus WS-801 digital recorder (Olympus Corporation, Tokyo, Japan). Air temperature at time of singing was also noted. A female from the same population was placed with males to encourage singing (male *Laupala* only have one kind of song). Songs were recorded as an MP3 and converted to .wav files using iTunes (Apple Inc.; Cupertino, CA, USA). Sound files were analyzed using RAVEN Pro v.1.5 (Bioacoustics Research Program, Cornell University Lab of Ornithology; Ithaca, NY). From each song, five nonconsecutive measurements were taken of pulse period, pulse duration, and carrier frequency. The pulse rate was calculated by taking the inverse of the pulse period (the time from the beginning of one pulse to the beginning of the next pulse). The carrier frequency is calculated as the dominant frequency of the pulse. Pulse duration is calculated from the beginning of each pulse to its end. Pulse rate and pulse duration were measured to the nearest 0.001s and carrier frequency was measured to the nearest 0.1Hz. The dimensionality of pulse rate, pulse duration, and carrier frequency were reduced using principal components analysis. Principal components with eigenvalues greater than 1 were input into a linear discriminant analysis (LDA) to determine if males could be assigned to their population based on their song. Data analysis was performed using R studio (v. 1.0.13, R Studio Team 2015).

CHC Preparation and Sample Analysis

All glassware was rinsed twice with hexane prior to use and forceps were cleaned with two washes of hexane between samples. Individuals were extracted in 300µl of HPLC grade hexane for 5min. Vials were swirled gently for ~5s at the beginning and end of the 5min period. Individuals were then removed from the hexane and frozen. Extracts were then run through a hexane-rinsed Pasteur pipet with glass wool to remove particulate matter from the bodies that would clog the GC column and injector. Samples were blown down under a gentle N2 stream and resuspended in 50µl of heptane.

A Shimadzu AOC-20i autoinjector was used to inject 1µl samples into a Shimadzu GC-2014 gas chromatograph with flame ionization detection (GC-FID) equipped with a HP-5 column (20m, 0.180mm diameter, and a film thickness of 0.18um. Runs consisted of a starting temperature of 60˚C held 1 min, a ramp 20˚C/min to 200˚C. The temperature was then ramped 5˚C/min to 320˚C and held for 15 min, yielding a total run time of 47 min. The FID was set to 340˚C and a sampling rate of 40 msec. The injection port was 300˚C with a pressure of 144.2 kPa. The total flow rate of gasses was 10ml/min with column flow of 0.80 ml/min, and purge flow of 3.0ml/min, yielding a linear velocity of 32.5 cm/sec. The split ratio was calculated to be 7.7 given the other parameters.

Peaks for all samples across all populations and species were first manually integrated and then binned by retention time and patterns of peaks were confirmed by eye within each population. Within each population, retention times that were shared by at least 75% of the individuals in each population were selected and the remaining

missing values for these selected peaks were manually integrated so that all individuals had a value for each retention time. Consequently, peaks were initially independently determined in each population. Bins for all populations were then combined, which resulted in presence/absence differences among populations. Because one of the objectives of this study was to quantify differences in CHC expression in *L. pruna*, I sacrificed the ability to identify the structure of individual peaks in order to be more quantitatively more accurate. Using GC-MS would allow for structural identification, but at the cost of accuracy of abundance (Pacchiarotta et al. 2010). Another difficulty in not being able to determine the identity of individual peaks was that if peaks co-eluted or were shoulder peaks, I was not able to separate them. Consequently, I took a more conservative approach of combining any peaks overlapping in retention time into a single value. Again, any missing values were manually integrated so that all individuals had a value at each retention time across all populations. To normalize the data, values were log transformed using the formula $Z_{i,j} = \log(X_{i,j}/g(X_j))$, (Aitchison 1986, Mullen et al. 2007)

I ran a principal components analysis (PCA) and a linear discriminant analysis (LDA) which included chromatographic data from *L. kohalensis* to provide a reference point for how variation in *L. pruna* populations compares with another closely related species. I then reran the PCA and LDA without *L. kohalensis* to examine how individuals were assigned among populations.

.To determine the level of sexual dimporphism within populations of *L. pruna*, one overall PCA and independent LDAs for each population were carried out in R Studio (R Development Core Team 208) for each population. Only populations with a

total sample size (i.e. both males and females) of 20 individuals were used. Principal components with eigenvalues greater than 1 were then input into an LDA to determine if males and females could be assigned to their sex based on their chemical profiles. Subsequently, what retention times might differ between sexes were tested by performing t-tests on the log-transformed values and using a Bonferroni correction for multiple comparisons.

Geographic distance, song variation and CHC expression

Using the PCA above, I performed a series of Mantel tests to explore if a relationship between song, CHCs, and geographic distance exists within *L. pruna,* Geographic distances (Euclidean) were calculated using the ruler tool on Google Earth. Collection locations were estimated within 1 s of the coordinate reported in Fig. 3.1. A Mantel test was performed separately on the mean population PC scores of song and geographic distance. Next, a series of Mantel tests (one test per principal component) were conducted with the mean population CHC PC loading scores and geographic distances. Two sources of PC scores were used to calculate population mean values. I first performed a PC analysis with both males and females together on all PCs with eigen values greater than 1. Because results (below) indicated that CHC composition is significantly different between males and females, I also conducted separate Mantel tests on males and females of *L. pruna* .

Comparing song and CHC expression

Several different phenotypic patterns might be observed across the range of *L. pruna*.

Four possibilities and their causes are 1) random drift (Fig. 3.2A), stabilizing selection (Fig 3.2B), isolation by distance or directional selection (Fig. 3.2C), or it may follow the age of volcanoes (Fig. 3.2D). The volcanic ages follow a non-linear progression (Fig. 3.1) and so if volcanic origins explain these differences, a step-like pattern might be observed. The distributions of these phenotypes may occur together or independently of one another. To determine if there is a geographic relationship between song and CHC expression, I tested for a correlation between male *L. pruna* PC1 scores to each song parameter (pulse rate, carrier frequency, and pulse duration). PC1 was selected because by definition it explains the greatest proportion of independent data and is therefore the most likely to capture the variation in CHC expression among males. A positive relationship between CHC PC1 and pulse rate would suggest that populations with similar CHC PC1 scores tend to have similar songs as well, whereas the lack of a linear relationship would suggest that CHCs and song evolve independently.

RESULTS

Song Analysis

Songs were recorded and analyzed from a total of 102 males and recording temperatures were kept relatively constant (20.07˚ ± 0.45 C, Table 3.1). An ANCOVA showed that there is significant heterogeneity in pulse rate among populations of *L. pruna*, when controlling for the effect of temperature ($F=30.79_{8,101}$, $p<2 \times 10^{-16}$; there was no temperature x population effect: Fig. 3.3). There was also significant heterogeneity in carrier frequency among populations of *L. pruna* when controlling for

temperature ($F=2.753_{8,101}$, $p=9.41 \times 10^{-3}$; there was no temperature by population interaction; Fig. 3.4). Lastly, an ANCOVA showed that there is significant heterogeneity in pulse duration among populations of *L. pruna* when controlling for the effect of temperature ($F=4.351_{8,101}$, $p=1.98 \times 10^{-4}$; there was no interaction between temperature and population; Fig. 3.5).

The principal components analysis of song resulted in two principal components with eigenvalues greater than 1, explaining 69.59% of total variation. The LDA correctly assigned 44 of 103 males to their population of origin based on their song characteristics (df=8 Wilks λ =0.302 approx. $F=9.52_{16,186}$, num df=16, den df=186, $p< 2.2 \times 10^{-16}$, Table 3.2a; Fig. 3.6).

Gas Chromatography Analysis

Population comparisons: I ran a total of 224 samples from 9 populations and 2 species through the gas chromatograph. One male and four female samples were excluded from further analysis because of markedly different CHC expression and were assumed to be *L. cerasina,* resulting in 219 samples for analysis (192 *L. pruna*, 27 *L. kohalensis*). Overall CHC abundance (i.e. the sum of all peaks before log transformation for each individual) differed among a few population and species comparisons (ANOVA, df=9,sum sq.= 1.883×10^{14}, mean sq.=2.093×10^{13}, F=3.854, p= 1.55×10^{-4}). A subsequent Tukey test revealed that the significant differences were between LK and AS (diff= -2488477.19, lower= -4516717.3, upper=-460237.1, p=4.56×10^{-3}) and LK and KH (diff= -2364748.04 lower= -4240473.4 upper= -489022.7 p=3.05×10^{-3}).

Cuticular hydrocarbon profiles of males and females combined varied markedly among populations and species (log transformed; Fig. 3.7). Considering both *L. pruna* and *L. kohalensis* together, principal components analysis yielded 5 PCs explaining 77.47% of the variation (Fig. 3.8). A LDA analysis on these 5 PCs found significant overall differences among populations and species in CHC constitution (MANOVA, Wilks $\lambda = 1.46 \times 10^{-4}$, approx F= 126.88, num Df= 45 den, Df =920.12, $p < 2.2 \times 10^{-16}$, Fig. 3.9) and a subsequent PCA and LDA only on *L. pruna* populations was correctly able to assign 172 out of 192 samples to their source population (Table 3.2b). All analyzed peaks differed between populations of *L. pruna* after multiple ANOVA tests with Bonferroni correction, with a maximum p-value of 0.014 (3.3).

Male-Female Comparisons

I next compared males and females within populations to determine whether sexual dimorphism exists among populations. Five of the nine populations had at least 20 sample individuals to perform male-female analysis: Halema'uma'u, Naulu Trail, Kea'iwa, Alili Springs, and Kaiholena. Nineteen females and 20 males were examined from Halema'uma'u. The LDA correctly assigned 16/19 females and 17/20 males with a statistical difference between males and females (Wilks $\lambda = 0.60238$, approx. F=3.5205, num Df= 6 , den Df=32, $p = 8.68 \times 10^{-3}$, Fig. 3.10A). Samples from the Naulu Trail consisted of 7 females and 16 males. The LDA was able to correctly assign 6/7 females and 15/16 males resulting in a statistical difference between them (Wilks $\lambda =$ 0.36308, approx. F= 4.6778, num Df= 6, den Df= 20, p=0.016, Fig. 3.10B). The LDA was also able to correctly assign all 11 males and all 9 females. A MANOVA test on

the identified PCs indicates that male and female Kei'iwa are significantly different in their CHC expression (Wilks λ= 0.3344, approx. F= 4.3127, num Df= 6, den Df= 13, p=0.013, Fig. 3.10C). The LDA correctly assigned 10 of 13 females and 13 of 14 males from Alili Springs with a statistical difference between males and females (Wilks λ =0.48793approx F= 3.4983, num Df= 6, den Df= 20, p= 0.016, Fig. 3.10d). For Kaiholena, the LDA correctly predicted 15 of 20 females and 13/18 males with a statistical difference between males and females (Wilks λ= 0.67282, approx. F= 2.5124, num Df= 6, den Df=31, p=0.042, Fig. 3.10E). Despite the statistical differences among males and females in these populations, multiple t-tests with Bonferroni corrections failed to indicate specific CHCs that differed between males and females of any given population.

Song, CHC Expression, and Geographic Distance

A PCA on song revealed 2 PCs with eigenvalues greater than one explaining 69.59% of the variation. Mantel tests on song PCs found that PC2 (but not PC1) was significantly associated with distance (Mantel R= 0.334, p=0.0269). When analyzed together, male and female CHCs resulted in 6 PCs with eigenvalues greater than one explaining 84.13% of the variation. A Mantel test on PC values of males and females combined found a near significant relationship between PC3 and distance (Mantel R=0.249, p=0.0631). Mantel tests examining males and females versus geographic distance independently found no relationship between any of the 6 PCs and geographic distance (p=0.12 to 0.74).

Relationship Between Song Parameters and CHC Profiles During Population Divergence

There was significant heterogeneity among males in PC1 (ANOVA, df=8 sum sq.=455.4 mean sq.=56.93 F=92.12, $p<2x10^{-16}$, Fig. 3.11). There was also a significant positive correlation between PC1 of the male CHC profile and pulse rate (linear model, Estimate= 1.45, multiple R^2= 0.6227, $F_{1,7}$=11.55, p=0.0130, Fig. 3.12). However, there was no such correlation between CHC PC1 and carrier frequency (linear model: Estimate= -0.729, Multiple R-squared= 0.223, SE= 0.5144, $F_{1,7}$= 2.01, p=0.311) or pulse duration (linear model: Estimate= 0.1048, Multiple R-squared= $4.61x10^{-3}$, SE= 0.5823, $F_{1,7}$= 0.032, p= 0.862).

DISCUSSION

Species can vary across many phenotypic dimensions, including morphology, ecology, and behavior. Comparing the phenotypes of closely related species gives us a window into the past of that taxon and allows us to better understand the processes that caused those phenotypes to diverge. Similarly, we can compare population-level phenotypic variation and its distribution to gain even finer and more recent temporal resolution to help link patterns to the processes that lead to initial divergence within species and ultimately to the differences we see among species. While it is not uncommon to investigate differences among populations along a single phenotypic axis, populations and species usually vary along multiple axes, often with coupled phenotypes (e.g. Podos 2001, Grace and Shaw 2011; López-Fernández et al. 2012, Conte et al. 2015). Therefore understanding the historical process of speciation in a

taxon requires a comprehensive determination of which phenotypes differ between closely related species and evaluating the impact of those phenotypes on reproductive isolation. As these phenotypes are identified, the forces causing their change, and the consequences of that change, can be assessed on finer scales.

In this chapter, I explored variation in two behavioral phenotypes relevant to mate choice in *Laupala* across two distinct sensory modalities, male song and CHC expression. Both of these behavioral phenotypes are known to have diverged rapidly among species of *Laupala* (Mendelson and Shaw 2005, Mullen et al. 2007, respectively) and both can have powerful consequences for gene flow, even among populations (Grace and Shaw 2011) or closely related species (see Chapter 2). To understand how these behaviors change over time and contribute to reproductive isolation, we must first make an assessment of the variation of these behaviors within and between populations. Further, quantifying the geographic variation in these behaviors may lead to predictions both about their historical patterns and influences on their future trajectories within and among species. I found significant heterogeneity in both song and CHC expression among populations (Fig. 3.3- Fig. 3.5, Fig. 3.9.) whose spatial distributions might inform us about the historical processes that have shaped these phenotypes.

Song variation

Pulse rates were distributed in a non-linear fashion across the range of *L. pruna*. Crickets from Kaiwiki in the north had a similar mean pulse rate to those from Alili

Springs and Kaiholena in the south (Fig. 3). Halema'uma'u, Glenwood Rd., Naulu Trail, and Stainback Hwy are all spread across the central portion of *L. pruna*'s range, and, on average, are characterized by a slower pulse rate. Among populations of *L. pruna*, pulse rate varies the most dramatically among populations. Similarly, pulse duration and carrier frequency do not show similar spatial patterns of variation to those shown by pulse rate (Fig. 3.4, Fig. 3.5), a pattern also reflected among species. In *L. cerasina*, a sympatric congener of *L. pruna*, female acoustic preference suggests that all three traits are targets of sexual selection (Oh and Shaw 2013). Nevertheless, only pulse rate plays out into broader diversification patterns among species with, as yet, no established explanation (Oh and Shaw 2013).

CHC expression

Cuticular hydrocarbons differed markedly, both qualitatively and quantitatively between populations. Every retention time examined differed significantly among populations and species in repeated ANOVAS (Table 3.3). Log transformed CHC abundances represent the relative abundance at each retention time. These results suggest the differences among populations are not simply due to changes in the relative expression of one or two compounds among populations, but rather complex permutations of CHC proportions, though follow-up with GC-MS will be necessary to further explore differences.

The spatial distribution of CHC expression approximated that of song in that KW in the north resembled AS, KI, and KH (Fig. 3.7). Profiles from Halema'uma'u, Glenwood, Naulu Trail, and Stainback are all centrally located geographically and

appear qualitatively similar (Fig 3.7) and cluster closely together (Fig. 3.9). Kaiholena and Kea'iwa, cluster closely with Alili Springs separating both along LD1 and LD2 (Fig. 3.9), despite its geographic location between these two populations (Fig 3.1).

In populations with moderate to large sample sizes, sexual dimorphism was detected by the LDA, though differences of individual compounds between males and females could not be determined using multiple t tests (Figs. 3.10). The lack of ability to determine individual compound differences may be for two reasons. First, using Bonferroni correction may be too conservative and therefore raise the threshold to detecting differences to levels higher than biological expression (thus, larger samples sizes will be needed). Alternatively, rather than single compounds differing among males and females, the relative proportions of a subset of compounds may be required for information transfer (e.g. Groot et al. 2014). Therefore, comparing abundances of single compounds may not reveal statistically significant differences. Lastly, the inability to detect differences may be a relic of my choice of retention times. For bins of retention times with overlapping ranges, I summed them to make them more easily comparable. However, it may have resulted in the combination of multiple chemical compounds and reduced my ability to detect differences among males and females. The last possibility seems least likely, as retention times that were not combined (i.e. single peaks), were also not different. If retention times that differ were randomly distributed, I should have detected some differences among isolated retention times, but this was not the case.

Song is a sex-limited trait in crickets, where only males sing. Cuticular hydrocarbons are thought to primarily function in desiccation resistance but may have

functional tradeoffs with their use as signals (Chung and Carroll 2015). Both males and females express CHCs and are likely using them to make mating decisions. Among CHCs, there was no statistically meaningful relationship between their expression and geographic distance either when comparing males and females together or separately. Populations that are closer together tend to have more similar CHCs, but this relationship is not absolute. For example, OF and KW are in relatively close geographic proximity, yet express distinct CHC profiles (Fig. 3.7). Kaiwiki clusters more closely with KI-AS-KH in multivariate space than with the GW-HM-NT cluster, which are geographically closer (Figs. 3.8 & 3.9). These patterns suggest additional processes may be influencing the distributions of phenotypic variation in *Laupala pruna* besides simple linear isolation by distance. Indeed, the relative ages of the volcanoes on which they inhabit may be an indicator of phenotypic history, given that Kawaiki is on the older volcano of Mauna Kea and KI-AS-KH cluster if found on comparably-aged substrates imbedded within Mauna Loa (see below).

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Relationship between song and CHC distributions

Laupala crickets are known for their variable pulse rates of male song, which have diverged rapidly between closely related species of *Laupala* (Mendelson and Shaw 2005). Given that CHCs are a distinct sensory channel from song production, one might expect song and CHC expression to diverge independently among populations. However, when comparing CHC expression to three different song parameters, a positive linear relationship was found between CHC PC1 and pulse rate (Fig. 3.12). Given the known role of pulse rate in the diversification of *Laupala*, it is surprising

that patterns of CHC PC1 distribution match those of song. Further, these differences appear to map to the ages of the volcanos on Hawaii, with Kawiki on the oldest volcano (Mauna Kea), followed by the Ninole hills on the southern slope of Mauna Loa (KI, AS and KH), and finally, the youngest volcano of Kilauea (GW, HM, and NT) (Carson and Clague 1995). Ola'a Flume and SB are found on the eastern side of Mauna Loa and appear to be phenotypically more similar to their geographic neighbors on Kilauea, though the sample sizes for these populations were smaller, making discrimination more difficult. Nevertheless, these results suggest a somewhat complex relationship between the phylogenetic history and relative geographic positions of these populations for predicting how song and CHCs may have changed over time. Genetic data may be employed to test these predicted relationships among populations.

Conclusion

Overall, I identified differentiation in two phenotypes known to play a role in mate discrimination and act as barriers to gene flow. Further, I identified that rather than following a linear or random pattern of divergence, these signals vary in a non-linear fashion that appears to be influenced by a combination of geographic distance and the substrate ages of the volcanoes. Males and females showed sexual dimorphism of CHC expression in many of the populations sampled but do not show a relationship between differences in CHC expression and geographic distance. Lastly, pulse rate and chemical signals diverge together rather than independently of each other, suggesting that elements of complex courtship behavior may evolve as a unit rather

than independently.

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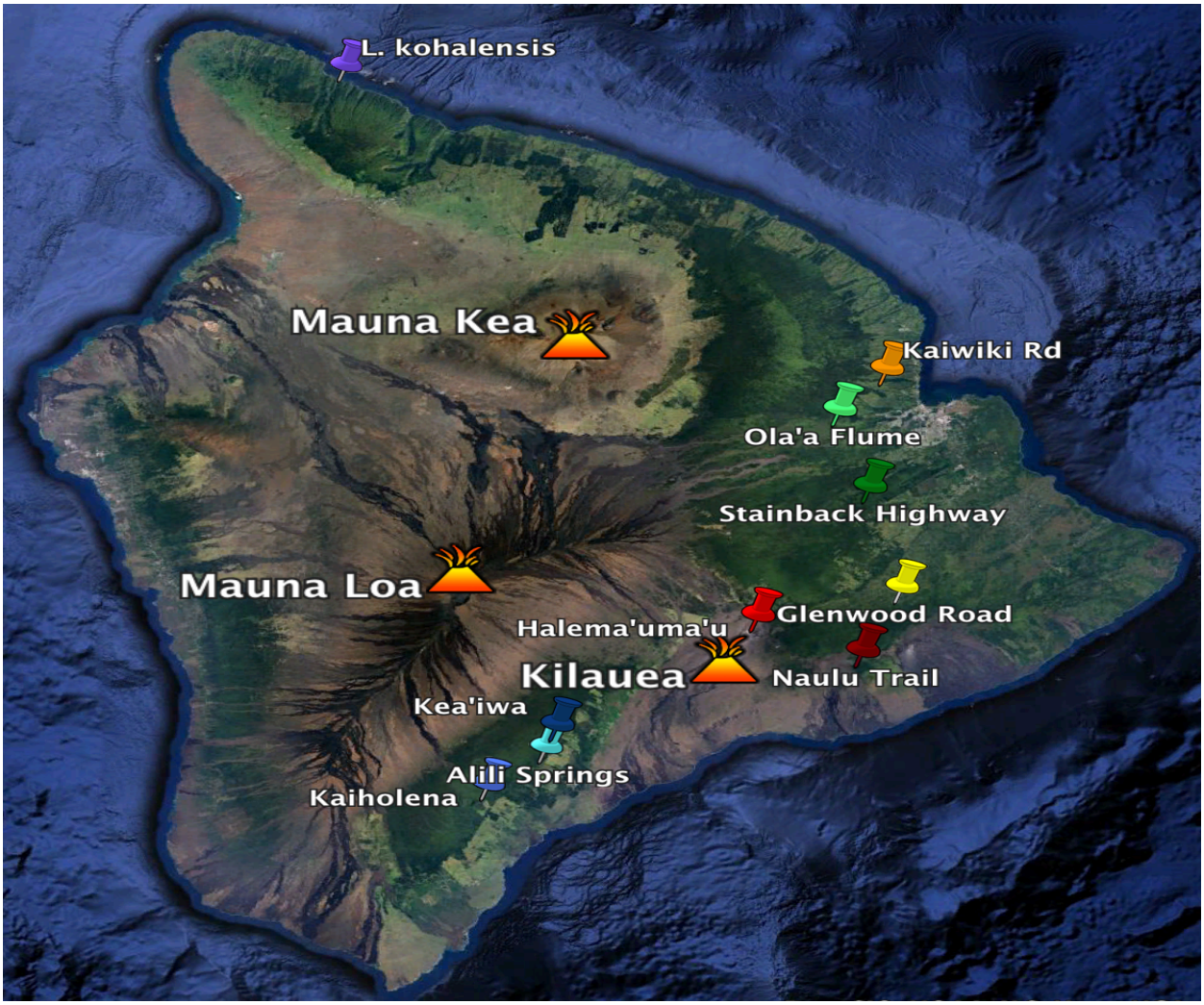
FIGURES AND CHARTS



Population	Latitude	Longitude
Kaiwiki	19° 45' 12.79" N	155° 7' 58.23"W
Ola'a Flume	19° 41' 50.07" N	155° 11' 6.50" W
Stainback Hwy	19° 34' 54.93" N	155° 10' 7.18" W
Glenwood Rd	19° 26' 49.45" N	155° 7' 20.85" W
Halema'uma'u Trail	19° 24' 36.10" N	155° 16' 44.60" W
Naulu Trail	19° 21' 30.0" N	155° 9' 59.00" W
Alili Springs	19° 13' 33.29" N	155° 31' 19.49" W
Kea'iwa	19° 16' 13.52" N	155° 29' 35.35" W
Kaiholena	19° 10' 34.26" N	155° 34' 35.52" W

Figure 3.1: Map of collection locations across the range of *Laupala pruna*. *L. kohalensis* is included in the north

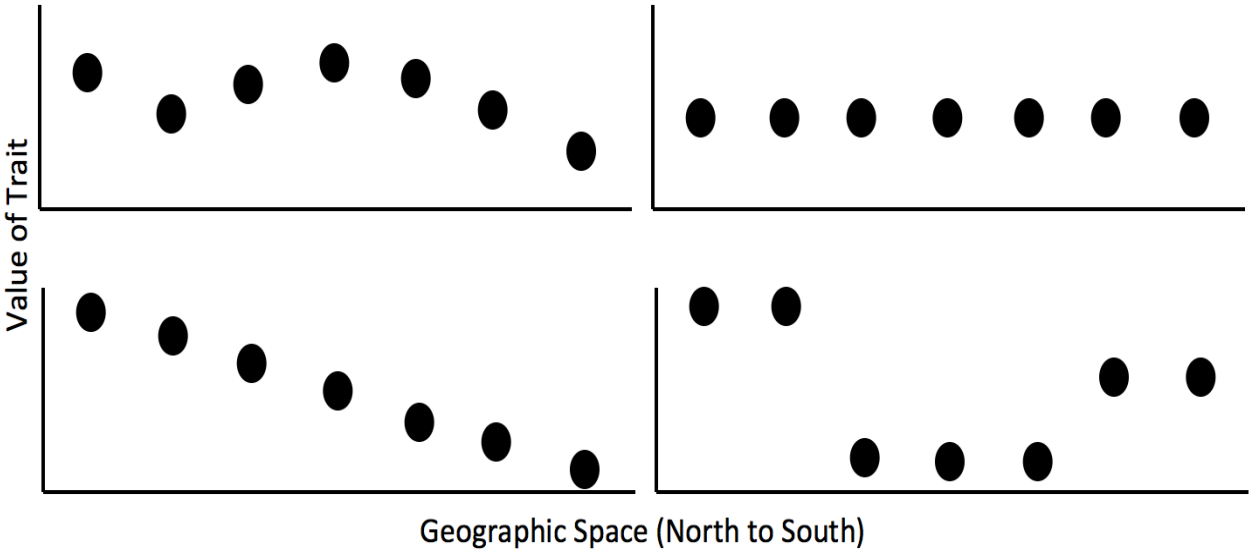


Figure 3.2: Alternative hypotheses for distribution of phenotypes and their causes (A) random drift (B) stabilizing selection (C) isolation by distance/ directional selection (D) volcanic age

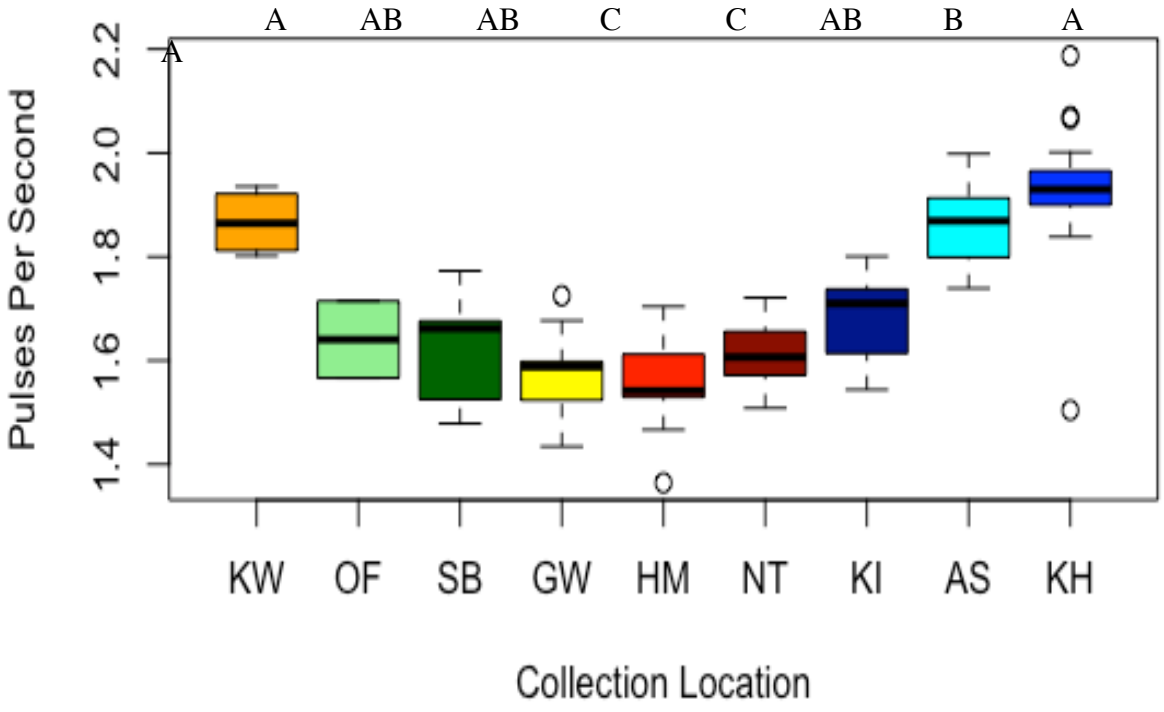


Figure 3.3: Boxplots of pulse rate across populations of *L. pruna*. (Kaiwiki (KW), Ola'a Flume (OF), Stainback Hwy. (SB), Glenwood Rd. (GW), Halema'uma'u (HM), Naulu Trail (NT), Kea'iwa (KI), Alili Springs (AS), and Kaiholena (KH))

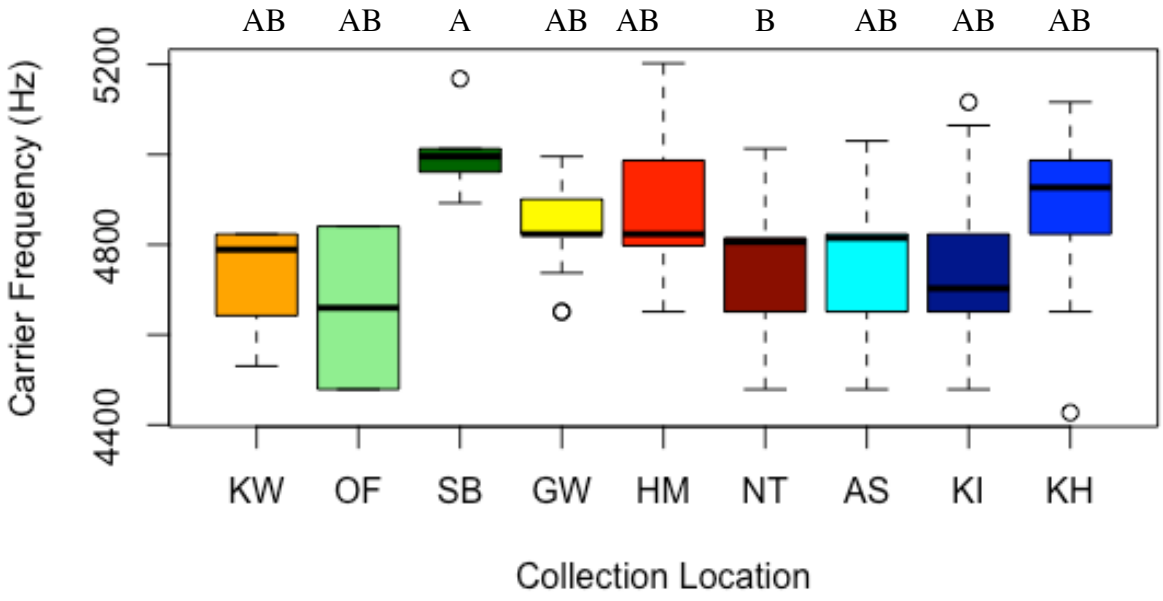


Figure 3.4: Boxplots of carrier frequency across populations of *L. pruna*.(Kaiwiki (KW), Ola'a Flume (OF), Stainback Hwy. (SB), Glenwood Rd. (GW), Halema'uma'u (HM), Naulu Trail (NT), Kea'iwa (KI), Alili Springs (AS), and Kaiholena (KH))

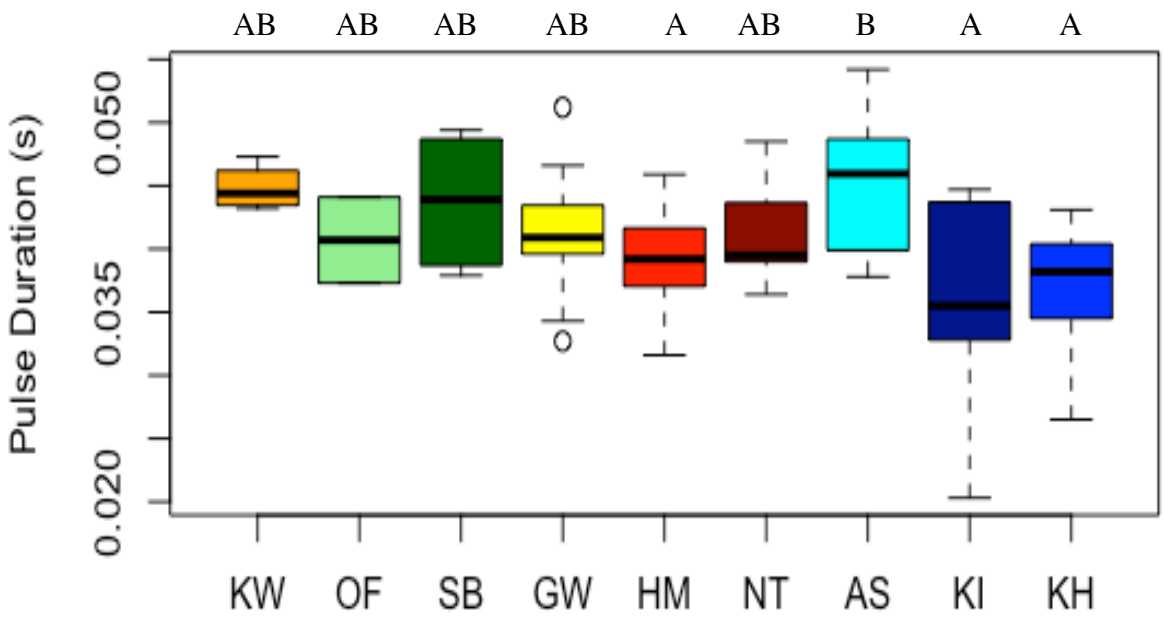


Figure 3.5: Boxplots of pulse duration across populations of *L. pruna* (Kaiwiki (KW), Ola'a Flume (OF), Stainback Hwy. (SB), Glenwood Rd. (GW), Halema'uma'u (HM), Naulu Trail (NT), Kea'iwa (KI), Alili Springs (AS), and Kaiholena (KH))

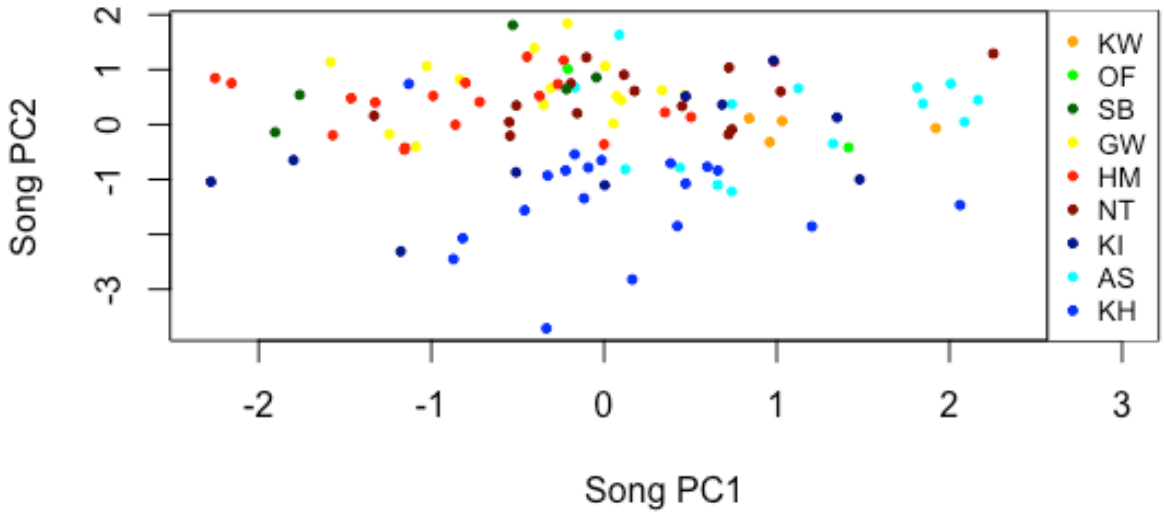


Figure 3.6: Comparison of principal components 1 and 2 of song parameters among populations of *Laupala pruna (*Kaiwiki (KW), Ola'a Flume (OF), Stainback Hwy. (SB), Glenwood Rd. (GW), Halema'uma'u (HM), Naulu Trail (NT), Kea'iwa (KI), Alili Springs (AS), and Kaiholena (KH)).

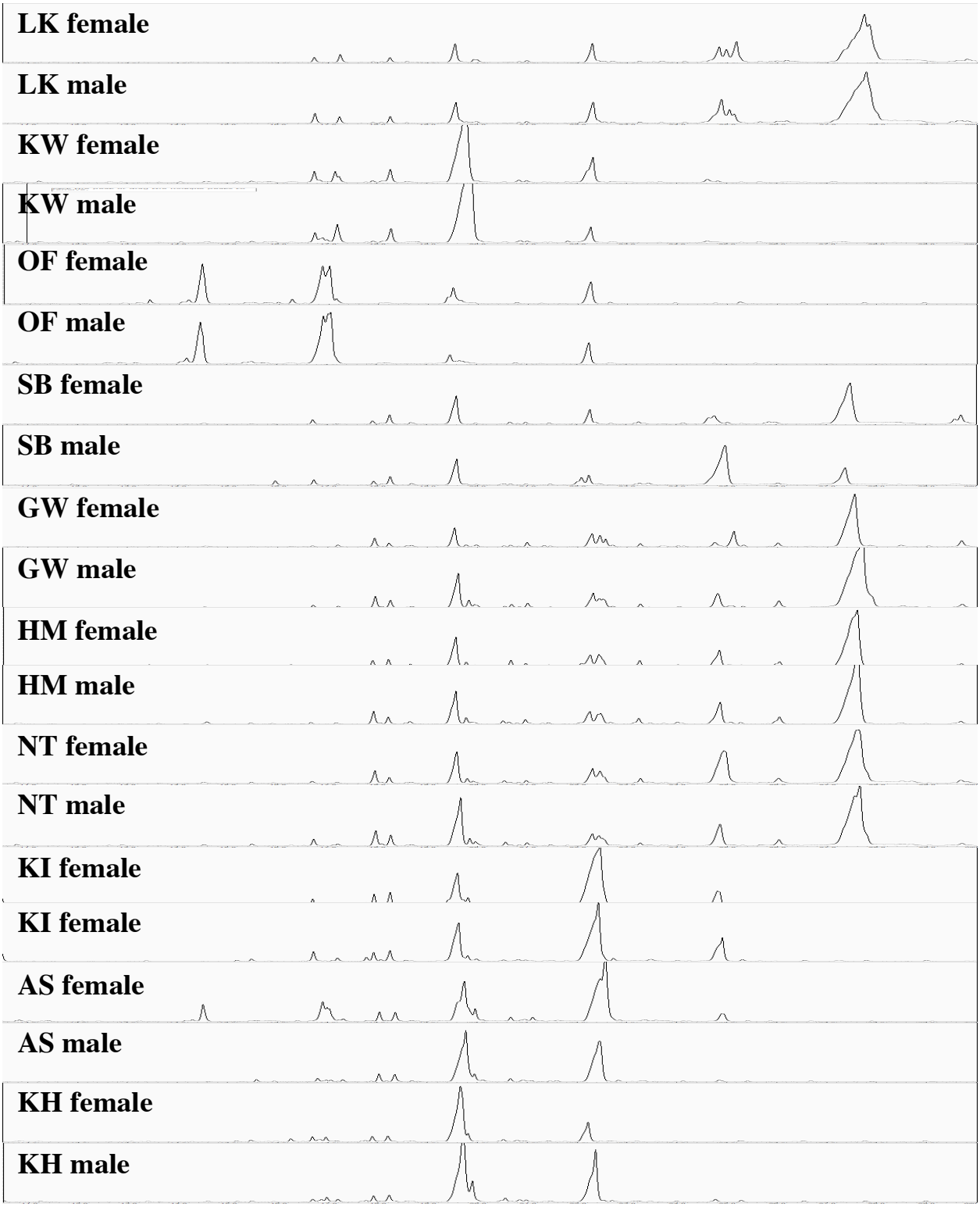


Figure 3.7: Chromatograms of *Laupala kohalensis* and populations of *L pruna* across its range: Kaiwiki (KW), Ola'a Flume (OF), Stainback Hwy. (SB), Glenwood Rd. (GW), Halema'uma'u (HM), Naulu Trail (NT), Kea'iwa (KI), Alili Springs (AS), and Kaiholena (KH).

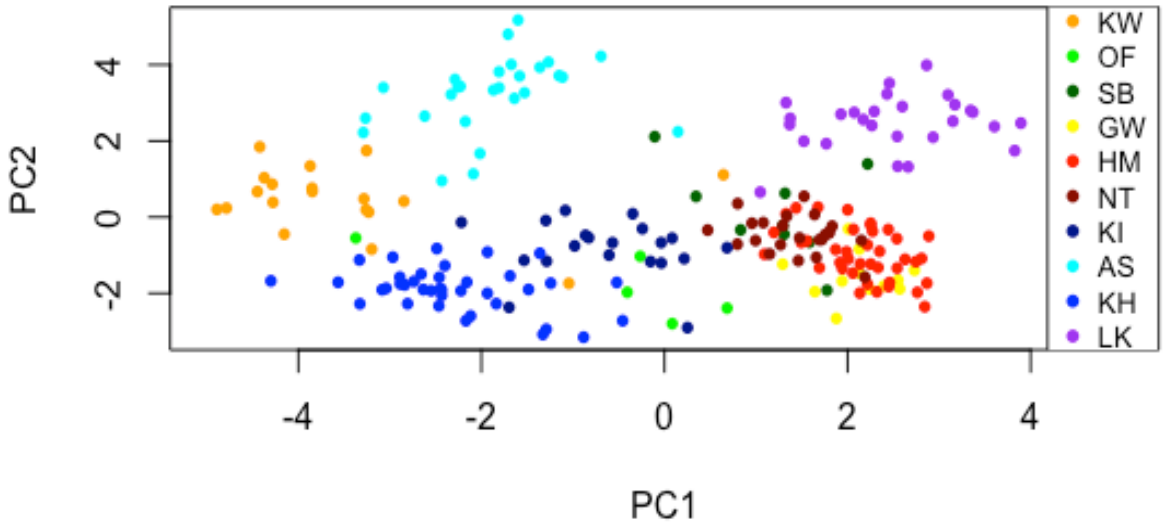


Figure 3.8: Comparison of principal components 1 and 2 of CHC expression among populations of *Laupala pruna (*Kaiwiki (KW), Ola'a Flume (OF), Stainback Hwy. (SB), Glenwood Rd. (GW), Halema'uma'u (HM), Naulu Trail (NT), Kea'iwa (KI), Alili Springs (AS), and Kaiholena (KH)). LK (purple) denotes *L. kohalensis*

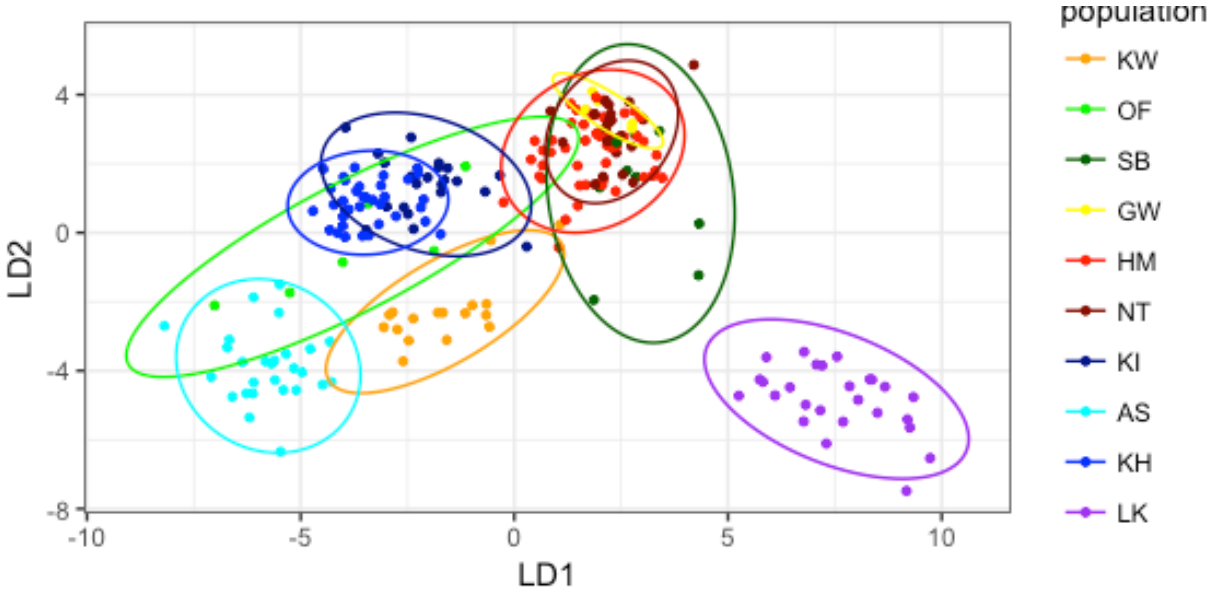


Figure 3.9: Comparison of linear discriminant scores 1 and 2 of CHC expression among populations of *Laupala pruna (*KW=Kaiwiki, OF=Ola'a Fluma, SB=Stainback Hwy., GW=Glenwood Rd., HM=Halema'uma'u, NT=Naulu Trail, KI=Kea'iwa, AS=Alili Springs, KH=Kaiholena). LK (purple) denotes *L. kohalensis*

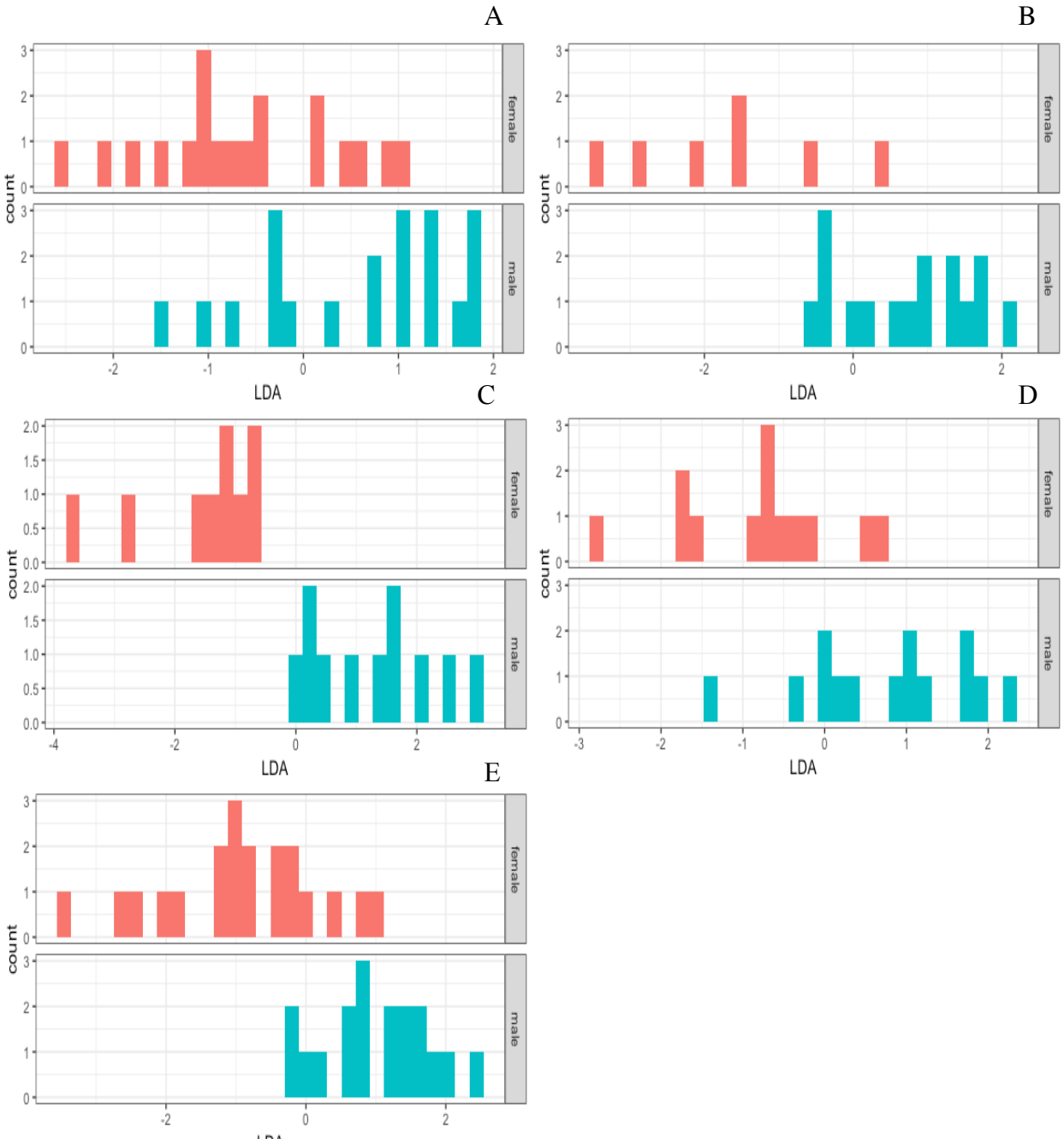


Figure 3.10: Linear discriminant analyses separating males and females based on CHC profiles from A) Halema'uma'u, B) Naulu Trial, C) Kea'Iwa D) Alili Springs, E) Kaiholena. Note that axes are not the same for all populations.

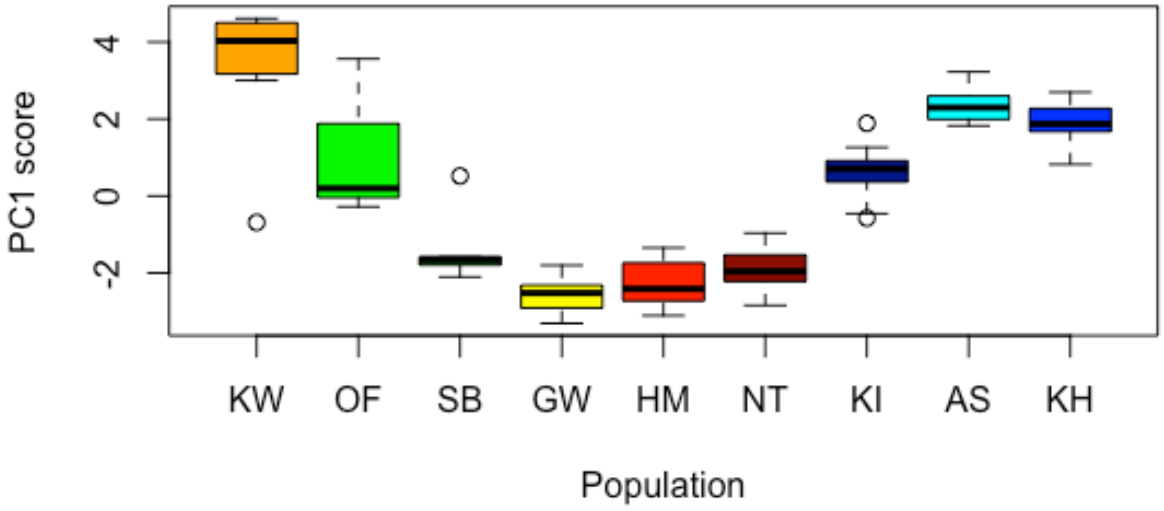


Figure 3.11: Boxplots of PC1 scores of males across populations of *Laupala pruna* (KW=Kaiwiki, OF=Ola'a Fluma, SB=Stainback Hwy., GW=Glenwood Rd., HM=Halema'uma'u, NT=Naulu Trail, KI=Kea'iwa, AS=Alili Springs, KH=Kaiholena).

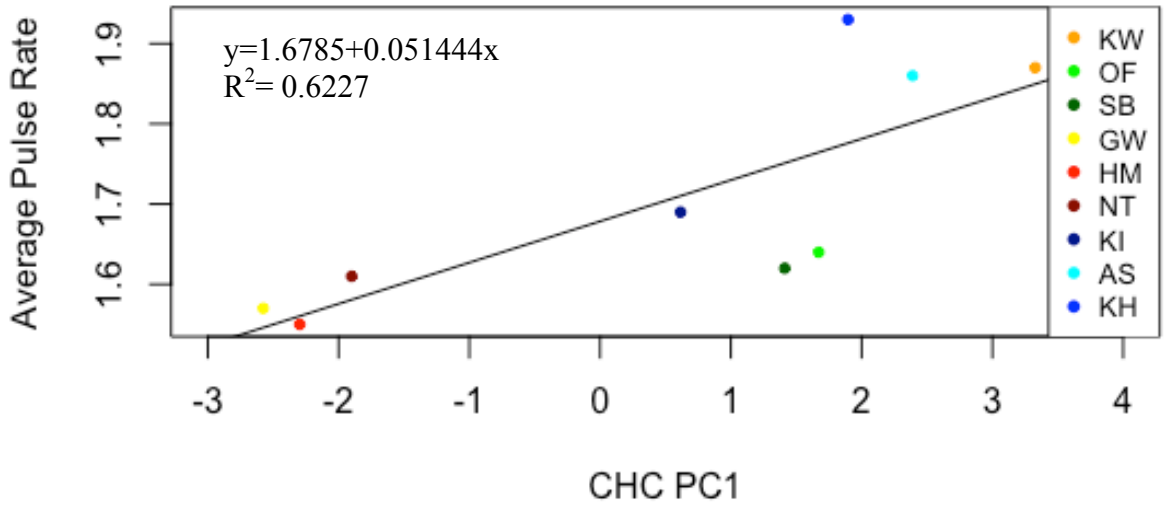


Figure 3.12: Linear model of cuticular hydrocarbon principal component 1 and pulse rate across populations of *L. pruna (*KW=Kaiwiki, OF=Ola'a Fluma, SB=Stainback Hwy., GW=Glenwood Rd., HM=Halema'uma'u, NT=Naulu Trail, KI=Kea'iwa, AS=Alili Springs, KH=Kaiholena),

Table 3.1: Average (sd) song parameters of 9 populations of *Laupala pruna* . pps= pulses per second, cf=carrier frequency (Hz), and dur= duration (ms) . The number of males for each population is noted next to the name of each population (n).

Population	pps (sd)	cf (sd)	dur (sd)
Kaiwiki (4)	1.87 (0.06)	4711.45 (166.71)	42.46 (4.91)
Ola'a Flume (2)	1.64 (0.10)	4677.00 (280.15)	41.21 (3.81)
Stainback Hwy. (5)	1.62 (0.12)	4988.81 (109.38)	41.75 (4.44)
Glenwood (15)	1.57 (0.08)	4828.02 (114.53)	43.09 (5.32)
Halema'uma'u (19)	1.55 (0.07)	4880.55 (157.57)	42.61 (5.89)
Naulu Trail (15)	1.61 (0.06)	4724.09 (136.52)	41.87 (4.53)
Kea'iwa (10)	1.69 (0.08)	4733.87 (184.25)	42.21 (4.81)
Alili Springs (14)	1.86 (0.08)	4750.83 (153.50)	43.57 (5.05)
Kaiholena (18)	1.93 (0.13)	4875.11 (124.22)	43.05 (5.28)

Table 3.2: (A) Observed (obs) versus expected (exp) assignment of male *L. pruna* from LDA analysis of song parameters. (B) Observed versus expected assignment of individuals from LDA analysis of CHC profiles. Values on the diagonal (bold) represent "correct" assignment of individuals from the location to which they were collected from on the island. Off diagonal values denote misassignment of an individual.

(a)	KW obs.	OF obs.	SB obs.	GW obs.	HM obs.	NT obs.	KI obs.	AS obs.	KH obs.
KW exp.	**0**	0	0	0	0	0	0	0	0
OF exp.	0	**0**	0	0	0	0	0	0	0
SB exp.	0	0	**0**	0	0	0	0	0	0
GW exp.	0	1	2	**3**	2	1	0	1	0
HM exp.	0	0	3	7	**13**	6	2	1	1
NT exp.	0	0	0	5	3	**4**	3	1	0
KI exp.	0	0	0	0	1	0	**0**	0	1
AS exp.	4	1	0	0	0	4	1	**7**	0
KH exp.	0	0	0	0	0	0	4	4	**17**

(b)	KW obs	OF obs	SB obs	GW obs	HM obs	NT obs	KI obs	AS obs	KH obs
KW exp.	**17**	0	0	0	0	0	0	0	0
OF exp.	1	**4**	0	0	0	0	0	0	0
SB exp.	0	0	**4**	0	0	1	0	0	0
GW exp.	0	0	0	**7**	2	0	0	0	0
HM exp.	0	0	0	4	**35**	1	0	0	0
NT exp.	1	0	4	1	2	**21**	0	0	0
KI exp.	0	1	1	0	0	0	**20**	0	1
AS exp.	0	0	0	0	0	0	0	**27**	0
KH exp.	0	0	0	0	0	0	0	0	**37**

Table 3.3: ANOVA results examining differences in retention times across populations of *L. pruna*. A Bonferroni correction yields an alpha value of = 0.00263

Retention time (min)	df	Sum sq.	Mean Sq.	F-value	p-value
14.54	8	30.42	3.80	20.76	$<2x10^{-16}$
15.48	8	38.79	4.85	39.94	$<2\ x10^{-16}$
17.09	8	43.80	5.47	65.41	$<2\ x10^{-16}$
17.94	8	57.42	7.177	98.75	$<2\ x10^{-16}$
18.04	8	32.49	4.061	222	$<2\ x10^{-16}$
18.17	8	12.89	1.61	57.26	$<2\ x10^{-16}$
18.54	8	0.649	0.081	2.437	0.014
19.68	8	19.95	2.49	93.64	$<2\ x10^{-16}$
20.60	8	9.42	1.18	22.89	$<2\ x10^{-16}$
20.77	8	16.18	2.02	29.69	$<2e\ x10^{-16}$
20.92	8	15.098	1.89	46.65	$<2\ x10^{-16}$
21.27	8	10.892	1.36	48.34	$<2\ x10^{-16}$
22.45	8	19.71	2.46	39.16	$<2\ x10^{-16}$
23.46	8	22.53	2.82	56.99	$<2\ x10^{-16}$
23.95	8	8.43	1.05	16.85	$<2\ x10^{-16}$
24.77	8	91.77	11.471	72.33	$<2\ x10^{-16}$
25.26	8	20.99	2.62	62.14	$<2\ x10^{-16}$
27.75	8	189.81	23.73	234.6	$<2\ x10^{-16}$
28.99	8	20.82	2.60	77.46	$<2\ x10^{-16}$