

## ABSTRACT

Title of Document: MATING COSTS, MALE CHOICE DISPLACEMENT, AND THE EFFECTS ON HYBRIDIZATION AND SPECIATION IN THE HAWAIIAN CRICKET LAUPALA (SUBFAMILY: TRIGONIDIINAE)

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Contact zones between two closely-related species provide unique laboratories for studying the processes of speciation. This is because, within these zones, species barriers will be reinforced and speciation will reach completion, or the barriers will break down, causing the two species to become one. Which of these two alternatives will occur depends on the degree of genetic differentiation and behavioral isolation between the species. If there is significant and non-combinable genetic variation between species, but behavioral isolation between the two incipient taxa is incomplete and allows hybrid offspring to be produced, these hybrid offspring will have lower fitness relative to parental types and selection should act directly to eliminate those offspring and indirectly against parents with broad mating preferences or traits. If however the genetic architecture is similar and behavioral isolation is incomplete, the populations would be expected to turn into a hybrid swarm and eventually become one species. Patterns of behavioral isolation and genetic variation in several *Laupala* species pairs suggest that

contact zones between closely related species are marked by conflicting patterns of behavioral isolation and genetic differentiation. Evidence also suggests that the complex courtship system of *Laupala* may allow male choice to play an important role in sexual selection and speciation. Therefore I tested several hypotheses about the genetic differentiation, sexual selection, and behavioral isolation in a contact zone between the closely-related and morphologically indistinguishable *L. tantalus* and *L. pacifica* species pair. First, by using the mitochondrial COI gene and AFLPs as genetic markers, I demonstrated that there appears to be mitochondrial DNA introgression between sympatric, but not allopatric congeners, which suggests contemporary hybridization in the contact zone. Next, I found that males experience post-mating resource-limitation and show a significant tendency to invest less into a second mating, however, their investment is dependent upon female size. Finally, I found that there is apparent displacement of male choice, decreased variation in spermatophore production, and asymmetrical mating isolation within the contact zone. This evidence all suggests that there is increased behavioral isolation in this contact zone, which may be consistent with a hypothesis of speciation by reinforcement. However, this evidence also suggests that male costs may result in male choice conflicting with other isolating mechanisms. If so, this study may be another putative case of reinforcement, or it may be an entirely novel report of conflicting selection pressures within a hybrid zone. I suggest that further studies are needed to measure hybrid fitness as well as to evaluate relative male and female mating costs within the complex mating system of this rapidly-diversifying genus.



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HYBRIDIZATION AND SPECIATION IN THE HAWAIIAN CRICKET LAUPALA  
(SUBFAMILY:TRIGONIDIINAE)

by

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## Preface

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

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## Introduction

Contact zones between closely-related species provide a unique opportunity to study the dynamics of speciation in action in a natural setting. Within such contact zones, depending upon the degree of genetic and behavioral degree of isolation between species, the size of the zone, and the time since first contact, one can make predications about the likely outcome of heterospecific mating and can test hypotheses about processes of speciation. Several outcomes can be predicted based upon these factors. For example, in some contact zones, micro-habitat changes between species with different phenotypic adaptations will prevent contact between the species, allowing them to remain distinct. In other cases, species might share a similar niche, but enough behavioral differences will have accumulated such that no hybridization will occur between them. However, in many other cases, the two species will have not accumulated enough behavioral or phenotypic differences to remain completely distinct in the face of frequent physical contact; this is when contact zone dynamics become particularly interesting.

When two incipient species are not yet totally isolated, several outcomes are possible. First, if the species are recently diverged sister taxa, with few accumulated genetic or behavioral differences, the two species may interbreed and form a hybrid swarm which may eventually become a new species. Alternatively, if one species has a slight selective advantage, then hybridization followed by a selective sweep could eventually eliminate one species in favor of the other. And finally, if few behavioral differences have accumulated, but there are significant incompatibilities in the genetic or phenotypic architecture of the species, we might expect to find either that the processes

of reproductive character displacement or reinforcement are at work in order to strengthen the behavioral isolation between the species.

Reinforcement is the process whereby natural selection strengthens prezygotic barriers to gene exchange between populations in response to reduced hybrid fitness. This is thought to proceed when a population is split, remains so for some amount of time while mutations affecting fitness accumulate within each subpopulation, and comes together again in a zone of secondary contact. Those individuals that choose mates from within their own subpopulation (mate assortatively) will produce offspring of higher fitness than those individuals which engage in hybrid matings. Over successive generations, assortative mating, and thus reproductive isolation, will increase until the populations no longer mate freely and may thus be classified as “biological species” (Mayr 1963). Reinforcement is fascinating because it provides a role for natural selection in generating and maintaining species boundaries. It can explain the evolution of these boundaries as a function of both allopatric and sympatric processes by describing how speciation is completed in sympatry as a result of incomplete postzygotic isolation in allopatry. Reproductive character displacement (RCD), is the pattern which is often a signature of reinforcement, or can independently evolve as a result of the strengthening of species barriers. First described by Brown and Wilson (1956), RCD is a pattern in which mate discrimination characters, such as plumage colors (Saetre *et al.* 1997), calling songs (Littlejohn and Loftus-Hillis 1965), or preference functions (McLain 1985), shift in sympatry such that there is no overlap in value of these characters between heterospecific populations. This results in reduced mating between these two sympatric populations relative to their allopatric counterparts. RCD may occur between populations that are

already distinct species as a result of selection against overlap in mating signals (Butlin 1989). This would happen when such overlap caused individuals from each species to engage in non-adaptive heterospecific matings that resulted in no insemination, no fertilization, or no viable offspring. However, many reported cases have found that RCD occurs between taxa that do successfully hybridize (Coyne and Orr 1989; Rundle and Schluter 1998; Kiang and Hamrick 1978), thereby implicating reinforcement as the underlying process that drives RCD.

Another important problem with the theory is that all tests of RCD have assumed that females are the choosy sex. First proposed by Darwin (1871), and later developed by Bateman (1948) and Trivers (1972), female choice has long been thought to be the primary force behind both sexual selection and the evolution of mating systems. A burgeoning number of reports on the importance of male choice would, however, suggest otherwise (Reinhold *et al.* 2002; Kvarnemo and Simmons 1999; Amundsen and Forsgren 2001; Pilastro *et al.* 2003; Werner and Lotem 2003; Bonduriansky 2001). The frequency of these reports suggests that male choice may be quite commonplace, and that in species with apparent high male mating investment, such as crickets (Kvarnemo and Simmons 1999) male choice may be just as, if not more, important than female choice in shaping the mating system. Many studies have reported that males in numerous insect species prefer larger females, since large female body size is usually indicative of egg mass, and therefore is a measure of fecundity (Togashi 2007; Honek 1993; Jimenez-Perez and Wang 2004). To date, almost all reports of reinforcement and reproductive character displacement have tested for shifts in female choice or male characters across a zone of contact; few have looked for the opposite patterns—shifts in male choice or female



sexual characters. However, the increasing evidence for male choice justifies its examination in the context of reinforcement.

This means that systems in which males provide females with resources during mating, such as nest sites, protection, or nuptial gifts, are good candidates for such reinforcement studies. This is because they have the potential, through displaced patterns of male choice, to have an impact on the evolution of mating systems. Thus, if reinforcement has occurred in such a system, it would be manifested by changes involving male, rather than female choice and would be a novel mechanism of reinforcement. However, the key to uncovering these relationships is to find a system in which details of the natural history and taxonomy are already somewhat defined, and tests of mating behavior and male choice are tractable. Crickets of the genus *Laupala* provide just such a model system.

#### ***LAUPALA AS A MODEL SYSTEM***

*Laupala* is a genus of crickets endemic to single islands of the Hawaiian island chain. *Laupala* is composed of 38 cryptic species (Otte 1994; Shaw 2000b), distinguishable only by song rate and morphometric differences in male genitalia. It is an instructive system in which to study processes of behavioral isolation because of its long and complex courtship system. Courtship in *Laupala* begins shortly after sunrise when a male begins to call from his perch on or near the forest floor. Once a female has located the male, they begin a series of copulations, in which the male circles the female, antennates her abdomen, intermittently sings, and finally ends each copulation with the transfer of a spermless “microspermatophore” (hereafter “micro”) to the female, which she consumes.

The number of microspermatophores passed varies from approximately 6-15, depending on the species (Shaw and Khine, 2004). The duration and timing of events during the mating sequence also varies between species, and even populations of the same species. At the culmination of an 8-10 hour mating sequence, the male passes the female a larger, sperm-filled “macrospermatophore” (hereafter “macro”), which she also consumes after the sperm has evacuated. While the costs of reproduction to the female are undoubtedly substantial (including egg production and mate searching), the costs to males may be just as high or higher, due to sperm production, spermatophore production, and the energy of song production. For *Laupala* females, mating “mistakes” by heterospecific males may actually be desirable. While the nutritional value of micros in *Laupala* is unknown, evidence from other cricket species suggests that females may receive life-span or direct nutritional benefits from consuming spermatophores (Hou and Sheng 1999; Simmons *et al.* 1999; Vahed 2007a, and references therein). If *Laupala* females receive a nutritional benefit, we might expect that females would be selected to manipulate all males into transferring micros to them. A high male mating cost coupled with potential female benefits could have a major effect on the strength and direction of patterns of reproductive isolation.

*Laupala* is additionally ideal for a study of contact zone dynamics because complicated phylogenetic patterns within the genus suggest that sympatric hybridization may have contributed to speciation. As noted above, *Laupala* was originally classified as a genus with 38 species. This phenetic hypothesis was proposed based upon ancestral relationships among song rates and male genitalia (Otte 1994). However, a later mtDNA phylogeny (Shaw 1996a) did not agree with this phenotypic classification, particularly in

contact zones between sympatric congeners. Conversely, later nuclear DNA (Shaw 2002) and AFLP phylogenies (Shaw 2004) conflicted with the mtDNA phylogeny, but confirmed the original phenogram. Interestingly, all of the conflicts between cladistic hypotheses are in areas of species overlap, which suggests that mtDNA introgression due to hybridization between sympatric species may be causing the disagreement. Moreover, a close examination of Otte's phenogram shows that in many geographic locales, individuals that might be classified as species A according to song type, are actually classified as species B, even though the song appears to differ significantly from the species B mean (Otte 1994). Otte attributes the somewhat arbitrary pattern of these groupings to reproductive character displacement in areas of sympatry between A and B (Otte 1989). Such a report suggests that RCD and reinforcement may be prevalent in *Laupala*.

However, reinforcement can be difficult to demonstrate because, in addition to finding RCD of a mating character in a contact zone relative to an allopatric conspecific, several other preconditions must first be met (Howard 1993). Briefly, one must show that 1) hybridization is possible, 2) selection acts against hybrid individuals, 3) differences in this character are detectable by the opposite sex, 4) this character is heritable, and 5) the RCD is not the result of ecological, or other selection. It can be difficult to identify populations that meet all of these criteria, however, two species on the island of Oahu, *L. tantalus* and *L. pacifica*, are ideal for such a study because they satisfy many of these conditions. First, they occur in both sympatric and allopatric populations, allowing one to set up the proper comparisons for a test of reinforcing selection. Next, they appear to satisfy the first condition, given that there are few to no habitat differences between

species, and therefore it is unlikely that hybrids would experience ecological selection. They also satisfy the second through fourth conditions because previous work on *Laupala* has found that song characteristics are heritable (Shaw 1996b; Shaw 2000a), and females discriminate against non- species specific pulse rates by choosing songs that are closest to the species mean (Shaw and Herlihy 2000; Mendelson and Shaw 2002); therefore it is likely that hybrids would suffer decreased mating success. Only hybridization and reproductive character displacement need to be demonstrated in order to provide substantial support for a hypothesis of speciation by reinforcement in this species pair. Hybridization appears to be likely given evidence for mtDNA introgression between these non-sister taxa in a sympatric population (Shaw 2002; Mendelson and Shaw 2005); additionally, evidence from recordings of intermediate singers (Otte 1994) suggests that hybridization may be ongoing. However, the extent of hybridization had not been confirmed in this species pair, nor has reproductive character displacement been confirmed.

Therefore, the overall objective of this study was to examine the extent of behavioral and genetic isolation in the *L. tantalus* and *L. pacifica* species pair and assess how male choice might be affecting the strength and direction of isolation. My general aims were 1) to uncover genetic evidence of hybridization 2) to demonstrate that males experience mating costs and may be choosy, and 3) to show behavioral isolation due to reproductive character displacement is present and stronger within in a contact zone relative to outside the zone. To demonstrate that hybridization is occurring between sympatric congeners, I analyzed patterns of shared genetic variation between and within sympatric and allopatric populations of these species. To demonstrate that males in

*Laupala* may be choosy, I examined male remating ability and differential male mating investment. To measure behavioral isolation, I compared mating frequencies and measured mating characters between allopatric and sympatric congeners.

In Chapter I, I use mtDNA and AFLP markers to investigate species and population boundaries, as well as evaluate the degree of mtDNA introgression both within and between species in sympatry and allopatry. I did this by using a 560bp sequence of the COI gene as a mtDNA marker and analyzing patterns of nuclear DNA variation in 656 polymorphic AFLP bands in sympatric and allopatric populations of *L. tantalus* and *L. pacifica*. By estimating relationships using phylogenetic methods, measuring genetic variation and population structure, and evaluating the ancestry of individuals within each population, I was able to demonstrate that mtDNA variation is shared across species boundaries in the contact zone, but not between allopatric conspecifics. I was also able to demonstrate that there is little shared nuclear variation between sympatric congeners; however, putative hybrid individuals were identified. Both results suggest recent, and perhaps contemporary hybridization is occurring between these sympatric congeners. Previous evidence suggested that hybridization may have occurred; however, this is the first time a widespread pattern of mtDNA introgression has been revealed.

In Chapter II, I document the costs of mating to males by measuring decreases in spermatophore production over subsequent days of mating, and I measure the mating response of males towards larger females. I tested the hypotheses that males will produce fewer spermatophores on a second consecutive day of mating, and that they will produce more spermatophores for larger females. I found results which positively supported both

of my hypotheses, and in addition found that males appear to modulate their investment based upon the relative size of consecutive mating partners. This is a novel finding not only in *Laupala*, but in insect mating systems in general, as it suggests that males might have a threshold that must be reached before mating investment is maximized.

Finally, in Chapter III, I document the patterns of mate choice and reproductive character displacement using no-choice tests from both a male and a female perspective. I also quantify population-level differences on several characters that may be candidates for sexual selection between *L. tantalus* and *L. pacifica*. I first test the hypotheses that sympatric *L. pacifica* and *L. tantalus* are incompletely isolated, and allopatric populations of *L. pacifica* are not isolated by comparing inter and intraspecific mating frequencies. I then test the hypotheses that there is reproductive character displacement of mating characters by comparing mating frequencies and mean values in potentially sexually-selected characters in sympatric and allopatric congeners. I found that there is significant, but incomplete behavioral isolation among sympatric and allopatric congeners that is consistent with a hypothesis of male choice displacement. Additionally, I found that there is asymmetrical mating isolation between the sympatric male and female congeners. Moreover, the asymmetry fits the pattern found in the previous study which suggests that males prefer large females. Finally, I found a trend suggesting a shift in spermatophore production times between allopatric conspecifics that fits a pattern of reproductive character displacement. I suggest that these results are both novel and important because not only does this indicate that there is asymmetric species isolation in *Laupala*, possibly driven by a shift in reproductive characters, but additionally, it suggests that there is potential for conflict between male and female sexual selection. If

this is the case, this is the first such report of such a conflict between male choice and female sexual selection, and has potential to change the way we study speciation in organisms with complex mating systems.

## **Chapter I: Contrasting molecular phylogenies provide evidence for introgressive hybridization between sympatric populations in *Laupala***

### **ABSTRACT**

Contact zones provide an unique opportunity to study mechanisms of speciation, such as the processes which shape mate preference and strengthen species barriers within the zone. The Hawaiian cricket genus *Laupala* presents such an opportunity in a contact zone between *L. pacifica* and *L. tantalus*. Behavioral evidence from this species pair suggests that behavioral isolation is stronger within the contact zone than between allopatric pairs; however, that isolation is incomplete.. Past studies have found disagreement in the patterns presented by mitochondrial, nuclear, and phenotypic data. The disagreements are all found in contact zones between one or more species, which suggests that there may be mtDNA introgression between these hybridizing taxa. However the extent of mtDNA introgression and the degree of nuclear gene flow remains unknown. In this study we sequenced 560bp of the mitochondrial cytochrome oxidase subunit I (COI) gene and analyzed 656 polymorphic AFLP bands for approximately 146 individuals from three sympatric and allopatric populations of *L. tantalus* and *L. pacifica*. We investigated the patterns in these data using four approaches. A neighbor-joining and haplotype network analysis of mtDNA haplotypes show shared mtDNA variation in sympatric congeners, while a neighbor-joining analysis of AFLP phenotypes supports the current classification of species. Gene flow estimates using F-statistics showed that reproductive barriers existed between the sympatric congeners, and an analysis of



**molecular variance showed that there is significant genetic divergence between the sympatric congeners, but not between the allopatric *L. pacifica* populations. Finally, a clustering analysis of AFLPs identified multiple individuals of mixed ancestry, indicating that hybridization is ongoing or occurred in the recent past. We discuss the phylogenetic results in relation to behavioral data showing incomplete isolation between this species pair, and suggest that they provide support for a hypothesis of recent or ongoing speciation.**

## **INTRODUCTION**

Contact zones studies have played a major role in the field of evolutionary biology because such areas act as natural laboratories of speciation, where one can test species concepts and therefore gain insights into the processes of speciation (Hewitt 1988). Likewise, research on interspecific hybrids in these zones has helped to uncover the mechanisms preventing or allowing interbreeding of species (Hewitt 1988; Mallett 2005; VanHerwerden *et al.* 2006; Croucher *et al.* 2007) and can help us to answer fundamental questions about how reproductive barriers evolve and whether gene flow between species affects adaptation. However, in order to answer these questions and understand the evolutionary processes acting on species in a contact zone, it is first necessary to determine if hybridization is occurring within the zone, and next, whether or not it is leading to species divergence, or homogenization of the two species into one. Which of these two results occurs should be dependent upon the degree of divergence that has already occurred. In very recently diverged sister taxa, one would expect that insufficient genetic incompatibilities have accumulated and therefore hybridization would have low

costs, or even confer fitness advantages (Pfennig 2007). Hybridization events may occur, leading to gene flow and potentially long-lasting consequences on the genetic architecture of the species. However, in cases where there is significant behavioral isolation between species in a zone of contact, one would expect that speciation occurred previous to secondary contact, the two species are reproductively distinct, and no phylogenetic evidence of hybridization would be found .

As has been recently observed (Counterman and Noor, 2006) many hypotheses of hybrid zones and the processes are based on behavioral evidence alone, often lacking genetic support for hybridization. Such genetic demonstrations can be challenging, for several reasons. First, sample sizes of individuals in many studies are small, and if hybridization is infrequent, it may be missed. Second, depending upon what type of marker is used, in what portion of the genome it is located, and whether or not it is under selection, results can lead to drastically different gene-trees (for recent examples see Masta *et al.*, 2002; Crochet *et al.*, 2003; VanHerwerden and Doherty, 2006; Roe and Sperling, 2007; Ray *et al.*, 2008), which may fail to confirm species status and lead to incorrect conclusions about hybridization (Funk and Omland 2003). And finally, closely-related species often share genetic variation for a long period after divergence (Tajima 1983; Wu 1991; Maddison 1997; Hudson and Coyne, 2002) in part because it may only be necessary that a small portion of the genome be differentiated in order for speciation to occur (Broughton and Harrison 2003). Demonstrating only that there is little genetic divergence between putatively hybridizing sister taxa does not distinguish between the causes of shared polymorphism, but only introgression could definitively support a hypothesis of hybridization (Funk and Omland 2003).

The Hawaiian crickets of the genus *Laupala* constitute an interesting case for such a study of hybrid zone dynamics because of their behavioral isolation and complicated phylogenetic history. Species of *Laupala* are all single-island endemics distributed throughout the Hawaiian archipelago, and are thought to be derived from a sword-tail cricket ancestor from the Western Pacific of the subfamily Trigonidiinae (Otte 1994). *Laupala* has rapidly diversified in the native montane rainforests of the islands, where they inhabit leaf-litter of both native and introduced plant species. They are phenotypically similar and have been described as a genus composed of 38 cryptic species (Otte 1994; Shaw 2000b), differentiated only by song rate and nearly indistinguishable morphometric differences in male genitalia. Otte (1994) published the first systematic hypothesis of *Laupala*, a phenogram which he based upon the variation in these characters. He divided *Laupala* into three major species groups: the *kauai* group, found only on the northwestern island of Kauai; and the *cerasina* and *pacifica* groups, found on Oahu, the Maui-Nui complex, and Hawaii, and hypothesized to have each diversified via independent radiations onto sequential islands of the Hawaiian chain. However, a mtDNA phylogeny (Shaw 1996a) did not support Otte's original hypothesis of three major clades, and found instead what appeared to be a pattern of multiple invasions by a single species. A further nested analysis of mtDNA haplotypes of Big Island species revealed geographically restricted haplotypes that were frequently shared between sympatric congeners, but not between allopatric conspecifics (Shaw 1999). Conversely, a later nuclear DNA sequence phylogeny (Shaw, 2002) as well as an AFLP phylogeny (Mendelson and Shaw, 2005) did support Otte's original phenogram, but not

the mtDNA phylogeny. As a result, the species boundaries and the extent of hybridization remain unclear.

An interesting pattern that has emerged from all of these analyses is that the major disagreements between these systematic hypotheses arise in areas of species overlap (Shaw 2002). This discrepancy suggests that gene flow between the two major groups has occurred subsequent to splitting of these lineages. Several species pairs in many geographically disparate locales show this pattern of putative mtDNA introgression. One such pair includes *L. tantalus* and *L. pacifica*. These two species are distributed throughout the Koolau mountain range on the eastern side of Oahu (Figure 1). *L. pacifica* occurs in many contiguous populations throughout this range, but the *tantalus* species is thought to be confined to a smaller area entirely within the *pacifica* range (Otte, 1994). Although these two species appear to be taxonomically distinct non-sister taxa based upon song rate, genitalia, and nuclear DNA, the mtDNA phylogeny shows that *pacifica* is more closely-related to *tantalus* in a mid-island contact zone relative to a geographically-distant allopatric population of *pacifica* (Shaw 1996a).

Behavioral data from laboratory matings give some insight into the evolutionary dynamic in this contact zone. Previous experiments have shown that mating is much more frequent between the *pacifica* populations than between the heterospecific pairs (J. Jadin, Ch.3); this pattern supports the species division shown in the nuclear and phenotypic data. Nevertheless, hybrids can be generated in lab (J. Jadin, unpublished), and intermediate singers have been identified in field recordings (Otte, 1994). Altogether these data suggest that hybridization is occurring in the contact zone between these species. However, the genetic consequences and patterns of hybridization are

unconfirmed, as the previously reported mtDNA and nuclear phylogenies were created using only a very small sample size (typically <4 individuals/species/population), and the number and degree of shared mtDNA haplotypes is still unknown. Further, the small sample size does not allow clear resolution of relationships in the nuclear DNA.. Many recent studies of rapidly diversifying organisms have found that incongruence among gene trees is a widespread problem, particularly in insects (e.g. Beltran *et al.* 2002; Downie *et al.* 2002; Kilman *et al.* 2000; Buckley *et al.* 2006; Egger *et al.* 2007; Belfiore *et al.*, 2008). However, in order to understand dynamics of this hybrid zone, this problem must be solved and a clearer resolution of the patterns of both mitochondrial and nuclear DNA data must be obtained.

Theory predicts that gene genealogies should vary between loci in rapidly-speciating taxa, due to the random nature of lineage sorting (Edwards and Beerli 2000). Therefore studies of such species benefit from comparisons of multiple loci. The objective of this study was to characterize the mitochondrial COI and nuclear AFLP phylogenetic patterns of sympatric and allopatric populations of *L. pacifica* and *L. tantalus* across a contact zone where behavioral evidence (J. Jadin, Ch.3), song data (Otte 1994), and previous DNA evidence (Shaw 2002) suggest that hybridization may be occurring. I examined the resulting mtDNA and nuclear phylogenies, first, in order to determine if previously proposed evolutionary partitions of the *L. pacifica* and *L. tantalus* species based on phenotypic and nuclear data (Mendelson and Shaw 2005) could be supported with the current larger data set. I next assessed whether these data corroborated the previous hypothesis that mtDNA genetic variation is shared primarily in sympatry, while nuclear variation is partitioned between species. Finally, using AFLPs I

investigated the genetic architecture of the populations in order to identify putative hybrid individuals. Results from AFLPs suggest that the two *L. pacifica* populations are nearly genetically indistinct, and that *L. tantalus* is a taxonomically distinct species that shares substantial mtDNA variation, but limited nDNA variation, with *L. pacifica* exclusively in a contact zone. These data corroborate evidence of incomplete reproductive isolation between these taxa, and provide phylogenetic support for a hypothesis of incomplete and possibly ongoing speciation.

## **METHODS**

### *Specimen Collection*

A total of 146 wild-caught males, approximately 49/population, were used for genetic analysis. Specimens used for this analysis were collected at two sites on the island of Oahu (Figure 1). The sympatric site located at Manoa Cliffs had both *L. pacifica* and *L. tantalus* individuals. The collection area was defined by a transect of approximately 100 meters on either side of the Manoa Cliffs trailhead (21.19.406 N, 157.48.846W, 1408 ft) on Mt. Tantalus. Individuals from *L. pacifica* were also collected at an allopatric site along a trail leading past the Pupukea Boy Scout Camp (~ 21.63010N, 158.02175W, 1200ft). This collection site was more than 25 miles north of the sympatric site. The entire extent of the *L. tantalus* range is unknown, but it does not visibly or audibly inhabit the forest for at least 1 mile surrounding this population.

Specimens were captured live and returned to the lab where the song rates of the males were counted with a stopwatch. They were assigned to the *pacifica* or *tantalus* species,

depending their song rate; all males sang at the approximate song rate of *pacifica* (0.6pps) or *tantalus* (2.1pps) (Otte, 1989). Individuals were placed in specimen cups and checked every two days for deceased individuals. These males were immediately frozen in individual tubes at -80C. The genitalia and one leg of each male used in the study was removed and saved as a voucher specimen, and the remainder of the male was used for DNA preparation.

#### *mtDNA collection and analysis*

DNA was extracted using standard phenol/chloroform protocols with phase-lock gel tubes. Extracted DNA was stored at -20C. The COI gene, which is commonly used for species barcoding was deemed a suitable marker because of the sufficient level of variation it contains between even closely-related species (REF). DNA was amplified in a standard PCR reaction with COI forward and reverse primers (COIF: 5'-GGTCAACAAATCATAAAGATATTGG-3'; COIR: 5'-TAAACTTCAGGGTGACCAAAAAA TCA-3') (Folmer *et al.*, 1994) to obtain an approximately 620bp fragment. PCR conditions entailed a 2 min denaturing period at 95°C followed by 35 cycles of 95°C for 30s, 50°C for 90s, and 72°C for 90s, and ending with a final 72C elongation period for 2 min. After confirmation of single band presence, the reaction was prepared for sequencing using EXOSAP (REF). Automated sequencing was performed using dye terminator chemistry (ABI, Foster City, CA, USA). Nucleotide sequences were automatically aligned, trimmed to 560bp, and then manually edited using the program Sequencher v4.8 (Gene Codes Corp, Ann Arbor, MI, USA).

### *AFLP collection and analysis*

Procedures for generating AFLP bands generally followed Vos, *et al.*, (1995). Restriction enzymes *EcoRI* and *PstI* were used to digest approximately 250ng of genomic DNA while the *PstI* and *EcoRI* adaptors were concurrently ligated. A 1:10 dilution of this restriction-ligation product was used as a template in a pre-selective PCR amplification. The primers for this amplification were the sequence of the restriction enzymes and one additional selective nucleotide (*EcoRI*: 5'GACTGCGTACCAATTC + A; *PstI*: 5'-GACTGCGTACATGCAG + A). A 1:40 dilution of this product was used as the template for the selective amplification, in which the primers were the same as for the pre-selective amplification, but with the addition of two extra nucleotides. The following primer pair combinations were used: Eaac-Pacg, Eaac-Paaa , Eaac-Paac, Eaac-Pata, Eagc-Paac, Eagc-Pacg; the first letter of each primer pair represents the restriction enzyme site it adheres to (Eco or Pst), the next letter designates the pre-selective (a) base pair, and the last two letters designate the selective base pairs. PCR conditions for selective amplification entailed first a denaturing step at 95° for 1:30, followed by 9 cycles of 95°C for 0:30, 65°C for 0:30 at -1° per cycle, and 72°C for 1:00. There were then 23 additional cycles of 95°C for 0:30, 56°C for 0:30, and 72°C for 1:00. The samples were then prepared for genotyping by resuspension in Hi-Di ROX(-500) (ABI, Foster City, CA). Automated fragment analysis was performed using an ABI3730 sequencer (ABI, Foster City, CA). The data were then visually scored for presence or absence of bands (1 or 0) using GeneMapper software v 4.0 (ABI, Foster City, CA). Scoring was optimized by setting bin width to 0.5base pairs (Holland *et al.*, 2008) and by ignoring all peaks under 100 Relative Florescence Units (RFU). Only bands from 100-



425bp were scored, as this was the range of lengths that showed the clearest peaks. The 6 primer pairs reactions resulted in 656 polymorphic bands. A complete technical replicate (from digestion–ligation through selective PCR) was performed on any individuals that had jagged or low peaks, or showed significantly mixed ancestry post Structure.

Repeatability was estimated for each marker as one minus the ratio of the number of differences between technical replicates to the total number of individuals genotyped (as in Bonin *et al.* 2004; Pompanon, *et al.* 2005). Putative markers with less than 90% repeatability and individuals with less than 90% repeatability were discarded from the analysis.

#### *Data Analysis*

For both the mtDNA and AFLP data, neighbor-joining trees were estimated in PAUP\*4.0b10 (Swofford, 2003). For the AFLP data, Nei-Li restriction site distance criteria were used; Nei-Li distance criteria weights the gain of a restriction site more heavily than the loss of a restriction site and is therefore most appropriate for AFLP data. For the mtDNA data, the neighbor-joining tree was produced using minimum total character difference distance criteria. Nodal support was calculated with 1000 bootstrap replicates on a neighbor-joining tree created using the same parameters. A maximum parsimony (MP) analysis was also performed on the COI sequence data. For this analysis, all characters were unordered and given equal weight. A heuristic search was begun with starting trees obtained by stepwise addition. Ten random sequence addition replicates were performed with TBR branch swapping, and one best tree was held at each step. One best tree was found, and nodal support was calculated with 1000 bootstrap

replicates using the same search parameters. Haplotype networks based on a statistical parsimony algorithm (Templeton *et al.*, 1992) were constructed for the mtDNA data using TCS1.21 (Clement *et al.* 2000). Briefly, TCS calculates haplotypes frequencies and uses them to estimate outgroup probabilities for those haplotypes; these probabilities should correlate with haplotype age. An absolute distance matrix is then calculated for all pairwise comparisons of haplotypes and the probability of parsimony is calculated for pairwise differences until the probability exceeds 0.95 (Clement *et al.* 2000). The most likely connections among haplotypes are then made and used to generate the network.

$F_{ST}$  values for the mtDNA and AFLP data were calculated using Arlequin, version 2000 (Schneider *et al.*, 2000). For the mtDNA data, distances between haplotypes were calculated using the Tamura and Nei model, which allows for different transition and transversion ratios, and  $\gamma$  was set to zero as no heterogeneity of mutation rates among sites was expected. For the AFLP data, distance between genotypes was calculated using simple minimum pairwise distance criteria. Estimates for significance levels for  $F_{ST}$  values were determined by performing 10000 permutations.  $F_{ST}$  analyses such as Arlequin were not originally designed to be used with dominant markers such as AFLPs, however, it is often used for this purpose (Svensson *et al.* 2004; Irwin *et al.*, 2005) and yields results similar to other methods (de Casas *et al.*, 2006). AFLP markers are dominant markers; therefore the  $F_{ST}$  calculated for the AFLP data is based on band frequencies rather than allele frequencies and should not be directly compared to  $F_{ST}$  values calculated from codominant markers.

In order to calculate assignment probabilities of individuals to a number of populations ( $K$ ) ranging from 1 to 5 and estimate admixture between populations,

Structure 2.2 (Pritchard *et al.*, 2000; Falush *et al.* 2007) was used. The program Structure uses a Bayesian model-based clustering method in order to infer population structure from genotypic data gathered from unlinked markers. The algorithm which Structure uses assumes a model in which there are  $K$  populations (where  $K$  is the number of populations, and can be known or unknown), and it defines  $K$  by a set of allele frequencies at each locus. Individuals in the sample are assigned to single populations with some degree of probability, or jointly to two or more populations if their genotypes appear to indicate admixture (Pritchard *et al.* 2000). It is assumed that within populations, the loci are unlinked and in Hardy-Weinberg equilibrium. In order to estimate the best model of ancestry, I performed 10 iterations each of an admixture and a no admixture model. For both models, each run consisted of a 10,000 step burn-in with 10,000 cycles of data collection, and for each value of  $K$ , the parameter set was run for 10 iterations. The log-likelihood,  $L(D)$ , values were averaged across each of the iterations and were used to calculate the ad hoc statistic  $\Delta K$ , which is related to the second order rate change in the log probability of the data (Evanno *et al.*, 2005). The  $K$  value with the largest  $\Delta K$  was identified as the true number of population clusters according to Evanno *et al.* (2005). I then calculated the Bayes factors for the fit of the data to each model, admixture or no admixture, following the methods of Fitzpatrick *et al.*(2008). After the model and  $K$  were chosen, I again ran 10 iterations of Structure using the USEPOPINFO model (Pritchard *et al.*, 2000). This model assumes that most individuals classified as being from a particular population will have pure ancestry from that population, but that a small fraction of them may have mixed ancestry. In this model I assigned individuals to one of two populations defined by species. I set the MIGRPRIOR = 0.05, which implies that the prior probability

that an individual has pure ancestry from a predefined population is 0.95. I set GENSBACK = 2, which calculates the ancestry of the individuals up to two generations back. This allowed me to determine, with a high probability, whether any of the individuals were likely either to be hybrids or to be misclassified.

To estimate the partitioning of the AFLP and mtDNA genetic variation between and within groups, analysis of molecular variance (AMOVA) was performed using Arlequin, version 2000 (Schneider *et al.* 2000). Genetic differentiation values ( $\Phi_{ST}$ ) between pairs of populations were calculated. These values are analogous to traditional  $F$  statistics: they are designed to estimate nucleotide diversity, rather than allelic diversity as  $F$  statistics do (Excoffier *et al.* 1992). Estimates of significance levels for  $\Phi$ -statistics were determined by performing 10000 permutations. The three fixation indices are defined as follows (Hartl and Clark, 1997):  $\Phi_{ST}$  measures fixation in the subpopulations relative to that of the total population,  $\Phi_{CT}$  measures fixation in a specified group of the subpopulations relative to the total population, and  $\Phi_{SC}$  measures fixation in the subpopulation relative to that within the group in which it is contained.

## **RESULTS**

A total of 146 individuals from 3 populations comprised of 2 taxonomic species were sequenced for 560bp of the mitochondrial COI gene and were analyzed for 656 polymorphic AFLP bands generated from 6 primer pair combinations.

Results show that the COI gene is highly conserved, as indicated by the small number of variable sites among the sequences, as well as by the number of silent and replacement substitutions observed (Table 7). Of the 560bp sequenced only 16 (2.8%) of

the sites were polymorphic. 15 of these mutations were transitions, and only one was a transversion, and was contained in only one *tantalus* individual. A total of 15 haplotypes were observed, and their relative frequencies are shown in Table 1. Of these 15 haplotypes, 8 were contained exclusively within the northern allopatric *pacifica* population, and the remaining 7 haplotypes shared between the *tantalus* and *pacifica* individuals within the sympatric populations. The number of individuals analyzed per locality was approximately 48 individuals per population. In the allopatric *pacifica* population, 34 (69%) of the individuals shared one haplotype, while in the sympatric mixed-species population, 86 (88%) individuals, 39 from *pacifica*, and 49 from *tantalus* shared one haplotype.

The primer pairs used in the AFLP analysis generated the following number of bands per primer pair: ac-cg (90), ac-aa (128), ac-ac (97), ac-ta (199), gc-ac (115), gc-cg (125). This resulted in a total of 656 polymorphic bands between 100-400bp. Overall, the vast majority (84.5%) of bands were polymorphic and shared between all three populations. However, 29 bands (4.4%) were polymorphic but exclusively in *tantalus* and 27 (4.1%) were polymorphic but contained exclusively in *pacifica*. Forty-one bands (6.2%) were polymorphic but contained only in the sympatric locality, and three bands were polymorphic but contained only within the allopatric population. None of these bands were fixed exclusively in any of the populations or species. Figure 6 depicts the frequency differences in bands between populations.

### *Phylogenetic analysis*

The unrooted haplotype network was used to define geographic structure (Figure 2) and showed that the northern allopatric *pacifica* population formed its own clade that was entirely distinct from the southern sympatric *pacifica* and *tantalus* populations. The haplotype estimated as most ancestral was H7, and the remaining haplotypes within the southern sympatric population are connected to it by no more than two steps. The vast majority of the sympatric individuals sampled (84) all shared haplotype H6, which was nearly evenly divided between species. Eight haplotypes were found in the allopatric *pacifica* population, and none were more than five mutational steps away from the H6 sympatric haplotype. The phylogenetic relationships among these 15 haplotypes are summarized by a neighbor-joining phylogram (Figure 3). Aside from the outgroup, three major clades were identified and clustering of haplotypes clearly corresponded to location and matched the results from the haplotype network. There was 53% bootstrap support for the seven sympatric *L. tantalus* and *L. pacifica* haplotypes forming one major branch of the tree, and 91% and 76% bootstrap support for the other two major branches that were comprised of the eight allopatric *L. pacifica* haplotypes. The MP analysis of the COI sequence data resulted in a tree of the same topology as the NJ tree, with similar bootstrap support for the same clades, therefore only the results from the NJ tree are presented here.

The AFLP analysis showed a much different pattern in the data, revealing that the majority (92%) of the *L. tantalus* individuals fell into a distinct clade with 100% bootstrap support (Figure 4). However, four individuals (tan 7150, 402, 420, 505) fell into a separate clade that appears to be a sister taxon to the major *tantalus* clade, with 69% bootstrap support. Its proximity to, but exclusion from, the larger *tantalus* clade

suggests that this is a group of putative hybrid individuals. The remaining *pacifica* individuals clustered onto the other half of the phylogram and were divided into several subgroups, all of which contained a mixture of individuals from sympatric and allopatric populations.

### *Genetic diversity*

Analyses of molecular variance (AMOVA) based on haplotype divergence were used to estimate variance components and fixation indices. For both the AFLP and mtDNA data sets, the populations were grouped by location, such that the sympatric *tantalus* and *pacifica* comprised one group and the allopatric *pacifica* population comprised another. The AFLP data showed that most of the genetic variation in the data set was due to variation within populations (59.5%), but that subdivision between populations within locations (28.0%) also contributed significantly to the overall variation (Table 2). The least genetic variation (12.6%) was due to subdivision among locations, and was not a significant contributor to the overall variation. Pairwise genetic distance data supported this pattern: Table 5 shows that all pairwise AFLP genetic distances were significantly different from zero, however the distances between *tantalus* and the sympatric and allopatric *pacifica* populations were similar (pd = 226.28, 221.84,  $P < 0.0001$ ), whereas the genetic distances between the *pacifica* populations (pd = 148.65,  $P < 0.0001$ ) were nearly the same as the within population genetic distance (pd = 148.87).

The mtDNA showed the converse pattern to the AFLP data (Table 2): when grouped by location, the main variance components come from the subdivision among populations (22.6%), as well as the subdivision among locations (77.6%) and the

subdivision within locations contributed the least to the overall variation (0.21%). The pairwise genetic distances between the sympatric congeners was very low (Table 5:  $pd=0.399$ , NS) and was close to within population genetic distances, whereas the distance between the allopatric *pacifica* population and the sympatric *pacifica* and *tantalus* populations was significantly higher (Table 5:  $pd= 8.274, 8.211, P<0.0001$ ). The results show that when grouping these three populations based upon nuclear data, the individuals within species are each others closest relatives, but that when grouping populations based upon mtDNA data, individuals within locations are closest relatives.

For the mtDNA, population pairwise  $F_{ST}$  values were significant for all comparisons between the sympatric and allopatric locations, but not between the *tantalus* and *pacifica* species within the sympatric location (Table 3). The AFLP data showed that population pairwise  $F_{ST}$  values were significant between all populations, but the  $F_{ST}$  values between the *pacifica* species (Table 3:  $F_{ST}=0.0347, P <0.0001$ ) were notably lower than the  $F_{ST}$  values between the *L. tantalus* species and either the allopatric ( $F_{ST}=0.3212, P <0.0001$ ) or sympatric ( $F_{ST}=0.3113, P <0.0001$ ) *pacifica* populations. These results show that while there is moderate nuclear genetic differentiation between the *pacifica* populations, there is very great differentiation between the *tantalus* and *pacifica* species, in sympatry and allopatry. Conversely, there is no significant mtDNA differentiation between the *tantalus* and *pacifica* populations in sympatry (Table 3:  $F_{ST}=0.0177, NS$ ), which indicates that there is significant sharing of alleles, a result which could be expected if there is hybridization between these populations.

### *Hybridization*



The  $LnK$  values were averaged over 10 iterations for each of the two models (admixture or no admixture with correlated allele frequencies). Calculation of  $\Delta K$  of each model using the  $LnK$  output from Structure showed that for each model there was a clear peak at  $K = 2$  (Table 4). The average marginal log likelihoods for the admixture and no admixture models at  $K = 2$  fitted using Structure were -47578.5 and -47972.0, respectively. Calculations of both Bayes factors showed that an admixture model was favored with a factor of  $8.0 \times 10^{170}$ . Given that Bayes factors greater than 100 can be considered “decisive” (Goodman, 1999), I concluded that the admixture model best fitted the data.

These results also mean that the data strongly support the hypothesis that *pacifica* and *tantalus* are admixed, albeit at a low degree. Averaged over 10 runs, 97% of the *pacifica* individuals and 93% of the *tantalus* individuals were assigned to one of these two population clusters with a very high probability. For the most part, assignments correlate well with the *a priori* designation of individuals to either of the two species. However, 13 individuals showed evidence of greater than 20% mixed ancestry, with four notable exceptions that had >50% mixed ancestry (Figure 5). One of these four individuals from the *tantalus* population (*tan7150*) had a roughly equal probability of being assigned to either population, which is consistent with the expectation for a first generation hybrid. However, all individuals were song-typed previous to analysis, and this individual, like all classified as *tantalus*, sang near the species mean pulse rate of 2.0pps. The remaining three *tantalus* individuals had varying degrees of assignment to the *tantalus* population, ranging from 23-35% averaged over 10 iterations, suggesting that they may be backcrossed hybrid individuals. There were several other individuals

from the *pacifica* populations which showed mixed probabilities of assignment. To further assess the statistical confidence for all of these possible instances of admixture, I performed a second clustering analysis (as in Kronfrost *et al.*, 2008); in this second analysis Structure estimated the posterior probability that each individual had pure ancestry by using information on the designated population of origin for each individual. I set the prior probability of pure ancestry to 0.95. Averaged over ten iterations, results from this second analysis showed that the probability that each of the previously-identified individuals belongs to their assigned populations falls outside of the 95% confidence interval. A summary of their ancestry probabilities is shown in Table 6. One of these individuals (*tan505*) appears to be misclassified, one appears to be a backcrossed hybrid individual (*tan7150*), and the other two individuals appear to have immigrant ancestry farther back. An additional four individuals from the allopatric *L. pacifica* population and four individuals from the sympatric population showed a high probability of assignment to the opposite clade two (or more) generations back (Table 6), and one individual (*mcp6145*) appears to be the result of a hybrid that has been backcrossed with a *pacifica* parent.

Contamination of DNA samples could also result in such a pattern, however, in order to determine that contamination did not cause the anomalous banding patterns in these individuals, the band totals for all individuals were assessed. Amplification with six primer pairs resulted in, on average, 251 bands per individual. Within *tantalus*, the mean band number was 238, and within *pacifica*, the mean band number was 257. If DNA from one species had contaminated the sample of another, that individual would be expected to have significantly more bands than average (because it would contain bands from both

species). None of the putative hybrid individuals had a significantly different mean band number than their species average, indicating that contamination from that other species was unlikely.

## **DISCUSSION**

Previous studies on the sympatric congeners *L. tantalus* and *L. pacifica* have found that behavioral isolation is incomplete, and have suggested that mtDNA has introgressed between these otherwise taxonomically-distinct species (Shaw 2008; Parsons and Shaw, 2001). Such findings suggest contemporary or recent hybridization has occurred. By comparing phylogenetic patterns at multiple genetic loci and across geographically disparate populations, I have been able to reveal a pattern of non-exclusive ancestry in nuclear and mitochondrial lineages between sympatric and allopatric populations of these species. Analysis of the mitochondrial COI gene sequence shows that the sympatric congeners have a high degree of shared variation, having mtDNA that is more similar than the mtDNA of allopatric conspecifics. The converse pattern is displayed in nuclear AFLP markers, which show significant differentiation in all populations, but have greater shared variation between allopatric *pacifica* populations than either does with *tantalus*. Finally, Structure analysis of AFLP data suggests that several individuals of *tantalus* have significantly mixed ancestry. Overall, the results confirm the pattern first reported by Shaw (2002) and suggest that sympatric hybridization allowing widespread mitochondrial introgression along with limited nuclear introgression is the most parsimonious explanation for the similarity in haplotype arrays, and differences in AFLP profiles, between sympatric populations of the *tantalus* and *pacifica* species.

### *mtDNA monophyly in sympatry*

The phylogenetic relationship among the mtDNA data was summarized by a neighbor-joining tree and a haplotype network, and the genetic variation within and between populations was analyzed with AMOVA and F-statistics. All analyses—phylogenetic, AMOVA, and a haplotype network—were concordant, showing that the two allopatric populations of *pacifica* were significantly differentiated and by all appearances, not each others closest relatives. This result is in agreement with previous findings (Shaw, 1996a) from this species pair, in which phylogenetic reconstructions of mtDNA data suggested that sympatric populations of *pacifica* and *tantalus* were sister species, not allopatric *pacifica* populations. Conversely, all analyses were also concordant in suggesting that sympatric *pacifica* and *tantalus* were each others closest relatives.

Several different processes could explain the shared mtDNA variation found in sympatric species of *Laupala*, including incomplete lineage sorting in the mtDNA, an ancient hybridization event followed by selection on mtDNA, or infrequent interspecific hybridization coupled with strong selection against hybrids allowing. The likelihood of any of these three hypotheses is discussed further below.

### *Nuclear genome population structure*

In contrast to the mitochondrial data, AFLP frequencies exhibited significant differences across the two species, indicating that nuclear genotypes of *pacifica* and *tantalus* do not support a hypothesis that they are sister taxa. Rather, phylogenetic analysis done on AFLP data using the more distantly related *L. kukui* as an outgroup indicate that previous

phenotypic groupings based on singing behavior and male genital morphology (Otte, 1989) represent distinct genetic lineages. First, the neighbor-joining analysis showed that 46 of the *tantalus* individuals fell into a single clade which had 100% bootstrap support. The remaining 4 individuals that did not group with the larger *tantalus* clade, instead came out as the next most closely-related group. The remainder of the *pacifica* individuals, from both sympatry and allopatry, formed a separate clade that was divided up into many mixed-locality groups, with few of them showing greater than 50% bootstrap support (Figure 4). The AMOVA revealed that there is more genetic differentiation between the sympatric congeners than between allopatric conspecifics (Table 3). The F-statistics also support this finding: the pairwise  $F_{ST}$  values are significant for all pairs, showing that there is significant genetic structuring which defines populations (Table 4). However, the  $F_{ST}$  values between the *pacifica* populations and *tantalus* are each  $>0.3$ , which is typically considered evidence of very great genetic differentiation (Wright, 1978), while the low pairwise  $F_{ST}$  between the *pacifica* populations indicates that while differentiation exists, it is moderate at best.

Finally, a population structure analysis of the banding patterns showed that the differentiation in the AFLPs was indicative of two distinct population clusters. Structure estimated that the band frequencies present in the sample could be best grouped into two population clusters, one of which contained all of the *pacifica* individuals and the other which contained the *tantalus* individuals. Averaged over 10 iterations, it showed that roughly 97% of the allelic ancestry of *pacifica* belonged within its putative group, and 93% of *tantalus* allelic ancestry came from the *tantalus* group. This result also strongly supports the phenetic grouping of these two species.

### *Interspecific hybridization*

If we accept that current species designations are correct, how do we explain the discrepancy between the mitochondrial and nuclear datasets? Both interspecific hybridization or incomplete lineage sorting may result in polyphyly (Funk and Omland, 2003), and distinguishing between these hypotheses is difficult due to the large variance in rates associated with lineage sorting and the fact that both processes can produce similar phylogenetic patterns (Holder *et al.*, 2001). Although historically cited as conclusive evidence for current hybridization (as in: Tegelstrom 1987; Ruedi *et al.* 1997; Goodman *et al.* 1999) data describing shared sympatric mtDNA variation does not on its own distinguish between recent hybridization and introgression versus shared ancestral polymorphism and incomplete lineage sorting. This is because these two types of shared variation can become blurred under some scenarios. For example, if the mtDNA molecules of two species diverged during a period of geographic separation, and a later hybridization event coupled with a selective sweep caused fixation of mtDNA variants with higher fitness, this would result in the appearance of sister taxa that in fact no longer hybridize and have long ago diverged (Shaw 2002). Alternatively, mtDNA similarities could persist without current hybridization if speciation is recent, due to the fact that phenotypic isolation can evolve before neutral markers become fixed (Coyne and Orr, 1989). This scenario is unlikely in *Laupala* because previous research based upon nuclear markers has estimated the split between these two taxa to have occurred approximately 3.7 million years ago (Mendelson and Shaw, 2004).

Therefore, I suggest that the data here provide substantial support for the hypothesis of hybridization. Two lines of evidence support this. First, while shared mtDNA variation in sympatry on its own cannot support a hypothesis of current hybridization, the analysis of a second divergent marker along with mtDNA lends additional support to such a hypothesis. Mitochondrial data coupled with information from other loci has often provided convincing evidence of introgression between sympatric species (e.g. Dowling, 1993; Gardner, 1996). However, even with the use of additional markers, contrasting genetic patterns between nuclear and mitochondrial markers can be explained by higher genetic drift of mtDNA rather than current hybridization (due to reduced effective size of mtDNA when compared with nDNA) (Palumbi & Baker 1994). It is however widely accepted that the addition of mtDNA sequence data from geographically disparate conspecifics that do not share this variation provides strong support for a hypothesis of secondary contact with introgression (Funk and Omland 2003; Mallett 2005). Many recent studies have successfully used this approach to show evidence of hybridization after secondary contact, including those on damselflies (Hayashi *et al.* 2005), *Heliconius* (Beltran *et al.*, 2002), *Liolaemus* (Morando *et al.* 2004), *Anopheles* (Besansky *et al.* 2003), *Bufo* (Masta *et al.* 2002), and *Drosophila* (Machado *et al.* 2002). Given that phenotypic data (Otte 1994), patterns of behavioral isolation (J. Jadin, Ch. 3), and AFLP data all suggest that the populations classified as *pacifica* are a single species, and *tantalus* is a separate species, these geographically disparate, and non-exclusive patterns of variation provide substantial support for a hypothesis of hybridization after secondary contact (Morrow 2000; Crochet *et al.* 2003; Ray *et al.* 2008; as well as those cited above).

A second line of evidence provides additional, albeit weaker, support for a hypothesis of current hybridization. This evidence comes from patterns in the data revealed by the Structure analysis, which suggest that contemporary, but limited, hybridization may be occurring. While most of the individuals in each population showed a high degree of ancestry within their populations, there were four *tantalus* individuals that displayed a pattern of significantly mixed ancestry (Figure 5). One of these individuals, designated *tan7150* (Table 5), had AFLP bands that appeared to be derived from an ancestor in the *pacifica* population. This pattern would be consistent with that of a first generation hybrid. Similarly, one sympatric *pacifica* individual (*mcp6145*) appeared to have a hybrid grandparent that had backcrossed with a *tantalus* individual. The remaining individuals showed mixed degrees of ancestry with the opposite clade, which could be consistent with predictions for hybrid individuals that had backcrossed several generations back. In addition, while significant clustering of alleles suggested that nuclear DNA falls into two population clusters, shared bands accounted for almost all of the bands scored. When two species are very closely-related, the expectation is that there will be few to no fixed bands (as in Lopez *et al.*, 1999). Because *tantalus* and *pacifica* are not thought to be sister taxa, more fixed bands between species are expected; in two other *Laupala* species that are estimated to have diverged at a date commensurate with that of the lineage split between *tantalus* and *pacifica*, nearly one quarter of the bands scored were fixed (Mendelson and Shaw 2002). The fact that there were no fixed bands between *tantalus* and *pacifica*, but instead an overwhelming majority of shared bands, suggests that gene flow may be causing this shared variation.



However, these individual ancestry patterns revealed by Structure should be interpreted with caution, for several reasons. First, the algorithm employed in Structure is based upon an assumption that band frequencies reflect alleles that are in linkage disequilibrium, selectively neutral, and in Hardy-Weinberg equilibrium. While it is true that AFLPs are considered to be selectively-neutral markers (Vos *et al.* 1995) because they sample broadly across the genome, they are likely to be sampling parts of the genome that are under selection, as well as ones that aren't, and therefore, it is unlikely that all bands are selectively neutral. Therefore, while Structure is widely used to analyze AFLPs, they violate the assumptions underlying the Structure algorithm.

AFLP data in general also has inherent difficulties. AFLPs have become a popular marker for genetic analyses because they can be rapidly developed, have a low cost, are highly repeatable, and allow one to collect large volumes of data in a relatively short amount of time, compared to other DNA marker techniques, such as direct sequencing, RAPDs, or microsatellites (Koopman 2003). However, this comes at the cost of data that can be difficult to interpret. Like all genetic techniques, the scoring procedures used on AFLPs can be subjective. While setting inflexible scoring criteria will help to eliminate some subjectivity, it cannot be eliminated. Because AFLPs are dominant markers, it is not possible to determine if a peak is the result of one or two copies of an allele. Likewise, if two restriction sites are spaced equidistant from each other on disparate sections of the genome, they will amplify and read as a single band in AFLP analysis. Therefore, it can be difficult to distinguish between AFLP bands that are identical by descent and those that are identical by state (Vos *et al.* 1995; Vekemans *et al.* 2002), however, to be suitable for phylogenetic analysis, marker fragments must have

evolved independently, and be homologous (Swofford *et al.* 1996). A recent simulation study found that fragment homology could be improved by eliminating small fragments from the analysis (Althoff *et al.* 2007). In order to avoid this problem, only fragments above 100bp were scored, however, it is not possible to determine if this was conservative enough of an approach. Despite this potential problem, the same study did nonetheless conclude that AFLPs are a useful tool for studying relationships among populations of a species of recently-diverged taxa like *Laupala* (Mendelson and Shaw, 2005).

#### *Implications for speciation*

If we accept that *tantalus* and *pacifica* are hybridizing in the contact zone, there are still many reasons to think that reproductive isolation between them is quite strong. First, as noted, the two species show significantly different structure in their nuclear genomes and these differences are just as strong between sympatric heterospecifics ( $F_{ST}=0.3113$ ,  $p < 0.0001$ ) as between allopatric heterospecifics ( $F_{ST}=0.3212$ ,  $p < 0.0001$ ). If hybridization were causing significant gene flow, we would expect sympatric populations to show more genetic similarity than allopatric populations, in both nuclear and mitochondrial genomes. Interbreeding is expected to result not in elimination of variation, but in randomization of variation (Slatkin 1987). Because we do not find this, we have reason to suspect that hybridization is infrequent. Second, the strong association previously found between nuclear DNA, male genital morphology, and song rate would not be expected if isolating barriers were weak (Shaw 2002). Third, while heterospecific matings do occur in the lab, they are both attempted and successfully completed significantly less

frequently than conspecific matings between either of the *pacifica* populations (J. Jadin, Ch. 3). However, the results suggesting that hybrid individuals were present in this study also suggests that intrinsic postzygotic isolation is not very strong, and thus, premating isolation may be leading to, and perhaps strengthening isolation.

Information on directionality would allow us to make further predictions about the patterns of isolation between this species pair. Other studies have found that the behavior of males relative to females can determine the direction of the outcome of hybridization (as in: Takami *et al.* 2007, Svensson *et al.* 2008; Van der Sluijs *et al.*, 2008; Stelkens *et al.*, 2008). The companion to the current study found that behavioral isolation between *tantalus* and *pacifica* is asymmetric, with the male *tantalus*-female *pacifica* pairs attempting significantly fewer, but completing slightly more matings than the reciprocal combination (J. Jadin, Ch. 3). In this case, one should expect to see a preponderance of *pacifica* alleles introgressed into a *tantalus* background. However, because *tantalus* and *pacifica* belong to separate taxonomic divisions within *Laupala*, are therefore not sister taxa, and the split between their groups is quite distant at 3.7 million years ago (Mendelson and Shaw, 2005), it is difficult to determine the direction of introgression without detailed data from the taxa connecting them. Also, data on hybrid fertility or viability would allow us to assess if hybrids from one cross had higher viability, fertility, or mating success than the other. From this we may be able to predict which species would be the recipients of genetic introgression. Hybrid data is not currently available, but should be the focus of further studies.

### *Conclusions*

Phylogenies of recently and rapidly-diverging species are always difficult to resolve because the results of ongoing gene-flow as well as incomplete lineage sorting of ancestral polymorphism become entangled. This study is significant because the data reported here proffer considerable support for the hypothesis that the high degree of mtDNA similarity between sympatric *tantalus* and *pacifica* is the result of hybridization with mitochondrial introgression. Additionally, this study has identified some putative hybrid individuals, which suggests that hybridization is not only recent, but may be currently ongoing. It would be predicted that the mtDNA is introgressing from *tantalus* into *pacifica*, given the divergence in *pacifica* mtDNA haplotypes between localities; however, in order to conclusively determine the directionality of introgression further genetic examination of intermediate taxa are needed. Further, future behavioral studies on and estimation of hybrid viability between reciprocally paired species will help determine the consequences and direction of hybridization. Overall, these results corroborate a previous phylogeny and provide support for a previous finding of incomplete behavioral isolation in sympatry. Speciation in this young genus has undoubtedly not been a linear process, and stochastic events in allopatry and sympatry have likely contributed significantly to its rapid rate of diversification.

## TABLES

**Table 1** Number and frequency of COI haplotype for each population. Two haplotypes, numbers 6 and 7, were shared between species. No haplotypes were shared between the allopatric (P) and sympatric (M) populations. Out of 560 bp, only 16 (2.8%) were variable; 15 of these mutations were transitions, and 1 (13S) had both a transition and transversion mutation.

haplotype#	total	Population		
		<i>L. pacifica</i> <sub>P</sub>	<i>L. pacifica</i> <sub>M</sub>	<i>L. tantalus</i> <sub>M</sub>
1	4	4	0	0
2	6	6	0	0
3	34	34	0	0
4	2	0	2	0
5	3	0	3	0
6	88	0	39	49
7	4	0	3	1
8	1	0	0	1
9	1	1	0	0
10	1	1	0	0
11	1	1	0	0
12	1	0	0	1
13	1	0	0	1
14	1	1	0	0
15	1	1	0	0

**Table 2** Analysis of molecular variance (AMOVA) results of three populations of the *L. pacifica* and *L. tantalus* species based on AFLP markers and mtDNA COI haplotypes.

These analyses were performed on approximately 48 individuals per population. For the AMOVA, the populations were grouped based on location, such that the sympatric heterospecifics comprised one group and the allopatric *pacifica* the other. For the AFLP data, Nei-Li restriction site distances were used to compute genetic variation, whereas pairwise distances was used in the mtDNA data.

Data	Source of variation	<i>df</i>	Variance components	% of variation	$\Phi$ -statistics	<i>P</i> -value
mtDNA	Among locations	1	1.52	77.59	$\Phi_{CT} = 0.776$	0.3327
	Among populations/locations	1	0.004	0.21	$\Phi_{SC} = 0.009$	0.0616
	Within populations	146	0.44	22.62	$\Phi_{ST} = 0.774$	<0.0001
AFLP	Among locations	1	15.85	12.55	$\Phi_{CT} = 0.168$	0.6657
	Among populations/locations	1	35.30	27.96	$\Phi_{SC} = 0.320$	<0.0001
	Within populations	141	76.12	59.49	$\Phi_{ST} = 0.206$	<0.0001

**Table 3.** Population pair-wise  $F_{ST}$  values for the three populations used in this study. The mtDNA values were estimated assuming the Tamura-Nei substitution model and the AFLP values were estimated by computing minimum pair-wise distances, both in Arlequin. An asterisk \* denotes significance (<0.0001).

		mtDNA		
		<i>L. pacifica</i> <sub>P</sub>	<i>L. pacifica</i> <sub>M</sub>	<i>L. tantalus</i> <sub>M</sub>
<i>L. pacifica</i>	PAC <sub>P</sub>	0.0000	+	+
<i>L. pacifica</i>	PAC <sub>M</sub>	0.7043*	0.0000	+
<i>L. tantalus</i>	TAN <sub>M</sub>	0.7257*	0.0177	0.0000
		AFLP		
		<i>L. pacifica</i> <sub>P</sub>	<i>L. pacifica</i> <sub>M</sub>	<i>L. tantalus</i> <sub>M</sub>
<i>L. pacifica</i>	PAC <sub>P</sub>	0.0000	+	+
<i>L. pacifica</i>	PAC <sub>M</sub>	0.0347*	0.0000	+
<i>L. tantalus</i>	TAN <sub>M</sub>	0.3212*	0.3113*	0.0000

**Table 4.** Averaged estimated log probability (LnP), variance (PD), and  $\Delta K$  at different clusters ( $K$ ) from *L. pacifica* and *L. tantalus* AFLP profiles from 10 iterations of an admixed ancestry model with correlated allele frequencies in Structure. The  $\Delta K$  has a mode at the true  $K$ , which is 2.

K	LnP	PD	$\Delta K$
1	-56425.1	2834.3	--
2	-47578.5	4508.82	84.75
3	-44981.1	5137.91	29.28
4	-44482.7	5436.61	6.40
5	-44077.4	5015.9	--



**Table 5.** Population pairwise uncorrected genetic distances based upon AFLP and mtDNA data. Values along the diagonal are average within population pairwise distances. An asterisk (\*) denotes a significant value.

			Pairwise distances: mtDNA		
	N	# haplotypes	<i>L. pacifica<sub>P</sub></i>	<i>L. pacifica<sub>M</sub></i>	<i>L. tantalus<sub>M</sub></i>
<i>L. pacifica<sub>P</sub></i>	49	8	0.42347	8.27486*	8.21063*
<i>L. pacifica<sub>M</sub></i>	48	4	+	0.44958	0.39904
<i>L. tantalus<sub>M</sub></i>	53	5	+	+	0.33817
			Pairwise distances: AFLP		
	N	# genotypes	<i>L. pacifica<sub>P</sub></i>	<i>L. pacifica<sub>M</sub></i>	<i>L. tantalus<sub>M</sub></i>
<i>L. pacifica<sub>P</sub></i>	46	46	138.10338	148.65217*	221.83565*
<i>L. pacifica<sub>M</sub></i>	48	48	+	148.87411	226.27833*
<i>L. tantalus<sub>M</sub></i>	50	50	+	+	162.70612

**Table 6** Structure results of 13 individuals which showed a significant probability (>95%) of being migrants (i.e. hybrid ancestry or misclassified). The first analysis used no prior population information and assigned individuals to clusters based solely on genotype. The second analysis used prior population assignments. It was set to investigate ancestry in the opposite clade up to two generations back and the assess probability of assignment per generation, as averaged over 10 iterations.

Species	site	ID#	Without prior information:		With prior population information:			
			P( <i>tant</i> )	P( <i>pac</i> )	P( <i>tant</i> )	P( <i>pac</i> )	P (immigrant parent)	P (immigrant grandparent)
1 ( <i>L. tantalus</i> )	M	tan7150	0.417	0.583	0	0.0321	0.02	0.9587
	M	tan420	0.313	0.687	0	0	1	0
	M	tan402	0.260	0.740	0	0	1	0
	M	tan505	0.401	0.599	0	0.9807	.006	0.0187
2 ( <i>L. pacifica</i> )	M	mcp6145	0.180	0.820	0	0.4961	0.5039	0
	M	mcp7025	0.169	0.831	0	0.0146	0.9854	0
	M	mcp7029	0.216	0.784	0	0	1	0
	M	mcp6143	0.314	0.686	0	0	1	0
	M	mcp646	0.292	0.708	0	0	1	0
	P	pup405	0.312	0.688	0	0	1	0
	P	pup544	0.246	0.754	0	0	1	0
	P	pup546	0.352	0.648	0	0	1	0
	P	pup551	0.217	0.783	0	0.0007	0.9993	0

**Table 7.** Variable sites in the 557bp portion of mitochondrial COI gene sequenced. There were a total of 15 variable sites,

	9	42	135	171	213	321	339	363	366	382	390	409	507	546	549
T6127	...A	...A	...C	...A	...C	...T	...C	...C	...T	...T	...G	...T	...A	...C	...A
T505	...A	...A	...C	...A	...C	...T	...C	...C	...T	...T	...G	...T	...A	...A	...A
P438	...G	...G	...C	...G	...T	...T	...C	...C	...T	...C	...G	...T	...A	...T	...A
P419	...G	...A	...T	...A	...C	...C	...C	...C	...C	...C	...G	...T	...A	...T	...A
P412	...G	...G	...C	...G	...T	...T	...C	...T	...T	...T	...G	...T	...A	...T	...A
P411	...G	...A	...T	...A	...C	...C	...T	...C	...T	...C	...G	...T	...A	...T	...A
P405	...G	...A	...T	...A	...C	...C	...C	...C	...T	...C	...G	...C	...A	...T	...A
4pup	...G	...A	...C	...G	...T	...T	...C	...C	...T	...T	...G	...T	...A	...T	...A
6pup	...G	...A	...T	...A	...C	...C	...C	...C	...T	...C	...G	...T	...A	...T	...A
34pup	...G	...G	...C	...G	...T	...T	...C	...C	...T	...T	...G	...T	...A	...T	...A
2mcp626	...A	...A	...C	...A	...C	...T	...C	...C	...T	...T	...G	...T	...C	...T	...A
2mcp412	...A	...A	...C	...A	...C	...T	...C	...C	...T	...T	...G	...T	...A	...T	...T
86mc	...A	...A	...C	...A	...C	...T	...C	...C	...T	...T	...G	...T	...A	...T	...A
4mc	...A	...A	...C	...A	...C	...T	...C	...C	...T	...T	...A	...T	...A	...T	...A

resulting in a total of 15 unique haplotypes out of 146 individuals.

## FIGURE LEGENDS

**Figure 1.** Map showing sample localities for *Laupala* species from mid-elevational rainforest floors on the island of Oahu. The dashed line represents the approximate putative distribution of *L. pacifica* and the dotted line represents *L. tantalus*, as proposed by Otte (1994). The square shows the location in the contact zone between *L. pacifica* and *L. tantalus* that was sampled for this study, and the circle represents the allopatric *L. pacifica* population.

**Figure 2.** Statistical parsimony haplotype network for COI data set. The haplotype node area is proportional to the number of individuals which have that haplotype. Links between nodes are all single mutational steps, regardless of length. Pupukea (allopatric) *L. pacifica* haplotypes are shown in black, Manoa Cliffs (sympatric) *L. pacifica* are shown in light gray, and Manoa Cliffs *L. tantalus* are shown in dark gray. *L. kokei* was used as an outgroup.

**Figure 3.** Phylogram (neighbor-joining tree) of 15 unique COI haplotype sequences from 146 individuals of *L. pacifica* and *L. tantalus*. Haplotypes names are shown preceded by an *H*. The relative size of each node is roughly representative of the number of individuals sharing each haplotype. Numbers in italics on branches indicate bootstrap support for each clade, based on 1000 bootstrap replicates; all clades with bootstrap values greater than 50 are indicated. The 8 haplotypes found in the northern allopatric *L. pacifica* population fall into two distinct clades (designated by the dotted line), whereas the 7 haplotypes found within the sympatric *L. pacifica* and *L. tantalus* populations (dashed line) show no distinct grouping within their clades.

**Figure 4.** Unrooted Neighbor-Joining tree for 656 unique AFLP bands from 144 individuals of *L. pacifica* and *L. tantalus*. Sample names consist of a three-letter abbreviation of sample site (tan = sympatric *tantalus*; mcp = sympatric *pacifica*; pup = allopatric *pacifica*), followed by the sample ID#. Branch lengths are reflective of genetic distances between genotypes; individuals highlighted in gray are those which Structure identified as putative hybrid individuals.

**Figure 5.** Genetic clustering based on 656 AFLP loci. Each individual is represented by a narrow vertical column with the proportion of the two colors indicating the genome proportion derived from each of the two populations. Three *L. tantalus* individuals exhibited strong evidence of ancestry belonging to the other clade (\*), while one individual (+) showed nearly evenly mixed ancestry.

Figure 6. Histogram showing number of bands with frequency differences between populations. Black bars represent differences between allopatric conspecifics (*L. pacifica*<sub>M</sub> and *L. pacifica*<sub>P</sub>), the dark gray bars represent differences between sympatric conspecifics (*L. pacifica*<sub>M</sub> and *L. tantalus*<sub>M</sub>), and pale gray represents differences between allopatric heterospecifics (*L. pacifica*<sub>P</sub> and *L. tantalus*<sub>M</sub>). Each value on the x-axis represents a 10 percentage point interval (i.e. 20% includes differences ranging from 11% to 20%).

**FIGURES**

**Figure 1.**

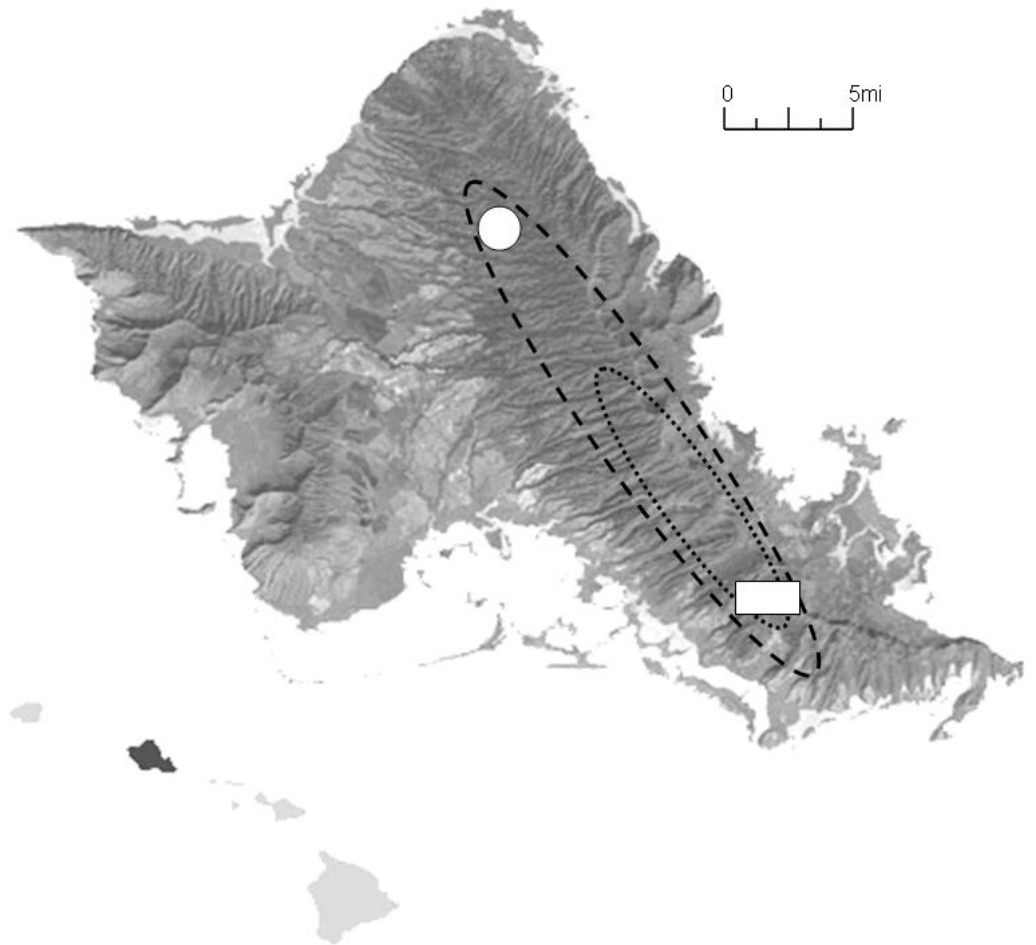


Figure 2.

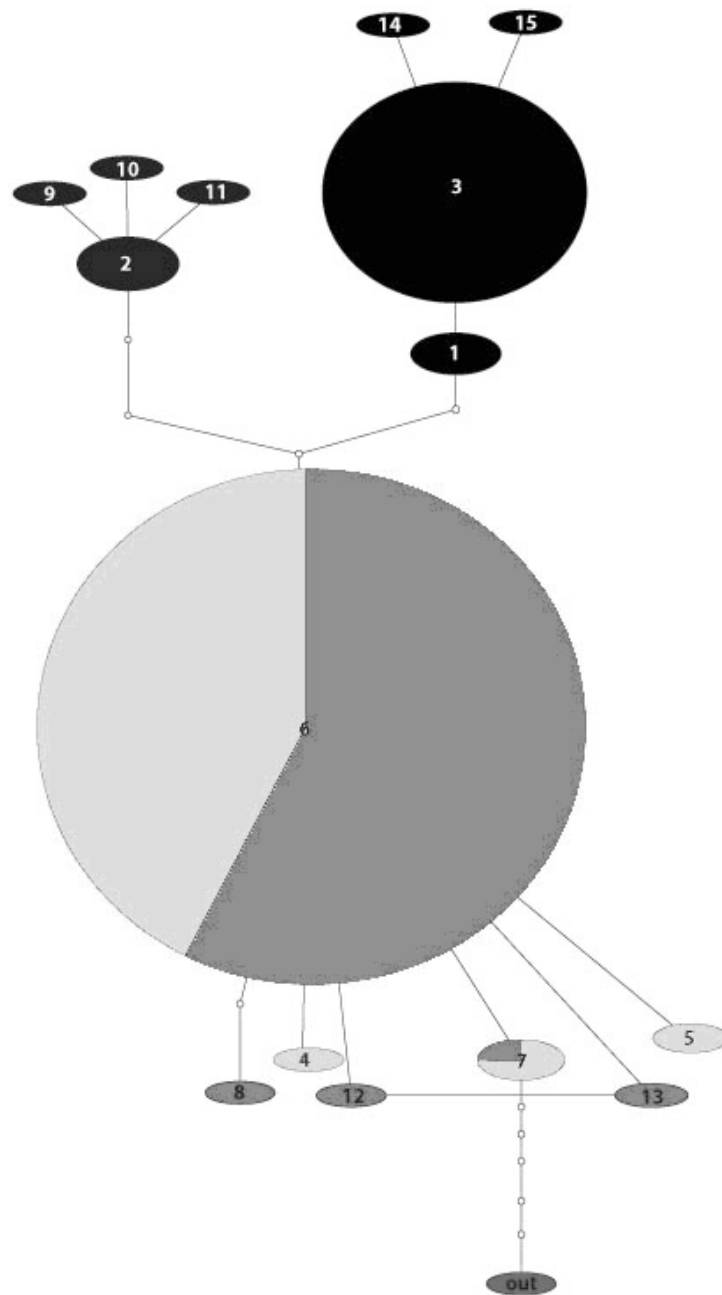


Figure 3.

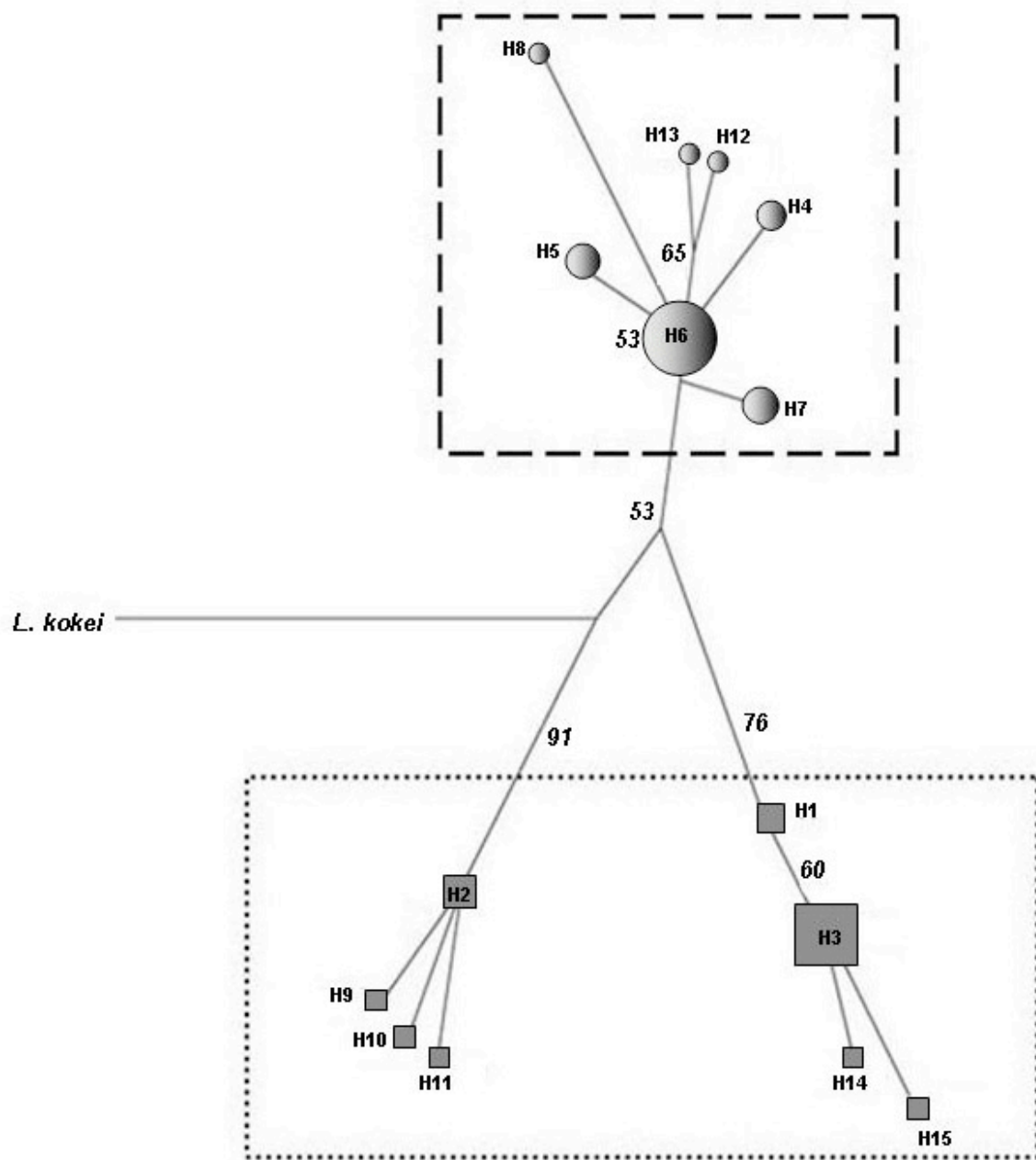
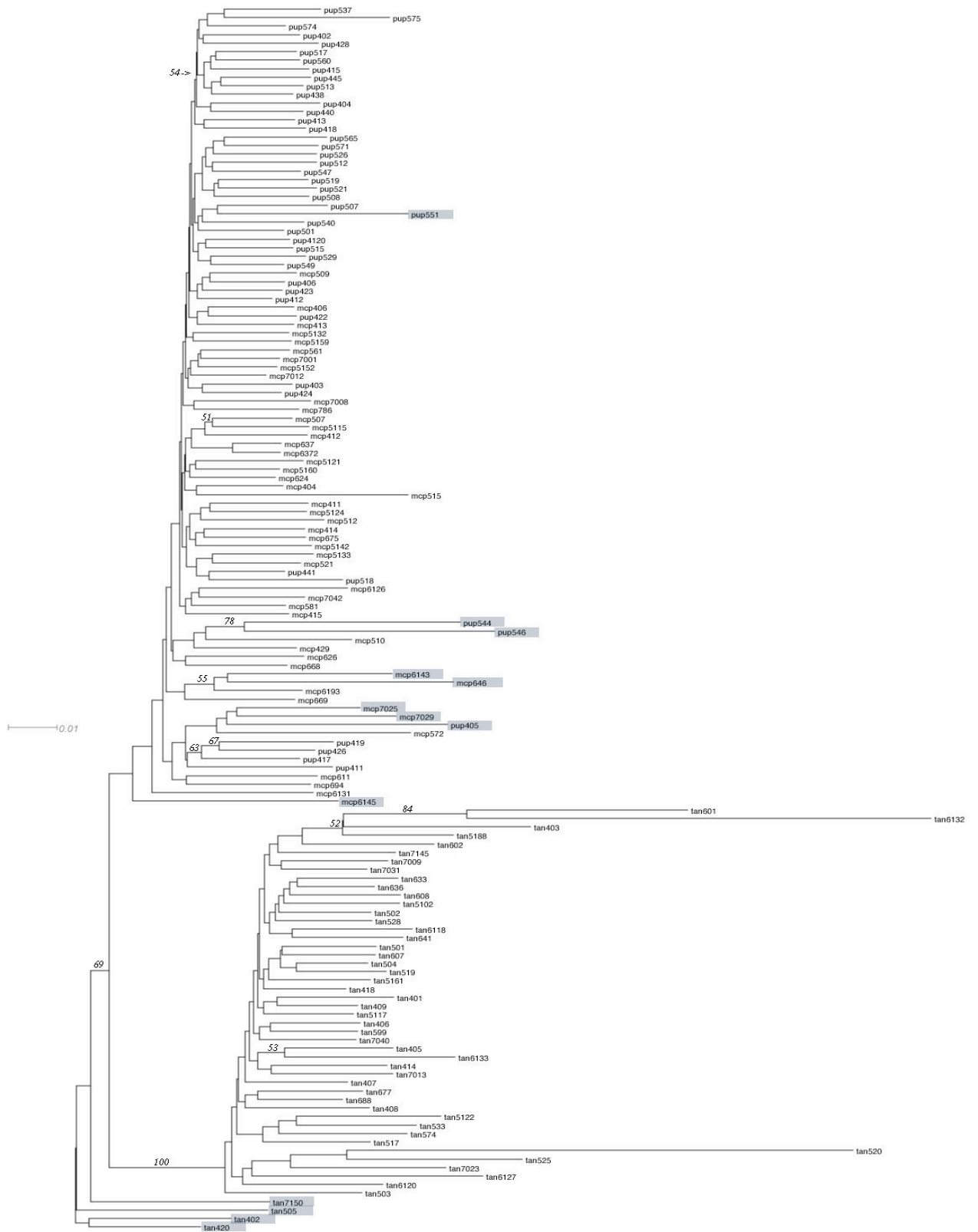
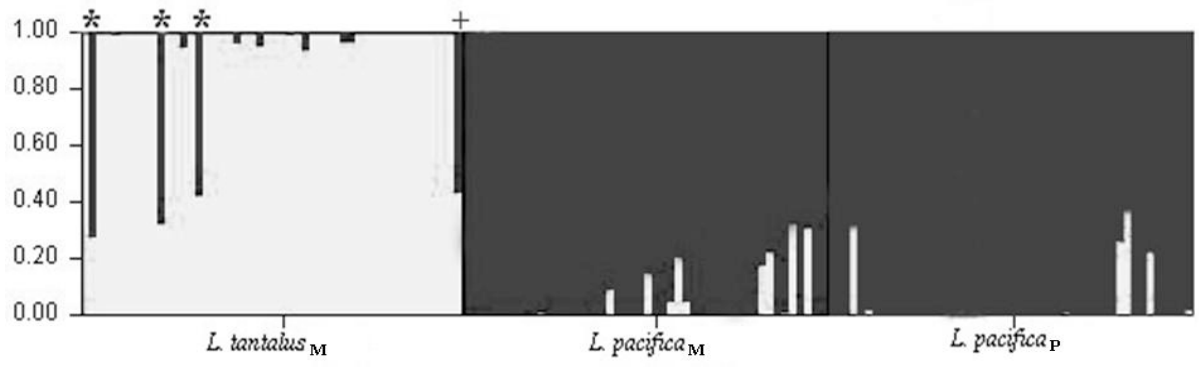




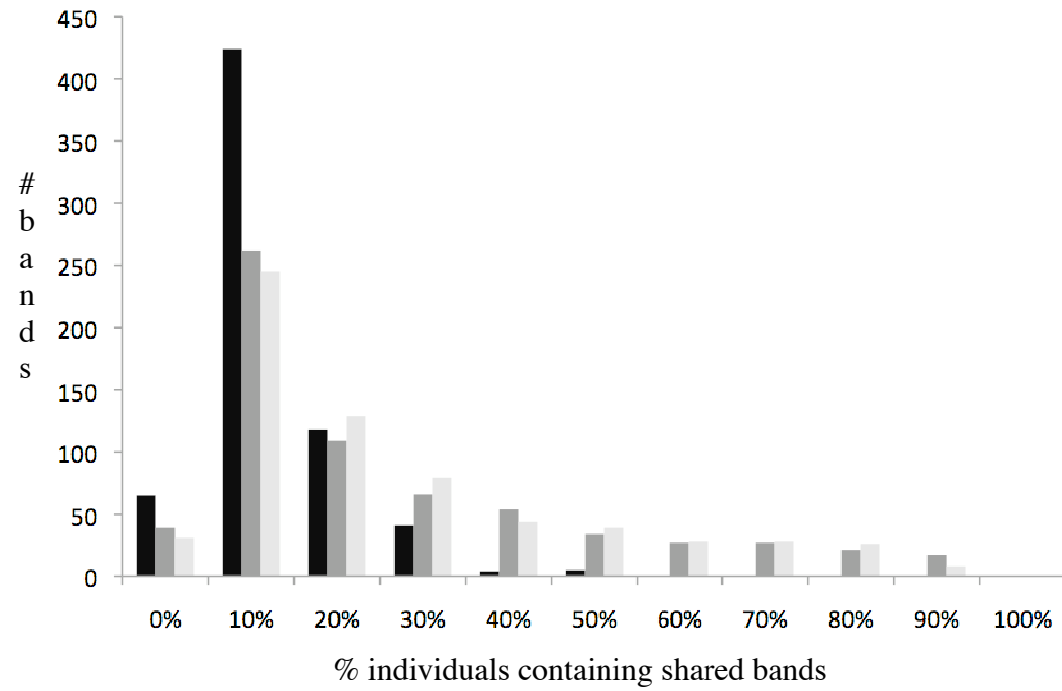
Figure 4.



**Figure 5.**



**Figure 6.**



## **Chapter II: Good things come in small packages: costly spermatophores and differential male mating investment in *Laupala***

### **ABSTRACT**

Female reproductive costs are thought to dictate the direction of sexual selection in most animal systems. However, male reproductive costs may play an equally, if not more important role in determining the direction of sexual selection. Mounting evidence suggests that they may be especially important in systems with high male investment into the mating process, such as some orthopterans. Crickets of the genus *Laupala*, have an elaborate and time-consuming mating process which may be as costly to males as females. Males produce numerous spermless spermatophores followed by a single sperm-filled spermatophore in the course of an all-day mating bout. If this system incurs significant costs for the males, they should show preference for particular female traits, such as body size, which indicate high female fitness. The aim of this study was, first, to quantify male mating costs by analyzing changes in spermatophore production after consecutive matings; and second, to determine if males displayed a preference for larger females by comparing timing and spermatophore production differences between female partners. Results show that males produce fewer spermatophores after multiple matings. Results also show that food-deprived males less frequently complete multiple matings than do food-supplemented males. Finally, results indicate that there are several correlates which predict spermatophore production: mating initiation and the number of spermatophores were positively predicted by female weight, and the decrease in

**spermatophore production over consecutive days is negatively correlated with a positive change in weight between consecutive females. These results suggest that males are resource-limited and can gauge the size of their partners, which allows them to choose which females will be the recipients of greater mating effort. I argue that high male mating costs are present and are therefore important in determining how sexual selection may be acting in this genus.**

## **INTRODUCTION**

The standard model of sexual selection predicts that males will be indiscriminate and females will be choosy. This model was first proposed by Darwin and was based upon his comparison of mating behavior across numerous taxa (Darwin 1871), in which he repeatedly observed that mate choice was either decided through male-male competition or through female choice for males with the showiest displays. The female choice model of sexual selection was later corroborated by Bateman's (1948) work which showed that females were likely to be the choosy sex because of the higher physiological/caloric cost of eggs compared to sperm. Trivers (1972) work lent further support to this model by showing that as a result of higher gametic costs and lower reproductive potential, females would invest more into offspring than would males. Together these papers ushered in an era of sexual selection research that focused on quantifying female costs and identifying female choices, and as a result, female reproductive costs are relatively well-understood. Male reproductive costs, conversely, were overlooked for many years because it was thought that male gametes were "cheap," could be produced in unlimited quantities, and therefore males paid few mating costs. Recent work on sexual conflict and ejaculates, however, has shown that the cost of male reproduction in many species approaches or

even surpasses the cost of female reproduction (Vahed 2007b; Wagner 2005; Parker and Ball 2005; Kondoh 2001).

Males have been predicted to pay particularly substantial costs for reproduction in systems with large ejaculates (Bonduriansky 2001; Vahed 2007a), or in systems with male parental investment and arduous or dangerous mating bouts (Bonduriansky 2001). Empirical evidence has thus far followed these predictions, showing that there are high male mating costs in many taxonomically diverse systems. Examples have been found in birds with male parental care (Torres and Velando 2005; Schamel *et al.* 2004; Amundsen 2000), in beetles with long mating bouts (Peterson *et al.* 2005), and in orthopterans, which are thought to have costly male gametes (Bateman and Fleming 2005; Gwynne 1993; Gwynne and Simmons 1990). These costs come in many forms, and are the additive effect of many factors, such as direct physiological costs that result in calorie loss. Physiological costs are incurred from vocalizations (Ward and Slater 2005; Thomas 2002; Hoback and Wagner 1997), mating displays (Hansen *et al.* 2008; Usherwood 2008; Sullivan and Kwiatkowski 2007, and references therein), search locomotion (Hack, 1998; Forsyth *et al.* 2005), ejaculate or gift production (Vahed 2006; Wagner 2005; Burpee and Sakaluk 1993), and feeding (Sakaluk *et al.* 2004). Mating also incurs behavioral costs, which are factors that affect the life-span of an individual as a direct result of their behavior. For example, in some bushcrickets, song attracts parasitic flies that acoustically locate the singing male and deposit larvae around him, drastically decreasing his fitness (Lehmann and Lehmann 2000). Predation is another behavioral cost of reproduction, which not only affects lifespan, but has also been found to affect the direction of sexual selection (Svensson *et al.* 2007). Finally, there are temporal costs that can act as

multipliers of the aforementioned costs. For example, a brief mate search will result in less caloric expenditure than would an extended period (Forsyth *et al.* 2005); or, longer copulations with infected individuals may lead to increased exposure to a parasite than would briefer or fewer copulations (Bouma *et al.* 2007; Read 1991).

It becomes important to understand not only the costs of mating but also their relative effects on an individuals' fitness if we hope to make predictions about sexual selection in any of these systems. This is because one of the consequences of high male mating costs is that male choice is likely to evolve. Male mate choice has been found in numerous systems with potentially high male mating costs, such as in wolf spiders (Roberts and Uetz 2005) and cichlids (Werner and Lotem 2003) where mating is dangerous, in field crickets where displaying is costly (Hunt *et al.* 2004) and in numerous other orthopterans, where gamete production is thought to be costly (Dewsbury 1982; Kvarnemo and Simmons 1999; Bateman and Ferguson 2004; Bateman and Fleming 2006). In addition, male choice is also predicted in systems with high variance in female quality, or a female-biased operational sex ratio (OSR) (Johnstone *et al.* 1996; Bonduriansky 2001). When variance in female quality is high and/or females are abundant relative to males, males should maximize their investment into mating by choosing the fittest females (Emlen and Oring, 1977), such as those that display the highest quality ornaments (Amundsen *et al.* 1997; Amundsen and Forsgren 2001), provide the most parental care (Bonduriansky 2001), or have the most potential fecundity (Parker 1983; Dunn *et al.* 2001; Bateman and Ferguson 2004). Across most insect taxa, large female body size is indicative of high egg carrying capacity and therefore, high fecundity (Honek 1993; Kazimirova 1996; Preziosi *et al.* 1996; Sokolovska *et al.* 2000).

As such, many studies have found that males choose the largest females to be the recipient of their greatest mating efforts (Fischer *et al.* 2000; Danielson-Francois *et al.* 2002; Rogers *et al.* 2006). Moreover, research has shown that the more resource-deprived the males are, the more strongly they prefer these large females (Byrne and Rice 2006).

Crickets are particularly useful systems in which to study the costs of male reproduction. This is because male crickets produce a sperm-containing mass known as a spermatophore which is presented to females during courtship. The spermatophore appears to have different functions for different species, perhaps acting as a food source for the female (Simmons 1990; Sakaluk 1985; Jia and Sakaluk 2000; Engels and Sauer 2006; Vahed 1998), a sensory trap (Vahed 2007b; Sakaluk 2000; Sakaluk *et al.* 2006), or as a gift to preoccupy the female while the male copulates with her (Heller *et al.* 1994; Vahed 1998; Will and Sakaluk 1994; Sakaluk 2000; Sakaluk *et al.* 2006). Much recent work also suggests that males use the spermatophore or seminal fluids to manipulate female receptivity or fecundity (Cordero *et al.* 1995; Arnqvist and Nilsson, 2000; Arnqvist and Andres 2006; Wedell 2005) and therefore the spermatophore is a source of sexual conflict between males and females (Vahed 2006). Some of this work suggests that larger spermatophores have greater manipulative power, but are also more costly for the males, in terms of longevity or remating ability (Simmons 1990, 1995; Hayashi 1993; Sakaluk *et al.* 2004)

Crickets of the genus *Laupala* are valuable for a study of male reproductive costs due to their large spermatophore output and elaborate mating system. In the *Laupala* mating system, males produce a series of 6-15 small spermless spermatophores (hereafter



“micros”) and 1-2 sperm-containing macrospermatophores (hereafter “macros”) per copulation. In addition to the large number of micros produced, a single copulation in *Laupala* can last up to 12 hours, resulting in only one insemination. This dual system of micros and macros is nearly unique among known cricket species, with only one other known example (DeCarvalho and Shaw 2005). The exact function of the micro is unknown, but recent evidence shows that it enhances sperm uptake into the females spermatheca (T. deCarvalho, in prep), and may also function as a form of assessment of female readiness (Shaw and Khine 2004). It superficially appears that this process may be quite costly to the males, given the time and number of micros involved. Previous studies in other Orthopteran systems have found that there is a positive correlation between spermatophore size and male costs as inferred by length of male refractory period (Vahed 2006). Therefore, because of the overall large investment in spermatophores in a given mating, *Laupala* may be an ideal system in which to study the consequences of male reproductive costs.

For the present study, I hypothesized that micro production in *Laupala* is costly to males and will lead to the evolution of male choice. I tested these hypotheses by assessing remating ability and preferential female investment. Males were divided into both high and low-diet treatments. Males were predicted to have attenuated remating abilities, in terms of number of micros produced and delay in production, after consecutive mating days. They were also predicted to invest more effort, increased micro production, into mating with larger females. Finally low-diet males were predicted to show a greater depression in remating ability relative to high-diet males. Overall results

suggest that mating does incur a “cost” for males, who may be able to modulate their mating investment based on their partners’ reproductive potential.

## **METHODS**

### ***Animal Collection***

The individuals used in this study were lab-reared offspring of crickets captured from two sites on the island of Oahu (site MC: sympatric *L. pacifica* and *L. tantalus*, site PU: allopatric *L. pacifica*) (MC: 21° 19'35", -157° 48'45"; PU: 21° 37'14", -158° 0'45"), one site on the island of Hawaii (site DH; sympatric *L. kona* and *L. hualalai*, site MN: allopatric *L. kona*) (DH: 19° 41'53", -155° 57'13"; MN: 19° 12'56", -155° 51'38"), and one site with allopatric *L. paranigra* (WR: 19° 27'45", -155° 14'54"). At all sites, *Laupala* live in the leaf litter on or near forest floors. Wild caught individuals were captured in the field using nets and plastic vials, and housed according to population in two quart plastic containers while in transport. Upon return to the laboratory, individuals from single-species sites were split up into opposite sex pairs of approximately the same developmental stage. Mature males from mixed-species sites were identified to species based upon song rate and paired with a mature female captured from the same site. Immature males were assigned species status once they reached maturity, and paired with a similarly-aged female in the same fashion. If females in mating pairs from mixed-species sites had not oviposited within two weeks, the male from each pair was removed and switched out with one from the other species at that site. Eggs from all pairs were collected, and upon hatching, nymphs were transferred to single-family jars. Upon reaching maturity, males and females were separated into individual holding cups, which were labeled with family ID#. Once a male reached maturity, his song rate was checked

in order to confirm his species identity as well as that of his siblings. All individuals used for the trials were first or second generation lab reared from wild-caught individuals.

### *Animal Care*

Lab conditions were set at a 12h day/night cycle and 20°C. Mating pairs were housed in a 250mL plastic sample cup which was moistened with a wet Kimwipe and liberally supplied with Flukers Cricket Chow treated with Tegosept, both of which were changed weekly. If eggs had not been produced within two weeks, a different female was placed with the male. Mated females oviposited into the wet kimwipes, which were collected weekly and kept in separate egg cups until nymphs hatched after ~30days. Nymphs were collected and housed in single family glass jars, moistened and fed in same manner as described above. Upon maturing, individuals were removed from the jars and housed individually in cups, and 20-25 days post-molt males were identified to species based on song rate (Otte 1994) in order to confirm species identity of family.

### *Feeding treatments*

I hypothesized that food-deprived males would be able to remate less frequently than well-fed males. Therefore, high and low diet treatments were applied to the males. Those in the high-diet treatment were fed ad libitum throughout their maturation period and were given fresh food and water the evening before a trial. Males in the low-diet treatment were transferred to a new cup 10 days previous to their trial and were held in that cup with fresh water, but without food, until their trials began. All females were fed and watered ad libitum.

### ***Mating System***

All *Laupala* species mate diurnally, and all species follow the same general courtship sequence, however, the timing of mating events vary somewhat between species (J. Jadin, Ch.3; Danley *et al.* 2007). Generally, males begin calling in the early morning hours, post-sunrise. Once a female has located a male in his perch in the leaf litter on the ground or just above it, individuals face each other while antennating each others abdomens. After some period of time, the male will produce a micro. He will then circle the female several times, back up under her, and insert a sperm tube into her urogenital tract, leaving the spermatophore external to the female. After successful insertion, the male again faces the female, and they remain in this position for approximately 5-20min. During this facing period, the male will produce another micro, and as soon as he begins to move again, the female will remove the micro attached to her and consume it. If the female removes the micro during the facing period, the male jumps rapidly, chases, and frequently even swipes at the female with his front legs (pers.obs.). This cycle of micro production, facing, and transfer gets repeated 6-15 times on average, depending upon the species (DeCarvalho and Shaw 2005). The mating culminates with the production and transfer of the sperm-containing macro. The sequence of events is the same for the macro as for the micros, however, the facing period before and after transfer are significantly longer, presumably to account for the approximately 3X larger mass of the macro as well as sperm-transfer time.

### ***Behavioral trials***

All individuals used in trials were 20-50 days post-final molt. As adults, *Laupala* live approximately 3-4 months, therefore, the 20-50 day old crickets used in these trials were thought to be within a reasonable window of fertility. The evening before a trial, the male was anesthetized briefly with CO<sup>2</sup>, weighed, and returned to his cup. Trials were performed in a mating dish (plastic Petri dishes, 85mm diameter, 28mm depth) moistened with a wet Kimwipe. A maximum of 14 trials were simultaneously performed. All dishes were visually, but not acoustically, isolated from each other with cardboard barriers. Trials were started 2 hours post “sunrise” (i.e. “lights on” in the observation room). On the morning of a trial, an observer placed a male and a female from the same population and species together in a viewing dish. The observer then sat nearby, quietly noting the time of production and transfer of each micro, or the micro consumption time if it was not transferred, and the production and transfer times for the macro. After the macro was transferred, the observer then quickly separated and anesthetized the pair using CO<sup>2</sup>, removed the macro from the female, and weighed each individual, to the nearest milligram, on a microbalance. The males were returned to their cups, given fresh food and water, and allowed to recover for the next day, when he was paired with a second female and the same behavioral manipulations and observations were performed. The males were again weighed after the second day of mating and subsequently stored in a -80C freezer. All species pairs were observed until macro transfer occurred, or until “light out” in the observation room. If a male did not produce a macro on the first mating day, he was not used in a second day of trials.

### *Statistical Analysis*

All statistical analyses were performed using JMP software (SAS Institute). All tests were performed with the data combined across all populations, as well as individually between populations. This was because while all *Laupala* species observed in the trials have the same general mating patterns, there are significant differences in timing and weight between species. While weight data was normalized across populations for the combined analysis, timing or other unknown differences between species may effect their ability or propensity to mate; therefore, all data was also analyzed by population. In order to determine if there may have been significant differences in behavior among populations or among location types (sympatric or allopatric), ANCOVAs were performed using population or location type as one of the predictor variables. When measuring change in micro number across populations, the response variables had to be normalized across populations because there are different average micro numbers produced by each species. To normalize micro number, the average number of micros per population was calculated, and the change in micro number was divided by this average, and multiplied by 100 in order to measure a percent change (hereafter referred to as species-adjusted percent change).

To test if there was a significant change in spermatophore number or spermatophore latency within males between the two days of mating in both high-diet and low-diet males, paired t-tests or Wilcoxon signed-rank tests were used. Chi-square tests were performed on proportions of high- and low-diet males initiating and completing mating on each day in order to determine if diet affected mating. Only males that completed mating on both days were used for these analyses.

Next, I wanted to test whether male or female weight or age positively or negatively affected micro or macro number and timing on the first day of mating. To evaluate this, several one-way ANCOVAs were performed with micro number, mating initiation (time of production of first micro), and mating completion (time of production of macro) set as the dependent variables, and male-female weight difference, male age, female age, male residual weight, or female residual weight set as the independent variables. Residual weights were obtained by subtracting the population mean from each individual's true weight. This transformation was done because male and female weights differ significantly between species, and residual weights allowed the analysis to avoid conflating species weight differences with micro production differences. Only males that mated to completion on day 1 were used for this analysis. In order to determine if these independent variables affected micro variance on mating day 2, a final set of ANCOVAs was performed using the same variables as above, plus two additional factors; the difference in weight between consecutive females (not transformed) as an independent factor and the difference in micro number between consecutive days as a dependent factor. For these analyses, only males that mated to completion on both days were used. Finally, logistic regressions were performed on binary response data in order to determine if residual male or female weight or age could predict mating initiation or completion. Once again, only males that completed mating on the day under analyses were used. Also, all of the above analyses were performed independently on both high-diet and low-diet males.

## **RESULTS**

I staged a total of 303 trials using high-diet virgin males and 117 trials using low-diet virgin males. The data from the trials, including the average timing of mating events, weights, and number of spermatophores produced and transferred is shown in Table 1.

***Estimating limits on spermatophore production: high-diet***

Overall the combined data showed that 177 of 303 (58%) high-diet pairings successfully mated (produced a macrospermatophore), and 248 of 303 (82%) high-diet pairings produced at least one microspermatophore on the first day of mating (Table 2). Of the 177 successful matings, 142 (80%) produced at least one micro, and 114 (64%) produced a macro on day 2 for a second successful mating. A chi-squared test on the combined data showed that there was no significant difference between number of males who produced micros or macros on either day.. Likewise, when data for the individual species was analyzed, there were no significant differences in the number of micros or macros produced on day 1 or day 2 within any species (Table 2).

Only those males that produced a macro on both days were used for spermatophore production difference analysis. There were a total of 114 males that produced a macro on both days. Results on the combined data showed that males produced significantly fewer micros on the second day of mating, relative to the first day (Figure 1:  $t = -6.93$ ,  $p < 0.001$ ); within species all populations except *L. kona<sub>A</sub>*, the decrease in spermatophores between day 1 and day 2 was significant (Table 3). There were no significant differences in the species-adjusted percent change in micro production between the populations, species, or locations. Additionally, the time of production of the first micro was significantly later on the second day of mating (Figure



1: Wilcoxon  $T = 4.01$ ,  $p < 0.0001$ ) for the combined data; within species the delay in mating initiation showed a positive trend within each population, but was significant only in *L. paranigra*<sub>S</sub>, *L. pacifica*<sub>A</sub>, and *L. kona*<sub>S</sub> (Table 3).

A contingency analysis showed that there were significant differences among populations ( $n=302$ ,  $df=6$ ,  $\chi^2: 20.95$ ,  $p < 0.01$ ) and locations ( $df=4$ ,  $\chi^2: 18.18$ ,  $p < 0.01$ ) in proportion of individuals that made micros or macros on either mating day. There were also significant differences between location types (sympatric or allopatric) ( $\chi^2: 9.13$ ,  $p < 0.01$ ), with a lower proportion of individuals at sympatric sites producing micros or macros than those at allopatric sites. There were no significant differences between any of these factors on the second day of mating.

#### ***Estimating limits on spermatophore production: low-diet***

82 of 117 total pairings (69%) produced at least one micro on the first day of mating. While there was a slight trend for fewer low-diet males to produce a micro on day 1, a Fisher's exact test showed that there were no significant differences between the low- and high-diet males for the combined data. 48 of 117 low-diet pairings (41%) produced a macro on day 1; a Fisher's exact test on the combined data showed that there was a significant difference between low- and high-diet males for day 1 macro production (Table 2: Fisher's  $p=0.04$ ). Of those 48 successful matings, 37 (77%) produced at least one micro and 18 (38%) successfully produced a macro on the second day. A chi-square test showed that there were no significant differences between the proportions of low-diet and high-diet males that successfully produced at least one micro on the second day of

mating, but did find there to be significantly fewer low-diet males than high-diet males who produced a macro two days in a row (Table 2:,  $p=0.04$ ).

Only those 18 males which produced a macro on both days were used for spermatophore production difference analysis, as in the high-diet experiments. Because of the small sample size, this data was not analyzed by species. Males produced significantly fewer spermatophores on the second day of mating, relative to the first day (Figure 1:  $t= -2.65$ ,  $p=0.016$ ). Additionally, the time of production of the first spermatophore was significantly later on the second day of mating (Figure 1: Wilcoxon  $T=3.94$ ,  $p<0.0001$ ).

#### ***Spermatophore production correlates: high-diet***

Only males which produced a macro on the day under analysis were used for the following production correlates analysis. The results showed that when the number of micros produced on day 1 was set as the response variable, larger positive female weight residuals significantly predicted number of micros produced across the combined data (Figure 2a:  $F_{1,175} = 54.78$ ,  $p<0.0001$ ), and predicted how early mating was initiated ( $F_{1,175} = 20.44$ ,  $p<0.0001$ ); for the population data, all showed positive trends for day 1, but two were not significant (Table 4). On day 2 of mating, the female weight residuals again predicted the number of micros produced (Figure 2a:  $F_{1,113} = 8.65$ ,  $p=0.0040$ ), and again showed a non-significant trend for predicting the time of mating initiation (data not shown); when analyzed by population, all again showed positive trends, but only two were significant (Table 4). An ANCOVA showed that there were significant differences on day 1 among the populations ( $F_{1,6} = 2.89$ ,  $p<0.01$ ) but not among location types in

pattern of response to female weight. On day 2, there were no significant differences on day 2 among populations, but there was a difference among location types in pattern of response to female weight, with the sympatric populations responding more strongly (producing more micros) than the allopatric populations in response to female weight ( $F_{1,1} = 4.03, p < 0.05$ ).

Additionally, in the combined data for those males that completed mating on both days, positive absolute weight difference between consecutive females negatively predicted the decrease in micro number between day 1 and day 2 (Figure 3:  $F_{1,113} = 23.17, p < 0.0001$ ). This means that if a male was paired with a larger female on day 2 than on day 1, he decreased the number of micros he produced less than he would have if presented with a similarly sized or smaller female; when broken down by individual species, such a pattern was only apparent in three populations (Table 5). An ANCOVA on the same data showed that there were no significant differences among the populations, but there was a slight significant difference between location types, with the sympatric populations having a stronger response to change in female weights than the allopatric populations ( $F_{1,1} = 4.11, p < 0.05$ ). And finally, a logistic regression of mating initiation on residual female weight showed that mating was initiated and completed more frequently with larger females on both day 1 (Figure 4a: initiation:  $\chi_1^2 = 5.00, p = 0.0253$ ; completion:  $\chi_1^2 = 32.36, p < 0.0001$ ) and day 2 (Figure 4b: initiation:  $\chi_1^2 = 11.93, p = 0.0006$ ; completion:  $\chi_1^2 = 5.56, p = 0.0184$ ). Once again, when broken down by population, although most populations showed a trend which mirrored that of the combined data, fewer than half were significant (Table 6).

### ***Spermatophore production correlates: low-diet***

Only those 18 males that completed mating on both days were used for statistical analyses. When the number of micros produced was set as the response variable, female weight residuals significantly predicted number of micros produced on day 1 (Figure 2b:  $F_{1,17}=6.53$ ,  $p < 0.03$ ), but there were no predictors for micro production on day 2. This data was analyzed by individual species but only two populations showed a significant pattern on either day (Table 4).

When all independent variables that might have predicted a decrease in micro production were used in an ANCOVA as above, none significantly predicted the drop in micro number. Additionally, a logistic regression did not find that female weight residuals could predict mating initiation or completion as it did for the high-diet males. However, it should be noted that the failure to find significant results may have been due to small sample size and low power even across the combined data.

## **DISCUSSION**

*Laupala* males appear to have a very costly mating system, given the material, energetic, and temporal investment that is put into each mating, supporting the hypothesis that *Laupala* males exercise mate choice. To obtain evidence for costs in this system, males were mated consecutively and the change in micro number between mating days was measured. Additionally, the frequency of mating events of both high-diet and low-diet males was compared to understand how resource limitation affects mating costs. Finally, to determine if males were choosy, the correlation between female size and spermatophore production was assessed. As predicted, *Laupala* males display attenuated

mating abilities in consecutive matings by producing fewer micros on the second day, generally regardless of species. Results also show that a significantly lower proportion of low-diet than high-diet males are able to produce macros on either day. Results also showed that males initiated mating sooner and produced more micros when paired with larger females, suggesting they can assess the size of their partners. Finally, results showed that males modulate micro production on consecutive days corresponding to the size of mating partners, suggesting that males assess partners and adjust mating effort from day to day. For all tests, the data within most individual species showed a similar trend to the data combined across all species, suggesting that despite average differences in timing or weight, the processes of sexual selection are similar across species.

Altogether, these data suggest that mating is costly to *Laupala* males, who choose partners that allow them to maximize their fertilization success.

### *Species differences*

Overall, while all analyses showed that there were some significant differences between populations, locations, and location types, overall positive or negative trends were consistent regardless of the breakdown. It has previously been observed (J. Jadin, unpublished; K. Shaw, pers.comm) that there are differences in mating propensity in the lab between species and populations, but why this occurs is unknown. The differences between sympatric and allopatric locations in the proportion of individuals that produced micros or macros on day 1 was interesting. Allopatric individuals were more likely to produce micros and macros on the first mating day than were sympatric individuals. One possible reason for this result is that because allopatric individuals will not make mating mistakes, they have broader mating preferences and are more likely to mate with any

individual. However, it may also be the result of the *L.hualalai* species, which only occurs in sympatry, bringing the average for all sympatric individuals down because of its low mating propensity. While the former is intriguing and provides support for a hypothesis of increased reproductive isolation in sympatry, it is not possible to eliminate either hypotheses without further study. However, we believe that the overall similarities among species in general mating patterns justifies the lumping in the analyses.

### *Costs*

As predicted, it appears that mating imposes a future “cost” on *Laupala* males. This cost results in males showing a decreased ability to produce micros in consecutive matings, and in a significantly lower proportion of mated males completing a second mating. This suggests that a mating depletes a male of enough resources that he cannot invest as heavily into a second mating. Previous studies on the costs of nuptial gifts have found similar results. Gwynne (1990), for example, found that when a male katydid engaged in multiple matings, it negatively affected his spermatophore size. Another study found that mated male bush crickets produced lower quality spermatophores in subsequent matings (Wedell and Ritchie 2004).

Additionally, if spermatophore production is costly, one would expect to find nutritional condition affecting production, resulting in low-diet males having a greater refractory period or producing fewer spermatophores than high-diet males. Our results show that a significantly smaller proportion of low-diet males produced macros on either mating day than did high-diet males, following this prediction. Previous studies in other gift-giving insects have reported similar results. In the study noted above, Gwynne (1990) also found that male katydids on high-quality diets mated more frequently than

those on low-quality diets, and the variance in spermatophore size significantly increased in males on low-quality diets. Wagner (2005) similarly found that field crickets fed a low quality diet took longer to produce a sperm-containing spermatophore than did those on a high-quality diet. A notable result from the present study is that the cost of mating may come from macro production, rather than production of the numerous micros, as originally predicted. As a result, the predication that food-limited males would be unable to produce micros at a level commensurate with that of high-diet males was unsupported; it appears that low-diet males are only limited in their macro production ability. It may be that the larger macros are significantly more resource-using than are micros. Previous studies have found that larger nuptial gifts in crickets do result in higher male costs (Sakaluk *et al*, 2004), and that low-diet males compensate for high costs not by decreasing spermatophore size, but instead by having a longer refractory period (Wagner 2005). Most recently Vahed (2007b) reported that larger ejaculates, but not larger spermatophylaxes (or “gifts”) cause a longer refractory period, and therefore it is the ejaculate, not the gift, which is costly. This may explain why low-diet males can produce micros but not ejaculate-containing macros.

The results here additionally reveal a significant delay in mating initiation on day 2. These data support the frequently-made assumption that there is a trade-off between resources spent on current reproduction and future remating rate (Parker and Ball 2005). The data also support the growing body of evidence showing that males experience a refractory period after mating, which is correlated with ejaculate size or quality (Hayashi 1993; Wedell 1994; Sakaluk *et al*. 2004; Vahed 2007b). One implication for a delay in initiating mating on day 2 is that males may miss the peak receptivity period of females

and lose out on a mating. Previous research in *Laupala* has shown that males of different species have peak mating activity periods (J. Jadin, Ch.3; Danley *et al.* 2007) and it is thought that female receptivity periods may mirror that of males. Results also show that *Laupala* males will mate with sympatric heterospecifics (J. Jadin, Ch.3). If males delay courtship initiation on day 2 of mating, they may miss the peak activity period of conspecific females and be increasing their probability of engaging in a heterospecific mating.

A delay in remating may also affect the operational sex ratio (OSR), resulting in more females that are ready to mate at any given time relative to males. A female-biased OSR would mean that males, the rarer sex, will be relatively choosy with the females they encounter, whereas females may be relatively less choosy. However, to address questions about the OSR it is first necessary to understand the female refractory period. The current study does not measure female refractory periods or female costs, however, it is likely that in *Laupala*, as in most animal systems, the females pay significant costs for mating. Females in the lab can lay hundreds of eggs over the course of their lifespans, which likely require significant resources to produce. We therefore do not suggest that the results from this study imply that males necessarily have higher costs than females, rather we interpret the results from this study as indicating that *Laupala* males, like males in many other Orthopteran systems, have substantial costs which may approach those of the female. Such an investment into mating should mean that a male would be selected to discriminate among mates as much as females do.

Finally, it should be noted that in addition to the direct physiological costs of spermatophore production, previous studies have found that calling and advertisement are



energetically draining and may also limit male remating ability, as for example in *Teleogryllus commodus*, where time spent calling is correlated with a reduced lifespan (Hunt *et al.* 2004). In our testing conditions, males were calling to a female for up to 9 hours, and in the wild, a single bout may take even longer (pers.obs.). Males do not sing continuously throughout this mating bout, but do sing at regular intervals after a spermatophore has been produced but before the female has accepted it. Males that were unable to transfer to females were often observed calling nearly continuously throughout the day (pers.obs); therefore, even when males were not able to secure a successful mating, considerable energy was spent on the mating effort. The decrease in mating effort, in terms of both micro production and remating delay, may also be affected by other energetic factors such as song production.

### ***Male choice***

A further implication of this study is that if males are limited in their remating ability, they should display preferences for the females that maximize their fitness. Results here also show that naïve males produce significantly more micros for larger females. Larger females typically contain more eggs and are therefore more fecund (Bonduriansky 2001 and refs therein; Knox and Scott 2005). While the relationship between female size and fecundity has not been tested in *Laupala*, it is likely that there is a similar relationship to other studies. Therefore, if males pay significant costs for mating, they would be selected to preferentially invest mating resources into those females with higher fecundity.

Results here showed that when males are sequentially presented with differentially sized females, they appear to modulate their investment in response to

female size. When presented with a larger female on day 2 they, on average, produce more micros than if they were presented with a smaller or equally-sized female. Given that males produce fewer micros on day 2 on average, this pattern emerges as a smaller decrease in micro production for large females than for small females. This suggests that males preferentially invest in larger females, and that they have a “memory” of previous mating experiences and can adjust accordingly. Similar results have been found before in pacific blue-eyed fish (Wong *et al.* 2004) and field crickets (Hunt *et al.* 2004). This may be because males which have already secured a mating may be more certain of their “attractiveness” to females and therefore are more sensitive to courtship costs (Hunt *et al.* 2004). Alternatively, males may have a set point for investment into a female of a given size and do their best to reach that set point during any given mating. Males presented with a small female on the day 1 may be less depleted for day 2. Overall, these results suggest that males are choosy, and are able to assess the relative size of their partners and invest accordingly. These results run contrary to the long-established paradigm of high female reproductive costs and female-limited reproductive rates (Bateman 1948; Trivers 1972), but agree with an increasing number of findings in gift-giving insects, suggesting that male costs can no longer be discounted (Sakaluk *et al.* 1987; Simmons *et al.* 1992; Wagner 2005; Vahed 2007b).

### ***Implications for evolution in *Laupala****

Ultimately, if mating is costly for males, one would expect selection to be acting upon male mating behavior, as well as female mating behavior. In a genus such as *Laupala*, which appears to have speciated rapidly (Mendelson and Shaw 2005), and may hybridize

in contact zones (J. Jadin, Ch. 3; Shaw 2002), this sort of selection could have profound implications. Female choice is an important factor in mating, in that females prefer species-specific song types (Shaw 2000a; Mendelson and Shaw 2002). However, females do have some flexibility in song choice, and will mate with heterospecifics (Shaw and Lugo 2001; J. Jadin, Ch.3). Given that males are able to modulate mating investment and effectively choose larger females, this could have consequences for evolution in *Laupala* contact zone where there are often significant weight differences between species (J. Jadin, Ch.3). If males are selected to invest most heavily into the largest females, mispairings may occur when there is a conflict between choice for ideal body size and choice for any species-specific sexual signals. Recent studies in other organisms (e.g. *Drosophila*: Boake, *et al.* 1997; frogs: Pfennig 1998; damselflies: Svensson *et al.* 2007) have found that this conflict between mate and species recognition is the result of high-quality conspecifics resembling heterospecifics. If *Laupala* males gain fitness benefits by mating with large conspecific females, males may choose large sympatric heterospecifics if mating signals overlap. Evidence of this pattern has been found in two *Laupala* species in a contact zone, where male choice for large females appears to conflict with female preference for conspecific males (J. Jadin, Ch.3).

Results of this study demonstrate that the mating system of *Laupala* is costly to males, resulting in the production of fewer spermatophores and a delayed initiation on mating day 2. Males also mate more frequently and produce more micros with larger females. This is consistent with a hypothesis that male costs in this genus lead to male discrimination, as well as with the more general prediction that large male ejaculates and or nuptial gifts are costly to males. Whether or not male costs and male choice in

*Laupala* have any significant effect on the OSR or the direction of evolution remains to be determined, and future research on the mating system in this genus should be directed towards quantifying female costs, refractory periods, and fecundity. If, as other results from *Laupala* suggest, male choice has the potential to transcend species boundaries and counteract female choice, *Laupala* could be a novel example of a system in which male choice results in selection that runs counter to the female sexual selection.

## TABLES

**Table 1.**

Species	♂ wt	♀ wt	Day 1			Day 2		
			Micro time	# micros	Macro time	Micro time	#micros	Macro time
<i>hualalai</i>	0.0256		5895	4.2	19957	9546	4.1	22130
<i>kona<sub>A</sub></i>	0.0248		7229	7.2	21300	8288	5.5	20797
<i>kona<sub>S</sub></i>	0.0233		4059	5.3	19939	7146	4.4	20252
<i>pacifica<sub>A</sub></i>	0.0261		1257	7.9	17529	2381	6.3	18199
<i>pacifica<sub>S</sub></i>	0.0248		3757	6.3	17413	3541	4.7	15550
<i>paranigra</i>	0.0236		6070	6.6	26083	15498	4.6	25789
<i>tantalus</i>	0.0284		10127	5.9	22855	14051	4.5	25685

**Table 2. Mating events in high-(H) and low-(L) diet individuals.  $\chi^2$  tests were performed and P values are shown in table.**

<b>EVENT</b>	<b>Day1-High</b>	<b>Day2-High</b>	<b>p</b>	<b>Day1-Low</b>	<b>Day2-Low</b>	<b>P</b>	<b>H vs. L day1 (p)</b>	<b>H vs. L day2 (p)</b>
Prop ♂s initiating mating	248/303 (82%)	142/177(80%)	NS	82/117(69%)	37/48(77%)	NS	NS	NS
<i>L. hualalai<sub>S</sub></i>	43/57	14/18	NS	--	--	--	--	--
<i>L. kona<sub>S</sub></i>	47/65	25/33	NS	20/26	8/11	NS	NS	NS
<i>L. kona<sub>A</sub></i>	36/39	22/27	NS	22/28	13/16	NS	NS	NS
<i>L.pacifica<sub>S</sub></i>	41/46	25/32	NS	5/5	5/5	NS	NS	NS
<i>L. pacifica<sub>A</sub></i>	31/33	22/24	NS	--	--	--	--	--
<i>L. paranigras<sub>S</sub></i>	37/49	22/31	NS	42/58	12/16	NS	NS	NS
<i>L. tantalus<sub>S</sub></i>	13/13	12/12	NS	--	--	--	--	--
Prop ♂s completing mating	177/303 (58%)	114/177(64%)	NS	48/117(41%)	18/48	NS	0.04	0.04
<i>L. hualalai<sub>S</sub></i>	18/57(32%)	8/18(44%)	NS	--	--	--	--	--
<i>L. kona<sub>S</sub></i>	33/65(50%)	21/33(64%)	NS	15/26	3/11	NS	NS	NS
<i>L. kona<sub>A</sub></i>	27/39(69%)	19/27(70%)	NS	16/28	4/16	NS	NS	NS
<i>L.pacifica<sub>S</sub></i>	32/46(70%)	21/32(66%)	NS	5/5	4/5	NS	NS	NS
<i>L. pacifica<sub>A</sub></i>	24/33(73%)	17/24(71%)	NS	--	--	--	--	--
<i>L. paranigras<sub>S</sub></i>	31/49(63%)	17/31(55%)	NS	16/58	7/16	NS	0.02	NS
<i>L. tantalus<sub>S</sub></i>	12/13(92%)	10/12(83%)	NS	--	--	--	--	--

Table 3. T-tests (#micros) and Wilcoxon signed-rank (time) tests measuring the decrease in micro production between mating days, as well as a delay in mating initiation (spermatophore production) time in high-diet males. For all populations except *L. kona<sub>A</sub>* the decrease in spermatophores between day 1 and day 2 was significant. The delay in mating initiation showed a positive trend within each population, but was significant only in *L. paranigra<sub>S</sub>*, *L. pacifica<sub>A</sub>*, and *L. kona<sub>S</sub>*.

High Diet only		# micros		time of first micro	
Species	n	T	P	T	p
<i>L. hualalai<sub>S</sub></i>	8	-3.02	<0.01	1.79	NS
<i>L. kona<sub>A</sub></i>	19	-1.98	NS	0.77	NS
<i>L. kona<sub>S</sub></i>	21	-4.76	<0.001	2.61	<0.05
<i>L. pacifica<sub>A</sub></i>	17	-2.29	<0.05	4.04	<0.001
<i>L. pacifica<sub>S</sub></i>	22	-3.94	<0.001	1.89	NS
<i>L. paranigra<sub>S</sub></i>	17	-5.47	<0.001	4.59	<0.001
<i>L. tantalus<sub>S</sub></i>	10	-2.53	<0.05	1.65	NS

Table 4. A one-way ANOVA showed that there was a significant positive relationship between residual female weight and number of micros produced on day 1 of mating for high-diet males in four of the seven populations. For day 2 of mating, the only significant relationship between residual female weight and number of micros was in the *L.kona<sub>A</sub>* and *L.pacifica<sub>A</sub>* populations. For low-diet males, only two populations showed a positive response to female weight on either day, but it is likely small sample size issues may have confounded the analysis.

species	High-diet: Day 1			Day 2			Low-diet: Day 1			Day 2		
	n	F	P	n	F	P	n	F	p	n	F	p
<i>L.hualalai<sub>S</sub></i>	15	0.34	NS	8	4.58	NS	0	--	--	0	--	--
<i>L.kona<sub>A</sub></i>	27	11.31	<0.01	19	6.47	<0.05	17	10.26	<0.01	13	0.73	NS
<i>L.kona<sub>S</sub></i>	32	9.91	<0.01	21	3.29	NS	12	0.65	NS	9	7.49	<0.05
<i>L.pacifica<sub>A</sub></i>	24	0.01	NS	17	29.66	<0.01	0	--	--	0	--	--
<i>L.pacifica<sub>S</sub></i>	30	15.06	<0.01	22	3.75	NS	6	1.48	NS	6	0.01	NS
<i>L.paranigra<sub>S</sub></i>	30	25.29	<0.01	17	2.89	NS	17	1.11	NS	13	1.26	NS
<i>L.tantalus<sub>S</sub></i>	11	6.727	<0.05	10	0.98	NS	0	--	--	0	--	--



Table 5. A one-way ANOVA on the high-diet data showed that weight difference between sequential females significantly predicted the change in number of micros a male produced in only the *L.pacifica*<sub>A</sub>, *L.pacifica*<sub>S</sub>, and *L.paranigra*<sub>S</sub> populations. In these populations, when presented with a larger female on the second day of mating, males produce more spermatophores than if presented with a smaller female. Only data for those males which completed mating on both days were used so as not to conflate spermatophore response with readiness to mate. Low-diet males were not analyzed due to the small sample size.

species	n	F	p
<i>L.hualalai</i> <sub>S</sub>	8	1.491	NS
<i>L.kona</i> <sub>A</sub>	19	2.729	NS
<i>L.kona</i> <sub>S</sub>	21	3.456	NS
<i>L.pacifica</i> <sub>A</sub>	17	11.079	<0.01
<i>L.pacifica</i> <sub>S</sub>	22	4.648	<0.05
<i>L.paranigra</i> <sub>S</sub>	17	8.351	<0.01
<i>L.tantalus</i> <sub>S</sub>	10	0.079	NS

Table 6. A logistic regression of female weight on mating initiation (micro produced) showed a significant positive effect in the *L. pacifica*<sub>S</sub>, *L. paranigra*<sub>S</sub>, and *L. pacifica*<sub>A</sub> populations only, and a logistic regression of female weight on mating completion (macro produced) showed significant positive effects in only the *L. paranigra*<sub>S</sub> and *L. kona*<sub>S</sub> populations.

On the second mating day only *L. hualalai*<sub>S</sub>, *L. pacifica*<sub>S</sub>, and *L. pacifica*<sub>A</sub> showed significant positive effects for initiation, none for completion. Female weight did not significantly predict mating initiation or completion on either day for the low diet males (data not shown), but in all cases, the lack of positive association may be due to small sample sizes.

species	day 1: initiated			completed		day 2; initiated			completed	
	n	$\chi^2$	p	$\chi^2$	p	n	$\chi^2$	p	$\chi^2$	p
<i>L.hualalai</i> <sub>S</sub>	57	1.222	NS	3.238	NS	18	4.133	<0.05	0.508	NS
<i>L.kona</i> <sub>A</sub>	39	0.045	NS	0.0120	NS	28	1.121	NS	3.003	NS
<i>L.kona</i> <sub>S</sub>	65	1.715	NS	7.048	<0.01	33	0.823	NS	0.439	NS
<i>L.pacifica</i> <sub>A</sub>	33	8.835	<0.01	2.601	NS	32	18.346	<0.01	1.638	NS
<i>L.pacifica</i> <sub>S</sub>	46	4.095	<0.05	1.040	NS	25	5.719	<0.05	0.898	NS
<i>L.paranigra</i> <sub>S</sub>	49	19.249	<0.01	12.519	<0.01	31	1.376	NS	1.853	NS
<i>L.tantalus</i> <sub>S</sub>	13	--	--	--	--	12	--	--	0.529	NS

## FIGURE LEGENDS

**Figure 1.** T-tests (#micros) and Wilcoxon signed-rank (time) tests showed a significant decrease in micro production between mating days (top), as well as a delay in mating initiation (spermatophore production) time (bottom) in both high-diet (A) and low-diet (B) males on the combined data. Error bars represent one standard error. The mean  $\pm$  1 s.e. is shown in the top graphs, the median  $\pm$  1 s.e. is shown in the bottom graphs

**Figure 2.** a) A one-way ANOVA showed that there was a significant positive relationship between residual female weight and number of micros produced on day 1 (left) ( $F_{1,300} = 12.66, p=0.0004$ ) and day (2) of mating for high-diet males (right) ( $F_{1,113} = 5.58, p=0.0199$ ) when data was combined for all species. Overall, it may be that more spermatophores were produced for larger females in part as a consequence of initiating mating earlier: on both days they showed a non-significant trend to mate earlier with larger females.

b) The same pattern was true on day 1 for low-diet males, who also produced more micros when paired with large females (left) ( $F_{1,46}=7.30, p <0.0096$ ). This can likely be explained by the fact that they initiated mating significantly earlier with larger females ( $F_{1,46}=30.06, p<0.0001$ ). There was no relationship between weight and number of micros on day 2 for low-diet males (right), but this may be due to the small sample size.

**Figure 3.** The X-axis represents the difference in weight (g) of day2–day1 females for each male. The Y-axis represents day2-day1 micro production. A one-way ANOVA on the combined data showed that weight difference between sequential females was the

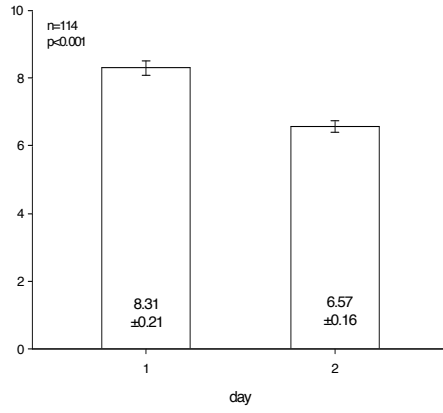
only significant predictor of the change in numbers of micros a male produced: when presented with a larger female on the second day of mating, males produce more spermatophores than if presented with a smaller female ( $F_{1, 113} = 23.17, p < 0.0001$ ). Only data for those males which completed mating on both days were used so as not to conflate spermatophore response with readiness to mate. This was significant for the high-diet males only; the low-diet sample size likely lacked power.

**Figure 4.** a) Mating was initiated (micro produced) and completed (macro produced) more frequently with larger females on the first mating day [hi-diet males only]. Box plot below shows median and the 90<sup>th</sup> percentile in female weight data as classified by whether they were offered  $\geq 1$  micro (mating initiated) (left) or offered a macro (mating completed) (right). A logistic regression of female weight on mating initiation ( $\chi_1^2 = 5.00, p = 0.0253$ ) and completion ( $\chi_1^2 = 32.36, p < 0.0001$ ) showed a significant positive effect in the combined data. b) The same pattern appeared for the second mating day for micros ( $\chi_1^2 = 11.93, p = 0.0006$ ) and macros ( $\chi_1^2 = 5.56, p = 0.0184$ ) in the combined data. Female weight did not significantly predict mating initiation or completion on either day for the low diet males (data not shown), but this may be due to small sample sizes.

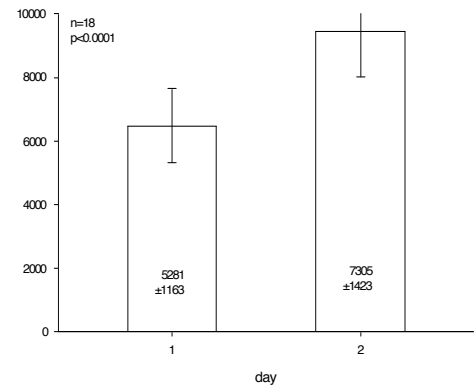
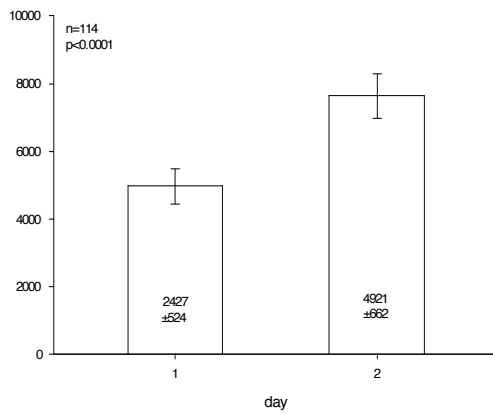
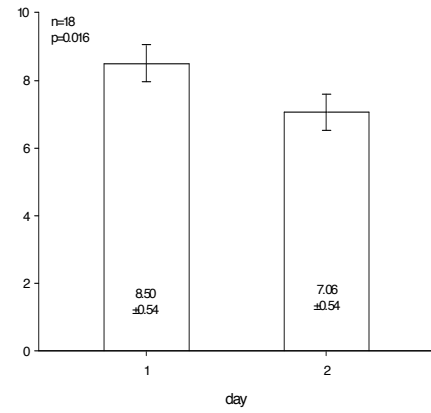
# FIGURES

## Figure 1.

### a) High-diet

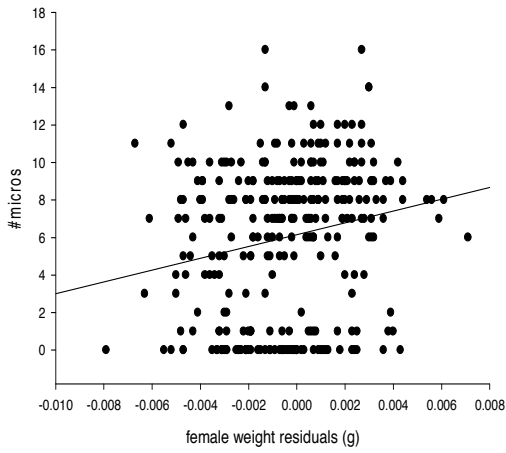


### b) Low-diet

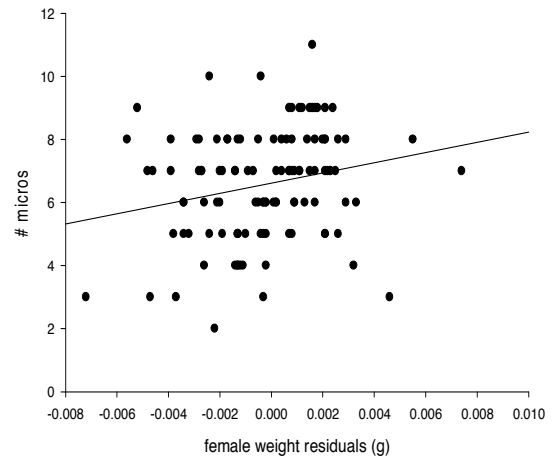


**Figure 2.**

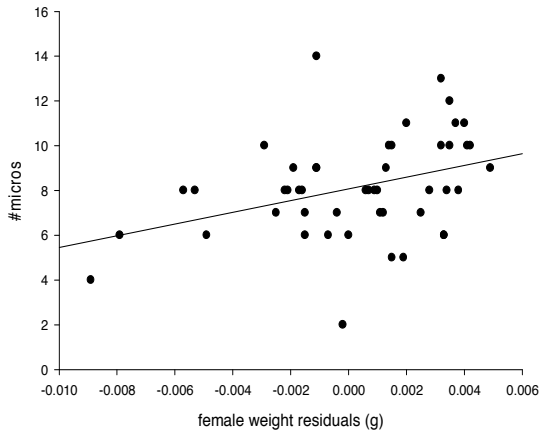
**a) high-diet, day 1**



**high-diet, day 2**



**b) low-diet, day 1**



**low-diet, day 2**

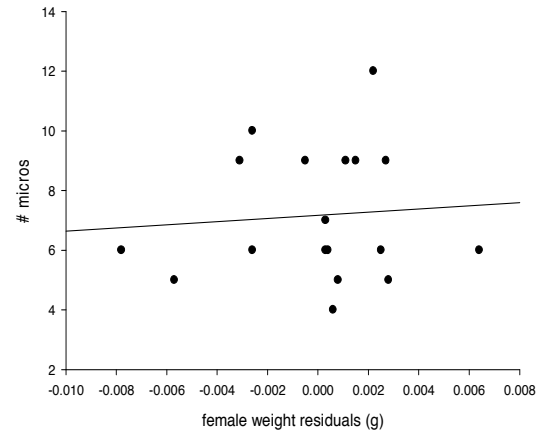
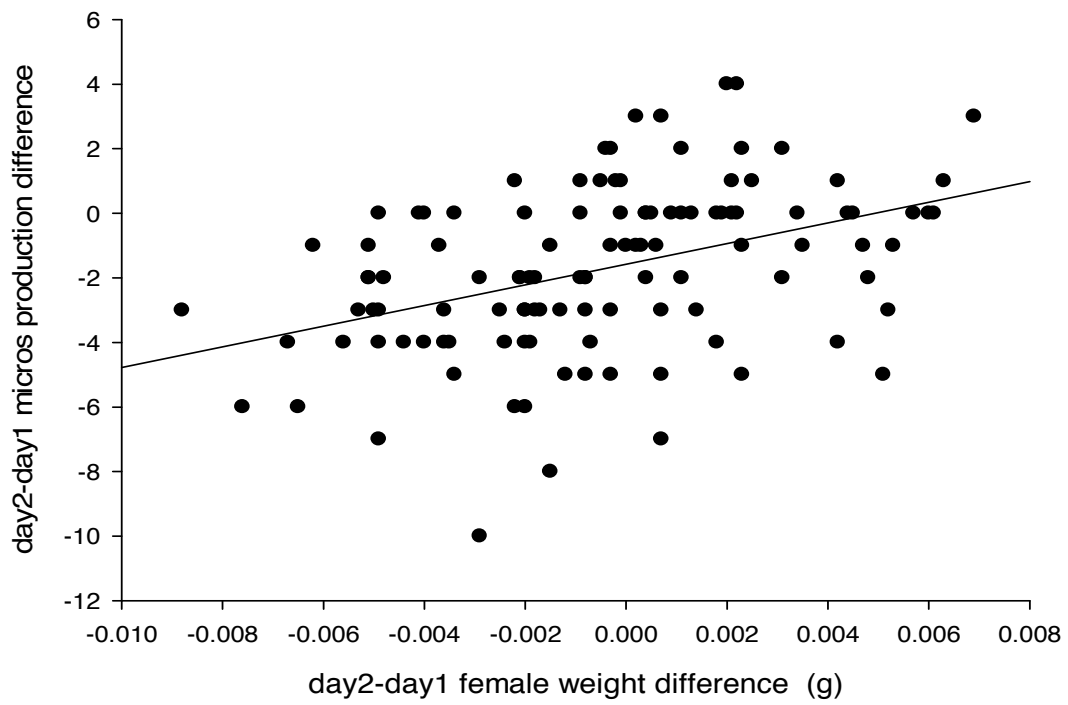
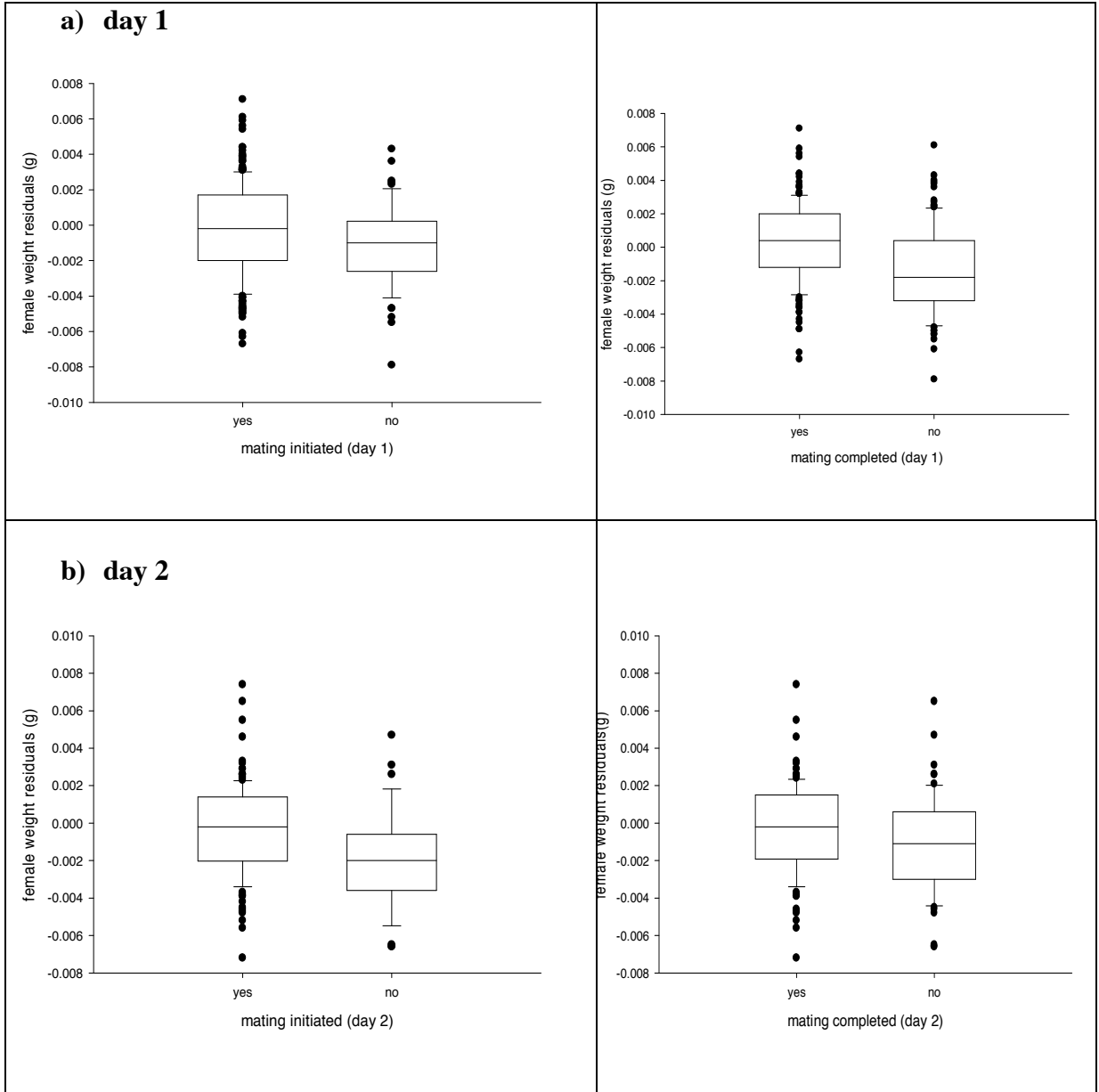


Figure 3.



**Figure 4.**





## **Chapter III: Asymmetric behavioral isolation in sympatric *Laupala* populations**

### **ABSTRACT**

The endemic Hawaiian cricket genus *Laupala* is an instructive model system for studying processes of speciation and sexual selection because of its complicated genetic history and its elaborate mating system that allows for selection at many steps of the mating process. Previous molecular data has shown that *L. tantalus* and *L. pacifica* on the island of Oahu may be hybridizing in a contact zone, but distinct species song rates and the finding of few to no hybrids suggests that the species pair remains distinct. However, the degree of behavioral isolation between these species both within and outside of the contact zone is still unknown. In this study, mating experiments were conducted to test for reproductive isolation and reproductive character displacement between sympatric and allopatric *L. tantalus* and *L. pacifica* populations. The mating frequencies within and between populations were compared in order to determine if the two species were reproductively isolated, and if so, if mate choice or mating character displacement were evident. Male and female weight, mean spermatophore number, mating sequence timing, and diel calling patterns were all measured in order to identify potential variables involved in reproductive isolation. Results first revealed the mean value of several phenotypic characters, including average spermatophore number, weight, peak singing period, and spermatophore production time, are significantly different between heterospecific populations, and that there is significant behavioral isolation between heterospecifics. Results also show that there is a significantly higher degree of

isolation between sympatric than between allopatric *L. tantalus* and *L. pacifica* pairs, suggesting displacement of mate choice or other mating character between populations of *L. pacifica*. Additionally, the isolation is asymmetric, in that males from the small *L. pacifica* species more frequently court the large *L. tantalus* species than do males in the reciprocal pair, and overall, large females are courted more frequently, regardless of species. Finally, results show that *L. pacifica* spermatophore number and timing may be undergoing displacement in the contact zone relative to the allopatric population. I therefore suggest that both singing activity and spermatophore timing patterns contribute to species isolation and result in reproductive character displacement of female choice, but male mate selection for large females may be counterbalancing species divergence. Altogether, it appears that this species pair represents a novel example of a system in which selection on male mate preferences conflicts with selection on female choice.

## INTRODUCTION

Contact zones between two incipient, recently-diverged, or other closely-related species are the ideal natural laboratory for setting up studies of reproductive isolation. Within recent contact zones, closely-related species may be currently undergoing speciation, allowing one to view the process in action. Often not just one, but multiple, processes are at work to either facilitate or undermine further isolation between species pairs, and the challenge can be to tease apart multiple competing selection pressures.

One topic that has been a popular focus of late is reproductive isolation that leads to reproductive character displacement. Reproductive character displacement (RCD) is a

pattern in which a mating character, be it choice, song type, or plumage color, is displaced in a zone of contact relative to those individuals of the same species outside of the zone. RCD can be the result of the process of reinforcement, a process of speciation where selection against hybrid offspring results in indirect selection on parental species mating preferences, causing divergence in preference or characters until speciation is complete. However, RCD can emerge even without hybridization, when there is overlap in mating signals or mate choice between species. If the overlapping species engage in heterospecific matings, and there is a cost to engaging in matings, then selection should act to cause further divergence in mating signals in the species pair. Those individuals that have stricter, more differentiated, criteria for mate choice, or more species-specific display traits will pay fewer costs due to mismatings, and contribute more and/or fitter offspring to subsequent generations. These derived characters should then spread throughout the sympatric population, leaving the ancestral characters persisting in the allopatric population. The allopatric population may still be able to engage in matings with the heterospecifics, but total isolation between sympatric congeneric species should result. In such a case, reproductive character displacement is not a signature of a process of speciation such as reinforcement, but rather reflects an evolutionary process of species divergence that functions to eliminate wasted reproductive effort (termed reinforcement in the “broad-sense” by Servedio and Noor 2003).

Many studies of late have tried to specifically address the distinction between traditional reinforcement and this “broad-sense” reinforcement, by looking not only for reproductive character displacement between populations, but also for sexual selection on reproductive characters within populations (Servedio and Noor 2003). As it turns out,

there are often multiple types of selection acting in hybridizing populations, and evidence suggests that conflicts may exist between the selection pressures leading to species divergence and those increasing sexual selection. This may occur when, for example, there is conflict between mate quality and species recognition characters within species (Boake *et al.* 1997), such as when high quality heterospecifics resemble conspecifics (Pfennig 1998, 2007), or when the costs of mating mistakes for males and females differ significantly enough to result in asymmetric isolation between species (Svensson *et al.* 2007). Such findings suggest that selection leading to reproductive isolation may be opposed by other forms of selection (Marshall *et al.* 2002), and therefore, when looking for evidence of reproductive isolation through reproductive character displacement, one may also have to evaluate the direction and costs of selection acting on the characters. Few studies to date have attempted to do this, and more are needed.

The Hawaiian cricket genus *Laupala* is an informative model system for studies of mating signal divergence and reproductive character displacement because of its complicated evolutionary history and elaborate mating system. First, the mating sequence is relatively long, with matings lasting 5-10 hours, depending on the species. Males first attract females with a long-distance calling song, and then mating begins when a female and male face each other while the male produces the first of a series of spermless “microspermatophores” (hereafter “micro”), circles the female, sings, and ends the copulation by transferring the micro to the female, which she eventually consumes (Shaw and Khine 2004). A series of 6-15 of these copulations then ensue, and at the culmination of the mating bout, the male passes the female a larger, sperm-filled “macrospermatophore” (hereafter “macro”), which she also consumes after the sperm

have evacuated. This elaborate mating system has significant male (J. Jadin, Ch.2) and presumably, female, costs, all of which could be subject to selection. Selection may be acting on female or male choice, or male mating characters, at any stage of the courtship process. In addition, if the costs for mating with a heterospecific are sufficiently high, selection may affect the strength and direction of patterns of reproductive character displacement.

*Laupala* is also an instructive system in which to study patterns of reproductive isolation because several groups of species occur in sympatric populations, have shared mtDNA, yet appear to be distinct species according to nuclear DNA. First, the geographic ranges of most of the 38 species of *Laupala* partially overlap with one or more congeners (Otte 1994) and it has been suggested that divergence in male calling song in several zones of overlap conform to a pattern of reproductive character displacement (Otte 1989). Indirect evidence additionally suggests that hybridization and introgression is a persistent feature of *Laupala* (Shaw 1999, 2002; Parsons and Shaw 2001; Mendelson and Shaw 2005), and viable and fertile hybrids can be formed in the laboratory between some species pairs (Shaw 1996b; Mendelson and Shaw 2006) although the extent of natural hybridization is unknown. Research has also shown that interspecific song and female preference differences have a genetic basis (Shaw 1996b; Shaw 2000a), that hybrids have intermediate songs (Shaw 2000a), and that females clearly favor conspecific over heterospecific songs in sympatry (Mendelson and Shaw 2002).

The present study was designed to determine if there is significant behavioral isolation between sympatric populations of *L. tantalus* and *L.pacifica*, as predicted from

the degree of genetic divergence between them. We also test for reproductive character displacement between allopatric *L.pacifica* populations, and measure characters that might be under selection within and between populations. It builds upon previous observations in the focal species *L. pacifica* and *L. tantalus*, which share similar mid-elevation, forest understory microhabitats, and occur in both sympatric and allopatric communities on the island of Oahu, Hawaii, USA. In sympatry, the songs of these two species are largely distinct (Otte 1994) suggesting distinct breeding populations, an inference corroborated by the finding that nuclear genetic data (amplified fragment length polymorphism) sort species into distinct lineages, regardless of geographical location. Yet, in sympatry, both species share mtDNA haplotypes extensively (J. Jadin, Ch.1), and Otte (1994) reports occasional intermediate singers based on field recordings.

Given these observations, I predicted that these species would show partial prezygotic isolation, making them ideal candidates to test for a pattern of reproductive character displacement in sympatric relative to allopatric populations. I collected data on numerous components of courtship, including micro and macro production, courting initiation, peak singing times, and male and female weights, in order to confirm species differences and determine which characters, if any, might be the target of displacement. I applied statistical tests to mating frequency data in order to measure the degree of isolation between different populations, to test for the presence of male-female asymmetric isolation between reciprocal crosses, and to identify if reproductive character displacement of choice had evolved in heterospecific matings between sympatric and allopatric pairs. Results show that there is displacement of mate preference between allopatric conspecifics, isolation is asymmetric between species, and that some

reproductive characters may be undergoing displacement. Overall these results suggest that there are two distinct, behaviorally-isolated species, and that selection for behavioral isolation between them may possibly in conflict with sexual selection within species.

## **METHODS**

### *Animal collection and maintenance*

All parents of individuals used in these experiments were collected at two sites on the island of Oahu. Individuals from two species, *L. tantalus* and *L. pacifica* were collected at the sympatric site (Mt. Tantalus, hereafter referred to as TAN<sub>M</sub> and PAC<sub>M</sub>, respectively), in appx. 20 X 30 meter patches both above and below the road at the Manoa Cliffs trailhead (21° 19'35", -157° 48'45"; 1408 ft)(Figure 1). This area is just above an urban center, but is protected and contains a large number of native plant species, in addition to introduced weedy species. *Laupala* were captured in the leaf litter on the ground, a mixture of decaying leaves from Palm grass (*Poaceae* spp.), Hapu'u (*Cibotium chamissoi*), Koa (*Acacia koa*), and bark from Royal palms (*Roystonea* spp.) Individuals from *L. pacifica* were also collected at an allopatric site (Pupukea, hereafter referred to as PAC<sub>P</sub>) along a trail leading past the Pupukea Boy Scout Camp (~21° 37'14", -158° 0'45", 1200ft) (Figure 1). This site experiences little human traffic, the vegetation is mostly native, and the crickets were collected in a 10m X 5m area on a approximate 35° slope above a desiccated stream bed. At this site the crickets were found both on the ground on the leaf litter, as well as in the decaying bark on the side of trees. Both sites appear to have a constant high humidity and ground temperatures of approximately 69°F throughout the year. Crickets were collected using a combination of

insect nets and small plastic vials. They were housed in 2qt plastic containers by population and fed *ad libitum*, until returned to the laboratory. Once in the lab, males were identified to species based upon song type and paired with a female that appeared, at best guess, to be the same species. Each mating pairs was contained in a 100ml plastic sample cup that contained a moist Kimwipe for use as an oviposition substrate. An ample supply of Fluker's cricket chow treated with Tegosept was provided for each pair. Food was changed weekly, and egg-containing tissues were collected bi-weekly and placed in a hatching cup, which contained only wet Kimwipes. If eggs were not oviposited by a mating female within two weeks of cohabitation, her male partner was switched out with a male from a different species. The lab temperature was maintained at 69°F and the light cycle was kept at a constant 12:12h cycle. Both species appear to have similar life-history characteristics, such as egg development periods, time to sexual maturation, and developmental stages.

### *Nymph generation*

After one month, cups were checked every three days for hatching nymphs. Nymphs were then collected and placed in one quart glass jars containing Kimwipes for moisture and bent paper toweling to use as a substrate during molting. All jars contained single-family mixed-sex crickets and contained no more than twenty individuals per jar. Each population used in this experiment produced 20-35 different families, ideally insuring sufficient genetic and/or behavioral diversity, if present, among the offspring. Nymphs remained in these jars for approximately four months, until they were near final molt, at which point they were transferred to individual sample cups. Species identity of



each family was confirmed by checking song rate of at least one mature male per family. Each cup was again moistened with a wet Kimwipe which was changed bi-weekly. Individuals were fed *ad libitum* weekly.

### *Pretrials*

All animals used in trials were recently matured (18-50 days), virgin, and paired with conspecifics in order to test for sexual maturity. Pretrials took place 2-3 days before the trial. At least one day of rest was given between the trial and the pretrial in order to insure that the male had not begun to exhaust his energy or spermatophore supply. Sexual maturity in the male was determined by the production of a single micro. Females were defined as sexually mature if they allowed a male with a micro to begin to back up beneath them. After a successful pretrial, the individuals were separated, the pretrialed pair was noted, and they were placed in separate fresh cups, and fed.

### *Mating Trials*

Standard no-choice asymmetrical (reproductive character displacement) mating trial set-ups were used for this experiment, with a goal of 40 trials of each of the six possible pairings (Figure 2). No-choice experiments were deemed appropriate for two reasons: first, when mating in the wild, *Laupala* males are located in small hidden perches and are unlikely to be approached by or be able to pair with more than one female at a time (J. Jadin, pers. obs); second, given that we wanted to measure behavioral isolation, not choice, allowing males to choose between females would not have given us an accurate measurement of absolute isolation. Males and females which were pretrialed

together were never used together in a trial. Trials began 2 hours after “sunrise” (i.e. lights on) when a male and female were placed together in a doubled petri dish with a wet kimwipe. Dishes were shaded from direct overhead light, and visually, but not acoustically separated from the neighboring pair. An observer sat quietly and manually entered behavioral events on a laptop computer using behavioral data recording software (Cricket Sex Logger, C.Anderson). In this way, up to 14 copulating pairs could be observed simultaneously. The observer noted age of cricket (+/- 2 days), and recorded time when each micro and the macro were produced and if and when they were passed. All species pairs were observed until macro transfer occurred, or until “light out” in the observation room. After trials, individuals were anesthetized with CO<sub>2</sub> and weighed on a microbalance.

### *Singing Behavior*

I gathered data on peak song times both in the field, and in the laboratory. The field study was conducted at the Manoa Cliffs (M) field site only. The presence or absence of singing males was scored hourly for 18 consecutive hours (4AM-9PM) at 10 focal positions, each approximately 20m apart along a transect on Manoa Cliffs trail. Additionally, the number of males singing was estimated into one of four categories: 0 (none), 1 (1-3 distinct and countable individuals), 2 (4-7 distinct but uncountable individuals), and 3 (7++, countless individuals). The study was performed on April 17, 2007. Morning twilight occurred at 0548 and evening twilight occurred at 1914h (US Naval Observatory data).

The laboratory study was conducted on wild-caught individuals one month after returning from the field, so as to give the individuals time to acclimate both to the lab temperature and humidity conditions as well as the change in light regimen. For the lab study, 15 individuals from each of the three populations were placed in separate overturned screen-topped plastic cups (100mL). Each cup was placed 6” from the next on a table in an Acoustic Systems anechoic chamber maintained at 69°F, and the placement of the cups was randomized with respect to population origin of individuals. The lights followed a standard 12:12 day:night cycle and were off from 1800h to 0600 h. Individuals were placed into the experimental position at 1500h and allowed to acclimate for one hour before the trial began. The total number of singing males per population was noted for five minutes at one hour intervals. They were observed in this way for 24 hours.

*Terminology Key*

Many comparisons, between multiple populations, at multiple stages of mating, in different directions were made in the course of analysis for this study. In order to facilitate easier interpretation of results, the following terminology for mating stages, comparisons, and characters will be used as shorthand through the remainder of this paper:

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**Mating pair combinations:**

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the *male* will be listed first, followed by the *female*, i.e., PAC<sub>M</sub>PAC<sub>P</sub> refers to the mating pair comprised of PAC<sub>M</sub> male and PAC<sub>P</sub> females

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**Mating pair:**

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<i>Homotypic</i>	within species and within population
<i>Heterotypic</i>	within species, but of different populations
<i>Heterospecific</i>	as typically defined, i.e. not of the same species

<i>Reciprocal pair</i>	the mating pair combinations in which the male-female origins are switched relative to each other, i.e. $TAN_M PAC_M$ is reciprocal to $PAC_M TAN_M$	
<b>Mating stage:</b>	<b>variable:</b>	<b>definition:</b>
<i>Micro time</i>	continuous	time of first micro (seconds after start)
<i>Courted, -ing</i>	binary	$\geq 1$ micro was/not produced
<i>Micro number</i>	integral	number of micros produced in a mating bout
<i>Passed, -ing</i>	binary	any micros were/not passed
<i>Rate</i>	continuous	rate of transfer of micros (micro/hour)
<i>Macro time</i>	continuous	time of macro (seconds after start)
<i>Macro production</i>	binary	macro was/not produced
<i>Macro pass</i>	binary	macro was/not passed, as % of macros produced
<i>Inseminated</i>	binary	macro was/not passed to female, as % of total pairs, and therefore, represents total number of matings successfully completed

### *Statistical Analyses*

Analyses were categorized and are presented as being within populations, among populations of the same species ( $PAC_M$  and  $PAC_P$ ), or between species ( $PAC$  and  $TAN$ ). For all statistical analyses SAS 9.1 or JMP v7.0.1 (SAS Institute) software was used.

### *Intrapopulation analyses*

Descriptive statistics for male and female weight (gram), micro time (sec), rate, micro number, and macro time (sec) were calculated in order to determine population averages for these phenotypic characters. For the diel pattern data, the number of field sites with singing males was noted and graphed (# sites vs. hour of day), and the peak activity period for each species was noted by eye. For the indoor song data, the number of males singing each hour was counted and graphed (# males vs. hour of day), and again, the peak activity period was noted. I then calculated intrapopulation frequencies for each

stage of the mating, including courting, passing, macro production, macro passing, and total inseminated.

In order to test for *a priori* hypothesized associations between mating characters, one-way ANOVAs and logistic regressions were run. First, initiation time was set as the independent variable and micro number as the dependent variable in order to test how timing affected the number of micros produced. Then, to test the effect of female size on mating effort, individual ANOVAs were run on each female population, with female weight and micro number set as the independent and dependent variables, respectively. Finally, female size was regressed on courting using logistic regressions.

In order to directly assess how males modulated spermatophore production in response to variability in conspecific female weight, individual one-way ANOVAs were run on each male population using the MIXED procedure in SAS. Female weight was set as the independent variable and micro number was set as the response variable. Because the response variable was not normally distributed, a randomization test with 5000 randomizations was performed and the P value was generated from the random distribution. Logistic regressions using female weight, female population, and the interaction effect as the independent variables, and courting and macro production as the dependent variables were also performed.

### *Intraspecific analyses*

In order to compare mean differences between conspecific populations, differences between the *L. pacifica* populations in male and female weight, micro number, rate, micro time, and macro time were analyzed with a one-way ANOVA using

the MIXED procedure in SAS. Levene's tests of unequal variance were used to compare differences in variance between the distributions of all characters (Levene 1960). For all characters except male and female weight, only the data from within population matings were analyzed in order to avoid the potentially confounding effects of mating isolation. For those response variables which were not normally distributed, 5000 randomizations of the data were performed. Means between populations were compared using individual contrasts and Tukey's adjustments were applied to the P values.

To examine the diel activity pattern of the two *L. pacifica* populations, I performed a generalized linear model repeated measures ANOVA on data from the indoor song trials using the SAS GLIMMIX procedure (Danley *et al.* 2007). For this analysis, it was necessary for the number of repeated measures ( $n=24$ ) to be fewer than the number of males per species ( $n=15$ ), otherwise the analysis would fail to converge upon a parameter estimate. As such, the 24h observation period was divided into twelve blocks consisting of 2h each, beginning at 0400h. The total number of singing males heard each hour was noted and an average for each block was used as the response variable in the repeated measures ANOVA model. Two contrast statements were used to test for population differences in male singing behavior; the first contrast assessed the peak period by comparing the number of singers during the apparent peak in activity (1200h-1400h) vs. all other 2h blocks; the second of which compared number of singers in PAC<sub>MC</sub> and PAC<sub>PU</sub> across this peak singing period (1200h-1400h).

I tested for sexual isolation and mating asymmetry between PAC populations using two different methods. There has been much controversy in the sexual selection literature regarding the appropriate statistics for measuring sexual selection, asymmetry,

and isolation: all commonly-used statistics can yield different results and have been shown to have differing degrees of reliability (Perez-Figueroa *et al.* 2005; Sugano and Akimoto 2007). For all analyses, the null model was random mating. I first compared mating probabilities within and between PAC<sub>M</sub> and PAC<sub>P</sub> pairs using log-likelihood ratio  $\chi^2$  on *a priori* planned non-orthogonal contrasts (Hoskin *et al.* 2005). Randomization tests were performed; the data were resampled 1000 times and the p value for the original log-likelihood ratio  $\chi^2$  statistic was derived from the resampled distribution (Manly 1994). I then calculated indexes of isolation (I<sub>PSI</sub> coefficients) and asymmetry (IA<sub>PSI</sub> coefficients) using JMATING software version 1.0.8 (Carvajal-Rodriguez and Rolan-Alvarez 2006). PSI indices run from 0 to  $\infty$ , with values lower than 1 indicating than there are fewer matings than expected under random mating (disassortative mating) and values higher than 1 indicating more matings than expected (assortative mating)(Rolan-Alvarez and Caballero 2000). I<sub>PSI</sub> indices calculate the deviations from random mating between homotypic and heterotypic pairs and estimate isolation between types. IA<sub>PSI</sub> indices calculate the deviations from random mating between two populations in reciprocal pairings and estimate asymmetry between types. JMATING performs 10,000 bootstrap replications of the observed values of the mating pairs in order to generate a distribution for the estimator. It then calculates a bootstrap average, standard deviation, and two-tailed probability of getting a sexual isolation estimate significantly different from zero (random mating). Currently there are no statistical methods developed to compare differences between conspecific controls and heterotypic treatments using the IA<sub>PSI</sub> approach (Rolan-Alvarez, pers.comm.), so only the IA<sub>PSI</sub> coefficients for homotypic or reciprocal pairings are presented.

### *Interspecific analysis*

The one-way ANOVA procedure as noted above was also used to compare means of the continuous phenotypic variables between PAC and TAN populations. As previously, 5000 randomizations of the data were performed and means between populations were compared using individual contrasts with Tukey's adjustments.

To examine the diel pattern of and species differences in male singing at the Manoa Cliffs field site, I used the SAS GLIMMIX procedure to perform an analysis similar to the one described above. For this analysis the 18h observation period was divided into nine periods consisting of 2h each, beginning at 0400h. The total number of transect sites with singing males present each hour was used as a measure of male response; an average for each block was used as the response variable in the repeated measures ANOVA model. Two contrast statements were used to test for species differences in male singing behavior at the visible peak of singing activity for each species; the first of which compared the two species across the peak singing period for PAC<sub>M</sub> (0600h-1100h) and the second compared across the peak time for TAN<sub>M</sub> (1600h-1800h). The indoor trials were again examined exactly as described in the previous section, except that contrast statements compared the number of singing individuals of PAC<sub>M</sub> and TAN<sub>M</sub> across the PAC<sub>M</sub> (0600h-1200h) and TAN<sub>M</sub> (1600h-1800h) peak singing periods.

Finally, I tested for sexual isolation and mating asymmetry between PAC and TAN populations using log-likelihood ratio tests and the I<sub>PSI</sub> estimator methods as described above. In this case I first compared mating probabilities within PAC<sub>P</sub>, PAC<sub>M</sub>,



and TAN<sub>M</sub> pairs using log-likelihood ratio  $\chi^2$  on *a priori* planned non-orthogonal contrasts for differences in mating frequency. I then calculated the I<sub>PSI</sub> coefficients and IA<sub>PSI</sub> coefficients between PAC<sub>P</sub>, PAC<sub>M</sub>, and TAN<sub>M</sub> using the JMATING software as described above.

## RESULTS

### *Intrapopulation comparisons*

All phenotypic variables (female weight, male weight, micro time, rate, micro number, macro time) had similar distributions within species and were significantly different between species. Descriptive statistics are shown in Figure 3.

The diel song pattern data is graphed in Figure 4 (transect data) and Figure 5 (indoor data). Each species appeared to have a distinct peak in singing behavior. The PAC<sub>M</sub> sang constantly throughout the 18h study period in the field, but shows a peak in calling behavior between 0600h and 1200h; in lab they sang between 0600h and 2100h, but showed a clear peak in activity at 1300h. The PAC<sub>P</sub> in lab sang from 0600h until 1700h and also showed a peak around 1200h, however, this population exhibited biphasic singing behavior with a smaller peak of singing activity centered around 0800h. The TAN<sub>M</sub> population was silent in the early morning hours, and did the majority of its singing between 1300h and 1900h, with a distinct peak between 1600h and 1700h. The repeated measures ANOVA showed that species and time of day had a significant effect on singing behavior [Table 1:  $F(8,18) = 18.78$ ,  $p < 0.0001$ ] in the field. Likewise, in the laboratory experiments, the repeated measures ANOVA again confirmed that there was a

significant species-specific effect on male calling in response to time of day [Table 2:  $F_{22,36}=7.92$ ,  $p<0.0001$ ].

Analysis of the micro production data revealed that later micro time had a significant negative effect on the micro number (Figure 6:  $F_{1,165}=190.24$ ,  $P<0.0001$ ) and that within each population, males produced more micros for larger females (Figure 7a: TAN<sub>M</sub>:  $R^2=0.22$ ,  $F_{1,23}=6.55$ ,  $P=0.0168$ ; PAC<sub>P</sub>:  $R^2=0.35$ ,  $F_{1,34}=18.64$ ,  $P<0.0001$ ; PAC<sub>M</sub>:  $R^2=0.21$ ,  $F_{1,38}=10.32$ ,  $P=0.0036$ ). This finding corroborates results from a previous study on male mating effort (J. Jadin, Ch. 2). There was no significant effect of female weight on micro time, macro time, macro production, or total inseminated.

#### *Intraspecific comparisons*

Female and male weights were distributed normally within and between all populations, however, micro number, micro time, macro time, and rate data were not. Therefore, a randomization test was applied to all data to deal with the non-normal distribution. A one-way ANOVA with 5000 randomizations was performed using the MIXED procedure; Tukey's adjusted least squares means contrasts showed there were no significant differences in male weight, female weight, micro number, micro time, macro time, or rate between the PAC populations (Figure 3). However, a visual examination of the data suggested that the variances in some of the characters were not equal between the PAC populations. Levene's tests for homogeneity of variance were applied to the ANOVA results and revealed that both micro time (Figure 3:  $F_{1,74}=10.20$ ,  $P=0.0021$ ) and micro number (Figure 3:  $F_{1,74}=5.17$ ,  $P=0.0258$ ) had significantly lower variances in the PAC<sub>M</sub> population. The decreased variance in micro number is likely a side effect of a

decreased variance in the micro time (Figure 6), since males that start courting later would have less time to produce before dark, assuming a constant rate was maintained.

In the laboratory diel pattern song experiments, the peak calling time (Figure 5) did not differ between the PAC<sub>P</sub> and PAC<sub>M</sub> populations: contrasts revealed that the period from 1200h-1500h was the period of greatest activity for both *pacifica* populations [PAC<sub>P</sub>:  $t(36) = 6.24$ ,  $p < 0.0001$ ; PAC<sub>M</sub>:  $t(36) = 12.29$ ,  $p < 0.0001$ ]. However, there were quantitative differences within the peaks: during the combined *pacifica* peak period from 1200h-1500h there were significantly more PAC<sub>M</sub> than PAC<sub>P</sub> population males singing [ $t(36) = 5.04$ ,  $p < 0.0001$ ]. This result may have potentially been the consequence of some sort of differing selection pressures within the populations. For example, it does appear that the sympatric population has a much higher population density than the allopatric population (J. Jadin, pers. obs.), which could result in selection for males to sing more frequently. However, this result may also have been an anomaly, and without repeated trials, it is not possible to distinguish between these two explanations.

Table 3 shows a summary of the frequency of occurrence of each stage of mating for all interspecific pairs. The log-likelihood  $\chi^2$  and the Fisher's tests (hereafter collectively referred to as the "exact tests") compare differences in frequencies of each stage of mating between any two mating pair combinations. With 9 different mating pair combinations, there are a total of 36 possible comparisons, i.e. PAC<sub>M</sub>TAN<sub>M</sub> v. PAC<sub>M</sub>PAC<sub>P</sub>, PAC<sub>M</sub>TAN<sub>M</sub> v. PAC<sub>M</sub>PAC<sub>M</sub>, etc. However, most of these comparisons would be redundant. Therefore, I decided to use six comparisons to detect mating asymmetry and isolation between the PAC populations. These comparisons (Table 4) reveal differences in frequency of completing any stage of mating by using  $\chi^2$  statistics to

estimate significant differences between conspecific homotypic control pairs and conspecific heterotypic test pairs (i.e.  $PAC_M PAC_M$  v.  $PAC_P PAC_M$ ) or between any two heterotypic test pairs (i.e.  $PAC_M PAC_P$  v.  $PAC_P PAC_M$ ). First, the homotypic control comparisons showed there were no significant differences in within-population frequency for courting, passing, macro production, macro passing, or total inseminated between the  $PAC_M$  and  $PAC_P$  populations (Table 4a). The  $I_{PSI}$  indices similarly test for frequency differences, but they are designed to test for differences in within-population mating propensity only, and therefore only compare conspecific homotypic controls to each other. This is important because differences in mating frequencies in heterotypic pairs may not be reflective of isolation if each type has different propensities for mating within their population. Results from the  $I_{PSI}$  indices corroborated the exact test results (Table 5) on the homotypic pairs. Finally, the exact tests also showed there were no significant differences in frequency of courting, passing, macro production, macro passing, or total inseminated when  $PAC_M$  and  $PAC_P$  were mated homotypically as compared to heterotypically (Table 4b). Altogether, these results mean that these populations are not isolated from each other, and also that both populations show inherently similar mating propensities. Any further differences I detected would be the result of asymmetry between populations.

The reciprocal  $PAC_M PAC_P$  vs  $PAC_P PAC_M$  contrasts, which measure male-female asymmetry in heterotypic matings, showed that there was some asymmetry between the two PAC populations at the level of courting (Table 4d;  $\chi^2=8.17$ ,  $p<0.05$ ), however, this asymmetry disappeared in further stages. It appears that slightly more courtings occur when the male in the pair comes from the  $PAC_M$  population than when he comes from the

PAC<sub>P</sub> population. The IA<sub>PSI</sub> indices did not corroborate the exact test results (Table 6), showing that there was no asymmetry based on male population when mating heterotypically.

### *Interspecific comparisons*

The one-way ANOVA procedure described above was also used to test for differences in the basic phenotypic characteristics between all of the species. Contrasts showed that male weight ( $F_{2,327} = 137.32$ ;  $p < 0.0001$ ;  $t_{327} = -16.53$ ; Tukey's adj.  $p < 0.0001$ ), female weight ( $F_{2,327} = 121.08$ ;  $p < 0.0001$ ;  $t_{327} = -15.55$ ; Tukey's adj.  $p < 0.0001$ ), micro number ( $F_{2,327} = 68.20$ ,  $p < 0.0001$ ;  $t_{327} = 11.54$ ; randomized  $p < 0.0001$ ), micro time ( $F_{2,327} = 32.98$ ,  $p < 0.0001$ ;  $t_{327} = -8.09$ ; randomized  $p < 0.0001$ ), and rate ( $F_{2,327} = 3.87$ ,  $p = 0.02$ ;  $t_{327} = 2.33$ , randomized  $p < 0.003$ ) were all significantly different between the *pacifica* and *tantalus* species. In addition, Otte (1994) showed that pulse rates are significantly different between these two species: *tantalus* sings at 2.0 pulses/sec (pps) at 20°C while *pacifica* sings at 0.5pps. Figure 3 graphically summarizes the differences in these variables by population.

Field measurements of singing behavior revealed further differences between the two species. At Manoa Cliffs, peak singing time was significantly different between the two species (Figure 4), with *pacifica* peaking between 0600h and 1100h *tantalus* peaking between 1600h and 1700h. The repeated measures ANOVA showed that the species differed in their calling behavior [Table 1:  $F(1,18) = 51.96$ ,  $p < 0.0001$ ], the time of day had a significant effect in the singing behavior of both species [Table 1:  $F(8,18) = 4.63$ ,  $p < 0.0001$ ], and a species-specific effect on male calling in response to time of day was

observed [Table 1:  $F(8,18) = 18.78, p < 0.0001$ ]. The contrast statements revealed that there were significantly more *pacifica* males singing during its period of greatest activity from 0600h-1100h [ $t(18) = 12.06, p < 0.0001$ ], and that *tantalus* had more males singing at its period of greatest activity from 1600h-1800h [ $t(18) = -4.76, p < 0.0001$ ], as expected. In the laboratory experiments, the peaks in calling time again differed between the species (Figure 5). The repeated measures ANOVA confirmed that the species differed in their calling behavior [Table 2:  $F(2,36) = 8.08, p < 0.0001$ ], the time of day had a significant effect in the singing behavior of both species [Table 2:  $F(11,36) = 16.75, p < 0.0001$ ], and a species-specific effect on male calling in response to time of day was observed [Table 2:  $F(22,36) = 7.92, p < 0.0001$ ]. The contrast statements revealed that there were significantly more *pacifica* males singing at its period of greatest activity from 1200h-1500h [ $t(36) = 7.56, p < 0.0001$ ], and that *tantalus* had more males singing at its period of greatest activity from 1600h-1800h [ $t(36) = -7.40, p < 0.0001$ ].

As reported above, males produced a larger number of micros for larger females, within populations. Micro production data could however not be compared across male species because such a comparison would be confounded by isolation between species; a preference, or lack thereof towards females of a particular size might reflect a preference for conspecifics rather than a body size preference. However, within females, a logistic regression of female weight, female population, and the interaction term on courting showed that larger females were courted significantly more often, regardless of the species origin of the male she was paired with (Figure 7b; Wald  $\chi^2_1 = 20.52, P = < 0.0001$ ). There was no significant effect of female weight on micro time, macro time, macro production, or total inseminated.

A summary of the frequency of occurrence of each stage of mating for all heterospecific pairs is shown in Table 3. The exact tests in Table 4(a) compared the mating propensities within vs. between species. All contrasts between conspecific pairs showed that there were no significant differences within populations in mating frequencies at any stage of mating between TAN<sub>M</sub> and either of the PAC populations. This means that any further differences in mating frequencies are the result of behavioral incompatibilities between the populations, rather than an artifact of different mating rates between populations.

The data from six heterospecific homotypic vs heterotypic controls were used to assess mating asymmetry and isolation between heterospecifics, and these are shown in Table 4(b-d). The exact tests showed that TAN<sub>M</sub> males (Table 4b; vs. PAC<sub>P</sub>: n=65,  $\chi^2=14.61$ , p<0.0001; vs PAC<sub>M</sub> n=65,  $\chi^2=31.77$ , p<0.0001) courted heterospecific females significantly less frequently than they did conspecific females. Both PAC<sub>M</sub> and PAC<sub>P</sub> males however showed only a non-significant trend to court TAN<sub>M</sub> females less frequently than conspecifics (Table 4b). At the stage of macro production, heterospecific pairings culminated in macros less frequently than did any conspecific pairings (Table 3; Table 4b). The total number mated, in all cases, showed the same frequency pattern as did the total number of macros produced; only in the PAC<sub>M</sub>TAN<sub>M</sub> pair was the proportion of macros passed significantly lower than any other combination. This pair is particularly interesting because courting and passing of micros occurs at a frequency indistinguishable from that of conspecific pairs (Figure 8), while macro production and passing occur at a frequency indistinguishable from other heterospecific pairs.

When testing for evidence of reproductive character displacement between the species by contrasting sympatric and allopatric heterospecific pairings ( $TAN_M PAC_P$  vs  $TAN_M PAC_M$ ,  $PAC_P TAN_M$  vs  $PAC_M TAN_M$ ) the analysis showed that  $TAN_M$  males courted significantly fewer sympatric  $PAC_M$  females than the allopatric  $PAC_P$  females (Table 4c;  $n=80$ ,  $\chi^2=6.24$ ,  $P<0.05$ ). This isolation was only apparent at the stage of courting. Both PAC populations courted  $TAN_M$  females with equal frequencies (see Table 4c for full summary). The  $I_{PSI}$  isolation indices in Table 5 also showed that at the stage of courting, there was significant isolation between  $TAN_M$  and  $PAC_M$  (Table 5:  $I_{PSI}=0.245$ ,  $p<0.01$ ) sympatric populations, but not between the  $TAN_M$  and  $PAC_P$  allopatric populations. At all further stages of mating, there was significant isolation between  $TAN_M$  and both PAC populations (Table 5).

Finally, male-female heterospecific mating asymmetry was measured using the exact tests shown in Table 4d; these indicated that of the two reciprocal heterospecific crosses only the  $PAC_M TAN_M$  pair showed any significant behavioral asymmetry, with the  $PAC_M TAN_M$  pair courting more frequently than the reciprocal  $TAN_M PAC_M$  pair (Table 4d:  $n=80$ ,  $\chi^2=27.65$ ,  $p<0.0001$ ). The  $IA_{PSI}$  asymmetry indices corroborated these results, also showing that the  $PAC_M TAN_M$  reciprocal comparison had marginally significant asymmetry (Table 6:  $n=80$ ,  $IA_{PSI}=1.27$ ,  $p=0.052$ ) at the stage of courting; no asymmetry was detected in any of the reciprocal comparisons at any of the other stages of mating.

## **DISCUSSION**



*L. tantalus* and *L. pacifica* coexist in sympatric populations, show evidence of mtDNA introgression, and yet still appear to maintain overall species integrity. In the laboratory, I paired conspecific and heterospecific individuals from three sympatric and allopatric populations and I compared mating frequency of all pair combinations in order to test for isolation and reproductive character displacement between these populations. In order to assess what factors might be contributing to behavioral isolation between these species, I estimated peak male calling times in lab and in the field, as well as average weights, average number of micros, timing of micro and macro production, and the rate of micro production in both species. Finally, I estimated the relationship between courting, micro production, and female weight within each population. Overall, I detected significant isolation between heterospecifics, an increase in spermatophore production for large females, and significant and asymmetric isolation between differentially-sized species pairs, all of which suggest a potential for conflicting selection pressures between species. I also found significantly different mating frequencies between sympatric and allopatric heterospecific pairs, as well as significant variation in micro number and timing of mating initiation between the *pacifica* populations, which shows that there is character displacement of mate characters and mate preferences within *pacifica*. These results overall demonstrate not only that these are two distinct behaviorally-isolated species, but also that this isolation is asymmetric and may be a novel example of a system in which male and female preferences are conflicting in such a way as to undermine diversifying selection.

### **Species differences**

I found that there were no interspecific differences in mating propensity, but there was significant isolation between the *tantalus* and *pacifica* species. Males from each species courted conspecifics significantly more frequently than heterospecifics, demonstrating that there is behavioral isolation between the two species. Both species also differ significantly in all measured phenotypic characteristics. *L. tantalus* has higher weight, produces fewer micros, has a later peak calling time, and starts micro and macro production later than either of the *pacifica* populations. These results were not surprising given that previous studies suggested that these are three populations of two distinct species (Shaw 2002; Parsons and Shaw 2001; Mendelson and Shaw 2005). However, this is the first time that patterns of behavioral isolation have been demonstrated.

It may be that these interspecific differences are integral to behavioral isolation between sympatric congeners. Body size, as discussed below, may be a barrier to mating between differentially-sized species (Nagata *et al.* 2007; Vigueira *et al.* 2008; Richmond and Jockusch, 2007), mating initiation and macro production may be timed for successful copulation (Brevault and Quilici 2000; Sakai and Ishida 2001), or diel differences in song production may be matched to the receptivity period of conspecific females (French and Cade 1987; Loher and Orsak 1985; Jacot *et al.* 2008). Likewise, if females are most responsive to micros during certain hours of the day, the difference in time of mating initiation could act as an isolating mechanism between the species (Danley *et al.* 2007). Additional results from this study, discussed below, suggest this hypothesis may be correct. However, to support this hypothesis, data on peak female receptivity is needed. An attempt was made at gathering such data, but it was

unsuccessful, and modifications of experimental design will be needed for future investigation.

### **Reproductive Character Displacement**

A comparison of the courting frequency of the allopatric *pacifica-tantalus* pairs to the sympatric *pacifica-tantalus* pairs revealed significantly fewer courtship attempts between the sympatric *tantalus* male-*pacifica* female pair than the allopatric pair, or either of the reciprocal *pacifica* male- *tantalus* female pairs (Table 3) . The hallmark of reproductive character displacement is greater discrimination against sympatric heterospecifics than allopatric heterospecifics. This results when individuals residing in sympatry mate with heterospecific individuals. If the matings incur a cost, either through a loss of energy, material resources, or hybrids that have decreased fitness, then theory predicts that selection indirectly causes divergence in mating characters or mate choice in the parental populations (Dobzhansky 1932; Howard 1993). The allopatric individuals not subjected to such selection will display ancestral mate preferences or characters. The results presented here are consistent with displacement of mate choice between the conspecific populations.

While “choice” is not traditionally considered a reproductive character in most studies of reproductive character displacement, it is a behavior (character) involved in reproduction, and several past studies have considered it as such (Gabor and Ryan, 2001; Gray and Cade, 2000; Gerhardt, 1994); we will also consider it a reproductive character here. Therefore there are two potential explanations for the displacement of choice that are worth considering. One hypothesis is that selection is acting on the preference

parameters of males. A previous study found that males produced significantly fewer micros on the second consecutive day of mating, and therefore it was hypothesized that mating in general, and micro production in particular, results in significant mating costs for *Laupala* males (J. Jadin, Ch.2). Assuming that heterospecific matings incur at least some fitness costs, one should therefore find reproductive character displacement affecting male choice. For example, since mating frequency displacement was significant only at initiation, the results make it appear that *tantalus* males are choosy. *L. tantalus* males could be choosing to court sympatric PAC<sub>M</sub> females less frequently than PAC<sub>P</sub> females due to displacement in female reproductive characters between sympatric and allopatric females. In the present study, no such character was identified, however, cuticular hydrocarbons are known to play an important role in mate identification and speciation in insects (Carde 1977; Coyne 1994; Ginzl 2003; Dietmann 2003), and have been found to be diverging in species of *Laupala* (Mullen *et al.* 2007). If female cuticular hydrocarbons in *pacifica* are ancestrally similar to those of *L. tantalus*, but have shifted away from the ancestral state in sympatry due to drift or selection, *tantalus* males may be discriminating against sympatric *pacifica* females. However, until a female character is identified and further testing is performed, this hypothesis cannot be confirmed.

An alternative explanation for decreased mating attempts between the sympatric heterospecifics is that selection is causing displacement of *pacifica* female choice. Displacement in female choice, rather than male choice, would be consistent with results from other previous studies reporting selection causing reproductive character displacement (Saetre *et al.* 1997; Coyne and Orr 1989, 1997; Noor 1995, 1997; Higgie *et al.* 2000; Nosil *et al.* 2003; Pfennig and Simovich 2002; Hoskin *et al.* 2005). Sexual

selection theory predicts that females have more to lose from an unsuccessful mating, resulting in selection on females to assortatively mate, thereby leaving choosy females and relatively indiscriminate males (Bateman 1948; Trivers 1972). Displacement of female choice is as likely as displacement of male choice in *Laupala*, and in fact, it could be mutual. Although micro production is apparently costly to males (J. Jadin, Ch.2), the costs of spermatophores may be insignificant relative to the costs of eggs. As such, the decrease in courting attempts between sympatric congeners may be the result of female discrimination against interested *tantalus* males.

The asymmetric courting frequency between reciprocal pairs of the *pacifica* populations provides further support for a hypothesis of displacement of female choice. The PAC<sub>M</sub>PAC<sub>P</sub> pair has a slightly, but significantly, higher courting frequency than the PAC<sub>P</sub>PAC<sub>M</sub> pair. If allopatric *pacifica* males have ancestral character states (Howard 1993), they may be similar to the heterospecific males. Selection for assortative mating in sympatric *pacifica* females would result in discrimination against allopatric *pacifica* males with ancestral mate characters. This would cause a pattern of increased discrimination against conspecific allopatric males similar to the pattern revealed here.

The differing variance in micro number and micro time between the *pacifica* populations is particularly interesting because this may be a male character that is undergoing displacement (Figure 3). There was decreased variance in both time and number of micros in the sympatric population, which is indicative of divergence as a result of selection against overlapping traits in sympatry. There is a strong positive relationship between earlier micro production and the number produced, so it is likely that decreased variance in micro number in sympatry is the result of decreased variance

in initiation time. Females of both species have peak receptivity periods, which the males may be attempting to match. Such a result has been found in other systems (French and Cade 1987; Loher and Orsak 1985; Jacot *et al.* 2008). It is difficult to distinguish whether such a shift is a result of selection directly on the male character or on female choice without data on the female receptivity period; however, this pattern provides most support for a hypothesis of female choice. This is because there is clear displacement of mate choice between the two *pacifica* populations, but a trait that may be important for mate choice decisions, is not yet significantly different. The decrease in variance in micro number alone suggests that this trait is in the process of catching up with the shift in choice (Lande and Arnold 1983; Grant and Grant 1989), and therefore, selection acting on female choice parameters and resulting in a subsequent shift in male characters.

### **Mating Investment and Asymmetry**

Finally, results of this study showed that there appears to be asymmetrical isolation between sympatric species. As noted above, when the mating frequency of male-female pairs of *tantalus* and *pacifica* were compared, results showed that PAC<sub>M</sub> males courted TAN<sub>M</sub> female more frequently than did the reciprocal pairing. In insects, larger females are frequently found to be more fecund (Honek 1993; Kazimirova 1996; Preziosi *et al.* 1996; Sokolovska *et al.*, 2000), have more eggs, and are therefore more preferred as mates (Fischer *et al.* 2000; Danielson-Francois *et al.* 2002; Rogers *et al.* 2006), because their partners will have higher fitness (Bateman 1948). As noted above, *Laupala* males behave in a similar fashion, producing more micros for larger females and courting large females more frequently (J. Jadin, Ch.2). Those results were replicated in the current

study, however, results here also show that the *tantalus* species is overall significantly larger than the *pacifica* species. The asymmetric courting pattern might therefore be due to male preference for large females that transcends species boundaries.

This is particularly interesting because it suggests a possible conflict between species recognition and mate quality selection: if males always prefer large females then interspecific gene flow from the smaller *pacifica* males-large *tantalus* females matings may counteract divergent selection. The likelihood of such a preference depends on the relative costs of courting for each sex. If males that had a strong preference for large females had significantly greater fitness than those with a weaker preference, and encounters with heterospecific females are either infrequent, or hybridization results in greater than zero fitness, then selection may not penalize males with strong female size preferences. We might instead expect that divergence would continue if female mistakes were costlier than those for males, or that a hybrid swarm would result if the male benefits of mating with a large heterospecific female outweighed any female costs.

It is important to consider that while asymmetry is primarily found at the stage of courting, there also appears to be a marginal degree of asymmetry at the final stage of passing (Table 4b). During the superficial stages of early courtship, all heterospecific pairs have a similar degree of isolation, while conspecific pairs do interbreed (Table 3; Figure 8)—excepting the  $PAC_M TAN_M$  pair, which courts at a similar frequency to conspecific pairs. At the stage of macro production occurs, this variation disappears (Figure 8). One hypothesis to explain this is that  $PAC_M$  males are courting the large  $TAN_M$  females in order to maximize fitness (Bateman 1948), but they desist once at the critical stage of gamete transfer. This may be because micros are relatively cheap to

produce, while macros may be more energetically costly (J. Jadin, Ch.2). Males may be hedging bets on a successful mating, but produce macros only when the female signals interest. During the *Laupala* courtship sequence, males appear to assess females: if a male passes a micro to a female and she rapidly removes it, he jumps and hits her with his legs (pers.obs.). Therefore, females that reject micros might trigger males to forgo macro production, resulting in a decrease in frequency of macro versus micro production.

This explanation may account for the drop in macro production, but still does not fully account for the asymmetry in passing success in the PAC<sub>M</sub>TAN<sub>M</sub> pair. To explain this pattern, I cautiously propose that in *Laupala*, selection on individuals to assortatively mate may affect each species and each sex differently, and it may depend upon the relative costs of failure for each stage of the mating. If allopatric *pacifica* retain characters that are similar to those of *tantalus* males and selection acts differentially on each stage of the mating process depending upon the relative cost of each stage, then we might predict that small sympatric *pacifica* males will vigorously court large *tantalus* females. However, selection acting on the critical stage of gamete transfer will result in *tantalus* females discriminating against sympatric *pacifica* males more strongly than allopatric males, who maintain ancestral mate characters (such as timing of micro production). Such a prediction corresponds with the pattern revealed by the PAC<sub>M</sub>TAN<sub>M</sub> pair. Strong male preference for large females may outweigh diversifying selection in the early stages of mating, resulting in *pacifica* males courting *tantalus* females. Females may not be decreasing their fitness substantially by accepting micros if acceptance does not cause a decrease in fertilization success. Microspermatophores may even be nutritious and provide direct benefits to females and/or their offspring (e.g. Simmons *et al.* 1999).



However, if heterospecific fertilization result in lower reproductive success, selection should act on females before gametes are transferred. Because the mating system of *Laupala* is complicated and allows selection to act at many stages, competing selection pressures can become entwined. The hypotheses put forth here begin to disentangle some of these processes, but should be taken as a guide for future studies rather than a definitive explanation.

## **Conclusions**

The results described here show that heterospecific matings can occur in the contact zone between the closely-related *tantalus* and *pacifica* species, but there is a clear difference in choice relative to areas outside the zone. Additionally, there is a clear decrease in variance in the number and initiation of micro production within, relative to outside, the zone. This pattern is consistent with a pattern of reproductive character displacement. It is likely that diversifying selection is acting on female choice in the *pacifica* species, causing displacement in male reproductive characters, but without further tests for displacement of female characters, it is difficult to rule out that the selection may instead be acting on male choice. Additionally, the pattern of mating frequency is asymmetric between species pairs, and may be the result of an innate male preference for larger females that transcends species boundaries.

These results overall suggest that mutual, and perhaps conflicting forms of selection are acting on *pacifica* and *tantalus* in their zone of contact: while females may ultimately be deciding whether or not a heterospecific mating will go to completion, males may also be choosing to mate with large females across species boundaries,

resulting in selection that conflicts with female choice. However, much more work is needed to clarify the results from this study. The *Laupala* mating system is elaborate and allows for selection at many stages, each of which may result in different costs being imposed upon the individual engaging in each stage. First, it will be necessary to thoroughly quantify the relative costs of mating for each sex. Second, a female size-controlled preference study is needed in order to categorically conclude that male body size preference also applies to heterospecific matings. And finally, in order to understand how speciation is being hindered or enhanced, it is necessary to quantify hybrid fitness. Such further studies will provide us with a better understanding of how reproductive costs in an unusual mating system can affect the direction and degree of evolution.

## TABLES

**Table 1.** A repeated measures analysis of variance that examines the effect of species, time of day, and the interaction of species and time of day on the number of sites with singing males of *L. tantalus* and *L. pacifica* at Manoa Cliffs on outdoor data. The p values were obtained from 1000 randomizations

effect	Df		F	p
	Numerator	Denominator		
Species	1	18	51.96	<0.0001
Time of day	8	18	4.63	0.007
Species*time of day	8	18	18.78	<0.0001

<b>Contrast estimates, <i>L. pacifica</i> vs. <i>L. tantalus</i></b>					
period	Estimate	Standard error	df	T	p
6am-11am	9.5	0.788	18	12.06	<0.0001
4pm-6pm	-6.5	1.364	18	-4.76	<0.0001

**Table 2.** A repeated measures analysis of variance that examines the effect of species, time of day, and the interaction of species and time of day on the number of sites with singing males of *L. tantalus* and *L. pacifica* at Manoa Cliffs, and *L. pacifica* at Pupukea, indoor data. The p values were obtained from 1000 randomizations of the data. Contrast estimates both compared number of singers singing at each designated peak time as well as periods throughout the day of highest activity.

effect	df		F	p			
	numerator	denominator					
Species	2	36	8.08	<0.0001			
Time of day	11	36	16.75	<0.0001			
Species*time of day	22	36	7.92	<0.0001			
<b>Contrast estimates</b>							
period	Contrast	species	Estimate	Standard error	df	T	p
12pm-3pm	# singers	PAC vs. TAN <sub>M</sub>	6.5	0.860	36	7.56	<0.0001
4pm-6pm		PAC vs. TAN <sub>M</sub>	-9.5	1.216	36	-7.40	<0.0001
12pm-3pm	peak period	PAC <sub>P</sub> vs PAC <sub>M</sub>	5.0	0.993	36	5.04	<0.0001
4pm-6pm		TAN <sub>M</sub>	9.5	1.0372	36	9.16	<0.0001
12pm-3pm		PAC <sub>P</sub>	9.6	1.5384	36	6.24	<0.0001
12pm-3pm		PAC <sub>M</sub>	18.9	1.5384	36	12.29	<0.0001

**Table 3.** Male and female population and species, sample size (n), number and proportion of males producing  $\geq 1$  micros, means  $\pm$  sd of micros produced (out of those that completed mating), number and proportion of males successfully passing  $\geq 1$  micros, number and proportion of all males that produced macros, number and proportion of those macros successfully transferred, and percent out of total sample that successfully mated (i.e. produced and transferred macro). Out of those males that completed a mating, they produced a similar number (n) of micros for any female, no matter what the population of origin of the female.

<i>Type</i>	<i>Male Pop</i>	<i>Fem Pop</i>	<i>n</i>	$\geq 1$ micros (% n)	<i>mean</i> <i>±s.d.</i>	$\geq 1$ passed (% of micro)	$\geq 1$ passed (% n)	<i>macros</i> (% n)	<i>passed</i> (% of macro)	<i>total mated</i> (% n)
<b>Inter</b>	TAN <sub>M</sub>	PAC <sub>M</sub>	<b>40</b>	16 40%	5.1 $\pm$ 1.10	14 87.5%	35.0%	10 25.0%	10 100%	<b>25.0%</b>
<b>Inter</b>	TAN <sub>M</sub>	PAC <sub>P</sub>	<b>40</b>	27 67.5%	4.4 $\pm$ 1.61	20 74.1%	50.0%	13 32.5%	13 100%	<b>32.5%</b>
<b>Inter</b>	PAC <sub>M</sub>	TAN <sub>M</sub>	<b>40</b>	37 92.5%	7.8 $\pm$ 3.36	33 89.2%	82.5%	14 35%	9 64.3%	<b>22.5%</b>
<b>Inter</b>	PAC <sub>P</sub>	TAN <sub>M</sub>	<b>37</b>	28 75.7%	7.7 $\pm$ 2.11	22 78.6%	59.5%	10 27.0%	10 100%	<b>27.0%</b>
<b>Intra</b>	PAC <sub>M</sub>	PAC <sub>P</sub>	<b>40</b>	40 100%	8.5 $\pm$ 1.58	37 92.5%	92.5%	29 72.5%	28 96.6%	<b>70.0%</b>
<b>Intra</b>	PAC <sub>P</sub>	PAC <sub>M</sub>	<b>35</b>	30 85.7%	9.3 $\pm$ 1.33	27 90.0%	77.1%	24 68.6%	24 100%	<b>68.6%</b>
<b>Control</b>	TAN <sub>M</sub>	TAN <sub>M</sub>	<b>25</b>	25 100%	4.8 $\pm$ 1.20	24 96.0%	96.0%	17 68.0%	17 100%	<b>68.0%</b>
<b>Control</b>	PAC <sub>M</sub>	PAC <sub>M</sub>	<b>40</b>	40 100%	8.2 $\pm$ 1.94	36 90.0%	36.0%	31 77.5%	29 93.5%	<b>72.5%</b>
<b>Control</b>	PAC <sub>P</sub>	PAC <sub>P</sub>	<b>36</b>	34 94.4%	8.2 $\pm$ 3.21	30 88.2%	83.3%	23 63.9%	21 91.3%	<b>58.3%</b>

**Table 4.** Summary of results for *L. tantalus* / *L. pacifica* mating frequency by comparison of frequencies of cross-population matings. The two population crosses being compared, the sample size, the likelihood ratio  $\chi^2$  values from randomized  $\chi^2$  tests are shown below. The “passed mics” and “passed macs” proportions used were “%of mic” and “%of mac” values from Table 1. All of the pass data contained at least one cell in the comparison that contained a count <5. Because of this, a  $\chi^2$  test was not appropriate and a Fisher’s exact test was used instead; therefore, only a p value is displayed. A sequential Bonferroni correction for 6, 3, 3, or 2 relevant comparisons was applied to all of the p values within categories (asymmetry control, interspecies control, reciprocal comparisons, or interspecies comparisons) obtained with the Fisher’s exact test. A \* denotes significance at the <0.05 level, \*\* denotes significance at the < 0.001 level.

male X fem	male X fem	N	Courted ( $\chi^2$ )	Passed Micros (p)	Macros Produced ( $\chi^2$ )	Passed Macros (p)	Mated ( $\chi^2$ )
a) Interpopulation controls							
PAC <sub>P</sub> X PAC <sub>P</sub>	PAC <sub>M</sub> X PAC <sub>M</sub>	80	3.05	1	1.710	1	1.693
PAC <sub>M</sub> X PAC <sub>M</sub>	TAN <sub>M</sub> X TAN <sub>M</sub>	65	0	0.641	0.709	0.533	0.150
TAN <sub>M</sub> X TAN <sub>M</sub>	PAC <sub>P</sub> X PAC <sub>P</sub>	80	2.16	0.384	0.111	0.499	0.592
b) Heterotypic controls							
PAC <sub>M</sub> X PAC <sub>M</sub>	PAC <sub>M</sub> X TAN <sub>M</sub>	80	4.20	1	<b>15.99**</b>	<b>0.02*</b>	<b>21.00**</b>
PAC <sub>M</sub> X PAC <sub>M</sub>	PAC <sub>M</sub> X PAC <sub>P</sub>	80	0	0.72	0.48	1	0.06
TAN <sub>M</sub> X TAN <sub>M</sub>	TAN <sub>M</sub> X PAC <sub>P</sub>	65	<b>14.61**</b>	0.06	<b>7.93*</b>	1	<b>7.93*</b>
TAN <sub>M</sub> X TAN <sub>M</sub>	TAN <sub>M</sub> X PAC <sub>M</sub>	65	<b>31.77**</b>	0.55	<b>10.90*</b>	0.21	<b>11.91**</b>
PAC <sub>P</sub> X PAC <sub>P</sub>	PAC <sub>P</sub> X TAN <sub>M</sub>	73	5.39	0.33	<b>10.25**</b>	1	<b>7.453*</b>
PAC <sub>P</sub> X PAC <sub>P</sub>	PAC <sub>P</sub> X PAC <sub>M</sub>	71	1.56	1	0.17	0.23	0.80
c) Interspecific pairings							
TAN <sub>M</sub> X PAC <sub>P</sub>	TAN <sub>M</sub> X PAC <sub>M</sub>	80	<b>6.24*</b>	0.446	0.36	1	0.55
PAC <sub>P</sub> X TAN <sub>M</sub>	PAC <sub>M</sub> X TAN <sub>M</sub>	77	4.46	0.306	0.47	0.053	0.21
d) Reciprocal pairings							
PAC <sub>P</sub> X TAN <sub>M</sub>	TAN <sub>M</sub> X PAC <sub>P</sub>	77	0.10	0.758	0.276	1	0.276
TAN <sub>M</sub> X PAC <sub>M</sub>	PAC <sub>M</sub> X TAN <sub>M</sub>	80	<b>27.65**</b>	1	0.815	0.495	0.069
PAC <sub>M</sub> X PAC <sub>P</sub>	PAC <sub>P</sub> X PAC <sub>M</sub>	75	<b>8.17*</b>	1	0.139	1	0.018

**Table 5.** Between population  $I_{PSI}$  estimates of sexual isolation at each step of a mating.  $I_{PSI}$  and one standard deviation given. The first column of variables compares the proportion of males, out of the total sample, that produced at least one micro. The second column represents the proportion of those which made micros and subsequently passed, whereas the third column represents the proportion of the total sample which passed a micro. The same explanation applies to the macro calculation in the next two columns. The final column shows the total proportion of males that successfully mated (i.e. produced and transferred a macro). Because JMating requires equal sample sizes, all proportions were estimated as a percentage of the lowest common sample size, which for total  $n=25$ , for total producing micros=16, and total producing macros=10. An \* denotes significance at  $<0.01$ , and \*\* denotes significance at  $<0.001$ .

$I_{PSI}$	<b>%micros made</b>	<b>%micro pass</b>	<b>%macros made</b>	<b>%macro pass</b>	<b>%total mated</b>
TAN <sub>M</sub> PAC <sub>P</sub>	0.155 ±0.07	0.094± 0.14	<b>0.427± 0.13**</b>	-0.024± 0.17	<b>0.369± 0.14*</b>
TAN <sub>M</sub> PAC <sub>M</sub>	<b>0.245 ±0.11*</b>	0.026± 0.14	<b>0.382± 0.14**</b>	0.097± 0.18	<b>0.498± 0.13**</b>
PAC <sub>P</sub> PAC <sub>M</sub>	0.0213 ±0.10	0.027± 0.14	-0.003± 0.12	-0.035± 0.17	-0.031± 0.12

**Table 6.** The Indexes of asymmetry ( $IA_{PSI}$ ) coefficients are shown below.  $IA_{PSI}$  coefficients are calculated using ratios from PSI pairwise coefficients. 1000 bootstrap replicates were performed on the data to generate the coefficient and the p value. The lowest common sample size, of 25 individuals for the  $TAN_M \times TAN_M$  cross was used for all analyses; all other populations were adjusted for sample sizes of 25. The † denotes marginal significance ( $p = 0.0516$ ) when a sample size of 25, the minimum common sample size, was used.

male X fem	male X fem	N	Micros $IA_{PSI}$ $\pm S.D.$	Passed micros $IA_{PSI}$ $\pm S.D.$	Macros $IA_{PSI}$ $\pm S.D.$	Passed macros $IA_{PSI}$ $\pm S.D.$	Total mated $IA_{PSI}$ $\pm S.D.$
Interspecific controls							
$PAC_P \times PAC_P$	$PAC_M \times PAC_M$	80	1.00 $\pm$ 0.03	1.00 $\pm$ 0.07	1.01 $\pm$ 0.06	1.00 $\pm$ 0.12	0.99 $\pm$ 0.05
$PAC_M \times PAC_M$	$TAN_M \times TAN_M$	65	1.00 $\pm$ 0.07	1.00 $\pm$ 0.07	1.023 $\pm$ 0.16	1.00 $\pm$ 0.10	1.04 $\pm$ 0.18
$TAN_M \times TAN_M$	$PAC_P \times PAC_P$	80	0.99 $\pm$ 0.05	1.00 $\pm$ 0.07	0.99 $\pm$ 0.14	1.01 $\pm$ 0.12	0.96 $\pm$ 0.13
Reciprocal pairings							
$PAC_P \times TAN_M$	$TAN_M \times PAC_P$	77	0.99 $\pm$ 0.07	1.01 $\pm$ 0.12	0.95 $\pm$ 0.29	1.00 $\pm$ 0.09	0.29 $\pm$ 0.69
$TAN_M \times PAC_M$	$PAC_M \times TAN_M$	80	<b>1.27<math>\pm</math>0.27</b> †	1.00 $\pm$ 0.07	1.25 $\pm$ 0.50	0.95 $\pm$ 0.15	0.48 $\pm$ 0.89
$PAC_M \times PAC_P$	$PAC_P \times PAC_M$	75	1.00 $\pm$ 0.04	1.01 $\pm$ 0.10	1.00 $\pm$ 0.05	1.00 $\pm$ 0.10	0.04 $\pm$ 0.89



## FIGURE LEGENDS

**Figure 1.** Populations (island of Oahu) used in this study. The circle indicates the sample site for the allopatric *L. pacifica* population (PAC<sub>P</sub>) and the square is sample site for the sympatric *L. pacifica*/*L. tantalus* population (PAC<sub>M</sub> and TAN<sub>M</sub>). The dashed line indicated approximate distribution of *L. pacifica*, and the dotted line represents the approximate *L. tantalus* distribution as described by Otte (1994).

**Figure 2.** a) Classical scheme for testing reproductive character displacement. Green matings are controls, green/red matings are experimental. The null hypothesis is that all species come from a common ancestor. If reinforcing selection is at work in the contact zone, then within the test species (in this case *L. pacifica*), the sympatric individuals will have the derived characteristics as a result of secondary contact with *L. tantalus*. The assumption is that the *sympatric* female and the heterospecific male will mate less frequently than the allopatric female and heterospecific male because of selection for discrimination in sympatry.

b) In this study these comparisons will also be tested from the male choice perspective, i.e. female heterospecifics (*L. tantalus*) will be mated to sympatric PAC<sub>M</sub> and allopatric PAC<sub>P</sub> *L. pacifica* males. Because previous research in *Laupala* has found that male mating is costly, an *a priori* hypothesis in this study was that male choice might be playing a role in reinforcement. Therefore, trials in both directions may help determine which sex is experiencing reproductive character displacement.

**Figure 3.** Population phenotype differences. The box plots show the median, the 5<sup>th</sup> and 95<sup>th</sup> percentiles, and the minima and maxima. The mean  $\pm 1$  S.D. is shown below each box; the sample size for each population is shown below the x-axis. All characters except male and female weight were analyzed based upon within population matings only so that the results would not be confounded by mating isolation effects. All variables were then compared across populations using one-way ANOVA randomization tests. All models had significant F values (in text above). Tukey-adjusted least squares means contrasts were performed between the *pacifica* and *tantalus* species; the t-value for that contrast is given in the upper left corner of each graph. For all variables, TAN<sub>M</sub> was significantly different from each *pacifica* population, but neither PAC<sub>M</sub> nor PAC<sub>P</sub> was significantly different from the other. However, both mating initiation (Levene's  $F_{1,74}=10.20$ ,  $P=0.0021$ ) and number of micros (Levene's  $F_{1,74}=5.18$ ,  $P=0.0258$ ) showed significantly different variance between the PAC populations.

**Figure 4.** Diel pattern of male calling behavior of sympatric PAC<sub>M</sub> (dotted line) and TAN<sub>M</sub> (solid line) at Manoa Cliffs trailhead, Oahu. The graph depicts number of sites with singing males plotted against time.

**Figure 5.** Diel pattern of male calling behavior of sympatric PAC<sub>M</sub> (dotted line) and TAN<sub>M</sub> (solid line) and allopatric PAC<sub>P</sub> (dashed line). The graph depicts number of singing males plotted against time.

**Figure 6.** Relationship between micro time and number of micros produced for TAN<sub>M</sub> (triangles), PAC<sub>M</sub> (filled circles) and PAC<sub>P</sub> (open circles). Only matings that resulted in the production of a macro were used for the analysis. The solid line represents the simple linear regression of micro number on time of mating initiation. Regressions were significant within each population as well, however, because there is a significant difference in initiation time between the species, an ANOVA was used instead to evaluate the overall pattern among populations. It showed that males that started mating earlier produced significantly more micros ( $F_{1,165}=190.24$ ,  $P<0.0001$ ).

**Figure 7. a)** Relationship between conspecific female weight and number of micros produced for TAN<sub>M</sub> (triangles and solid line), PAC<sub>M</sub> (circles and dotted line) and PAC<sub>P</sub> (squares and dashed line). The solid line represents the simple linear regression of micro number on female weight. Males of all populations produced significantly more micros for large females within populations (ANOVA TAN<sub>M</sub>:  $R^2=0.22$ ,  $F_{1,23}=6.55$ ,  $p=0.0168$ ; PAC<sub>P</sub>:  $R^2=0.35$ ,  $F_{1,34}=18.64$ ,  $p<0.0001$ ; PAC<sub>M</sub>:  $R^2=0.21$ ,  $F_{1,38}=10.32$ ,  $p=0.0036$ ).

**b)** Box plot diagram depicting relationship between female weight and courting, paired by female population of origin. Because nearly 100% of homotypic pairings resulted in courtship, those pairs were removed from the analysis, and the graph below represents only results from heterotypic pairings. Overall, a logistic regression showed that female weight predicted whether or not she would be courted Wald  $\chi^2_1=20.52$ ,  $P=<0.0001$ ).

**Figure 8.** Comparison of mating stages between all combinations of species pairs. The x-axis shows stage of the mating sequence as percentage of total paired, not percentage of

total, the y-axis represents frequency as percentage of time each stage of the mating sequence was successful. On the left side of the graph, 5 of the 6 lines in the top group are conspecific mating pairs. However, the thick dashed line represents the heterospecific  $PAC_M$  male- $TAN_M$  female pair. This pair initiates mating (produces a micro) and passes micros at the same frequency as do conspecific pairs. However, once the stage of gamete transfer (macro production and transfer) is reached, it drops down to, and in fact below, heterospecific levels.

**FIGURES**

**Figure 1**

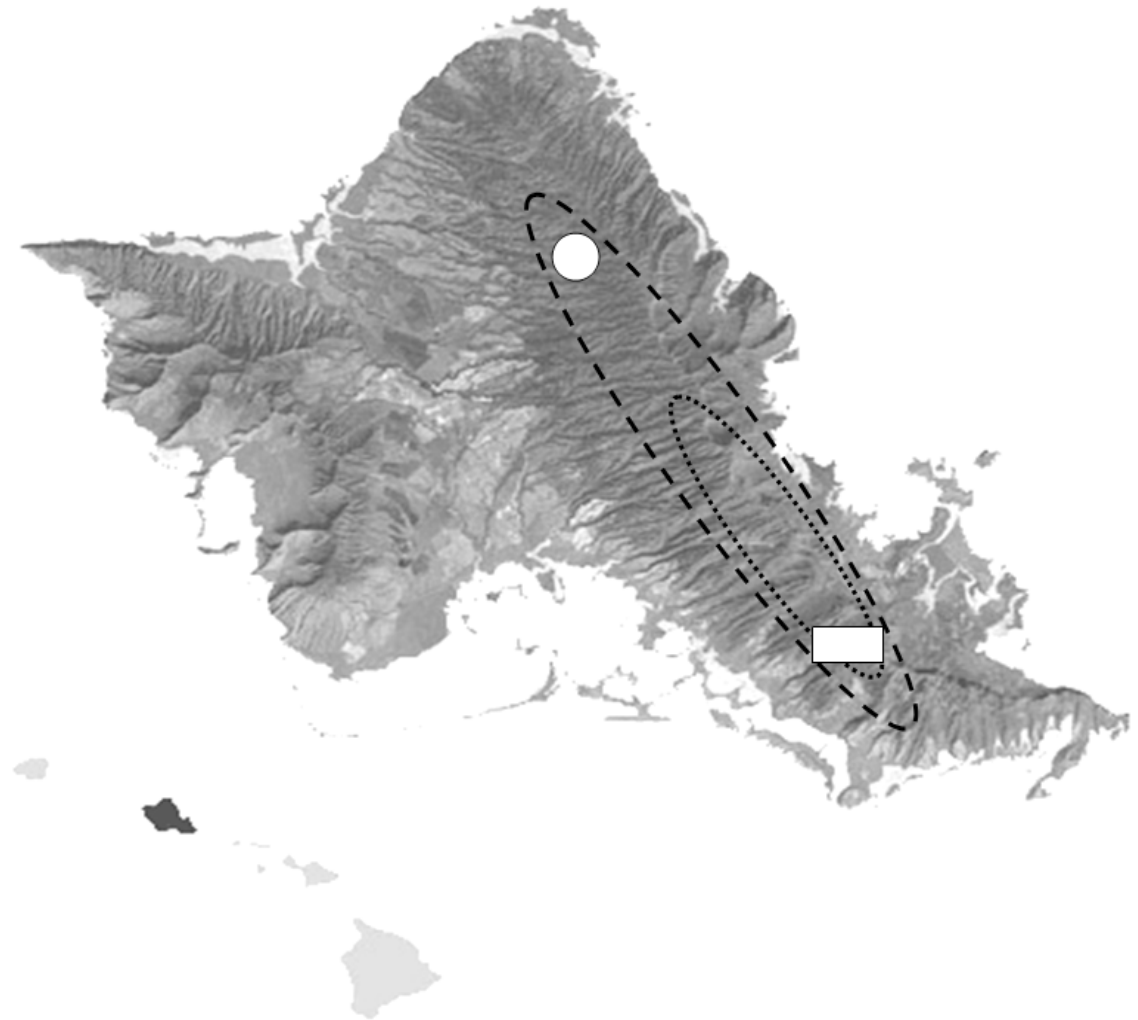
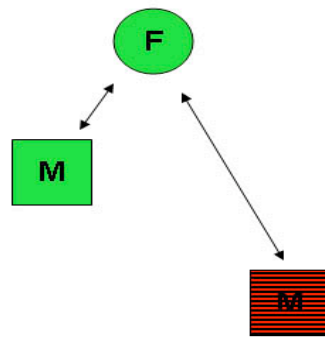
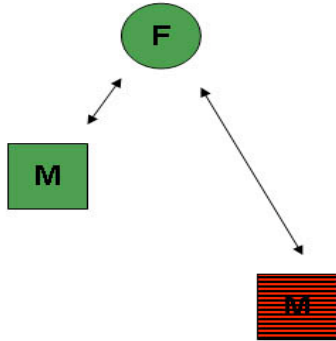


Figure 2

a)

sympatric *L. pacifica*

allopatric *L. pacifica*

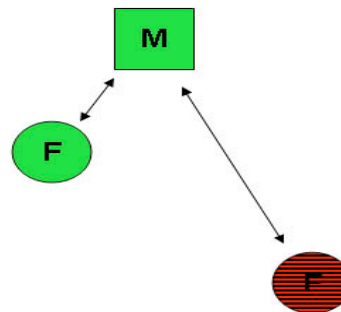
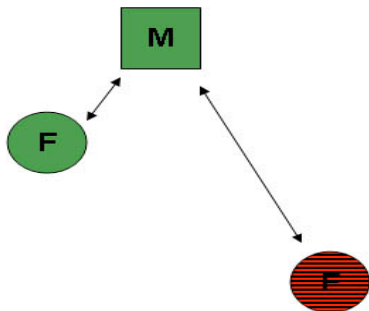


 = *L. tantalus* male (sympatry)

b)

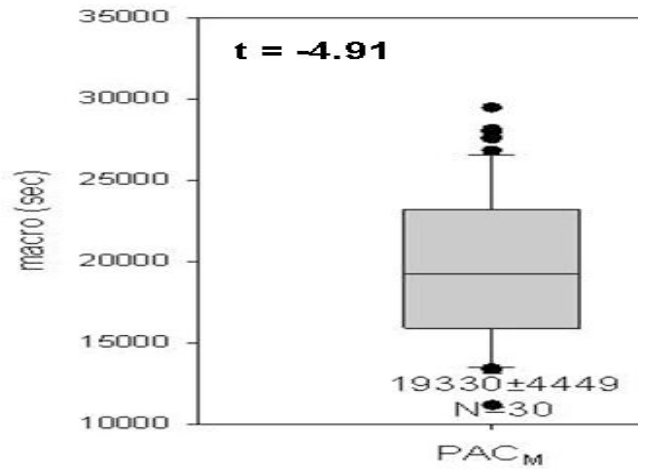
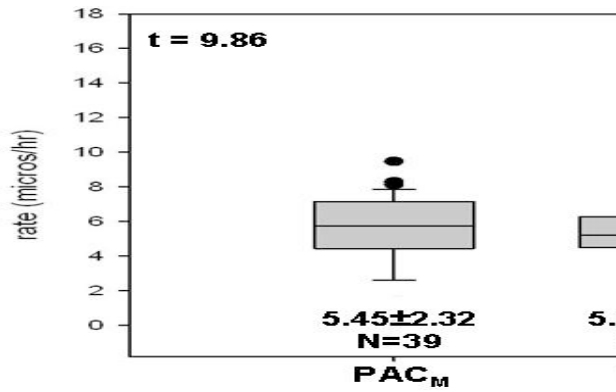
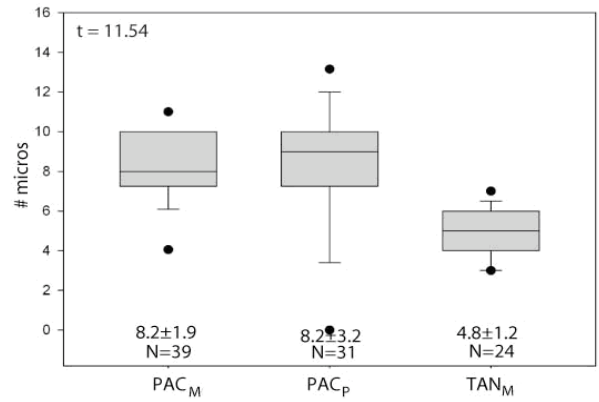
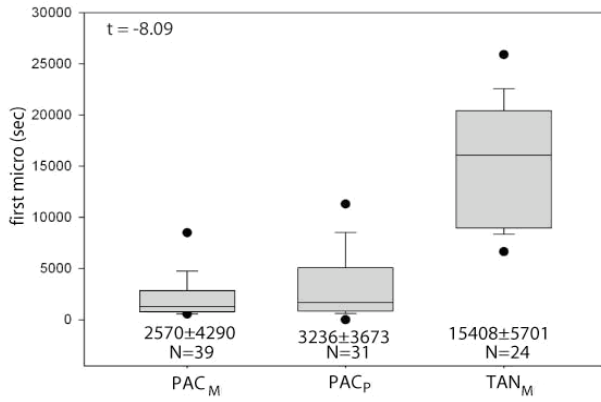
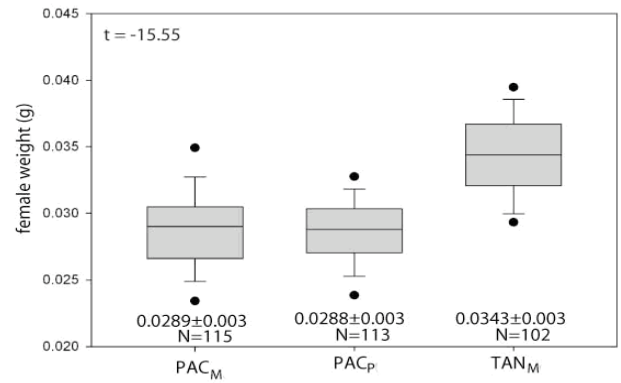
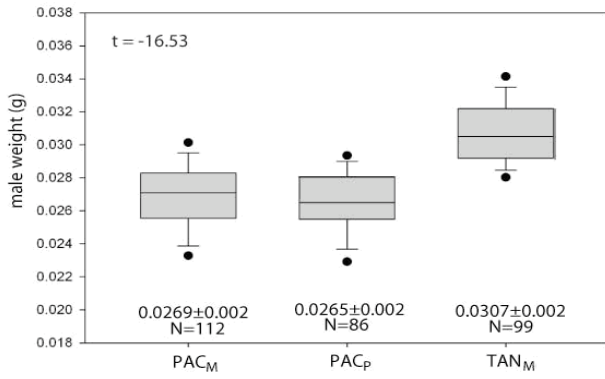
sympatric *L. pacifica*

allopatric *L. pacifica*

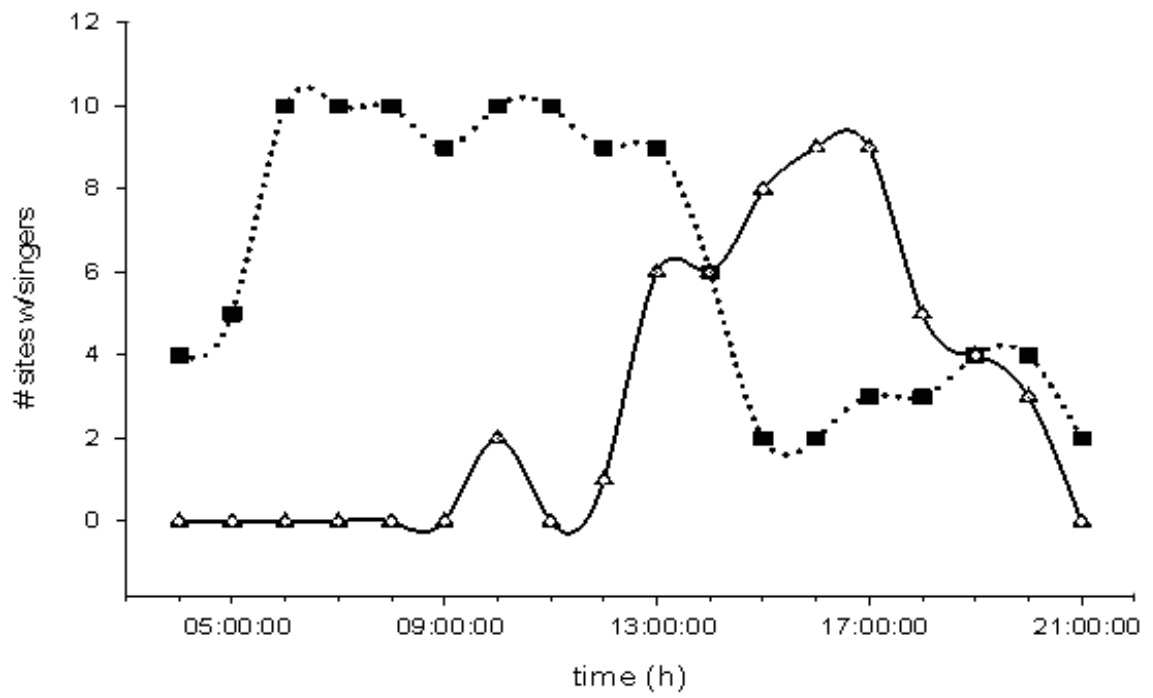


 = *L. tantalus* female (sympatry)

**Figure 3**



**Figure 4**





**Figure 5**

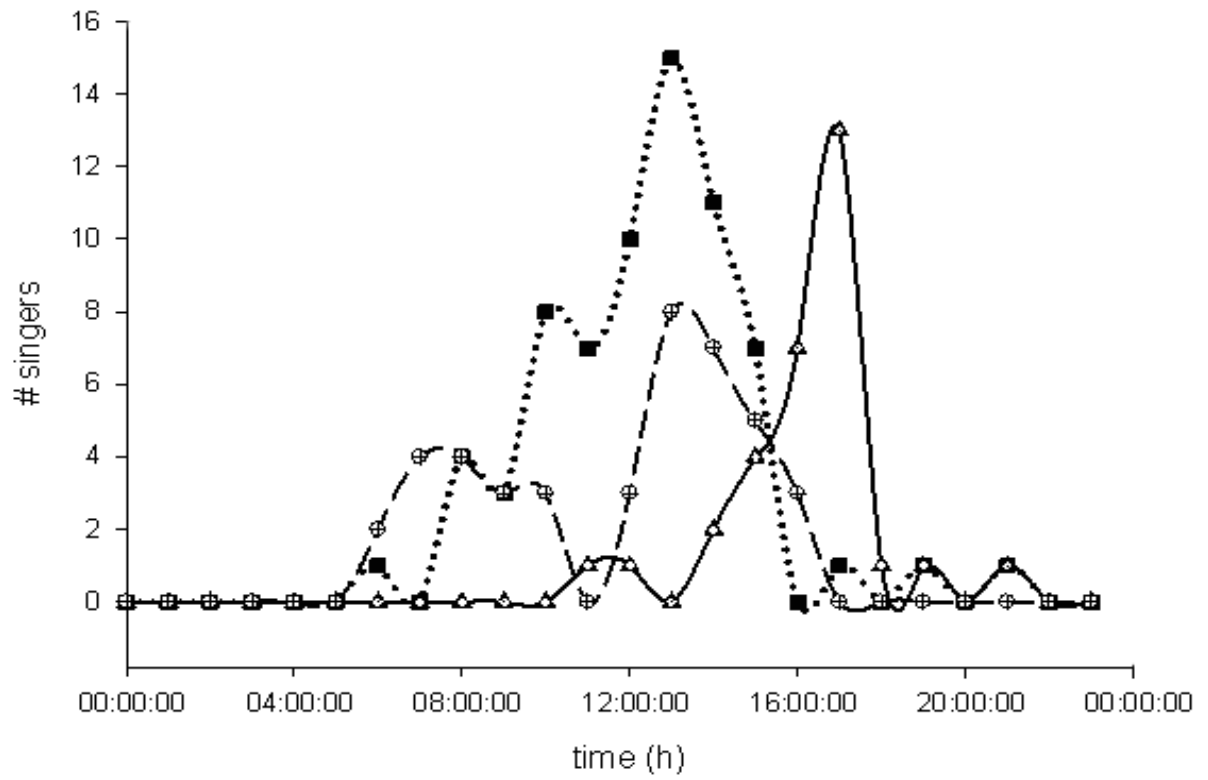
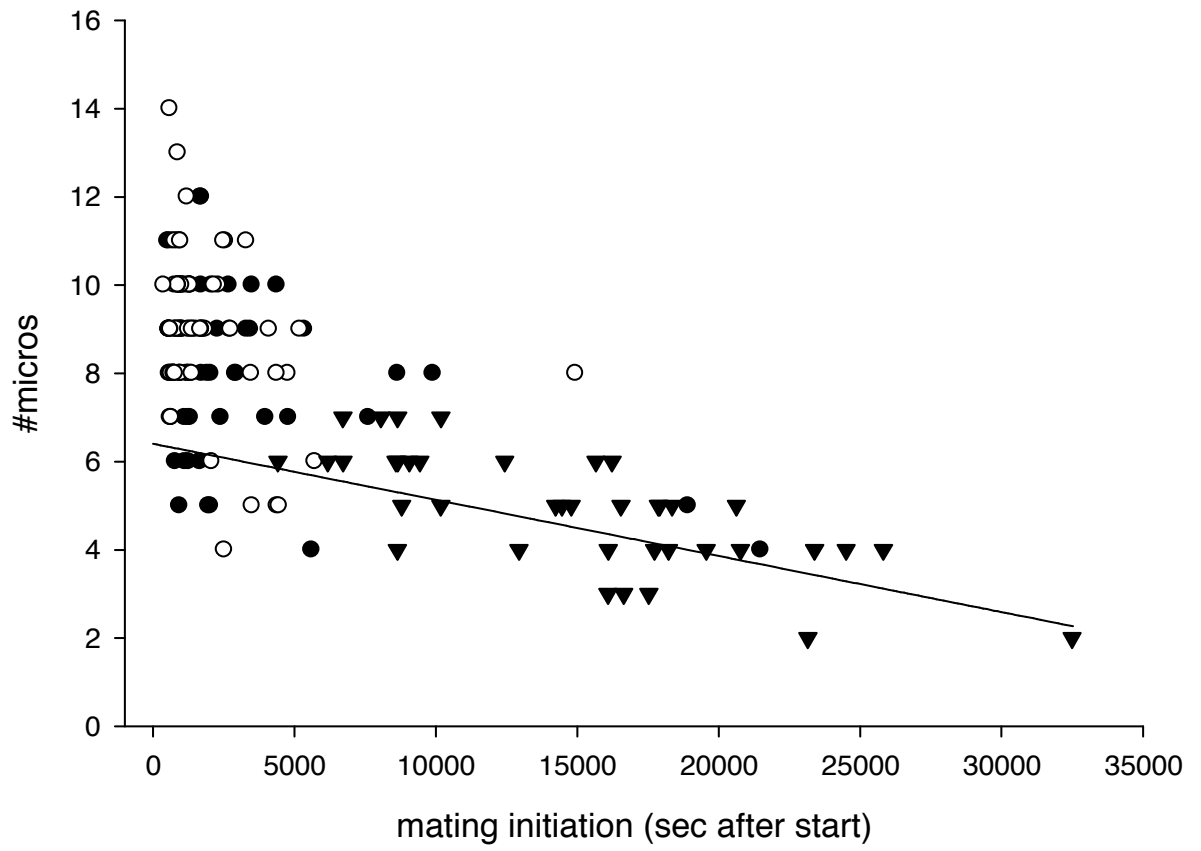
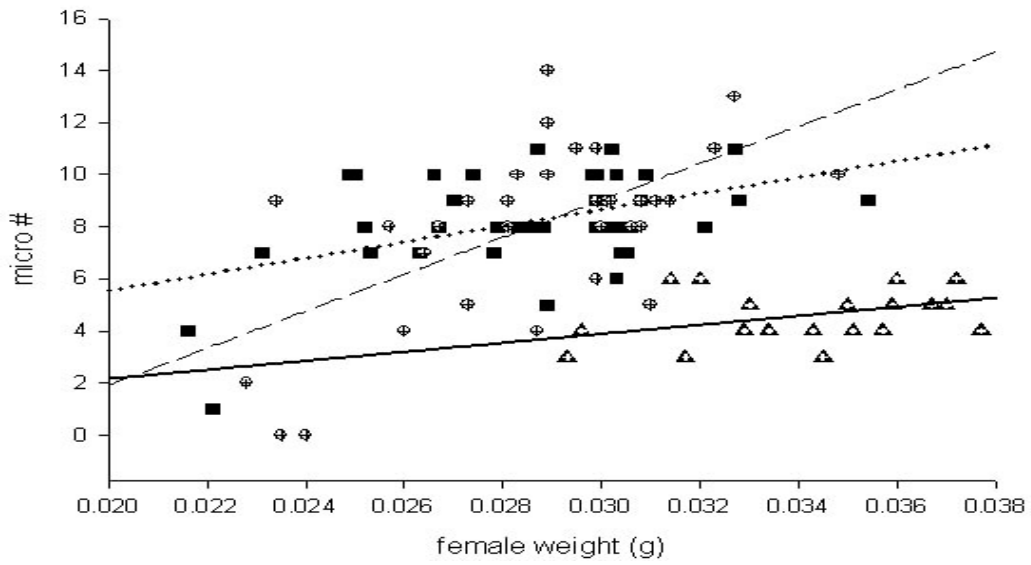


Figure 6



**Figure 7**  
**a)**



**b)**

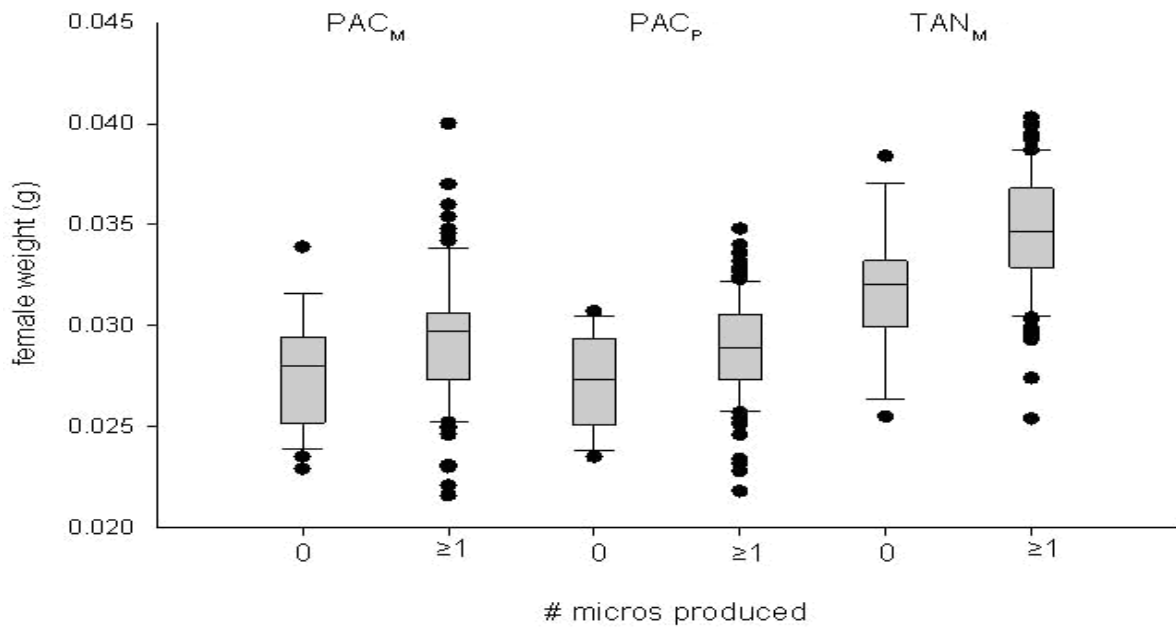
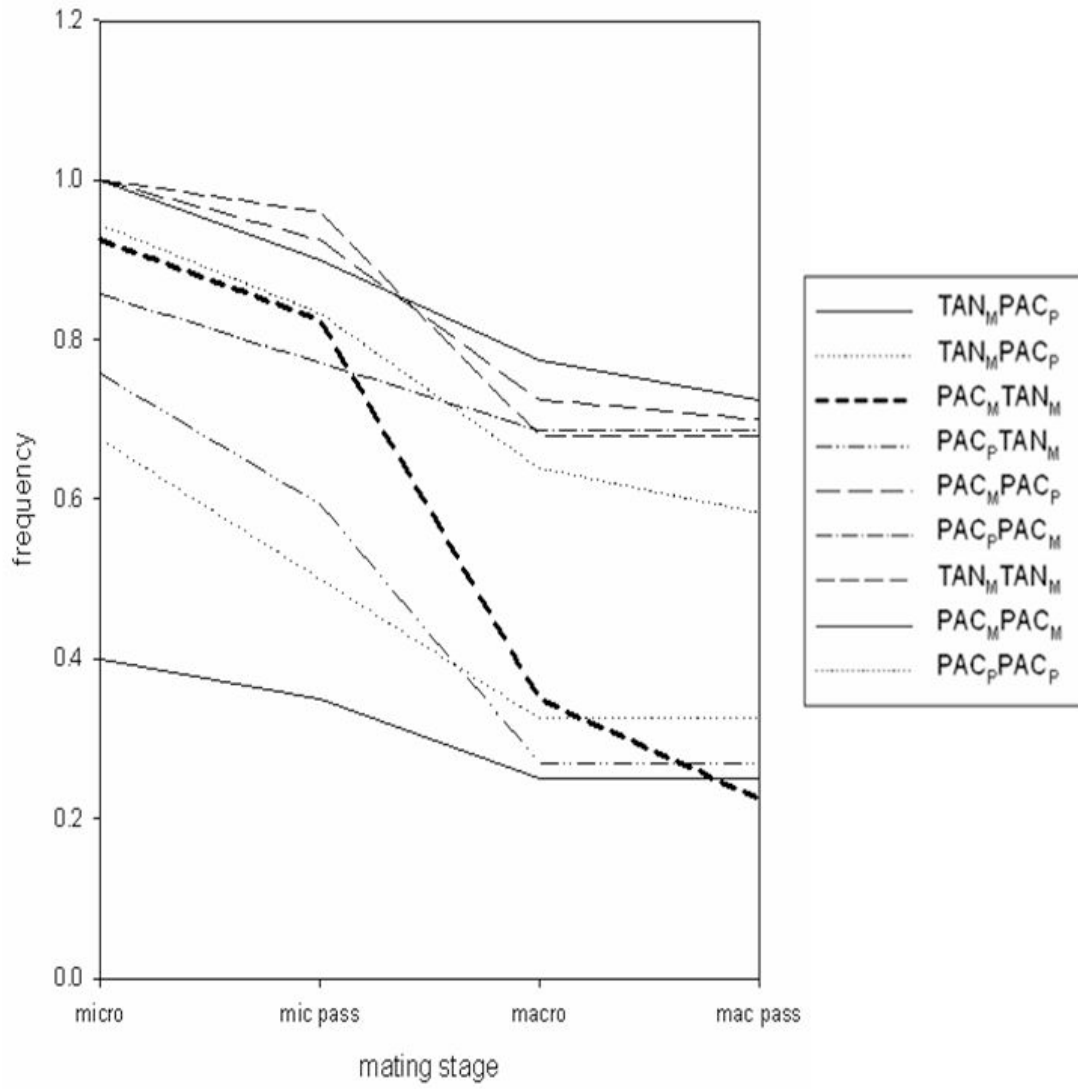


Figure 8



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