Assessing Differences in Desiccation Tolerance in Two Species of Hawaiian Swordtail Cricket

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Abstract

Two species of Hawaiian swordtail cricket, *L. orientalis* and *L. makaio*, inhabit different locations on the island of Maui in the Hawaiian archipelago, generally with *L. orientalis* residing in a region of higher, and *L. makaio* inhabiting a region of lower, rainfall. This difference in habitation exposes the two species to different levels of humidity, with *L. orientalis* being subjected to greater levels of moisture than *L. makaio,* when controlling for elevation. It may be possible that these species' distinct habitats are a result of different humidity preferences. These distributions in turn may be influenced by the different compositions of cuticular hydrocarbons (CHCs) that coat the surface of their bodies and prevent desiccation. To test whether such differences in desiccation resistance may exist, individuals from each species were subjected to a series of desiccation tolerance experiments, which measured the length of time in which each species could survive arid conditions. Additionally, the CHC profiles of each species were analyzed by extracting the CHCs from individuals of each species and running them through gas chromatography. The results of these experiments do not reveal a statistically significant difference in the survivability of *L. orientalis* and *L. makaio* in arid environments, though a difference in CHC profiles has been observed.

Introduction

The genus *Laupala* are comprised of flightless crickets that populate forests across the Hawaiian archipelago. These insects are of great interest in the field of evolutionary biology due to their remarkably high speciation rate, which is one of the highest among arthropods (Mendelson and Shaw, 2005). To date, 38 species of *Laupala* have been identified. Despite this large degree of divergence in the genus, these species have homogenous morphological characteristics, pointing to differences in secondary sexual characteristics as the primary source of their differences as well as their main driver of speciation (Mullen et al., 2007). One such characteristic is the pulse rate

of the courtship songs *Laupala* males produce, quantified as the number of pulses produced per second. These rates span from 0.71 pulses per second in *L. paranigra* to 3.72 pulses per second in *L. kohalensis* (Shaw and Lesnick, 2009). Pulse rates have a significant role in the selection of male mates by female *Laupala*, with females generally preferring males with pulse rates close to that of their own species. While pulse rates are the primary means by which females select males, female *Laupala* do not engage in any form of corresponding auditory communication. Instead, male selection of females in the genus is hypothesized to be influenced by the composition of lipids that comprise the cuticles of *Laupala* crickets (Stamps and Shaw, 2019).

Cuticular lipids are compounds that coat the outer surfaces of insects. They serve a variety of roles ranging from providing protection against environmental hazards (Jackson and Baker 1970) to mediating interactions between an insect and other organisms (Espelie et. al, 1991). The composition of cuticular lipids that a particular individual has is largely dictated by genetics. Hydrocarbons are among the host of cuticular lipids that can be found on insects. These compounds exclusively consist of carbon and hydrogen atoms connected by a single chain of 19 to 35 carbon atoms (Drijfhout et al., 2013). Each carbon atom is bound to another carbon atom in the chain, with the remainder of its bonds generally being taken up by hydrogen atoms. Hydrocarbon chains can vary in their degree and type of saturation as well as in the presence and degree of methyl-branching. Saturated hydrocarbons are those in which every carbon in its carbon chain is attached to one another by single bonds. Conversely, unsaturated hydrocarbons are characterized by the presence of at least one double bond in its carbon chain. Unsaturated hydrocarbons can contain one, two, or three double bonds in their carbon chains. Unlike unsaturated hydrocarbons, saturated hydrocarbons can be methyl-branched – that is, they can have methyl groups (CH_3) attached to the carbons in their carbon chains. Variations in the carbon chain length as well as in the presence of

double bonds and methyl groups in hydrocarbons can result in variation in physical properties, such as melting point and flexibility. CHC profiles, the particular sets of CHCs that individuals possess, vary between insect species and sometimes between individuals of different sexes and life stages.

CHCs provide a means by which insects can engage in chemical communication. The form that this communication takes is largely dependent on the volatility of an insect's CHCs. In the case of *Drosophila melanogaster*, certain circumstances may allow individuals to produce CHCs that are volatile enough to be sprayed at other flies through wing movements (Ferveur et al., 2005). However, CHCs often have low volatilities and remain in a solid, waxy form (Drijfhout et al., 2013). In cases such as this, chemical communication with CHCs is performed through physical contact between individuals. These types of exchanges are observed in crickets including those in the *Laupala* genus, who utilize antennal contact to transmit information provided by CHCs (Stamps and Shaw, 2019). The detection of CHCs by volatile or surface expression provides insects with a means of discriminating the sex and species of other individuals, thus facilitating the identification of potential mating partners. Chemical communication with CHCs has also been shown to extend to an array of other contexts, including the recognition of nestmates and signaling fertility (Smith et al., 2013).

Beyond chemical communication, CHCs also serve an important role in maintaining water balance in insects. The hydrophobic character of CHCs provides them with low permeability to water, endowing the compounds with waterproofing properties (Drijfhout et al., 2013). The light weight of insects relative to water droplets renders them susceptible to losses of mobility in situations in which excessive amounts of moisture accumulates on their bodily surfaces. The waterproof character of CHCs thus limits the extent to which moisture can restrict movement.

Furthermore, these same properties prevent the excessive loss of moisture through cuticular surfaces. This function is particularly important in insects, as their large surface area-to-volume ratio confers an otherwise high rate of desiccation. This notion has been validated in the laboratory, as insects who have had their CHCs experimentally removed demonstrate much higher rates of desiccation compared to insects with intact CHCs (Chung and Carroll, 2015). Given the great variety of CHC profiles that can be observed across different insect species, there should be a correspondingly broad range of desiccation rates that insects can demonstrate. CHC features that are conducive to higher levels of impermeability to water are generally those that increase their boiling point and thus reduce their volatility. Such features include longer chain lengths, saturation, and a lack of methyl-branching (Gibbs and Pomonis, 1995). Differences in CHC profiles can be observed between populations of insects exposed to environments with different levels of humidity. This is evident in the African mosquito species *Anopheles gambiae* and *Anopheles coluzzii*, who reside in environments with dry seasons and thus fluctuating humidity levels. Their CHC profiles have been shown to differ between dry and wet seasons, with the effect of increasing their desiccation tolerance levels during dry seasons and lowering them during wet seasons (Arcaz et al., 2016). Furthermore, it has been demonstrated that the humid rainforest-dwelling *Drosophila* species *D. birchii* have fewer CHCs than *D. serrata*, which reside in the less humid outskirts of the rainforest. This difference in CHC concentration has rendered *D. birchii* more susceptible to desiccation than their *D. serrata* counterparts (Chung et al., 2014). Similar differences in CHC profiles are expected to be seen in insect populations that reside in geographic regions with different expected levels of humidity.

The role of CHCs in chemical communication among species in the *Laupala* genus has been supported experimentally (Stamps and Shaw, 2019). However, the role of CHCs in

desiccation tolerance in *Laupala* has not yet been demonstrated. The Hawaiian archipelago in which the species of this genus reside exhibits great variation in relative humidity levels. This broad span of moisture exposure is hypothesized to have brought about significant, region-specific differences in CHC profiles across the *Laupala* genus. Of interest in the study described here are the *Laupala* species *L. orientalis* and *L. makaio*, which differ in the environments in which they reside. *L. orientalis* reside along the windward side of East Maui, as shown in the map depicted in Figure 1. The distribution of *L. makaio* on the other hand, is known only from Kipahulu Valley on the southeastern end of East Maui. The known distribution of *L. makaio* extends into upper elevations, whereas available evidence suggests that *L. orientalis* is restricted to relatively lower elevations (Shaw, 2000). The difference in regional and elevational distribution has likely resulted in the two species being exposed to differing levels of relative humidity, with the regions in which *L. orientalis* reside being considerably more humid than the lower regions that *L. makaio* inhabits.

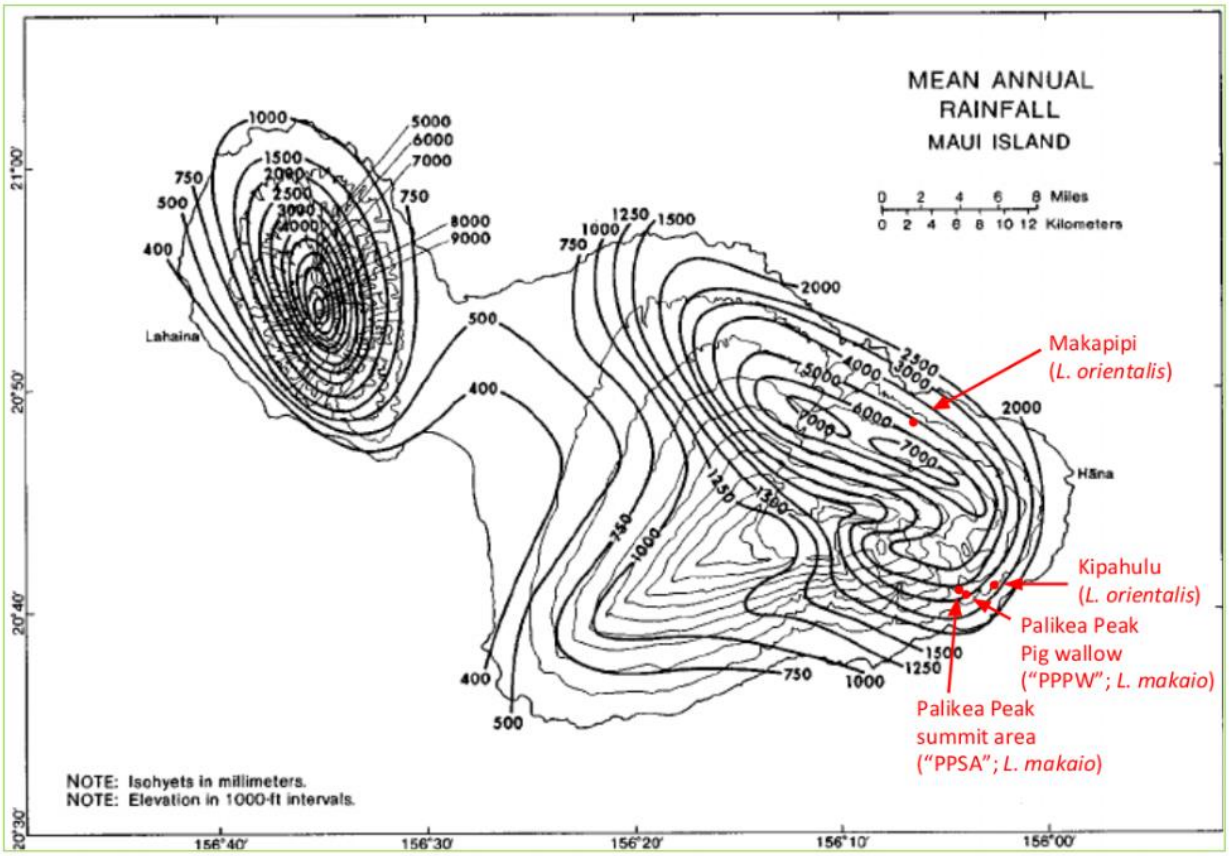

Figure 1 - Isohyets of mean annual rainfall for the island of Maui, Hawaiian Islands (from Giambelluca, T. W., Nullet, M. A., and Schroeder, T. A.: 1986, Rainfall Atlas of Hawaii, Report R76, Hawaii Dept. of Land and Natural Resources, Division of Water and Land Development, Honolulu, Hawaii, p. 267.

The goal of this project is to determine whether the different distributions of *L. orientalis* and *L. makaio* reside is coincident with a difference in the species' cuticular hydrocarbon profiles and desiccation tolerance. If there appears to be evidence for a difference in desiccation tolerance, a secondary goal for this project would be to determine whether such preferences may be mediated by differences in the CHC profiles of the two species. To that end, CHC samples were extracted from individuals of each species. Gas chromatography was applied to these samples, with the results being analyzed computationally to assess the extent to which the CHC profiles of the two species differ. Furthermore, individuals of each species were subjected to rounds of experimentation to compare their relative rates of desiccation. It is hypothesized that *L. makaio*,

which reside in drier conditions than *L. orientalis*, show higher desiccation tolerance, while *L. orientalis*, which are exposed to wetter conditions, show lower desiccation tolerances. The former's expected tolerance for drier habitats will be related to its CHC profiles that might enhance their survivability in drier environments.

As CHC profiles are largely species-specific, they may provide a means by which otherwise similar species can be distinguished (Kather and Martin, 2012). In elucidating the CHC profiles of *L. orientalis* and *L. makaio*, insight may be gleaned regarding how CHC compositions may differ between different species of *Laupala*. The use of characteristics such as CHC profiles to identify species boundaries is particularly useful in *Laupala*, whose species are both numerous and nearly identical morphologically. Furthermore, by establishing a connection between desiccation tolerances and CHC profiles, it may be possible to make inferences on whether two populations of *Laupala* possess similar collections of CHCs and thus whether two populations may be able to interbreed.

Methods

Animal Collection and Rearing

Individuals from the *L. orientalis* or *L. makaio* species were caught in the wild in 2021 from three locations on East Maui, Hawaii (FIGURE X): Palikea Peak summit area ("PPSA"; c. 2200 feet in elevation; *L. makaio*), Kipahulu Valley; Waimoku Trail, Kipahulu Valley ("Kipahulu"; c. 1,000 feet in elevation; *L. orientalis*); and Makapipi Road (c. 1,350 feet in elevation; *L. orientalis*). The animals used for CHC extractions were housed in jars containing other members of the same species. Dampened Kimwipes were placed in each jar to provide a source of moisture, and crickets in each jar subsisted on a diet of cat kibble (Organix Chicken and Rice formula). Animals selected

to partake in desiccation tolerance experiments were housed in individual plastic cups and were provided with the same diet and source of moisture. These animals were all juveniles prior to being selected and their maturity was assessed several times per week. All crickets used in this study were reared at a constant temperature of 20º C and were kept on a day-night cycle consisting of 12 hours of light exposure and 12 hours of darkness.

Cuticular Hydrocarbon Extraction

Cuticular lipids were extracted from *L. orientalis* and *L. makaio* crickets gathered from PPSA, Kipahulu regions of Hawaii. Lab-bred hybrids (F_2 of an F_1 intercross) of the two species were also included in this sample of crickets (N = 46). These crickets were randomly sampled from collections of mature wild-caught crickets belonging to either species. The crickets were either dead or anesthetized with carbon dioxide. All steps involved in CHC extraction were carried out in a fume hood. All glassware used was rinsed with clean hexane to remove any potential contaminants. An individual glass vial was prepared for each cricket used in extraction, with each vial being filled with 300 µL of liquid-chromatography grade hexane. Each cricket was placed into a vial for five minutes to allow adequate time for the elution of their CHCs into the hexane, after which the crickets were stored and frozen for later use. The contents of each vial were filtered into clean vials through Pasteur pipettes packed with glass wool to remove any potential contaminants that may have accumulated during CHC extraction. The pipettes were rinsed with two 100 µL aliquots of clean hexane prior to the addition of the 300 µL samples of CHC-containing hexane to the pipettes. The 500 µL of hexane in each vial was subsequently evaporated with nitrogen gas. The resulting dry isolated CHC samples were stored and frozen for later analysis.

Gas Chromatography

Gas chromatography was carried out with a Shimadzu GC-2014 gas chromatograph with flame ionization capabilities, which was coupled to a Shimadzu AOC-20i autoinjector used to insert samples into the machine. The chromatograph was equipped with a HP-5 capillary GC column (20 m, 0.180 mm diameter, 0.18 μm film thickness) through which samples were run. To prepare the previously extracted CHC samples for insertion, 60 μL of hexane was added to each collection vial to re-elute the CHCs. The hexane containing the re-eluted CHCs were then transferred to plastic inserts which were then placed in autoinjector vials. These vials were placed in the chromatograph's coupled autoinjector. In addition to the CHC samples, the autoinjector was also loaded with two control vials corresponding to alkane ladders containing alkanes with carbon chains of known lengths. A 1 μL sample from each vial was withdrawn by the chromatograph's autoinjector before being injected into the chromatograph itself. Each 1 μL CHC sample corresponding to each unique vial was subjected to a 47-minute run through the chromatography column consisting of three phases: a one-minute phase in which the sample was held at 60º C, a 7-minute ramp-up phase in which the temperature was elevated to 200º C at a rate of 20º C/min, and a final phase consisting of a slower temperature elevation of 5º C/min to a temperature of 320º C, which was maintained for 15 minutes. The flame ionization detector operated at a temperature of 340º C and sampled once every 40 ms. Once every CHC sample was run through the machine, the resulting chromatographs for each sample were assessed with *LabSolutions* software, and any peaks corresponding to CHCs below the length of C19 or above the length of C35 were manually removed.

Analysis of Chromatography Data

Chromatographs were analyzed using software packages in R. To standardize the data, the GCalignR package was used to align individual sets of peaks from the chromatographs, reducing

the amount of variability in the results that were a product of noise. Principal component analysis (PCA) was applied to the standardized chromatography data to reduce its dimensionality to two components that could be plotted and visualized with the ggplot2 package. The resulting plots provided a means of determining whether *Laupala* crickets of different locations could be distinguished by their CHC profiles.

Assessing Interspecies Desiccation Tolerance Differences

L. makaio crickets from Maui's PPSA region and *L. orientalis* crickets from Maui's Makapipi Road were subjected to two rounds of experimentations to compare interspecies desiccation tolerances. The crickets selected were randomly sampled from collections of wild-caught individuals belonging to either species that were known to have not yet matured at the time of selection. Desiccation tolerance was measured as the relative rate at which an individual dies in the absence of a source of moisture. Each round consisted of 6 crickets of each species, with 3 of each sex, for a total of 12 crickets per round. The experiment was adjusted in the second round so that the 6 crickets selected matured no more than four days apart from each other, as to account for potential age-based differences in desiccation rates. Each cricket was placed in an individual container absent of both food and a source of moisture. The mortality of the crickets was tracked at irregular intervals throughout the day across the span of multiple days. The time of each check-in, along with the temperature and relative humidity of the containers at the time of each check-in, were recorded. The resulting survival data was analyzed using Kaplan-Meier survivorship analysis and a Log-rank test Chi-Square.

Results

Locational Differences in CHC Profiles

Principal component analyses performed on *Laupala* CHC chromatography data resulted in the segregation of CHC profiles into at least three clusters corresponding to different regions of the Maui, along with F_2 hybrids. The "PPSA" and "PPPW" region in which *L. makaio* resides makes up one of these clusters. The Waimoku trail, "Kipahulu" region in which *L. orientalis* resides makes up the second cluster. The third discernible cluster corresponds to the F_2 hybrids between these two parental species. There does not appear to be much intersex variation in CHC profiles within these three clusters.

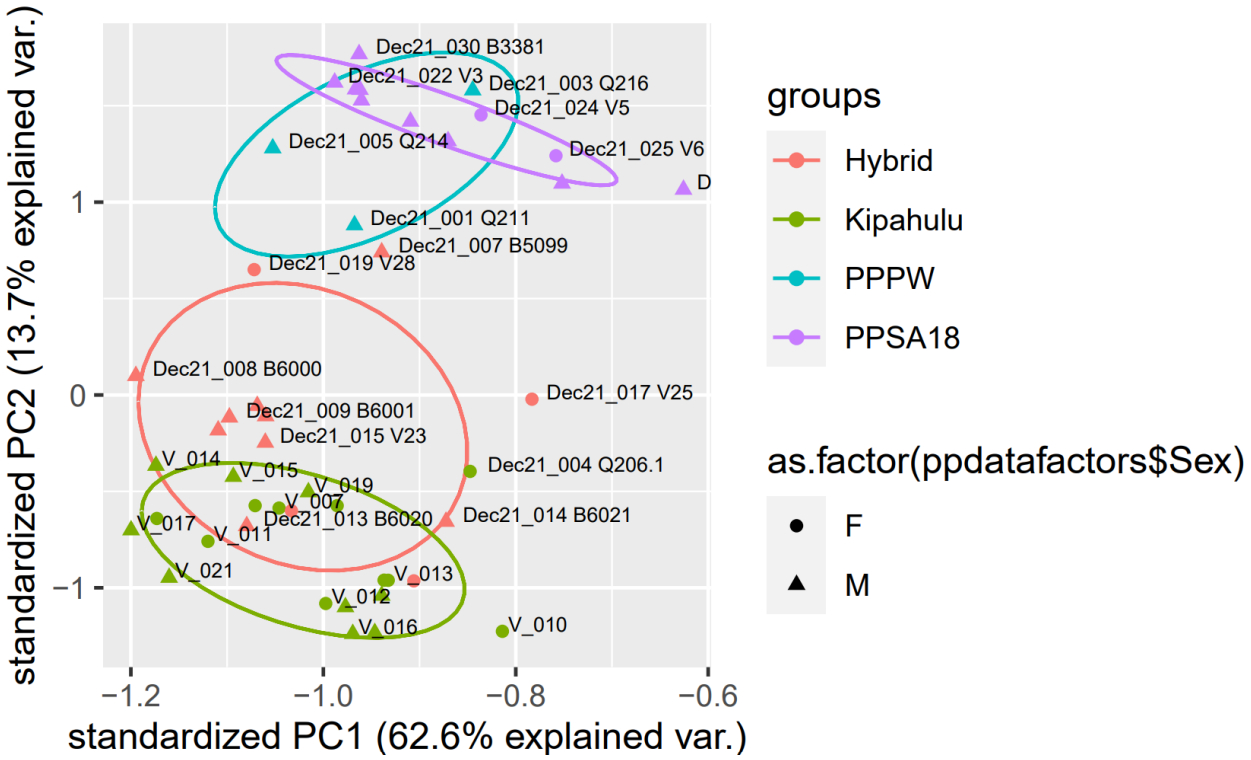

Figure 2 - Principal component analysis of CHC gas chromatography data. Different colors delineate different locations and different shapes delineate the sex of the cricket whose CHCs were extracted. The colored boundaries are 95% confidence intervals that capture the range of principal component 1 and principal component 2 combinations typical of CHC profiles belonging to crickets of a particular region.

Species Differences in Survival under Desiccation Conditions

The exposure of *L. orientalis* and *L. makaio* crickets to desiccation conditions suggests that the survival rate of *L. makaio* under such conditions is greater than that of *L. orientalis.* The median survival times (ST50) for *L. makaio* were estimated from the survival plots in Figure 2 to be 48.75 and 45.33 hours for rounds 1 and 2 of the experiment respectively, compared to ST50s of 39.5 and 41.12 hours for *L. orientalis.* One *L. makaio* cricket had escaped during the first round of experiments, as indicated by the censor cross in figure 2A. The results of Log-rank Test Chi-Square tests performed on each round of experiments produced values of $X^2 = 1.3$ and $p = 0.25$ with a test power of 0.1688 for round 1 and values of $X^2 = 0.87$ and $p = 0.35$ with a test power of 0.1799. Neither sets of values suggest that the species differences observed in desiccation rates in either round of experimentation were significant.

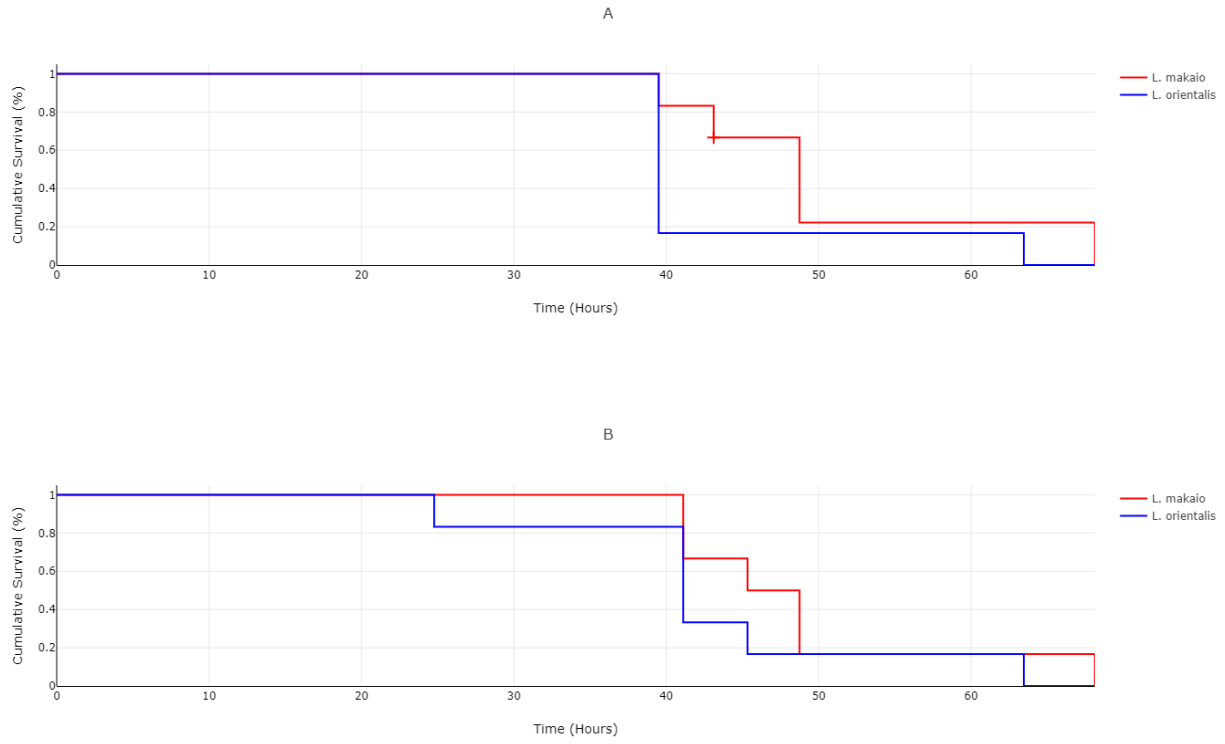

Figure 3. Kaplan-Meier plots depicting the survival of samples of L. orientalis and L. makaio exposed to desiccation conditions. Plots A and B depict the results of the first and second rounds of experiments respectively. Time is expressed as the number of hours elapsed since the onset of the exposure of the cricket samples to desiccation conditions. Cumulative survival refers to the percentage of crickets in a sample that are still alive at a particular point in time.

Discussion

If different CHC profiles vary in the degree of desiccation and moisture resistance they provide, we would expect such profiles to vary among insect populations residing in environments with different levels of relative humidity. This expectation holds when comparing the CHC profiles of *L. orientalis* and *L. makaio*, which reside at elevations exhibiting different humidity levels. When reduced to two principal components, CHC profiles belonging to each species separate into distinct nonoverlapping clusters, validating the use of their CHC profiles as a means of distinguishing between the two species and possibly other insect species that differ in humidity exposure. While CHC profiles are known to differ between sexes, intersex variation was not particularly prominent in the samples of the two species studied.

L. orientalis's habitation of a wetter region of East Maui suggested that the species would fare worse than their *L. makaio* counterparts in laboratory desiccation conditions. This assumption seemed to be validated in the desiccation tolerance experiments performed in this study. In both rounds, *L. makaio* individuals appear to have a slight advantage over *L. orientalis* in survival rate of desiccation conditions. This is consistent with the role that CHCs are known to play in water balance in insects, with drier environments generally selecting for CHC profiles or abundances that provide greater protection against desiccation. Similar results have been noted in *A. coluzzi* living across dry and wet seasons (Arcaz et al., 2016) as well as in *Drosophila* species living in humid and arid environments (Chung et al., 2014). In both cases, populations residing in drier environments exhibited lower desiccation rates that were tied to their CHC profiles. Nonetheless, the advantage we observed in this regard has not been deemed significant by statistical tests, suggesting a small or nonexistent difference in survivability. This is inconsistent with our hypothesis that *L. makaio* would have greater survivability than *L. orientalis*, and that this

difference in survivability would be a result of different CHC profiles. If no such preference exists, the differences we observe in the CHC compositions between *L. orientalis* and *L. makaio* may be a result of sexual isolation between the two species, as *Laupala* species are known to prefer mates with CHC profiles characteristic of their own species. It is worth noting that the power of the tests indicating a low significance is low. Therefore, their results cannot be reliably used to discount the originally presented hypothesis. That there is a discernible higher survival rate among *L. makaio* in desiccation conditions and that this apparent advantage is consistent across both rounds of experimentation invites further investigation into possible differences in desiccation tolerances.

Future iterations of this study would of course benefit from a higher sample size that would address the low power of the statistical tests previously described. However, it is worth acknowledging that even if a statistically significant difference is found between the desiccation tolerances of two *Laupala* species, chromatography analyses such as those we performed in this study would not be sufficient in establishing a connection between CHC profiles and desiccation tolerance in *Laupala*. As CHC profiles are known to be used in species discrimination, it is possible that the CHC profiles of any two *Laupala* species would be different regardless of any differences or lack thereof in their exposures to moisture. It is also possible for a species to simply have a greater concentration of CHCs on their cuticles than another, and that this difference in abundance alone would endow the species with a higher level of desiccation tolerance regardless of any differences in CHC profiles themselves. As such, future investigations should incorporate a means of measuring and controlling for CHC abundance. Additionally, gas chromatography could be coupled to mass spectrometry in order to identify the particular CHC species a *Laupala* species has. This would enable an assessment of the extent to which a species' CHC profile is populated with long-chained, saturated, and unbranched CHCs, which could be used as an indirect measure

of the amount of desiccation resistance a species' CHCs provide. Such data would be more informative of a potential connection between CHCs and *Laupala* desiccation tolerance than simply knowing that CHC profiles vary across ranges of moisture exposure.

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References

Medelson, T. C. and Shaw, K. L. 2005. Sexual behaviour: rapid speciation in an arthropod. *Nature.* 433,7024: 375-6.

Mullen, S. P. et al. 2007. Rapid evolution of cuticular hydrocarbons in a species radiation of acoustically diverse Hawaiian crickets (*Gryllidae: trigonidiinae: Laupala*). *Evolution; international journal of organic evolution.* 61,1: 223-31.

Shaw, K. L. and Lesnick, S. C. 2009. Genomic linkage of male song and female acoustic preference QTL underlying a rapid species radiation. *Proceedings of the National Academy of Sciences.*106 (24) 9737-9742.

Stamps, G. F. and Shaw, K. L. 2019. Male use of chemical signals in sex discrimination of Hawaiian swordtail crickets (genus *Laupala*). *Animal Behaviour.* 156:111-120.

Jackson, L. L., Baker, G. L. 1970. Cuticular lipids of insects. *Lipids* 5: 239–246

Espelie, K. E., Bernays, E. A. and Brown, J.J. 1991. Plant and insect cuticular lipids serve as behavioral cues for insects. *Arch. Insect Biochem. Physiol*. 17: 223-233.

Drijfhout, F., Kather, R., and Martin, S. 2013. The role of cuticular hydrocarbons in insects.

Behavioral and Chemical Ecology. 91-114.

Ferveur, J. F. 2005. Cuticular Hydrocarbons: Their Evolution and Roles in Drosophila Pheromonal Communication. *Behavior Genetics.* 35: 279

Smith A. A., Millar J. G., Hanks L. M., Suarez A.V. 2013. A conserved fertility signal despite population variation in the cuticular chemical profile of the trap-jaw ant *Odontomachus brunneus*. *The Journal of Experimental Biology*. 216: 3917–24

Chung, H. and Carroll, S. B. 2015. Wax, sex, and the origin of species: Dual roles of insect cuticular hydrocarbons in adaptation and mating. *BioEssays*. 37: 822-830.

Gibbs, A., and Pomonis, J. G. 1995. Physical properties of insect cuticular hydrocarbons: The effects of chain length, methyl-branching and unsaturation. *Comparative Biochemistry and Physiology Part B*: *Biochemistry and Molecular Biology*. 112: 243-249

Arcaz, A. C., et al. 2016. Desiccation tolerance in Anopheles coluzzii: the effects of spiracle size and cuticular hydrocarbons. *The Journal of Experimental Biology.* 219: 1675–1688.

Chung H., et al. 2014. A single gene affects both ecological divergence and mate choice in Drosophila. *Science.* 343: 1148–1151.

Shaw, K. L. 2000. Further acoustic diversity in Hawaiian forests: two new species of Hawaiian cricket (*Orthoptera: Gryllidae: Trigonidiinae Laupala*), *Zoological Journal of the Linnean Society*. 129: 73-91.

Kather, R. and Martin, S. J. 2012. Cuticular hydrocarbon profiles as a taxonomic tool: advantages, limitations and technical aspects. *Physiological Entomology*. 37: 25-32.