INVESTIGATION OF THE GENETIC BASIS OF TEMPORAL VARIATION OF BEHAVIORS IN THE HAWAIIAN SWORDTAIL CRICKET GENUS *LAUPALA*

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INVESTIGATION OF THE GENETIC BASIS OF TEMPORAL VARIATION OF BEHAVIORS IN THE HAWAIIAN SWORDTAIL CRICKET GENUS *LAUPALA*

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In this dissertation I investigate the genetic basis of temporal variation among species of the cricket genus *Laupala*. *Laupala* species display interspecific variation in temporal characters on ultradian and circadian scales, allowing for comparative analyses of temporal variation. The species are primarily delineated by male song pulse rates and female pulse rate preferences. Interspecific variation has also been identified in the daily timing of singing and mating in wild-caught animals. Here I examine the interspecific differences in the daily timing of mating-related behaviors using lab reared *L. cerasina* and *L. paranigra.* Raising the species under environmentally homogeneous conditions demonstrated that differences in daily timing of mating-related activities and locomotion are genetically regulated. I examined the hypothesis that circadian variation underlies the daily timing differences and found a significant difference in the circadian free-running periods of singing between *L. cerasina* and *L. paranigra*. However, the difference was in the opposite direction than was expected, suggesting that either circadian variation is not sufficient to explain daily timing differences or that the association between circadian rhythms and daily timing is different in *Laupala* than in other taxa. Examining the circadian clock gene *period*, I found eight differences in the deduced amino acid sequence but no significant difference in the timing of transcript accumulation. Finally, to identify

candidate genes for temporal song variation, I used suppressive subtractive hybridization to isolate differentially expressed gene transcripts between *L. cerasina* and *L. eukolea*. These sister species express substantially different song pulse rates (~2.4 pps and ~4.0 pps, respectively). I identified ten candidate genes and further characterized these genes by using qPCR to look for interspecific differential expression in the thoracic ganglia and head, regions involved in song regulation in crickets. All the genes showed differential transcript abundance in these regions, making them strong candidates for variation in song pulse rates. Having demonstrated a genetic basis to variation in timing and identified candidate genes for temporal variation provides a critical foundation for further efforts toward understand the genes and mechanisms underlying temporal variation in *Laupala*.

BIOGRAPHICAL SKETCH

Dan Fergus was born on March 24, 1975, to Lois and Ray Fergus in Cedar Rapids, Iowa. He was the youngest of six children. As a child his mother frequently took Dan hiking at the nature center and the family would go on camping trips most summers. Through this exposure to the outdoors Dan gained his interest in the natural world and biology.

Dan attended the University of Iowa, where he earned a BS with honors in biology in May of 1998. His focus during his undergraduate career was in ecology and freshwater biology. Working in the lab of Steve Heard, he examined the effects of detrital processing by stream invertebrates.

After completing his BS degree, Dan worked for two years with Sarah England at The University of Iowa, College of Medicine. In the England lab he examined the role of alternative splicing of the BK channel in smooth muscle contractility. He then moved to Denver, Colorado were he worked with Christina Leslie at National Jewish Medical and Research Center on understanding the trafficking and function of the protein, cPLA2. After a year in Denver, Dan returned to The University of Iowa to take a position as lab manager for Sarah England and continue his work on alternative splicing of the BK channel.

In 2002 Dan began graduate school at the University of Maryland with Kerry Shaw. For his graduate research he combined his experience with molecular biology and an interest in behavior to examine the genetic basis of behavioral variation in the Hawaiian swordtail cricket genus *Laupala*. Dan transferred to Cornell University in 2007 when Kerry Shaw took a faculty position at in the department of Neurobiology and Behavior. He completed his PhD at Cornell University in October of 2009.

For my parents, Lois and Ray Fergus, and Holly Menninger.

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Thank you to my family, especially my parents, Lois and Ray Fergus. They have always supported me and worked hard to help me overcome difficulties early in my academic career. Without their assistance I could not have made it this far.

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CHAPTER 1

Introduction

Common experience tells us that a wide array of organisms keep track of time. Roosters call in the morning, fireflies are active at night, tulips flower in spring, chrysanthemums flower in the fall, and when we change time zones it can take days to adjust. However, it is not so obvious how organisms know what time it is. The question of how organisms keep track of time has been a subject of study for decades. In 1951 Lashley described temporal processing as one of the most important and yet least understood aspects of neurobiology [1]. Since this time tremendous strides have been made in understanding temporal regulation across different time scales. Central pattern generators, networks of neurons that are intrinsically capable of producing rhythmic motor outputs such as walking, chewing, respiring, or stridulating, have been examined physiologically and genetically to help us better understand how these neuronal networks produce rhythmic outputs that typically cycle on the order of milliseconds to seconds [2-4]. Circadian rhythms, which regulate activity patterns and physiological changes on a daily scale, have been extensively studied in a wide array of taxa, including fruit flies (*Drosophila)*, mice (*Mus musculus)*, golden hamsters (*Mesocricetus auratus*), German cockroaches (*Blattella germanica*), several species of moths and crickets [5-11]. However, despite all of the advances that have been made in understanding temporal regulation, the mechanisms of time keeping and the relationships of temporal processing within and across temporal scales have not been fully elucidated [12-14]. Understanding the genetic basis of temporal variation across taxa and time scales will provide insight into how organisms keep track of time and whether temporal mechanisms are evolutionarily conserved.

In this dissertation I examine the genetic basis of temporal behavioral variation between species of the Hawaiian swordtail cricket genus *Laupala*. The *Laupala* genus consists of 38 single island endemic species living in mid-elevation rainforests [15]. All of the species have arisen within the last 5 million years [16]. Variation in temporal characters across closely related species of *Laupala* provides an exceptional opportunity for comparative analyses of the genetic basis of temporal behavioral variation. The species of *Laupala* are morphologically and ecologically cryptic, with male song pulse rate and female song preference being the primary characters delineating species boundaries [15]. Song pulse rates range from approximately 0.5 to 4.0 pulses per second (pps) [15]. The interspecific variation in song pulse rates is polygenically regulated [17-19]. Recent work has uncovered further variation in mating related characters between the species, with variation identified in the daily timing of singing and mating [17]. Furthermore, courtship in *Laupala* is an elaborate and lengthy process lasting several hours and involving the transfer of multiple spermless spermatophores (microspermatophores) and a single sperm-filled spermatophore (macrospermatophore) [18-20]. This novel protracted courtship process creates the opportunity for extensive temporal variation, such as variation in the duration of courtship or the timing of the production and transfer of spermatophores.

Several aspects of *Laupala* help make the genus a more genetically tractable system than other non-genetic model systems. The rapid and recent speciation of *Laupala* has produced species with very low sequence divergence, with a maximum interspecific nuclear sequence divergence of 1.6% [21]. This low sequence divergence suggests that genetic drift has not caused extensive variation among the species, and thus confounding background variation that is not related to phenotypic differences should be low. Furthermore, many of the species can be hybridized in the lab which

has facilitated our understanding of the polygenic nature of song variation among *Laupala* [22-24]. Finally, resources, including an EST library [25] and QTL maps [23, 24, 26], have been developed in *Laupala*, and the successful use of RNAi in other crickets [27] suggests that it could be implemented in *Laupala*. Such resources and tools can greatly facilitate both the identification and analysis of candidate genes for behavioral variation among *Laupala* species.

Identifying the genetic basis of temporal variation in *Laupala* is a forward genetic process. The first step in the process is to verify that the variation is indeed genetically regulated. Variation in habitat or rearing conditions can have major effects on behavior. For example, temperature variations can have important short-term and long-term effects on behavior and gene expression in different taxa [28-31]. Thus, after the identified behavioral variation has been identified, a genetic basis must be confirmed by approaches such as common-garden experiments or heritability analyses.

After confirming the genetic basis of the behavioral variation,, there are a number of approaches to identify the genetic variation underlying the phenotypic variation. In both genetic model systems like *Drosophila* and non-genetic model systems like *Laupala*, this typically begins by identifying candidate genes. Given varying definitions of candidate genes in the scientific literature [For example:32, 33, 34], it is important to note that I define a candidate gene broadly as a gene which has been identified as potentially important in determining the phenotypic variation of interest. As outlined in a number of reviews [32-36], a gene may be selected as a candidate based on its functional role in another system, linkage of a particular gene to genetic markers of phenotypic variation, or divergence of sequence or expression between phenotypically distinct groups. There are a wide variety of techniques, such as literature searches, QTL analyses, microarrays, and suppressive subtractive

hybridization, to identify candidate genes in both genetic model and non-genetic model systems.

Finally, once candidate genes have been identified for behavioral variation, further analyses are needed to confirm whether the candidate gene is likely to play a role in the behavioral variation. In genetic model systems this frequently involves transgenic methods, establishing a link between gene and behavior by altering the expression level or the allele of the gene in tissues of interest. In non-genetic model systems establishing a link between a gene and behavioral variation is more difficult. However, using a QTL map a researcher can determine whether a gene falls within a chromosomal region that has been linked to the phenotypic variation. Additionally, it may be possible to introgress a candidate gene between genetic backgrounds to see how the gene effects behavioral variation. Finally, the development of RNAi has allowed researchers to perform knock-out or knock-down experiments in non-model systems. Reducing gene expression can establish whether a candidate gene is involved in regulating a particular phenotype.

My objective in this dissertation was to examine the genetic basis of natural temporal variation among species of the genus *Laupala*. My general aims were to: 1) confirm the genetic basis of and further characterize interspecific differences in daily timing of mating-related behaviors; 2) examine circadian variation and determine whether differences in circadian rhythms are likely to play a role in daily temporal variation; and 3) identify candidate genes which may play a role in behavioral temporal variation, specifically song pulse rate variation among *Laupala*. For aims 1 and 2, I performed comparative analyses with the sympatric species *Laupala cerasin*a and *Laupala paranigra*. For aim 3, I performed analyses with the sister species *L. cerasina* and *L. eukolea*.

In Chapter Two, I examine variation in the daily timing of courtship and mating-related behaviors between *L. cerasina* and *L. paranigra*. Field and lab work with wild-caught individuals of these species demonstrated differences in the peak singing and mating times of these species [17]. I have looked more closely at the mating-related behavior in lab-reared animals raised in a common environment to test the hypothesis that the daily variation in timing of mating-related behaviors is genetically regulated, as opposed to resulting from environmental differences. Additionally in this chapter I examine the timing of specific courtship elements to better characterize variation in courtship between sympatric *Laupala* species. I found temporal variation in several mating-related behaviors, including the rate of microspermatophore production and the overall duration of courtship. Consistent with findings from the wild-caught crickets, *L. paranigra* began courtship and mated significantly later than *L. cerasina*.

In Chapter Three, I measured the circadian rhythms of *L. cerasina* and *L. paranigra*. It has been seen in *Drosophila* and *Bactrocera cucurbitae* that later activity phases under light:dark conditions are associated with longer circadian free-running periods under conditions of constant lighting [37, 38]. In this chapter I test the hypothesis that the later activity times of *L. paranigra* indicate a longer circadian free-running period than *L. cerasina*. I test this hypothesis by examining the free-running periods of both song and locomotion in each species. Interestingly, while the free-running periods based on locomotion did not significantly differ between the species, the free-running period of song was significantly shorter in *L. paranigra* than in *L. cerasina*. This is the opposite of what I hypothesized. In this chapter I also sequenced the *period* (*per*) gene transcript from both species to examine sequence variation and to test the hypotheses that 1) *per* transcript abundance cycles through the day and 2) there is an interspecific difference in the timing of *per* transcript abundance that

corresponds to the difference observed. The transcript does indeed cycle, with a peak around the time of lights out, and there is interspecific coding sequence variation. However, I found no significant difference in the timing of *per* transcript expression between the species. The results of this study indicate that circadian variation is likely not the source of daily temporal variation in mating-related behaviors; however, the sequence differences in the *per* transcript are intriguing and the potential role of *per* in temporal variation requires further investigation.

In Chapter Four, I examine differential gene expression between *L. cerasina* and *L. eukolea*, sister species that have diverged within the last 0.5 million years [16]. These species are morphologically cryptic and will readily hybridize when housed together in the lab. However, the species' song pulse rates are very different (*L. cerasina*: ~2.4 pps; *L. eukolea*: ~4.0 pps). In this study I used suppressive subtractive hybridization to identify genes that are differentially expressed between the species and may serve as candidate genes for phenotypic variation, particularly song variation. I further examined transcript abundance within the head and thoracic ganglia, two regions that are likely to play a role in song variation, to determine whether any of the transcripts are differentially expressed in these regions. I identified 10 differentially expressed transcripts between the two species, all of which were differentially expressed within the head, the thoracic ganglia, or both regions. Most of these transcripts could not be functionally identified; however, several appear to be proteases and one appears to be a zinc-finger transcription factor homolog of the *Drosophila* gene *glass.* The localization of differential expression and the putative functions of the identified genes make them intriguing candidates of song variation in *Laupala*.

I conclude from this research that *Laupala* have extensive interspecific temporal variation. The differences range from less than a second, for song pulse rate,

to hours for the daily timing of behaviors. While I found a significant circadian difference between two of the species, this difference does not seem likely to account for the difference in the daily timing of mating related behaviors. Additionally, I have identified several candidate genes for the interspecific differences, a critical step for elucidating the genetic basis of behavioral variation.

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CHAPTER 2

A genetic analysis of novel courtship elements in the Hawaiian cricket *Laupala*

ABSTRACT

The Hawaiian swordtail cricket genus *Laupala* (Gryllidae: Trigonidiinae) has an elaborate and protracted courtship system which last several hours and includes the transfer of several spermless spermatophores from the male to the female. Recent work has identified interspecific differences in peak calling and mating times among wild and wild-caught *Laupala* species. The protracted nature of *Laupala* courtship generates the possibility for variation in novel behavioural elements which do not exist in most other cricket species. Here we present a quantification of the courtship process, from initiation of courtship through mating, in first generation lab-reared individuals of two sympatric species, *Laupala cerasina* and *Laupala paranigra*. We find support for the hypothesis that interspecific differences in the onset and duration of courtship, spermatophore production rate, and the time of mating have a genetic basis. Establishing that the relative timings of these behavioural differences have a genetic basis justifies further study of their origins and evolution, how temporal variation is related among behaviours at different time scales, and whether timing is involved in mate choice or sexual isolation in this rapidly evolving genus.

INTRODUCTION

Divergence in the timing of activities or life history traits can reduce competition, reproductive interference or interbreeding, and consequently promote species coexistence in sympatry [1-3]. For example, temporal separation of feeding or adult emergence may partition food resources among insects [4, 5] and daily timing differences of pollen release may partition pollinator species among plants [6], thereby

reducing reproductive competition between closely related species. Reduced interbreeding may result from divergence in the timing of activities important for reproduction, such as development [7-9], breeding season [10-13], daily mating activities [3, 14] or ultradian (<24 h) courtship behaviors [15-17].

Species of the Hawaiian swordtail cricket genus *Laupala* are extremely closely related as well as morphologically and ecologically cryptic. Yet members of this group frequently live in congeneric communities of two or more species. Evidence suggests that reproductive behavior, which varies on a number of time scales, plays an important role in facilitating the coexistence of species of *Laupala*. Species differ in the song pulse rates of males and females exhibit acoustic preference for rates characteristic of their own species [18-20]. Daily timing of behaviors also differs among *Laupala* species. Danley et al. [21] reported that the peak singing time of the sympatric species *L. cerasina* and *L. paranigra* differ in the field, with corresponding differences in the timing of mating in laboratory experiments among wild-caught individuals. If the timing of these daily reproductive behaviors reduces the probability of reproductive interference or interbreeding, it may have important implications for the coexistence of sympatric species. Thus, the timing, *per se*, of reproductive behaviors, becomes an important candidate phenotype in need of study.

The production and transfer of spermatophores in *Laupala* is part of an elaborate and lengthy courtship lasting several hours [22-24]. Throughout the day, males of *Laupala* produce a series of small, spermless spermatophores (microspermatophores) prior to a larger, sperm-filled spermatophore (macrospermatophore) in each mating [22]. While extensive work has gone into examining the timing of mating-related characters in several other cricket species [25-30], sperm-less spermatophores have rarely been observed [13]. The complex and temporally protracted nature of *Laupala* courtship behavior suggests that adaptive divergence may have caused the differences

in timing of reproductive events, resulting in timing phenotypes that apparently have not arisen in other cricket mating systems. The potential for selection or other evolutionary forces to shape the differential timing of reproductive and courtship events such as those observed in *Laupala* depends upon an underlying genetic contribution to these phenotypes.

Support for the hypothesis that genetic differences underlie variation in the daily reproductive rhythms between the sympatric *Laupala* species discussed above would mean that these phenotypes could evolve in response to selection. Alternatively, however, species may show behavioral responses that differ due to variable environmental cues (e.g. light, temperature) associated, for example, with different microhabitat use. By rearing two species of *Laupala* under controlled laboratory conditions, we tested whether there is a genetic component to the differences in the daily timing of mating. In addition, we examined the timing of specific courtship elements to better characterize variation in courtship between sympatric *Laupala* species.

METHODS

In this study we examined two *Laupala* species, *L. paranigra* and *L. cerasina*, which are endemic to the Big Island of Hawaii. These species have overlapping ranges and have an estimated divergence time of approximately 3.7 Myr [31]. *L. paranigra* nymphs were collected along Kaiwiki Road (19˚45’ N, 155˚10’ W) and *L. cerasina* were collected at Kalopa State Park (20˚02’ N, 155˚26’ W). The nymphs were stored with damp Kimwipes (Kimberly-Clark) and Cricket Chow (Fluker Farms), and transported back to the University of Maryland.

At the University of Maryland all crickets were housed at 20˚ C on a 12:12 light:dark cycle. Wild-caught nymphs were kept in plastic cups with damp Kimwipes

and fed Cricket Chow (Fluker Farms) treated with methyl paraben (Methyl Paraben; USB Corporation) to inhibit mould growth. Upon maturing, male/female pairs were housed together in cups to allow mating and oviposition into moistened Kimwipes. Kimwipes with eggs were collected, placed in clean cups, and kept moist. As F_1 nymphs emerged they were housed under the same conditions as the parental individuals and reared to sexual maturity, approximately 5 months post hatching.

Sexually mature pairs of virgin F_1 males and females were placed in plastic Petri dishes with moistened Kimwipes within 15 min of the lights coming on. The Petri dishes were separated from one another with cardboard dividers such that each mating pair within a Petri dish was visually isolated from adjacent mating pairs. Pair establishment time was recorded to ensure that there was no significant difference between species in the starting time.

Courtship is initiated via long distance acoustic communication, followed by the approach of the female and mutual antennation. Upon contact, a mating pair will typically engage in a face-to-face positioning with antennation, while the male produces a microspermatophore. Several minutes later, the male transfers the microspermatophore to the female via copulation. After a variable period of time following transfer, the female eats the microspermatophore. This process of transferring microspermatophores is repeated several times before the male produces and transfers a single, sperm-filled, macrospermatophore [22-24]. Because the transfer of a macrospermatophore typically results in insemination, we considered macrospermatophore transfer to indicate a successful mating.

Mating pairs were observed from the time they were established through mating (the transfer of the macrospermatophore) or until the onset of the dark cycle for pairs that did not court. In each trial we recorded the times of first song production; initial face-to-face positioning; each spermless microspermatophore production and

transfer; and macrospermatophore production and transfer. In many cricket species males produce a distinct courtship song when in close proximity or antennal contact with a female (reviewed in: [32, 33], which could be considered the initiation of courtship. However, *Laupala* possess only one song type, which is expressed in several contexts [34]. Shaw and Khine [23] use the onset of male-female circling behavior as the onset of courtship. Because we did not consistently observe this behavior, we report both the onset of song and the first face-to-face positioning with antennal contact as proxies of courtship initiation.

We set up equal numbers of each species on each day that observations were performed, randomizing the order in which pairs were established to eliminate effects of timing or order of set up. Student's two-tail t-tests were used to determine significance of interspecific differences in the timing of behaviors as well as the number of microspermatophores produced and transferred. We investigated both the overall rate of microspermatophore production and transfer as well as the change in rate throughout mating. The overall rate was calculated by dividing the number of microspermatophores produced or transferred by the time from the first to the final microspermatophore. The change in rate of microspermatophore production during courtship was examined by determining the period from the production of one microspermatophore to the next and comparing successive periods. We performed one-way ANOVAs to determine whether the time between microspermatophore production and transfer varied within each species over the duration of courtship. Tukey corrected pairwise comparisons were employed to examine the significance of variation in the periods from one microspermatophore to the next within species over the duration of courtship. Differences between species in mean microspermatophore production rates as well as variation in the periods between microspermatophores

throughout courtship were examined with a two-way ANOVA. Unless otherwise noted, means are reported ± SE.

RESULTS

There was no significant difference between species in the time that pairs were set up (mean times were 0h 6min ± 1.4min and 0h 5min ± 1.5min after lights on for *L. cerasina* and *L. paranigra* respectively). In *L. cerasina*, approximately 82% (14/17) of pairs that produced microspermatophores went on to successfully produce and transfer a macrospermatophore, while in *L. paranigra* approximately 93% (13/14) of pairs that produced microspermatophores successfully produced and transferred a macrospermatophore. The data reported below, as well as in the table and figures, are based solely on observations from those pairs that successfully mated.

We observed a significant difference between species in the timing of both the initiation of the first song (Table 2.1; $t = 2.83$, $df = 25$, $p < 0.01$) and of the first instance of face-to-face positioning (Table 2.1; $t = 3.36$, $df = 23$, $p < 0.01$). The timing of both of these behaviors was earlier in *L. cerasina* than in *L. paranigra*. There was a significant difference between *L. cerasina* and *L. paranigra* in the time of production ($t = 6.51$, $df = 24$, $p << 0.01$) and transfer ($t = 6.97$, $df = 24$, $p << 0.01$) of the first microspermatophore (Table 2.1), with *L. cerasina* performing these behaviors approximately 4 hours earlier than *L. paranigra* (Figure 2.1). The macrospermatophores were also produced ($t = 12.53$, $df = 25$, $p << 0.01$) and transferred ($t = 10.39$, $df = 25$, $p << 0.01$) approximately 2 to 2.5 hours earlier in *L. cerasina* than *L. paranigra*. The timing of the production and transfer of macrospermatophores were highly stereotyped within each species, with intraspecific standard errors of less than ten minutes.

Table 2.1. The mean times ± SEM after lights on that various courtship and mating behaviors were performed by both species are shown as hrs:min. As described in the text, all the behaviors occurred at significantly different times between *L. cerasina* and *L. paranigra*.

			1st Microspermatophore		Macrospermatophore	
Species	1st Song	1st Facing	Production	Transfer	Production	Transfer
L. cerasina	0:15±0:02	0:19 ±0:02	0:59 ±0:11	1:37 ±0:13	7:17 ±0:09	8:28 ±0:09
L. paranigra	1:14 ±0:21	2:12 ±0:37	5:06 ±0:38	6:02 ±0:35	9:52 ±0:07	10:38 ±0:07

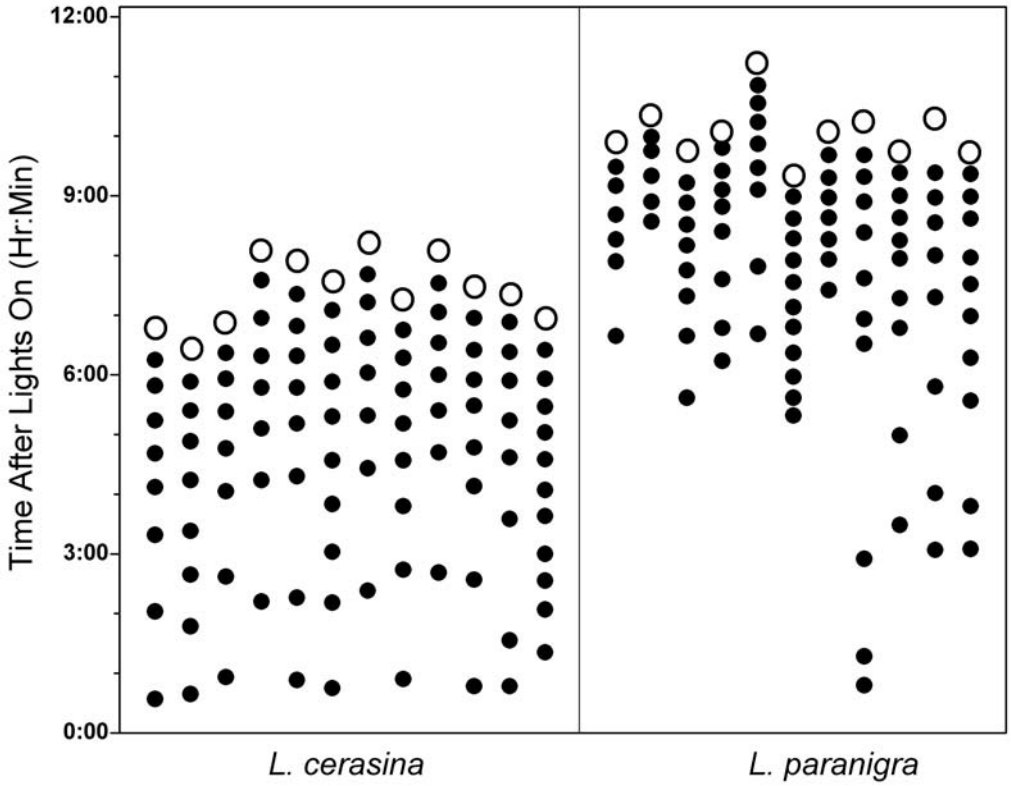

Figure 2.1. Spermatophore production times. Productions of spermatophores are plotted against time after lights on. Each column displays data from a single individual. Smaller black dots represent microspermatophores while larger unfilled dots represent macrospermatophores.

There were no significant differences between species in the number of microspermatophores produced or transferred. *L. cerasina* produced 8.0 ± 0.28 and transferred 6.3 ± 0.57 microspermatophores while *L. paranigra* produced 8.3 ± 0.43 and transferred 6.9 ± 0.45 microspermatophores. It was not unusual that some microspermatophores failed to be transferred to the female. In these cases the male generally made several unsuccessful transfer attempts, after which he ate the microspermatophore himself. When microspermatophore transfer did occur, there was negligible intraspecific variation in the time between production and transfer of microspermatophores (*L. cerasina*: 0h 25min ± 0.4min; *L. paranigra*: 0h 19min ± 0.6min). Thus, we only analyzed the time between production, and not transfer, of successive microspermatophores in the analyses described below.

We found a significant difference between species in the length of time between the transfer of the first microspermatophore and the transfer of the macrospermatophore ($t = 3.87$, $df = 24$, $p << 0.01$). This interspecific difference in the duration of courtship is reflected in the overall rates of microspermatophore production, which also differed significantly between *L. cerasina* and *L. paranigra* ($F = 8.45$, $df = 1, 178$, $p < 0.01$). The period from production of one microspermatophore to the next varies as courtship proceeds (Figure 2.2) in both *L. cerasina* ($F = 18.87$, $df = 9, 95$, $p << 0.01$) and *L. paranigra* ($F = 5.31$, $df = 9, 82$, $p << 0.01$). Additionally, there was a significant interaction effect of species and number of microspermatophores produced on the rate of microspermatophore production ($F = 2.56$, $df = 9, 178$, $p < 0.01$). In *L. cerasina* there was a significant decrease in the period between successive microspermatophore until the fourth microspermatophore, while in *L. paranigra* there was an initial non-significant increase in the period of successive microspermatophores rate from the first to second period, followed by a significant decrease in the period from the second to third interval between

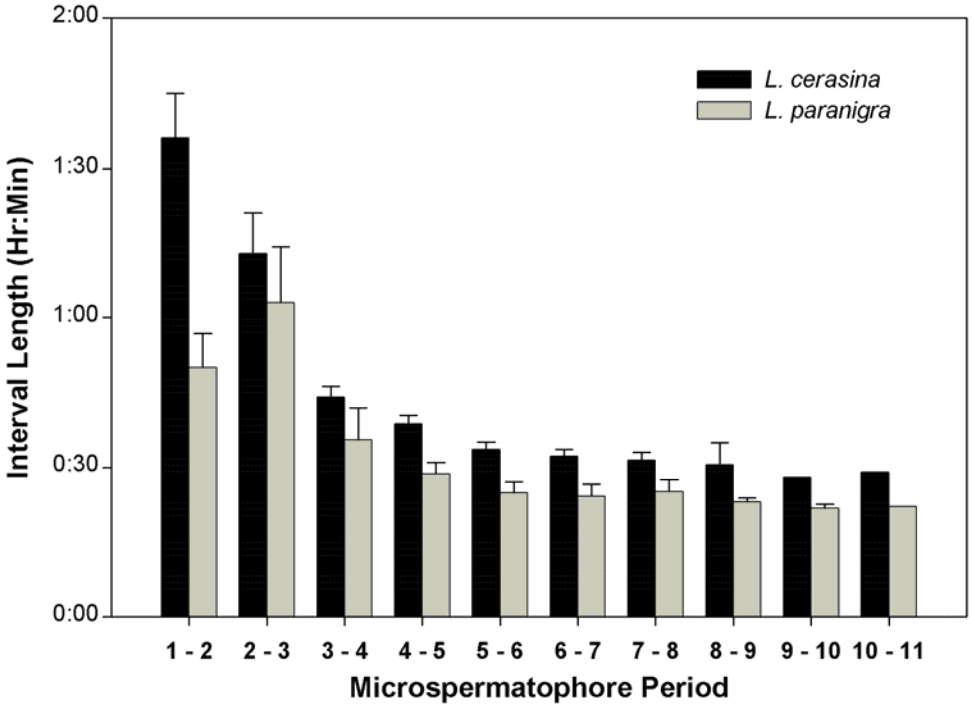

Figure 2.2. Microspermatophore production rates. The time period between productions of successive microspermatophores is potted for successive pairs of microspermatophores. The small standard errors in the last two periods for *L. cerasina* and last three periods for *L. paranigra* are due to very small sample sizes for those periods.

microspermatophores (Figure 2.2). Thus, we found a difference between species in both the rate of microspermatophore production and in the change in rate of microspermatophore production as courtship proceeds.

DISCUSSION

Establishing that temporal patterns of particular behaviours are phenotypes *per se* should enable tests of hypotheses about 1) whether adaptive differentiation may have generated species differences in such phenotypes, 2) how variation in such phenotypes contributes to reproductive barriers, and 3) how timing phenotypes deployed at different time scales may be correlated or pleiotropically controlled [35-38]. For example, in *D. melanogaster*, variation in the *period* gene has been shown to pleiotropically regulate both circadian rhythms and the ultradian cycling of courtship song interpulse intervals [35]. In addition, variation in timing has been implicated as a barrier to gene flow and a potential factor in speciation on ultradian [19, 39, 40], circadian [41-43], seasonal [9-11, 44-46], and multi-year time scales [7, 47].

Here we have demonstrated interspecific variation in the timing of elements of *Laupala* courtship and mating. Variation in production time for the first microspermatophore in the courtship series and the final sperm-containing macrospermatophore was significantly greater between than within species (Table 2.1). Additionally, while we observed high variation within species in the time period between successive early microspermatophores, this variation falls dramatically by the third or fourth microspermatophore. Overall the periods between microspermatophores are shorter in *L. paranigra* than in *L. cerasina* to a statistically significant degree (Figure 2.2). The first microspermatophore is produced and transferred four hours later in the day in *L. paranigra* than in *L. cerasina*, which could

constrain the rate of microspermatophore production in *L. paranigra* in order to fit the same number in before dark.

The protracted courtship prior to mating enables a context for unique temporal variation in mating-related behaviors between *Laupala* species. In most cricket species, including *Laupala*, courtship and mating includes female orientation to male calling song, pair formation and a brief courtship, followed by the transfer of a sperm-containing spermatophore (i.e. mating). In *Acheta domesticus* and *Teleogryllus commodus*, the expression of the calling song is associated with the presence of a spermatophore [27, 48]. Furthermore, in *T. commodus*, species typical daily mating time appears to be a correlated with the timing of the calling song. However, mating time appears unrestricted, as pairs of *T. commodus* housed together in the laboratory will readily mate throughout the day, with only the rate at which males produce new spermatophores varying by time of day [32]. In *Laupala*, the expression of calling song and the display of a sperm-containing spermatophore are temporally decoupled. Between initial pair formation and mating the pairs engage in a lengthy courtship involving the prior transfer of multiple spermless microspermatophores. Our study demonstrates interspecific variation on the order of minutes for the rate of microspermatophore production to hours for the duration of courtship and the time of day at which mating occurs. The production and transfer of spermless spermatophores appears to be relatively common in endemic Hawaiian swordtail crickets [22, 23], but has only been reported in one species outside of this group, *Nemobius sylvestris* [13]. Thus the unusual courtship system of *Laupala* has provided characters which differentiate species, and provide a substrate upon which selection might act.

In this study we examined lab reared F_1 individuals, demonstrating a genetic basis to the observed variation in timing. The use of such common garden experiments dates back Mendel's pioneering work with garden peas in the mid 19th century

(reprinted as: [49]). While molecular technology has advanced to the point of allowing molecular examination of the genes underlying behavior [50], the common garden experiment remains a useful technique with which to establish a genetic contribution to natural or mutagenically induced behavioral variation [51-55]. Establishing this genetic contribution indicates that the timing of these courtship and mating behaviors can evolve in response to selection or other evolutionary forces.

Our demonstration of a genetic contribution to interspecific variation in these novel temporal phenotypes, coupled with the functional context of their expression, suggests that selection may have played a role in their origins and recent evolution. Some of the traits examined here are necessarily correlated, and any future study of selection acting of these traits should take this into account. For example, directional selection to increase microspermatophore number could achieve this by increasing the rate of microspermatophore production or prolonging the duration of courtship. An examination of additional species with known phylogenetic relationships could help elucidate which of these characters (courtship duration, microspermatophore number, or microspermatophore rate) is a direct target of selection.

Regardless of which measured element might be the direct target of selection, several selective pressures could potentially contribute to its evolution and the patterns of divergence we have observed. For example, selection to reduce reproductive interference between sympatric species could explain some of the observed differences, resulting in reproductive character displacement [56, 57]. A comparison of the daily timing of singing between sympatric and allopatric populations of *L. cerasina* by Danley et al. [21] found no significant difference between sympatric and allopatric (with respect to *L. paranigra*) localities and thus character displacement of singing time was not supported. Character displacement of the other mating-related behaviors therefore seems unlikely, but requires further investigation. It may be the

case that the differences in timing characterize the deeper split, evident in the phylogenetic history of *Laupala*, between the *cerasina* and *pacifica* species groups represented by the species in this study. Testing the hypothesis of reproductive interference may be addressed more appropriately through comparative analyses of a broader array of species.

Alternatively, sexual selection may have shaped the timing of reproductive characters in *Laupala*. Females within each species may have a window of receptivity and/or timing preferences; therefore female choice may exert divergent selective pressure on the timing of male behaviors. Male-male competition could also drive the timing variation between *L. cerasina* and *L. paranigra*. The courtship process has been demonstrated to increase ejaculate uptake by females (deCarvalho T. N. & Shaw K. L., In prep). Consequently, the interspecific difference in overall rates of microspermatophore production may result from the necessity to transfer a certain number during a species-specific courtship period.

Based on the results of this study, it is clear that there is a genetic contribution to the interspecific timing of mating related behaviors. Taken together with previous work in *Laupala* we are finding that behavioral timing appears to be evolutionarily plastic across time scales and behaviors, from how fast a male sings or provides microspermatophores on the ultradian scale, to the time of day of singing or mating on the circadian scale. With such a wide range and array of variation in temporal phenotypes across the genus, *Laupala* will provide a useful model for examining how temporal divergence effects mate selection and understanding how of temporal variation is controlled across different time scales and behaviors.

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CHAPTER 3

Behavioral and molecular examination of circadian rhythms in the Hawaiian cricket genus *Laupala*

ABSTRACT

The daily activity times and circadian rhythms of crickets have been a frequent subject of behavioral and physiological study for decades. Little is known about the molecular basis of circadian rhythms or temporal variation in crickets. However, analyses of circadian clock proteins in crickets indicate that the underlying mechanism differs from the *Drosophila* model of circadian rhythm generation. In this study I examined the circadian free-running period of two species of *Laupala* crickets, *Laupala cerasina* and *Laupala paranigra*, that are known to differ in the daily timing of mating related activities. Additionally, I cloned the *period* (*per*) gene transcript from both species to examine daily cycling and sequence variation of this candidate gene transcript. The species differed significantly in free-running period of singing, but did not differ significantly in the free-running period of locomotion. The *per* transcript abundance showed significant daily variation in both species, but the timing of transcript abundance variation did not differ significantly between species. Several amino acid coding differences were identified between species. These differences demonstrate the potential for *per* to play a role in interspecific behavioral variation in *Laupala*.

INTRODUCTION

Circadian rhythms, endogenously generated oscillations occurring on approximately a 24 hour scale, have been found in organisms ranging from eubacteria to animals, plants and fungi [1]. The endogenous clock underlying circadian

oscillation is an adaptation that makes it possible for organisms to predict daily and seasonal changes in their external environments [2]. The circadian clock allows an organism to maintain circadian cycles, or free-run, in the absence of external cues. A great deal of our understanding of circadian clocks comes from *Drosophila*. Shortly after Benzer pioneered the use of *Drosophila* to examine the genetic basis of behavior [3], three mutations of an X-linked gene, later named *period* (*per*), were found to alter the endogenous circadian free-running periods of flies carrying the mutations [4]. This work opened the door for subsequent investigations of the molecular basis of circadian rhythms. The predominant model of a circadian clock, based on *Drosophila*, consists of interlocked transcriptional/translational feedback loops, the *period/timeless* (*per/tim*) loop and the *clock* (*clk*) loop, with other genes involved in regulating and entraining cycling [5-7]. Circadian cycling is maintained by an approximately 24 hour cycle of protein expression, phosphorylation, trafficking between cytoplasm and nucleus, and degradation [8-12]. The feedback clock mechanism appears to be somewhat conserved, with an extremely similar mechanism using different genes operating in mammals [6]. Studies in a wide variety of organisms including cyanobacteria [13, 14], *Neurosopora cressa* [15, 16], several plants [17-19], mammals [20-22], and a wide array of insects [23-29] have further elucidated the behavioral and molecular basis of circadian rhythms.

Here I investigated circadian rhythms as well as sequence variation and transcription of *per* in two species of the Hawaiian swordtail crickets genus *Laupala*. Given the commonly observed daily cycling in cricket song, the circadian rhythms of crickets have been a frequent subject of study, from early behavioral and physiological studies [26, 30-35] to more recent molecular analyses [36-41]. Like other organisms, they typically possess an endogenous circadian clock [30, 31, 35]; however, there are physiological differences in the endogenous clock of crickets relative to *Drosophila*.

For example, the daily variation in expression of circadian clock proteins, including PER, CLK and TIM, which occurs in *Drosophila* [1, 5, 6, 42] appears to be lacking in crickets [36, 37, 40, 41]. Additionally, CLK is the only one of these proteins which has been observed to translocate into the nucleus in crickets [36]. However, it was recently demonstrated that *per* transcript abundance undergoes daily variation in *Gryllus bimaculatus* and that inhibition of PER expression leads to arrhythmia [27, 38]. Thus, the genetic underpinnings of circadian rhythms in crickets are likely somewhat conserved relative to *Drosophila*, but a clear understanding of the circadian mechanism has not been elucidated.

The genus *Laupala* is particularly exciting for the study of variation in timing because closely related species in the genus differ in several temporal characters, providing a compelling system of comparative analyses. The most conspicuous character differentiating the species is pulse rate of the male calling song, a sexually selected trait that is genetically regulated [43-45]. Species of *Laupala* have also been shown to display daily behavioral rhythms whereby males sing and courting pairs mate at predictable times during daylight hours [46]. Moreover, significant differences have been observed in the daily timing of singing and of mating between the species *L. cerasina* and *L. paranigra*, with *L. paranigra* exhibiting later timing of both activities [46, Chapter 1].

In this study I examined the daily timing of singing and locomotion in visually and acoustically isolated males to verify the later activity phase of *L. paranigra* relative to *L. cerasina*. I further test the hypothesis that these behaviors are expressed with circadian free-running cycles (the cycling of activity in the absence of external cues, or zeitgebers) to determine whether these activities are regulated by an endogenous circadian clock. In conjunction with these measurements, I ask whether *L. paranigra* has a longer free-running period than *L. cerasina*, a hypothesis inspired by

the suggestion that later activity patterns correspond to longer free-running periods [47, 48]. Finally, I cloned and sequenced the *per* transcript from both species and measured transcript abundance at different times throughout the day to measure circadian expression level. With these results, I address whether interspecific differences exist in the timing of *per* expression and assess its correspondence to interspecific differences in circadian rhythms. I conclude with a discussion of circadian rhythms, several aspects of the *per* gene sequence, and temporal correlations that speak to the possibility of pleiotropic regulation.

METHODS

Experimental Animals

L. cerasina were collected from Kalopa State Park (20°02'N, 155°26'W) and *L. paranigra* were collected from Kaiwiki Road (19°45'N, 155°10'W) in 2005 from the Big Island of Hawaii. Fifth generation lab reared individuals were maintained at 19.4° to 20.1°C under 12:12 light:dark (L:D) cycle. Crickets were provided cricket feed (Fluker Farms, Port Allen, LA) and water on a moistened Kimwipe. Care was taken to avoid sibling matings to reduce inbreeding, thus maintaining some genetic diversity in the laboratory populations. Adult males were used in all parts of this study. The mean ages (days past final molt) on the initial trial day in this study were 41.5 ± 18.9 days for *L. cerasina* and 41.3 ± 9.3 days for *L. paranigra*.

Examination of Daily Timing and Free-Running Periods

The songs of 26 *L. cerasina* and 20 *L. paranigra* adult male crickets were digitally recorded at temperatures between 19.4° and 20.8°C and pulse rates, in pulses per second (PPS), were measured. The song recording and pulse rate measurements were performed using Raven Interactive Sound Analysis Software (Cornell Lab of

Ornithology, Ithaca, NY). To determine the song pulse rates, five pulse periods were measured (±0.5 ms) and averaged for each individual. The inverse of the mean period was calculated to produce the pulse rate. A regression analysis was used to assess the relationship between pulse rates and recording temperatures.

To measure daily activity times and endogenous free-running circadian periods, the males from which songs had been recorded were placed into individual, visually and acoustically isolated chambers with moistened Kimwipes and methylparaben treated food (Figure 3.1). Each chamber contained an omnidirectional condenser microphone (RadioShack Corporation, Fort Worth, TX) and an infrared photomicrosensor (Omron Industrial Automation, Schaumburg, IL) to detect motion. Each chamber was placed in a larger enclosure made of Quiet Barrier HD sound-insulating acrylic (American Micro Industries, Inc, Chambersburg, PA). Both the inner and outer chambers had 0.25 cm thick acrylic glass windows to allow light into the chambers. Up to six chambers were run simultaneously. The sound attenuation between chambers was estimated at over 100 dB at 5 kHz (the carrier frequency of *Laupala* song) while the maximum observed volume of a *Laupala* song was under 80 dB (personal observation).

Song and locomotor activity were recorded continuously with the omnidirectional microphone and infrared photosensor, respectively, transmitted via a DI-720-USB Data Acquisition System (DATAQ Instruments, Inc, Akron, OH) to a computer which recorded the data using WinDaq Lite data acquisition software. The data were collected in one second bins by using the intelligent oversampling feature of WinDaq to sample all the inputs at 240 Hz and record either the maximum sample for the sound data or the mean of the samples for the locomotion data at one second intervals. By recording in this manner song pulses, which are less than one second, are

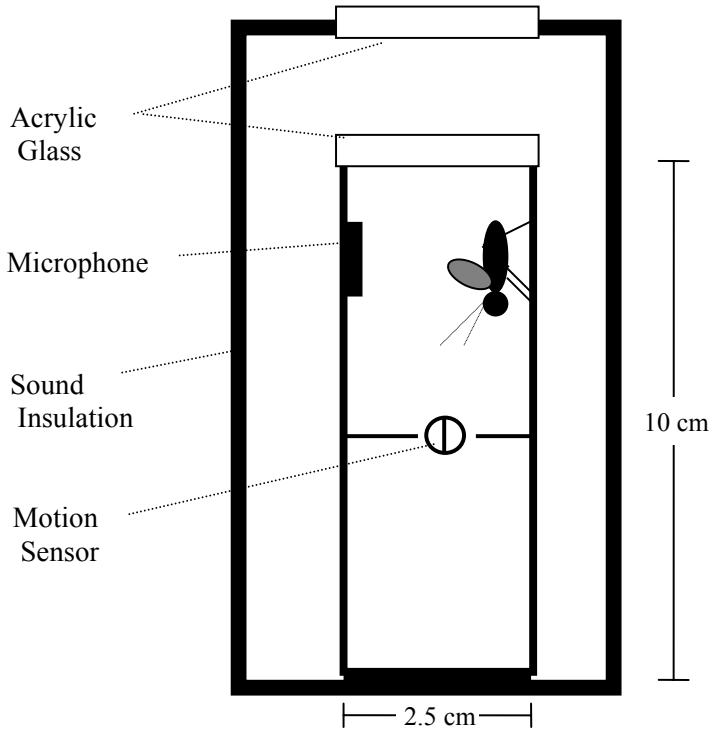


Figure 3.1. Individual circadian monitoring chambers. Each chamber contained and omnidirectional microphone and infrared photomicrosensor to detect sound and motion. Individual chambers were placed in a larger sound reducing enclosure. The chambers had 0.25 cm thick acrylic glass windows to allow room light in.

still detected in the one second bins, while short motion signals, such as an antenna moving past the infrared sensor, do not produce signals that differ substantially above background. The crickets were recorded in the chambers for three days under 12:12 L:D conditions before switching to constant light (L:L) for at least 10 days. The light reaching the inner chambers in both L:D and L:L was low light (<15 lux). Temperatures during the trials remained quite steady at 19.9°C and did not vary on a daily cycle during the constant lighting conditions.

To examine the daily timing of singing and locomotor activity I measured the presence or absence of each activity within 0.1 hour bins for each individual over a two day (48 hour) period during the L:D portion of the trials. I combined data from these two days to determine whether singing or locomotion occurred during a given time period on either day for each individual. For statistical analysis, the 24 hour L:D cycle was divided into six hour periods, the total song or locomotion activity was calculated for each six hour period and normalized to the maximal activity for each individual. Student's t-tests were used to compare activities between species for each of the six hour periods. To plot the results, I calculated the mean activity for each species in 0.5 hour bins, normalized to the maximal activity for each species, and plotted this against zeitgeber time (ZT).

I estimated the endogenous free-running period from both the song and locomotion data using the Lomb-Scargle periodogram method [49] with a resolution of 0.1 hour. When the Lomb-Scargle method was not able to identify a significant free-running period estimate those data were excluded from further analyses; this situation occurred in seven of 92 total estimates. Double-plotted actograms, in which each line represents two days, were produced to visualize the data. I used a correlation analysis and a paired t-test to verify that the free-running periods of song and locomotion are positively correlated and did not differ significantly from one another.

Regression analyses were used to determine whether free-running period is related to the maturity age (number of days past final molt) of the crickets or the start date of the trial, a factor that would account for any effects of temperature variation during rearing or trials. Interspecific differences in the free-running periods were tested using *t*-tests assuming unequal variances. An analysis of covariance (ANCOVA) was performed to examine song pulse rate as a function of circadian free-running period, species, and the interaction between free-running period and species. Regression analyses were performed with *L. paranigra* data to further examine the relationship between song pulse rate and circadian free-running period. Furthermore, because there was a significant effect of trial start date on the free-running period of song, I performed within trial regression analyses for each of the three trials containing more than two *L. paranigra* individuals. The statistical software JMP, version 7.0 (SAS Institute, Cary, NC) was used to conduct all statistical tests.

Identification and Sequencing of per

To identify the *Laupala per* sequence I isolated total RNA from the head of a *L. cerasina* male using a PureLink Total RNA Purification System (Invitrogen, Carlsbad, CA). First-strand cDNA was synthesized from RNA with SuperScript II (Invitrogen, Carlsbad, CA). I designed degenerate PCR primers (GGATACAGAATGGTCCGCTTT**YR**T**N**AA**Y**CC and CGAAGAATCGCTGAATGTTTTC**R**TT**R**TA**R**TT) based on regions of high amino acid conservation between the cricket *G. bimaculatus* and other insects using the CODEHOP method. Using these primers I performed PCR and amplified a 470 bp region of the *per* transcript from the cDNA. This fragment was cloned using a TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA) and sequenced. Based on the initial sequence, rapid amplification of cDNA ends (RACE) was performed from *L. cerasina*

head total RNA using a GeneRacer kit (Invitrogen, Carlsbad, CA) to amplify and sequence the 5' and 3' portions of the transcript.

To obtain high quality sequence from both species, total RNA was isolated from heads of *L. cerasina* and *L. paranigra* males, and reverse transcribed into first-strand cDNA as above. PCR primers were designed based on the RACE sequences to amplify most of the *per* transcript. The forward primer, ACGCATGTGTCAAGAGCTGA, binds to a site in the 5' untranslated region (UTR) of *per*. The reverse primer, ATGGGTCGAGGTTTGTTGTG, binds near the 3' end of the coding region of *per*. First-strand cDNA products from each species were used as templates for PCR reactions with Platinum Taq HiFi polymerase (Invitrogen, Carlsbad, CA), producing products of approximately 3200 bp. Each PCR reaction was performed in duplicate so that each portion of the PCR product was amplified, cloned, and sequenced at least twice from each species, with most regions being sequenced several times to reduce the presence of PCR and sequencing errors. Because there was not a good 3' UTR priming site, the 3' end of the transcript was amplified using 3' RACE in both species. The sequences were assembled into contiguous sequences (contigs), aligned, and analyzed using BioEdit sequence analysis software (Ibis Therapeutic, Carlsbad, CA), ClustalW [50], and NetPhos 2.0 phosphorylation site prediction software [51]. The BLOSUM 62 matrix was used for determining amino acid similarities. The deduced amino acid sequences of *Laupala* PER were compared with the PER sequences from *G. bimaculatus* (GenBank Accession: BAG48878) and *D. melanogaster* (Accession: AAF45804).

Alternative Splicing of per

Two possible alternative splice sites were identified within the coding region of *per* based on presence or absence of regions of DNA within different clones.

However, cloning reactions can create artifacts that resemble alternative splicing, so I verified alternative splicing by splice-site specific PCR. Primer pairs flanking each of the putative splice sites were designed (Table 3.1). If a site is not alternatively spliced, PCR with the flanking primers produces a single band, whereas two bands are seen if alternative splicing does occur. PCRs were performed using cDNA from heads of both *L. cerasina* and *L. paranigra* using the primers flanking each of the putative splice sites, as well as an apparently non-spliced control region of *per* as a control.

Analysis of Daily per *Cycling*

To determine whether daily cycling of the *per* transcript occurs in *Laupala* and whether there are differences between the species, I performed quantitative PCR (qPCR) with total RNA from the heads of *L. cerasina* and *L. paranigra* males maintained under 12:12 L:D and sacrificed at each of six times of day. Three males from both species were sacrificed at each of the following times: ZT 3, 9, 11, 13, 15 and 21 (ZT 0 = lights on). The time course focused heavily around lights out because *per* transcript abundance is expected to peak around this time[38]. Crickets were sacrificed by CO_2 anesthesia, decapitated, the heads submerged in RNAlater (Ambion, Inc, Austin, TX), and stored at 4°C until all tissues were collected and could be processed. Total RNA was extracted from each of the heads using the PureLink kit, and quantified using a Nanodrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA). Two µg of each RNA sample was treated with DNase (Turbo DNA-free; Ambion, Inc) in 50 µl reactions according to the manufacturer's protocol to remove contaminating DNA. The RNA samples were reverse transcribed with random hexamer primers using AffinityScript reverse transcriptase (Stratagene, La Jolla, CA).

Table 3.1. Splice site specific *per* primers. Primers were designed to flank the alternative splice sites. The control primers are from a region of *per* which is not expected to be alternatively spliced.

Splice Site	Forward Primer	Reverse Primer
Control	CCTTCGACGCTTGGTAGTGGAA	ACACGATCTTTGGGGTGGACAA
Splice Site 1	GCGTCTCAACGCTGCAAAGATT	CTTCAGTGAGGTGGGGTGGTTG
Splice Site 2	CACCCCACCTCACTGAAGCACT	CTCCACTTCTCTCGGAGGTCCA

I performed qPCR using the relative standard method with Power SYBR PCR Master Mix (Applied Biosystems Inc, Foster City, CA) and a 7900 HT Sequence Detection System (Applied Biosystems Inc, Foster City, CA). The *per* primers used were ATGGGTGGTTCACAGTCTCCACAT and TGACCAGGAAGAAGCATGCCAGTA, with 16S rRNA as an internal control. For the standard curves, cDNA samples were pooled and diluted to produce a curve ranging from 0.25x to 4x the amount used in the experimental reactions. I standardized the *per* and 16S control results to their respective standard curves, and then normalized the *per* results to the 16S internal control for that sample. The *per* transcript abundances normalized to the peak abundance were plotted against time using SigmaPlot 10 (SPSS Inc, Chicago, IL). A two-way ANOVA was used to test for statistically significant daily variation or interspecific differences.

RESULTS

Examination of Daily Timing and Free-Running Periods

I found significant differences in the timing of daily activities between *L. cerasina* and *L. paranigra* under L:D conditions (Figure 3.2). *L. cerasina* performed a significantly greater portion of their song activity in the first six hours of light ($t = 3.96$, $df = 33$, $p < 0.01$), while *L. paranigra* sang primarily during the last six hours of light ($t = 4.54$, $df = 33$, $p < 0.01$). The locomotion data indicate that *L. paranigra* are less active early in the dark phase than they are late in the dark phase, with a gradual increase in activity as the dark phase progresses. During two time periods, the first six hours of light ($t = 2.55$, $df = 33$, $p = 0.016$) and the first six hours of dark ($t = 5.66$, $df = 33$, $p < 0.01$), *L. cerasina* were significantly more likely than *L. paranigra* to show locomotory activity, with no significant differences at other times of day.

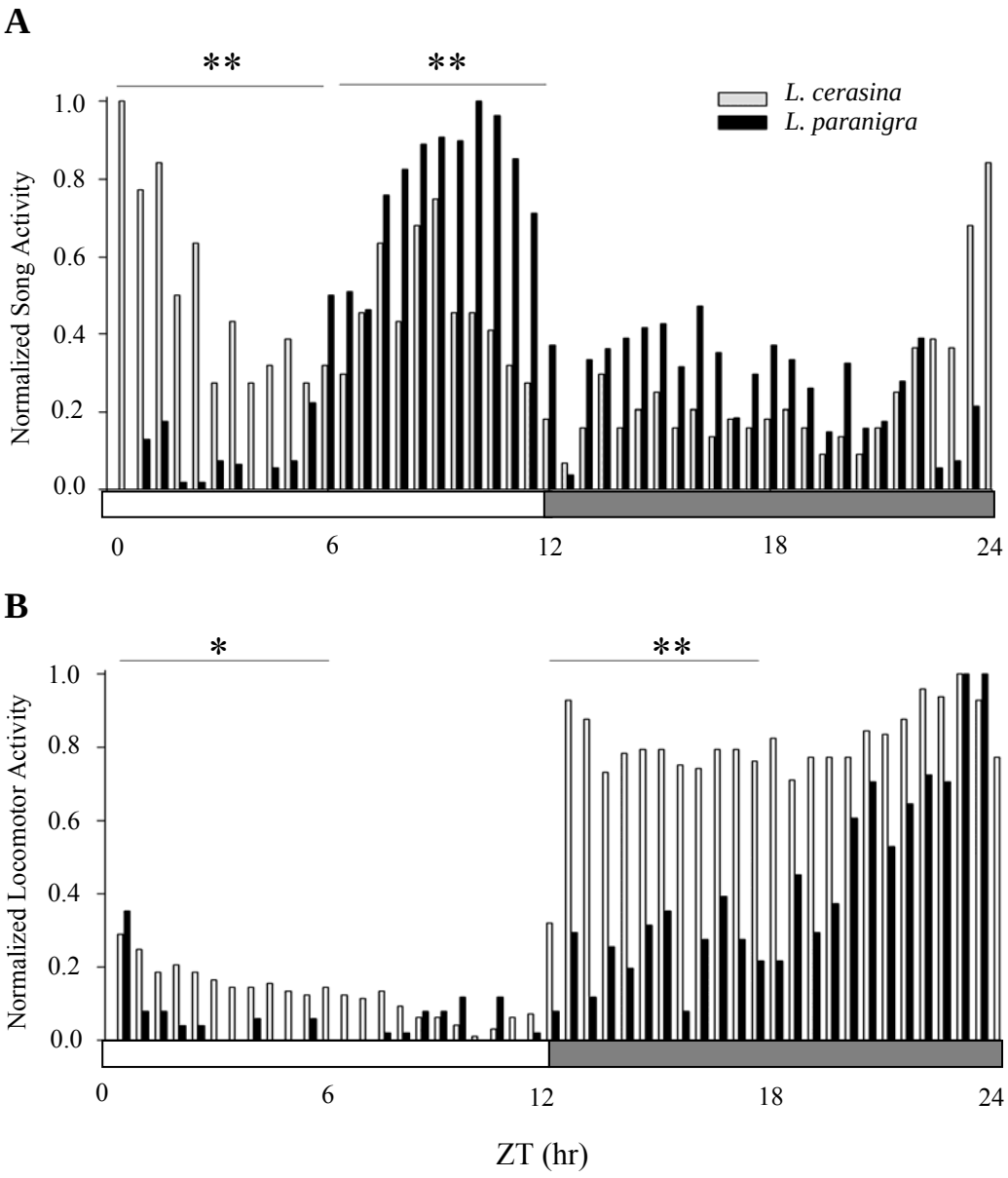


Figure 3.2. Daily song and locomotor activity. The normalized activity of *L. cerasina* and *L. paranigra* under L:D conditions is plotted against ZT hour. The horizontal lines with asterisks above the graphs indicate significant interspecific difference in activity over the six hour period. (* $p<0.05$; ** $p<0.01$)

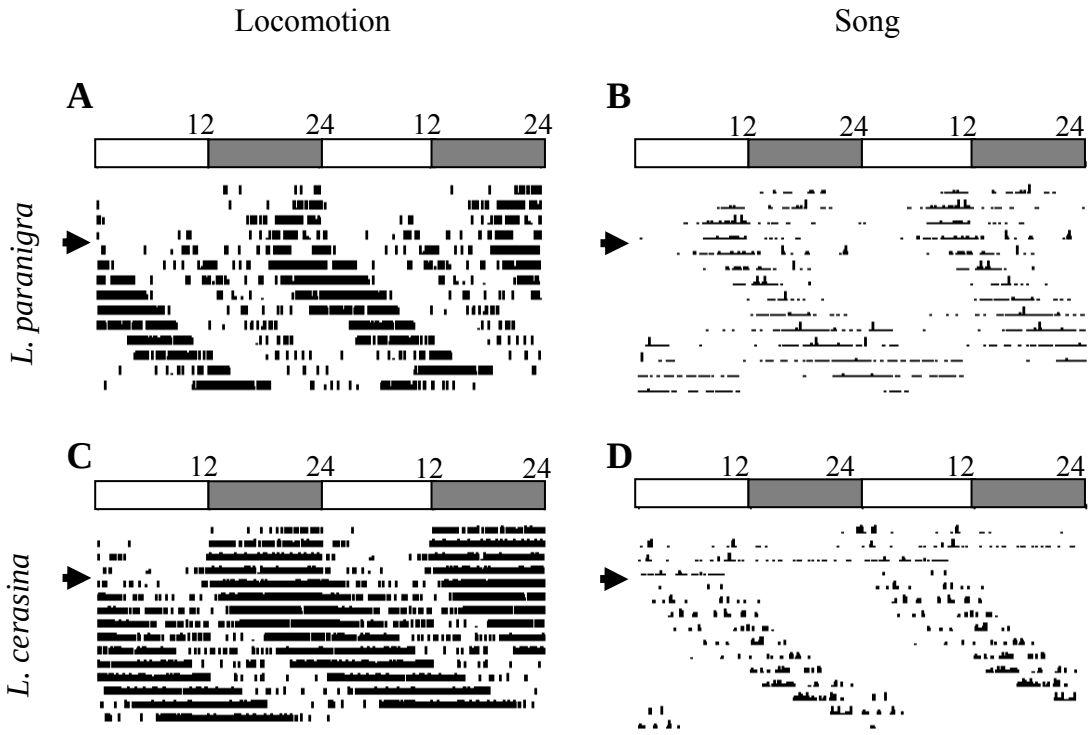


Figure 3.3. Representative double-plotted actograms of locomotor and song activity. The white and gray bars and ZT hour indicate the timing of the initial L:D regime and arrows indicate the change to constant low light . Each actogram displays data from one individual. All individuals had free-running periods greater that 24 hour for both locomotion and song, with *L. cerasina* generally having a longer free-running period than *L. paranigra*.

Males of each species exhibited endogenous circadian free-running periods for both singing and locomotion over a minimum of 10 days of constant low light (Figure 3.3). Using Lomb-Scargle periodogram analyses, I identified significant free-running periods (at $\alpha = 0.01$) in 81% and 96% of *L. cerasina* for song and locomotion respectively and in 100% and 95% of *L. paranigra* for song and locomotion. A paired t-test showed no difference in the length of an individual's free-running period as estimated by singing or by locomotion ($t = 0.37$, $df = 38$, $p = 0.71$). Additionally, free-running period estimates based on song were positively correlated with free-running period estimates based on locomotion for both *L. cerasina* ($p < 0.01$, $r = 0.63$) and *L. paranigra* ($p < 0.01$, $r = 0.67$).

I used regression analyses to examine the effect of age and trial start date on the estimated free-running periods. The ages of the crickets used in this study had no significant effect on the free-running estimates based on song or locomotion for either species (song: *L. cerasina*, $p = 0.65$; *L. paranigra*, $p = 0.90$; *locomotion*: *L. cerasina*, $p = 0.79$; *L. paranigra*, $p = 0.09$). There was a significant negative relationship between the trial start day and the free-running period estimate based on song in *L. paranigra* ($p = 0.013$, $R^2 = 0.29$), with individuals in later trials having shorter free-running periods than those in earlier trials. However, no significant relationship was found in the locomotion estimate from *L. paranigra* ($p = 0.11$) or either of the *L. cerasina* free-running period estimates (song: $p = 0.23$; locomotion $p = 0.50$). A 0.5°C increase in the rearing temperature of the crickets during the course of this experiment may account for the shorter song free-running period in later trials of *L. paranigra*.

The mean free-running period estimates based on both song and locomotion were longer in *L. cerasina* than in *L. paranigra* (Table 3.2). The estimates based on

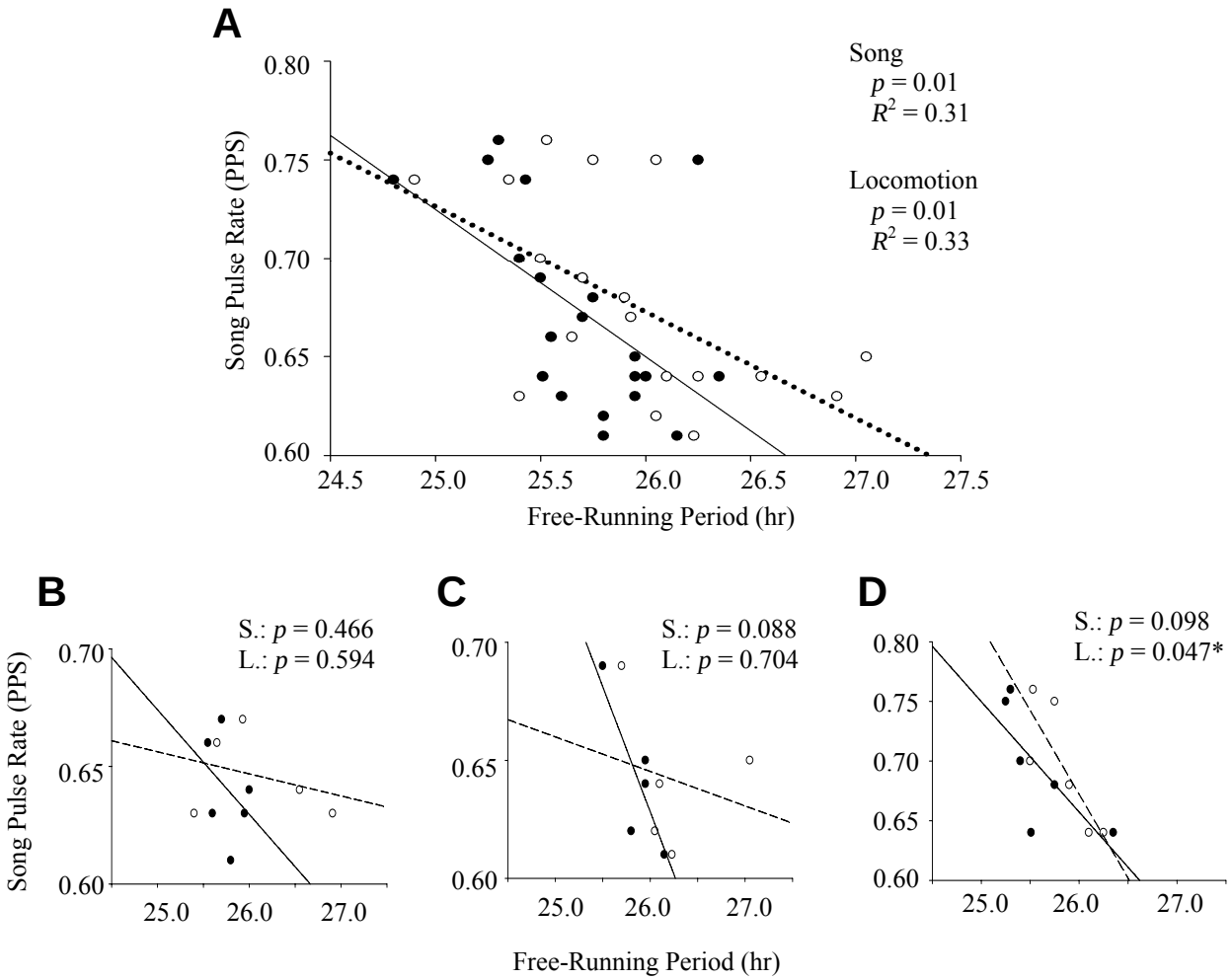
Table 3.2. Song pulse rates (PPS) and endogenous free-running periods were calculated based on song and locomotion. The mean of the song and locomotion free-running period estimates were also determined for each individual. The species averages are displayed as mean ± SD (n). The variation in "n" within species is due to exclusion of non-significant free-running period estimates.

		Free-Running Periods (hr)		
Species	Pulse Rate	Song	Locomotion	Mean
L. cerasina	2.38 ± 0.02 (26)	26.43 ± 0.22 (21)	26.06 ± 0.17 (25)	26.25 ± 0.18 (26)
L. paranigra	0.67 ± 0.01 (20)	25.70 ± 0.08 (20)	25.94 ± 0.12 (19)	25.82 ± 0.09 (20)

song were significantly different ($F_{1,39} = 9.60$, $p < 0.01$) while the difference based on locomotion was not statistically significant ($F_{1,42} = 0.28$, $p = 0.58$). The estimated interspecific difference in free-running periods ranged from 0.12 hour (locomotion) to 0.73 hour (song; Table 3.2).

Males' songs were recorded and the pulse rates were measured for both species (Table 3.2). Regression analyses suggested no significant relationships between song pulse rate and trial start date (*L. cerasina*: $p = 0.75$; *L. paranigra*: $p = 0.25$), age (*L. cerasina*: $p = 0.66$; *L. paranigra*: $p = 0.48$), or song recording temperature (*L. cerasina*: $p = 0.21$; *L. paranigra*: $p = 0.92$). ANCOVAs demonstrated statistically significant interaction effects between species and song free-running period as well as species and locomotion free-running period on song pulse rate (song*species: $F_{1,37} = 4.41$, $p = 0.043$; locomotion*species: $F_{1,40} = 4.83$, $p = 0.034$), indicating that the relationship between pulse rate and free-running period differed by species . To this end, I found significant relationships between song pulse rate and free-running period of both song ($p = 0.011$, $R^2 = 0.31$) and locomotion ($p = 0.011$, $R^2 = 0.33$) in *L. paranigra*, with longer free-running periods corresponding to slower pulse rates (Figure 3.4A). No such relationship between song pulse rate and free-running period was observed in *L. cerasina* (song: $p = 0.36$; locomotion: $p = 0.30$). To account for the effects of trial start date, I performed within trial regression analyses for each of the three trials containing more than two *L. paranigra* individuals (Figure 3.4B-D). Five of these six regression analyses demonstrated non-significant negative relationships, while one (Figure 3.4D) showed a significant negative relationship with the locomotion free-running period ($p = 0.047$, $R^2 = 0.67$).

Figure 3.4. Linear regressions of *L. paranigra* song pulse rate by endogenous circadian free-running period of song (closed circle, solid line) and of locomotion (open circles, dashed line). (A) The overall data shows a significant negative relationship between song pulse rate and the free-running periods of song and locomotion. (B-D) To account for the variation between trials, regressions were performed for each the three trials that included more than two *L. paranigra* individuals. Most of these regressions showed non-significant negative relationships between pulse rate and free-running periods. (D) There was a significant relationship between song pulse rate and the free-running period of locomotion during one trial.


A
Song
p = 0.01
R2 = 0.31
Locomotion
p = 0.01
R2 = 0.33
Song Pulse Rate (PPS)
0.80
0.75
0.70
0.65
0.60
24.5
25.0
25.5
26.0
26.5
27.0
27.5
Free-Running Period (hr)
B
S.: p = 0.466
L.: p = 0.594
C
S.: p = 0.088
L.: p = 0.704
D
S.: p = 0.098
L.: p = 0.047*

Identification and Sequencing of per

I obtained the 3770 bp full length transcript sequence of the *L. cerasina* transcript (GenBank accession No. GU053569), and 3702 bp of the *L. paranigra* transcript of *per* (GenBank accession No. GU053570), which included all but a short region of the 5' untranslated region of the transcript. The nucleotide sequences were 99.6% identical between species, with only 14 interspecific nucleotide differences in the 3702 bp which was sequenced from both species. The deduced *Laupala* PER amino acid sequences had eight non-synonymous (amino acid coding) differences (Figure 3.5). Two predicted serine phosphorylation sites were differentially present / absent between the *L. paranigra* and *L. cerasina* sequences.

Laupala PER amino acid sequences differed substantially from both *G. bimaculatus* and *D. melanogaster* (Table 3.3; Figure 3.5). The divergence between *G. bimaculatus* and *Laupala* resulted largely from regions of low sequence identity interspersed with regions of high identity (Figure 3.5). Amino acid divergence within *Laupala*, and between *Laupala* and *G. bimaculatus*, primarily occurred outside of the identified functional domains as identified in *G. bimaculatus* [38].

Alternative Splicing of per

Two potential alternative splice sites were detected in the process of cloning and sequencing the *per* transcript (Figures 3.5 & 3.6). Splicing of the first alternative splice site (splice site 1) removes a 75 bp region from the transcript. The second alternative splice site (splice site 2) removes a 123 bp segment. Splice site specific PCR products of both the expected spliced and non-spliced sizes were observed in the heads of *L. cerasina* and *L. paranigra*, while the control PCR of a non-spliced region showed a single band in each species. These results confirm that alternative splicing of *per* occurs in *Laupala*.

Table 3.3. Interspecific amino acid identity and similarity of PER. The numbers given for the comparisons of *Laupala sp*. to the other species are accurate for both *Laupala* species.

Species Comparison	Identity	Similarity
L. cerasina – L. paranigra	99%	99%
Laupala sp. – G. bimaculatus	65%	74%
Laupala sp. – D. melanogaster	29%	43%

Figure 3.5. Alignment of *L. cerasina* (*Lc*), *L. paranigra* (*Lp*), and *G. bimaculatus* (*Gb*; BAG48878) PER proteins. Alternative splice sites are italicized and underlined. Functional protein domains (PAS-A, PAS-B, NLS, and CLD), based on [38], are indicated below the alignment. Arrows designate variable predicted phosphorylation sites.

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Lc  MEESDTSTHKVSDSGYSNSCNSQSQRSSGSSKSHHSNSSGSSGYGCHPSTLGSGTEAFRQPPVTKRNKDKEHKKKKLKST 80
Lp  MEESDTSTHKVSDSGYSNSCNSQSQRSSGSSKSHHSNSSGSSGYGCHPSTLGSGTEAFPQPPVTKRNKDKEHKKKKLKST 80
Gb  MEESDTSTHKVSDSGYSNSCNSQSQRSSGSSKSHHSNSSGSSGYGGHPSTVGSGTEVFPQPHVTKRNKDKEHKKKKAKST 80

Lc  LVAATSDNHVESKNLAVSPSIAHESTISDNSKPITKASSKTVSKSPSKLPGQISSNAPSIGVNNLVIEVNANSQPSLSTV 160
Lp  LVAATSDNHVESKNLAVSPSIAHESTISDNSKPITKASSKTVCKSPSKLAGQISSNAPSIGVNNLVIEVNANSQPSLSTV 160
Gb  LTSATTDHHVDSKNVAASP-VTETTLCTEGVKSANKTVSKTTSKSTAKVGVQNST-CSAPGVN---TETTEDVQLPLTCL 155
                                                  ↑

Lc  SQSPDTDVTPVINEEKDGSS-LVGDPEAQET-LECLRNSEEPVPQIEDEFSTIVSLHDGVVMYTTSTITKVLGFPKDMWL 238
Lp  SQSPDTDVTPVINEEKDGSS-LVGDPEAQET-LECLRNSEEPVPQIEDEFSTIVSLHDGVVMYTTSTITKVLGFPKDMWL 238
Gb  SQSSNKELPPVITEENECATGGIADPEEQEINQNCVRNDISPI-QIENEFSAIVSLHDGVVMYTTTSITSVLGFPKDMWL 234
                                                 (*******************PAS-A*****

Lc  GRSFIDFVHPKDRVAFASHITTGFSLPVEENRCKVSLTAKESFYCCLRQYRGLKANGYGVTEKKVTYLPFHLTMTFRDVK 318
Lp  GRSFIDFVHPKDRVAFASHITTGFSLPVEENRCKVSLTAKESFYCCLRQYRGLKANGYGVTEKKVTYLPFHLTMTFRDVK 318
Gb  GRSFIDFVHPKDRMAFASHITTGVALPVEENRCKVSLTAKESFYCCLRQYRGLKSSGYGVTEKKVTYLPXHLTXTFRDVT 314
    ***********************)

Lc  NSEKMNLAGEETGIQGSFLIIEATLVKSSYTHPEETKNSSKFIMQHQASCQLSSVSSDVVQYLGYLPQDMVNHSIFEFYH 398
Lp  SSEKMNLAGEETGIQGSFLIIVATLVKSSYTHPEEMKNSSKFIMQHQASCQLSSVSSDVVQYLGYLPQDMVNHSIFEFYH 398
Gb  STEKMGLAEEEPGIQGSFLIILASRIKPAYTHPDETKISSKFIIRHQASCELSHVDSDIVQYLGYMPQDMIGRSVFEFYH 394
    ↑               (*********************************PAS-B************************

Lc  PDDTPYLKEVYERVVKAQGKPFRSKPYRFQVQNGDYVLLDTEWSAFINPWSRKLEFVIGQNRVLKGPSNPDVFAPPKEAD 478
Lp  PDDTPYLKEVYERVVKAQGKPFRSKPYRFQVQNGDYVLLDTEWSAFINPWSRKLEFVIGQNRVLKGPSNPDVFAPPKEAD 478
Gb  PEDSPYLKEVYEGVIKAQGQPFRSKPYRFKAQNGGFVLLDTEWSAFINPWSRKLEFIIGQNRVLKGPPNPNVFVTQTSED 474
    *****************) (************************CLD*****************************)

Lc  NIQISEEVLKKRNVIEQEIEHLLNETIQRTTEAAKQLASQRCKDLATFMENLMEEVAKPELKVDLPTEEQSFS------E 552
Lp  NIQISEEVLKKRNVIEQEIEHLLNETIQRTTEAAKQLASQRCKDLATFMENLMEEVAKPELKVDLPTEEQSFS------E 552
Gb  CLQISEEVLKESKVIQHEIENLLKETIQRTTGAAKQVASKRCKDLATFMEILMDEVTKPELKVDLPSEEQSFSKNIILQE 554

Lc  RDSVMLGEISPHHDYYDSKSSSGTPPTYNQLNYNENIQRFFESKPKTTVSDES---KMEANRS-NSTDEEGKSMPVADSS 628
Lp  RDSVMLGEISPHHDYYDSKSSSGTPPTYNQLNYNENIQRFFESKPKTTVSDES---KMEANRS-NSTDEEGKSMPVADSS 628
Gb  RDSVMLGEISPHHDYYDSKSSSETPPSYNQLNYNENIQRFFESKPKTTLSDESGESKTDANRSHNSTDEEGKSMPVADSS 634

Lc  LDSSNRFVSDSYRKRHMRIKETTLRCKFKKRKCCSPINGSGSG--GSSGSAGMPGSAASRGDTSATNTSRGSYQPPHLTE 706
Lp  LDSSNRFVSDSYRKRHMRIKETTLRCKFKKRKCCSPINGSGSG--GSSGSAGMPGSAASRGDTSATNTSRGSYQPPHLTE 706
Gb  LNSSNR------------------------KCCSPVNGSGSGSGGSSGSAGMPGSAASRGDTSATNTSHGSYKPPHLTE 689

Lc  ALLCRHNEDMEKQMVQKHREQRSKGERDNKKKFPQEKMQEANHGVKRCGSHSWESEPFKASKYPHVENLLATGNAVPLPN 786
Lp  ALLCRHNEDMEKQMVQKHREQRSKGERDNKKKFPQEKMQEANHGVKRCGSHSWESEPFKASKYPHVENLLATGNAVPLPN 786
Gb  ALLCRHNEDMEKQMVQKHRELRSKG--DSKKKMSHEKLQEQNHGVKRSGSHSWEGEPFKASKHPHVENLLASGNAVPMPN 767
                                      (************NLS************)

Lc  IATMGGAAAVPSMFPGSPNVNLWPPFSVTVTPHLSSQPCFAHSTYTGANMGGSQSPHLASMIPMYYIPTGSHQTNLPSRG 866
Lp  IATMGGAAAVPSMFPGSPNVNLWPPFSVTVTPHLSSQPCFAHSTYTGANMGGSQSPHLASMIPMYYIPTGSHQTNLPSRG 866
Gb  VAALGGATQMSPMYPGSPNVNLWPPFSVTVTPLQSTQPCLAHNSFPGRHNGKFTVSSFGQHDSCLLHPHWVTAGQFAPSR 847

Lc  LTPQEHPGPPHTGMLLPGQPQYIPSQVPVINPMPSMLYHPMQPMYGAPQMLYSSIMLQPSTILPAPLSQAGMLPATSRAL 946
Lp  LTPQEHPGPPHTGMLLPGQPQYIPSQVPVINPMPSMLYHPMQPMYGAPQMLYSSIMLQPSTILPAPLSQAGMLPATSRAL 946
Gb  LSTSGAPWXSSNRHAPAWQAQYILSAVHDK-FIPSMLYHPVHQMYGLLPMMYSSVMLQPSTILPAPMSQAGLLSASSRSM 926

Lc  VKQGKPMTESGTPSGVGAASKFQRPASQATSVKAEPGSAMGSIASASIKRALSECSKKDKSLCSPGAPTSSPGPDEEKPR 1026
Lp  VKQGKPMTESGTPSGVGAASKFQRPASQATSVKAEPGSAMGSIASASIKRALSECSKIDKSLCSPGAPTSSPGPDEEKPR 1026
Gb  MKQDKPPNENGTPNGVGPTTKFQRPASQATSVKAEPGSAMGSIASASIKRAMSECSKKDKSLCSPGAQTSSPCPEEDKTK 1006

Lc  EVE-NRDFGPPREVENTTGDDSSYSSFYSFLRTDNTDDSMNSYPRDKECLYPCKSEDMNWERSENCKKSHNKPRPILKDP 1105
Lp  EVE-NIDFGPPREVENTTGDDSSYSSFYSFLRTDNTDDSMNSYPRDKECLYPCKSEDMNWERSENCKKSHNKPRPILKDP 1105
Gb  EGHGNLDFGPLREIENTTGDESSYSSFYSFLRTDKSDESMKSSPRDKDFPYPCKPEVRVL                      1066

Lc  PWLEHVNVTPDLVYRYQINEKNLESVLENDLQTLKEIQQVTSLQ 1149
Lp  PWLEHVNVTPDLVYRYQINEKNLESVLENDLQTLKEIQQVTSLQ 1149
```

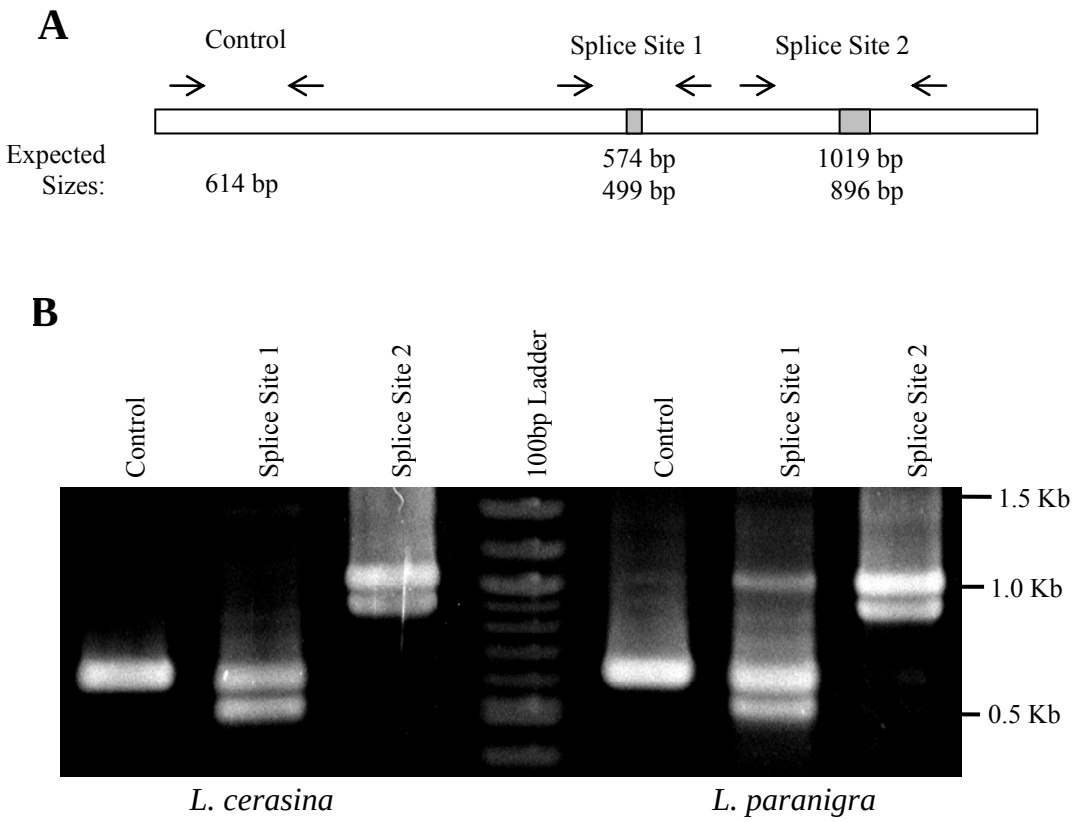

Figure 3.6. Alternative splicing of the *per* transcript. (A) The schematic representation of the *per* transcript demonstrates the location of primers (arrows) relative to the alternative splice sites. Expected PCR product sizes with and without the alternative exons (gray regions) are given below each site. (B) The PCR results which show bands at the expected sizes indicate that both sites are alternatively spliced in the heads of *L. cerasina* and *L. paranigra*.

Analysis of Daily per *Cycling*

Transcript abundance of *per* varied significantly through the day ($F_{5,24} = 4.41$, $p < 0.01$, Figure 3.7) in an apparent daily cycle. As expected, the *per* transcript was most abundant close to lights out (ZT: 12), with the peak observation occurring one hour before lights out (ZT 11) in both *L. cerasina* and *L. paranigra*. The two species did not differ from one another across times ($F_{1,24} = 0.15$, $p = 0.70$).

DISCUSSION

Daily Activity Times and Free-Running Periods

Circadian rhythms are an important adaptation [2] that allow an organism to anticipate and prepare for daily and seasonal environmental variation. Tremendous effort has gone into examining circadian rhythms in insects [52-58], with *Drosophila* providing the most well characterized molecular model of a circadian clock [4-8, 59-62]. However, even before the first clock mutant was reported in *D. melanogaster*, researchers had been investigating daily variation and circadian rhythms in crickets [26, 63-65]. The circadian mechanism in crickets appears to differ from that of *Drosophila*, both in the molecular basis of the endogenous clock as well as the localization of the circadian pacemaker neurons [33, 35-37, 39-41, 66]. Understanding both the behavioral variation in circadian rhythms among crickets and mechanisms underlying cricket circadian clocks will provide insight into the evolution and plasticity of circadian rhythm generation, as well as further our understanding of the molecular basis of circadian timing.

In this study I found significant differences in the daily timing of both song activity and locomotion under L:D conditions between *L. cerasina* and *L. paranigra*. For both activities the onset of activity is earlier in *L. cerasina* than in *L. paranigra*,

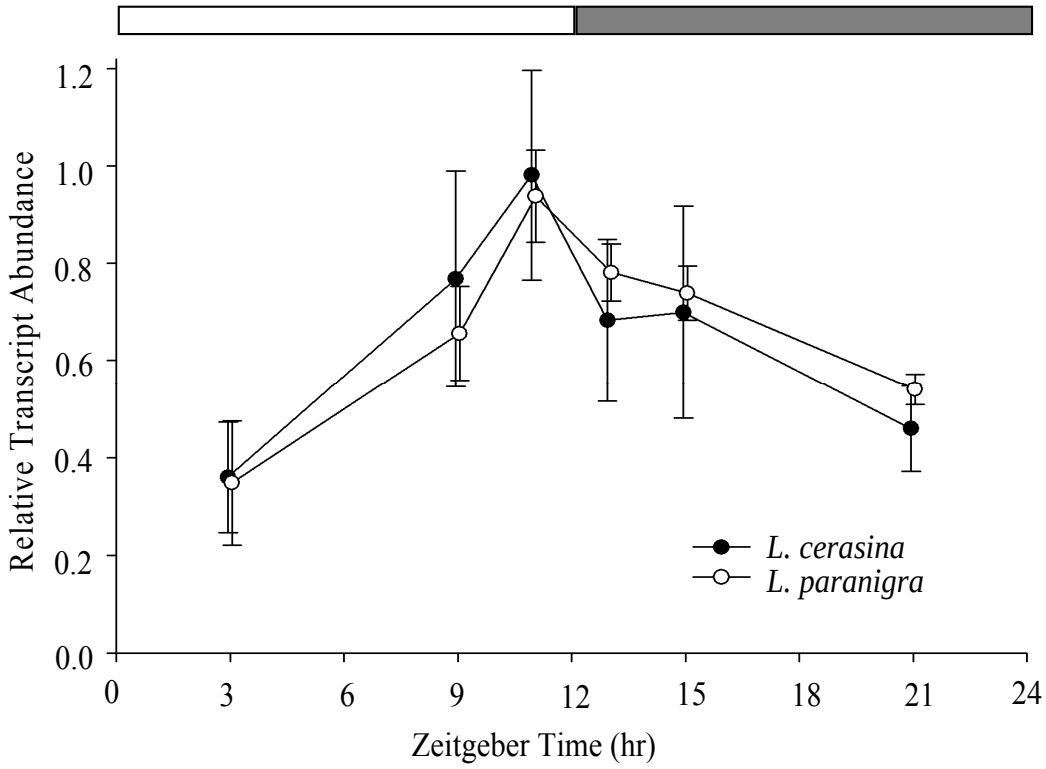

Figure 3.7. qPCR of *per* transcript abundance in the heads of *L. cerasina* and *L. paranigra* throughout the day. The white and gray bar at the top indicates periods of light and dark. The results demonstrate daily cycling of *per* transcript abundance in both species, but no interspecific difference in timing. The data points are offset slightly for ease of viewing.

consistent with previous reports of daily timing in these species [46, Chapter 1]. Interestingly, the interspecific differences in activity times appear to result from both a shift in the timing of activity as a whole, as well as variation in the underlying temporal pattern of activity. Most notably, while the locomotion activity of *L. cerasina* is high immediately at lights-out and remains high throughout the dark phase, the locomotion activity of *L. paranigra* appears to increase throughout the dark phase to peak just before lights-on (Figure 3.2B).

I have demonstrated free-running periods for both song and locomotion activity in *Laupala*. The positive correlations and lack of significant differences within species between the free-running period of song and of locomotion is consistent with previous work from *T. commodus* suggesting a single circadian pacemaker regulates the timing of both song and locomotion in crickets [32].

There was a statistically significant difference in the circadian free-running period of song between *L. cerasina* and *L. paranigra*, while the difference in the free-running period of locomotion was not significant. The observed difference of approximately one half-hour in the mean free-running periods is smaller than the roughly two hour difference in mating times observed between these species [46, Chapter 1] or the 10 hour difference seen here in peak song time (Figure 3.2A). However, the timings of these characters are not likely to scale exactly with one another due to other non-circadian temporal regulation and zeitgeber cues. More notably, the direction of the difference in free-running periods is the opposite of what I hypothesized. In *D. melanogaster* and *B. cucurbitae,* flies with longer free-running periods have later phases of locomotor activity and mating than flies with short free-running periods [47, 48]. While *L. cerasina* has a longer free-running period than *L. paranigra*, singing, mating and locomotion occur earlier in *L. cerasina* than *L. paranigra*. Thus, while song and locomotion are under circadian regulation, the basis

of the difference in daily timing between the two *Laupala* species may be independent of the variation in circadian rhythms, or the molecular mechanism regulating both behaviors may be different than that seen in other species.

I observed a significant negative relationship between free-running periods and song pulse rate in *L. paranigra*. This relationship is consistent with the relationships observed in *T. oceanicus* [67], *D. melanogaster* [68] and *B. cucurbitae* [69], but was not seen in *L. cerasina*. It is important to note that the relationship between pulse rate and song free-running period in *L. paranigra* may be confounded by the significant effect of trial start date on song free-running period. However, the significant negative relationship also exists between song pulse rate and locomotion free-running period, which was not significantly related to trial start day. Furthermore, examinations of within trial regression analyses were consistent with the negative relationship between pulse rate and free-running periods. The six within trial regressions displayed five non-significant negative relationships and one significant negative relationship. Given the small within trial sample sizes, the consistent trends and significant result provide strong evidence that the relationship between pulse rate and free-running period is not accounted for by trial start date.

Extrapolating the best-fit line of the pulse rate regression for *L. paranigra* to the mean song rate of *L. cerasina* (2.38 pps) would predict an endogenous circadian free-running period of less than 3 hours for *L. cerasina*. Such a short free-running period would obviously be maladaptive. If there is a common genetic basis to the variation in song and circadian rhythms, there is likely a constraint on how much a gene affecting both can vary.

Analysis of the per *Gene*

In *D. melanogaster*, circadian free-running period and modulation of courtship song interpulse interval are both affected by mutations of the *per* gene [8]. Furthermore, natural variation in *per* accounts for differences between *Drosophila* species in courtship and the daily timing of locomotion and mating [70-72]. While PER protein abundance has not been shown to cycle in crickets [40, 41], *per* transcript abundance has been found to cycle on a daily basis in the cricket *G. bimaculatus* and was important for proper expression of circadian rhythms in that species [27, 38]. The temporal variation in circadian rhythms, daily activity, and song pulse rate observed in *Laupala* make *per* a strong candidate for *Laupala* behavioral variation.

Using qPCR I tested the hypotheses that *per* transcript abundance varies across the day, and that the *per* transcript will accumulate later in *L. cerasina* than *L. paranigra*, consistent with the longer free-running periods of *L. cerasina*. I confirmed that there is daily cycling of the *per* transcript in *Laupala* heads with a peak around lights out, similar to what was observed in the cricket *G. bimaculatus* [38]. This is consistent with a role for this gene in maintaining circadian rhythms. Additionally, there was no significant difference between *L. paranigra* and *L. cerasina* in their temporal expression of the *per* transcript. Transcript abundance tended to rise and fall more quickly in *L. cerasina* than in *L. paranigra*, which may suggest an earlier shift in cycling in *L. cerasina*. However, because *L. cerasina* had longer free-running period estimates than *L. paranigra*, a later timing of *per* transcript expression is expected in *L. cerasina*. Thus, the putative earlier timing of *per* transcription seems unlikely.

I observed sequence variation in the *per* gene between *L. cerasina* and *L. paranigra*, which may play a role in behavioral differences between the species. I found eight amino acids differences between these species, two of which would alter predicted serine phosphorylation sites within the PER protein. According to the

Drosophila model, PER phosphorylation is involved in setting the pace of the circadian clock by inducing degradation of PER [73, 74]. It has been proposed that cycling of phosphorylation of clock genes, as opposed to cycling of protein abundance, may be key to maintaining the circadian clock [73]. Such a model may be consistent with the circadian clock of crickets, in which circadian clock protein levels are constant throughout the day [36, 40, 41]. In addition to the changes in putative phosphorylation sites, two non-conserved amino acid changes between *L. cerasina* and *L. paranigra* occurred within the PAS-B domain, a protein interaction domain. One of these PAS-B substitutions is between a negatively charged, polar residue (glutamate; *L. cerasina*) and a neutral, non-polar residue (valine; *L. paranigra*) while the other substitution is between a neutral residue (threonine; *L. cerasina*) and a positively charged residue (histidine; *L. paranigra*). Such differences in polarity and charge at a protein binding site could alter protein binding affinities and may thus have consequences for PER function. The remainder of the amino acid substitutions fall outside of known functional domains and are not predicted to alter phosphorylation; however, most of these substitutions are also non-conserved and may affect the conformation or function of the protein.

In addition to the molecular variation and cycling identified in *Laupala per*, I identified two alternative splice sites of the *per* gene. Both of these sites are alternatively spliced in the heads of both *Laupala* species. Alternative splicing of a single gene produces different isoforms with different properties. The potential function of these alternative splice isoforms of *per* is intriguing. Though the total abundance of PER protein has not been found to cycle throughout the day in crickets, the cycling of different splice forms has not been examined. Cycling of alternative splice isoforms may be key to maintaining circadian cycling in crickets.

The results presented here suggest that the daily variation in behavior observed between *L. cerasina* and *L. paranigra* is not due to a simple shift in endogenous circadian timekeeping. The complex nature of the daily temporal variation suggests that is more likely the result of multiple genetic factors. The potential for comparative analyses makes *Laupala* a tractable system with which to investigate temporal variation and elucidate the genetic underpinnings of temporal regulation. The variation observed in the *per* gene demonstrates the potential for a role of *per* in behavioral variation, and provides the foundation for further analyses of this candidate gene.

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CHAPTER 4

Identifying candidate genes for interspecific behavioral variation

ABSTRACT

The use of genetic model systems has provided valuable insights into the genetic basis of behavior; however, the genes identified in these systems do not always correspond to genes involved in natural behavioral variation. Examining non-model systems provide valuable insights into the genes underlying natural variation. However, studying natural variation in non-model systems presents challenges resulting from the lack of sequence information and molecular tools. In this study I examine sister species of the cricket genus *Laupala*. A key differentiating characters between these species is the pulse rate of the male song. I use suppressive subtractive hybridization (SSH) to identify candidate genes with differential transcript abundance between the species. I further examine transcript abundance in the head and thoracic ganglia, which are involved in cricket stridulatory regulation, to determine whether expression varies in regions likely to be involved in the natural behavioral variation.

INTRODUCTION

Understanding the genetic basis of natural behavioral variation is a major goal of neurobiology. Most studies aimed at identifying genes underlying behavior have used genetic model systems to do so, largely because of the availability of genome sequences, genetic tools and mutational variants in such systems. However, genes identified by mutational analyses may be different than those involved in natural variation. For example, comparisons of mutational studies and quantitative trait loci in *Drosophila* have suggested little overlap in the genes implicated in the genetics of courtship song [1, 2]. In addition to examinations of genetic model systems, it is

important to understand the genetic bases of behavioral phenotypes in non-genetic model systems. These systems present natural variation, which can allow us to determine whether results from model systems can be generalized, and whether the results apply to naturally occurring variation. Consequently, many non-genetic model systems can serve as behavioral, neurobiological, and evolutionary models to can provide insight into the evolution and functional genomics of behavior [3, 4]. For example, work with voles (*Microtus* spp.) demonstrated an important role of the vasopressin receptor V1aR in regulating naturally occurring variation in social interactions [5, 6]. Cloning and analysis of the *period* (*per*) gene from the German cockroach (*Blattella germanica*) has provided information about the molecular evolution of this well-studied circadian clock gene [7]. Channel localization within the lobster stomatogastric ganglion central pattern generator [8, 9] has provided insight into neuronal firing properties and informed models of neural function [10]. While the limited genetic resources of non-genetic model systems can present a challenge, continuing advancements in molecular methods and the insights to be gained from many of these systems makes the investigation of non-genetic model systems both feasible and important to a full understanding of behavioral genetics.

Identifying candidate genes is an important step in elucidating the genetic basis of phenotypic variation [11-13]. A rapid, unbiased, and economic means of identifying candidate genes underlying phenotypic variation can facilitate the investigation of naturally occurring behavioral variation in non-genetic model systems. In this study I adapted the method of suppressive subtractive hybridization (SSH, [14]) to identify differentially transcribed genes as candidates for behavioral variation between sister species of the cricket genus *Laupala*. The SSH method selects for sequences that are rare or absent in one cDNA library relative to a second. The selected differentially expressed transcript fragments are then sequenced. SSH has

been used to identify genes that are differentially regulated among bacterial strains (*Helicobacter pylori*) [15], castes of honeybees (*Apis melifera*) [16], and during diapause of northern house mosquitoes (*Culex pipiens*) [17]. SSH is typically used to compare different body parts from the same individual or different individuals of the same species because it requires high sequence identity so that DNA from the two sources will hybridize efficiently. Here I have applied SSH to two species of crickets, *Laupala cerasina* and *Laupala eukolea*. The recent divergence of these species [18] and low sequence divergence across the genus [19] makes SSH feasible in this system, and will facilitate the identification of candidate genes that are differentially transcribed between the species.

Crickets have served as models for a wide array of biological processes, including behavior [20-23], neurobiology [24-28], and evolution [29-32]. In particular, cricket song has been a focus of research for many decades [33]; physiological examination has demonstrated that the central pattern generator (CPG) for stridulatory singing in crickets is located in the 3rd thoracic ganglion (metathoracic ganglion; TG 3), while the 2nd thoracic ganglion (mesothoracic ganglion; TG 2) plays a role in coordination of the wing movements during stridulation [34]. The firing rate of command neurons from the brain can also influence the chirp rate in crickets [35]. While there has been extensive examination of a wide range of behavioral variation in crickets, little is known about the genetic basis of song variation. Furthermore, genetic and genomic resources are poorly developed in crickets. The only large-scale genetic resources from crickets are 8575 unique expressed sequence tags (ESTs) from *Laupala kohalensis* [36] (http://compbio.dfci.harvard.edu/tgi/) and approximately 11,000 EST sequences from *Gryllus bimaculatus* on the NCBI GenBank database, not all of which are unique. While valuable, these genetic resources are very limited compared to those of genetic model systems. In order to examine the genetic basis of

phenotypic variation in crickets it is necessary to employ methods that do not require extensive *a priori* sequence information. SSH provides such an approach.

Laupala is a genus of Hawaiian swordtail crickets that serves as a behavioral and evolutionary model. *Laupala* have undergone rapid speciation [18], with 38 species diverging within the last 5 million years [19]. While the genus consists of morphologically and ecologically cryptic species [37], there is well-characterized behavioral variation between the species. *Laupala* possess genetically regulated interspecific variation in mating related behaviors including song pulse rate variation [37-39], and daily time of singing and mating [23, Chapter 1]. Thus, *Laupala* are particularly well-suited for comparative studies examining the molecular basis of variation in temporal and rhythmic behaviors. In this study I used SSH to identify candidate gene transcripts that are differentially transcribed between *L. cerasina* and *L. eukolea*, sister species of *Laupala* from the Big Island (Hawaii) and Maui, respectively, that have diverged in their song pulse rates (*L. cerasina*: ~2.4 pulses/sec; *L. eukolea*: ~4.0 pulses/sec). The primary aim of this study is to identify candidate genes that may be involved in behavioral variation and speciation within the genus *Laupala*.

METHODS

Housing and Rearing

All crickets in this study were reared at the University of Maryland (College Park, MD) under 12:12 light:dark at 20º C. Crickets were housed in either quart-sized glass jars or 250 ml plastic specimen cups with moist Kimwipes and provided a diet of Fluker's Cricket Feed (Fluker Farms, Port Allen, LA). Male-female pairs of *L. cerasina* or *L. eukolea* were allowed to mate and the females oviposited into the moistened Kimwipes. Eggs were held until hatching, and nymphs were collected and

maintained under the same conditions as the parents in same-sex containers to prevent unwanted mating.

Suppressive Subtractive Hybridization

L. cerasina and *L. eukolea* were reared to adulthood and one male from each species was sacrificed by flash freezing in liquid nitrogen. Total RNA was isolated from the whole body of each individual using an RNeasy Mini Kit (QIAgen, Inc., Valencia, CA) and the quality was verified using an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA). The SMART cDNA synthesis method (Clontech Laboratories, Inc., Mountain View, CA) was used to reverse transcribe the RNA into full-length cDNA transcripts. The SMART cDNA synthesis method employs a poly-T primer and template switching to produce full length first strand product with adapter sequences on either end that allow for PCR amplification. This first-strand cDNA was then amplified by PCR to produce cDNA for use in the subtraction protocol, while staying within the linear amplification stage of the reaction to maintain the relative abundance of the transcripts. The cDNA products were purified by phenol:choloroform extraction and passed through Chroma Spin 1000 gel filtration columns (Clontech Laboratories, Inc., Mountain View, CA).

I employed SSH to select differentially transcribed genes using the PCR-Select cDNA subtraction kit (Clontech Laboratories, Inc., Mountain View, CA). Briefly, the cDNA from the two species were digested with *Rsa* I to produce blunt-ended fragments. In order to perform subtractions in both directions, samples of each pool of cut cDNA were used as testers, the cDNA from which candidates will be identified, and as drivers, the cDNA to which the tester will be hybridized to remove transcripts common to both pools. Tester cDNA was subdivided into two equal pools and different adapters, ‘1’ and ‘2R’, provided with the subtraction kit, were ligated to the

blunt fragment ends of the two subsamples. Each tester pool was mixed with an excess of driver cDNA from the other species, heated to denature, and then allowed to hybridize. This step binds transcript fragments that are common to both samples. The two tester subsamples, '1' and '2R', were then mixed and hybridized in the presence of an excess of denatured driver. Those cDNA fragments that are present in the testers but rare or absent in the driver hybridize between tester pools '1' and '2R' to produce fragments that could be amplified by PCR. In order to create libraries of transcript fragments that are most abundant in each species, I performed subtractions in both directions; that is, SSH was repeated alternatively using *L. cerasina* and *L. eukolea* as the tester. Both libraries were PCR amplified and cloned with a T/A cloning kit (Invitrogen Corporation, Carlsbad, CA).

Screening of Subtractive Libraries

Screening was performed to reduce the number of false positives present in the subtractive libraries. Probes for screening were produced by digoxigenin (DIG) labeling of both subtractive library products using the DIG DNA Labeling and Detection Kit (Roche Applied Science, Indianapolis, IN). Individual colonies were picked from both subtractive libraries, placed in sterile H_2O, heated to 95˚ C, and used as templates for PCR. The PCR products were spotted, in duplicate, onto two positively charged nylon membranes and cross-linked in a UV cross-linker. One of the duplicate membranes was probed with one subtractive-hybridization product while the other duplicate membrane was probed with the reverse subtracted product in DIG Easy Hyb solution at 42º C overnight. The membranes were washed four times in 2X SSC / 0.5% SDS and two times in 0.2X SSC / 0.5% SDS at 66 ˚ C. They were then incubated with alkaline phosphatase conjugated anti-DIG antibodies, followed by incubation with the chemiluminescent substrate CDPStar according to the DIG DNA

Labeling and Detection Kit. The blots were imaged with a CCD camera imaging system (UVP, Upland, CA). Those clones that showed substantial differential abundance based on the screening process were sequenced, contiguous sequences (contigs) were assembled using the program BioEdit (Ibis Therapeutic, Carlsbad, CA), and the contigs were then compared via BLAST to sequences in the NCBI GenBank database. A BLAST hit E-value cutoff of 10^{-5} was used to detect sequences with relatively high identity while accepting the high sequence divergence expected between *Laupala* and the taxa in the Genbank database.

Verification of Differential Expression

To verify the interspecific differential expression of the candidate genes identified by SSH and screening processes, I used quantitative reverse-transcription PCR. Primers were designed based on the sequences from the SSH clones. The primers were designed to amplify fragments between 50 and 250 base pairs (bp) with optimal primer melting temperatures of 60º C. I extracted total RNA from the whole bodies of two *L. cerasina* males and two *L. eukolea* males using the RNeasy Mini Kit and quantified the RNA by Nanodrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA). Equal amounts of RNA from each species were treated with Turbo DNA-free DNase (Ambion, Inc., Austin, TX) to remove contaminating mitochondrial or genomic DNA. Equal volumes of total RNA were reverse transcribed to produce first-strand cDNA using random hexamer primers with the AffinityScript reverse transcriptase (Stratagene, La Jolla, CA). The first-strand cDNA was used as a template in relative quantitative PCR (qPCR) using Power SYBR PCR Master Mix with a 7900 HT Sequence Detection System (Applied Biosystems, Inc., Foster City, CA). Each qPCR reaction was performed three times, using 16S rRNA as an internal control. Relative interspecific transcript expression was calculated for each candidate transcript

using the $2^{-\Delta\Delta C_T}$ method [40]. The $2^{-\Delta\Delta C_T}$ approach normalizes the experimental threshold cycle results (C_T) from one sample to the C_T of the internal control (16S rRNA) for that sample, and then compares the normalized experimental results between samples.

Analysis of Differential Transcripts

To investigate the possibility that identified candidate transcripts may play a role regulating behavioral variation between species, I dissected out body parts likely to be involved in behavioral regulation. Specifically, I isolated the head (including brain and subesophogeal ganglion [SEG]), the mesothoracic and metathoracic ganglia (TG 2-3; Figure 4.1), and the legs from five males of each species and pooled tissues by body part and species. The head and TG 2-3 are involved in many behaviors in crickets including singing, [34, 35]. The legs are not expected to be directly involved in known behavioral variation and were used as a control for normalizing the results from the other body parts. Total RNA was isolated from each pool of body parts and reverse-transcribed into cDNA, as described above. Only candidate transcripts that appeared to be differentially expressed in the whole-body were examined by qPCR with 16S rRNA as an internal control. Relative transcript abundance for each candidate transcript was calculated between species for each body part as well as between body parts within each species using the $2^{-\Delta\Delta C_T}$ method.

Differences observed in both SSH and qPCR may result from differential transcription or from sequence divergence leading to differences in the efficiency of hybridization or primer binding. To determine whether interspecific sequence variation is influencing the results, transcripts that showed differential abundance in

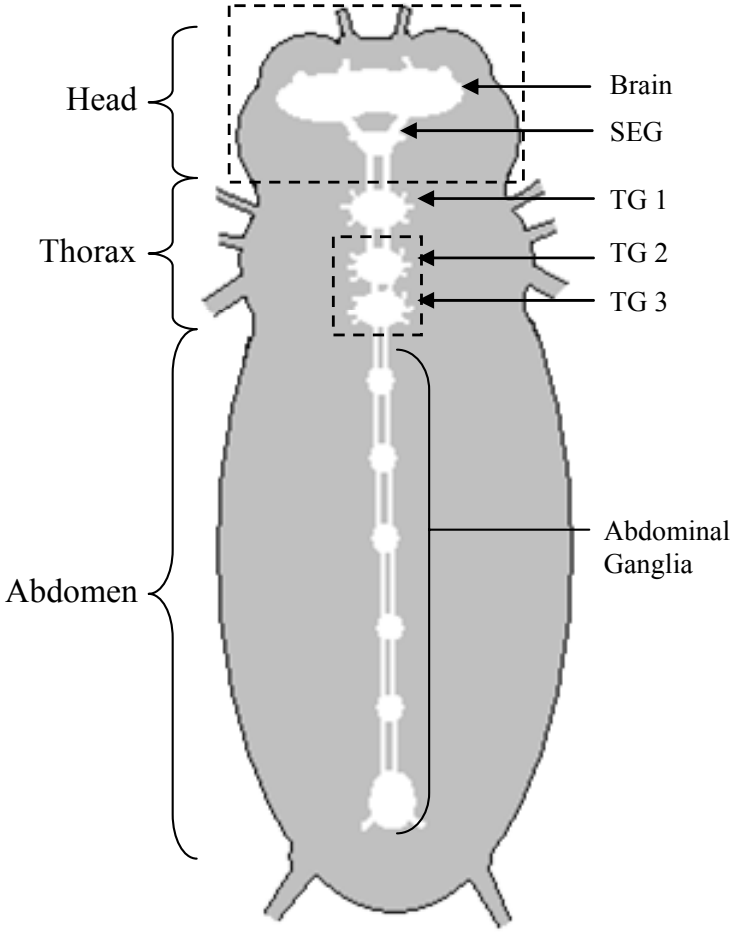

Figure 4.1. Schematic of cricket nervous system. The major divisions of the nervous system include the brain (or supraesophageal ganglion), the subesophogeal ganglion (SEG), the three thoracic ganglia (TG), and the abdominal ganglia. The dashed boxes indicate the head and TG 2-3 which, along with the legs, were examined in this study.

the same direction in all of the examined body parts were PCR amplified and sequenced from both species using primers that flanked the qPCR priming sites.

Assessment of Differential Transcript Identification

To estimate the number of unique transcript clones that would be identified by further sampling, and the expected number of candidate transcripts that are differentially expressed between species, I adapted rarefaction and the Chao estimator, ecological methods for examining species richness. Rarefaction uses random sub-sampling of data to produce a curve of the number of unique samples expected relative to sample size [41-43]. It has recently been employed in a similar analysis to estimate gene discovery based on the depth of sequencing coverage[44]. The Chao estimator is a nonparametric estimator of species richness based on the abundance of previously sampled species, which provides an estimate of the expected species richness [45, 46]. To apply these methods to differentially expressed candidate transcripts, each differentially expressed transcripts were treated as a unique "species" and the remaining, non-differentially expressed transcripts were grouped together and treated as a single abundant species. The rarefaction curve was plotted using SigmaPlot 10 (Systat Software, Inc.) and a hyperbolic curve (modified hyperbola II, SigmaPlot) was fit to the data to extend the estimate and determine the expected return on further sampling of this SSH library.

RESULTS

Subtractive Library Screening and Verification

From the initial SSH libraries, 384 clones were selected and screened (192 from each direction of subtraction (Figure 4.2). Of these clones, 252 (66%) were eliminated due to lack of differential transcript abundance based on the initial

screening. The remaining 132 clones, which appeared to be differentially expressed, were sequenced and checked for contigs, revealing 32 unique transcript fragment sequences. Whole-body qPCR comparing transcript abundance between *L. cerasina* and *L. eukolea* showed that 15 of the 32 identified transcripts showed interspecific variation in qPCR results.

Analysis of Differential Transcripts

To further characterize interspecific variation, I examined the head, TG 2-3, and the legs of five males from each species using qPCR with the 15 candidate transcripts identified above. All of the transcripts showed differential qPCR results in at least one of the body parts examined. However, differences observed in both SSH and qPCR may result from differential transcription or from sequence divergence. To distinguish between these possibilities, potential candidate, transcripts which showed interspecific differences in the same direction for all of the examined body parts were sequenced from both species to assess sequence divergence within and outside of the qPCR primer sites. I found that five of the transcripts had sequence divergence both inside and outside the qPCR priming sites, which likely interfered with hybridization during SSH and primer annealing in qPCR. All of these transcripts were homologs of mitochondrial genes (Table 4.1). I obtained high quality sequence for 1405 bp of these mitochondrial transcripts and found a mean sequence divergence of 4.4%, in contrast with the 0.15% sequence divergence that I found for the 654 bp of high quality sequence obtained from the nuclear EST transcripts.

After eliminating the five transcripts with identified sequence divergence, 10 candidate transcripts remained that showed differential transcript abundance between

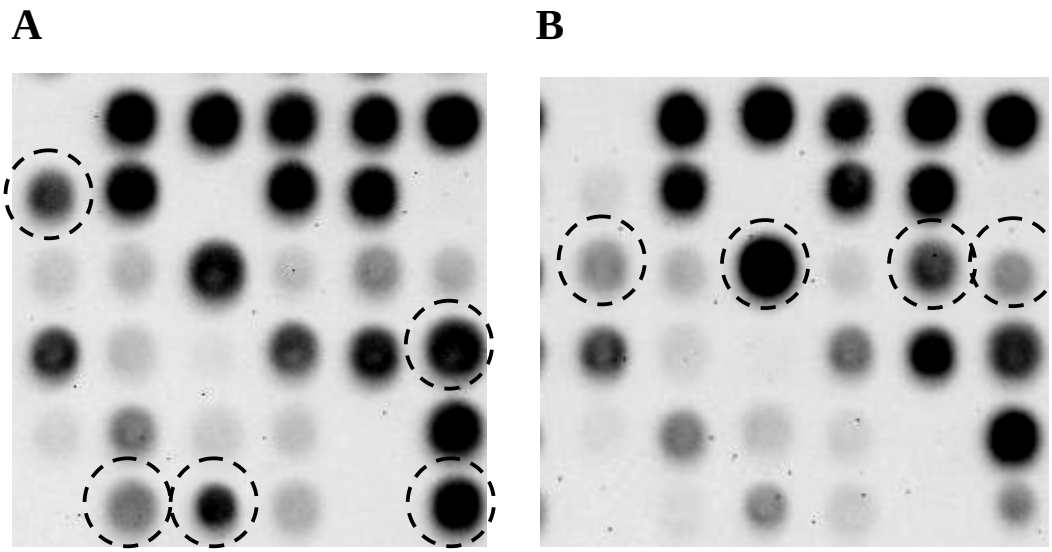

Figure 4.2. Screening of suppressive subtractive hybridization clones. Clones from the subtractive libraries were selected, amplified by PCR, and spotted onto two nylon membranes in the same pattern. Each membrane was incubated with digoxigenin labeled probes from either (A) the *L. cerasina* minus *L. eukolea* library or (B) the *L. eukolea* minus *L. cerasina* library. The probes were immunologically detected by chemiluminescence. Dashed circles indicate example clones with differential signals relative to background. The images have been inverted for easier viewing.

L. eukolea and *L. cerasina* (Table 4.2). All of these transcripts showed differential abundance in TG 2-3 and six of them showed differential abundance in the head, making them strong candidates for involvement in behavioral variation. Three of the differential transcripts showed substantial identity to sequences from the *L. kohalensis* EST database. Additionally, I assigned putative functions to four of the candidate transcripts based on homology with annotated sequences from other species.

I further characterized the 10 differentially transcribed candidates by calculating the relative transcript abundance within each species relative to the legs (Table 4.2). Normalizing transcript abundance in this way provides information regarding which transcripts are expressed highly in regions of interest for behavioral variation relative to other regions. For example, transcript 2-f12 (GT128474) is much more abundant in TG 2-3 of *L. cerasina* than *L. eukolea* (Table 4.2), and the 2-f12 transcript is also more abundant in TG 2-3 than the legs of *L . cerasina* (Table 4.3). A transcript, such as 2-f12, that is both highly expressed in a tissue of interest with regard to behavioral variation and differentially expressed interspecifically stands out as a particularly interesting candidate gene

Assessment of Differential Transcript Identification

I used rarefaction analysis to estimate the return on further sampling of the SSH library. The hyperbolic curve fitted to the rarefaction results suggests that sampling an additional 384 clones from the SSH library is likely to identify only two to three more differentially expressed transcripts. The return on further sampling therefore greatly diminishes with increasing numbers of identified differentially

Table 4.1. Divergent mitochondrial sequences. The candidates listed in this table were identified by SSH, but their presence in the SSH library was due to sequence rather than expression divergence. All of the transcripts in this table are mitochondrial, which were found to have a much higher overall interspecific divergence than the nuclear genes. This table also lists the GenBank accession numbers as well as the species from which the sequence was identified. Accession numbers are given for the four *L. kohalensis* ESTs with the highest bit scores and E values less 10^{-5}.

Clone ID	GenBank Accession	Species of Origin	Homologs	*L. kohalensis* EST homologs
DF-c2	GT128472	*L. cerasina*	Cytochrome c oxidase subunit III	EH636889, EH631432
32-c3	GT128469	*L. cerasina*	NADH dehydrogenase 4	
96-c25	GT128470	*L. cerasina*	Cytochrome c oxidase subunit I	EH633468, EH637295, EH634922, EH637085
4-G4	GT128480	*L. eukolea*	NADH dehydrogenase subunit 1	
2-f4	GT128471	*L. eukolea*	Cytochrome b	EH632030

Table 4.2. Differential candidate transcript abundance. This table shows the interspecific transcript ratios of *L. cerasina* to *L. eukolea* in mesothoracic and metathoracic ganglia (TG 2-3), head, and legs. The Ø symbol indicates that no qPCR product was observed for that species and body part. The GenBank accession numbers for these sequences are listed, as well as putative functions and the strongest *D. melanogaster* gene hit based on BLAST results with E values less than 10^{-5}. Accession numbers are given for the four *L. kohalensis* ESTs with the highest bit scores and E values less 10^{-5}.

Clone ID	GenBank Accession	Transcription Ratios (*L. cerasina : L. eukolea*)			Putative functions / homolog	*L. kohalensis* EST homologs
		TG 2-3	Head	Legs		
2-e8	GT128473	1: Ø	2:1	2:1	Unknown	None
2-f12	GT128474	281:1	1:1	1:1	Unknown	None
3-a5	GT128475	8:1	1:2	1:1	Serine protease / CG32808	None
3-B9	GT128481	6:1	1:1	1:1	Serine protease, Trypsin / *Trypsin29F*	None
3-H10	GT128482	1:133	1:876	1:929	Unknown	EH634262
4-F9	GT128483	11:1	1:7	15:1	Zinc-dependent metalloprotease / CG6696	None
96-c26	GT128476	11:1	3:1	1:1	Zinc-finger transcription factor / *glass*	EH630018, EH641029, EH641866, EH629961
96-c7	GT128477	4:1	1:1	1:1	Unknown	EH631572, EH640880
DF-C3	GT128478	3:1	1:Ø	1:2	Unknown	None
DF-C7	GT128479	4:1	1:1	1:1	Unknown	None

Table 4.3. Body part specific transcript abundance. The results of qPCR for the interspecifically differentially transcribed candidates were used to look at body part specific transcription. Within each species, the transcript level from TG 2-3 and the head were normalized to the level in the legs to determine the relative amount of transcription in each body part within each species. The Ø symbol indicates that no qPCR product was observed for that transcript in that body part and species.

Clone ID	GenBank Accession	*Species*	TG 2-3	Head	Legs
2-e8	GT128473	*L. cerasina*	2.1	1.0	1
		L. eukolea	Ø	3.0	1
2-f12	GT128474	*L. cerasina*	27.9	5.8	1
		L. eukolea	0.07	3.1	1
3-a5	GT128475	*L. cerasina*	3.6	0.7	1
		L. eukolea	0.5	1.0	1
3-B9	GT128481	*L. cerasina*	3.9	1.7	1
		L. eukolea	0.6	1.0	1
3-H10	GT128482	*L. cerasina*	5.4	1.3	1
		L. eukolea	0.8	1.3	1
4-F9	GT128483	*L. cerasina*	1.2	0.1	1
		L. eukolea	1.6	10.7	1
96-c26	GT128476	*L. cerasina*	1.3	0.5	1
		L. eukolea	0.2	0.2	1
96-c7	GT128477	*L. cerasina*	1.1	0.8	1
		L. eukolea	0.4	0.8	1
DF-C3	GT128478	*L. cerasina*	1.0	0.8	1
		L. eukolea	0.2	Ø	1
DF-C7	GT128479	*L. cerasina*	0.3	0.8	1
		L. eukolea	0.06	0.8	1

expressed transcripts. Based on the Chao estimator, with unique transcripts treated as species, 16 differentially expressed transcript candidates are expected to a degree detectable by SSH. Thus, the ten identified transcripts represent 62.5% of the expected differentially expressed transcripts, given the power of this technique.

DISCUSSION

Extensive efforts have gone into identifying and characterizing genes underlying behavioral variation in animals. Genetic model systems, such as *mus* and *Drosophila*, have provided insights that help us better understand a wide array of behaviors, like endogenous circadian rhythms [47-49], epilepsy [50], attention deficit/hyperactivity disorders [51], and depression and anxiety [52]. These model systems have a tremendous amount of further unrealized insight to provide. However, much of the knowledge gained from these systems is based on mutational analyses of behavior or genetic dysfunction rather than naturally occurring behavioral variation. Recent developments in molecular biology have allowed for more in-depth molecular investigation of non-genetic model systems [11], which can serve as useful models for elucidating the genetic basis of natural behavioral variation. Indeed, a number of studies in non-genetic models have provided insights into variation in circadian rhythms [53, 54], affiliative behavior [5, 6], and swarming [55].

Differentially Expressed Transcripts

Using SSH I have identified 10 candidate gene transcripts that may be important for song differences between *L. cerasina* and *L. eukolea* in particular, and in temporal variation in general. Most of the candidate transcripts identified in this study showed weak or no similarity to previously annotated genes. Only three of the 10 candidate transcripts had possible homologs among available *Laupala* EST sequences

(Table 4.1) and only four show similarity to previously annotated genes from other species. The six candidate transcripts for which I was not able to assign putative functions could not have been identified from a literature search for candidate genes. Seven transcripts, which are not present in the published *Laupala* EST library, could not have been identified by microarray analysis of this library. The high proportion of previously unidentified candidates underscores the value of a method which provides an assessment of the whole transcriptome to identify transcriptional variation which would be missed with other approaches.

An examination of the relative abundance of the 10 differentially expressed transcripts provides insight into whether the candidates may be involved in known behavioral variation. The brain and SEG of the head and TG 2-3 are likely to be involved in regulating behaviors which vary between *L. cerasina* and *L. eukolea*. All 10 of the transcripts show a three-fold or greater interspecific difference in abundance in the head or TG 2-3, while six differed by over 10-fold in these body parts (Table 4.2). Looking further at the relative tissue specific expression, six of the transcripts are at least twice as abundant in the head or TG 2-3 as the legs in one or both species (Table 4.3). The combination of high interspecific differences in expression and elevated expression in the head or TG 2-3 points to transcripts 2-e8, 2-f12, 3-H10 and 4-F9 as strong candidates for behavioral variation between *L. cerasina* and *L. eukolea*. In spite of the strong differences in some of these transcripts, all of the identified transcripts show interspecific variation in the head or TG 2-3, and thus should all be considered candidates for behavioral variation.

Additional information about the candidate transcripts and the likelihood that they might play a role in behavioral variation can be gained from identifying homologs and putative functions. I found that one of the transcripts, 96-c26 (GT128476), had strong sequence similarity to a variety of zinc-finger RNA

polymerase II transcription factors, particularly to the *glass* (*gl*) gene. The *gl* gene is expressed predominantly in the eyes in *Drosophila*, but is also expressed in the adult brain and larval CNS, according to the FlyAtlas database [56, 57]. Mutations of *gl* have been associated with neuroanatomical and behavioral defects [58, 59], consistent with a role for 96-c26 in *Laupala* behavioral variation. Two of the identified transcripts, 3-a5 (GT128475) and 3-B9 (GT128481), which had high similarity to the *Drosophila* genes CG32808 and *trypsin 29F* (*try29F*), respectively, are likely serine proteases. Both of these *Drosophila* genes are found within the CNS, with CG32808 showing particular enrichment in the brain and thoracicoabdominal ganglion. Serine proteases are found throughout the nervous system and play critical roles in neural development, synaptic plasticity and neuropeptide processing [60, 61]. The 4-F9 (GT128483) transcript has high sequence similarity to a number of genes of another protease family, zinc-dependent metalloprotease, especially the *Drosophila* gene CG6696. Phenotypes for alleles of CG6696 have not been identified, but it and other metalloproteases are expressed in the CNS of *Drosophila* and many are involved in neural development and axonal guidance [62], among other biological processes. All of the putative functions for these candidates have the potential to effect neural or behavioral variation, and the top *Drosophila* homologs are all expressed within the nervous system. Thus, both the relative transcript abundance and the putative functions indicate that the transcripts identified in this study are important candidates for behavioral variation in *Laupala*, and may ultimately inform our understanding of the divergence that occurs early in speciation.

Interspecific Sequence Divergence

In addition to the 10 candidate gene transcripts which are differentially expressed, I identified five transcripts for which sequence divergence likely explains

both their presence in the SSH library and interspecific differential qPCR results. Interestingly all five of these transcripts appear to be homologs of mitochondrial genes. The 4.4% sequence divergence observed in these transcripts, relative to 0.14% in other transcripts, is consistent with previous work in *Laupala* showing greater maximal interspecific divergence in mitochondrial (4.0%) compared to nuclear (1.6%) DNA sequences [19, 30]. The high sequence divergence of these transcripts likely reduced both interspecific hybridization during the SSH process and primer binding during qPCR, thus producing differential results between species each of these methods. The fact that these transcripts appear to be mitochondrial, and that they are present due to high mitochondrial sequence divergence, suggests that these five transcripts are not particularly strong candidates for behavioral variation. However, the identification of these genes demonstrates the sensitivity of SSH to sequence variation, which can help identify rapidly evolving genes with high sequence divergence.

Future Candidate Gene Analyses

There are a number of ways that relationships between the candidate genes identified here and known behavioral variation might be further examined. The genus *Laupala* consists of many closely related species with well characterized song rate variation. Expression of these genes could be examined across the genus, plotting expression against song rate to determine whether a correlation exists across the genus between expression and song rate. *L. cerasina* and *L. eukolea* will readily hybridize in the lab and F_2 hybrids display high song pulse rate variation. An analysis of gene expression and song pulse rate variation within the F_2 generation could provide a powerful means of examining the role of each transcript in song variation. Alternatively, QTL maps for song pulse rate and female preference variation between

L. kohalensis and *L. paranigra* have been published [63, 64], and QTL maps from other interspecific pairs are likely to be produced. Identifying polymorphisms, such as SNPs, within these transcripts will allow the genes to be placed on the QTL maps and to determine whether any of them fall within the identified QTL regions. Such a physical correspondence between an gene and a QTL region would strengthen the candidate status of that gene for involvement in song pulse rate or preference variation. Finally, RNA interference (RNAi) is being employed in an expanding array of taxa. RNAi was recently utilized in the cricket *G. bimaculatus* to examine the behavioral effects of reducing PER expression [53]. Developing and utilizing RNAi in *Laupala* would allow us to selectively reduce expression of particular candidates and examine the effects on phenotypes of interest. Such an approach would provide the most direct test of the relationship between the candidate gene and the phenotype. Each of these approaches would bring us steps closer to a better understanding of natural behavioral variation.

Use of SSH for Interspecific Comparisons

In this study I have demonstrated that SSH is a low cost and rapid means of identifying candidate genes for behavioral variation between closely related species with low sequence divergence. This method requires no pre-existing sequence information, making it ideal for non-genetic model systems. SSH has most often been used within species to examine variation in gene expression between tissues or treatments, but here I have demonstrated the potential for broader application of SSH to identify candidate genes for behavioral variation between closely related species. This approach has great advantages over three common approaches for identifying such candidate genes. Mining the literature for genes that may influence the phenotype of interest is an approach that has been successful in a number of taxa [For example: 5,

65, 66-69]. However, using available literature limits the scope of genes considered and requires conservation of gene sequence and gene function. A second approach is to create an EST library, and screen the library by microarray to find ESTs for which expression levels correspond to phenotypic variation. This approach is unbiased and the library can often be utilized in future studies. Drawbacks to the EST library / microarray approach are the high development cost and the fact that the microarray is limited to examining only those genes in the EST library. To be comprehensive a very large EST library is necessary. The third approach is genome wide quantitative trait locus (QTL) mapping. QTL mapping examines the whole genome in an unbiased manner and has facilitated identification of genomic regions associated with behavioral variation in non-genetic model systems, including *Laupala* [63, 64, 70]. However, QTL analysis typically identifies large regions of the genome containing many genes. Without a genome sequence or shared synteny with a well sequenced organism, identifying the genes within the QTL region is not trivial. While literature based gene searches, EST sequencing/microarray analyses, and QTL mapping all provide useful information, none of them provide a comprehensive, low cost, and rapid means of producing sequences from candidate genes for behavioral variation in a non-model system. The application of SSH to examine recently diverged species appears to be a useful alternative to common methods of identifying candidate genes for behavioral variation.

Identifying the genes underlying the variation among *Laupala* species will give us insights into behavioral genetics and speciation. The candidate transcripts identified in this study represent a critical step in examining natural behavioral variation by identifying novel candidates for behavioral variation and more specifically song rate variation in *Laupala* crickets. The stridulatory CPG and neural processes controlling wing coordination during singing have been localized to the 2nd

and 3rd thoracic ganglia in crickets [34] The fact that all of the differentially expressed transcripts identified here show variation in this region, some with variation limited specifically to TG 2-3, increases the likelihood of their involvement in song variation. Identifying these candidates is a critical first step in elucidating the genetic underpinnings of natural behavioral variation.

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CHAPTER 5

Conclusions and Future Directions

In this dissertation I have employed both behavioral and molecular approaches to examine the genetic basis of temporal variation among species of the cricket genus *Laupala*. Previous work has identified interspecific differences in the time of day that singing occurs in the field and the time of mating of wild-caught individuals in the lab [1]. Furthermore, additional work has demonstrated a genetic basis to interspecific variation in the pulse rates of *Laupala* male calling songs [2, 3] and female song pulse rate preferences [3, 4]. However, a genetic basis has not been established for the differences in daily timing, and candidate genes for temporal variation have not been examined among *Laupala.* These are critical steps for elucidating the genes underlying natural behavioral variation, a major goal of neurobiology.

Behavioral Analysis

In Chapters Two and Three of this dissertation, I examined daily activity times of lab-reared individuals, observing the courtship process, from initiation of courtship through mating in male/female pairs as well as the timing of song and locomotion in visually and acoustically isolated males in *Laupala cerasina* and *L. paranigra*. I have documented interspecific differences in the daily timing of locomotion, singing, onset of courtship, and the time of mating, with *L. paranigra* expressing a later phase of activity than *L. cerasina* for each behavior. Furthermore, the duration of courtship and the rate of spermatophore production also differ between the species, indicating that the variation between species is not just a simple shift in the time of day of activities, but also a shift in the temporal patterns of these behaviors. By rearing both species in a controlled laboratory environment, reducing environmental variation, I have

demonstrated a genetic contribution to the behavioral variation, a necessary prerequisite for evolution of these characters. Having established that the timings of these behaviors are phenotypes that vary interspecifically opens the door for studies of the genetic basis of this temporal variation and whether timing is involved in mate choice or species isolation in this rapidly evolving genus [5].

Work with *Drosophila* and melon flies (*Bactrocera cucurbitae*) have found associations between later activity phases of locomotion and mating under light:dark conditions as well as longer circadian free-running periods under constant lighting [6, 7]. Because of the later activity phase of *L. paranigra* relative to *L. cerasina*, in Chapter Three I tested the hypothesis that *L. paranigra* has a longer circadian free-running period. I placed individual males in visually and acoustically isolated chambers and monitored song and locomotion activity for at least 10 days under constant low light (<15 lux). I found that singing and locomotion are indeed under circadian regulation; however, contrary to what I hypothesized, *L. cerasina* had a significantly longer free-running period based on song and a longer, but not statistically significant, free-running period of locomotor activity. Thus, the basis of the difference in daily timing between the two *Laupala* species may be independent of the variation in circadian rhythms, or the molecular mechanisms regulating both behaviors may be different than that of other species.

An additional behavioral observation in Chapter Three was a significant relationship between song pulse rate and circadian free-running period of both song and locomotion in *L. paranigra*. Such a relationship was not observed in *L. cerasina*. The significant relationship in *L. paranigra* may indicate pleiotropic temporal regulation across behaviors and time scales; however, because the relationship does not exist in *L. cerasina*, future studies are needed to investigate whether this relationship is unique to *L. paranigra*, or exists within and between other *Laupala*

species. A frequent association between circadian rhythms and song pulse rate variation among *Laupala* species would indicate a common genetic basis and may point to circadian clock genes as candidates for song pulse rate variation.

Molecular Analyses

In addition to the examination of circadian rhythms in Chapter Three, I cloned and sequenced the *period* (*per*) gene from *L. cerasina* and *L. paranigra*. The *per* gene is a circadian clock gene which has also been implicated in song variation in *Drosophila* [8, 9]. Using qPCR I found that *per* transcript abundance varies on a daily basis in both species, with no significant interspecific differences in the timing of transcript abundance. I did identify eight interspecific differences in the deduced amino acid sequences, and two alternative splice sites within the coding region of *per*. Whether and how sequence variation or splicing affect behavioral differences between *Laupala* species remains to be studied. Future work should examine the sequence variation of *per* across multiple *Laupala* species that vary in temporal behaviors. Additionally, by look for associations between sequence and behavior and determining the timing and localization of the alternatively spliced isoforms of *per*, we will gain insights into the roles that sequence variation and differential splicing may play in behavioral variation.

In Chapter Four, I used suppressive subtractive hybridization to identify genes that are differentially expressed between *L. cerasina* and *Laupala eukolea*. These sister species are recently diverged [5] and have polygenically regulated song variation (Personal observation; *L. cerasina*: ~2.4 pps; *L. eukolea*: ~4.0 pps). I identified ten transcripts that are differentially expressed between these two species that serve as candidate genes for the variation in song pulse rate differences. I used qPCR to examine expression in the mesothoracic and metathoracic ganglia and the head,

regions that are likely to be involved in song regulation. All ten genes were differentially expressed in at least one of these regions, suggesting they are all strong candidates for involvement in song pulse rate variation.

Several methods are available to further assess the role of candidate genes in temporal variation. Here I list and briefly describe five approaches which could be applied in future studies to the ten candidate genes identified in Chapter Four as well as the *per* gene sequenced in Chapter Three. These analyses will help determine the likelihood that a candidate gene plays a role in the behavioral variation of interest. The first approach for examining the candidate genes is to utilize available QTL maps for song variation [3, 10, 11] to determine whether any candidate genes are located within the known QTL regions. A physical link between a candidate gene and QTL will bolster the case for the role of the candidate in the behavioral variation. The second approach is to utilize the large number of closely related *Laupala* species for comparative sequence analyses. Sequencing the candidate genes from multiple *Laupala* species will allow for the calculation of the ratio of non-synonymous to synonymous sequence changes (dN/dS) to test for positive selection and to determine whether there is a correlation between gene sequences and trait variation. The third method is to perform qPCR in tissues relevant to the behavioral variation across many *Laupala* species. Having a continuous range of variation in behavior and expression will allow for a regression analysis to determine whether there is a significant relationship between expression level and behavior. The fourth approach, RNA interference (RNAi), has recently been employed in crickets [12, 13]. RNAi can be used to reduce expression of each of the candidate genes, and then the temporal behaviors can be examined to look for the effect of knock-down of gene expression on the behavior. Such an effect would indicate a role of the gene in behavioral regulation. A final approach to further assess the role of candidate genes in behavioral variation is

to introgress alleles between closely related species and measure the effect of the introgression on temporal variation. This approach, which has recently been used with *Laupala* QTL (Wiley & Shaw, In prep), would provide the clearest means of demonstrating a role of a particular gene in behavioral variation. However, the clean introgression of a single gene between species would take several years. While most of the approaches outlined, other than introgression, are not sufficient when used alone to demonstrate a role for a particular candidate in natural behavioral variation, when used in concert they can make a strong case for the role of a candidate gene in the observed variation. For example, demonstrating that a differentially expressed gene is localized to a QTL region, has undergone positive selection, and the knockdown of that gene via RNAi alters the behavior of interest would provide compelling evidence that the gene is involved in natural behavioral variation.

In conclusion, the work presented here identifies behavioral variation in daily timing in *Laupala* crickets and demonstrates that this variation is genetically regulated. Furthermore I have identified and begun to examine a number of candidate genes for interspecific differences in daily activity times and in song pulse rates. The identification of these candidate genes lays the groundwork for future studies to identify the genes underlying natural behavioral variation and to understand the genetic mechanisms regulating temporal variation.

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