ABSTRACT

Title of Document: THE ADAPTIVE SIGNIFICANCE AND PREVALENCE OF COURTSHIP FEEDING BEHAVIOR IN HAWAIIAN SWORDTAIL CRICKETS

Tagide deCarvalho, Doctor of Philosophy, 2008

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Males of many insect species feed their partner during courtship and mating. Studies of male nutrient donation in various systems have established that nuptial feeding has evolved mostly through sexual selection. Although there is extensive diversity in form, the function of nuptial gifts is typically limited to either facilitating copulation or increasing ejaculate transfer, depending on the time at which the gift is consumed by females. Unlike other insects, the Hawaiian swordtail cricket *Laupala* (Gryllidae: Trigonidiinae) exhibits serial transfer of nuptial gifts. Males transfer multiple spermless 'micro' spermatophores over several hours before mating at the end of the day (i.e. before the transfer of a single sperm-containing 'macro' spermatophore). By experimental manipulation of male microspermatophore donation, I tested several hypotheses pertaining to the adaptive significance of nuptial gifts in this system. I found that microspermatophore transfer improves insemination, by causing the female reproductive tract to take in more sperm. This result reveals a previously undocumented function for premating nuptial gift donation among insects. Enhanced sperm transfer due to

microspermatophore donation may represent male manipulation or an internal mechanism of post-copulatory choice by females. I also performed experimental manipulation of male photoperiod to investigate how time and gender influence nuptial gift production and mating behavior. I found that the timing of mating is limited in males but not females and that the time of pair formation has consequences for the degree of nuptial gift donation, which suggests that both mating timing and microspermatophore number is important for male reproductive success. Finally, I observed the mating behavior of several trigonidiine taxa for a comparative analysis of sexual behavior and found that other genera also utilize spermless microspermatophores, which suggests that microspermatophore donation may be a common nuptial gift strategy among swordtail crickets. The elaborate nuptial feeding behavior of Hawaiian swordtail crickets prior to mating represents a newly discovered strategy to increase male insemination success rather than mating success. Based on this unexpected result, it is worth exploring whether courtship behaviors in other cricket or insect mating systems have also evolved to increase sperm uptake.

THE ADAPTIVE SIGNIFICANCE AND PREVALENCE OF COURTSHIP FEEDING BEHAVIOR IN HAWAIIAN SWORDTAIL CRICKETS

By

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Preface

This dissertation contains a single introduction section and three chapters. Each chapter is presented in manuscript form, with abstract, introduction, methods, results, and discussion, followed by tables, figure legends, and figures. A single bibliography section occurs at the end for references cited throughout the dissertation.

Dedication

To my grandfather, Dr. Sergio deCarvalho, who started me on the path of scientific inquiry and inspired me to pursue this degree.

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INTRODUCTION

Males of many insects provide food to females during courtship and mating. Nuptial gifts encompass any form of male nutrient donation that females consume before, during or after copulation. Insects exhibit a remarkable diversity of nuptial gifts, which include glandular secretions, salivary masses, regurgitated food, sexual cannibalism, prey items, seminal products and spermatophores (reviewed in Thornhill, 1976b; Vahed, 1998). Because nuptial feeding involves the transfer of nutrients, nuptial gifts have been hypothesized to be a form of paternal investment that increases the fitness of the donating male's offspring (Thornhill, 1976b; Trivers, 1972). However, most studies have demonstrated that sexual selection rather than natural selection is the primary selective pressure responsible for male nuptial feeding adaptations, which result in greater mating or fertilization success for the donating male (reviewed in Vahed 1998).

Depending on the timing of nuptial gift donation, males experience sexual selection either in the context of premating or post-copulatory female choice. Nuptial gifts provided during courtship have been hypothesized to increase male mating success by attracting females (e.g. Thornhill, 1976b). For example, in hangingflies, males provide captured prey to females during courtship and females were more likely to mate with males that present larger or more palatable food gifts (Thornhill, 1976a). In other systems, nuptial feeding appears to maintain the female in a position which improves the ability of the males to initiate copulation. For example, in the fruit fly *Drosophila subobscura*, males provide a regurgitated drop of food which

females consume while the male begins mounting (Steele, 1986). Males that were experimentally restricted from providing this nuptial gift suffered reduced mating success. Support for the mating success hypothesis has been demonstrated for a number of other nuptial gift systems, such as edible hind-wings in Orthoptera (Eggert and Sakaluk, 1994) and cephalic glands in Coleoptera (Eisner et al., 1996) and Zoraptera (Choe, 1995).

Nuptial gifts provided during or after copulation have been hypothesized to increase fertilization success through increased sperm transfer. In insects, females are able to discriminate against males after mating by limiting copulation duration (Thornhill 1976b), removing spermatophores (Sakaluk 1984; Simmons 1986) and manipulating sperm within the reproductive tract (Ward 1993; Eberhard 1996). Nuptial feeding adaptations such as nuptial prey in Mecoptera (Thornhill, 1976a) and Diptera (Svensson et al., 1990) have been demonstrated to increase copulation duration, while various forms of nuptial gifts delay premature removal of external spermatophores in Orthoptera (Sakaluk, 1984; Simmons and Gwynne, 1991; Wedell and Arak, 1989). By increasing the quantity of sperm and/or ejaculate substances that alter female remating or oviposition behavior, males are able to gain an advantage in sperm competition if females multiply mate.

While donating males benefit from increased mating or fertilization success, choosy females have been predicted to benefit directly by obtaining nutrition or indirectly through advantageous genes for their offspring (Andersson, 1994). Support for the direct benefits hypothesis has been demonstrated in many taxa (recent review in Gwynne, 2008). Less evidence exists for the indirect benefits hypothesis; however

one robust example is the scorpionfly *Panorpa vulgaris.* In this species, males that were able to provide nuptial gifts sired offspring with superior fighting ability than the offspring of males which could not provide nuptial gifts (Thornhill and Sauer, 1992).

An alternative hypothesis is that males that provide nuptial gifts may be exploiting a gustatory response by females, wherein females do not benefit from their preference (Sakaluk, 2000). Support for the sensory exploitation model of female choice has been demonstrated in a non-gift-giving species of cricket, *Acheta domesticus*. *A. domesticus* females accepted an extra-spermatophore component from the gift-giving species, *Gryllodes sigillatus,* which consequently altered *A. domesticus* female remating behavior in a manner that would reduce sperm competition for a donating male (Sakaluk, 2000).

My dissertation is a study of the evolution and adaptive significance of nuptial feeding in Hawaiian swordtail crickets (Gryllidae: Trigonidiinae). Males in the genus *Laupala* exhibit elaborate nuptial feeding behavior, which differs in several aspects from other insect mating systems, including other crickets. Males transfer a series of spermless 'micro' spermatophores via copulation, which are consumed by females. Protracted donation of microspermatophores (hereafter referred to as micros) occurs over several hours and culminates in the transfer of a single sperm-containing 'macro' spermatophore, which is produced by males at a certain time of day regardless of nuptial feeding duration. Unlike other crickets and insect taxa that provide gifts before mating, the production of two types of spermatophores is unusual for crickets and the timing of nuptial gift transfer occurs well before mating (i.e. the transfer of

the sperm-containing spermatophore). Furthermore, the serial transfer of nuptial gifts is unique, necessitating a considerable time investment, and appears to be regulated by photoperiod. Due to the divergence in characteristics from other nuptial gift systems, swordtail crickets offer an excellent opportunity to study selection hypotheses in an extremely elaborate system that may offer insights into new behavioral strategies used by both sexes.

The objectives of this study were to examine male nuptial feeding behavior in the context of both sexual and natural selection. My general aims were to: 1) investigate the adaptive significance of micro production; 2) examine how photoperiod affects male micro production and mating behavior and 3) investigate the pervasiveness of the dual spermatophore system in Hawaiian cricket taxa. For the first two aims, I performed experiments on the focal species, *Laupala cerasin*a. For the third aim, I observed and compared the behaviors of several Hawaiian swordtail cricket genera.

In Chapter I, I investigated the benefits of nuptial gift donation for males. I tested both the male mating and fertilization success hypothesis by experimental manipulation of micro donation. For the fertilization success hypothesis, I examined three strategies by which micro donation could increase male fertilization: 1) delayed female macro removal to increase sperm transfer; 2) delayed female remating to reduce the risk of sperm competition and 3) enhanced sperm uptake from the macro into the female reproductive tract. Although micro donation occurs before mating, I did not find that nuptial gifts improve male mating success. Instead, I demonstrated that nuptial gift donation enhances sperm uptake by the female reproductive tract, a

novel mechanism by which male insects increase sperm transfer. Furthermore, this type of post-copulatory choice, in which females favor males via internal manipulation of ejaculate, has not been documented before in crickets. I found that most females remate, therefore increased sperm transfer due to micro donation likely improves male fertilization success under the risk of sperm competition.

In Chapter II, I investigated mating behavior in the context of the light environment by experimental manipulation of pair establishment time and male photoperiod. I tested the hypothesis that mating is time restricted and examined the relationship between nuptial gift transfer and photoperiod. I also tested the hypothesis that male circadian rhythm determines the timing of mating. I found that among males and females on the same photoperiod, pairs established later in the day had a reduction in both the initiation of courtship and a significant reduction in mating success. I also found a significant reduction in micro number as pairs were established at later points in the photoperiod, and a correspondingly small but significant delay in macro production. I found that male rhythm generates the boundaries of the mating period and that females have a wide window of receptivity. These results show that males will adjust both micro number and macro transfer time within certain ranges, suggesting that both factors play an important role in male reproductive success.

In Chapter III, I compared courtship and mating for a comparative analysis across the endemic swordtail cricket genera *Trigonidium*, *Prolaupala,* and an introduced genus, *Anele* to serve as an outgroup. I found that *Prolaupala* shared a derived spermatophore system with *Laupala.* *Trigonidium* do not produce micros,

but exhibit other behavioral strategies that have been demonstrated to improve fertilization success in other cricket taxa. *Anele ulia* produces micros, but many other behavioral elements are not similar to *Prolaupala/Laupala.* Furthermore, I observed behavioral characters that were previously considered unique to other crickets in the family Gryllidae. These results suggest that many behavioral strategies have arisen independently within the gryllid lineage, including microspermatophore production, demonstrating that sexual selection has resulted in many convergent adaptations within this group.

CHAPTER I

Premating nuptial gifts enhance sperm transfer in the Hawaiian swordtail cricket, *Laupala cerasina*

ABSTRACT

In many cricket species, males provide females with food gifts during the mating process. Such nuptial gifts primarily improve male mating effort, either by increasing mating or fertilization success. Nuptial gifts provided directly before mating facilitate copulation, but gifts provided during insemination increase ejaculate transfer by preventing premature consumption of the external spermatophore by females. The Hawaiian swordtail cricket, *Laupala cerasina,* has an elaborate courtship system in which males transfer many relatively small, spermless 'micro' spermatophores that females consume before the production and transfer of the final, sperm-containing 'macro' spermatophore. Micro donation is unusual and their function is unclear because feeding occurs far in advance of the mating event. Here, we examined the function of micros by testing the mating and fertilization success hypotheses of micro function, including several strategies through which males may influence female behavior to improve fertilization. We test the predictions that micros function to: 1) delay macro removal; 2) inhibit remating; and 3) enhance sperm transfer. We also performed field observations of the mating process to document micro production

characteristics, which informed our experimental design. We performed laboratory experiments consisting of two treatment groups: females that received a natural amount of micros followed by the sperm-containing macro or females that received only the macro. We found that females who received micros acquired more ejaculate than females who did not. This finding supports the fertilization success hypothesis of micro function. However, micro transfer did not result in delayed macro removal by females. Rather, micro transfer stimulated greater sperm uptake from the female reproductive tract, which we consider a 'sperm transfer enhancement' strategy. These results reveal that in addition to spermatophore removal, female crickets may have an internal mechanism to favor males after copulation, as exhibited in other insects and animals.

INTRODUCTION

Males of many insect species provide nuptial gifts that females eat during mating such as body parts, glandular secretions, regurgitated food, prey items and spermatophores (for a review see Vahed, 1998). Nuptial gifts are a form of male mating effort: their presence increase a male's opportunity to mate or the number of eggs he fertilizes (Simmons and Parker, 1989). In crickets and katydids (Orthoptera: Ensifera), nuptial gift function is related to the stage at which it is offered to the female (Gwynne, 1997). Gifts are typically offered immediately preceding mating (e.g. Alexander and Otte, 1967b; Brown and Gwynne, 1997; Ono et al., 1989), during

mating when spermatophores are transferred by copulation (e.g. Alexander and Otte, 1967a; Bidochka and Snedden, 1985; Mays, 1971) or postmating while insemination via the spermatophore occurs (e.g. Brown and Gwynne, 1997; Gwynne, 1995; Gwynne, 1997). Nuptial gifts provided directly before copulation typically improve male mating success, while those provided during or after mating increase fertilization success (for reviews see Gwynne, 1997; Vahed, 1998).

Nuptial gifts provided before mating have been shown to increase male mating success by enticing females into mating. Several species provide food items to females directly before copulation to ensure that females stay in a position to facilitate spermatophore transfer. For example, in the sagebrush cricket, *Cyphoderris strepitans*, females mount males and feed on specialized wing pads while males extrude the spermatophore (Dodson et al., 1983). Males that lack hindwing material are less likely to have a female remain mounted for spermatophore transfer and, consequently, such males suffer lower mating success (Eggert and Sakaluk, 1994). Similarly, metanotal gland secretions in tree crickets also appear to promote copulation. The female mounts the male and feeds on glandular secretions while the male transfers a spermatophore (Gwynne 1997; Brown 1997). However, because metanotal feeding continues after spermatophore transfer, its function also has been investigated in the context of insemination enhancement (Brown, 1994).

Nuptial gifts provided during insemination are considered an 'ejaculate protection' strategy because they reduce female interference with ejaculate transfer. The ejaculate is enclosed within a capsule, called the ampulla, which remains external to the female (Loher and Dambach, 1989). Sperm flows from the spermatophore

ampulla into the female at a gradual rate, therefore spermatophore attachment duration determines the amount of sperm a female receives (Sakaluk, 1984; Sakaluk, 2000; Simmons, 1986). Males that provide nuptial gifts during or after copulation benefit by prolonging copulation or delaying spermatophore removal by females. For example, female ground crickets in the genus *Allonemobius,* feed on special spurs located on the male's legs during mating (Mays, 1971). This feeding results in prolonged genital coupling after spermatophore transfer, which maintains the female in a position that makes spermatophore removal difficult (Bidochka and Snedden, 1985; Fedorka and Mousseau, 2002). Many katydid and certain gryllid species provide a food gift after mating called a spermatophylax, a gelatinous substance that coats the spermatophore (Brown and Gwynne, 1997; Gwynne, 1995; Gwynne, 1997). Females are distracted from prematurely consuming the spermatophore capsule while they eat the spermatophylax, which results in fuller insemination (Gwynne et al., 1984; Sakaluk, 1984; Simmons, 1995; Wedell, 1993; Wedell and Arak, 1989).

Increased ejaculate transfer is predicted to improve male fertilization success. Cricket females typically mate with more than one male. Consequently, sperm competition has resulted in adaptations that either improve a male's numerical representation of sperm or alter female reproductive behavior (Parker 1970). By transferring a greater number of sperm, a male increases his paternity success when a female multiply mates (Sakaluk 1986b; Simmons1987; Wedell 1991; Sakaluk & Eggert 1996). Additionally, some species transfer ejaculate substances that manipulate female behavior, e.g., causing reduced postmating receptivity (Gwynne, 1986; Simmons and Gwynne, 1991) or increased rates of oviposition (Murtaugh and

Denlinger, 1985; Stanley-Samuelson and Loher, 1986; Wedell and Arak, 1989). These chemicals typically act in a dosage-dependant manner (Stanley-Samuelson et al., 1986); therefore nuptial gifts that result in greater ejaculate transfer may also function to increase substances other than sperm.

The Hawaiian swordtail cricket genus, *Laupala,* has an elaborate mating system, which includes nuptial feeding in the form of spermless 'micro' spermatophores. A series of microspermatophores (hereafter referred to as micros) are transferred to the female over the course of several hours. The final 'macro' spermatophore (hereafter referred to as the macro) is approximately three times larger and contains sperm (deCarvalho and Shaw, 2005; Shaw and Khine, 2004). The transfer of both types of spermatophores involves copulation and insertion of the sperm tube into the female. Following each type of spermatophore transfer, males and females typically antennate for a period of time before the female removes and consumes the spermatophore. This behavior is referred to as post-copulatory guarding. *Laupala* nuptial feeding is unusual because it occurs long before mating (i.e. the transfer of a sperm-containing macro) and insemination.

Because of the timing of their donation, micros necessarily utilize a different mechanism to influence female behavior, though it is not known whether micros function to increase a male's mating or fertilization success. Two hypotheses could explain the function of micros. *The mating success hypothesis* predicts that microspermatophore transfer results in greater macrospermatophore transfer success. While micros are not directly used to entice females into mounting, females may prefer to receive the macro from males that produce micros. Alternatively, *the*

fertilization success hypothesis predicts that micros function to improve male fertilization success following female acceptance of the macro.

In order to understand the adaptive function of microspermatophore nuptial feeding, we investigated both hypotheses of micro function through experimental manipulation of micro donation in *Laupala cerasina*. To investigate the mating success hypothesis, we compared macro transfer success in two treatment groups: females that received a natural number of micros followed by the sperm-containing macro, and females that received only the macro. Using these same treatments, we also investigated the fertilization success hypothesis by measuring correlates to fertilization success. We considered three hypothetical strategies by which micros could alter female behavior to improve fertilization success. First, the 'ejaculate protection' hypothesis predicts that micro transfer delays female macro removal. If sperm flows from the macro at a gradual rate as in other crickets, an increase in the duration of macro attachment would result in fuller insemination. Although premating micro donation cannot directly protect the macro, micros might influence females to delay macro removal through some other means (e.g. gustatory satiation after micro consumption). The second hypothesis, 'remating inhibition', predicts that micros delay female remating, thereby reducing the potential for sperm competition. Micros are spermless, but their donation might represent a strategy to increase the transfer of non-sperm substances that inhibit subsequent receptivity. The third hypothesis, 'enhanced sperm uptake' predicts that micros induce greater sperm uptake from the macro into the female reproductive tract. This hypothesis was based on our own preliminary observations of macro sperm drainage patterns and represents an

alternative to the 'ejaculate protection' hypothesis because it does not involve female macro removal behavior.

Prior to laboratory experiments, we observed natural courtship behavior at Kalopa State Park, Hawaii. Field observations were performed to document the onset of courtship relative to the photoperiod and number of micros transferred. In the lab, the number of micros produced by a male depends on the time at which pairs are introduced; late introductions result in fewer micros before mating. Therefore, the field data were used to determine when to introduce pairs relative to the photoperiod, to achieve a natural number of micros for experimental treatments.

METHODS

Field study

Field observations of mating behavior were conducted every day from July 21st to 29th, 2004 at Kalopa State Park, Hawaii. Morning twilight occurred at 05:29, dawn occurred at 05:53 while the sun set at 19:01 and evening twilight ended at 19:24 according to the United States Naval Observatory.

To observe the courtship sequence between a single pair of mating individuals, we marked the location of focal males found under tree bark in *Metrosideros polymorpha* (Ohi'a) and in the recesses of *Psidium guajava* (Guava). In order to ensure that we observed the onset of mating, we checked each site for female

presence shortly after dawn and then every 10-20 minutes. Once courtship was established at a particular site, we recorded the timing of the following behaviors: onset of courtship (designated by a face-to-face body position), micro production, and macro production and transfer.

Laboratory experiments

L. cerasina nymphs were collected on the island of Hawaii in 2004-2006 and studied at the University of Maryland. Crickets were contained in quart-sized glass jars or plastic specimen cups lined with moist paper towels and Kimwipe tissues. Crickets were maintained on a diet of Fluker's Cricket Feed, at 20˚-22˚ C on a 12:12 light: dark cycle. Nymphs were isolated to same-sex containers to remain virgins for mating experiments.

All three experiments shared the same basic methods and experimental design. Males and females were paired in plastic Petri dishes with water-saturated Kimwipes. Pairs were established approximately 3 ½ hours after the onset of the photophase (8 ½ hours before the dark phase), similar to natural courtship observed in field portion of study. Two mating treatments were established for each experiment: 1) "Micros absent" females received only the macro; 2) "Micros present" females received a series of micros and the macro. Females were randomly assigned to each treatment. Treatment 1 was arranged by pairing a male with a non-experimental female, who accepted a series of micros. Courtship was allowed to progress until the macro was formed by the male, at which point the female who received micros was

taken away and replaced with a focal female. In the second treatment, mating pairs were allowed to progress to macro transfer. To control for the effect that Petri dish disturbance may have on female behavior in the first treatment; the dish containing the mating pair was separated and reconnected after macro formation in the second treatment.

Experiment 1: Do micros increase mating opportunities or macro retention time by females?

Following the mating treatment described in the general methods, we recorded the number of females that accepted the macro (i.e. mated) for both treatment groups. After macro transfer was complete, we recorded female macro removal behavior. Females remove spermatophores using their hind leg and it usually takes multiple attempts to complete sperm tube removal. Therefore, we recorded the time interval (mins) between macro transfer and both the female's initial attempt and complete removal of the macro in order to assess both the duration the female intended to retain the spermatophore and the actual duration.

Experiment 2: Do micros delay female receptivity and remating?

Following the mating treatments described in the general methods, females were prevented from removing the macro in order to standardize macro attachment time across females. Immediately after macro transfer, females were coaxed down a plastic funnel into a 0.2 ml microcentrifuge tube, which was narrow enough to restrict leg movement. After 60 minutes, females were removed from the tube and anesthetized with $C0_2$. The macro was removed with forceps and examined under a dissecting microscope for sperm.

To measure the female remating period, females were paired with a different male each day subsequent to the initial mating until they remated. We recorded the interval (in days) between the initial mating and both the acceptance of a subsequent micro and macro. It was important to record both of these behaviors because females may accept one or more micros from a certain male and then terminate courtship, therefore remating (macro acceptance) may not occur until another day. We considered micro acceptance a signal of receptivity and macro acceptance remating. Females were introduced to males using the same methods as the initial mating and remained paired with males for at least six hours per day. Females were paired with males every day for at least 16 consecutive days and then at least twice a week. Non-virgin males were used for remating trials, which had prior mating success (defined by the transfer of a macro) at least 3 days before the remating trial. Work on other *Laupala* species has demonstrated that well-fed, laboratory-reared males are able to produce a macro the next day subsequent to mating (Jadin & Shaw, in preparation). To determine the onset of oviposition, containers housing individual females were

checked every day for eggs, beginning after the initial mating and ending when females remated.

Experiment 3: Do micros increase sperm transfer into the female spermatheca?

Following the mating treatments described in the general methods, females were observed until they attempted to remove the macro. At this time, females were immediately anesthetized with CO_2. To assess the amount of sperm that drained from the macro into the reproductive tract, macros were quickly removed from the female and photographed on a hemacytometer without a coverslip (see Figure 1). We then dissected the female to remove the spermatheca, which was placed on a hemacytometer, compressed with a cover slip and photographed (see Figure 1). The hemacytometer design is such that raised edges hold the coverslip 0.1 mm off the marked grid; therefore each spermatheca was flattened to the same degree. After photography, we dissected spermathecae and noted whether sperm cells were present. We also measured female femur length as a measure of body size.

To evaluate whether micro substances add significant volume to the spermatheca, we added two treatment groups: 1) virgin females, and 2) females that received only micros. The "micro only" treatment group was arranged by pairing crickets using the same protocol as the other experiments, the key alteration being that females were removed from the Petri dish as soon as a male produced a macro. Spermathecae from these additional treatment groups were measured using the same protocol for mated females.

Digital photographs of macro ampullae and spermathecae were taken with a JVC TK-1280U color video camera mounted to an Olympus CH2 compound microscope. Measurements were performed using image-analysis software (ImageJ 1.39). To assess the amount of sperm drainage from the macro, the area of sperm remaining in the ampulla was circumscribed and subtracted from the area of the entire macro ampulla. We refer to the calculated area of ampulla empty space as the "sperm drainage" area. To assess the amount of sperm transferred to the female, the area of the flattened spermatheca was measured. The spermatheca is elastic and spherical, which allowed us to indirectly measure sperm volume. Unfortunately, we were unable to perform direct measures of sperm number despite extensive efforts to do so. *Laupala* sperm agglutinate to a degree that precluded individual cell counts and are not readily dissociated by standard methods or several methods that we attempted to develop.

Statistical Analyses

Analyses were performed using JMP 7.0 for Windows (SAS Institute, Inc). All tests of significance were two-tailed and results are reported as means ± SE. Each laboratory experiment was based on two treatments; therefore Student's t-tests and Pearson chi-square test (or Fisher's Exact) analyses were used for treatment comparisons when possible.

In the experiment to compare the remating interval between the two treatment groups, many females remated the next day which resulted in a non-normal, right-

skewed distribution. We performed a permutation randomization test involving 10,000 iterations in SAS 9.1.3 (SAS Institute, Inc). This data set also included remating intervals of varying precision. There were two females in each treatment group that accepted spermatophores much later than the rest (23-32 days); therefore after a certain point they were assayed for receptivity only twice a week, rather than every day. This resulted in a 2-4 day interval during which they regained receptivity; rather than a 1 day interval as with most females. We scored these particular data points in two different ways for analysis. First, we used the earliest possible date females could have regained receptivity, which biased these data towards the shortest refractory period. Second, we used the day that females actually accepted spermatophores, which biased these data towards the longest refractory period. Neither approach resulted in significant *p* values; therefore we only report the results biased toward the shortest refractory period.

To test the sperm transfer enhancement hypothesis, we performed two replicate experiments in the spring and fall of 2006. To determine whether it was appropriate to pool data from both experiments, we performed an ANCOVA to evaluate interactions between our treatments and experiments. There was no significant interaction between treatment and experiments for any of the dependant variables in our study; therefore, we combined the two data sets for the final analyses. To evaluate the relationship between sperm transfer and time, we performed regression analyses of sperm drainage and spermatheca area on macro attachment duration. We used macro attachment duration as a covariate when it had a significant relationship with dependant variables. To determine whether spermatheca area

indicates the amount of sperm a female has received, we performed a regression analysis of spermatheca area on macro sperm drainage area. We found a significant relationship between sperm drainage and spermatheca area, therefore we compared spermatheca area between the two treatment groups using ANCOVA with macro attachment duration in the model. We did not include female femur length (i.e. body size) because it did not have a significant effect.

RESULTS

Field study

Twelve mating pairs of *L. cerasina* were observed in the field, though some variables were not collected for certain pairs. Courtship began at 11:03 ± 0:25 hr:min (n = 10), which was approximately 5 hours after sunrise (8 hours before dark). Macro production occurred at 15:50 ± 0:09 hr: min (n = 8) and macro transfer occurred at 16:48 ± 0:07 hr: min (n= 8). The total number of micros produced was 6.6 ± 0.32 (n = 8).

We compared the number of micros produced by field males to males from Experiment 1 (6.9 ± 0.20, n = 24), which were paired 3 ½ hours after the beginning of the laboratory photophase (8 ½ hours before dark). There was no significant difference between the two groups in the number of micros (t = 0.654, p = 0.524).

Experiment 1: Do micros increase mating opportunities or macro retention time by females?

We found no significant difference in the number of females that mated between the two treatment groups (Fisher's Exact test, $p = 0.354$; micros present = 77.8% mated (14/18); micros absent = 93.3% mated (14/15)). Two females from the micros present group overtly rejected macro transfer; rejection was indicated by the male's failure to transfer the spermatophore after moving into the copulation position. The three other mating failures (across both groups) were due to early termination by the male, indicated by the male's removal of his macro without attempting to copulate with the female.

After copulation, there was no significant difference between the two groups in the amount of time before females attempted to remove the spermatophore (Table 1; $t= 0.745$, $p = 0.463$) or to successfully remove the macro (Table 1; $t = 0.715$, $p = 0.480$).

Experiment 2: Do micros delay female receptivity and remating?

The transfer of micros did not have a significant effect on female remating behavior (see Figure 2 for remating interval distribution). Most females remated (95.2 %; 60/63). A large proportion of females in each treatment group remated on the subsequent day, but there was no significant difference between the two groups ($\chi^2 = 2.61$, $p = 0.106$; micros present = 48.4 % females mated (15/31) and micros absent = 69.0 % females mated (20/29)). There was no significant difference between

treatment groups in the number of days to accept a micro (Table 2; randomization test, $p = 0.329$) and the macro (Table 2; randomization test, $p = 0.308$). There was also no significant difference between the treatment groups in the number of females that laid eggs before remating ($\chi^2 = 1.68$, $p = 0.195$; micros present = 27.6 % of females oviposited (8/29); micros absent = 13.8% of females oviposited (4/29)).

Experiment 3: Do micros increase sperm transfer into the female spermatheca?

We compared spermatophore removal behavior between the two treatment groups and there was no significant difference in the length of time until the first attempt to remove the macro (micros present females: 33.9 ± 2.2 minutes, $n = 23$; micros absent females: 37.6 ± 3.0 minutes, $n = 25$; t = -1.06, $p = 0.294$). Furthermore, there was no relationship between the duration of macro attachment and the amount of sperm drained from the spermatophore for either treatment group (micros present: $r^2 = 0.0021$, $p = 0.8354$, n = 23; micros absent: $r^2 = 0.069$, $p = 0.2356$, n = 22). However, there was a significant relationship between duration of macro attachment and spermatheca size within the micros present group ($r^2 = 0.226$, $p = 0.0217$, n = 23) but not within the micros absent group ($r^2 = 0.107$, $p = 0.1579$, n = 20).

To assess the effect that micros had on sperm transfer into the female, we compared the amount of sperm that drained from the macro ampulla between treatment groups. Micros present females had significantly larger sperm drainage areas in the macro ampulla than in the micros absent treatment group (t = 4.44, $p < 0.0001$; Table 3). We also compared the number of females that failed to receive any

sperm between the two treatment groups. Sperm transfer failure was identified by a full macro in combination with an empty spermatheca. All females in the micros present group received sperm (0/24 failures). However, 31.8% (7/22) of females in the micros absent group failed to receive sperm, which was significantly more sperm transfer failures than the micros present group (Fisher's Exact test, $p = 0.0038$).

To determine if spermatheca size is affected by the amount of sperm drained from the ampulla, we assessed the relationship between the sperm drainage area in the ampulla and spermatheca area. Spermatheca area increased significantly with sperm drainage area ($r^2 = 0.244$, $p = 0.0019$, n = 37; Figure 3). To assess the effect that micros had on sperm uptake by the female reproductive tract, we compared the spermatheca size between the treatment groups. Females had significantly larger spermatheca areas in the micros present group than in the micros absent treatment group (ANCOVA $F_{2, 40} = 4.27$, $p = 0.0208$; treatment $p = 0$ .0376; macro attachment duration $p = 0.0126$; Table 3). To evaluate whether micro substances add a significant amount of material to the female sperm storage organ, we compared spermatheca size between the virgin and micro only treatment groups. There was no significant difference in spermatheca area between virgins and females that received only micros ($t = 1.05$, $p = 0.3014$; Table 3).

DISCUSSION

Male crickets typically donate nuptial gifts prior to mating or during insemination to increase their mating effort. However, in the swordtail cricket *L. cerasina,* males provide specialized spermless spermatophores during the courtship stage, which is distinct from the mating event. Although micros are provided before mating, we found that their transfer does not increase mating success. Instead, we found that micros increase the amount of sperm transferred to the female. This increase in sperm transfer is not a result of an ejaculate protection strategy, typical for other crickets. Rather, we found that micro donation results in greater sperm uptake by the female reproductive tract. We also found that females usually remate and do so before oviposition, which indicates that males engage in sperm competition. Therefore, fuller insemination due to micro donation likely improves a male's fertilization success in the context of sperm competition.

We found that microspermatophores were not necessary for females to mate, nor did they function to increase male mating success. Most females accepted the macro with or without the prior transfer of micros and there was no significant difference in the number of mating failures between the two treatments. In the unsuccessful matings, failure was either due to male termination or female rejection of macro transfer.

We demonstrated that micro transfer resulted in fuller insemination, which supports the fertilization success hypothesis. By comparing the amount of sperm that drained from the macro between the treatment groups, we found that females who

received micros had macros that were more fully drained of sperm, on average, than females who did not receive micros. Females that received micros also had larger spermatheca, which is further evidence of fuller insemination. By dissecting spermathecae, we were able to verify that sperm cells substantially contributed to spermatheca size. We corroborated this statistically, via the positive correlation between macro sperm drainage area and spermatheca area, which indicates that spermatheca size varies depending on the amount of sperm transferred. We were also able to rule out that micros contributed any significant volume of material to the spermatheca, because there was no significant difference in spermatheca size between virgin females and females that received only micros. Therefore we conclude that micro transfer has a positive effect on the deposition of sperm from the macro into the female spermatheca.

We found that micro donation did not delay macro removal behavior by females; therefore the increase in sperm transfer due to micro donation was not the result of an ejaculate protection strategy. In the first experiment, we found no significant difference in the amount of time until the female's first attempt to remove the macro or how long it took females to completely remove the macro between the two treatment groups. This demonstrates that micro donation does not function to delay female macro removal behavior or increase difficulty of removal. In the second experiment, there was also no significant difference in the mean time of macro attachment duration, yet we found a significant difference in the average amount of sperm transferred between the two groups. This phenomenon could be attributed to either a change in the rate of sperm transfer between the groups or a change in the

amount of sperm permitted to enter the reproductive tract. The lack of a relationship between macro attachment duration and the amount of sperm drainage suggests that sperm flow from the macro does not occur at a gradual rate. This was an unexpected finding, because other studies of ejaculate transfer in crickets show a linear relationship between sperm transfer and time. Due to the absence of a relationship between sperm transfer and time, we were not able to assess whether micro donation results in an increase in the rate of sperm transfer. Macro sperm drainage may be punctuated, instead of gradual. Our anecdotal observations of sperm transfer from the macro support this idea. For example, sperm sometimes appeared to rapidly drain from the ampulla into the female and then abruptly stop in some instances. The 'leftover' sperm may be draining at a slower rate or not at all, suggesting that females may allow a certain amount to drain and block the rest from entering the reproductive tract. The idea that sperm transfer may be blocked by the female reproductive tract is statistically supported by our result that all females in the micros present group received some amount of sperm, while approximately one third of the micros absent females did not receive any sperm.

In many other insects and animals, females have a certain amount of control over sperm movement through the reproductive tract via muscle control or chemotaxis (see reviews of Eberhard, 1996; Simmons, 2001). There is indirect evidence that crickets may have muscle control over sperm transfer, therefore it is possible that *L. cerasina* females are able to enhance or impede sperm transfer within the reproductive tract. Crickets in the family Gryllidae have a long, convoluted spermathecal duct through which sperm travel when the female is inseminated and

the eggs are fertilized (Sturm, 2005). In the field crickets *Teleogryllus commodus* and *Gryllus bimaculatus*, the spermathecal duct has been observed to be surrounded by a muscular envelope (Yasuyama et al., 1988). These muscles have been observed to generate peristaltic contractions (Essler et al., 1992; Sturm, 2005), and transport sperm from the spermatheca to the area of egg fertilization (Sugawara, 1993). Peristalsis used in fertilization may also be used in insemination, though this idea has not been directly investigated. However, indirect evidence provided by Simmons and Achmann (2000) supports this idea. They found that anesthetized female bushcrickets, *Requena verticalis*, receive lower sperm numbers than unmanipulated females. Anesthetization inhibits active muscle control, which suggests that female muscle control is involved in sperm transport into storage.

If female muscle activity is largely responsible for sperm transport, micro donation may stimulate peristalsis. This response could be a direct result of the process or properties of micro transfer. Chemical manipulation of the female reproductive tract is one potential mechanism by which micros could directly stimulate a peristaltic response. In the *G. bimaculatus*, a gonadotropic substance present within the seminal fluid was demonstrated to increase muscle contraction rate in the region of the spermathecal duct (Kimura et al., 1988), which suggests that seminal fluids may induce the female reproductive tract to transport sperm.

Mechanical stimulation of the female reproductive tract from the act of micro transfer is another potential mechanism that may elicit greater sperm uptake. In some species of mammals and spiders, genital movements by males have been demonstrated to increase sperm transport (Eberhard, 1996). This effect is considered

"copulatory courtship" by Eberhard (1996); therefore greater sperm uptake may represent female post-copulatory choice. Micro transfer may enable females to assess aspects of male phenotype and thus females may outwardly accept the macro but reject the male sperm by inhibiting sperm transport into the spermatheca.

We found that almost all *L. cerasina* females remated, which suggests that micro donation evolved in the context of sperm competition. We suggest that micros are an adaptation to sperm competition by increasing the numerical representation of sperm from a given male. However, micro donation does not function to alter certain female behaviors that often affect male fertilization success. Micro transfer did not significantly extend the female remating period, demonstrating that micros do not contain receptivity-inhibiting substances. In this experiment, we manipulated macro attachment time to control for the amount of ejaculate received between the treatments. In the micros present group, females likely received more sperm due to micro transfer but this did not significantly alter the remating period. These results suggest that *L. cerasina* macros also do not contain receptivity-inhibiting substances. Although there are examples of katydids and other non-orthopteran insects (Cordero, 1995; Eberhard, 1996; Gillott, 2003) that transfer receptivity-reducing substances in their ejaculate, this effect has not been demonstrated in the family Gryllidae (Fleischman and Sakaluk, 2004; Orshan and Pener, 1991). Additionally, we found that micros did not increase the occurrence of oviposition before remating. One of the potential benefits that males might gain from delaying female remating is that females may lay more eggs before remating. Therefore a male could increase the likelihood that eggs are fertilized by his own sperm before a female acquired sperm from

another male. In this study, most females remated before laying eggs; however there was no significant difference between the two treatment groups in the number of females that did lay eggs before remating.

The field component of our study revealed that most courtships begin late morning, approximately 9 hours before dark. We set up our laboratory experiments 8 ½ hours before the dark phase, resulting in no significant difference in micro number between field and experimental matings. We most likely observed the higher end of the natural range of micro production number in the field due to our method of scanning focal males in the early morning until a female arrived. However, the field data do not represent the upper limit of micro production. Males produce more micros in the laboratory when they are paired with females earlier in the photophase and micro number is a function of the time of day that males begin the courtship (deCarvalho, Ch. 2). The variation in micro number seen in the field is likely a result of the timing of pair formation. Because of the artificial nature of the treatment in which females did not receive any micros, we likely observed an extreme sperm transfer response. We did not explore the effects that natural variation in micro number has on sperm transfer. Therefore, the relationship between the time of pair formation, micro number and insemination success will be an important avenue to explore in future studies.

The discovery that sperm transfer may not occur in a linear rate in *Laupala* is an avenue that warrants future investigation. This may be an effect of female internal manipulation of the ejaculate, which may be unique to *Laupala.* Alternatively, internal manipulation of sperm transfer may be a common phenomenon in other

cricket species, though perhaps not the primary form of female influence over the insemination process. Investigation of female control over insemination has focused on spermatophore removal behavior, but the idea that females may have internal control was put forward by Eberhard (1996). His suggestion was based on Simmons's (1986) data in *G. bimaculatus* that showed variation in the rate that spermatophores emptied sperm. If most cricket females do have internal control, this represents another mechanism of post-copulatory choice that females may employ.

In summary, micros represent a unique form of nuptial gift provided before mating but our data suggest that they do not function to improve mating success. Instead, our results support the fertilization success hypothesis of nuptial gift function. We have demonstrated that the donation of micros has a positive effect on sperm transfer in *L. cerasina*. However, the mechanism by which they stimulate sperm uptake could be related to their consumption or another aspect of their donation. In addition, it remains to be investigated whether the effect of micro donation on sperm transfer is male or female mediated. Males may be manipulating female physiology or females may be favoring males through cryptic choice.

TABLES

Table 1. Mean ± standard error of interval (mins) between macro transfer and first removal attempt and complete macro removal by females in each treatment group

	1st removal attempt	**Complete removal**
Micros absent $n = 14$	30.2 ± 4.6	41.5 ± 5.0
Micros present $n = 14$	25.6 ± 4.0	36.5 ± 4.8

Table 2. Mean ± standard error of interval (days) from initial mating to subsequent acceptance of a micro (i.e. receptivity) and macro (i.e. remating) for each treatment group

	Micro acceptance	**Macro acceptance**
Micros absent $n = 29$	2.8 ± 1.1	3.4 ± 1.1
Micros present $n = 31$	4.8 ± 1.5	5.5 ± 1.6

Table 3. Mean ± standard error of area (mm^2) of sperm drainage from macro ampullae and spermathecae for each treatment group

	Macro sperm drainage	**Spermathecae**
Micros absent	$n = 22$ 0.254 ± 0.022	$n = 20$ 0.737 ± 0.057
Micros present	$n = 23$ 0.365 ± 0.010	$n = 23$ 0.822 ± 0.042
Micros only	ϕ	$n = 12$ 0.338 ± 0.051
Virgins	ϕ	$n = 17$ 0.367 ± 0.017

ϕ Macros not measured for these treatment group

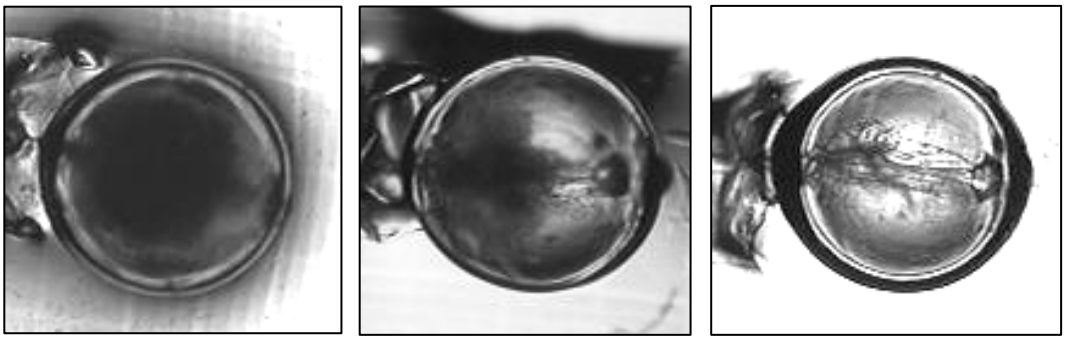
FIGURE LEGENDS

Figure 1. Representative images of macro ampullae and spermathecae measured for sperm transfer experiment. The macro photos represent consecutively larger sperm drainage areas (i.e. smaller amounts of sperm) from left to right. The final macro on the right was devoid of sperm. The spermatheca photos portray consecutively smaller spermathecae from left to right. The final spermatheca on the right was devoid of sperm.

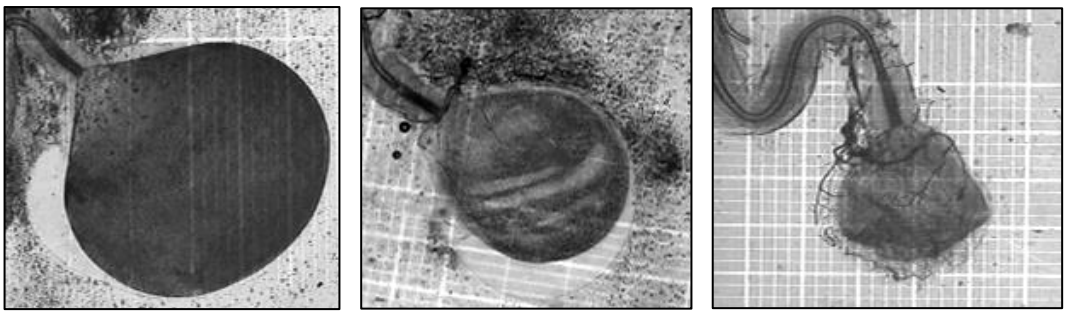
Figure 2. The distribution of female remating intervals for both treatment groups.

Figure 3. Macro sperm drainage area is positively related to spermatheca area, suggesting that greater sperm transfer results in the increase of spermatheca size

FIGURES



a) Macrospermatophores removed from females



b) Female spermathecae

Full ⟶ Empty

Figure 1

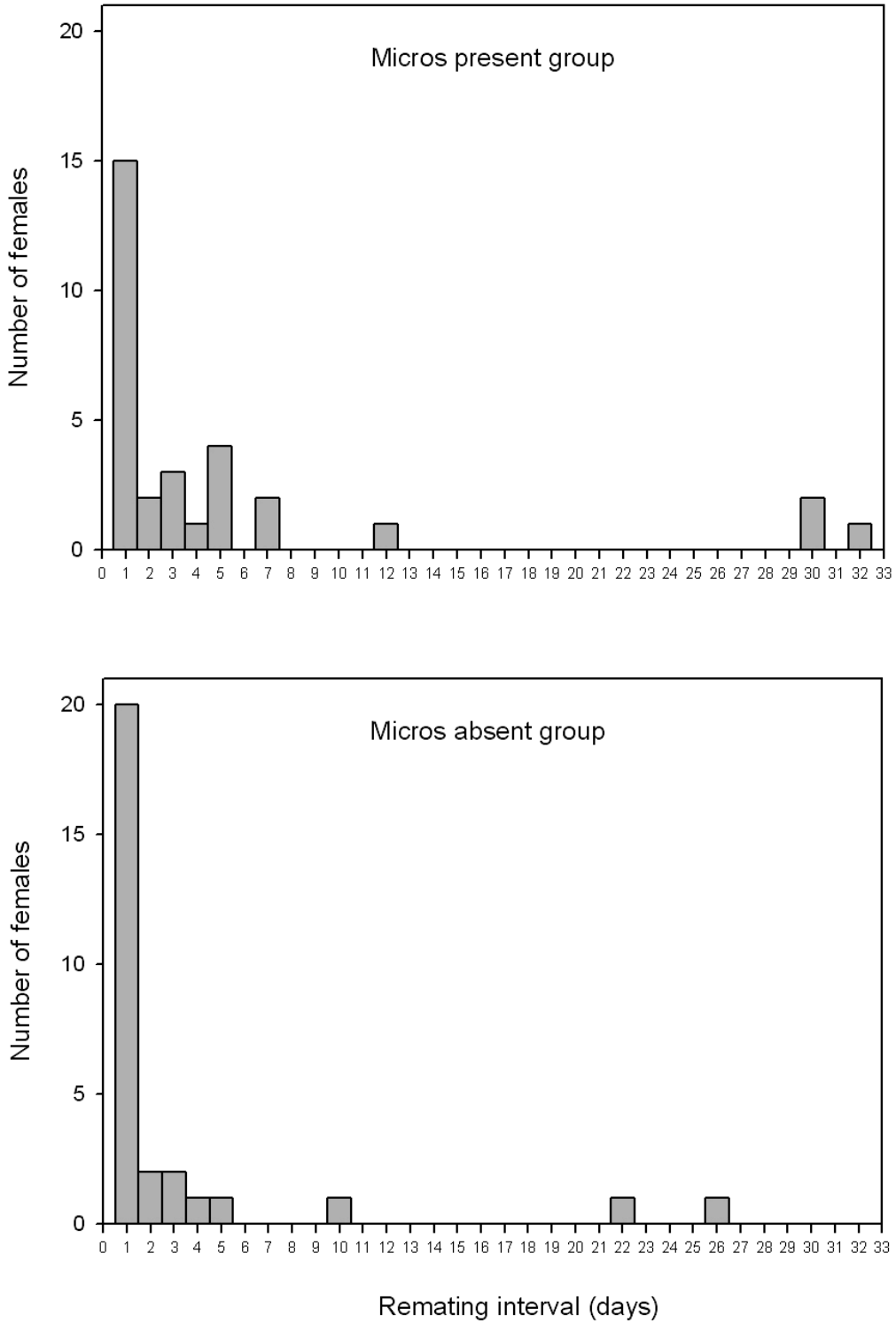

Micros present group
Number of females
0
5
10
15
20
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33
Micros absent group
Number of females
0
5
10
15
20
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33
Remating interval (days)

Figure 2

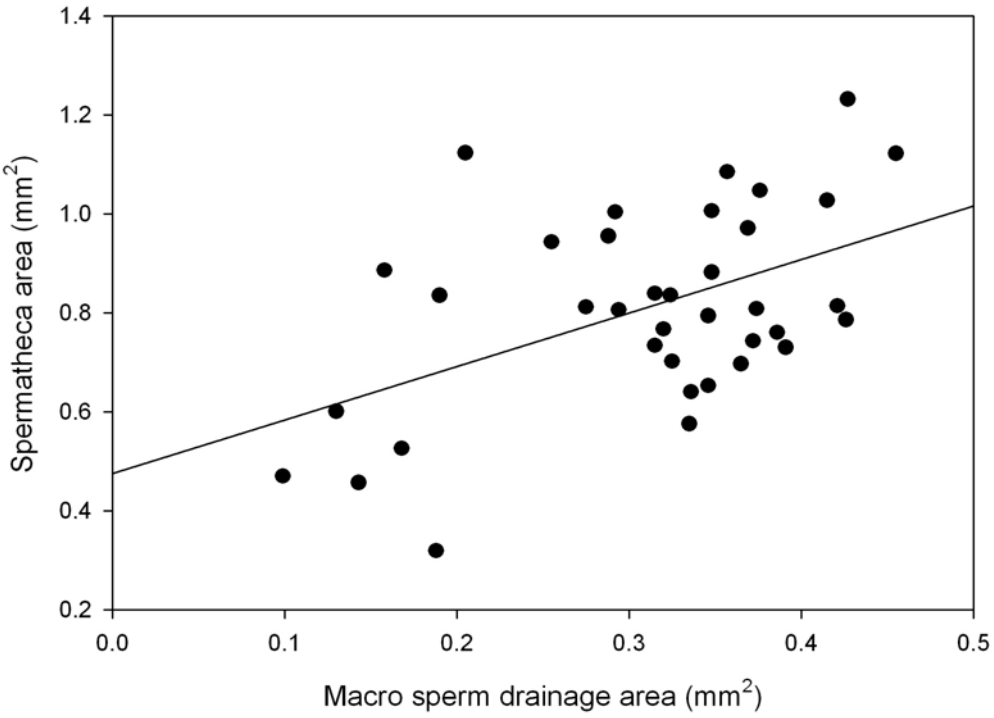

1.4
1.2
1.0
0.8
0.6
0.4
0.2
Spermatheca area (mm2)
0.0
0.1
0.2
0.3
0.4
0.5
Macro sperm drainage area (mm2)

Figure 3

CHAPTER II

Males drive nuptial gift number and timing of mating activity in the Hawaiian swordtail cricket, *Laupala cerasina*

ABSTRACT

In many insects, courtship and mating is restricted to a specific time of day and the rhythm of mating activity may be generated by one or both sexes. The Hawaiian swordtail cricket, *Laupala cerasina,* courts during the day over the course of several hours during which males transfer nuptial gifts in the form of spermless 'micro' spermatophores to females. Courtship culminates with the transfer of one sperm-containing 'macro' spermatophore which, in natural populations, has been observed to occur only within a specific one hour period regardless of courtship start time. We tested the hypothesis that mating in *Laupala* is time restricted by pairing males and females at four different times. We also recorded micro number to examine the relationship between nuptial gift transfer and photoperiod. To assess whether male rhythm determines the mating period, we shifted male photoperiod out of phase to the female light cycle; one early and one late shifted treatment. We found that among males and females on the same photoperiod, pairs established later in the day had a reduction in both the initiation of courtship and many courtships failed to progress to mating; 85% of the pairs failed to mate in the latest treatment group. We also found

that macro production was delayed to accommodate micro production in the later treatments. In the male photoperiod shifting experiment, we found that male rhythm generates the boundaries of the mating period and that females have a wide window of receptivity. Our results show that males will adjust both micro number and macro transfer time within certain ranges, which suggests that both factors play an important role in male reproductive success. The *Laupala* mating system is unusual for crickets in that male mating ability is time limited. Although the adaptive significance of mating time specificity is not understood, these results have important implications for mating barriers between sympatric species.

INTRODUCTION

Mating behavior follows a daily cycle in many animals. The temporal organization of mating-related activities such as mate searching, courtship and copulation reflects selection on these behaviors to occur at a favorable time with respect to social or environmental conditions. Several insect groups have been the focus of study on the daily rhythm of mating activity, including flies (Brevault and Quilici, 2000; Hardeland, 1972; Ikeda, 1976; Sakai and Ishida, 2001b; Suzuki and Koyama, 1980), bugs (Bento et al., 2001) moths (Bento et al., 2001; Giebultowicz and Zdarek, 1996; Konno and Tanaka, 1996; Silvegren et al., 2005), cockroaches (Rymer et al., 2007), bees (Koeniger and Wijayagunasekera, 1976; Koeniger et al., 1996), beetles (Wang

and Li-Yuan, 2005; Wang et al., 2002) and crickets (French and Cade, 1987; Rost and Honegger, 1987; Sakaluk, 1987; Zuk, 1987).

The timing of mating activity is related to the period of sexual ability or receptiveness of each sex. In some systems, both males and females have restrictions on the time at which they are able to mate. For example, in the moth *Spodoptera littoralis,* females release pheromones at a certain time of day and males have a corresponding rhythm to their responsiveness to pheromones (Silvegren et al., 2005). Thus both sexes are synchronized in their daily rhythm of receptivity. Furthermore, males and females are restricted to only mating at this time, which was demonstrated by pairing males and females with out-of-phase photoperiods. In contrast, only females are responsible for the daily rhythm of mating activity in the fruit fly genus *Drosophila* (Sakai and Ishida, 2001a). Through the use of behavioral mutants, *D. melanogaster* females were demonstrated to have a limited period in which they are willing to mate; however, males courted females at any time. The timing of mating activity in *Drosophila* has been hypothesized to be a result of a rhythm to female pheromone production or receptivity to male courtship song (Sakai and Ishida, 2001a).

In crickets, the timing of mating activity appears to be the result of circadian rhythms of several premating behaviors (Loher, 1989). In most species, males prepare for mating by forming a spermatophore which is present when males begin song production to attract females. Both spermatophore production (Loher, 1974; McFarlane, 1968; Zuk, 1987) and calling song have daily rhythms (e.g. Loher, 1972; Wiedenmann and Loher, 1984). Female locomotion involved in mate searching also

has periodicity, which typically coincides with the peak time of male calling song (French and Cade, 1987; Loher, 1979; Loher, 1989; Loher and Orsak, 1985) Upon pairing, males perform brief courtship behaviors before mating. During mating one or more spermatophores are transferred to the female's genitalia via copulation. The peak time of mating activity in the field typically corresponds to the timing of premating behavior. However, both sexes are able to mate at any time when artificially paired in the laboratory (Loher, 1974; Nowosielski and Patton, 1963; Zuk, 1987), unlike some insects that have a time restriction in sexual receptivity or ability. Although crickets may be able to mate at any time, males appear to be more affected by the time of day in their sexual ability than are females. For example, in *T. commodus*, females are receptive 24 hours of the day but males are slower in their rate of successive spermatophore production at certain times of the day (Loher, 1989).

The Hawaiian swordtail cricket, *Laupala,* has an elaborate mating process that occurs during the day. In *L. cerasina,* field observations show that premating and mating activity peaks at a certain time of day (Danley et al. 2007). Like other cricket species, *L. cerasina* males exhibit some levels of calling activity throughout the 24 hour period, but calling peaks at a certain time. Song production is concentrated in the first half of the photophase and females arrive within the period of maximum calling activity (Danley et al., 2007; deCarvalho, Ch. 2). Upon arrival of the female, the pair engages in courtship that lasts from 4-7 hours (deCarvalho, Ch. 1). Courtship involves the transfer of a series of spermless 'micro' spermatophores, which are considered nuptial gifts (Shaw and Khine, 2004). Following the courtship stage, males transfer a single sperm-containing 'macro' spermatophore towards the end of

the photophase within, approximately, a one-hour time period (Shaw and Khine, 2004).

In *Laupala,* the timing of mating (macro production and transfer) appears to have a rhythm in addition to the rhythm of premating behaviors, unlike other cricket taxa. In field observations of *L. cerasina*, pair establishment coincides with peak calling activity (Danley et al., 2007; deCarvalho, Ch. 1). Timing of the sperm-containing macro did not correlate with the onset of courtship. These observations suggest that macro production may have its own daily rhythm, similar to other crickets which have a rhythm to spermatophore production. However, while male field crickets posses a circadian rhythm to spermatophore production in the absence of females, they are capable of producing spermatophores at any time in the presence of females; thus, they apparently are not restricted, physiologically, to mating at a certain time of day. In contrast, *Laupala* males appear to be constrained in the time at which macros are formed, which has a direct impact on the time at which mating occurs. This restriction to the mating period may reflect the limits of male ability, female receptivity, or a combination of the two.

Determining which sex has constraints on the timing of mating will lend insight into the selection pressures responsible for the mating period. Diel timing of macro production may reflect an evolutionary response in males to reduce sperm competition. For example, a male may benefit by monopolizing a female via micro transfer in order to delay macro transfer until just before the egg laying period, so that his partner does not have the opportunity to mate with another male before she uses his sperm for fertilization. Alternatively, macro timing may reflect the time of female

receptivity. Females may have a limited period of receptivity to insemination, possibly due to the temporal organization of other reproductive behaviors, or to avoid times that are dangerous for mating. Because the number of micros transferred from the male to the female affects the male's insemination success, the timing of macro production may have important consequences for other male behaviors. For example, the daily rhythm of male calling to attract mates may need to begin sufficiently early to accommodate the protracted micro transfer necessary to ensure high insemination success. In addition to intraspecific interactions, the role that males and females play in the timing of mating has interspecific consequences. Danley *et al* (2007) proposed that interspecific differences in the timing of mating may be a mechanism of reproductive isolation between sympatric species of *Laupala.* The sympatric species *L. cerasina* and *L. paranigra,* have non-overlapping periods of macro production and transfer, when individuals of both species are paired at the onset of the photophase. However, timing differences to mating will only be an effective barrier if both genders possess limited temporal windows of mating activity.

In this study we investigate the gender specific time limits to mating in *L. cerasina*. We test the hypothesis that pairs are limited in the time at which they mate. To test this hypothesis, we paired males and females at progressively later points in the photoperiod to determine if there are time limits to macro production or transfer. We also test the hypothesis that males generate the rhythm of mating. In other crickets, males have a rhythm to spermatophore production; therefore we focused on males as the gender limited in sexual ability over time. To test this hypothesis, we performed

an experiment in which males were shifted out of phase to the light cycle of their potential female mates.

METHODS

L. cerasina were collected at Kalopa State Park, Hawaii (20˚02' N, 155˚26' W). At the University of Maryland all crickets were housed at 20˚ C on a 12:12 light: dark cycle in 120 ml plastic cups with damp Kimwipes. Crickets were fed *ad libitum* Fluker's Cricket Feed which was treated with Methyl Paraben (USB Corporation) to inhibit mold growth. Males and females were housed separately until they reached sexual maturity. Breeding pairs were housed together in a single cup to allow mating and oviposition into the moistened Kimwipe. Cricket nymphs were housed under the same conditions as the parental individuals and reared to sexual maturity. Virgin F_1 males and females were used for the following experiments.

Mating time limit experiment

Sexually mature pairs were placed in plastic Petri dishes with moist Kimwipes at four periods: 0, 6, 8 and 10 hours after the beginning of the photophase (12, 6, 4, 2 hours before the scotophase, respectively). Any pairs that failed to mate were re-paired at the beginning of the photophase on the following day to assess whether failure to

mate was based on the time of day or male/female incompatibility. We used only the pairs that succeeded in mating for our analyses.

A Fisher's exact test was used to compare the proportion of pairs that engaged in courtship (scored when males that produced one or more micros) and successful mating (scored when males produced and transferred the macro) between consecutive treatment groups, in order to assess whether there was a decline in the number of pairs that engaged in mating activity at successively later points in the photoperiod. A one-way ANOVA followed by a Tukey corrected multiple comparisons test was used to compare macro production time between groups.

Male phase-shift experiment

Part a: Pair establishment at the beginning of the female photophase

In order to test the hypothesis that males drive the timing of macro production, two treatments groups of males were shifted out of phase relative to the female light cycle, 5 hours earlier and 5 hours later (see Table 1). Both treatment groups were housed in incubators 14 days prior to the mating experiment on their respective light cycles. A control group of unmanipulated males were housed in the same incubator as females.

After a minimum of 14 days under the respective light regime, pairs were placed in plastic Petri dishes with moist Kimwipes within 15 min of the onset of the female photophase. Treatment group males were held in their incubators until the onset of the experiment (therefore, early-shifted males had already experienced the

onset of the photophase 5 hours earlier and late-shifted males were in their scotophase prior to the experiment; see Table 1). Mating pairs were observed from the time they were established through insemination, or until the end of the female photophase. Several mating related behaviors were observed and recorded including: micro production, macro production and transfer. This experiment was conducted on three separate days and data from each day were pooled for the analysis.

Courtship and mating behaviors were analyzed using observations from only those pairs that successfully transferred a macro. A one-way ANOVA followed by a multiple comparisons test with Tukey adjustment was used to test differences in macro production time between groups. Because males were expected to produce the macro at a specific time on their light cycle, we predicted a significant difference between the three groups in the time at which the macro is produced.

Part b: Pair establishment at the beginning of the late-shifted male photophase

Because late-shifted males were paired with females within the male scotophase in the first experiment, an additional experiment was performed to test whether pairing late-shifted males with females at the beginning of the male photophase would affect the time of macro production. Sexually mature pairs were placed together within 15 min of the beginning of the late-shifted male photophase. Control group males were included in the experiment (therefore, control males were paired 5 hours into their photophase). Mating pairs were observed from the time they were established through insemination, or until the end of the late-shifted male photophase (see Table 1). This

experiment was conducted on three separate days and data from each day were pooled for the analysis.

Student's two-tailed t-tests were used to compare macro timing between groups. Based on the hypothesis that males are driving the timing of macro production, we predicted that late-shifted males would produce macros significantly later than control males when both groups were paired at the onset of the late-shifted male photophase. We also examined the hypotheses that either light or female presence is a zeitgeber (external cue) for a male-driven daily rhythm in macro production. If late-shifted males paired at the onset of their photophase produce macros significantly later than late-shifted males paired at the onset of the female photophase, it would suggest that light or female presence plays a role in the daily rhythm of macro production.

RESULTS

Mating time limit experiment

We compared the mating activity of pairs that were set up at 4 different points in the photoperiod. There was a significant relationship between the time of pair establishment and the number of micros produced (Figure 1; $F_{3, 48} = 94.24$, $p < 0.0001$) and mean micro number was significantly different between all four treatment groups (Table 2; Tukey-Kramer HSD: $p < 0.05$). Likewise there was an increase in the number of males that failed to begin courtship (i.e. failed to produce micros) in the 10 hour group compared to the 8 hour group; however, this difference was non-significant (Table 3; Fisher exact contingency table test [one –tailed], $p = 0.13$).

There was also a significant relationship between the time of pair establishment and the time of macro production (Figure 1; $F_{3, 31} = 41.43$, $P < 0.0001$) and mean macro time was significantly different between all four treatment groups (Table 2; Tukey-Kramer HSD: $p < 0.05$). In addition, there was a significant increase in the proportion of males that failed to produce a macro in the 8 hour group compared to the 10 hour group (Fisher exact contingency table test [one –tailed], $p = 0.005$). Every male that produced a macro was successful in transferring it to the female.

Male phase-shift experiment

Part a: Pair establishment at the beginning of the female photophase

There was a significant relationship between the timing of pair establishment in the light-cycle phase and macro production time ($F_{2, 29} = 83.93$, $p < 0.0001$). All three phase groups were significantly different from one another in the mean time of macro production (Figure 2, Table 3; Tukey-Kramer HSD: $p < 0.05$). There was also a significant relationship between the timing of pair establishment in the light-cycle phase and macro transfer time ($F_{2, 26} = 89.61$, $p < 0.0001$) and all three groups were significantly different from one another in the mean time of macro transfer (Table 2; Tukey-Kramer HSD: $p < 0.05$).

Part b: Pair establishment at the beginning of the late-shifted male photophase

There was a significant difference in the time of macro production (Figure 3, Table 3; $t = 18.72$, $p < 0.0001$) and transfer (Table 3; $t = 16.41$, $p < 0.0001$) between late-shifted and control males. There was also a significant difference in the time of macro production (Table 2; $t = 7.09$, $p <0.0001$) and transfer (Table 2; $t = 7.84$, $p < 0.0001$) between late shifted-males in parts ‘a’ and ‘b’ of this male phase-shift experiment.

DISCUSSION

Timing of mating follows a regular daily cycle in many animals, often related to receptivity or physiological ability of one or both sexes. In cricket systems the circadian rhythm of premating behaviors has been suggested to regulate the timing of mating. While many crickets appear to be physiologically capable of mating whenever the opportunity arises, typical timing of mating in the field seems to correspond to the timing of female locomotory activity and male calling, which in turn affects the time of pair establishment. Previous work suggested that *Laupala* also possess species-typical mating times, with mating preceded by an unusually extensive courtship involving serial microspermatophore transfer. We have found that production of the single sperm-containing macro appears to be restricted to a narrow window of time regardless of the onset of courtship. However, as pairs were established later in the photophase, macro production time was delayed to a certain degree, possibly to facilitate micro production and transfer. We have also demonstrated that macro production time limits are based on the male light cycle and that females do not play a significant role in this timing once pairs are established. That males adjust both micro number and macro production time based on when they begin courtship suggests both factors are important to male reproductive success.

In the mating time limit experiment, we discovered that macro production was limited to a period of approximately three hours towards the end of the photophase. All pairs established early in the day, represented by the first two sample points in the photoperiod, successfully mated. However, courtship and mating activity decreased

later in the day, represented by the latter two sample points. Fifteen percent of the pairs established eight hours into the photophase and 43 % established ten hours into the photophase, did not engage in micro transfer. Of the pairs that did engage in initial courtship through micro transfer, many failed to progress to macro production and transfer. Overall, 30.8% and 85.7% of pairs established eight and ten hours into the photophase, respectively, failed to mate. Because we used only the pairs that successfully mated when reintroduced early the following day in our analyses, we are able to conclude that time of day was the primary cause for the severe reduction in mating towards the end of the photoperiod.

We found that macros were produced at a significantly later time between each successive treatment group in the mating time limit experiment, which suggests that males delayed macro production in order to produce more micros. Further evidence to support this idea is the short courtship duration by pairs established at 8 and 10 hours into the photoperiod, which indicates that at least some males are physiologically able to produce a macro within 1 to 2 hours after pairing with a female. However, pairs established at 6 hours into the photoperiod court for approximately 3 hours, which is approximately 45 minutes later than the macro production time of males paired at the onset of the photophase. Therefore, the later time at which macros are produced in the 6 hours group compared to the earliest group does not reflect a physiological constraint on how long it takes males to form a macro. Rather, these data suggest that males are delaying macro production for another purpose, such as increasing micro production. Because males appear to adjust the macro production time to accommodate micro production, this suggests that micro

number is important to male reproductive success. Micro transfer has been demonstrated to increase sperm uptake by the female (deCarvalho, Ch. 1) , therefore the number of micros transferred may affect the degree of sperm transfer enhancement.

Although males delay macro production to a certain extent when pairs are established later in the day, late pair establishment has negative consequence for the allocation of nuptial gifts. The negative relationship between the time of establishment and micro number demonstrates that males are not able to produce as many micros when they pair with females later in the day. We have observed in the current study and previous experiments (Shaw and Khine 2004) that the rate of micro production increases towards the end of the photoperiod. Males appear to reach the upper limit on micro replenishment rate after being established 6 hours into the photoperiod. This upper limit of micro production rate together with the limit on the timing of macro production precludes late paired males from producing the same number of micros as males that have paired early in the photoperiod. Therefore, if micro number is important to male reproductive success, there may be selection on males to attract females early enough in the day to accommodate adequate or optimal micro number.

The results from the male phase-shift experiment demonstrate that once pairs are established, the timing of mating in *L. cerasina* is driven by males. Males that were shifted out of phase from the female light cycle produce and transfer macros based on the timing of their own light cycle, rather than on that of the female's light cycle. In part 'a' of the experiment, males that were early-shifted by 5 hours produced

their spermatophores approximately 4 hours earlier than control males. This one hour discrepancy between the two groups is due to pair establishment for early males occurring 5 hours into their own photoperiod. In the mating time limit experiment, we observed that males established 6 hours into the photoperiod produced their macros approximately one hour later than males established at the beginning of photoperiod. Therefore, the timing of macro production for early males is what we would expect if mating activity occurs on the male and not the female light cycle. In part 'b' of the experiment, late-shifted males produced their macros approximately 5 hours later than control males, which corresponded to their 5 hour phase-shift. Therefore, we conclude that the time of macro production and transfer is entirely based on the male light cycle.

The timing of male macro production appears to be under both endogenous and exogenous control. The results from part 'a' of the male phase-shift experiment suggest that macro production may be under endogenous circadian regulation. When established at the beginning of the photophase for the control females, late-shifted males produced macros one hour later than control males, though both groups experienced the onset of light and female interactions at the same time on the day of the experiment. If exogenous cues alone controlled the timing of macro production, we would see no significant difference between these two groups, while a complete endogenous regulation would have resulted in macros of the late shifted group being produced 5 hours later than control males. The one hour difference in macro production time between the control and 5 hour late-shifted males indicates an important role for external cues, while simultaneously giving evidence for some sort

of endogenous circadian timing. This discrepancy in the time of macro production may have been an effect of rapid resetting of the internal clock by early light cues or female presence.

Knowing the gender responsible for the time of mating activity lends insight into the selection pressure for the mating period. Because macro timing is primarily male limited, we can rule out the hypothesis that the time of macro production is a consequence of female preference. Instead, there may be selection on males to mate at a certain time of day due to intra-sexual (e.g. sperm) competition. Evidence from laboratory experiments demonstrates that females usually remate the day after their first mating (deCarvalho, Ch. 1). If females begin laying eggs during the scotophase, males may benefit by delaying macro transfer until as late as possible in the photophase to prevent his partner from mating with another male before she begins oviposition. Therefore, his sperm would gain exclusive access to his partner's eggs until the next day; this effect is referred to as the first male advantage (Calos and Sakaluk, 1998). However, in the laboratory, females typically oviposit at least a few days after mating (deCarvalho, Ch. 1) , although this may not reflect female behavior under natural conditions.

Alternatively, the timing of mating could be an indirect affect of selection on the timing of other male behaviors. For example, the timing of mating and age of first reproduction are linked via genetic pleitropy in the melon fly, *Bactrocera cucurbitae* (deCarvalho, Ch. 1; Miyatake, 2002). Selection on reproductive age could play a role in the particular rhythmicity of macro production in *Laupala.* However, it would not

explain why males are restricted to producing spermatophores only during that time period, unlike other crickets.

The discovery that males only are responsible for the timing of mating activity has implications for interactions between sympatric species of *Laupala.* Danley et al (2007) discovered a significant difference between *L. cerasina* and *L. paranigra* in the timing of macro production and transfer, which was suggested to be a potential premating barrier between these two species. In *L. cerasina*, female receptivity does not appear to constrain the time when established pairs mate, which suggests that the time of day would not preclude female *L. cerasina* from accepting spermatophores from *L. paranigra* males. The average time of macro transfer for *L. paranigra* is approximately 2.5 hours later than *L. cerasina*; in this study *L. cerasina* females accepted a macro from phase-shifted males up to 7 hours later than their own species' average macro transfer time. Furthermore, *L. cerasina* females accepted macros from early phase-shifted males up to 4.75 hours earlier than the average macro transfer time of unmanipulated males. If *L. paranigra* females have a similarly wide window of receptivity, it would overlap with the time that *L. cerasina* males produce macros. Therefore, it does not appear that a shift in male macro timing would be an effective premating barrier to reproduction between these two species. However, female locomotion or mate searching may have a circadian rhythm, which might preclude females from encountering males of other species that call at different times. As Loher (1989) suggested, premating activities such as female locomotion, not female receptivity, may be temporally constrained. We are currently studying the diel rhythm of female locomotion in several sympatric species pairs. These data will shed light on

whether asynchrony in premating behaviors can be an effective barrier to reproductive isolation among species of *Laupala.*

TABLES

Table 1. Graphical representation of the photophase portion of the light/dark cycle between the control and treatment groups. The two treatment groups are phase-shifted by five hours in either direction from the control male and female light regime.

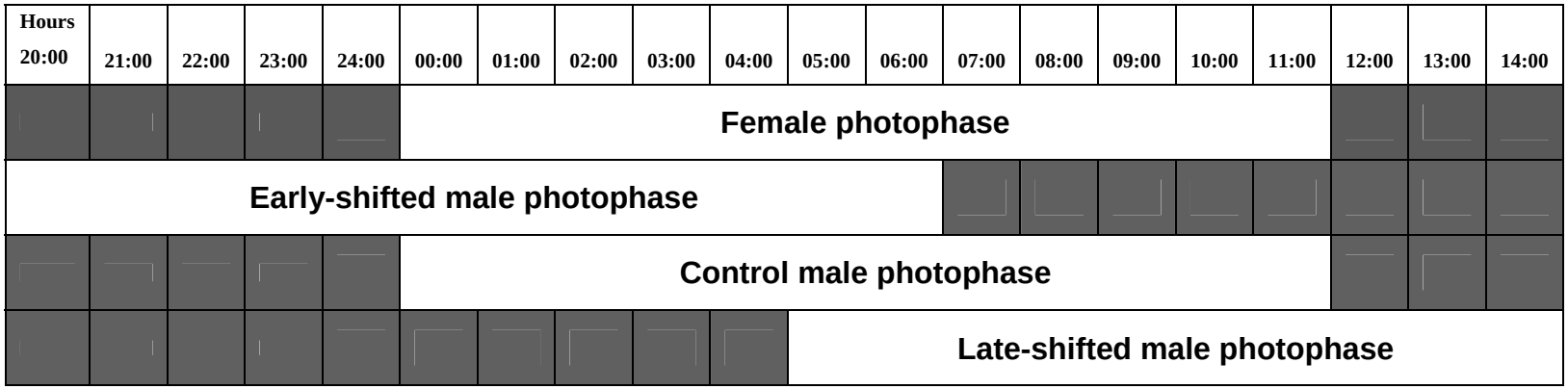


Table 2. Percentage of pairs that failed to engage in courtship (transfer of one or more micros) and percentage of pairs that failed to mate (transfer macro) for the mating time limit experiment. Mean ± standard error of micro number and macro production time (hr: mins) are presented for each treatment group. Times are the number of hours elapsed after the beginning of the photophase.

Establishment time	**% courtship failure**	**% mating failure**	**Micro number**	**Macro production time**
0 hours	None (0/11)	None (0/11)	8.0 ± 0.36	8:14 ± 00:08
6 hours	None (0/13)	None (0/13)	5.6 ± 0.25	8:54 ± 00:06
8 hours	15.4 % (2/13)	30.8 % (4/13)	2.9 ± 0.43	8:52 ± 00:07
10 hours	42.9 % (6/14)	85.7 % (12/14)	0.8 ± 0.21	10:56 ± 00:06

Table 3. Mean ± standard error of macro production and transfer time for both part 'a' and part 'b' of the phase-shift experiment are presented for each treatment group. Times are number of hours elapsed after the beginning of the female photophase. Lower sample sizes for macro transfer times compared to macro production times reflect that certain transfer observations were not precise (therefore dropped from the analyses) and that some males failed to transfer macros. Numbers in parentheses represent transfer failures.

Treatment	**Experiment**	**N**	**Macro production time**	**N**	**Macro transfer time**
Control	a: Female light cycle	13	8:02 ± 00:12	12	8:57 ± 00:10
Early-shifted	a: Female light cycle	10	4:04 ± 00:08	9 (1)	5:03 ± 00:09
Late-shifted	a: Female light cycle	9	9:17 ± 00:29	8	10:00 ± 00:27
Control	b: Late-shifted male light cycle	12	8:29 ± 00:08	9	9:40 ± 00:08
Late-shifted	b: Late-shifted male light cycle	11	13:00 ± 00:11	9 (2)	13:59 ± 00:13

FIGURE LEGENDS

Figure 1. Micro and macro production times from the mating time limit experiment. Each row consists of data from a single pair and each dot represents the production time of a spermatophore (small dot = micro; large dot = macro).

Figure 2. Micro and macro production times from male phase-shift experiment, part a: pair establishment at the beginning of the female photophase. Each row consists of data from a single pair and each dot represents the production time of a spermatophore (small dot = micro; large dot = macro). All males were paired with females at the beginning of the female photophase.

Figure 3. Micro and macro production times from male phase-shift experiment, part b: pair establishment at the beginning of the late-shifted male photophase**.** Each row consists of data from a single pair and each dot represents the production time of a spermatophore (small dot = micro; large dot = macro). All males were paired with females at the beginning of the late-shifted male photophase.

FIGURES

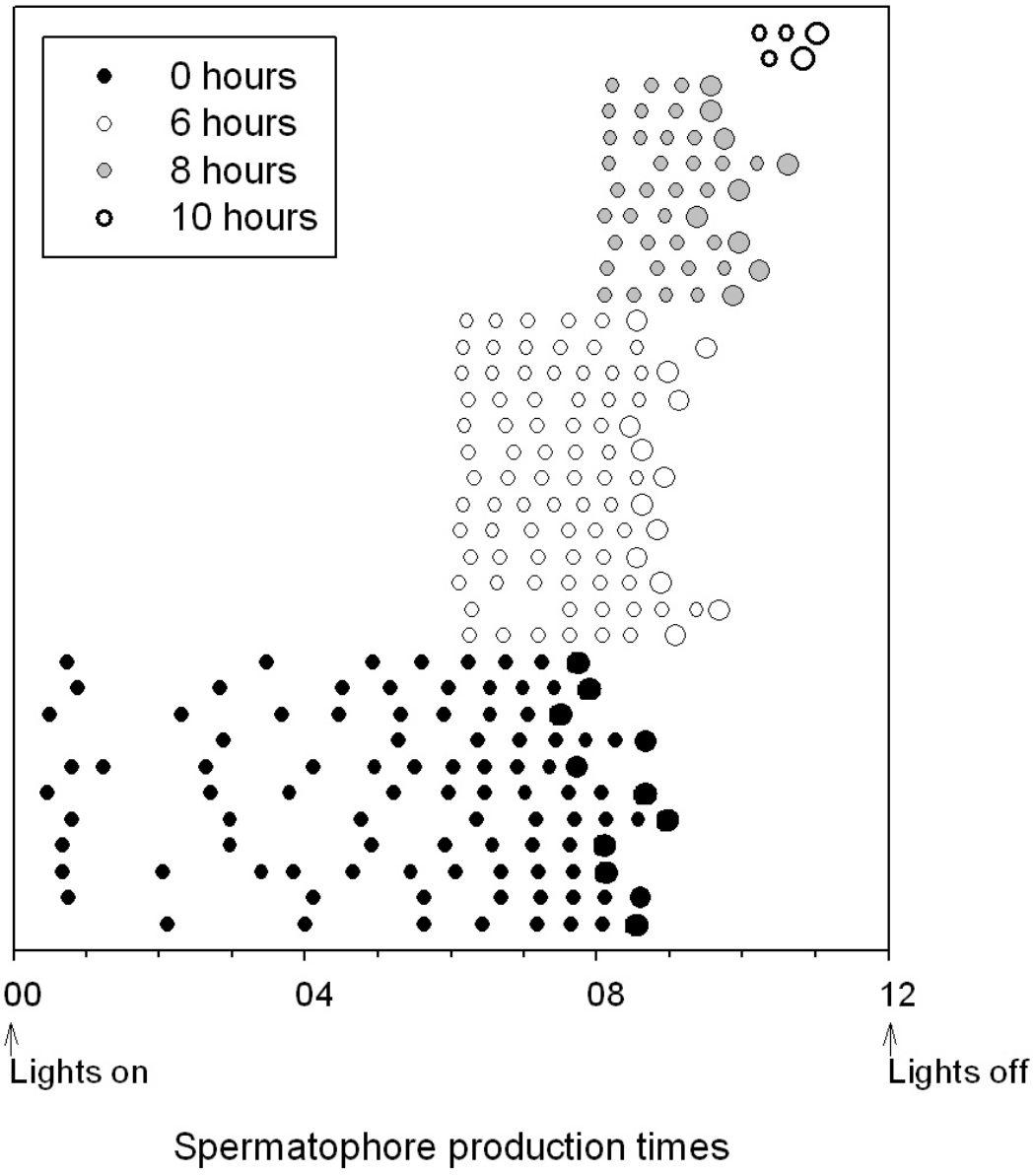


Figure 1

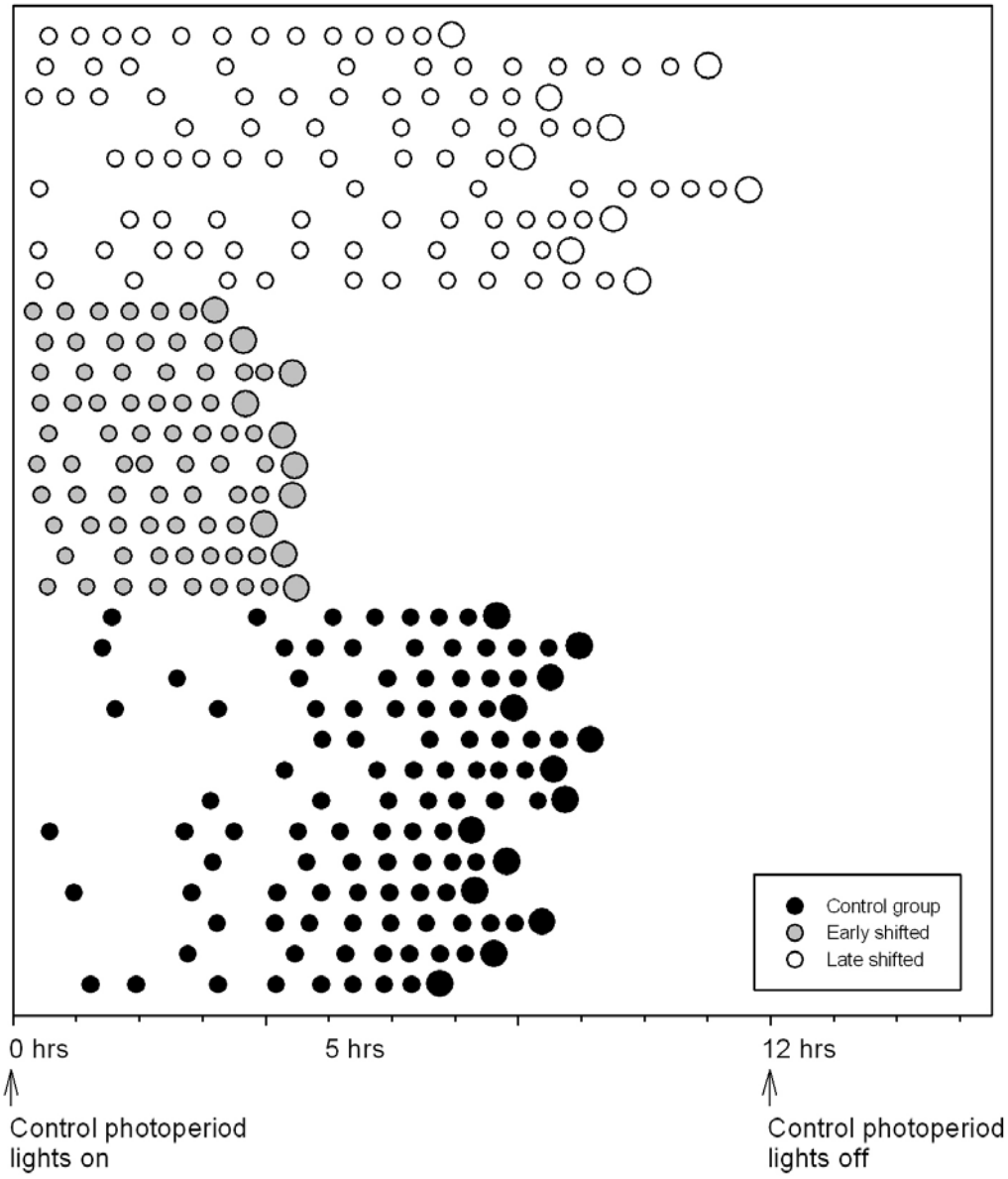

Control group
Early shifted
Late shifted
0 hrs
5 hrs
12 hrs
Control photoperiod lights on
Control photoperiod lights off
Spermatophore production time

Figure 2

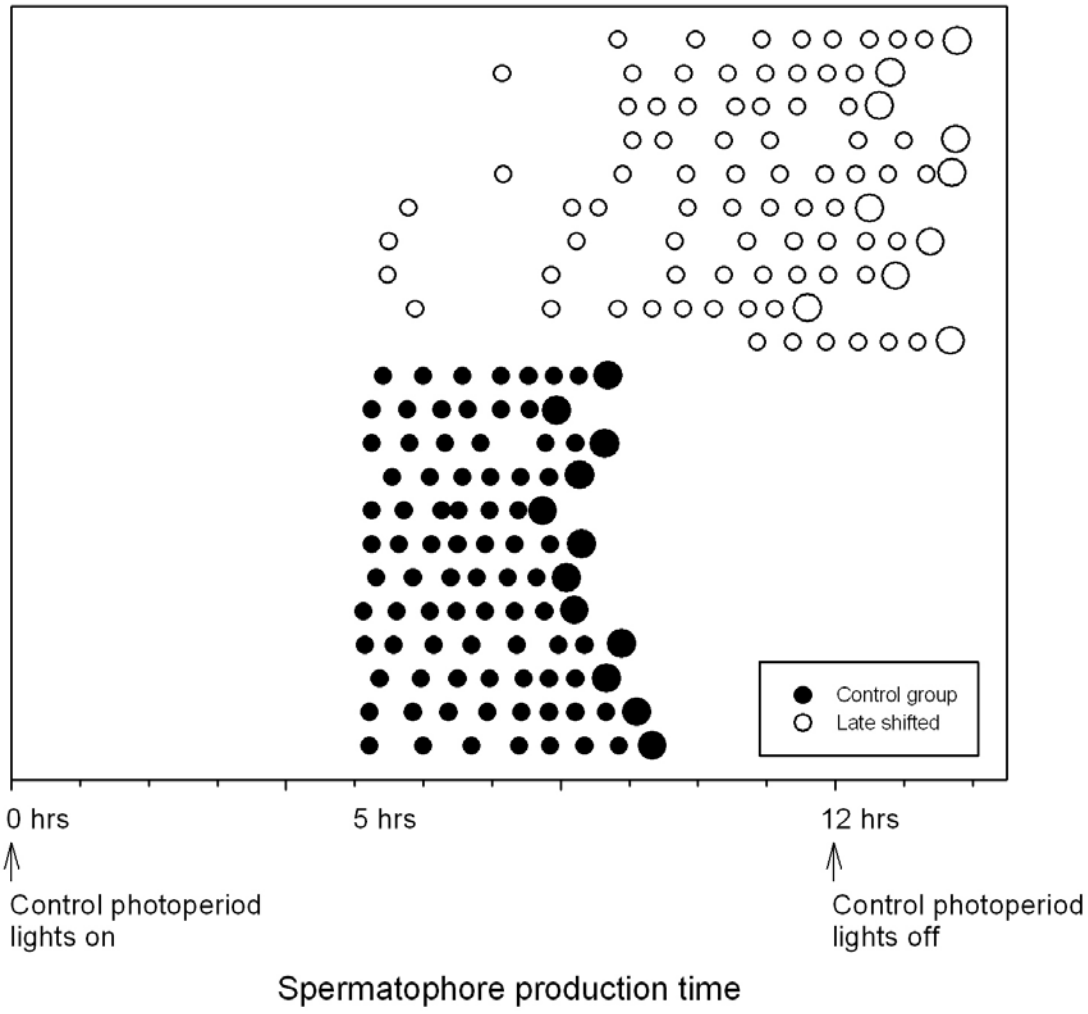

Control group
Late shifted
0 hrs
5 hrs
12 hrs
Control photoperiod
lights on
Control photoperiod
lights off
Spermatophore production time

Figure 3

CHAPTER III

A comparative analysis of Hawaiian swordtail cricket mating behavior

ABSTRACT

Crickets in the family Gryllidae are well known for their elaborate mating behaviors; however, the sexual behavior of the subfamily Trigonidiinae is poorly understood. A large portion of this clade is endemic to Hawaii, which is a speciose lineage comprised of three genera: *Trigonidium, Laupala* and *Prolaupala.* The genus *Laupala* is the only Hawaiian group in which sexual behavior has been studied and these species exhibit an unusual form of nuptial feeding. Males transfer a series of spermless 'micro' spermatophores, which are consumed by females before a larger sperm-containing 'macro' spermatophore is transferred. In *Laupala,* micro transfer has been demonstrated to increase the uptake of sperm from the macro into the female reproductive tract, which constitutes a novel mechanism of mating effort in crickets. We observed the courtship, copulation and post-copulatory behaviors of *Trigonidium* and *Prolaupala* to investigate the diversity of mating behavior strategies in the endemic swordtail cricket fauna. We also observed the behavior of an introduced genus to Hawaii, *Anele*, to serve as an outgroup for character polarity. We found that *Prolaupala* species produce micros during a behavioral sequence very similar to *Laupala.* *Trigonidium* do not produce micros, but

exhibit other behavioral strategies that likely function to improve insemination, such as prolonged copulation and post-copulatory guarding. *Anele ulia* produces micros, which suggests that a dual spermatophore system may be ancestral to the endemic fauna. We also observed behavioral characters that were previously considered unique to other crickets, such as an 'extra spermatophore component' in *T. mokuleia* and 'preliminary mounting' in an undescribed *Prolaupala* species. Behavioral diversity among the Hawaiian fauna reflects the general labile nature of gryllid mating behavior and the convergence of behavioral strategies in response to sexual selection. Furthermore, our results suggest that micro production may be a common mating effort strategy within Trigonidiinae, which warrants further investigation.

INTRODUCTION

True crickets (Orthoptera: Gryllidae) exhibit exceptional diversity in mating behavior and have been a valuable system for examining questions regarding the evolution of reproductive strategies. Much of the diversity in courtship and mating reflects different behavioral strategies by males to maximize ejaculate transfer (for reviews see Gwynne, 1997; Vahed, 1998; Zuk and Simmons, 1997). Studies of male mating effort in crickets have significantly advanced our understanding of sperm competition (Simmons, 2001), cryptic female choice (Eberhard, 1996), inter-sexual conflict (Sakaluk et al., 2006) and sensory exploitation (Sakaluk, 2000). However, compared to the large number of

classified species, relatively few mating systems have been studied in detail (but see below), which suggests that our knowledge of cricket behavioral strategies is limited.

In crickets, the typical mating sequence involves brief courtship, spermatophore transfer through copulation and post-copulatory guarding behaviors (Alexander and Otte, 1967b; Gwynne, 1995; Loher and Dambach, 1989). During courtship, the male cerci are directed towards the female. The male performs vibratory movements and sings before moving into the copulatory position. A spermatophore is transferred during copulation such that the sperm-containing portion of the spermatophore, the ampulla, remains external to the female and the sperm tube is inserted into her genital tract. After copulation, the pair engages in a stationary position, during which the male guards the female. After a period of time, females remove and consume the spermatophore. All crickets species copulate repeatedly if not interrupted, thereby transferring several spermatophores.

In their landmark paper, Alexander and Otte (1967) studied components of sexual behavior among nine subfamilies of Gryllidae and documented extensive variation between subfamilies and genera; however these characteristics did not greatly differ between closely related species. Alexander and Otte (1967) reached conclusions about the polarity of several characters. For example, in many taxa, males provide nuptial gifts that females feed upon at different points in the mating sequence including dorsal secretions, extra spermatophore components and body parts. They suggest that the male dorsal gland is an ancestral character for gryllids, which has been subsequently lost in several taxa. Copulation position also greatly varies and they suggest that the ancestral state is the female positioned above the male. While many species maintain this initial

female above position throughout the copulatory event, there are several derived copulatory positions in which males subsequently rotate backwards or upside down. Copulation duration ranges from less than one second to ninety minutes; short copulatory acts are considered to be the ancestral state. Regarding spermatophore transfer, the condition in which spermatophores are freely attached to the female's genitalia after copulation appears to be ancestral for Gryllidae. In contrast, males of some species hold the ampulla during copulation and thus the spermatophore does not remain with the female afterwards. Following the work by Alexander and Otte (1967), Gwynne examined the origins of several mating characters including female spermatophore removal behavior, post-copulatory mate guarding and nuptial feeding behavior (Gwynne, 1997). Gwynne concludes that female spermatophore consumption and male guarding behaviors are ancestral for Gryllidae. Furthermore, Gwynne (1997) concludes that male nuptial feeding adaptations arose several times within the lineage, differing with Alexander and Otte's (1967) assessment that dorsal secretions are ancestral.

Since the work of Alexander and Otte (1967) many contributions to the study of cricket sexual behavior have established functional roles to particular elements of courtship and mating. One of the most intensively studied behaviors is nuptial feeding (Gwynne, 1997; Vahed, 1998). Female polyandry is typical for crickets and male sperm competition success depends on the proportion of sperm that females use relative to other males (Simmons, 2001). Because females tend to eat spermatophores before sperm transfer is complete (Gwynne, 1997), several nuptial gifts have been studied in the context of an 'ejaculation protection' function (for review see Vahed, 1998). The ejaculate protection hypothesis predicts that nuptial feeding deters the female from

premature spermatophore removal, thereby facilitating an increase in the amount of sperm transferred (Boldyrev, 1915). Experiments on nuptial feeding behavior in gryllid taxa support this hypothesis. For example in *Gryllodes sigillatus* (subfamily Gryllinae), males provide an extra spermatophore component, the spermatophylax, which is attached to the ampulla. Spermatophylax feeding has been demonstrated to distract the female from ampulla consumption, thereby increasing sperm transfer (Sakaluk, 1984). Nuptial feeding that occurs during copulation, such as dorsal gland secretions in *Oecanthus* species, (subfamily Oecanthinae) has been demonstrated to delay female dismounting from the male after copulation (Hohorst, 1937). Females do not remove spermatophores until after they dismount, therefore dorsal feeding increases spermatophore retention and sperm transfer (Brown, 1994).

Post-copulatory mate guarding is another male adaptation thought to minimize sperm competition. Guarding is characterized by a stationary position that includes mutual antennation; males often behave aggressively toward females that attempt to prematurely disengage in antennation or remove the spermatophore (Alexander and Otte, 1967b; Loher and Dambach, 1989). There are three non-mutually exclusive hypothesis by which post-copulatory guarding behavior serves to increase sperm transfer: spermatophore (ejaculate) protection, spermatophore renewal and rival exclusion (Loher and Rence, 1978; Sakaluk, 1991). In some species, guarding increases spermatophore retention time by females, which supports the ejaculate protection hypothesis (Bateman and MacFadyen, 1999; Evans, 1988; Hockham and Vahed, 1997). Spermatophore renewal is defined as post-copulatory guarding that keeps the female near the male so that he can transfer another spermatophore. This strategy depends upon the duration of

guarding being longer or equal to the amount of time it takes a male to produce a subsequent spermatophore (Loher and Rence, 1978). Alternatively, mate guarding has been demonstrated to delay or prevent copulation attempts by rivals, thereby decreasing the risk of the spermatophore becoming prematurely dislodged and sperm competition (Bateman and MacFadyen, 1999; Sakaluk, 1991).

Little is known about the mating behaviors of most Hawaiian swordtail crickets. The Hawaiian swordtail cricket clade is a speciose group composed of three genera, *Trigonidium, Laupala* and *Prolaupala.* Hawaiian *Trigonidium* includes 134 species and is composed of two major groups, Sections A & B, which are further divided into smaller species groups (deCarvalho and Otte, 2006; Otte, 1994). *Trigonidium* species have diverse habitat preferences such as tree-foliage, bark, grasses, ferns and vines. *Laupala* includes 38 species and is composed of three species groups, Kauiensis, Pacifica and Cerasina (Otte, 1994; Shaw, 2000). *Laupala* species have similar habitat preferences and are found in leaf-litter and low-lying vegetation. *Prolaupala* includes 4 species and is the sister group to *Laupala* (Otte 1994; Shaw unpublished data). *Prolaupala* are very similar in morphology and habitat to *Laupala,* but are differentiated mainly by genitalia characteristics and song (Otte, 1994). *Anele* is a monotypic swordtail cricket genus which inhabits non-native vegetation at roadside locations on Maui (Otte et al., 2003) and Kauai (deCarvalho & Otte, 2006). The phylogenetic relationship of *Anele* to other Hawaiian trigonidiines has yet to be formally investigated, but it is considered an introduced species (Otte et al., 2003).

A few *Laupala* species have been observed to display unusually elaborate mating sequences including the serial transfer of spermless ‘micro’ spermatophores (Shaw and

Khine, 2004; Shaw and Lugo, 2001). Courtship involves a relatively stationary face-to-face position, during which a micro is formed. When a male is ready to transfer the micro, he sings and performs 'pumping' (a body movement that consists of rapidly lowering and elevating the body); micros are transferred to the female via copulation. After a period of post-copulatory guarding during which the male extrudes another micro, the female removes the transferred micro and consumes it. Courtship, copulation and post-copulatory behaviors are repeated over the course of several hours during which time 2 to 16 micros may be transferred, depending on the species and time of courtship initiation (deCarvalho & Shaw, unpublished data; deCarvalho & Shaw 2005, Shaw & Khine 2004). The sequence of micro transfer ends when the male produces and transfers a single 'macro' spermatophore, which is approximately three times larger in diameter than the preceding micros and contains sperm (deCarvalho and Shaw, 2005; Shaw and Khine, 2004). Mating is followed by post-copulatory guarding and female consumption of the macro.

In *Laupala cerasina*, micro transfer has been demonstrated to enhance sperm flow from the macro into the female reproductive tract, which is a novel mechanism to increase sperm transfer in crickets (deCarvalho & Shaw). Although it is unknown whether the copulatory act or the consumption of micros stimulates the female to take in more sperm, micro transfer could be a strategy that other species employ to improve fertilization success. Only one other cricket species is known to produce spermless micros, *Nemobius* sylvestris (subfamily Nemobiinae) (Campan and Demai, 1983). However, it is unclear whether micro production is an uncommon strategy or just

underrepresented in study. Prior to the present work, few trigonidiine species have been observed to mate (Alexander and Otte, 1967b; Evans, 1988; Ingrisch, 1977).

Here, for the first time, we document some of the behavioral variation among species of endemic Hawaiian swordtail crickets. We describe courtship, copulation and post-copulatory behaviors, as well as spermatophore size and presence or absence of sperm in the spermatophore. We sampled across the breadth of the endemic taxa, including species from *Prolaupala* and the A and B lineages of *Trigonidium.* We also observed the mating behavior of the introduced species, *Anele ulia,* to serve as an outgroup to infer character evolution. In light of Alexander and Otte's (1967) observations and the relatively young age of the radiation, we expected to see divergence between genera, but little variation between species. In certain species that performed post-copulatory mate-guarding behaviors, we tested the spermatophore protection hypothesis of mate guarding. We interpreted our observations of other behaviors in light of the functional hypotheses, where possible.

METHODS

Collection localities

Crickets were collected during the day by locating singing males and sweeping a net through vegetation. Collection localities and dates are summarized in Table 1. Figure 1 illustrates the relationships of taxonomic groups included in this study.

Laboratory culture and behavior trials

Both nymphs and mature individuals were transported to the University of Maryland for study. Crickets were contained in plastic specimen cups lined with moist paper towels and Kimwipe tissues and maintained on a diet of Fluker's Cricket Feed at 20˚ C on a 12:12 light/dark cycle. Prior to their final molt, nymphs were isolated to same-sex containers to preserve virginity for mating experiments. In most cases, virgin adults were used for experiments. A smaller number of wild-caught adults were also used. In order to allow wild-caught males to maximize their sperm reserves and increase receptivity of previously mated females, adults were not mated for at least two weeks after capture.

For each mating trial, single male and female crickets were placed into a plastic petri dish containing a moist Kimwipe at the bottom. Pairs were introduced to each other approximately 3 hours after the onset of the laboratory photophase. Mating was observed under fluorescent light. Pairs were retained together for at least 6 hours or until mating

ceased. Cessation of mating was determined by the lack of antennal contact by both partners for at least an hour.

We observed the courtship, copulation and post-copulatory stages of the mating sequence. During courtship, the following variables were recorded: (1) male and female body position during antennal contact; (2) presence of 'pumping' or other body vibrations; (3) presence of song before spermatophore transfer. The position and duration of copulation was recorded. The duration of the following post-copulatory behaviors were recorded: (1) mate guarding; (2) spermatophore attachment. We also report the number of spermatophores that males produce and note how many were successfully transferred.

Multiple pairs were observed simultaneously and behaviors were scored during direct observation. A few of the mating pairs were not directly observed and their behavior was recorded using a Sony Handycam Hi8 analog video camera. Occasionally, the precise time of behavioral events could not be determined from the videotape (mainly due to the position of the crickets) and these events were not included in descriptive statistics.

Spermatophore analysis

Spermatophores were obtained from mated pairs placed together a few days after their observed mating trials. To acquire spermatophores, males were stopped as they attempted to back under females into the copulation position. Subjects were cold anesthetized at -20 degrees in a freezer and spermatophores were carefully removed with forceps under a

dissecting microscope. Spermatophores were measured under a dissection microscope using the eyepiece reticle. Diameter measurements were taken at the widest point of the ampulla. Digital photographs of spermatophores for figures were taken with a JVC TK-1280U color video camera mounted to a Leica MZ 8 light stereomicroscope or an Olympus CH2 light microscope.

Spermatophores were crushed in 10 µl of PBS buffer (pH 7.4) on a glass slide and examined under an Olympus CH2 light microscope at 200X and 400X for sperm presence. If sperm cells were not visible under the light microscope, we continued the examination by using fluorescent microscopy to verify that sperm cells were absent. We prepared the dye solution by adding a 1:10 ratio of propidium iodide (kit L–7011; Molecular Probes) to PBS buffer (pH 7.4). We added 10 µl of the dye solution directly to the sample and examined at 200X and 400X under ultraviolet light using a Nikon Eclipse E600 microscope fitted with two fluorescence filter cubes (Nikon B-2E/C and G-2E/C).

Character evolution

To examine the direction of character state change among the endemic fauna, we constructed a character matrix of discrete behavioral and spermatophore characters. *Anele ulia* character states were coded as ancestral. We present the character state codes alongside Otte's (1994) hypothesized relationships and make inferences at which nodes the character state changes may have occurred.

RESULTS

The mating behavior of *Prolaupala*

The mating sequence in both *Prolaupala* species were similar to each other, and to the sister genus *Laupala*. *Prolaupala* males courted females in the face-to-face position during which a spermatophore was extruded; males sang and performed pumping behavior when ready to back into the copulatory position. Copulation position consisted of the female above the male. After a period of post-copulatory guarding, the female ate the spermatophore and pairs returned to courtship after a variable period of time. Males transferred a series of micros before one final macrospermatophore. Unlike *Laupala*, males did not extrude a successive spermatophore during the post-copulatory guarding phase; rather, their spermatophores were formed during the next courtship phase. Copulation duration and post-copulatory guarding position also was divergent from *Laupala.* Spermatophore production number, post-copulatory guarding duration and female spermatophore removal times are summarized in Table 2. We describe particular details and species-specific behaviors below.

P. kukui (4 pairs)

The male produced a micro while either in the face-to-face position or after separating from the stationary position. To transfer the micro, the male quickly backed under the female and genitalia were coupled for less than one second. After copulation, the female walked off of the male's body and moved around the viewing dish while the male

followed. When the female became stationary, the male positioned himself behind or to the side of the female.

Prolaupala undescribed species **(4 pairs)**

Courtship and mating behaviors were very similar to *P. kukui*, with the exception that *Prolaupala sp.* males also perform a "preliminary mounting" before producing the initial micro. Preliminary mounting consisted of the male moving into the copulation position much slower than during an actual copulation; however genitalia were never coupled. After preliminary mounting, the male produced a micro within one minute. Also different from *P. kukui* was the absence of post-copulatory guarding.

The mating behavior of *Trigonidium* lineage A

Like the other endemic genera, males in this lineage courted females in a face-to-face position, during which a spermatophore was extruded. In contrast to the other genera, both males and females performed pumping behaviors during the stationary courtship position or in the absence of antennal contact. Like other endemic genera, males sang and performed pumping behavior when they were ready to back into the copulatory position, which consisted of female above the male. The males of most species (but not all) engaged in a face-to-face post-copulatory guarding position, after which the female consumed the spermatophore. However, males did not extrude a spermatophore until the next courtship phase like *Prolaupala.* Unlike the *Prolaupala/Laupala* clade, male *Trigonidium* A did not produce micros and instead transferred a series of uniformly sized

spermatophores during a rapid copulation of 2-3 seconds. Spermatophore production number, post-copulatory guarding duration and female spermatophore removal times are summarized in Table 2. We describe particular details and species-specific behaviors below.

T. mokuleia **(6 pairs)**

After spermatophore transfer, the female walked forward off of the male's back and began to wander around the viewing dish. The male performed vigorous pumping to engage the female in the face-to-face position, but the female did not always allow the male to guard her (the inconsistent presence of guarding was observed in all pairs). Guarded females retained the spermatophore significantly longer than unguarded females (Table 2; student's $t = 1.92$, $p = 0.04$, one tail).

T. haawina **(1 pair)**

Similar to *T. mokuleia,* the male and female engaged in post-copulatory guarding; though the male did not guard the female after every spermatophore transfer. The female retained the spermatophore longer when guarded, compared to when she was unguarded (Table 2).

T. paraspilos **(1 pair)**

The male did not successfully transfer the first two spermatophores, due to a lack of female receptivity to copulation. The third spermatophore was successfully transferred and post-copulatory guarding followed.

T. spilos **(1 pair)**

This pair did not exhibit any post-copulatory guarding behaviors.

T. kukui **(2 pairs)**

This species exhibited a different courtship position than the other endemic species, in which the female faced the male's posterior while the initial spermatophore was extruded. However, the male and female also engaged in the face-to-face position. After spermatophore transfer, males did not guard and the female ate the spermatophore within 1 minute. Though both males produced 7 spermatophores, only one and three spermatophores were successfully transferred between each pair, respectively.

T. kewai **(1 pair)**

There were no post-copulatory guarding behaviors. Similar to *T. kukui*, the female ate spermatophores quickly after transfer (female ate the spermatophores <1 minute, except for one instance in which the spermatophore was retained for 4 minutes).

The mating behavior of *Trigonidium* lineage B

Like the other endemic genera, males in this lineage courted and formed a spermatophore in a face-to-face position. Males also sang and performed pumping behavior when preparing to back into the copulatory position. Unlike the other endemic groups, copulation resulted in an end-to-end position and the spermatophore ampulla was

retained by the male. These species produced fewer uniformly sized spermatophores like the A lineage and did not engage in post-copulatory mate guarding. Spermatophore production number is summarized in Table 2. We describe particular details and species-specific behaviors below.

T. napau **(5 pairs)**

Copulation began by the male backing underneath the female. Females then moved forward and the male was pulled behind her until they were positioned end to end. The male often thrust towards the female during copulation. Copulation duration was variable, the male and female remained together for 9.8 minutes, on average (SD = 2.7, n=7). To separate, the male and female walked away from each other, during which the male retained the ampulla and pulled the sperm tube from the female. The process of sperm tube removal usually took a few seconds, although in one instance it took 5 minutes for full removal. After separation, the male removed the spermatophore with his hind leg and consumed it.

In contrast to the relatively long copulations described above, two copulations were exceptionally brief and genitalia remained coupled for only a few seconds. In these two circumstances, the female pulled away from the male immediately. After one of these copulations, the spermatophore remained with the female and she immediately consumed it.

T. manuka **(1 pair)**

Courtship and copulation behaviors were similar to *T. napau.* Only one spermatophore was transferred and the male and female remained in copula for 26 minutes. Sperm tube removal took approximately one minute; the male removed the spermatophore with his hind leg and consumed it.

The mating behavior of *Anele ulia* (6 pairs)

The male and female engaged in a back-to-back position during courtship, during which the male intermittently drummed the substrate with his palpi. Like the endemic fauna, males formed a spermatophore in the stationary courtship position. Males sang and performed pumping to signal their readiness to copulate. The position of copulation was like other endemic species, with the female above the male. The duration of copulation was similar to *Trigonidium* lineage A, in that it took several seconds. After copulation, the male walked out from underneath the female. The female turned around to resume the back-to-back position. Post-copulatory guarding appeared to be maintained until the next spermatophore transfer, during which the female typically remained motionless except to remove and eat the spermatophore. However, males were more active than females and occasionally repositioned themselves; therefore it was difficult to assess whether post-copulatory guarding concluded and a new courtship phase began or if guarding was continuous. Males produced a single micro before a single macro and this sequence was usually repeated. Spermatophore production number and female spermatophore removal times are summarized in Table 2.

Spermatophore characteristics

The micros of *P. kukui* and *P. sp*. did not contain sperm and were approximately three times smaller in diameter than macros (Figure 2). *A. ulia* micros did not contain sperm and were approximately 2.5 times smaller in diameter than macros (Figure 2).

All *Trigonidium* spermatophores contained sperm and sequential spermatophores were of relatively uniform size, therefore we do not report measurements. Unlike other endemic species, the spermatophores of *T. mokuleia* appeared to have an extra spermatophore. When the male extruded the spermatophore, his genitalia were surrounded by gelatinous material (illustrated with video frame, Figure 2), but the material was not present on the spermatophore after copulation when removed from the female by the experimenter (Figure 2). The extra material appeared to remain on the male.

Character evolution

Discrete courtship, copulation, post-copulatory and spermatophore characteristics are presented in a character matrix (Table 3). Pumping and singing behaviors exhibited before copulation are present in all taxa; therefore, they were excluded from the matrix. Figure 3 illustrates the hypothesized relationships among the endemic fauna with all of the character state codes presented next to each species. On Figure 3, we have labeled the nodes where character state changes may have occurred for certain characters. Figure 4 illustrates the direction of character state change for spermatophore size (micro/macro or

uniform) based on using two taxa that exhibit uniform-sized spermatophores as the ancestral condition.

DISCUSSION

We discovered substantial variation between species, as well as among genera, a finding we did not expect based on 1) the close phylogenetic relationships of the endemic Hawaiian trigonidiine fauna, and 2) commensurate levels of phylogenetic diversity and the associated, relatively low, levels of behavioral diversity reported in Alexander and Otte's (1967) seminal paper. On the generic level, *Trigonidium* and the *Prolaupala/Laupala* clade were found to exhibit major divergence in spermatophore type. Within *Trigonidium,* the two main species groups exhibit divergence in several aspects of copulatory behaviors. We also discovered new characters within genera, such as the extra spermatophore component in *Trigonidium* and preliminary mounting in *Prolaupala.* Many of the derived character states and new characters that have originated within the Hawaiian lineage have also been observed in other gryllid taxa.

We found that the fauna endemic to Hawaii possess similar courtship behaviors. All three genera share a derived courtship position, in which the male stands face-to-face with the female, except for *T. kukui* which exhibits the ancestral position. In the stationary courtship position, endemic males form the spermatophore, unlike many other gryllids where the spermatophore is usually formed before contact with the female (Loher and Dambach, 1989). Males of all species perform vibratory communication (pumping) and sing when they are ready to transfer the spermatophore. In *Trigonidium* lineage A,

females also perform pumping behaviors, which appears to be in response to male pumping. In contrast to the endemic species, *A. ulia* have a different courtship position which is derived for Gryllidae. The pairs stand back-to-back and in this position, the male extrudes a spermatophore and palpates the substrate.

The type of spermatophores that males produce differs between *Trigonidium* and the *Prolaupala/Laupala clade*. In *Trigonidium,* males produce only a single size of spermatophore that contains sperm and that usually lacks extra components. T. *mokuleia* was an exception; males produce an extra-spermatophore component in the form of a gelatinous material. A similar type of extra-spermatophore component has been observed in the Australian trigonidiine, *B. gidya* (Evans, 1988). In *T. mokuleia*, the extra material appeared to remain on the male, though its placement after copulation was not thoroughly investigated. In contrast to *Trigonidium*, *Prolaupala* and *Laupala* both produce multiple derived, spermless 'micro' spermatophores before a single 'macro' spermatophore. The macro appears to be similar to *Trigonidium* spermatophores, in relative size and shape.

Because we lack information on the relationships between trigonidiine genera, it is difficult to assess the polarity of micro production in regard to the Hawaiian fauna. *A. ulia* produces micros and their spermatophore system may represent the ancestral condition for the endemic fauna and, if so, the uniform size spermatophores produced by Hawaiian *Trigonidium* are derived. Alternatively, *Anele ulia* could have an independently derived microspermatophore system from the endemic Hawaiian fauna. *Anele's* spermatophore production sequence differs from *Prolaupala/Laupala*, which suggests that this character state may not be homologous. *Anele* males produce a single micro before a single macro and repeat the sequence, which is similar to the spermatophore

production sequence of a North American species of trigonidiine, *Phyllopalpus pulchellus* (Dave Funk, unpublished data). The micro/macro spermatophore system is absent in other trigonidiine species for which there are published observations: *Balamara gidya* (Evans, 1988), *Crytoxipha columbiana* (Alexander and Otte, 1967b), and *Trigonidium cincindeloides* (Ingrisch, 1977). When the uniform-sized spermatophores are used to represent the ancestral condition for the endemic Hawaiian fauna, the simplest pattern of character evolution indicates that micros are derived independently in each; *Anele* and *Prolaupala/Laupala.* Under this scenario, the micro/macrospermatophore system has arisen multiple times within Gryllidae.

In regard to spermatophore transfer, the endemic fauna differ in copulation duration, position and the manner of spermatophore attachment. *Anele ulia* and most endemic species retain the ancestral characteristics for the family Gryllidae, with the female mounted above the male and the spermatophore ampulla directly attached to the female. However, the duration of copulation varies among species. *Laupala* have relatively long copulation duration, in which micro transfer occurs in approximately one minute and macro transfer is twice as long in duration. In *Prolaupala*, the duration of copulation for the transfer of both the micro and macro does not appear to be bimodal and occurs in less than a second. In the middle of this range, *Trigonidium* lineage A copulation lasts for several seconds. Diverging from the copulation characteristics of the A lineage, pairs in the B lineage engage in prolonged copulation in which the pair is positioned end-to-end. The average copulation duration across both B species is approximately 18 minutes. Males retain the spermatophore ampulla; therefore the male removes and eats the spermatophore after copulation, rather than the female. Prolonged

copulation in which the male retains the spermatophore is also evident in the genus *Amphiacusta* (subfamily Phalangopsinae), although the crickets in this genus retain the ancestral copulation position for Gryllidae (Alexander and Otte, 1967b).

In addition to copulation, the undescribed *Prolaupala* species exhibits a derived behavior that we refer to as 'preliminary mounting'. This behavior precedes the production of spermatophores. A similar behavior has been observed in *Pteronemobius* (subfamily Nemobiinae); pairs briefly copulate without spermatophore transfer approximately an hour before a relatively prolonged copulation that includes spermatophore transfer (Mays, 1971). However, unlike *Pteronemobius*, *P. undescribed sp.* males move into the copulation position but do not copulate.

Mate-guarding is considered an ancestral character for Gryllidae (Gwynne, 1997) which *Anele ulia* and certain endemic species have retained. *A. ulia* utilizes a derived post-copulatory position for Gryllidae in which the male and female are positioned back-to-back. Of the endemic species that engage in post-copulatory guarding, all exhibit the face-to-face guarding position, which is the ancestral character state for Gryllidae (Alexander and Otte, 1967b), but considered derived when compared to *A. ulia.* In *Laupala,* males always engage in post-copulatory guarding after spermatophore transfer. In contrast, some of the *Trigonidium* A species that display mate guarding, such as *T. mokuleia* and *T. haawina,* are not always successful at engaging females after spermatophore transfer, therefore guarding behavior is not consistently present. Several *Trigonidium* A species and all B species have lost post-copulatory mate guarding behaviors.

The Hawaiian fauna appear to use different behavioral strategies as ejaculate protection mechanisms. If a behavior functions as ejaculate protection, we would expect it to prolong spermatophore retention and not co-occur with other ejaculate protection mechanisms (Gwynne, 1997). Furthermore, we might also expect to see a reduction in the transfer of spermatophores, because multiple spermatophore transfer has been proposed to compensate for premature spermatophore removal by female.

The combination of several mating characteristics suggests that the derived copulation behaviors of the *Trigonidium* B lineage function as ejaculate protection. In the two species observed, pairs engage in prolonged copulation during which the male holds the spermatophore ampulla. The fact that males remove the spermatophore sperm tube directly after copulation indicates that sperm transfer must occur during copulation. Females are not able to remove spermatophores while in copula; therefore the ejaculate is protected during copulation. *Trigonidium* males do not provide nuptial gifts to delay female decoupling like certain other gryllids (Bidochka and Snedden, 1985); therefore some aspect of this derived position may allow males to exert physical control over females. The absence of post-copulatory guarding further supports the ejaculate protection hypothesis in lineage B *Trigonidium*. Additionally, a reduction in spermatophore number is associated with these derived copulatory characteristics. The average spermatophore number of lineage B males was 1.5, which is much lower than the average number of spermatophores transferred in the A lineage (5.9).

Post-copulatory mate guarding also appears to function as ejaculate protection in the endemic fauna. We found evidence to support this hypothesis on both the individual and species level. Among the *Trigonidium* Section A species, most Oahu species exhibit

mate guarding, although males do not guard females after every spermatophore transfer. Within individual pairs wherein males only guarded females after some copulations, males appeared to delay female spermatophore removal during successful guarding bouts. For example, in *T. mokuleia,* when females were guarded they retained their spermatophores significantly longer than when males failed to guard them, which is consistent with the ejaculate protection hypothesis. Likewise in *T. haawina,* the female retained spermatophores approximately 4 times longer when she was guarded. Comparing spermatophore number between Oahu and Maui clades, Oahu species produced fewer spermatophores than Maui species (on average, 3.8 versus 8.0, respectively), which suggests that mate guarding increases the effectiveness of sperm transfer, as we observed that Maui species generally lack mate-guarding. Concomitantly, Maui females exhibit an extremely short spermatophore retention time (< 1 minute, on average), which may force males to transfer more spermatophores, similar to *Ornebius aperta*, in which males transfer an extreme number of spermatophores to compensate for the rapid removal of spermatophores by females (Laird et al., 2004).

Two alternative hypotheses exist for the function of mate-guarding, rival exclusion and spermatophore replenishment. We were not able to examine the rival exclusion hypothesis with our data. However, we were able to reject the spermatophore replenishment hypothesis for the function of mate-guarding in the endemic species. Support for this hypothesis requires that the duration of guarding being longer or equal to the amount of time it takes a male to produce a subsequent spermatophore (Loher and Rence, 1978). In *Trigonidium* and *Prolaupala*, spermatophores are produced by males after guarding; therefore the spermatophore renewal function is not applicable. *Laupala*

males produce a spermatophore while in the mate-guarding phase; however guarding ends well before males are ready to transfer the spermatophore (Shaw and Khine, 2004). Unlike the endemic fauna, *A. ulia* males appear to guard females during the entire inter-copulatory interval, therefore mate guarding may facilitate spermatophore renewal in this system.

Another behavioral strategy to improve insemination is referred to as 'sperm transfer enhancement' (deCarvalho, Ch. 1). In *Laupala,* micros are considered a sperm transfer enhancement strategy because their transfer stimulates greater sperm uptake from the sperm-containing macro (deCarvalho, Ch. 1). Micro transfer may have a similar function in *Prolaupala* and *Anele*, although we were not able to evaluate the sperm transfer enhancement hypothesis with our data. However, the extreme reduction in the number of sperm-containing spermatophores in a given mating bout is a common feature to all genera that utilize micros, which suggests that micros increase insemination success in *Prolaupala* and *Anele*. Enhanced sperm transfer may have allowed males to decrease mating costs via reducing sperm allocation to 1-2 macros. In the *Prolaupala/Laupala* clade, one micro is approximately $1/27^{th}$ the volume of a macro, therefore a typical number of micros constitutes less than half the material of a single macro.

An alternative, though not mutually exclusive, hypothesis for micro function is a test of female receptivity (Shaw and Khine, 2004). In *Laupala*, if a male fails to transfer several micros; he usually does not proceed with macro production (personal observation). Females will often allow a male to court, but then reject the male as he tries to move into the copulatory position to transfer a micro. Thus, through micro transfer attempts, the male may be able to assess female receptivity to accept the more costly

sperm-containing spermatophore. Mays (1971) suggested that micro transfer in *N. sylvestris* and preliminary mounting *Pteronemobius* may be necessary for the production of a sperm-containing spermatophore. He considered preliminary mounting in *Pteronemobius* a derivative of the closely related *N. sylvestris* micro/macro spermatophore system. We observed a similar pattern of character evolution in *Prolaupala,* in which *P. kukui* exhibits micro production and *P. undescribed s*p. exhibits preliminary mounting before micro production. In *Prolaupala,* both micros and preliminary mounting may function to test a female's receptivity, which is not mutually exclusive with the sperm transfer enhancement function of micros.

It is clear that mating behaviors within Gryllidae are relatively labile and species respond to sexual selection with convergent adaptations. We found that within the Hawaiian clade and between other gryllids, certain aspects of cricket mating behaviors appear to be relatively variable, such as the position and duration of copulation and the presence of post-copulatory guarding. We also observed behaviors that were previously considered relatively unusual or unique in other crickets, such as the preliminary mounting in *Prolaupala sp.* and the gelatinous extra spermatophore component in *T. mokuleia*. We also found that the trigonidiines of Hawaii utilize behavioral strategies that serve as ejaculate protection in other gryllid species, such as mate-guarding and prolonged copulation. The presence of micros in multiple genera suggests that sperm transfer enhancement may also be a common and important behavioral strategy to increase insemination success. Our findings reveal an excellent opportunity to investigate the sperm enhancement hypothesis in other species through both an experimental and comparative framework.

TABLES

Table 1. The collection locality for each species included in this study.

Species	**Island**	**Locality**
Anele ulia	Maui Kauai	Hana Highway, mile 5.5 Kuamo`o Road, near Arboretum
Prolaupala kukui	Maui	Pu`u Kukui Watershed Preserve
Prolaupala undescribed	Maui	Palika Peak, Kipahulu Valley
Trigonidium mokuleia	Oahu	Mokuleia Trail, Pahole Natural Area Reserve
Trigonidium paraspilos	Oahu	Kolowalu Trail, Mount Tantalus
Trigonidium spilos	Oahu	Kolowalu Trail, Mount Tantalus
Trigonidium haawina	Oahu	Pupukea
Trigonidium kukui	Maui	Pu`u Kukui Watershed Preserve
Trigonidium kewai	Maui	Hana Highway, mile 8.1
Trigonidium napau	Hawaii	Napau Trail, Volcanoes National Park
Trigonidium manuka	Hawaii	Manuka

Table 2. The mean ± standard error of spermatophore production number, post-copulatory guarding duration and female spermatophore retention duration are presented for each species.

Species	**N pairs**	**Spermatophore number**	**N obs**	**Guarding duration (mins)**	**N obs**	**Spermatophore retention (mins)**
A. ulia	6	Micro 1.8 ± 0.8 Macro 1.4 ± 0.5	5 7	†	4 7	Micro 34.5 ± 19.0 Macro 45.6 ± 3.3
P. kukui	4	Micro 4.8 ± 0.5 Macro 1	9 1	Micro 9.5 ± 9.6 Macro >53	8 1	Micro 21.8 ± 5.5 Macro > 53
P. sp.	4	Micro 4.5 ± 0.6 Macro 1	ϕ	ϕ	3 3	Micro 17.6 ± 4.2 Macro 55.0 ± 20.5
T. mokuleia	6	5.3 ± 1.0	16	20.4 ± 15.9	7 5	Guarded 21.0 ± 15.7 Unguarded 6.6 ± 6.3
T. paraspilos	1	3	1	3	1	4
T. spilos	1	3	ϕ	ϕ	2	14.0 ± 8.5
T. haawina	1	4	3	31.3 ± 6.5	3 1	Guarded 36.3 ± 9.6 Unguarded 4
T. kukui	2	7.0 ± 0.0	ϕ	ϕ	4	>1
T. kewai	1	9	ϕ	ϕ	6	>4
T. napau	5	2.0 ± 0.7	ϕ	ϕ	ϕ	ϕ
T. manuka	1	1	ϕ	ϕ	ϕ	ϕ

ϕ Variable of interest absent from the species behavioral repertoire
† Duration of behavior difficult to establish, qualitative description in results section.

Table 3. Character matrix for endemic taxa observed in this study. Only characters that exhibited variable characters states are included. Each character state was assigned a polarity code based on *A. ulia* representing the ancestral character states (0 = ancestral, 1 = derived, 2 = derived).

Species	**1 Courtship position (♂ to ♀)**	**2 Sequential spermatophore sizes**	**3 Extra spermatophore material**	**4 Copulation position**	**5 Copulation duration**	**6 Ampulla attachment**	**7 Post-copulatory guarding**
A. ulia	0, Back-to-back	0, Dual	0, Absent	0, ♀ above	0, Short	0, Attached to ♀	0, Present
P. kukui	1, Face-to-face	0, Dual	0, Absent	0, ♀ above	0, Short	0, Attached to ♀	0, Present
P. sp.	1, Face-to-face	0, Dual	0, Absent	0, ♀ above	0, Short	0, Attached to ♀	1, Absent
T. mokuleia	1, Face-to-face	2, Uniform	1, Present	0, ♀ above	0, Short	0, Attached to ♀	0, Present
T. paraspilos	1, Face-to-face	1, Uniform	0, Absent	0, ♀ above	0, Short	0, Attached to ♀	0, Present
T. spilos	1, Face-to-face	1, Uniform	0, Absent	0, ♀ above	0, Short	0, Attached to ♀	1, Absent
T. haawina	1, Face-to-face	1, Uniform	0, Absent	0, ♀ above	0, Short	0, Attached to ♀	0, Present
T. kukui	2, Back-to-face	1, Uniform	0, Absent	0, ♀ above	0, Short	0, Attached to ♀	1, Absent
T. kewai	1, Face-to-face	1, Uniform	0, Absent	0, ♀ above	0, Short	0, Attached to ♀	1, Absent
T. napau	1, Face-to-face	1, Uniform	0, Absent	1, End-to-end	1, Long	1, Held by ♂	1, Absent
T. manuka	1, Face-to-face	1, Uniform	0, Absent	1, End-to-end	1, Long	1, Held by ♂	1, Absent

FIGURE LEGENDS

Figure 1. Otte's (1994) hypothesized relationships among the endemic swordtail cricket taxa included in this study. Relationships were based on morphological characters such as head and pronotum coloration; wing color; wing and file size; leg coloration and size and genitalia.

Figure 2. Spermatophore ampulla photographs and range of diameter measurements. a) *P. kukui* micro (n = 2; 0.30 mm) and macro (n = 2; 0.86-0.96 mm); b) *A. ulia* micro (n = 6; 0.27- 0.28 mm) and macro (n = 2; 0.72-0.73 mm); c) *P. undescribed sp.* micro and macro; d) *T. mokuleia* spermatophore on the male after extrusion (arrow indicates the extra material surrounding the male genitalia) ; e) *T. mokuleia* spermatophore removed from a female after copulation.

Figure 3. Character states from the Table 3 character matrix presented on the tips of Otte's (1994) hypothesized phylogenetic relationships. Character state polarity was inferred by using *A. ulia* as ancestral character states. A solid line denotes the node at which character 2 (sequential spermatophore size) may have changed from dual sized spermatophores (i.e. micros and macros) to uniform spermatophores. The dashed line represents the node at which characters 4-6 (copulation position, copulation duration, ampulla attachment) may have changed from the ancestral female-above, short duration copulation with the ampulla attached to the derived female to end-to-end, long duration copulation where males hold the ampulla.

Figure 4. Character states for character 2 (sequential spermatophore size) are presented on Otte's (1994) hypothesized phylogeny. Character state polarity was inferred by using *Balamara gidya* (Gryllidae: Trigonidiinae; Evans 1988) and *Teleogryllus commodus* (Gryllidae: Gryllinae; Loher & Dambach 1989) for the ancestral condition. A solid line denotes the nodes at which the change from the ancestral state of uniform spermatophore size to the derived state of dual size spermatophores (i.e. micros and macros) may have occurred.

FIGURES

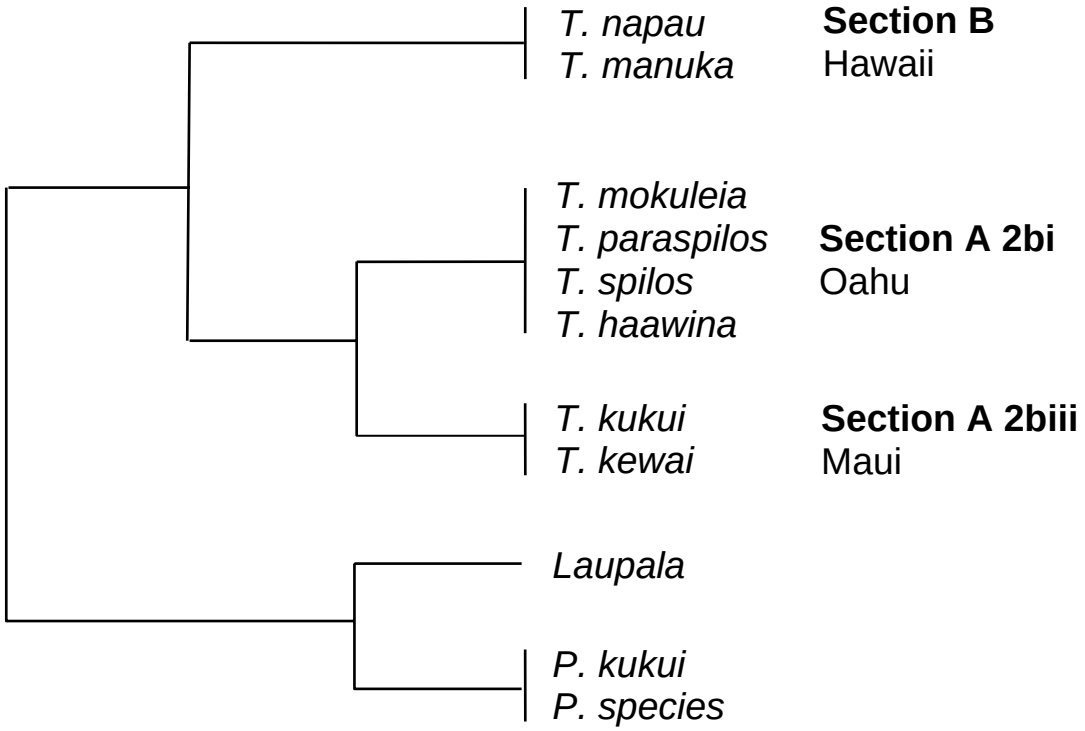
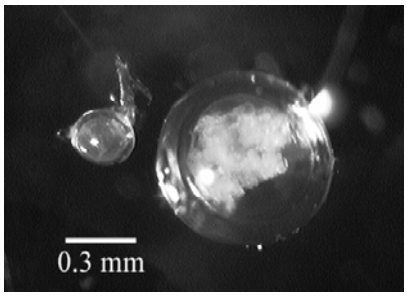
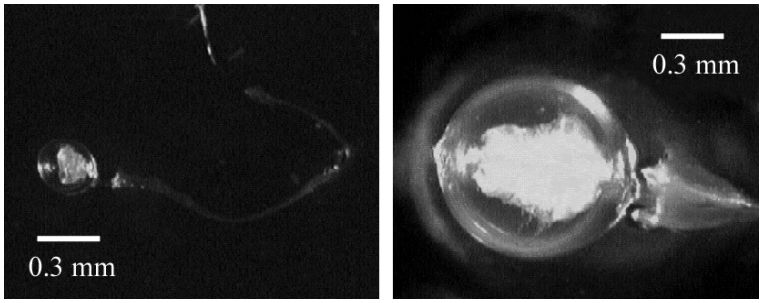


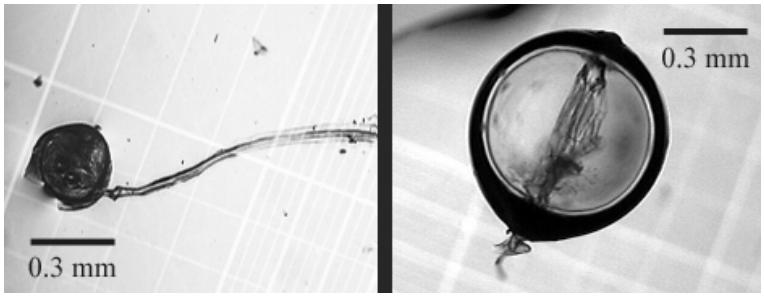
Figure 1



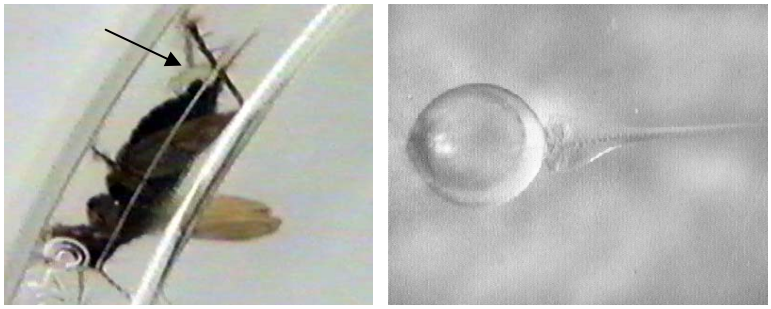
a) *A. ulia* micro and macro



b) *P. kukui* micro and macro

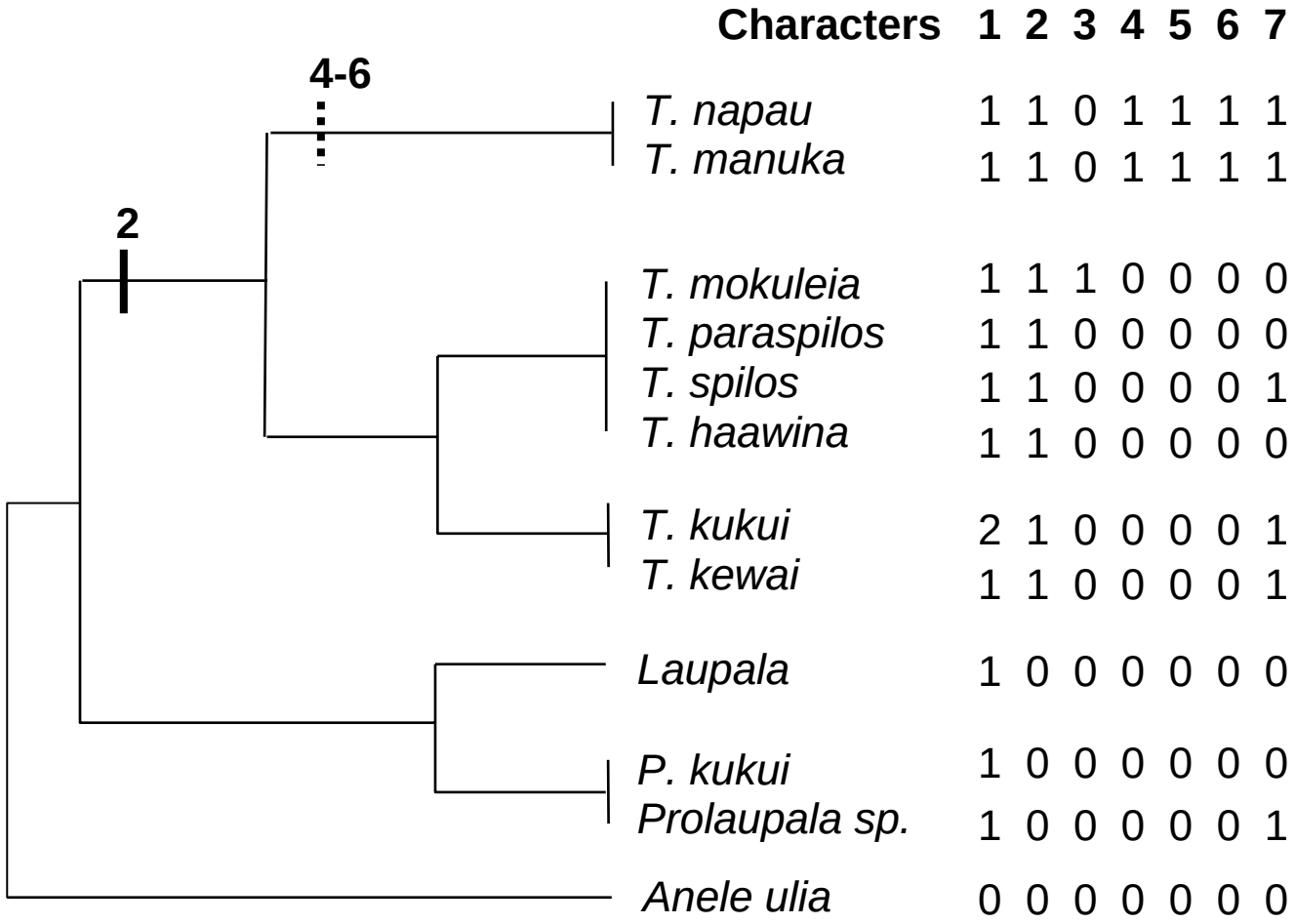


c) *Prolaupala sp.* micro and macro



d) *T. mokuleia* macro on male **e)** macro taken from female

Figure 2


4-6
2
Characters 1 2 3 4 5 6 7
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T. manuka 1 1 0 1 1 1 1
T. mokuleia 1 1 1 0 0 0 0
T. paraspilos 1 1 0 0 0 0 0
T. spilos 1 1 0 0 0 0 1
T. haawina 1 1 0 0 0 0 0
T. kukui 2 1 0 0 0 0 1
T. kewai 1 1 0 0 0 0 1
Laupala 1 0 0 0 0 0 0
P. kukui 1 0 0 0 0 0 0
Prolaupala sp. 1 0 0 0 0 0 1
Anele ulia 0 0 0 0 0 0 0

Figure 3

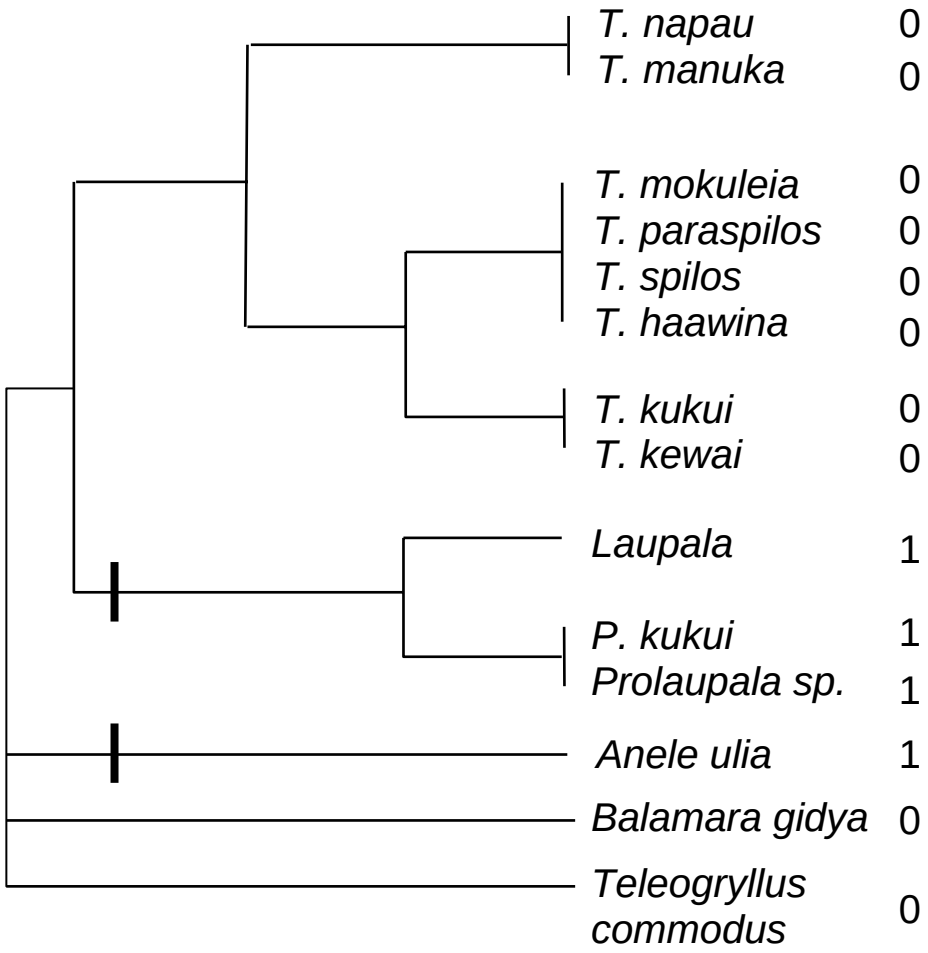
T. napau
T. manuka
T. mokuleia
T. paraspilos
T. spilos
T. haawina
T. kukui
T. kewai
Laupala
P. kukui
Prolaupala sp.
Anele ulia
Balamara gidya
Teleogryllus commodus
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Figure 4

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