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A Tale of Two Butterflies: The Effect of Larval Social Environment and Circadian Rhythms on Mating Behavior in Bicyclus anynana and Heliconius hewitsoni

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A Tale of Two Butterflies: The Effect of Larval Social Environment and Circadian Rhythms on Mating Behavior in *Bicyclus anynana* and *Heliconius hewitsoni*

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Abstract

Two key components of mate choice research focus on: 1) who an organism mates with, which may be influenced by any number of factors from sexual ornamentation to male-male competition; and, 2) when an organism courts, be it daily, monthly, or seasonally. Both aspects are especially important for gregarious species as mistakes in either can incur high costs to overall fitness. My research focuses on using butterflies to explore kin recognition from the larval stage and its possible impacts on adult mate choice and if courtship is circadian in *Heliconius hewitsoni*. My first experiment concerned kin recognition. When inbred, *Bicyclus anynana* are known to suffer from inbreeding depression, however populations can recover lost fitness within just a few generations when allowed to mate freely. It has been shown that *B. anynana* can recognize and choose against inbred individuals, however it is unknown whether they can detect siblings. I demonstrated that larval rearing condition (isolated or gregarious) did not influence adult mate choice in that female *B. anynana* did not innately detect or learn to detect and avoid sibling males during mate selection. Thus, in *B. anynana*, kin recognition may not be important to reproductive fitness. Through analysis of recorded behavior, I also showed that male harassment did not influence female mate choice. In my second experiment I examined circadian rhythms, specifically regarding courtship. I demonstrated that *H. hewitsoni* exhibits circadian rhythms, including a period of peak courtship around noon, and that some behaviors are sexually dimorphic in these butterflies. Recorded peak activity closely matches diurnal behavior in *H. hewitsoni*'s primary food source, which may influence overall behavior patterns in this species. My findings broaden our understanding of the mechanisms behind mate choice and provide valuable information for future research in these two systems, including the importance of female choice versus male harassment and sexual dimorphism in behavior. With

my research I have improved our overall understanding of kin recognition and circadian rhythms to address the “who” and “when” of mate choice.

Acknowledgements

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Owl. Harley and AJ, you kept my feet warm, provided me with all the snuggles I could handle, and helped me get up in the morning. My angry old lady and my big neurotic mess. I love you both!

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To everyone who is listed here and everyone who is not, I just want to say one more time, from the bottom of my heart:

Thank you.

Deonna Nicole "Nikki" Robertson

Dedication

This thesis is lovingly dedicated to the memory of Regina Edwards, Kenneth Robertson, and Bernice Brown. Regina, you inspired me. Papa, you encouraged me. Grandmother, you influenced me. You all believed in me. Thank you. I miss you.

Contents

Introduction

Mate choice is one of the most important elements of reproduction. It can be the pivot point between reproductive success and failure, especially for females, the sex generally investing the most in their gametes (Tregenza & Wedell, 2000). If a female chooses an optimal mate, be that decision made on any number of traits from the quality of the male's territory to the number and size of some characteristic (Robertson & Monteiro, 2005; E. L. Westerman, Hodgins-Davis, Dinwiddie, & Monteiro, 2012), it can confer direct fitness benefits to her offspring, thus increasing their chance for survival. Should a female choose poorly, the fitness costs can reduce reproductive output through juvenile death and suboptimal body condition in offspring. Thus, mate selection cues, such as pheromone production or sexual ornamentation, and courtship synchrony within a species, which confers the greatest amount of choice for both sexes, are vital to an organism's genetic fitness.

For gregarious species (species that form social groups), such as mice and social spiders, mate choice is especially important. Gregariousness provides species with multiple benefits, such as predator defense and increased offspring care, and has been documents in many animal taxa, including marine and terrestrial invertebrates (Bilde et al., 2007; Burnet, 1971), birds (Sharp, McGowan, Wood, & Hatchwell, 2005), and mammals (Porter & Moore, 1981; Porter, Wyrick, & Pankey, 1978). However, gregarious species also must be more diligent during courtship and mate selection, lest they mate with related individuals (which may cause inbreeding depression) or miss valuable courtship time periods. Therefore, gregariousness has associated costs and benefits.

I have chosen to use two gregarious species of butterfly, *B. anynana* and *H. hewitsoni*, in my research. My first chapter involves my work with *B. anynana* concerning larval social experience and kin recognition. Kin recognition reduces the likelihood of inbreeding, which in turn reduces the risk of inbreeding depression (Waldman, 1987). Prior work has shown that *B. anynana* suffers from inbreeding depression, including reductions in survival and male sex pheromone production (Saccheri, Brakefield, & Nichols, 1996; van Bergen, Brakefield, Heuskin, Zwaan, & Nieberding, 2013; van Oosterhout, Zulstra, van Heuven, & Brakefield, 2000). We also know that this species of butterfly learns as adults (E. L. Westerman et al., 2012). My research sought to determine if kin recognition based on innate preference or larval experience was the method by which *B. anynana* avoids inbreeding depression. My second chapter examined whether *H. hewitsoni* follow circadian rhythms, specifically regarding peak courtship times. Circadian rhythms allow organisms to synchronize their behavior, both within and outside of their species, to optimize aspects important to survival, including foraging success and avoidance of predators (Bell-Pedersen et al., 2005; Edery, 2000). While we have learned much about mimicry systems from *Heliconius* butterflies (James Mallet & Gilbert, 1995; Merrill et al., 2015), we still do not understand their daily behavioral patterns. My research sought to map activity patterns in these butterflies, with a particular emphasis on when they court.

Mate choice is a key component of natural selection. With my research, I have improved our understanding of kin recognition and circadian rhythms, two factors that greatly affect mate choice, in two gregarious butterfly systems. This body of work provides a starting point for future studies in both systems, including evidence for the importance of female choice over male harassment and sexual dimorphism in behavioral patterns.

Chapter One: Lack of kin recognition in the gregarious butterfly *Bicyclus anynana*

Abstract:

Gregarious species susceptible to inbreeding depression are hypothesized to combat this problem through either dispersal or kin recognition. For species with kin recognition, it is often unknown if filial recognition is innate or due to prior juvenile experience with siblings. Here, I test these two hypotheses in the gregarious butterfly *Bicyclus anynana*, a species that suffers from inbreeding depression when forcibly inbred but can recover quickly (within a few generations) when allowed to breed freely. I evaluate whether the quick recovery from inbreeding depression is associated with either innate or learned filial recognition. I first determined whether females innately prefer unrelated over sibling males using females reared in isolation and then given a choice between an unrelated and a sibling male. Then, I determined if females raised with siblings learned to detect and avoid mating with siblings as adults when given a choice between an unrelated male and a sibling male. Finally, I determined if females raised with siblings could learn to detect and avoid mating with familiar siblings when given a choice between familiar and unfamiliar siblings. I found that females mated randomly in all three choice combinations. Male behavior also did not influence female mate preference. These findings suggest that adult females do not innately avoid or learn to avoid siblings during mate selection, and that filial detection may not be as important to reproductive fitness in *B. anynana* as previously thought.

Introduction

Inbreeding depression refers to the reduction of fitness (adult size, fecundity, pheromone production, etc.) experienced by many organisms when related individuals mate and produce

offspring (Crnokrak & Roff, 1999; Hedrick & Garcia-Dorado, 2016). This reduction in fitness is due to increased homozygosity in inbred individuals, which means that the chance of deleterious or lethal alleles being expressed in inbred offspring is much higher than in outbred individuals (Hedrick & Garcia-Dorado, 2016; Hedrick & Kalinowski, 2000; Keller & Waller, 2002; D. H. Reed, Lowe, Briscoe, & Frankham, 2003). The resulting fitness losses and increased mortality can be detrimental to populations, especially those that are isolated or near extinction (Hedrick & Kalinowski, 2000; D. H. Reed et al., 2003).

Plants and animals have evolved numerous ways of avoiding inbreeding with closely related individuals to reduce the impact of inbreeding depression on offspring fitness (Blouin & Blouin, 1988; Gigord, Lavigne, & Shykoff, 1998; Matton, Nass, Clarke, & Newbigin, 1994; Pusey & Wolf, 1996; Williams, Clarke, & Knox, 1994). For example, some plants with the potential to self-fertilize, such as allspice (*Pimenta dioica*) and ribbonwood trees (*Plagianthus betulinus*), have evolved mechanisms of self-incompatibility, meaning that when pollen reaches the stigma of the parent plant it will be rejected (Charlesworth & Charlesworth, 1987; Waser, 1993). This rejection may be due to chemical messages or haploid gene expression in the pollen grain (Waser, 1993). In some vertebrates, the major histocompatibility complex (MHC) plays a large roll in deterring inbreeding, and thus inbreeding depression in offspring (Burnet, 1971; Monroy & Rosati, 1979; Stevens, Yan, & Pray, 1997). The MHC influences odors and/or pheromones organisms produce, which facilitate mate selection of individuals with differing MHC in species such as mice (*Mus* sp.), cattle (*Bos phylli*), chickens (*Gallus gallus*), and humans (*Homo sapiens*) (Brown & Eklund, 1994; Eggert, Müller-ruchholtz, & Ferstl, 1998; Klein et al., 1993; Porter & Moore, 1981; Potts, Manning, & Wakeland, 1991; Wedekind, Seebeck, Bettens, & Paepke,

1995; Zavazava & Eggert, 1997). Some invertebrates, such as ascidians (*Molgula provisionalis*, *Ciona intestinalis*, and *Botryllus schlosseri*), the rough periwinkle snail (*Littorina saxatilis*), and the Mediterranean sponge (*Scopalina lophyropoda*), rely primarily on dispersal to avoid inbreeding depression, and studies have shown that species with low dispersal tend to be more resilient against inbreeding depression (Blanquer & Uriz, 2010; Ng & Johannesson, 2015; Phillippi & Yund, 2017; Zimmer & Schneider, 2016). Other invertebrates, such as the red flour beetle (*Tribolium castaneum*), have been shown to simply not suffer from inbreeding depression, or to circumvent inbreeding depression by undergoing intense bottlenecks that purge deleterious alleles from populations, causing massive die offs followed by increased fitness in the remaining population (the invasive ladybug (*Harmonia axyridis*)) (Facon et al., 2011; Stevens et al., 1997).

The likelihood of inbreeding is increased in species that form social groups (gregarious species), especially those with groups primarily composed of familial individuals (Majolo, Huang, & Lincoln, 2018; Parreira & Chikhi, 2015). However, gregarious species also benefit from readily available potential mates (Majolo et al., 2018; Parreira & Chikhi, 2015). Many different mechanisms have evolved to facilitate outbreeding in gregarious species (Parreira & Chikhi, 2015). Female Ethiopian wolves (*Canis simensis*) avoid inbreeding by participating in extra-pack copulation with males from adjoining packs while retaining membership in familial packs (Sillero-Zubiri, Gottelli, & Macdonald, 1996). Many species of subsocial spiders stay in family groups until sexual maturity, at which time they disperse (Yip & Rayor, 2014), while the wood-feeding cockroach (*Cryptocercus punctulatus*) lives in familial “galleries” (tunnel systems) in rotting logs that are coinhabited by other families, providing ample mate opportunities outside of the immediate family (Garrick, 2017). Therefore, social structure can allow for outbreeding, and

gene flow between social groups can prevent inbreeding depression (Chesser, 1991; Parreira & Chikhi, 2015).

One common way gregarious species avoid inbreeding is dispersal before sexual maturity (Avilés & Bukowski, 2006; Moore & Ali, 1984). Prior to maturity, some or all individuals in an offspring group may disperse to other groups to avoid or minimize contact with related individuals, thereby reducing the likelihood of breeding with them (Avilés & Bukowski, 2006; Moore & Ali, 1984). When offspring dispersal is low, such as in wild dogs or social spiders, it is hypothesized that the benefits of group living outweigh the cost of potential inbreeding or not breeding altogether (Bilde et al., 2007; Ebensperger, 2001; Moore & Ali, 1984). For example, more food may be obtained by members of a group working in concert with each other, and while a given organism may not itself breed, the benefits to the survival of a sibling or half-sibling's offspring may offset the cost of passing down that organism's own genes (Bilde et al., 2007; Ebensperger, 2001). Alternatively, all members of a social group may breed and produce offspring when inbreeding depression is low or negligible compared to the benefits of social living, such as predator avoidance or thermoregulation (Stevens et al, 1997; Ebensperger, 2001; Bilde et al, 2007).

Another way gregarious species can avoid inbreeding depression is through kin or familial recognition (Brown & Eklund, 1994). Kin recognition can be learned or innate (genetically determined), and has been documented in many species, ranging from pigs (*Sus scrofa*) to social insects like honeybees (*Apidae* sp.) and wasps (Singer, 1998; Zavazava & Eggert, 1997; Crozier,

1988). Kin recognition is separate from individual recognition as it divides conspecifics into classes rather than individuals (Crozier, 1988). Examples of species with innate kin recognition include damselfish (*Acanthochromis polyacanthus* and *Amphiprion melanopus*) and sweat bees (*Lasioglossum zephyrum*) (Atherton & McCormick, 2017; Greenberg, 1979). Learned kin recognition requires exposure to related individuals in order to form preferences based on phenotypes detected by sensory systems (olfaction, visual, etc.) and can sometimes extend to familiar individuals, as has been seen in spiny mice (*Acomys cahirinus*), humans (*Homo sapiens*), paper wasps (*Polistes fuscatus*), and long-tailed tits (*Aegithalos caudatus*) (Porter & Moore, 1981; Porter et al., 1978; Sharp et al., 2005; Sheehan & Tibbetts, 2011). Different *Drosophila* species have evolved multiple kin recognition systems, including innate preference, preference based on larval diet, preference based on familiarity, and reduced female investment in offspring when mating with a relative (Lizé, McKay, & Lewis, 2014), illustrating that innate and learned kin recognition can occur within the same genus. While kin recognition has been studied in many insects, it is less understood in butterflies, though there is evidence that *Heliconius erato phyllis* larvae can detect and avoid cannibalizing sibling eggs (De Nardin & de Araújo, 2011).

One species of butterfly known to suffer from inbreeding depression is *Bicyclus anynana* (family Nymphalidae) (Saccheri et al., 1996; van Bergen et al., 2013; van Oosterhout, Zulstra, et al., 2000). Previous work in this system has identified four reproductive attributes affected by inbreeding depression: percentage of sterile eggs per clutch, zygote survival, juvenile survival, and adult lifespan (van Oosterhout, Zulstra, et al., 2000). Inbreeding depression also reduces genetic variation and heritability of wing pattern and size (Saccheri, Nichols, & Brakefield,

2001), and reduces the amount of male sex pheromone males produce, which leads to reduced mating success for inbred relative to outbred males (van Bergen et al., 2013). Female *B. anynana* are able to detect and choose against mating with inbred individuals of two known degrees of inbreeding (inbreeding coefficient = $F_{0.25}$ (offspring of siblings) and $F_{0.375}$ (offspring of two generations of related siblings)), meaning that outbred individuals are preferred over inbred individuals (van Bergen et al., 2013). Evidence suggests that inbred males also have decreased flight capabilities, which is reflective of reduced general condition (van Bergen et al., 2013). Females cannot detect inbred males based solely on body condition, as females with their antenna blocked were unable to distinguish outbred from inbred males in choice assays (van Bergen et al., 2013). While these studies demonstrate that female *B. anynana* butterflies detect and avoid mating with inbred males, it remains unclear whether they also detect and avoid mating with siblings (i.e. recognize kin).

B. anynana could have innate kin recognition, or they could learn to recognize kin through social interactions. Currently, there are no studies on larval learning in this species, but we do know that adult naïve females can learn appearance-based mate preferences after initial exposure to a novel phenotype, therefore this species is capable of learning (Westerman et al., 2012). To determine if kin recognition affects mate choice, I reared *B. anynana* larvae under two conditions (socially and in isolation) and conducted mate choice assays with adult individuals to determine: 1) if *B. anynana* females innately recognize and avoid mating with siblings; 2) if females learn to recognize and avoid mating with siblings; and, 3) if females avoid mating with familiar individuals in general.

While kin recognition is one factor that can influence female mate choice, male activity level is another (Fusani, Barske, Day, Fuxjager, & Schlinger, 2014). Some species, such as golden-collared manakins (*Manacus vitellinus*) and green swordtails (*Xiphophorus helleri*), choose mates based on higher male activity level (Fusani et al., 2014). Recent work in *B. anynana* suggests a link between female choice and male activity level, specifically regarding male harassment and sexual conflict (Karl & Fischer, 2013; Kehl et al., 2014; Kehl, Dublon, & Fischer, 2015). Other research has demonstrated a relationship between male pheromone production and female mate choice independent of male behavior (Nieberding et al., 2008; Nieberding & Holveck, 2018). These competing hypotheses have sparked a debate about which male trait (male aggression versus male pheromone) is more important to mate choice in *B. anynana* (Fischer, Karl, Dublon, & Kehl, 2018; Nieberding & Holveck, 2018). In this debate, Kehl et al (2015) demonstrated that young male (3-day old) pheromone level had no effect on female choice, but credited male persistence as more important to mating success. Research conducted by Nieberding et al (2008), however, showed that male sex pheromone and wing pattern was effective at close-range courtship and that males with their androconial structures blocked were significantly less successful at obtaining mates despite courtship behavior, thus behavior had no effect on female choice. It is important to note that male sex pheromone production increases with age in this species, therefore young males produce relatively small amounts when compared to males even a few days older (Nieberding et al., 2012). To specifically test the effect of male activity on female mate choice I used young males that were the same age (and consequently pheromone production matched (Nieberding et al., 2012)), recorded all male activity for the first hour of each mate choice assay, and assessed whether male behavior was predictive of mating success.

Methods

Study Organism and Animal Husbandry:

Bicyclus anynana is a subtropical African butterfly with both a dry season and a wet season form, which differ in morphology and behavior (Brakefield & Reitsma, 1991; Prudic, Jeon, Cao, & Monteiro, 2011). The adult form is dependent on rearing temperature (cool *versus* warm). In the dry season form, males are the choosy sex, while females are the choosy sex in the wet season form (Prudic et al., 2011). This species has been maintained in the laboratory since 1988 when the original population was established in Leiden, the Netherlands, from 80 gravid females collected in Malawi (Brakefield & Reitsma, 1991b). These 80 gravid females produced between 8,000-10,000 eggs, potentially with multiple fathers per clutch, which would have maintained genetic diversity (Saccheri et al., 2001). The population used in this study was established in Fayetteville, AR from the serial translocation of approximately 1,000 eggs from the original population in Leiden via Buffalo, NY, New Haven, CT, then Singapore.

I reared all butterflies in mesh cages (100 cm x 160 cm or 25.4 cm x 50.8 cm) in a greenhouse at 27^{o}C, 60-80% relative humidity, to induce the wet season *B. anynana* phenotype (Brakefield & Larsen, 1984). Male and female virgin adults were chosen from newly emerged virgin stocks from breeding colonies containing hundreds of individuals to establish two or three mating pairs, and subsequent families, per week. These mating pairs were fed banana on top of damp cotton, and kept in mesh cages 39.88 cm x 39.88 cm x 59.94 cm. To ensure that pairs mated, virgin females were dusted with PF-33 clownfish orange UV powder (Risk Reactor) and males were examined 24 hrs later for transfer indicating copulation. After copulation occurred, I provided

pairs with a host plant (*Zea mays*-corn) for the female to lay eggs on for six days. After 6 days, I collected larvae and eggs and divided them into three rearing and mate choice treatments. Caterpillars in treatment 1 (detailed below) were reared in isolation (Figure 1A) (n = 5 individuals per family for treatment 1), while caterpillars in treatment 2 and 3 were reared in groups of 15 individuals (Figure 1B). When there were larvae, these larvae were automatically sorted into social groups. Only eggs were used for isolated individuals to prevent potential sibling learning in 1st nstar caterpillars. I provided larvae with corn plants, *ad libitum*, and kept them in larvae sleeves until pupation. Pupa were gathered and sexed using a dissecting microscope every four days (Ferkau & Fischer, 2006), then divided into emergence cages based on sex, family, and treatment. I continued this rearing regime until I completed 30 choice tests per treatment, sample size determined via *a priori* power analyses (described in Statistical Analyses below), for each treatment described below, from July 2017-May 2018 (Figure 2).

Treatment 1- Unfamiliar Relative Vs Unfamiliar Unrelated (T1):

Females in this treatment were reared in isolation. After eclosion, I dusted females with orange UV powder (as described above), which was ultimately used to indicate female choice. These females were placed in 39.88 cm x 39.88 cm x 59.94 cm mesh cages, in isolation, with food (banana) 24 hrs before testing. On day one (day of eclosion designated as day zero), I gave females a choice between an unfamiliar related male and an unfamiliar unrelated male. I used choice males that were between two and five days old, but matched in age (i.e. two three-day-old males used in the same test). I marked choice males with black dots on either ventral hindwing for identification 24 hrs prior to testing; M1 on the left and M2 on the right (Figure 1D).

Designation as “M1” or “M2” alternated between the familiar and unfamiliar males to rule out black dot placement as a factor in female mate choice.

To determine whether initial male behavior influenced mate choice outcome, I observed the first hour of each choice assay, and documented all behavior using Behavioral Observation Research Interactive Software (BORIS). The behaviors observed are described in detail below. Food was removed and males were given a 15 min acclimation period before recorded observation, which started within an hour of sunrise. After one hour, the observation period ended, food was returned to the cage, and all three butterflies were left in the cage for 24 hrs. I then used a UV light to detect powder transfer to the chosen male and the choice was recorded (as described in Joron & Brakefield, 2003).

Treatment 2- Familiar Relative Vs Unfamiliar Unrelated (T2):

Females in this treatment were reared in family groups. Upon eclosion, I placed females in mesh cages 39.88 cm x 39.88 cm x 59.94 cm, in isolation, 24 hrs prior to testing and provided food. Females were dusted with orange UV powder (as described above) to indicate choice. On day one, I gave females a choice between a familiar male she had been reared with and an unfamiliar male from a different family. Choice males were between two and five days old (with the exception of one trial using one day old males), and matched in age. I marked males with a black dot on either ventral hindwing for identification (as described above). I conducted mate choice assays and determined female choice as described in treatment one.

Treatment 3- Familiar Relative Vs Unfamiliar Relative (T3):

Females in this treatment were also reared in family groups. Newly emerged virgin females were placed in mesh cages 39.88 cm x 39.88 cm x 59.94 cm, in isolation, and provided food 24 hrs before testing. I dusted females with orange UV powder. On day one, females were given a choice between two males, one familiar and one unfamiliar. Both males were from the same family as the female, but one male had been reared with the female and the other male had not. Choice males were always matched in age but varied between two and five days old. I marked choice males with black dots on either ventral hindwing for identification 24 hrs prior to testing; M1 on the left and M2 on the right (as described above). I conducted mate choice assays and tested for final female mate choice as described in treatment one.

Behavioral Observations:

To determine whether male behavior during the first hour of a choice assay influenced mating outcome, I documented all behavior of the males and female in each choice assay for the three treatments described above using BORIS observational software for one hour following the 15-minute acclimation time. Behavioral watches were conducted during peak morning activity for these butterflies (Westerman et al., 2014). Documented behaviors included: *Flying, Resting, Courting* (as described in Nieberding et al., 2008), *Basking, Antenna Wiggle, Walking, Fluttering, Sitting Near,* and *Copulating.* I considered a subject *Resting* if it sat for a minimum of three seconds with its wings closed, while *Basking* was documented similarly but with wings open. *Antenna Wiggle* consisted of the subject moving one or both antenna a minimum of 45^{o}, in any direction. I documented opening and closing of the wings without flight (such as while

resting or walking) as *Fluttering*, and I noted two subjects as *Sitting Near* if they were resting or basking within one wingspan of each other.

Statistical Analysis:

I performed all statistical analyses using R (ver 3.4.1, "single candle" within Rstudio), except power analyses and pairwise $\chi 2$ tests, which were conducted with JMP Pro (ver 13). To determine whether females mated more often with: 1) unfamiliar relatives or unfamiliar unrelated individuals; 2) familiar relatives or unfamiliar unrelated individuals; or, 3) familiar relatives or unfamiliar relatives, I used $\chi 2$ tests. I used logistic regression models to assess whether male behavior (overall activity, first courtship, and courtship duration) during the first hour of the choice assay influenced female mating outcome using R package "lme4". Individuals with incomplete data (unrecorded or lost during data transfer) were excluded prior to analysis (reducing sample from size n = 182 to n = 174). I used Principal Component Analyses (PCA) on my behavioral data for each sex to assess correlations between behaviors and to define composite behaviors for further analysis. I excluded copulation in the calculation of my principal components because of its direct relationship to female choice. Principal components are comprised of multiple variables, in this case the behavioral instances and durations, that are most strongly correlated. Principal component analyses are used to reduce a large number of correlated variables into a smaller number of uncorrelated composite variables (Jolliffe, 2011). The effects of these new composite variables on female choice were then assessed using logistic regression.

I used *a priori* power analyses to determine treatment sample sizes. I chose sample sizes (n =30) that allowed me to detect female preferences of ~72:26 in all mate choice treatments (see Supplementary Table 1). With 30 mate choice assays per treatment, I was able to record the first hour of activity for 180 males (n = 60/treatment) and 90 females (n = 30/treatment) total, which allowed me to detect small effects of male behavior on mating outcome (down to differences of 0.63 s for behaviors with a standard deviation of 1.5 s, for example, see Supplementary Table 2) and small effects of rearing condition on female behavior (as small as 0.9 with an approximated standard deviation of 1.5, see Supplementary Table 2).

To determine if there was any effect of rearing condition on butterfly behavior, I performed one-way analysis of variance (ANOVA) on the recorded data for PC1, PC2, PC3, first courtship, courtship duration, and sitting near duration in males and PC1, PC2, PC3, and sitting near duration in females. I also performed a pairwise $\chi 2$ test for treatment 1 and 2 females to determine if there was an effect of social rearing condition on female mate preference for unrelated males.

Ethics Statement:

All *B. anynana* butterflies were maintained in laboratory conditions as specified by U.S. Department of Agriculture Animal and Plant Health Inspection Service permit P526P-17-00343. All caterpillars were reared within mesh larval bags in a climate-controlled, walk-in chamber maintained at wet season conditions and provided with ample food and water until pupation or death. All pupa and adult butterflies were maintained in cylindrical mesh cages within a climate-

controlled, walk-in chamber and provided with ample food and water until death or too old for my experiment, at which point they were frozen for later study. Food was removed from the behavioral assay cage prior to the start of the observation period, but returned upon completion of the behavioral assay. After mate choice trials were complete, all butterflies used were frozen for later study.

Results

Familiarity and/or Relatedness did not Influence Female Mate Choice:

I found that females reared in isolation did not have an innate mate preference for unrelated individuals (n = 30, sibling chosen = 15; unrelated chosen = 15, $\chi 2 = 0$, p = 1, Figure 3). Female *B. anynana* reared socially also did not dislike siblings as mates (n = 31, sibling chosen = 14; unrelated chosen = 17, $\chi 2 = 0.29032$, p = 0.59, Figure 3). Thus, there was not an effect of rearing condition (being reared with siblings) on female ability to detect and avoid mating with relatives (n = 60, $\chi 2 = 0.067$, p = 0.7961). Socially reared females also did not prefer unfamiliar siblings over familiar siblings, suggesting that familiarity did not influence female mate choice (n = 30, familiar sibling chosen = 12; unfamiliar sibling chosen = 18, $\chi 2 = 1.2$, p = 0.2733, Figure 3). While my experimental design only allowed us to detect strong preferences, I would have needed 787 treatment trials for the observed 55:45 difference in female mate preference for unfamiliar unrelated males to be deemed significant, and 191 treatment trials for the observed 60:40 difference in female mate preference for unfamiliar related males to be deemed significant (see Supplementary Table 3), therefore if there is an effect of larval experience on filial mate avoidance, it is a small one.

Male Courting Behavior did not Influence Female Mate Choice:

First courtship was observed in 34 of the 91 choice assays, allowing me to assess whether females ultimately mated with the male who courted her first. I found that males who courted first were not preferred during female mate choice when data was analyzed as a whole or by treatment (All treatments n = 34, $\chi 2 = 0.11765$, p = 0.7316; T1 n = 11, $\chi 2 = 2.2727$, p = 0.1317; T2 n = 9, $\chi 2 = 0.11111$, p = 0.7389; T3 n = 14, $\chi 2 = 2.5714$, p = 0.1088, Figure 4). Courtship duration also had no effect on female choice, either when data was analyzed as a whole or by treatment (Logistic regression, all treatments n= 174, z = 0.662, p = 0.508; T1 n = 58, z = 1.757, p = 0.0789; T2 n =57, z = 0.655, p = 0.512; T3 n = 59, z = -1.590, p = 0.112, Figure 5). Given my results, I would have needed 2263 female mate choice trials that included courtship for the observed difference in successful and unsuccessful male courtship duration to be deemed statistically significant (observed effect of 6.5 s, with a standard deviation of 55.16 s, see Supplementary Table 4).

Principal Component Analysis

In my study, the first three principal components of my principal component analysis account for 66% of behavioral variance in males (see Supplementary Table 5). Principal component one (PC1) is comprised primarily of *fluttering*, *antenna wiggling*, *walking* and *flying*, or "high energy movements" (so called for increased metabolic output (Fritzsche McKay, Ezenwa, & Altizer, 2016)), and explains 37% of the behavioral variance observed in males. Principal component two (PC2) is composed primarily of *courting*, *flying*, and *sitting near*, or "courting movements", and

explains 15% of behavioral variance observed in males. Finally, principal component three (PC3) is comprised primarily of positive *resting* and *sitting near*, or "low energy movements", and negatively of *courting*, and explains an additional 14% of observed variance in male behavior.

In females, the first three principal components in my principal component analysis account for 76% of total recorded behavioral variance (see Supplementary Table 6). Principal component one (PC1) is comprised primarily of the same high energy movements seen in males (*antenna wiggling*, *walking*, *fluttering*, and *flying*) and explains 47% of behavioral variance observed in females. Principal component two (PC2) is composed primarily positively of *sitting near*, *resting*, *fluttering* or "cordial movements", and negatively of *basking*, and explains 16% of behavioral variance observed in females. Finally, principal component three (PC3) is comprised primarily positively of *resting* and *basking* or "motionless movements", and negatively of *sitting near*, and explains 13% of behavioral variance observed in females.

Male Activity Levels did not Influence Female Mate Choice

The three composite male behaviors (high energy movements (PC1), courting movements (PC2), and low energy movements (PC3)) were not significantly correlated with female choice in any of the treatments (All Treatments: PC1 n = 174, z = -1.641, p = 0.101; PC2 n = 174, z = 0.019, p = 0.985; PC3 n = 174, z = 1.367, p = 0.172; Figure 6. T1: PC1 n = 58, z = -0.936, p = 0.349; PC2 n = 58, z = 0.090, p = 0.928; PC3 n = 58, z = 1.221, p = 0.222; Supplementary Figure 1. T2: PC1 n= 57, z = -0.312, p = 0.755; PC2 n = 57, z = -0.342, p = 0.732; PC3 n = 57, z = 0.149, p =

0.882; Supplementary Figure 2. T3 PC1 n = 59, z = -1.519, p = 0.129; PC2 n = 59, z = 0.833, p = 0.405; PC3 n = 59, z = 1.289, p = 0.197; Supplementary Figure 3). I would have needed 446 males for the observed difference in successful and unsuccessful male high energy movements to be deemed statistically significant (observed effect of 0.46, standard deviation of 1.73, see Supplementary Table 4), over 2,000,000 males for the observed difference in successful and unsuccessful male courting movements to be deemed statistically significant (0.004, with a standard deviation of 1.10, see Supplementary Table 4), and 787 males for the observed difference in successful and unsuccessful male low energy movements to be deemed statistically significant (0.22 with a standard deviation of 1.05, see Supplementary Table 4), suggesting that, if there is an effect of male activity on female mate choice, it is small, and may not be biologically relevant.

Rearing Conditions did not Influence Adult Behavior

Rearing condition (social or in isolation), did not have an effect on adult male behavior (PC1 n = 172, isolated = 23, social = 150, f = 0.096, p = 0.757; PC2 n = 172, f = 0.27, p = 0.604; PC3 n = 172, f = 2.545, p = 0.112; Number of Courting Events n = 172, f = 1.874, p = 0.173; Courtship Duration n = 174, f = 1.431, p = 0.233; Sitting Near Duration n = 174, f = 0.004, p = 0.951).

Nor did I find an effect of rearing condition on adult female behavior (PC1 n =84, f = 1.653, p = 0.202; PC2 n = 84, f = 0.52, p = 0.473; PC3 n = 84, f = 0.729, p = 0.396; Sitting Near Duration n = 84, f = 1.077, p = 0.302).

Discussion:

My results suggest that females do not innately prefer unrelated males. In addition, they do not learn to avoid mating with brothers during social interactions as caterpillars, and they do not learn to prefer unfamiliar males based on larval social interactions. Male behavior also did not have any effect on female mate choice, either when treatments were pooled or analyzed separately.

My results did not support my hypothesis that female *B. anynana* would exhibit innate mate preferences for unrelated individuals to prevent observed costs of inbreeding depression (Saccheri et al., 1996; van Oosterhout, Zulstra, et al., 2000). This was unexpected, as innate kin recognition has been demonstrated in other life stages of butterfly species belonging to the same family as *B. anynana* (Nymphalidae) (De Nardin & de Araújo, 2011). *Heliconius erato phyllis*, caterpillars identify and avoid eating siblings, which suggests kin recognition, though it is unknown how they recognize siblings, or whether *H. erato* adults recognize siblings (De Nardin & de Araújo, 2011). Future research should examine *B. anynana* caterpillar and *H. erato* adult kin recognition to evaluate whether similar recognition systems exist in both species. This lack of innate dislike of sibling pheromones is also dissimilar to what we see in mice and humans, where individuals prefer mates with odors unlike their own (Potts et al., 1991; Wedekind et al., 1995). Humans also use olfaction to detect sibling and offspring scents on clothing and correctly identify which clothes were worn by their relatives (Porter & Moore, 1981). Similarly, we know female *B. anynana* can detect an inbred male due to lower male sex pheromone production (van Bergen et al., 2013), but my study shows they do not select against related males during mate choice. Previous studies have demonstrated that *B. anynana* females differentiate between age

and detect inbreeding using male sex pheromones during mate choice (Nieberding et al., 2012; van Bergen et al., 2013) and that they suffer from inbreeding depression (Saccheri et al., 1996; van Oosterhout, Zulstra, et al., 2000), therefore I hypothesized that they might also detect and choose against kin. We have demonstrated that females are able to detect male sex pheromones of individual males in our experimental setting (Tim Sullivan personal communication), therefore we know that they can respond to a specific male's scent in our environment. Males, however, do not develop male sex pheromones until after eclosion (Nieberding et al., 2012), thus females in my study did not have exposure to these pheromones prior to my mate choice assays. Perhaps under natural conditions they would use that exposure to learn to avoid siblings as mates.

Alternatively, cuticular hydrocarbons are well known to be important for both innate and learned kin recognition in insects, and may play a role in kin recognition in *B. anynana* (Howard & Blomquist, 1982; Lahav, Soroker, & Hefetz, 1999; Thomas, Parry, & Allan, 1999). Diet can alter the production of cuticular hydrocarbons which can cause kin recognition errors (related individuals treated with hostility, unrelated individuals treated favorably) in *Drosophila melanogaster*, Argentine ants (*Linepithema humile*), *Myrmecaphodius proseni* (a beetle), and salticid spiders (*Cosmophasis bitaeniata*) (Elgar & Allan, 2004; Liang & Silverman, 2000; Lizé et al., 2014; Meer & Wojcik, 1982). *Drosophila melanogaster* reared on different foods (ASG (Agar, sucrose, yeast) and banana-medium) preferentially mate with individuals that had been given a different food and preferred related individuals that had been given a different food over unrelated individuals with the same diet as them (Lizé et al., 2014). Therefore, dietary effects on cuticular hydrocarbon production were more important kin recognition to mate choice. Similarly,

when nestmates were fed different species of prey items in Argentine ants, nestmate recognition broke down and those that had consumed prey that the colony had not were attacked (Liang & Silverman, 2000). This form of kin recognition also influences predator success. Host ant species (*Oecophylla smaragdina* and *Solenopsis* sp., respectively) could not distinguish predatory salticid spiders or *M. proseni* from nestmates after their cuticular hydrocarbon profiles were altered to match specific colony profiles by the consumption of nestmates (Elgar & Allan, 2004; Meer & Wojcik, 1982). Therefore, diet can alter kin recognition at multiple levels (different species of food, different colony origin). *Bicyclus anynana* produces different cuticular chemicals based on age and sex, however, we do not currently know if diet affects cuticular hydrocarbon production in this species (Heuskin et al., 2014). Adult female *B. anynana* lay their eggs on host plant grasses in the wild, and multiple females may use the same host plant (Kooi, Brakefield, & Rossie, 1996). This has also been documented in laboratory populations throughout the literature. Wild *B. anynana* larvae are known to feed on a variety of grass host plants (Kooi et al., 1996), which could foster the production of unique cuticular hydrocarbons and allow for kin or group recognition in adults. Similar food intake between groups in my laboratory population could have masked the effect usually provided by cuticular hydrocarbons in the wild. Future research should assess the effect of host plant variation and adult female exposure to filial pheromones on sibling avoidance.

High levels of dispersal is an alternative mechanism to kin recognition for avoiding inbreeding (Avilés & Bukowski, 2006; Moore & Ali, 1984). As we do not know this species dispersal pattern and it is predicted that it is highly variable (Saastamoinen et al., 2012), there could be a similar effect as what is seen in a number of both sessile and mobile invertebrates, in which

species with low dispersal are more resistant to inbreeding depression (Ng & Johannesson, 2015; Phillippi & Yund, 2017). *B. anynana's* quick recovery from inbreeding depression (Saccheri et al., 1996) supports this hypothesis. Based on my results, *B. anynana* females do not detect siblings during mate choice. Therefore, I would expect lower fecundity among mated siblings as opposed to an outbred pair, but rapid fitness recovery following outbreeding in the next generation. Future work is needed to determine dispersal patterns for this species, and the relationship between dispersal and inbreeding depression.

In addition to not exhibiting innate kin recognition and avoidance, female *B. anynana* also did not learn to detect and choose against kin based on larval experience in my study. This is different from what we see in cooperatively breeding long-tailed tits (Sharp et al., 2005), but more similar to what has been observed in Banggai cardinalfish (*Pterapogon kauderni*), which are gregarious but do not give any indication of kin recognition as the mechanism behind grouping behavior (Kolm, Hoffman, Olsson, Berglund, & Jones, 2005). These differences could be due to the fact that survival may not be dependent on kin recognition. Long-tailed tits use auditory cues in contact calls to distinguish kin from non-kin, however chicks learn these calls over time and through parental and sibling care (Sharp et al., 2005). Increased food and vigilance by kin improves survivorship in these birds (Sharp et al., 2005). Banggai cardinalfish young also receive parental care (paternal mouthbrooding), but adult fish use local sea urchins to hide when threatened (Kolm et al., 2005). Consequently, knowledge of local social environment may be more important than kin recognition to the formation of groups, even though groups may predominantly consist of kin (Kolm et al., 2005). We know that *B. anynana* are gregarious, however my results suggest that kin recognition may not be an important component of this

gregariousness. *B. anynana* may receive benefits outside of mating opportunity, such as predator avoidance or foraging information, that drive gregarious behavior. Future work should investigate possible fitness benefits of gregariousness in this species.

In addition to demonstrating that female *B. anynana* do not have an innate or learned preference for unrelated males as mates, I have shown that adult female *B. anynana* do not choose mates based on familiarity. This is dissimilar from what we see in both paper wasps and spiny mice. Adult paper wasps learn visual cues (distinct yellow facial markings) to identify individuals in their groups (Sheehan & Tibbetts, 2011). Paper wasps live in strict social hierarchies, therefore individual recognition is important to stabilizing social interactions and reducing aggressiveness between groupmates (Sheehan & Tibbetts, 2011). In the spiny mouse study done by Porter et al. (1978), spiny mouse sibling pairs and non-sibling pairs raised together were more likely to huddle together than siblings or non-siblings raised apart. While they did not study mate choice in these mice, they did find a significant effect of familiarity on social behavior. Therefore, group recognition is important to a variety of social situations, such as mediating aggression between group members and social nesting (conspecifics sleeping together for benefits such as thermoregulation or moisture conservation (Madison, FitzGerald, & McShea, 1984)). Prior work in *B. anynana* has shown that the effective population size of males is roughly 32% (P. M. Brakefield et al., 2001) and that interrupted courtship (male-male competition) increases as sex ratio becomes more male biased (Holveck, Gauthier, & Nieberding, 2015). In my study, I showed that female choice is not affected by familiarity. However, given effective population sizes and male-male competition, males might be able to recall former competitors and adjust

aggressive behaviors accordingly. Future research will be needed to determine if males differentially interrupt courtship based on past experiences.

I did not detect signs of kin recognition during mate choice, and none of the male behaviors I analyzed (first courtship, courtship duration, or total behavior) increased the male's likelihood of mating. These findings shed new light on the current discussion on the importance of male activity level (and perhaps sexual harassment) versus female choice (for male sex pheromone and wing pattern) on mating outcome in *B. anynana* (Fischer et al., 2018; Nieberding & Holveck, 2018). My results do not support the male activity and persistence hypotheses, where male activity (including harassment) encourages female mate choice, proposed in Kehl et al. (2015) and Fischer et al. (2018). While these two studies found a link between highly active males and mating success, they used high density populations which may have restricted overall movement (Fischer et al., 2018). In my study, I used low density mate choice assays with ample room for activity (three butterflies within a 39.88 cm x 39.88 cm x 59.94 cm enclosure). In other species, such as yellow dung flies (*Scathophaga stercoraria*), guppies (*Poecilia reticulata*), and solitary bees (*Anthophora plumipes*), at high densities females are much more likely to encounter multiple males at once and risk reduced body condition via higher energy expenditure and potential damage (Chapman, Arnqvist, Bangham, & Rowe, 2003; Darden & Croft, 2008; Magurran & Seghers, 1994; Stone, 1995). At high density, low cage volume, and/or male-biased sex ratios, female choosiness in *B. anynana* (designated as the proportion of rejected matings) decreases (Holveck et al., 2015). Additionally, under these same conditions male-male competition in the form of interrupted courtship increases (Holveck et al., 2015). Therefore, the observed importance of male activity and female choosiness to mating outcome may not

accurately reflect natural relative importance when high densities are used in this system (Holveck et al., 2015). The low densities used in my study more closely mimicked natural conditions which would afford a female more escape and avoidance opportunities, thereby facilitating female choice. I also age-matched males to eliminate any confounding effect of pheromones to specifically test the hypothesis that male behavior (and courting persistence) influenced female mating outcome. Given that first courtship, courtship duration, and total activity were not associated with male mating success, I can say that when males are matched in age, the male activities I recorded had no effect on mating outcome. Therefore, female choice is more important to mating outcome than male activity and persistence.

Theory suggests that organisms either avoid inbreeding when fitness costs are too high, or allow it when fitness costs are negligible (Pusey & Wolf, 1996; Stevens et al., 1997). *B. anynana* suffers from inbreeding depression via lower fecundity, lower rates of egg hatching, and decreased adult condition (wrinkled wings), however they also experience rapid fitness recovery with outbreeding (Saccheri et al., 1996). My research found that *B. anynana* females did not innately prefer to mate with unrelated males, nor did they learn to prefer unrelated or unfamiliar males based on larval experience. One of the differences between my study and that of Saccheri et al. (1996) is that I used first generation siblings, while Saccheri et al. used multigeneration inbred lines ($F_2 – F_7$). While females in this species are able to detect inbred individuals and select against them (van Bergen et al., 2013; van Oosterhout, Zulstra, et al., 2000), my study demonstrated that *B. anynana* females do not preferentially avoid mating with siblings, at least when said females are not from an already highly inbred line. When females are from a highly inbred line, they outbreed easily due to inbred males being less appealing (Saccheri et al., 1996;

van Bergen et al., 2013). My research eliminated adult female familial or group recognition as a mechanism for inbreeding avoidance. When taken with van Oosterhout et al. (2000) and Bergen et al. (2013), my results suggest that female detection of male sex pheromone abnormalities in inbred individuals, which deters female acceptance, may be sufficient to prevent inbreeding depression in the absence of kin recognition in this species. Future research should assess when females can no longer detect inbreeding in different degrees of inbred individuals during mate choice.

Conclusions

Here I show that *B. anynana* females do not choose mates based on larval familiarity or relatedness. Furthermore, I show that larval social environment does not affect adult male or female activity level and that male activity level does not influence female choice between age matched males. Given that *B. anynana* recovers from inbreeding depression quickly in the absence of a kin recognition system, my findings support the hypothesis that kin recognition is not the only mechanism by which species avoid inbreeding depression. Additionally, my findings support the hypothesis that high male activity levels may not be as important to female choice as other fitness indicators in this system.

Figures

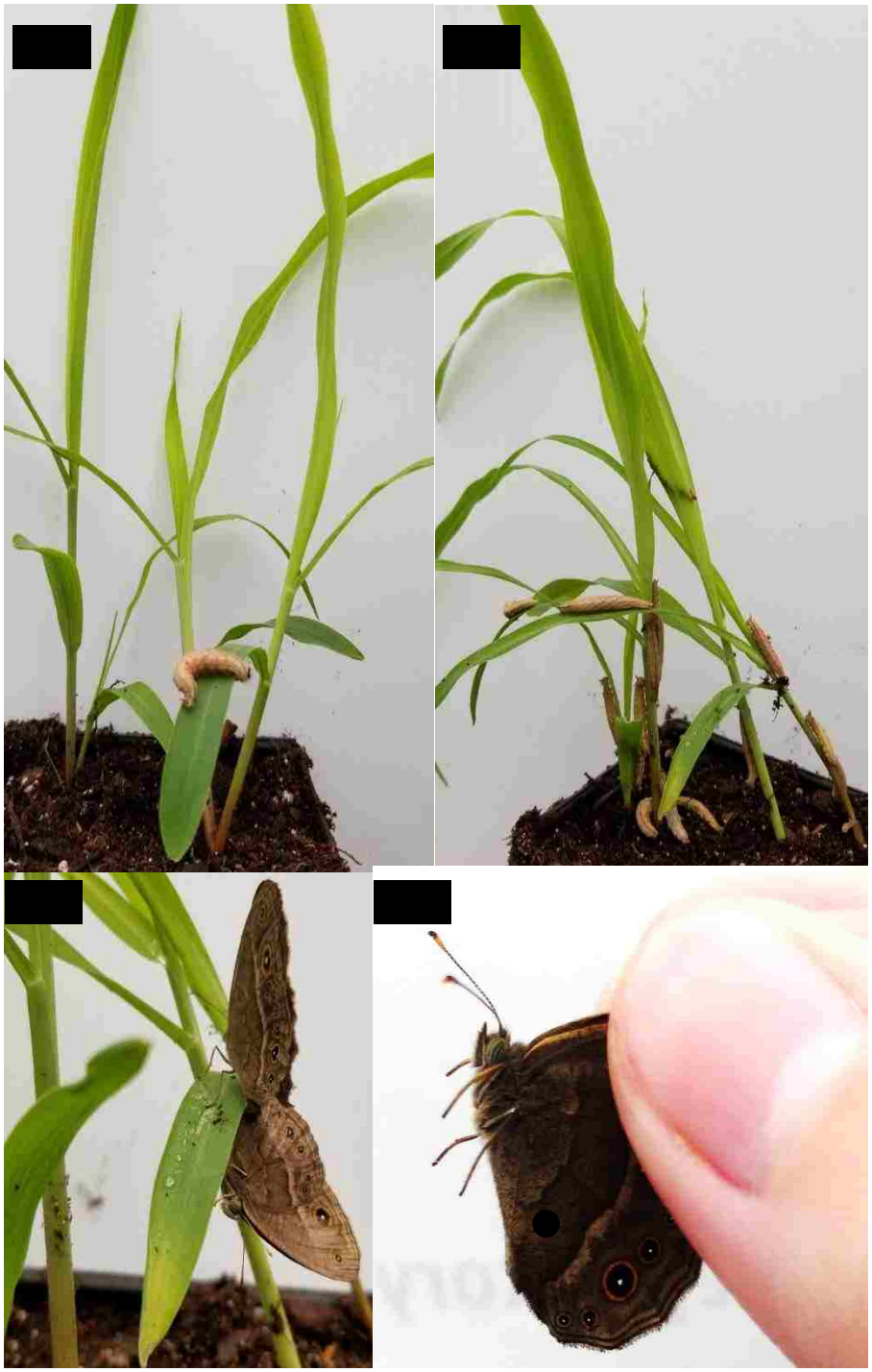


Figure 1: *Bicyclus anynana*. A) Isolated Treatment. B) Social Treatment (n=15). C) Adult *B. anynana* copulating. D) "M1" *B. anynana*, as indicated by the black dot on the left ventral hindwing.

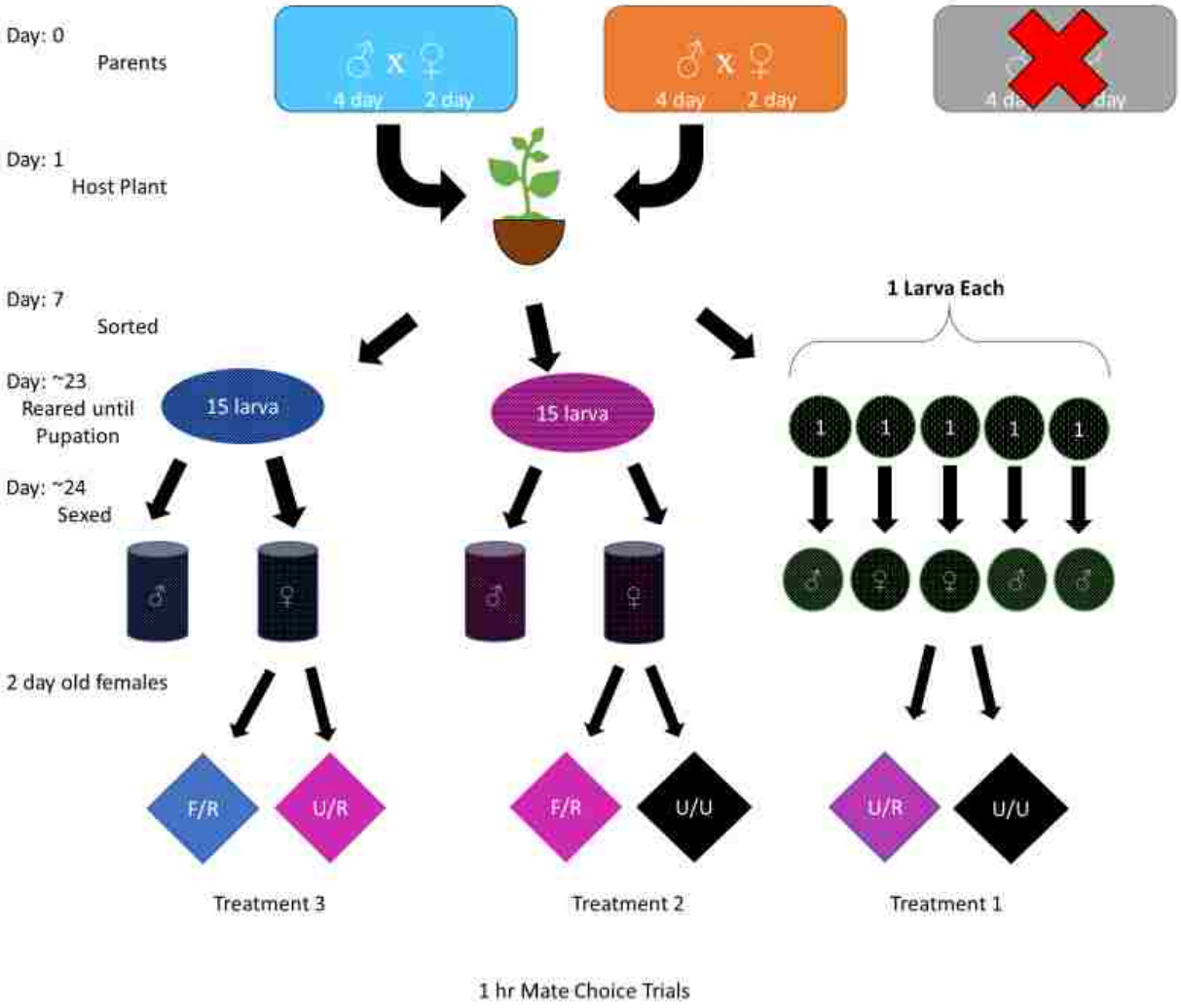


Figure 2: Experimental design schematic. If three mating pairs and families were established, only two of those families were used in the experiment due to low available space.

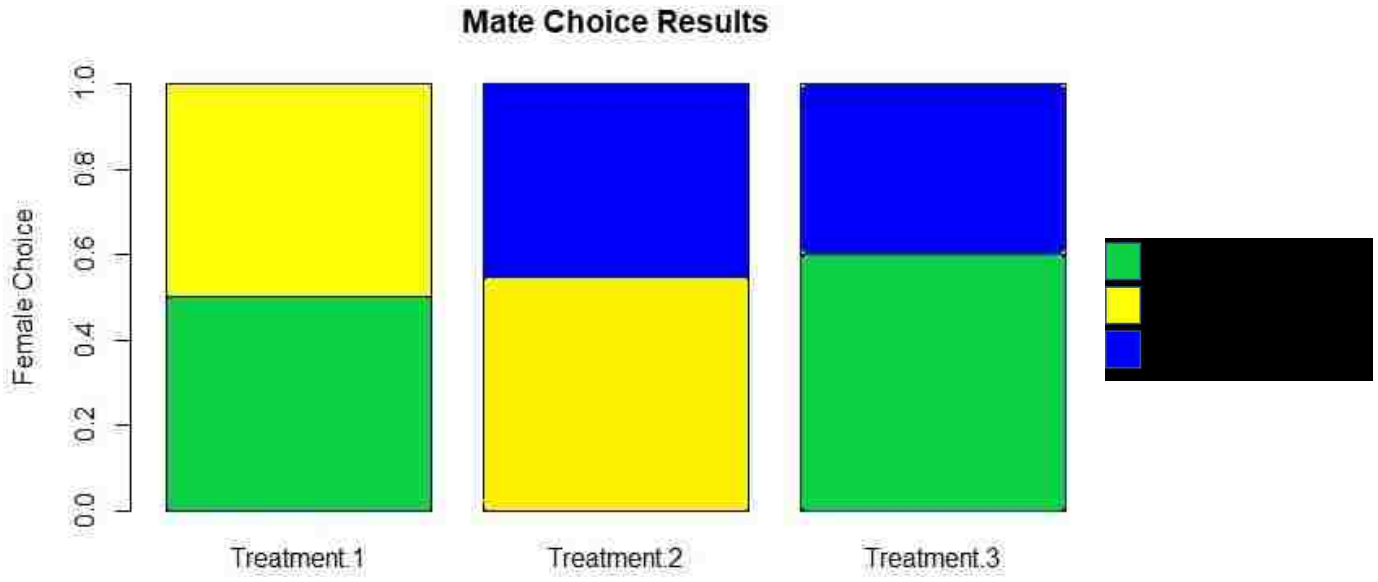


Figure 3: *B. anynana* do not choose mates based on relatedness or familiarity. *B. anynana* females do not innately prefer unrelated males (Treatment 1: n = 30, $\chi 2 = 0$, p = 1). They also do not choose mates based on kin recognition or group recognition (Treatment 2: n = 31, $\chi 2 = 0.29032$, p = 0.59; Treatment 3: n = 30, $\chi 2 = 1.2$, p = 0.2733). Green indicates the proportion of unfamiliar and unrelated males chosen, yellow indicated the proportion of unfamiliar and related males chosen, and blue indicates the proportion of familiar and related males chosen.

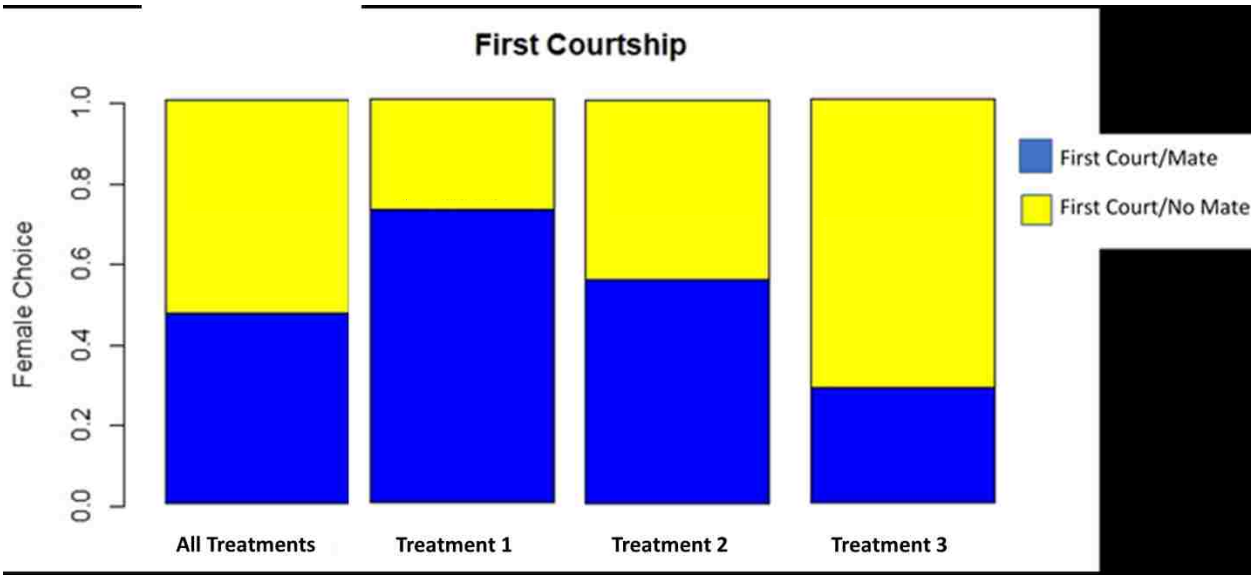


Figure 4: There is no copulatory advantage to courting first in *B. anynana*. Proportion of males for which I observed first courtship which ultimately mated with the female and those that did not (all treatments n = 34, $\chi 2 = 0.11765$, p = 0.7316; T1 n = 14, $\chi 2 = 2.5714$, p = 0.1088; T2 n = 9, $\chi 2 = 0.11111$, p = 0.7389; T3 n = 11, $\chi 2 = 2.2727$, p = 0.1317). Blue indicated the males that were first to court and were chosen while yellow indicates the males that courted first but were not chosen.

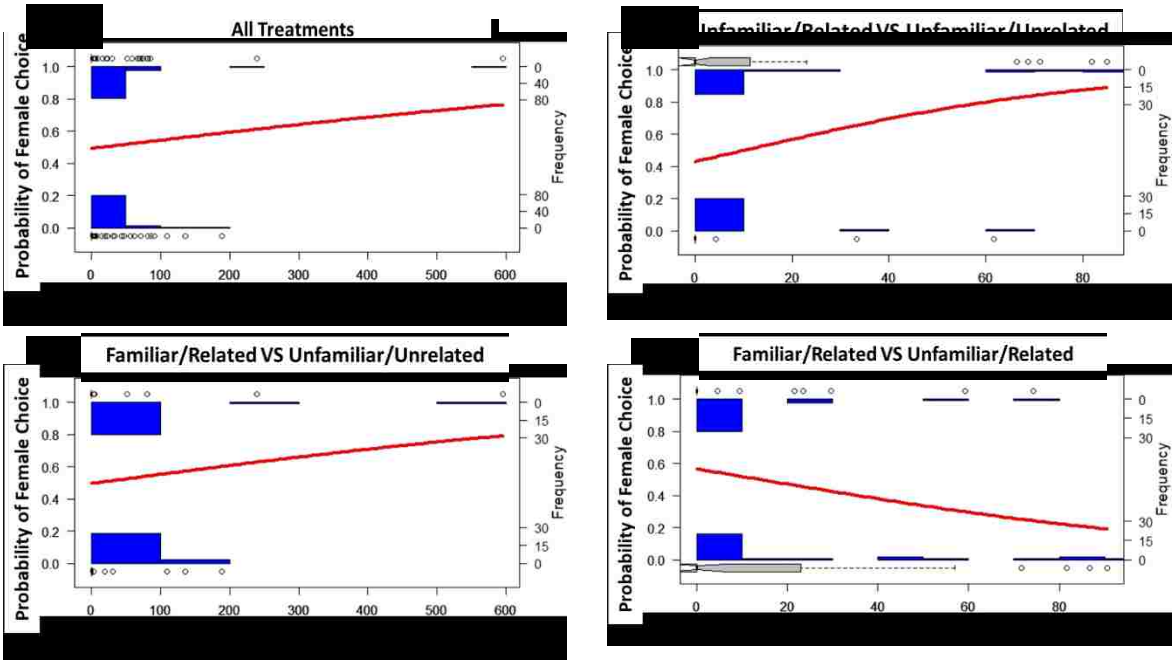


Figure 5: Male courting duration has no effect on female mate choice. Logistic regression of courtship duration for A) all treatments, B) treatment 1, C) treatment 2, and D) treatment 3 (all treatments n= 174, z = 0.662, p = 0.508; T1 n = 58, z = 1.757, p = 0.0789; T2 n =57, z = 0.655, p = 0.512; T3 n = 59, z = -1.590, p = 0.112).

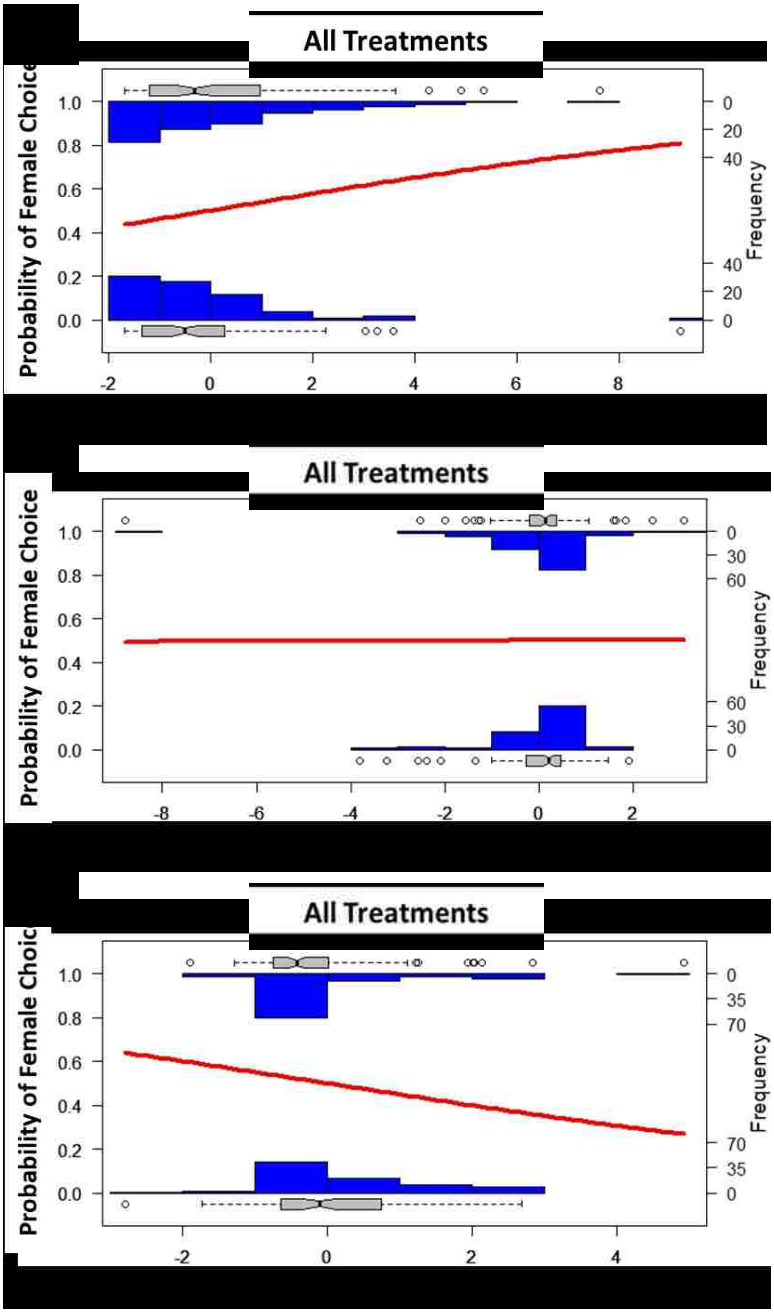


Figure 6: High energy movements, courting movements, and low energy movements have no effect on female mate choice. Logistic regression of mating outcome versus principal components for all treatments (A) PC1 n = 174, z = -1.641, p = 0.101; B) PC2 n = 174, z = 0.019, p = 0.985; C) PC3 n = 174, z = 1.367, p = 0.172).

Chapter Two: Evidence of circadian courtship in the neotropical butterfly *Heliconius hewitsoni*

Abstract

Circadian behavior may allow animals to optimize foraging, avoid periods of peak predation pressure, or optimize signal transmission for intraspecific competition and mate attraction. Consequently, one behavior that is often circadian is courtship, though courtship can also be seasonal or triggered by other environmental cues. Here, I test the hypothesis that the neotropical butterfly *Heliconius hewitsoni* exhibits circadian courtship patterns, as has been documented in many other lepidopteran species. I observed male behavior throughout the day across multiple days to determine if *H. hewitsoni* butterflies courted at specific times during the day, or if their courting was more dependent on weather conditions. I also documented other behaviors of both males and females to determine peak times of daily activity for these butterflies. I found that males court the most around solar noon, independent of weather conditions, and court the longest around 12:40. I also identified peak times of activity for all but two of our recorded behaviors. *H. hewitsoni* males and females exhibit the most antenna wiggling, flying, basking, and walking during the morning. They exhibit the most fluttering during the morning and noon hours, and the most resting during the evening. Fluttering is the only behavior that peaks at the same time as courtship. My results show that the circadian rhythm of *H. hewitsoni* matches observed nectar and pollen production patterns reported in its food plant (*Psiguria* species) and is a partial mismatch of peak activity times of avian predators (such as jacamars and flycatchers). These findings suggest that adult *H. hewitsoni* have a circadian rhythm that takes advantage of food sources and may lower the risk of predation.

Introduction

Circadian rhythms have been found across taxa, including bacteria (Bell-Pedersen et al., 2005; Lakin-Thomas & Brody, 2004), fungi (Bell-Pedersen et al., 2005; Lakin-Thomas & Brody, 2004), plants (McClung, 2006), invertebrates (Bloch, Hazan, & Rafaeli, 2013; Sandrelli, Costa, Kyriacou, & Rosato, 2008), and vertebrates (Aschoff, Daan, & Groos, 2012), and are vital to survival and reproduction in many species. Circadian rhythms are roughly 24-hour cycles of activity that correspond to the earth's rotation, but which may be maintained by responses to external cues (or zeitgebers) (Bloch et al., 2013; Edery, 2000; Groot, 2014; Sandrelli et al., 2008). For a cycle of activity to be considered a circadian rhythm it must fulfill three conditions: 1) It must free-run (continue) over a 24-hour period in the absence of external time cues; 2) it must be reset by changes in environmental condition, most commonly by light-dark or temperature fluctuations; and 3) it must not vary across a range of natural temperatures (Bloch et al., 2013; Edery, 2000; Groot, 2014). While their evolutionary origin is unclear, circadian rhythms are important to maintaining steady activity in organisms even when environmental conditions, such as periods of harsh weather or anthropogenic disturbances, force them to seek shelter in inadequately lit places (Edery, 2000).

Circadian rhythms can be organized into three categories: diurnal, crepuscular, and nocturnal (Blanchong, McElhinny, Mahoney, & Smale, 1999). Diurnal organisms, such as cotton (*Gossypium hirsutum*) and honey bees (*Apis mellifera*), experience peak activity during the daylight hours (Kaiser & Steiner-Kaiser, 1983; Loughrin, Manukian, Heath, Turlings, & Tumlinson, 1994). Crepuscular organisms, such as the sweat bee (*Megalopta genalis*) and common degu (*Octodon degus*), are most active during dawn and dusk (Kas & Edgar, 1998;

Kelber et al., 2006). Nocturnal organisms, including Geoffroy's tailless bat (*Anoura geoffroyi*) and the flowers it feeds on (*Markea* sp.), are primarily active at night (Muchhala & Jarrin -V., 2002). These periods of peak activity influence when organisms interact with each other on an intraspecies and interspecies level (Edery, 2000; Gilbert, 1975; Loughrin et al., 1994). Circadian rhythms influence everything from foraging success to predator avoidance, and adaptations, such as eye pupil shape and periods of pollen production, are often reflective of peak activity times (Edery, 2000; Kelber et al., 2006; Sandrelli et al., 2008).

Matched circadian rhythms within a species (intraspecific) allow organisms to synchronize their physiology and behavior to times that convey the most fitness benefit, which can impact survival and mate choice (Bloch et al., 2013; Edery, 2000). For example, *Drosophila* eclose in the early morning when relative humidity is high (Pittendrigh, 1954). This is important because newly emerged *Drosophila* are more prone to desiccation upon eclosion and their wings will not expand if humidity is too low, thus eclosion time has evolved to become synchronized with high humidity cues to reduce mortality (Pittendrigh, 1954). Circadian rhythms can also act as temporal reproductive isolation barriers. For example, two species of plume moth (*Platyptilia carduidactyla* and *P. williamsii*) are attracted to the same sex pheromones, however female *P. williamsii* release their pheromones during the first six hours of the night while male *P. carduidactyla* do not begin to seek mates until the second six hours of the night (Haynes & Birch, 1986). Therefore, the timing of species-specific behaviors maintain species barriers and prevent copulation errors.

Interspecific interactions are also heavily influenced by either matching or decoupling circadian rhythms in both mutualistic and predatory relationships. Many flowers, such as snapdragon (*Antirrhinum majus*) and tobacco (*Nicotiana suaveolens*), release the highest levels of volatile compounds when their respective pollinators, in this case bees and moths, are most active (Pichersky & Gershenzon, 2002; Yakir, Hilman, Harir, & Green, 2007). Thus, flowers improve their likelihood of pollination by matching their volatile production to their pollinators circadian rhythms. Conversely, zooplankton (*Diaptomus* sp. And *Daphnia* sp.) adjust their vertical position in the water column by light cued circadian rhythms to hide from visually dependent planktivorous fish, thereby avoiding predation (Zaret & Suffern, 1976). Thus, interspecific interactions are also important for shaping the evolution of species specific circadian rhythms.

Butterflies often have diurnal circadian rhythms due to food source availability, mate availability, and predator avoidance (Niepoth, Ke, de Roode, & Groot, 2018). While we know the circadian rhythms of many species of butterflies, such as the monarch (*Danaus plexippus*) and the large white butterfly (*Pieris brassicae*) (Froy, Gotter, Casselman, & Reppert, 2003; Veerman, Beekman, & Veenendaal, 1988), the circadian rhythms of many *Heliconius* species are largely unknown. *Heliconius* are long lived butterflies (three to six months) that oviposit on passionflower vines (*Passiflora* species) (Gilbert, 1975; Merrill et al., 2015). The leaves of these vines produce cyanoglucosides, which *Heliconius* larvae sequester and use to make themselves unpalatable to predators even as adult butterflies (Gilbert, 1975; Hay-Roe & Nation, 2007; Merrill et al., 2015). As adults, these butterflies have formed a mutualistic relationship with cucurbit (*Psiguria* species), wherein *Heliconius* feed on the pollen but also transport pollen to other cucurbit flowers (Gilbert, 1975; D. A. Murawski & Gilbert, 1986; Darlyne A. Murawski,

1987; New, 2017). Birds are thought to be the primary predator of these butterflies, and, while predation data are relatively rare, gregarious roosts are most often disturbed during crepuscular hours (James Mallet & Gilbert, 1995). *Heliconius* roost gregariously as a form of anti-predator defense caused by the aggregation of many unpalatable individuals sending the same repellent signal through color, however aggregations that are too large may attract the attention of naïve predators (Finkbeiner, Briscoe, & Reed, 2012). We do not know when courtship is most likely for many of these butterflies, or if it is circadian. In this study, I observed *Heliconius hewitsoni* (Figure 1) over multiple days in large flight cages in the lab to assess the presence of circadian rhythms in this species, with a specific focus on when courtship was most likely to occur.

Methods

Study Organism and Animal Husbandry:

Heliconius butterflies have been heavily studied as mimicry models since 1862 when Henry Walter Bates used them to develop his mimicry theory (Bates, 1862; Mallet, Jiggins, & McMillan, 1998; Merrill et al., 2015). These butterflies form intricate Müllerian mimicry rings to deter predators, meaning that all *Heliconius* species possess some level of toxicity though the degree of unpalatability varies between species (Mallet et al., 1998; James Mallet & Gilbert, 1995; Merrill et al., 2015; Müller, 1879). *Heliconius hewitsoni* is a Central American butterfly species from the "yellow" *Heliconius* mimicry ring (Mallet & Gilbert, 1995). It is native to lowland rainforests from southwestern Costa Rica to western Panama, and individuals are reported to have small home ranges with predictable daily movement (DeVires, 1987; Longino,

1984; R. D. Reed, 2003), but no one has documented specifics of their circadian rhythm in terms of courting activity and other behaviors.

Our laboratory population was provided by Suministros Entomologicos (Costa Rica Entomological Supply) in Alajuela, Costa Rica. Male and female adult *Heliconius hewitsoni* were kept in a large mesh communal cage 1.83 m X 0.86 m within a greenhouse with an average temperature of 27ºC and an average relative humidity of 60-80%. New individuals from breeder stocks were added upon emergence, and changes to colony composition were recorded. I marked all males with a silver dot on the left, ventral hindwing for identification purposes, and all butterflies were marked with individual silver numbers on the hindwing. Butterflies were fed Birds Choice Butterfly Nectar and trained to eat from artificial flowers, which were refilled every day between observations.

Behavioral Assays:

I observed the *H. hewitsoni* colony using SpectatorGO! behavioral software over 12 hrs/day from May 12, 2017 to May 31, 2017, from 7:20-19:20, which translates to starting and ending within an hour of sunrise and sunset at the latitude of my study location. Every 15 mins during this 12 hr period I conducted five-minute focal watches, during which I recorded the activity of three semi-randomly chosen individuals, with watches composed of either two males and one female or one male and two females to compare differences in activity between sexes. Population cages contained an average of 19 butterflies during my experiment, with the maximum number of individuals used per day to build this data set peaking at 23 and the minimum being 10. I

conducted 1,665 focal watches across 37 time points (later broken down into four time categories as well; see below), resulting in a minimum of 45 watches per time point (minimum 15 males or females per time point; maximum 30 males or females per time point) over the 15-day observation period.

Documented behaviors included: *Flying, Resting, Basking, Walking, Fluttering, Antenna Wiggle, Courting, Sitting Near,* and *Copulating*. Two individuals were considered *Sitting Near* if they were stationary and within one wingspan of each other. This may or may not have included one of the other focal animals. *Resting* and *Basking* were recorded as a subject stationary with its wings closed, or open, respectively, for three seconds or more. Opening and closing of the wings while not in flight was denoted as instances of *Fluttering*, and any antenna movement of approximately 45^{o} or greater was marked as *Antenna Wiggle*. *Fluttering* and *Antenna Wiggling* were recorded as instances (single movements at a time) while all other behaviors were recorded as durations, or measures of time a given behavior was observed. Additionally, overall colony activity was observed and recorded as "High" (marked movements from all or most of the colony), "Moderate" (movement from approximately half of the colony), or "Low" (little to no movement from the colony). Weather, documented as sunny, partly cloudy, cloudy, or rainy, and additional factors, such as movement from another person in proximity to the colony cage, were also recorded.

Statistical Analyses:

I conducted statistical analyses using R (ver. 3.4.1, "single candle" within Rstudio) except for my generalized linear mixed models on sex and time of day on activity, and my χ^2 analysis of colony behavior by time category, for which I used JMP (ver. 13 Pro). I compared behaviors across days to assess whether *H. hewitsoni* colony behavior changed over the duration of the experiment, independent of time of day, using repeated measures analysis. To determine if courtship was circadian in *H. hewitsoni*, I first divided the overall recorded time into four categories, morning (7:20-10:20), noon (10:40-13:20), afternoon (13:40-16:20), and evening (16:40-19:20) to make slight changes in activity more noticeable between periods of time. I then used repeated measures analysis to determine if time of day influenced courtship abundance and duration, when considering weather a random effect in my model. I used Pearsons χ^2 on colony activity level to determine if colony activity matched individual activity across time category. I used Principal Component Analysis (PCA) on my behavioral data to assess correlations between behaviors and define composite behaviors for further analysis. I included *copulation* and *lifted abdomen* durations in these calculations, however due to low sample size in both ($n = 2$ and $n = 3$, respectively) they did not contribute to overall structure of my principal components. I then used repeated measures analysis to assess the effect of time of day, with weather as a random effect, on these new composite variables (principal components). I used full factorial generalized linear mixed models to determine if there was an effect of sex or an interaction between sex and time of day on my behaviors. With Bonferroni correction for multiple testing, my significance threshold was $p = 0.005$ for all behavioral comparisons.

Ethics Statement:

All *H. hewitsoni* were maintained in laboratory conditions as specified by U.S. Department of Agriculture Animal and Plant Health Inspection Service permit P526P-17-00343. All butterflies used in these focal watches were kept in a large colony cage within a climate controlled, walk-in chamber. All butterflies were provided with ample food until natural death.

Results

Courtship is Circadian:

I found very small effects (R^2 ranging from 0.00079 to 0.026) of date on behavior that are statistically significant due to my large sample size (LGM n = 1665; see Supplementary Table 1), but unlikely to be biologically significant, thus data for all days are pooled for the remaining analyses. I found that courtship occurs most often at noon (RM F = 5.174, p = 0.001), but not in any specific 20-minute window during the broader noon time period (RM F = 1.623, p = 0.0117; Figure 2). I did not find a significant effect of time category on courtship duration (RM F = 2.998, p = 0.0297), however I did find a significant time point for long courtship, with peak courtship duration occurring around 12:40 (RM F =2.406, p = <0.001; Figure 2).

Diurnal Circadian Rhythm:

I found circadian activity in both behavioral instances (*fluttering* and *antenna wiggling*), and in four behavioral durations, including *resting*, *basking*, *flying* and *walking*. *H. hewitsoni* flutter most during the morning and noon time categories (RM F = 13.373, p = <0.0001), while *antenna*

wiggling occurs most often during the morning (RM F= 4.730, p = 0.0027; Figure 3). Resting duration in *H. hewitsoni* peaks in the evening (RM F = 16.899, p = <0.0001), while basking duration is highest during the morning (RM F = 13.909, p = <0.0001; Figure 4). *H. hewitsoni* did not vary in sitting near each other in any time category (RM F = 3.733, p = 0.0109; Figure 4). Flight duration peaked in the morning (RM F = 15.067, p = <0.0001), as did walking duration (RM F = 8.183, p = <0.0001; Figure 4). Due to low sample size in copulation events (n = 2) and *abdomen lifting* (n = 3), I was unable to calculate the effect of time category on these behaviors. Average behaviors across time category and time point are listed in Supplementary Table 2 and Supplementary Table 3, respectively. Colony activity levels mirrored individual activity levels for all time categories, in that activity was highest in the morning and decreased throughout the day (n = 1278, Pearson χ^2 = 384.474, p = <0.000; Figure 5).

Principal Component Analysis:

In my study, the first three principal components of my principal component analysis account for 50% of total recorded behavioral variance (see Supplementary Table 4). Principal component one (PC1) is comprised primarily of positive fluttering, walking and flying, and negative amounts of resting, or "high energy movements" (so called for increased metabolic output (Fritzsche McKay et al., 2016)), and explains 26% of the behavioral variance observed. Principal component two (PC2) is composed primarily of positive resting, fluttering, and walking, and negative amounts of basking, or "closed wing movements", and explains 13% of behavioral variance observed. Finally, principal component three (PC3) is comprised primarily of positive courting and sitting near, or "courting movements", and explains an additional 11% of observed variance.

Composite Behaviors and Circadian Rhythm:

I found that time category with weather as a random effect had a significant effect on all of my composite behaviors. High energy movements (PC1) lasted the longest during the morning time category (RM F = 18.553, p =<0.0001; Figure 6). Closed wing movements (PC2) lasted the longest during the afternoon (RM F = 6.136, p = 0.0004; Figure 6). Courting movements (PC3) did not change across time category, though this may be because PC3 contained courting and sitting near, two behaviors that did not have synchronous circadian rhythms (RM F = 3.760, p = 0.0105; Figure 6).

Differences in Activity Between Sexes:

I found that males and females exhibited behavioral differences. My data show that females performed fluttering (LGM F = 31.9657, p = <0.0001) and antenna wiggling (LGM F = 9.6701, p = 0.0019), more often than males. Male resting duration was longer than that of females (LGM F = 17.4628, p = <0.0001), however females basked longer than males (LGM F = 24.7056, p = <.0001). Males also sat next to other butterflies longer than females did (LGM F= 34.5624, p <0.0001). Time spent flying was not different between the sexes (LGM F = 0.0227, p = 0.8803), nor did I record a difference in walking duration between the sexes (LGM F = 4.4726, p = 0.0346). In a Principal Components Analysis on behavior by sex, PC1 and PC2 for both sexes were comprised of the same behaviors, but PC3 in females was primarily comprised of only sitting near with negative amounts of copulating. Slightly more of the total behavioral variance was explained when behaviors were assessed by sex than when both sexes were calculated together (56% in each sex instead of 50% together). I compared male and female composite

behaviors using the combined PCA. High energy movement duration was longer in females than in males (LGM F = 29.4849, p = <0.0001). There was no difference in closed wing movement duration (LGM F = 0.8453, p = 0.3580) or courting movement duration (LGM F = 7.2912, p = 0.0070) between the sexes (see Supplementary Table 5). There was no interaction effect of sex with time category on any behavior (see Supplementary Table 6).

Discussion

My results suggest that *H. hewitsoni* have circadian courtship patterns. Peak courting occurs around solar noon, which is between 12:30 and 1:30 in May in Arkansas. Additionally, I found that fluttering, antenna wiggling, resting, basking, flying, and walking are also circadian in these butterflies. The amount of fluttering and antenna wiggling, and duration of resting, basking, and sitting near, and high energy movement were found to be sexually dimorphic. I also found that overall colony activity level matched the activity level seen in my focal butterflies, with activity being highest in both during the morning and decreasing throughout the day.

My results supported the hypothesis that courtship is circadian in *H. hewitsoni*. My study was conducted in a laboratory setting in the absence of predators, therefore results from field studies may differ, however circadian behavior patterns tend to manifest in both laboratory and field conditions (Bloch et al., 2013; Edery, 2000; Lakin-Thomas & Brody, 2004; McClung, 2006). Circadian courtship that occurs around noon has also been documented in other butterflies, such as in three species of sulfurs (*Colias philodice*, *C. eurytheme*, and *Eurema hecabe*), while owl butterflies (*Caligo* and *Opsiphanes* species) and the squinting bush brown (*Bicyclus anynana*)

court at dawn and dusk, and the New Zealand leafroller moth (*Cnephasia jactatana*) experiences circadian courtship during the second half of the night (Jiménez-Pérez, Wang, & Markwick, 2002; Marshall, 1982; Rutowski & Kemp, 2017; Srygley, 1994). Diurnal versus crepuscular and nocturnal behavioral patterns appear to account for the difference in peak courtship times between these species of lepidoptera. Peak courtship activity is also influenced by other biological processes, such as development and reproduction. Similar to *Drosophila*, female sulfur butterflies eclose in the morning, meaning that their wings are not dry enough for flight yet (Marshall, 1982; Rutowski & Kemp, 2017). While male sulfurs will court females during this time, females are more easily found in the afternoon, the time period that corresponds to peak courtship rates and duration in these butterflies (Marshall, 1982; Rutowski & Kemp, 2017). Similarly, my study showed peak *H. hewitsoni* courtship to be around midday. This would give female *H. hewitsoni* (that also eclose in the morning) the opportunity to unfurl and dry their wings, and assume flight, thus making them more conspicuous to mate seeking males. It is of note that *H. hewitsoni*, and some other *Heliconius* species such as *H. charithonia and H. sara*, are thought to use pupal mating, meaning that males search larval host plants for female pupa, then mate with them as soon as they eclose (Beltran, Jiggins, Brower, Bermingham, & Mallet, 2007; Gilbert, 1991). Increased courtship activity around noon when newly emerged and dried females could be flying may be an alternate mating strategy for male *H. hewitsoni*. Alternatively, there could be some effect of female age on courtship after eclosion. Future research should determine how likely it is for a male to find a female pupa, and if time from female eclosion affects male courtship activity. Circadian rhythmicity in courtship could also increase potential reproductive output for female lepidoptera. Female New Zealand leafrollers oviposit early in the night, then will accept mating with courting males closer to the end of the night (Jiménez-Pérez

et al., 2002). Future research should determine if oviposition is also circadian in *H. hewitsoni*, and if there is a temporal link between oviposition and copulation events.

In addition to courtship, my research also demonstrated that high energy movements (which included walking and flying) occurred most during the morning and decreased throughout the day. This matches the diurnal pattern seen in cucurbit plants while being somewhat offset from peak activity times for tropical birds (Gilbert, 1975; James Mallet & Gilbert, 1995; D. A. Murawski & Gilbert, 1986; Darlyne A. Murawski, 1987; New, 2017). Cucurbit pollen is a prominent component of the adult *Heliconius* diet, and these butterflies are key to pollen transfer between plants (Gilbert, 1975; D. A. Murawski & Gilbert, 1986; Darlyne A. Murawski, 1987; New, 2017). Both species are diurnal, which facilitates the interaction of plant and pollinator (D. A. Murawski & Gilbert, 1986; Darlyne A. Murawski, 1987; New, 2017). Conversely, many *Heliconius* predators, such as jacamars (*Galbula* species) and flycatchers (Tyrannidae), are crepuscular (James Mallet & Gilbert, 1995), which means that my study found an overlap of high activity around dawn between these butterflies and their predators. This may be evidence of trade-off between peak pollen access and peak predation thereat for these butterflies, or it may be a trade-off between thermoregulation needs and peak predation threat. *Heliconius* are ectothermic and leave their roosts to bask in the morning, meaning that while they are active they are also slower than they would be later in the day, making them easier prey for birds (James Mallet & Gilbert, 1995). My study supports this observation by Mallet and Gilbert (1995) as my observed peak basking time was also during the morning. This may be the time period most responsible for birds learning that these butterflies are unpalatable. *Heliconius* adults roost gregariously to deter predators, and the most successful roosts for individual fitness are

comprised of approximately five individuals (Finkbeiner et al., 2012). When experimental roosts were comprised of ten individuals, signs of predation (beak marks in clay models) increased threefold. This was thought to be due to naïve predators spotting aggregations that were too conspicuous (Finkbeiner et al., 2012). Future research should determine whether peak pollen production or thermoregulation is most responsible for high activity during a time that also risks a high chance of predation.

My study shows that peak courtship coincides only with peak fluttering, which may be an artifact of courtship itself. Peak high energy movement duration occurs during the morning, not at noon. My results are different from what has been shown in the butterfly, *Bicyclus anynana*, and the fruit fly (*Drosophila melanogaster)*, both of which experience peak activity along with peak courtship (Bear & Monteiro, 2013; Bear, Prudic, & Monteiro, 2017; De, Varma, Saha, Sheeba, & Sharma, 2013; Westerman, Drucker, & Monteiro, 2014). *B. anynana* are crepuscular, and courtship takes place most often during dawn and dusk (Bear & Monteiro, 2013; Bear et al., 2017; Westerman et al., 2014). *B. anynana* are native to grasslands in Africa, while *H. hewitsoni* are native to the tropical rainforests of Central America(Brakefield & Reitsma, 1991; Longino, 1984). Research has shown that flight increases heat production in butterflies, and that high intensity light causes more heat production than low intensity light (Liao et al., 2017). Therefore, *B. anynana* might match peak courtship with peak activity to avoid becoming too hot during periods when light intensity is at its highest, while *H. hewitsoni* does not experience the same light intensity due to shade provided by trees (Endler, 1993). Future research should examine possible correlations between light intensity and circadian rhythms in butterflies. In *Drosophila melanogaster*, peak courtship is the defining behavior of morning peak activity (De et al., 2013).

My analyses showed that courtship was not one of the defining behaviors of high energy movements in *H. hewitsoni*.

Conclusions

Here I show that courtship, both occurrence and duration, is circadian, and occurs most often around solar noon in *H. hewitsoni*. Furthermore, I show that the other behaviors I recorded experience varying peak activity times throughout the day, such as basking occurring most often in the morning while resting occurs most often in the evening. My results, when taken in concert with other studies, further demonstrate that different species of butterfly court at different times of day. Additionally, my findings support the hypothesis that circadian rhythms are often synchronized with factors that increase survival, such as predator avoidance and increased foraging success.

Figures

Figure 1: *Heliconius hewitsoni.* A) Dorsal view; B) Ventral view.

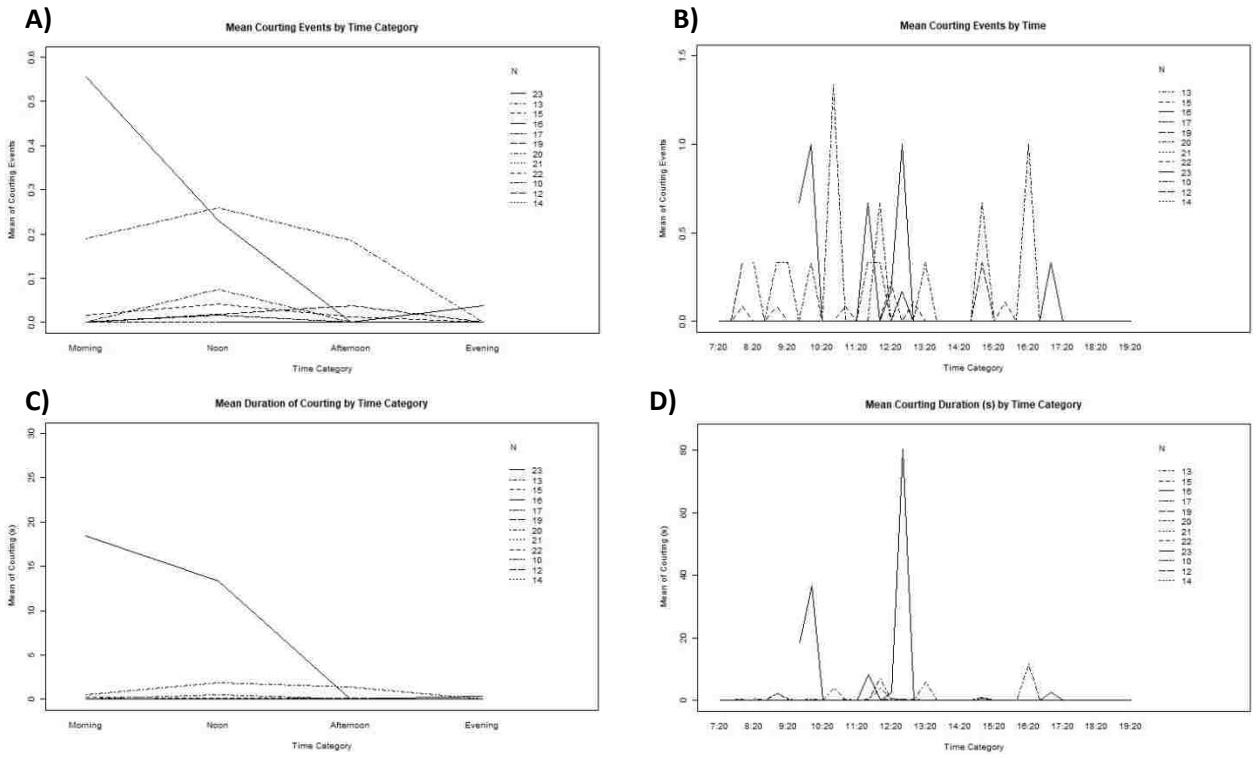
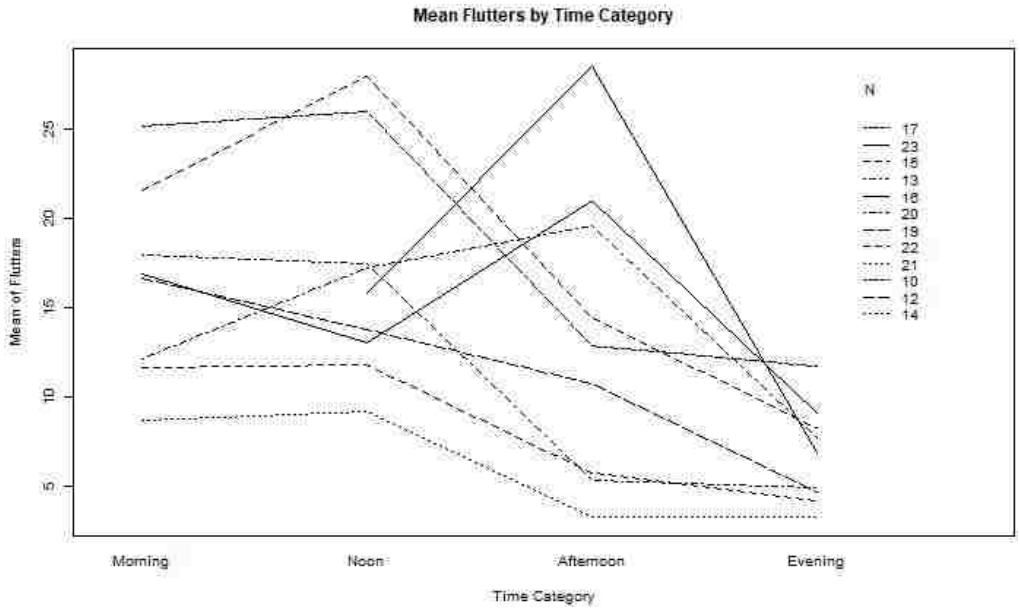


Figure 2: *Heliconius hewitsoni* experience circadian courtship. Courtship instances occur most during the noon time category (RM F = 5.174, p = 0.001), but not during any 20-minute window around the noon time period (RM F = 1.623, p = 0.0117). I did not see a difference in courtship duration peaks by time category (RM F = 2.998, p = 0.0297), however courtship duration was significant around the 12:40 time point (RM F =2.406, p = <0.001). A) Courtship instances by time category; B) Courtship instances by time; C) Courtship duration by time category; D) Courtship duration by time. N = total number of butterflies in the colony at that time.

A)



B)

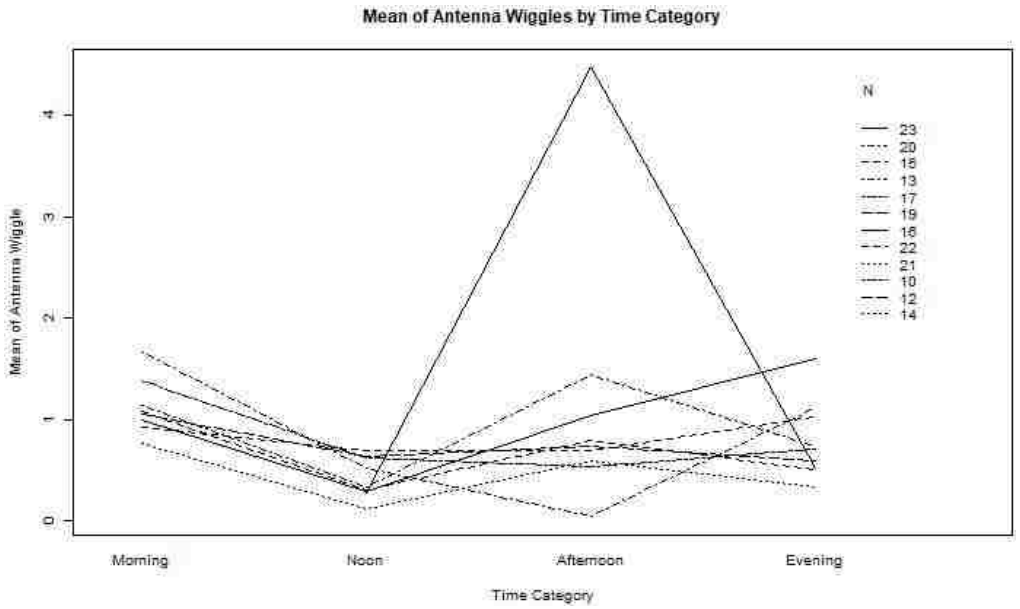


Figure 3: Times of peak activity for behavioral instances, *Fluttering* (A) and *Antenna Wiggling* (B). *Fluttering* takes place most often in the morning and at noon (RM F = 13.373, p = <0.0001). *Antenna Wiggling* occurs most during the morning (RM F= 4.730, p = 0.0027). N = total number of butterflies in the colony at that time.

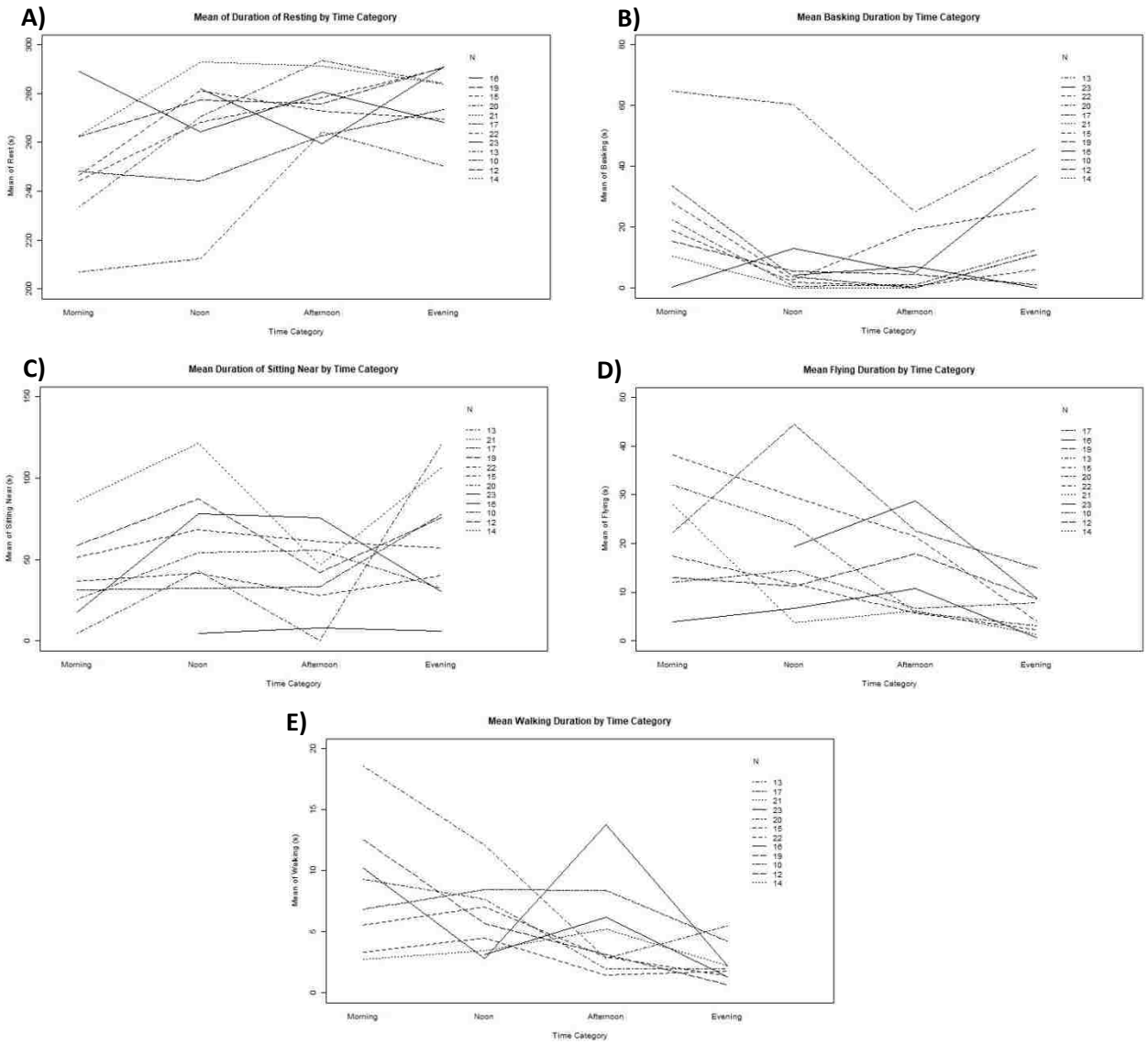


Figure 4: Time of peak duration for resting (A), basking (B), sitting near (C), flying (D) and walking (E). Resting duration is highest in the evening (RM F = 16.899, p = <0.0001). Basking duration is highest during the morning (RM F = 13.909, p = <0.0001). *Heliconius hewitsoni* do not sit near each other significantly differently in any time category (RM F = 3.733, p = 0.0109). Flight duration (RM F = 15.067, p = <0.0001) and walking duration (RM F = 8.183, p = <0.0001) are longest in the morning. N = total number of butterflies in the colony at that time.

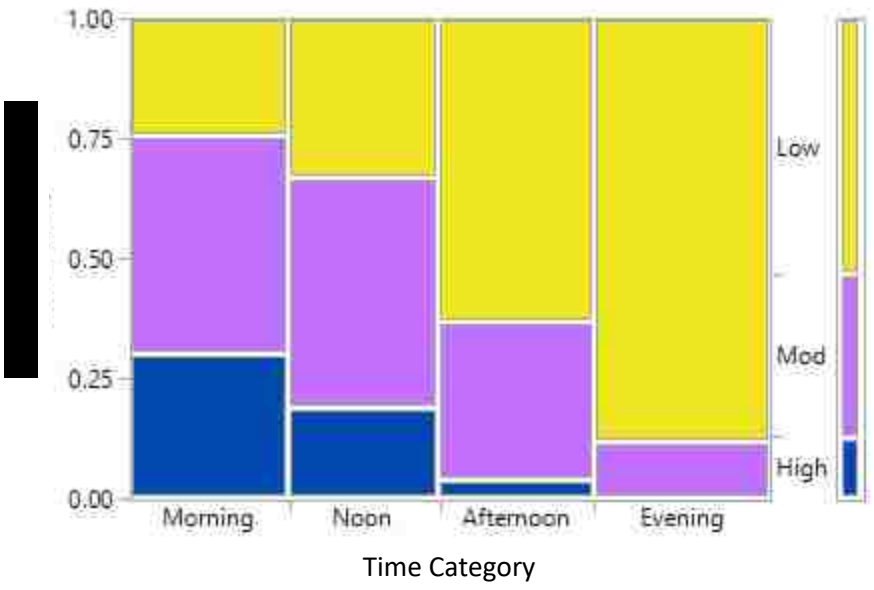


Figure 5: Mosaic plot of contingency analysis of colony activity by time category. Colony activity levels mirrored observed individual activity levels, with butterflies being most active in the morning and least active in the evening (n = 1278, Pearson χ^2 = 384.474, p = <0.0001).

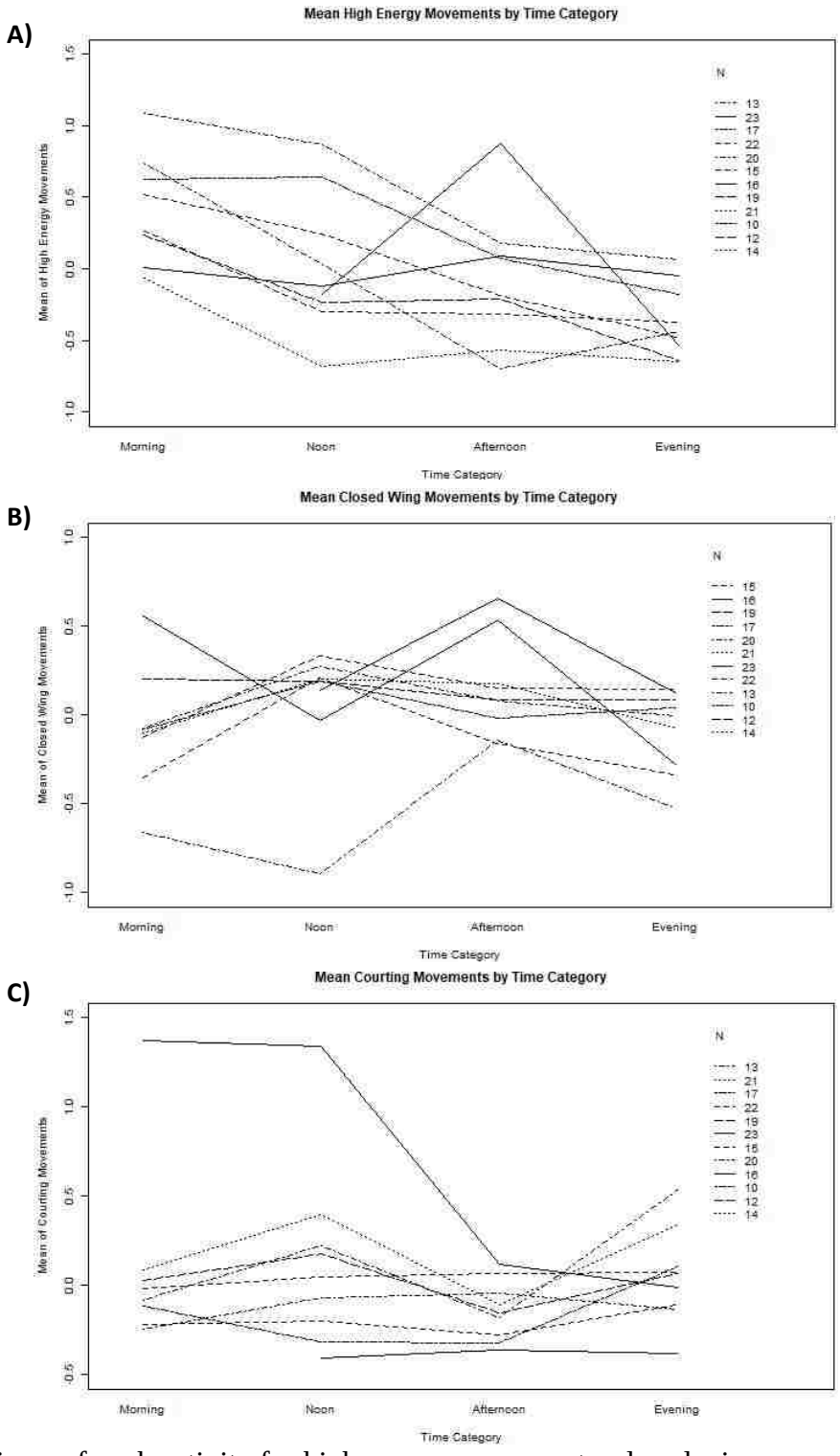


Figure 6: Times of peak activity for high energy movements, closed wing movements, and courting movements. High energy movements (A) have the longest duration during the morning (RM F = 18.553, p =<0.0001), while closed wing movements (B) have the longest duration during the afternoon (RM F = 6.136, p = 0.0004). Courting movements (C) did not vary with time category (RM F = 3.760, p = 0.0105). N = total number of butterflies in the colony at that time.

Conclusions

The studies described in this thesis demonstrated that *B. anynana* do not innately prefer or learn to prefer unrelated or unfamiliar males over sibling or familiar males based on larval experience, that male harassment does not influence female mate choice in *B. anynana*, that *H. hewitsoni* exhibits circadian rhythms, including a midday preference for courtship, and that behavior in *H. hewitsoni* is sexually dimorphic. Regarding the first study, my results suggest that kin recognition is not the mechanism behind avoiding inbreeding depression, however other work has suggested that lower male sex pheromone production in inbred males over outbred males may be sufficient to deter mate choice for inbred males (van Bergen et al., 2013). Therefore, kin recognition is not the only method by which inbreeding depression is avoided. Furthermore, I was able to demonstrate that female choice influences mate selection more than male harassment in conditions that are closer to natural densities and provide ample opportunity for the female to escape. The results from my second study provide valuable information for future research in this system, and hint at a circadian rhythm that may be related to either thermoregulation or foraging success.

We did not find evidence of larval learning that affected adult mate choice in *B. anynana*, but that does not mean that *B. anynana* do not learn other cues as larva, such as what might exist for optimal host plants or larval densities that threaten cannibalism, that have an effect on adult behavior. Future research that particularly interests me is the notion of cuticular hydrocarbons and their effect on kin recognition in insects (Elgar & Allan, 2004; Liang & Silverman, 2000; Thomas et al., 1999). Many laboratory settings that work with *B. anynana* rear their larva solely on corn plants (Heuskin et al., 2014; Prudic et al., 2011; van Oosterhout, Smit, van Heuven, &

Brakefield, 2000; Westerman et al., 2014), including our own. However, if diet effects the cuticular hydrocarbon output in other insect species, it is reasonable to hypothesize that it could also affect the cuticular hydrocarbons of *B. anynana*. This means that a potentially important factor that could affect behavior could be getting masked in laboratory populations. I feel this bares examination, and I hope to one day have the answer, either through continued work in this system by myself or by colleagues.

If I were to continue in the *Heliconius* system, I would want to explore the sexual dimorphism we found in overall adult behavior in greater detail. Specifically, I am interested in the total metabolic output in males versus females, and whether courtship requires a significant enough energy expenditure to explain the behavioral dimorphism I observed, as females tended toward higher amounts of activity in most other behaviors, such as fluttering and high energy movements. This would also give me an opportunity to expand my experimental knowledge as I have never performed experiments in energy expenditure.

The two studies presented here, though in different butterfly species, highlight both how far we have come in our research in mate selection and how far we still have to go. For every question we answer, it seems that two more take its place. Future research will hopefully explore potential juvenile learning and sexually dimorphic behavior more thoroughly, which will help us place pieces in the puzzles that are mate choice and behavior.

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Appendix

Chapter One: Supplementary Materials

Supplemental Table 1: *A priori* power analysis for mate choice assays. I calculated that I could detect female preferences of ~72:26 (~23% different from 50:50).

Treatment	**N**	**Power**	**Effect to Detect**
Unfamiliar Related/Unfamiliar Unrelated	30	0.8	0.51
Familiar Related/Unfamiliar Unrelated	30	0.8	0.51
Familiar Related/Unfamiliar Related	30	0.8	0.51

Supplemental Table 2: *A priori* power analysis for male and female behavior. Given my sample size, I can detect small effects of (A) male behavior on female mate choice, and (B) rearing condition on behavior in females (B).

A)

Behavior	**Est Std Dev**	**N**	**Power**	**Effect to Detect**
PC	1.5	180	0.8	0.63
Number of Courts	1.5	180	0.8	0.63
Time Courting	60	180	0.8	25 s

B)

Behavior	**Est Std Dev**	**N**	**Power**	**Effect to Detect**
PC	1.5	90	0.8	0.9

Supplemental Table 3: *Post hoc* power analysis of mate choice data to calculate effective sizes for detecting statistically significant effect of relatedness and familiarity.

Treatment	**Power**	**Observed Effect**	**N**
Unfamiliar Related/Unfamiliar Unrelated	0.8	0	N/A
Familiar Related/Unfamiliar Unrelated	0.8	0.1	787
Familiar Related/Unfamiliar Related	0.8	0.2	191

Supplemental Table 4: *Post hoc* power analysis for male behavior. I calculated that I would need very large sample sizes to detect the very small effects of male behavior on female mate choice that I observed.

Behavior	**Chosen Avg**	**Non Avg**	**Std Dev**	**Power**	**Observed Effect**	**N**
PC1	0.22	-0.22	1.73	0.8	0.46	446
PC2	-0.002	0.002	1.10	0.8	0.004	2374283
PC3	-0.11	0.11	1.05	0.8	0.22	787
Number of Courts	0.4	0.59	1.23	0.8	0.19	1318
Time Courting	12.46	18.95	55.16	0.8	6.5 s	2263

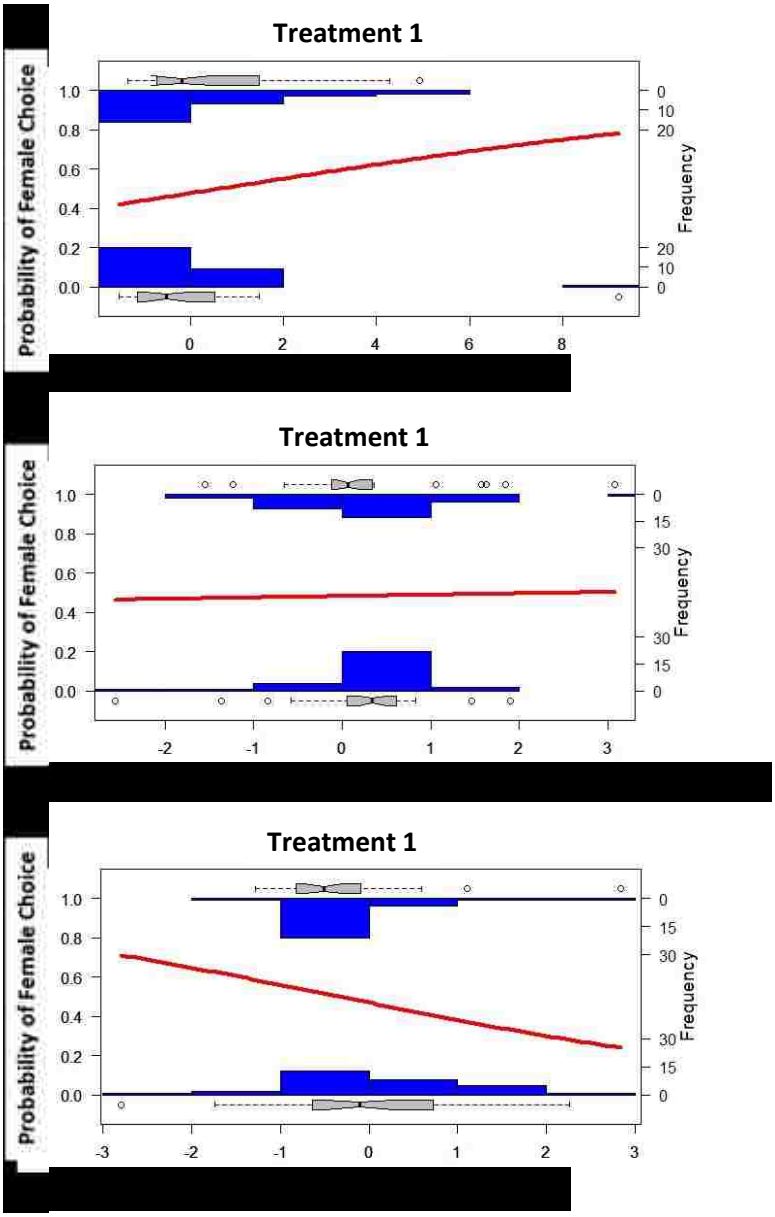
Supplemental Table 5: Principal component loadings for the principal component analysis on male behavior.

	Comp.1	Comp.2	Comp.3	Comp.4	Comp.5	Comp.6	Comp.7	Comp.8
Flutter	0.253109	-0.11229	-0.03794	0.039545	-0.2528	0.708848	13.55391	-9.75782
Antenna Wiggle	0.2526	-0.18875	0.074995	0.03967	-0.29236	0.333905	7.405895	11.43593
Resting Duration	0.039678	0.17164	1.592366	-0.04881	0.631084	1.801493	-0.18364	-0.00979
Courting Duration	0.056671	0.668188	-0.84959	0.030063	0.243725	2.579967	0.769495	1.432103
Basking Duration	-0.0013	-0.23598	-0.26544	0.578892	1.010916	-0.0174	1.101756	0.111443
Walking Duration	0.246184	-0.10229	-0.06396	0.047498	-0.12884	0.391565	-22.7138	-2.0369
Flying Duration	0.166494	0.372592	-0.00883	-0.14745	0.717887	-3.56994	1.499858	-0.00245
Sitting Near Duration	-0.01344	0.426892	0.558393	0.460589	-0.92961	-1.22844	-0.43349	-0.17251
Percentage Explained	**37%**	**15%**	**14%**	**13%**	**11%**	**6%**	**3%**	**1%**

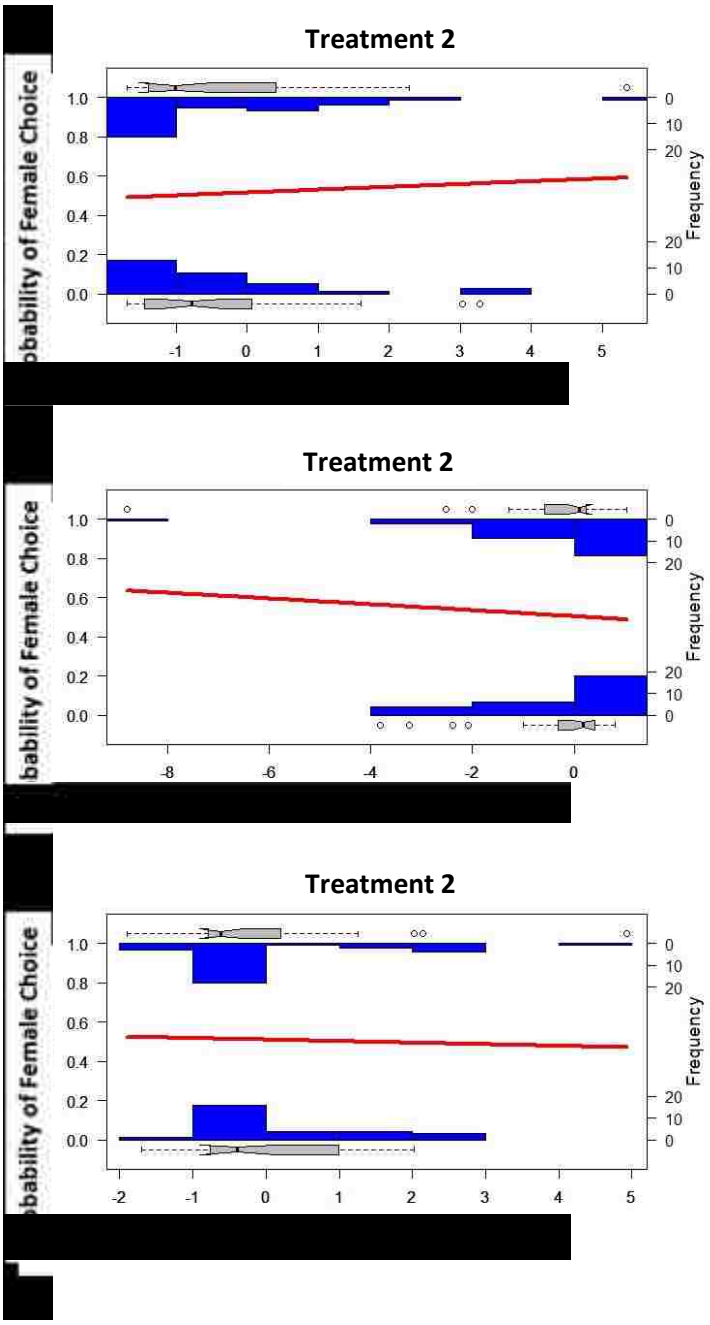
Supplementary Table 6: Principal component loadings for the principal component analysis on female behavior.

	Comp.1	Comp.2	Comp.3	Comp.4	Comp.5	Comp.6	Comp.7
Flutter	0.202864	0.195706	-0.18638	-0.14537	-4.11821	6.998478	0.976524
Antenna Wiggle	0.252854	-0.00091	0.107094	-0.0333	0.194784	-10.5345	-4.59716
Resting Duration	-0.05692	0.404731	1.359731	0.141952	-0.20061	-6.90494	0.278317
Basking Duration	0.146954	-0.16658	0.334876	1.330948	0.384143	13.6879	0.395933
Walking Duration	0.239516	-0.09213	-0.0142	-0.01283	1.003903	-22.1686	3.290709
Flying Duration	0.198578	0.106686	0.285234	-0.77831	2.288667	20.98052	0.675331
Sitting Near Duration	0.016157	0.552496	-0.88635	0.496902	1.447317	-1.05881	-0.01965
Percentage Explained	**47%**	**16%**	**13%**	**12%**	**6%**	**4%**	**1%**

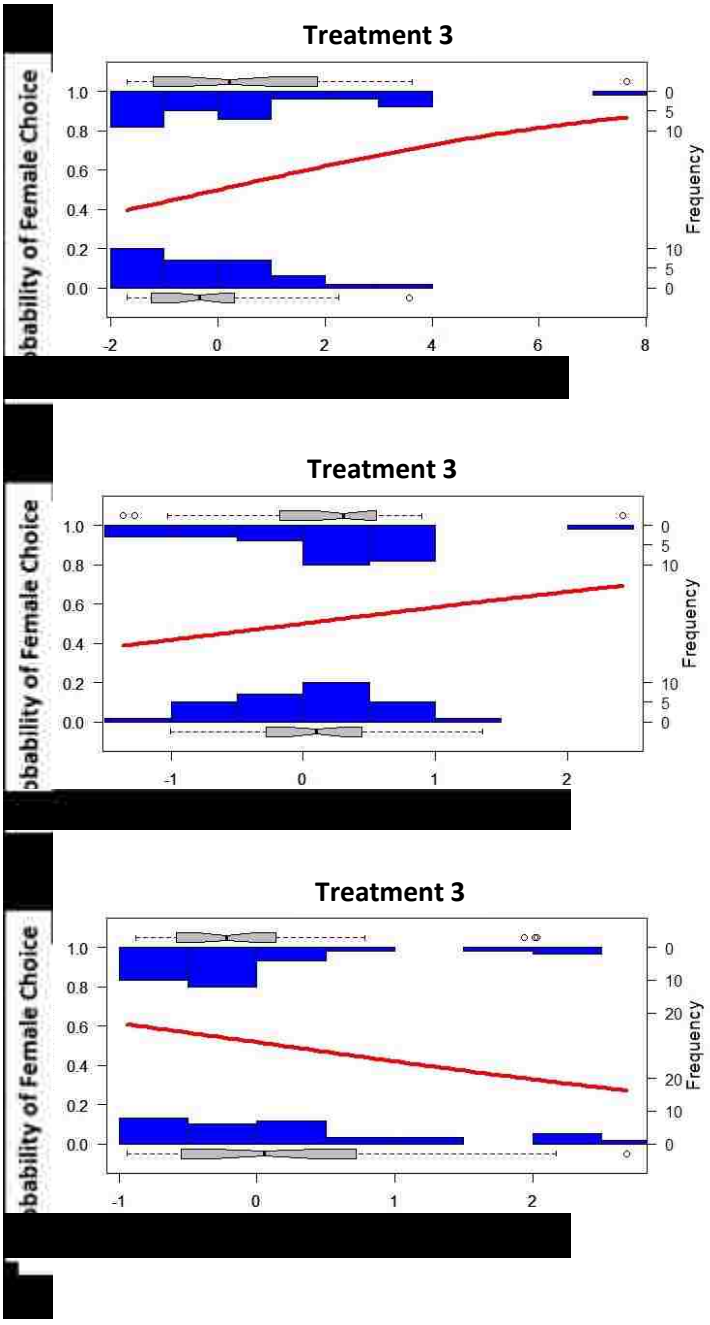
Supplemental Figure 1: Composite behaviors had no effect on female choice in Treatment 1 (PC1 n = 59, z = -1.519, p = 0.129; PC2 n = 59, z = 0.833 p = 0.405; PC3 n = 59, z = 1.289, p = 0.197).



Supplemental Figure 2: Composite behaviors had no effect on female choice in Treatment 2 (PC1 n= 57, z = -0.312, p = 0.755; PC2 n = 57, z = -0.342, p = 0.732; PC3 n = 57, z = 0.149, p = 0.882).



Supplemental Figure 3: Composite behaviors had no effect on female choice in Treatment 2 (PC1 n = 58, z = -0.936, p = 0.349; PC2 n = 58, z = 0.090, p = 0.928; PC3 n = 58, z = 1.221, p = 0.222).



Chapter Two: Supplementary Materials

Supplementary Table 1: Linear regression totals for all behaviors by date.

Behavior	**r^2 =**	**p =**
Fluttering	0.008886	0.0001
Antenna Wiggling	0.000789	0.2492
Resting	0.003711	0.0129
Basking	0.025722	<0.0001
Walking	0.002759	0.0321
Flying	0.02169	<0.0001
Sitting Near	0.002686	0.0345
Courtship	0.008288	0.0002
Copulation	N/A	N/A
Lift Abdomen	N/A	N/A

Supplemental Table 2: Averages for each behavior across time category. Flutters and antenna wiggling are instances; all other behaviors are in seconds.

Time Category	Flutter Avg.	Antenna Wiggle Avg.	Rest Avg.	Bask Avg.	Walk Avg.
Morning	16.35777778	1.117777778	247.4488889	25.08444444	8.226666667
Noon	17.18024691	0.44691358	267.2938272	7.696296296	6.286419753
Afternoon	12.78024691	0.995061728	274.7802469	7.474074074	5.222222222
Evening	6.491358025	0.730864198	277.6790123	16.5037037	2.145679012

Time Category	Fly Avg.	Courting Avg.	Copulate Avg.	Sitting Near Avg.	Lifted Abdomen Avg.
Morning	21.32889	0.468888889	1.326666667	43.76222222	0
Noon	19.19259	1.491358025	0	61.00493827	0.054320988
Afternoon	14.02222	0.10617284	1.479012346	43.32345679	0.041975309
Evening	5.797531	0.019753086	0	60.60987654	0

Supplemental Table 3: Averages for each behavior across time. Flutters and antenna wiggling are instances; all other behaviors are in seconds.

Time:	Flutter Average:	Time:	Antenna Wiggle Average:	Time:	Rest Average:
7:20	20.68888889	7:20	1.6	7:20	209.1555556
7:40	15.73333333	7:40	1.4	7:40	223.5333333
8:00	16.4	8:00	1.4	8:00	237.8888889
8:20	14.68888889	8:20	0.777777778	8:20	254.2444444
8:40	11.75555556	8:40	1.066666667	8:40	252.8222222
9:00	24.86666667	9:00	1.8	9:00	256.0444444
9:20	13.91111111	9:20	0.955555556	9:20	253.4222222
9:40	17.17777778	9:40	0.755555556	9:40	255.6666667
10:00	13.88888889	10:00	0.533333333	10:00	260.0888889
10:20	14.46666667	10:20	0.888888889	10:20	271.6222222
10:40	14.77777778	10:40	0.555555556	10:40	271.0666667
11:00	17.68888889	11:00	0.4	11:00	263.7555556
11:20	19.73333333	11:20	0.444444444	11:20	247.2
11:40	15.04444444	11:40	0.333333333	11:40	268.3777778
12:00	13.97777778	12:00	0.466666667	12:00	279.5777778
12:20	15.51111111	12:20	0.466666667	12:20	272.7777778
12:40	18.62222222	12:40	0.377777778	12:40	257.0222222
13:00	17.62222222	13:00	0.466666667	13:00	265.2444444
13:20	21.64444444	13:20	0.511111111	13:20	280.6222222
13:40	13.82222222	13:40	1.222222222	13:40	249.0666667
14:00	17.57777778	14:00	2.066666667	14:00	268.4
14:20	14.77777778	14:20	1.555555556	14:20	284.0888889
14:40	17.37777778	14:40	0.8	14:40	283.9111111
15:00	14.77777778	15:00	0.333333333	15:00	284.2444444
15:20	7.022222222	15:20	0.555555556	15:20	288.3111111
15:40	10.33333333	15:40	0.733333333	15:40	265.0222222
16:00	6.022222222	16:00	0.933333333	16:00	276.0444444
16:20	13.31111111	16:20	0.755555556	16:20	273.9333333
16:40	10.64444444	16:40	1.288888889	16:40	262.3555556
17:00	9.622222222	17:00	1.044444444	17:00	281
17:20	4.844444444	17:20	0.4	17:20	290
17:40	3.511111111	17:40	0.511111111	17:40	280
18:00	6.488888889	18:00	0.511111111	18:00	286.4444444
18:20	9.733333333	18:20	1.888888889	18:20	254.1777778
18:40	2.933333333	18:40	0.266666667	18:40	291.5111111
19:00	6.155555556	19:00	0.377777778	19:00	272.8222222
19:20	4.488888889	19:20	0.288888889	19:20	280.8

Supplementary Table 3 (cont.)

Time:	Bask Average:	Time:	Walk Average:	Time:	Fly Average:	Time:	Court Average:
7:20	54.77777778	7:20	15.17777778	7:20	22.75555556	7:20	0.088888889
7:40	50.8	7:40	8.911111111	7:40	12.26666667	7:40	0
8:00	31.84444444	8:00	10.91111111	8:00	22.48888889	8:00	0.066666667
8:20	10.02222222	8:20	10.6	8:20	14.68888889	8:20	0.044444444
8:40	20.04444444	8:40	4.066666667	8:40	27.44444444	8:40	0
9:00	15.51111111	9:00	15.11111111	9:00	21.2	9:00	0.755555556
9:20	21.8	9:20	2.6	9:20	22.64444444	9:20	0.022222222
9:40	23.53333333	9:40	6.688888889	9:40	21.31111111	9:40	1.244444444
10:00	14.22222222	10:00	3.222222222	10:00	27.86666667	10:00	2.466666667
10:20	8.288888889	10:20	4.977777778	10:20	20.62222222	10:20	0
10:40	0.755555556	10:40	9.533333333	10:40	19.66666667	10:40	0.266666667
11:00	11.51111111	11:00	4.933333333	11:00	21.8	11:00	0.111111111
11:20	11.82222222	11:20	7.466666667	11:20	33.75555556	11:20	0
11:40	2.888888889	11:40	9.311111111	11:40	23.93333333	11:40	0.577777778
12:00	6.688888889	12:00	2.911111111	12:00	10.77777778	12:00	0.733333333
12:20	4.177777778	12:20	4.777777778	12:20	20.88888889	12:20	0.488888889
12:40	13.28888889	12:40	6.466666667	12:40	11.11111111	12:40	10.77777778
13:00	12.02222222	13:00	5	13:00	19.4	13:00	0.066666667
13:20	6.111111111	13:20	6.177777778	13:20	11.4	13:20	0.4
13:40	16.48888889	13:40	5.222222222	13:40	13.31111111	13:40	0
14:00	12.8	14:00	4.977777778	14:00	18.33333333	14:00	0
14:20	0.222222222	14:20	4.533333333	14:20	13.82222222	14:20	0
14:40	4.866666667	14:40	4.911111111	14:40	7.066666667	14:40	0
15:00	2.533333333	15:00	3	15:00	15.31111111	15:00	0.155555556
15:20	6.133333333	15:20	2.977777778	15:20	6.511111111	15:20	0
15:40	15.77777778	15:40	9.777777778	15:40	15.2	15:40	0.022222222
16:00	3.866666667	16:00	2.666666667	16:00	20.93333333	16:00	0
16:20	4.577777778	16:20	8.933333333	16:20	15.71111111	16:20	0.777777778
16:40	25.68888889	16:40	3.733333333	16:40	10.82222222	16:40	0
17:00	4.933333333	17:00	3.288888889	17:00	10.4	17:00	0.177777778
17:20	1.466666667	17:20	1.533333333	17:20	8.622222222	17:20	0
17:40	18.84444444	17:40	0.866666667	17:40	2.066666667	17:40	0
18:00	11.53333333	18:00	2.933333333	18:00	2.244444444	18:00	0
18:20	38.55555556	18:20	4.022222222	18:20	4.066666667	18:20	0
18:40	9.488888889	18:40	0.777777778	18:40	1.377777778	18:40	0
19:00	20.24444444	19:00	1.755555556	19:00	7.8	19:00	0
19:20	17.77777778	19:20	0.4	19:20	4.777777778	19:20	0

Supplementary Table 3 (cont.)

Time:	Copulate Average:	Time:	Sit Near Average:	Time:	Lift Ab. Average:
7:20	0	7:20	31.55555556	7:20	0
7:40	0	7:40	31.33333333	7:40	0
8:00	0	8:00	22.46666667	8:00	0
8:20	13.26666667	8:20	49.48888889	8:20	0
8:40	0	8:40	59.53333333	8:40	0
9:00	0	9:00	35.86666667	9:00	0
9:20	0	9:20	37.24444444	9:20	0
9:40	0	9:40	70.97777778	9:40	0
10:00	0	10:00	45.48888889	10:00	0
10:20	0	10:20	53.66666667	10:20	0
10:40	0	10:40	40.48888889	10:40	0
11:00	0	11:00	26.84444444	11:00	0
11:20	0	11:20	61	11:20	0
11:40	0	11:40	35.55555556	11:40	0
12:00	0	12:00	69.17777778	12:00	0
12:20	0	12:20	73.31111111	12:20	0
12:40	0	12:40	77.13333333	12:40	0
13:00	0	13:00	75.62222222	13:00	0.488888889
13:20	0	13:20	89.91111111	13:20	0
13:40	13.31111111	13:40	11.66666667	13:40	0
14:00	0	14:00	48.66666667	14:00	0.022222222
14:20	0	14:20	57.17777778	14:20	0
14:40	0	14:40	71.13333333	14:40	0
15:00	0	15:00	22.8	15:00	0
15:20	0	15:20	45.82222222	15:20	0.355555556
15:40	0	15:40	21.82222222	15:40	0
16:00	0	16:00	53.75555556	16:00	0
16:20	0	16:20	57.06666667	16:20	0
16:40	0	16:40	60.75555556	16:40	0
17:00	0	17:00	52.93333333	17:00	0
17:20	0	17:20	59.71111111	17:20	0
17:40	0	17:40	76.37777778	17:40	0
18:00	0	18:00	63.91111111	18:00	0
18:20	0	18:20	71.75555556	18:20	0
18:40	0	18:40	70.46666667	18:40	0
19:00	0	19:00	60.22222222	19:00	0
19:20	0	19:20	29.35555556	19:20	0

Supplemental Table 4: Principal component loadings for the principal component analysis on behavior for both sexes.

	Comp.1	Comp.2	Comp.3	Comp.4	Comp.5	Comp.6	Comp.7	Comp.8	Comp.9	Comp.10
Flutter	0.445575	0.401691	0.055666	0.005909	0.091412	0.009982	0.007094	0.114644	0.784188	0.019327
Antenna Wiggle	0.33645	0.297132	0.017358	0.193553	0.133502	0.367796	-0.19378	-0.70854	-0.2609	-0.00299
Rest Duration	-0.53118	0.405461	-0.04391	0.079027	0.065141	0.040613	-0.06836	-0.04857	0.114013	-0.72066
Courtship Duration	0.071267	0.023948	0.682714	-0.17454	0.108475	-0.43005	-0.53381	-0.00698	-0.09929	-0.07882
Copulation Duration	0.041808	-0.25383	-0.09982	-0.61287	0.688196	0.206661	0.055354	-0.05089	0.045887	-0.15546
Sitting Near Duration	-0.1082	0.024835	0.673708	-0.02655	-0.03461	0.1966	0.689888	-0.13055	0.015423	0.003505
Basking Duration	0.304266	-0.59234	0.149193	0.284914	-0.16627	0.342907	-0.14942	0.119692	0.129105	-0.50455
Flying Duration	0.360771	-0.06804	-0.20452	-0.23738	-0.25506	-0.56405	0.337342	-0.336	-0.0612	-0.39164
Walking Duration	0.405927	0.379399	0.007489	0.007227	0.113618	0.096355	0.143446	0.578891	-0.52093	-0.20474
Lift Abdomen Duration	0.00846	-0.14456	-0.02409	0.641796	0.614318	-0.38942	0.192507	-0.02122	0.000795	0.001155
Percentage Explained	**26%**	**13%**	**11%**	**10%**	**10%**	**9%**	**9%**	**7%**	**4%**	**<1%**

Supplementary Table 5: Differences between behavioral output between the two sexes as calculated by generalized linear mixed models.

Behavior	**Female Least Square Mean**	**Male Least Square Mean**	**Mean Standard Deviation**	**F =**	**p =**
Fluttering	17.39	9.88	0.94	31.97	<0.0001
Antenna Wiggling	1.06	0.64	0.10	9.67	0.0019
Resting	258.90	273.10	2.40	17.46	<0.0001
Basking	20.66	9.04	1.65	24.71	<0.0001
Walking	6.53	4.63	0.64	4.47	0.0346
Flying	14.92	15.19	1.28	0.02	0.8803
Sitting Near	35.45	65.45	3.60	34.56	<0.0001
High Energy Movements	0.23	-0.20	0.06	29.48	<0.0001
Closed Wing Movements	-0.03	0.03	0.04	0.85	0.358
Courting Movements	-0.08	0.06	0.04	7.29	0.007

Supplementary Table 6: Differences between behavioral output between the interaction of sexes and time category as calculated by generalized linear mixed models.

Behavior	**Sex F=**	**Sex p =**	**Time Category F =**	**Time Category p =**	**Sex X Time Category F =**	**Sex X Time Category p =**
Fluttering	31.97	<0.0001	14.11	<0.0001	1.64	0.178
Antenna Wiggling	9.67	0.0019	5.42	0.001	1.62	0.1822
Resting	17.46	<0.0001	18.01	<0.0001	2.32	0.074
Basking	24.71	<0.0001	13.91	<0.0001	0.67	0.5702
Walking	4.47	0.0346	8.35	<0.0001	0.68	0.5648
Flying	0.02	0.8803	14.98	<0.0001	1.41	0.2383
Sitting Near	34.56	<0.0001	4.06	0.0069	0.31	0.8173
High Energy Movements	29.48	<0.0001	19.86	<0.0001	2.21	0.0846
Closed Wing Movements	0.85	0.358	6.03	0.0004	0.13	0.9398
Courting Movements	7.29	0.007	3.69	0.0116	0.44	0.7278