

AGE EFFECTS ON REPRODUCTIVE BEHAVIOR IN THE
TREEHOPPER *UMBONIA CRASSICORNIS* (HEMIPTERA: MEMBRACIDAE)

A Dissertation presented to the Faculty of the Graduate School
University of Missouri-Columbia

In Partial Fulfillment of the Requirements for the Degree
Doctor of Philosophy

by

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DECEMBER 2007

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TREEHOPPER *UMBONIA CRASSICORNIS* (HEMIPTERA: MEMBRACIDAE)

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..... Thanks for everything, Mom and Dad.

ACKNOWLEDGMENTS

It became quite apparent to me not long after I started at Missouri that this was going to be a team effort involving a large number of people. I can say with all honesty that I would not have survived these past 5 ½ years if it was not for the support and encouragement of many genuinely caring individuals. And so, without further a due, I would like to extend my heartfelt thanks to the following souls:

First and foremost, to my advisor Rex. He took a chance on me not long after I had spent the previous 2 years living on a beach (more or less). I owe much to his faith in my abilities, especially when I came close to packing it in. I would also like to thank each of my committee members, Carl, Johannes, Ray and Mark. Their advice and criticisms kept me honest, and each of them always challenged me to become a better scientist. I will never forget it.

The Division of Biological Sciences here at Mizzou is quite simply the best department I have ever been affiliated with. I appreciate all the help I received from every office in Tucker Hall and beyond. The staff was always there to answer any question I had, and they never turned me away. Pat, Amy, Robin, Julie, and Johnette in 105, Tyecee in 107, the computer gurus Alan, Josh, and Matt, the boys in the workshop, Barb out at the greenhouse, and finally Nila, who made sure I had a winter coat when I visited during recruitment 2002; I thank you all from the bottom of my heart.

The camaraderie among the EEB graduate students and post-docs was a constant source of comfort and support for me. The laughter (and alcohol) I shared with so many people will not be forgotten. In particular I would like to acknowledge the friendship of Noah, Casey, Malvika, Gerlinde, Oli and Tony. Breakfast at Ernie's with Gabe, Josh, and

Rob every Friday since November 2002 was simply the best, and our regular attendance ‘no matter what’ must be a record somewhere.

I would also like to thank my peers in the Cocroft Lab, Gabe, Laura, Karthik, Rob, Jen, Rafa, Holly and Lin. This was the best group to grow with, and without their emotional support (especially from Gabe and Laura), I would not have made it this far. From wine-tasting parties to fishing to bowling, we certainly had fun. Please keep ‘The Cocroft Lab Book of Quotes’ safe, and always remember to ‘Never Surrender.’ Best of luck to you all.

I must also thank my colleagues Dr. Ethan Freid and Dr. Luc Bussière. Their friendship was invaluable, and they continually set a standard for scientific and academic excellence that keeps me envious. I am a better person for knowing them. In addition, my friends in The Bahamas, and back home in Toronto (BP4L), were always there to offer words of encouragement and kindness, and I appreciate all their support.

I sincerely appreciate the funding sources that enabled me to conduct my dissertation research, most notably the National Science and Engineering Research Council of Canada, and the Sigma-Xi Research Society.

And finally, I would like to thank my family, without whose constant and unwavering support in all aspects of my life this would not have been possible. *Mille grazie per sempre.*

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Chapter 1

Introduction

Female mate choice is the subject of a large area of research in behavioral ecology. Over the past three decades many theoretical and empirical studies have investigated the patterns and consequences of female mating decisions (Andersson, 1994; Barbosa and Magurran, 2006; Bateson, 1983; Emlen and Oring, 1977; Shuster and Wade, 2003). The processes that influence the evolution of mate choice continue to receive much attention by researchers (Kokko et al., 2003). One empirical approach used to evaluate these processes investigates the role of male sexual behavior in affecting patterns of mate choice.

The success of males in obtaining matings is often linked to the reproductive benefits females derive (Bussière et al., 2005a; Jennions and Petrie, 1997). Males typically vary in their ability to provide benefits, and determining how females detect differences among males in the benefits they offer has revealed much about the processes that drive the evolution of mate choice. Females often use phenotypic cues that serve as indicators of mate choice benefits (Andersson and Simmons, 2006; Bussière et al., 2005b; Kokko et al., 2006). A wide diversity of male secondary sexual traits are known to influence female mating decisions. These traits can be found in many sensory modalities (e.g., visual, chemical, acoustic and vibrational), and often provide females with a reliable means of distinguishing between alternative males. Females that mate with the most ornamented males, or those with trait values that exceed the population mean,

often receive the best benefits (reviewed in Andersson, 1994; Gerhardt and Huber, 2002; Greenfield, 2002; Ryan and Keddy-Hector, 1992).

In addition to inter-sexual signals, traits or behaviors used in intra-sexual competition may also be used as indicators of mate choice benefits. Here, those males best able to successfully defend a group of females, or guard an important resource females require for offspring production, are usually the ones with the highest mating success (Bateman, 2000; Borgia, 1979; Le Boeuf and Reiter, 1988; Wong and Candolin, 2005). Note that traits may function in both inter-sexual and intra-sexual competition. For example, in many lekking species males compete against one another using sexual traits or displays that are directed at both male and female observers (Höglund and Alatalo, 1995; McGhee et al., 2007). In many species male competition has led to the evolution of alternative tactics. For example, small males unable to compete against larger males may adopt “sneaky” behavior to gain a mating (Gross, 1996). Our understanding of the processes affecting mate choice evolution has been greatly advanced by the study of alternative mating strategies, especially regarding their effect in maintaining variation in mate choice within a population (Brockmann, 2001; Shuster and Wade, 2003).

Another factor that may influence mate choice is male age; and its effect has been studied in great detail, both theoretically and empirically (reviewed in Brooks and Kemp, 2001). Preferences for old, young, and even middle-aged males have been demonstrated in a variety of species, and much research has been devoted to understanding the benefits females derive from mating with males of a particular age. Trivers (1972) and Halliday (1978) offered the first verbal arguments for age as an important factor affecting female mate choice. They stated that all else being equal, females should prefer older males since

they have demonstrated their capacity for survival. A few years later, Manning (1985) developed a theoretical argument in support of their idea. He proposed that older males on average, likely carry fewer somatic mutations than younger males since selection would remove individuals with deleterious mutations early in life. Thus, older males are genetically superior and females who mate with such males would benefit by passing on these genes to their offspring. He also cited some empirical studies in birds that showed preferences for older males, whom he argued were probably more experienced at raising young or holding a territory (Selander and Hauser, 1965; Wiley, 1974). However, his ideas were challenged by some who found no evidence for superior parental abilities by older males across several species of birds (see Alatalo et al., 1986; Weatherhead, 1984).

The first theoretical challenge to the idea that older males make better mates was put forth by Hansen and Price (1995). These authors included a life-history perspective in their model to show that older males do not have on average higher genetic quality, and thus are not genetically superior to younger males. They provided four arguments: 1) there typically are negative genetic correlations between early and late fitness components, 2) males usually suffer a decrease in fertility with age, 3) younger males are better adapted to the current environment, and 4) older males have accumulated more germ-line mutations. They concluded by stating that females choosing older males are probably not doing so for genetic benefits, although they did not offer any alternative hypotheses.

In the years following Hansen and Price (1995), more theoretical studies incorporated additional life-history variables into models of age-based mate choice. When factors such as the allocation of resources to sexual traits, variation in male

condition, and age-specific survival probabilities were included, the models showed that preferences for older males could evolve (Beck and Powell, 2000; Beck et al., 2002; Kokko, 1998; Kokko and Lidström, 1996). However, these models also predicted that preferences for younger males are equally possible, depending upon the conditions specified. One important conclusion from these studies was that the assumption of equating longevity with fitness that was made in early verbal arguments of age-based mate choice is not always correct (Brooks and Kemp, 2001). As a result, rather than attempting to produce a general explanation of age-based mate choice, researchers now propose that what is really important is an understanding of the various factors that cause male sexual behavior to vary with age.

Different empirical approaches can be taken to investigate the relationships between male age, sexual behavior and mate choice. One method studies potential trade offs that occur between male survival and investment in reproductive effort, and its effect on female mating decisions (Hunt et al., 2004b; Miller and Brooks, 2005). For example, recent studies in guppies and crickets have demonstrated that preferred high quality males may invest so heavily in sexual display that they suffer higher mortality at a young age (Brooks, 2000; Hunt et al., 2004a). Another approach studies how age affects the tactics males use to find and solicit matings from females. The focus here is not in linking variation in male sexual behavior with mate choice benefits *per se*, but rather in identifying potential causal factors that result in age-dependent male mating success, such as mate searching behavior or alternative mating strategies (Caro and Bateson, 1986; De Fraipont et al., 1993; Johnsen et al., 2003; Plaistow and Siva-Jothy, 1996).

This dissertation will explore the effect of male age on patterns of female choice, and on variation in male sexual behavior in the treehopper *Umbonia crassicornis* (Hemiptera: Membracidae). Using a combination of behavioral observations and manipulative experiments, I address three main questions:

- 1) What are the patterns and potential benefits to females of age-based mating?
- 2) How does male sexual advertisement vary with age and influence female choice?
- 3) How does male mate searching behavior vary with age and potentially affect male mating success?

Previous research with *U. crassicornis* suggested that females prefer to mate with older males (Wood and Dowell, 1985). This preference may be related to certain aspects of their social environment that subject females to high levels of inbreeding risk with their same-age brothers (Masters, 1997). However, these studies have some limitations, and thus additional experiments are required to address the role of age in affecting patterns of mate choice in this species. In addition, there are some gaps in our knowledge about many aspects of the natural history of *U. crassicornis* that could be important in order to fully understand the role age plays in affecting both male reproductive behavior and female choice.

The experiments conducted in this dissertation have been designed not only to investigate age, but also to provide additional information about the behavior and ecology of *U. crassicornis*. In Chapter 2 I investigate how the social environment experienced during mate searching influences age and relatedness-based patterns of female mating decisions. In Chapter 3 I explore the effect of age on variation in male mate-advertisement signals, and I also conduct a playback experiment evaluating age-based

signal variation on female choice. In Chapter 4 I examine age-based variation in certain male behaviors related to mate acquisition, in order to determine potential factors affecting male mating success. In Chapter 5 I discuss the implications of my results, and offer some suggestions for future research. It is hoped that this work will contribute to a better understanding of age as an important component of mate choice, and as a significant factor shaping male sexual behavior.

The study animal

Umbonia crassicornis ranges from Colombia to Mexico, with naturalized populations as far north as southern Florida (Mead, 1962). Individuals use a variety of host plants, primarily in the Mimosaceae (McKamey and Deitz, 1996). Females are monogamous and semelparous (Wood, 1974). Females care for their nymphs by making feeding slits on the host plant and protecting them from predators until they reach adulthood (Cocroft, 1996; Wood, 1976). After the eggs hatch nymphs undergo 4 instars, each lasting about one week, prior to eclosing to adulthood (Wood, 1974). Multiple family groups are often found on the same plant within 1 m of each other, or on separate plants that are within 2-3 m of one another (Cocroft RB, unpublished data; Masters, 1997; Wood and Dowell, 1985). All of the 50-80 siblings in a family eclose to adulthood within 2-3 days of one another (Wood and Dowell, 1985), then remain aggregated for 15-20 days before dispersing from their natal plant (Wood, 1974). Males and females reach sexual maturity in about 10 and 20 days, respectively, after eclosing to adulthood. Males disperse earlier than females: approximately 25% of males and 50% of females remain in their aggregation by the time females reach sexual maturity (Wood and Dowell, 1985).

Average dispersal distances for males and females are 6 and 3 m, respectively (Wood and Dowell, 1985). There is an overlap of generations in *U. crassicornis*, and it is common for older post-dispersal males to be found at aggregations of younger individuals during the period when females reach sexual maturity (Wood and Dowell, 1985). The lifespan of adult individuals in the field is unknown, but in our greenhouse colony adult males and females live to a maximum age of about 60 and 100 days, respectively (unpublished data). In order for father-daughter matings to occur, a male would have to live to about 85 days, which is well beyond the maximum age recorded for males, even under greenhouse conditions. Thus, father-daughter matings do not occur.

Pair formation is facilitated by substrate-borne vibrational signals produced by males, and female reply calls elicit searching behavior by males (Cocroft and McNett, 2006). Once a female is located the male initiates courtship by climbing onto one side of the female's body (Wood, 1974), and emits a series of vibrational signals while attempting genital contact (unpublished data). A female will often reject a courting male by shaking him off and walking away. Mating follows courtship only when a female raises the distal end of her abdomen to expose her genitalia. Female cooperation is thus a requirement during all stages of the mating sequence (i.e., mate attraction, courtship and copulation).

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Chapter 2

The influence of age and relatedness on mating patterns in *Umbonia crassicornis*

Abstract

The social environment of many species includes synchronous maturation of siblings in family groups, followed by limited dispersal of adults from their natal site. Under these conditions, individuals may experience high encounter rates with same-age siblings during mate searching, increasing their risk of inbreeding. Two hypotheses proposed to explain how females avoid inbreeding in such species are kin discrimination and age-based mating. We evaluated these hypotheses in the treehopper *Umbonia crassicornis*, a semelparous species in which females experience high levels of inbreeding risk with their same-age brothers. Female *U. crassicornis* that mate with a brother suffer from inbreeding depression. We used a free-choice mating design that offered females simultaneous mating opportunities with three groups of males: siblings, same-age non-siblings, and older non-siblings. These groups represent the types of males typically encountered by females in natural populations. There was no difference in the proportions of females mating with siblings vs. same-age non-siblings, suggesting an absence of kin discrimination. However, females mated with a greater proportion of older vs. younger non-sibling males. Given that sibling mating leads to inbreeding depression, and that females do not show kin discrimination per se, inbreeding avoidance may be an important benefit of mating with older males. We discuss some factors that may have contributed to the mating advantage of older males.

Introduction

In species characterized by synchronous maturation of siblings in family groups, followed by limited dispersal of adults from their natal site, individuals often face an increased risk of inbreeding when they begin searching for mates (Bateson, 1983; Lambin et al., 2001; Pusey and Wolf, 1996). This kind of social environment is found across a diverse group of taxa including some insects (McKamey and Deitz, 1996; Tallamy and Schafer, 1997; Wood, 1993), spiders (Aviles and Bukowski, 2005; Riechert and Roeloffs, 1993), copepods (Palmer and Edmands, 2000; Windsor and Innes, 2002), and amphibians (Waldman and McKinnon, 1993). In species where inbreeding depression occurs, behavioral mechanisms that reduce such costly mating errors are expected to evolve (Thornhill, 1993).

One widespread mechanism of inbreeding avoidance is kin discrimination (Arnold, 2000; Frommen et al., 2007; Hepper, 1991). Many studies have shown the ability of individuals to avoid kin as mates in species where siblings develop together in family groups (reviewed in Pusey and Wolf, 1996). Another proposed mechanism is age-based mating (Wood and Dowell, 1985). Where siblings mature in synchrony, a female could reduce her inbreeding risk by mating with an older male, since such a male (assuming he was not her father) would not be part of her immediate natal family.

Age-based mate choice has received much attention in recent years (Brooks and Kemp, 2001). Younger males are expected to be preferred if older males are of poorer quality as a result of age-associated declines in fertility, or from the accumulation of deleterious germ-line mutations later in life (Beck and Powell, 2000; Hansen and Price, 1995). In contrast, because older males have demonstrated their vigor by surviving,

mating with them could be beneficial for females because their offspring would inherit the male's genes for longevity (Halliday, 1983; Manning, 1985; Trivers, 1972). An important assumption of models of age-based mate choice is that significant differences in quality between different age-classes are the driving force maintaining the age preference (Beck and Powell, 2000; Hansen and Price, 1995; Kokko, 1998; Kokko and Lidström, 1996). However, the age-based mating hypothesis does not require any differences in quality between males of different ages; it only requires that the older male is not a sibling.

In the treehopper *Umbonia crassicornis* (Hemiptera: Membracidae), females have a high probability of encountering siblings during mate assessment. Siblings mature synchronously in family groups, and although individuals begin to disperse as they reach sexual maturity, most only disperse a few meters (Masters, 1997; Wood and Dowell, 1985). When sibling mating does occur, it results in significant inbreeding depression. In a population in Costa Rica, females mated to their brothers suffered a 32% reduction in adult offspring survival, compared to females mated to non-brothers (Masters, 1997). This drastic reduction in reproductive success for females (which mate only once) suggests that there may be strong selection favoring inbreeding avoidance (but see Kokko and Ots, 2006).

Here we test predictions of the kin discrimination and age-based mating hypotheses for inbreeding avoidance in *U. crassicornis*. Both hypotheses have been tested previously in this species. Masters (1997) tested the kin discrimination hypothesis in a Costa Rican population of *U. crassicornis* and found that females did not discriminate between siblings and non-siblings as mates, although she did find kin

discrimination in the closely related *U. ataliba* in the same study. Wood and Dowell (1985) tested the age-based mating hypothesis in a field study of *U. crassicornis* in Florida, and found that females were courted significantly more by older than by younger males. Taken together, these results suggest that any inbreeding avoidance by female *U. crassicornis* occurs through mating with older males, and not through kin discrimination *per se*. However, in Masters' (1997) study, mating trials were conducted under conditions that females were unlikely to experience in the field. The lack of kin discrimination, therefore, may not have accurately reflected mating patterns that would be expressed in a complex natural setting (Jennions and Petrie, 1997). In Wood and Dowell's (1985) study, it was not determined whether the females that were courted by older males eventually mated with them. Furthermore, in a field study it is difficult to determine the age structure of the available pool of mates. As a result, it was unclear whether females were courted by a larger proportion of older males than expected based on their representation in the mating pool. What is needed, therefore, is a study that examines both kin discrimination and age-based mating in a single experiment with known proportions of available mates, where female mating decisions can be evaluated under conditions that reproduce the social environment of mate choice in the field.

We tested predictions of the kin discrimination and age-based mating hypotheses concurrently, as both may influence the outcome of mate choice in *U. crassicornis*. We used a free-choice mating experiment that offered females simultaneous mating opportunities with males differing in age and relatedness. Our experimental design incorporated key aspects of the social environment of *U. crassicornis* critical for our assessment of female mating patterns: i) the onset of sexual maturity of siblings while

still aggregated in their family group; ii) the close spatial proximity of different family aggregations; and iii) the presence of courting older males while females are still sexually immature. Incorporating these social factors in a semi-natural setting provided females with the types of males typically present during mate searching (Wood, 1977; Wood and Dowell, 1985), and allowed us to monitor both male courtship behavior and female mating decisions. If kin discrimination is important for inbreeding avoidance in *U. crassicornis*, then females should mate less often with siblings than with non-siblings of the same age. Similarly, if age-based mating is important for inbreeding avoidance, then females should mate more often with older males than with younger males, when both are unrelated.

Methods

Insect maintenance

The individuals used in this experiment were greenhouse-reared descendents of insects collected in Miami and Key Largo, Florida in 2003 and 2004. Individuals were collected from the field as late-instar nymphs or teneral adults that were still aggregated in their family group. A total of 19 different families (each family comprising ~50 insects) was collected. Families were kept in a greenhouse at the University of Missouri and maintained on their own potted *Albizia julibrissin* (Mimosaceae) host plant isolated within a screen cage. When a family eclosed to adulthood the sexes were separated after about 10 days to prevent sibling mating. The colony was maintained through selective mating of unrelated individuals from different families every generation.

Study design

We conducted the experiment within a greenhouse at the University of Missouri. We used 2 cages, each measuring 183 x 122 x 76 cm (L x W x H). Within each cage we placed 5 potted *A. julibrissin* plants (approximate height: 70 cm). Three groups of adults were placed into each cage, with each group set on its own *A. julibrissin* plant. The 3 plants containing insects were placed in the middle of the cage, set at a distance of 50 cm from one another in a triangle. The proximity of the 3 groups of insects relative to one other falls within the range of inter-family distances measured in the field for *U. crassicornis* (RB Cocroft, unpublished data; Wood and Dowell, 1985). The 2 unoccupied plants were provided as additional dispersal locations, and were each situated to the left and right of the 3 central plants at a distance of 50 cm. Greenhouse temperatures ranged between 25-30°C, and the photoperiod was set to a 13:11 light: dark cycle.

Each group of insects within a cage was drawn from a separate family (3 groups = 3 different families in a cage). Two family groups were similar in age, with each composed of 10 male and 10 female siblings between 5-7 days old post-eclosion. These individuals were randomly selected from their respective larger family group and re-aggregated on their own *A. julibrissin* plant within the cage. Teneral adult *U. crassicornis* from the same family group under 10 days old can be re-aggregated onto a different host plant without affecting their behavior (Wood, 1974). The third family group was composed of only 10 sibling males, which were randomly drawn from a family that was 15-17 days old post-eclosion. These males were mate searching older males that had already dispersed, and thus their sisters were not included with them. Females in each cage thus had free choice of 3 groups of potential mates in an equal ratio: 10 siblings, 10

same-age non-siblings, and 10 older non-siblings. Our experimental unit for analyses of courtship and mating patterns was one group of 10 females from the same family.

This experiment was replicated over 3 time periods in 2004 (July, September and November). For each new study period, individuals were drawn from new sets of families and placed in each of the 2 cages as described above, on a new set of potted *A. julibrissin* host plants. Cages were washed with a mild detergent soap between replicates to eliminate any potential chemical cues left by the previous groups of insects.

Individuals were uniquely marked with a 3 mm² numbered tag glued to the left side of the pronotum. Each study period commenced on the day both cages were stocked with insects. Hourly observations were conducted daily from 08:00-18:00. We ended a study period once all the females in both cages mated. Study periods lasted 12 (July), 15 (September), and 17 (November) days. During observations we recorded the date, plant location and individual identities of insects engaging in courtship and mating behavior.

For the July study period we had available only 2 families that were identical in age, plus 1 older family of males of the appropriate age, to stock the cages. As a result, these 3 families were divided into the 2 cages according to the design outlined above, but for statistical analyses of courtship and mating patterns we pooled the data from both cages to avoid pseudoreplication of the 3 families (Hurlbert, 1984). For the September and November study periods we had available 6 families of the appropriate ages, and thus we were able to stock each of the 2 cages with their own unique complement of 3 families. Thus, in total 10 families were used to supply the younger males and females (2 in July, 4 in September, 4 in November), and 5 families were used to supply the older males (1 in July, 2 in September, 2 in November).

Courtship behavior

To compare the number of courtships exhibited by the 3 different groups of males (siblings, same-age non-siblings, and older non-siblings), we included in our analysis only those courtships that were observed occurring from the day the first female in each cage mated. This standardized our comparisons of the different groups of males in each cage relative to the onset of female mating. This was done because in natural populations, mate-searching older males arrive at aggregations and begin courting females several days earlier than sibling males. Thus, our analysis focused on relative differences during the period of female sexual receptivity.

Statistical analyses were conducted using JMP IN (SAS Institute Inc., 2003). We used an ANOVA to compare the proportion of females from each family (i.e., our experimental unit) that were courted by: (1) siblings vs. same-age non-siblings, or (2) younger vs. older non-siblings. Each analysis was conducted separately. The first analysis excluded the courtship data from older males in order to test the effect of relatedness. The second analysis excluded the courtship data from siblings in order to test the effect of age. Eliminating one group of males in each analysis also removed the non-independence of the response variable which otherwise is constrained to add to 1.0 (Quinn and Keough, 2002; Underwood, 1997). Proportion values were arcsine transformed to satisfy assumptions of normality and homogeneity of variance (Zar, 1984). Factors in both models included the study period (i.e., July, September, and November), cage (nested within study period), female family (nested within cage), and either the relatedness of the female to the male (sibling or same-age non-sibling), or the age of the male (younger or older non-sibling). The transformed data for both comparisons met assumptions of

normality (relatedness: Shapiro-Wilk $W=0.947$, $p=0.24$; age: Shapiro-Wilk $W=0.935$, $p=0.13$), and homogeneity of variance (relatedness: Bartlett test, $p=0.22$; age: Bartlett test, $p=0.68$).

Female mating patterns

To assess female mating preferences we compared the proportion of females in each family that mated with either: (1) siblings vs. same-age non-siblings, or (2) younger vs. older non-siblings. As with courtship behavior, we used separate ANOVAs for each comparison. Factors were the same as those used in the courtship analyses. The transformed data for both comparisons met assumptions of normality (relatedness: Shapiro-Wilk $W=0.973$, $p=0.77$; age: Shapiro-Wilk $W=0.949$, $p=0.27$), and homogeneity of variance (relatedness: Bartlett test, $p=0.49$; age: Bartlett test, $p=0.79$).

For all analyses descriptive statistics are reported as the mean \pm SE.

Results

Courtship behavior

Older males moved from their plant and began courting females from both younger family groups on their respective plants on the first day of observations during all 3 study periods. Both groups of younger males started to disperse and court females 4.7 ± 0.4 days later ($N=3$ study periods). A total of 328 courtships was observed when we included only those that occurred from the day the first female in each cage mated. Older males performed 44 ± 3.6 % of the courtships, while sibling and same-age non-sibling males performed 24 ± 2.2 %, and 32 ± 3.2 %, of the courtships respectively. Females began mating about 8 days after the start of observations (see Female mating patterns

below); thus, despite the difference in the onset of courtship behavior between older and younger males, females in each cage experienced courtship from all 3 groups of males for about 4 days prior to the onset of their sexual receptivity.

The proportions of females courted by siblings vs. same-age non-siblings were not significantly different, although there was a trend for females to be courted more by non-siblings ($F_{1,12}=4.503$, $p=0.06$, Fig. 1a). The proportions of females courted by siblings vs. same-age non-siblings did not differ among the study periods, cages and female families (study period: $F_{2,12}=0.169$, $p=0.85$; cage: $F_{3,12}=1.922$, $p=0.184$; female family: $F_{6,12}=0.449$, $p=0.831$).

The proportions of females courted by older vs. younger non-siblings were not significantly different, although there was a trend for females to be courted more by older males ($F_{1,12}=4.036$, $p=0.07$, Fig. 1a). The proportions of females courted by older vs. younger non-siblings did not differ among the study periods, cages and female families (study period: $F_{2,12}=0.261$, $p=0.78$; cage: $F_{3,12}=0.114$, $p=0.95$; female family: $F_{6,12}=0.061$, $p=0.98$).

Female mating patterns

Females began mating 8.1 ± 0.8 days ($N=10$ families) after the start of our observations. A total of 106 matings was observed. Older males obtained 44 ± 4.5 % of the matings, while younger sibling and non-sibling males obtained 28 ± 6.5 %, and 28 ± 3.7 % of the matings, respectively. Of the 14 females with no mating record, 9 died before having mated. The other 5 females were observed sitting on a clutch of eggs at the start of a new observation day and so were inferred to have mated. Our observation regime was thus successful in capturing 95% (106/111) of the matings. Average age at

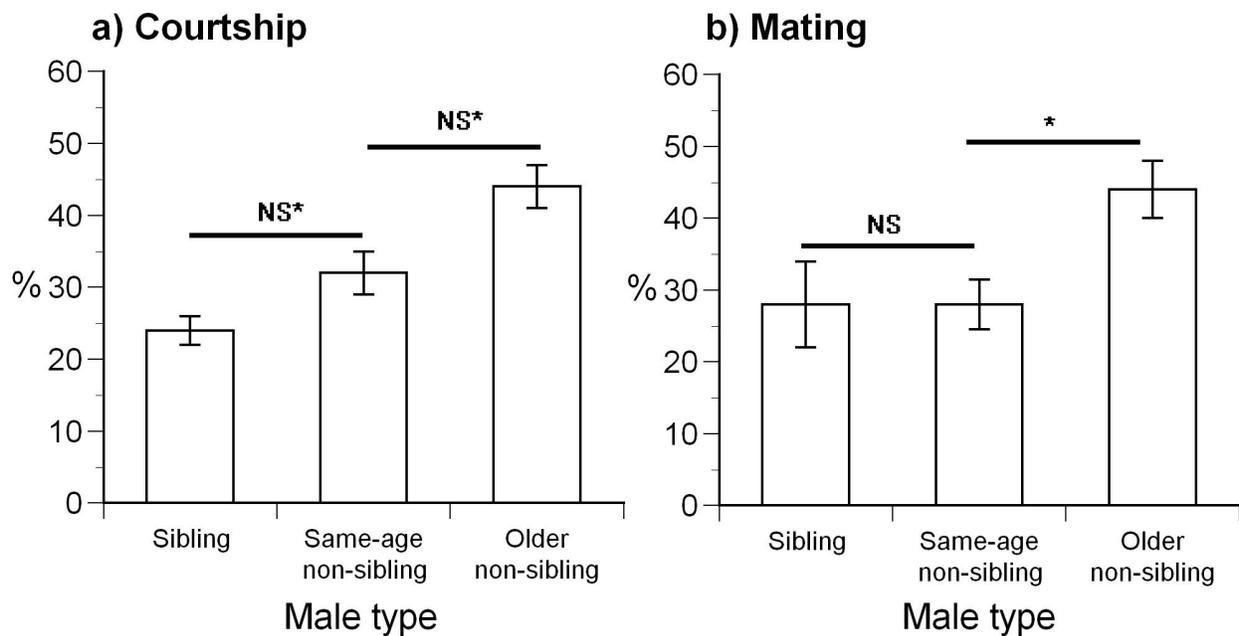


Figure 1. Mean proportions (\pm SE) of females from each family (a) courted by and (b) mating with the 3 different groups of males. Lines connect groups that were compared with an ANOVA. A significantly greater proportion of older vs. younger non-sibling males mated with females. There was no significant difference in the proportions of females mating with siblings vs. same-age non-siblings (*: $p < 0.05$; NS*: $0.1 < p < 0.05$; NS: not significant).

mating for females was 21.6 ± 0.3 days ($N=106$). Average age at mating for younger (siblings + same-age non-siblings) and older males was 21.7 ± 0.2 ($N=41$), and 29.8 ± 0.5 days ($N=30$), respectively.

Although there was a trend for females to be courted more by non-siblings, the proportions of females that actually mated with siblings vs. same-age non-siblings were not significantly different ($F_{1,18}=0.023$, $p=0.88$, Fig. 1b). The proportions of females that mated with siblings vs. same-age non-siblings did not differ among the study periods, cages and female families (study period: $F_{2,18}=0.057$, $p=0.94$; cage: $F_{3,18}=0.139$, $p=0.93$; female family: $F_{6,18}=0.898$, $p=0.53$).

Females mated significantly more often with older males ($F_{1,18}=5.165$, $p=0.04$, Fig. 1b). The proportions of females that mated with older vs. younger non-siblings did not differ among the study periods, cages and female families (study period: $F_{2,18}=0.023$, $p=0.97$; cage: $F_{3,18}=0.213$, $p=0.88$; female family: $F_{6,18}=0.445$, $p=0.83$).

Discussion

Females of species like *U. crassicornis* that suffer from inbreeding depression are expected to evolve behavioral mechanisms that reduce such costly mating errors (Pusey and Wolf, 1996; Thornhill, 1993). In our study, offering females different choices of males using an approach that re-created their social environment enabled us to simultaneously evaluate the kin discrimination and age-based mating hypotheses. The only indication of kin discrimination was a trend for females to be courted more by same-age non-siblings than by siblings. This difference in courtship rates though, did not translate into a significant difference in mating rates, which suggests females do not

discriminate kin from same-age non-kin. However, females mated significantly more often with older males, which supports age-based mating as a factor that reduces inbreeding in *U. crassicornis*.

Although mating with older males will lead to a reduced risk of inbreeding for *U. crassicornis* females, it may also provide other fitness benefits. Many empirical studies have documented increased reproductive fitness from mating with older (or middle-aged) males. These advantages include both direct benefits (Cote and Hunte, 1993; Hasselquist, 1998; Jones and Elgar, 2004; Mountjoy and Lemon, 1996; Srivastava and Omkar, 2004; Zuk, 1988), and indirect benefits (Conner, 1989; Dickinson, 2001; Jones et al., 2000; Woodhead, 1986). Determining if male age per se in *U. crassicornis* affects female reproductive fitness requires an experiment in which females are mated to unrelated males of varying ages and their reproductive success compared. The use of field-caught males (i.e., males of varying ages exposed to selection under natural conditions) in such an experiment would provide a test of whether mating with older males, independent of outbreeding, confers fitness advantages to females in this species.

The results of this study do not conclusively demonstrate active female choice for older males as mates. Indeed, females may have just mated with older males because they were the most prolific at courtship. However, I have observed females actively rejecting the courtship advances of males, which suggests that females are capable of exerting some form of choice with respect to their courting partner. Thus, females may have actively favored older males in this context, possibly through preferential response to the mate advertisement signals of older males. I will investigate the role of male mate advertisement signals on female choice in Chapter 3.

Two proximate factors may explain the mating advantage of older males. First, older males are predicted to have increased levels of sexual advertisement (Kokko, 1997; Proulx et al., 2002), and an older male courtship advantage has been observed in a range of species (Clutton-Brock and Albon, 1979; Felton et al., 2006; Kemp, 2002a; Le Boeuf and Reiter, 1988; Marden and Wagge, 1990; Otronen, 1995; Plaistow and Tsubaki, 2000; Woodhead, 1986). A field study of *U. crassicornis* in Florida found that older males performed significantly more courtships than younger males (Wood and Dowell, 1985), and there was a similar trend in our experiment. If female mate selection mechanisms in *U. crassicornis* include a preference for high courtship rates, then older males would have an advantage over younger rivals.

Second, preference for older males might have occurred as a result of sexual imprinting, a phenomenon whereby sexually immature females learn the cues of specific males in a population and then mate preferentially with them when they reach sexual maturity (Hebets, 2003; Kendrick et al., 1998; Slagsvold et al., 2002). Recall that older *U. crassicornis* males are typically found courting unreceptive females at their natal aggregation for several days prior to the onset of female mating (Wood and Dowell, 1985). In our study, even though females were still sexually immature during the period when only older males were courting, they might have imprinted upon older male courtship behavior and subsequently preferred them as mating partners. Sexual imprinting has recently been demonstrated in an invertebrate (Hebets, 2003), but additional studies are required to determine whether sexual imprinting can explain the older male mating advantage in *U. crassicornis*.

Although there was a significant mating advantage for older males, age per se did not explain all of the variation in mating success. Females typically use multiple cues in mate choice (Candolin, 2003; Hebets and Papaj, 2005), and it is likely that variation in other male traits influences attractiveness independently of age. Additional studies with *U. crassicornis* are under way to determine other factors (e.g., vibrational signals, courtship duration, and mate-searching behavior) that may contribute to a male's mating success. Also, females in this study were frequently courted simultaneously by two or more males, a situation that occurs commonly in natural populations (Wood, 1974; Wood and Dowell, 1985). Although we did not assess the extent of male competition in this study, the presence of several males competing simultaneously can affect a female's ability to effectively evaluate alternative male options (Bateson and Healy, 2005; Jennions and Petrie, 1997; Uetz and Norton, 2007; Wong and Candolin, 2005).

The lack of kin discrimination in mating by *U. crassicornis* observed in our study and that of Masters (1997) was surprising. This is a common method of avoiding siblings during mate choice in many species where siblings mature in family groups (Mateo, 2004; Paz y Mino and Tang-Martinez, 1999; Simmons, 1989). Many empirical studies have shown that females prefer non-siblings in mate choice experiments (reviewed in Pusey and Wolf, 1996), and kin discrimination is usually invoked as the process that enables relatives to avoid each other as mates. For singly mated *U. crassicornis* females, the lack of kin discrimination despite a significant cost to inbreeding strongly suggests that alternative mechanisms that reduce inbreeding should be favored by selection. Mating with older males may thus represent a consequence of selection that favors outbreeding.

Although females of many species discriminate against kin during mate choice, the model of Kokko and Ots (2006) showed that under different kinds of life history and parental investment patterns, selection could still favor females that mate with siblings even if inbreeding depression values reach as high as 60%. Those authors argue that such high levels of inbreeding depression should be accepted by females because it increases the reproductive success of their brothers. Interestingly, their simulation for a semelparous species where females encounter sibling and non-sibling males simultaneously and at high rates (as occurs in *U. crassicornis*), predicted that females would tolerate an inbreeding level of about 30%, which is similar to the value of inbreeding depression reported for *U. crassicornis* by Masters (1997). If females inbreed to increase the reproductive success of their brothers, then one intriguing prediction of the Kokko and Ots (2006) model is that a low quality male that is rejected by an unrelated female should be accepted by his sister. Although our data do not allow us to test this prediction, the Kokko and Ots (2006) model offers an interesting theoretical argument for why inbreeding persists in some species.

A common mechanism of inbreeding avoidance that we did not evaluate in our study is dispersal from the natal site prior to mating (Le Galliard et al., 2006; Lehmann and Perrin, 2003). This typically, but not always, involves one sex dispersing earlier and farther than the other, which separates opposite-sex siblings both spatially and temporally in the environment (Moore and Ali, 1984; Motro, 1991). In situations where dispersal distances for both sexes overlap (as in *U. crassicornis*), post-dispersal mating by females is thought to reduce inbreeding risk through a dilution effect that decreases sibling encounter rates (Perrin and Goudet, 2001; Perrin and Mazalov, 1999). Although our

experiment did not test this hypothesis, a field study that monitored dispersal behavior in *U. crassicornis* in their natural habitat in Florida found that 13% of the total courtships that were observed occurred between siblings away from their natal site (Wood and Dowell, 1985). This indicates that females are still vulnerable to inbreeding following dispersal.

In conclusion, the social environment of *U. crassicornis* exposes females to a high level of inbreeding risk. Dispersal reduces this risk, but mating with older males provides further protection that operates at both natal and non-natal locations. Studies are under way to determine the proximate mechanisms underlying the older-male mating advantage, as well as to assess the extent to which age-dependent variation in different aspects of male reproductive behavior affects male mating success.

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Chapter 3

Age-based variation in male vibrational signals, and its effect on female choice in *U. crassicornis*

Abstract

The structure of male mating signals is often influenced by age. The causes and consequences of age-based signal variation have been much studied in vertebrates, but are less explored in invertebrates. In the treehopper *Umberia crassicornis*, males produce vibrational signals to advertise for mates. Females mate more often with older males but the mechanism underlying older male mating success is unknown. My goals in this study were to determine whether mate advertisement signals contain cues that might enable females to identify older males. I recorded signals from 66 adult males over four consecutive weeks beginning at the onset of signaling, and measured 10 temporal, spectral, and energy-related parameters within each signal. Discriminant function analysis showed that most signals could be correctly classified according to age, and thus contain potentially useful age cues. I then tested whether females preferred the signals of older males in a playback experiment. When females were presented with signals representing differently aged males, there was no difference in their responses, indicating that females do not favor older males on the basis of long-range mate advertisement signals. Nonetheless, the fact that several signal traits varied significantly with age reveals a level of plasticity not usually associated with insect mating signals. These changes may be linked with age-related variation in other aspects of male reproductive behavior, such as the development of flight and mate searching tactics.

Introduction

Females in many animal species have mating preferences based on male age. In a recent review, Brooks and Kemp (2001) evaluated the conditions under which preferences for older or younger males are expected to evolve, and provided several empirical examples of age-based mate choice across a diverse group of taxa. Age-based mate choice is often linked to the kinds of reproductive benefits females derive. For example, by choosing males on the basis of age females may obtain direct benefits, such as increased fecundity or hatching success of offspring (Cote and Hunte, 1993; Hasselquist, 1998; Jones and Elgar, 2004; Mountjoy and Lemon, 1996; Srivastava and Omkar, 2004; Zuk, 1988); or indirect benefits, such as increased offspring survivorship (Conner, 1989; Dickinson, 2001; Jones et al., 2000; Woodhead, 1986).

Age-based mate choice usually requires cues for assessing age. Mating signals are typically used in this context, and in many species of vertebrates females will often evaluate multiple signal traits when choosing mates on the basis of age (Burley, 1981). Examples include odors in lizards and mice (Lopez et al., 2003; Osada et al., 2003); ornamentation or color patterns in birds and fish (Buchholz, 1991; Johnstone, 1995; Miller and Brooks, 2005); body size or courtship behavior in anurans (Felton et al., 2006; Howard et al., 1994); and acoustic signals in birds (Forstmeier et al., 2005; O'Loughlen and Rothstein, 2003; Poesel et al., 2006). In addition to providing a source of age information, these cues are also used as indicators of mate quality, and thus reflect a male's ability to provide mating benefits (Brooks and Kemp, 2001).

The effect of age on mating signals in invertebrates such as arthropods is less well known. One possible reason for this is that since many arthropods exhibit determinate

growth, mating signals are thought to be fixed after adult eclosion, and thus their expression stereotyped (Ewing, 1989). In addition, because individuals in many arthropod species are short-lived and have a breeding season that is limited to only a few weeks, the importance of age as a source of signal variation may be overlooked. However, arthropods are ideal subjects for investigation here since their short generation times make them well-suited for experimental manipulation. In fact, two studies in insects have shown that a trade off between reproductive effort and longevity affected age-dependent patterns of signal expression (Cordts and Partridge, 1996; Hunt et al., 2004a).

Many arthropod species use substrate-borne vibrational signals for mate attraction, and there are good reasons to expect a significant effect of age on these signals. Many vibrational signals are structurally complex, often containing distinctive components that are generated by different mechanisms simultaneously (Cocroft et al., 2006; Cökl and Virant-Doberlet, 2003; Elias et al., 2006; Virant-Doberlet and Cökl, 2004). The expression of these complex signals may thus be sensitive to age-related developmental processes that affect the underlying neural and muscular systems controlling signal production (Elias et al., 2006). In addition, as is common for other types of mating signals, variation among males in the allocation of resources to different signal traits over their lifetime may result in age-dependent patterns of signal expression (Cotton et al., 2004; Höglund and Sheldon, 1998; Kokko, 1997; LeBas et al., 2004; Scheuber et al., 2003b; Simmons, 1995). However, despite the widespread prevalence of vibrational signaling in arthropods – an estimated 195,000 species of insects (Cocroft and Rodríguez, 2005), and 35,000 species of spiders (Barth, 1998) – only four studies have

investigated the influence of age on the expression of vibrational mating signals (Kumar and Saxena, 1985; Moreira, 1993; Sattman and Cocroft, 2003; Zeigler and Stewart, 1985).

Most studies examining age effects on arthropod mating signals have been limited to Orthoptera, where males predominantly use acoustic signals to attract females (Gerhardt and Huber, 2002). Here, traits such as signaling effort, carrier frequency, and pulse pattern have been found to vary with age in some species (Hunt et al., 2006; Jacot et al., 2007; Ritchie et al., 1995; Simmons, 1995; Stiedl et al., 1991; Zuk and Simmons, 1997). However, in other orthopteran species age has no effect on signal variation (Murray and Cade, 1995; Souroukis et al., 1992; Walker and Cade, 2003). These contrasting results, along with the paucity of examples in other arthropod taxa and signaling modalities, highlight the fact that no consensus regarding age effects is currently available for invertebrate mating signals, and thus additional research is warranted.

Longitudinal (within-male) analyses offer a useful approach for assessing age-based changes in signal structure (Blumstein and Munos, 2005; Forstmeier et al., 2005; Garamszegi et al., 2007). Although cross-sectional (across-male) analyses provide a snapshot view of how signal variability correlates with the current population age distribution, they are limited because sampling across males provides few clues about the extent of within-male variability (Miller and Brooks, 2005). Longitudinal analyses, however, examine how traits change within individuals, and thus can offer a starting point for investigating intrinsic processes affecting age-based signal expression (Jacot et al., 2007). In addition, this approach has the potential to account for variation that

otherwise might not be captured from cross-sectional analyses that sample males only once in their life.

Males of the treehopper *Umberia crassicornis* (Hemiptera: Membracidae) produce complex multicomponent vibrational signals during mate advertisement, and female replies elicit searching behavior from males (Cocroft and McNett, 2006). Females mate more often with older males under free-choice conditions (Chapter 2), but the mechanism underlying older male mating success is unknown. One possibility is that age-based variation in male advertisement signals enables females to identify older males during pair formation. In this study I use a longitudinal approach to explore age-based changes in signal structure occurring over four consecutive weeks of adult life, beginning at the onset of male signaling behavior. After characterizing the effect of age, I then conduct a playback experiment to test whether females prefer the signals of older males. If *U. crassicornis* females use mate-advertisement signals to identify older males, then: 1) signals should contain reliable age cues, and 2) females should prefer the signals of older males.

Methods

Insect maintenance

Insects were derived from a naturalized population of *U. crassicornis* in southern Florida. From 2001-2006 repeated collections were made from five field sites in Miami and Key Largo, FL, USA. I collected individuals as late-instar nymphs or teneral adults when they were still aggregated in their family group (~50-80 individuals per family). Approximately 1000 individuals from 20 families were used to establish a greenhouse

colony at the University of Missouri (Columbia, MO, USA). Each family was enclosed within its own screen cage on a potted *Albizia julibrissin* (Mimosaceae) host plant. Approximately one week after individuals in a family eclosed to adulthood I separated the sexes by placing them on their own host plant to prevent sibling mating. I produced new families of insects by mating individuals from unrelated families every generation (about 3-4 months).

Recording of vibrational signals

In 2003 and 2005 I recorded signals from adult males reared from our greenhouse colony. I used males from 8 families (4 in 2003 and 4 in 2005). Males from 2005 were descended from insects collected in Florida in 2004, and thus were not related to males used in 2003. I randomly selected 8-9 males from each family for a total 66 males. I recorded each male once per week over 4 consecutive weeks, beginning at the start of signaling behavior (~10-12 days after adult eclosion). During each recording session I obtained a minimum of 3 calls from each male. If more than 3 signals were recorded I randomly chose 3 for analysis.

I used a potted *A. julibrissin* plant to make recordings. The plant was placed on a vibration isolation table that insulated recordings from low frequency building vibrations (Vibraplane 9100, Kinetic Systems, Inc., Boston, MA, USA). Males were recorded between 09:00-17:00 hours and at temperatures ranging between 22-27°C. I placed one male at a time on the plant on a narrow stem (diameter = 6 mm) and allowed it to settle for a few minutes. To stimulate male calling I played a pre-recorded *U. crassicornis* male-female duet from a computer through a speaker located about 2 m away from the plant. Air-borne vibrations imparted into the plant were sufficient to induce males to

produce mate-advertisement signals. Upon broadcast of the duet males would immediately begin signaling. Males remained stationary and usually called in bouts of 2 – 5 signals. If a male gave fewer than 3 signals the duet was immediately broadcast again to obtain more signals.

Signals were transduced with a Polytec CLV1000 laser vibrometer with a CLV M030 decoder module (Polytec PI, Inc., Auburn, MA, USA) set at 5 mm/s/V sensitivity. A small piece (~1 mm²) of reflective tape was affixed to the plant to increase the reflectance of the laser. To minimize substrate-related variation the laser was focused on a point ≤10 cm from the signaling insect (Cocroft et al., 2006). I placed each male with his dorso-ventral axis parallel with the laser beam to ensure that the major axis of stem vibration was measured correctly (McNett et al., 2006). Recordings were low-pass filtered at 5 kHz with the laser control module and high-pass filtered at 60 Hz with a Krohn-Hite 3202 filter (Krohn-Hite Corporation, Brockton, MA) before being input into a Macintosh computer using SoundEdit16 v.2 software (Macromedia, Inc., San Francisco, CA, USA). I monitored signals during recording using both an oscilloscope (Hameg HM 203-7) and a speaker (RCA Pro10W).

In 2005 I used the same recording protocol described above; however, a different *A. jilibrissin* plant was used (stem diameter where males were recorded = 4 mm). Signals were acquired using a National Instruments (NI) data acquisition board connected to a Dell computer. Custom software developed in LABVIEW v. 7 (National Instruments, Austin, TX, USA), and MATLAB v. 6.5 (The Mathworks, Inc., Natick, MA, USA), allowed me to quantify age-related variation in the peak amplitude (i.e., velocity) of vibrational signals.

Measuring signal parameters

The vibrational signals of *U. crassicornis* males contain two components produced simultaneously in different frequency ranges: a frequency- and amplitude-modulated downward sweep from 200-130 Hz; and a series of broadband ‘clicks’ in the 500-2000 Hz range (Table 1; Fig. 1). The low frequency (LF) component is generated using direct muscle contractions that vibrate the abdomen, transmitting the signal into the substrate via the legs (Ossiannilsson, 1949). The high frequency (HF) component is produced using a frequency-multiplier system, likely a tymbal located on the abdomen (Cökl and Virant-Doberlet, 2003; Gogala, 1985).

I used Canary v. 1.2.4 in 2003, and Raven v. 1.2 in 2005 (both from the Cornell Laboratory of Ornithology, Ithaca, NY, USA), to quantify 4 temporal and 5 spectral signal parameters (Table 1). I also measured peak amplitude for signals obtained in 2005. Of the 10 parameters measured, 7 were significantly correlated with temperature, and thus were corrected to a common temperature of 25°C. Three parameters (absolute amplitude, proportion of signals containing amplitude modulation (AM), and HF click rate) did not vary significantly with temperature (all, $p \geq 0.19$).

In total, 711 signals from 66 males were analyzed (312 in 2003, and 399 in 2005). In 2003, mortality reduced the number of males so that sample sizes at each age class were 32, 31, 25, and 16. In 2005, all 34 males survived but at 12 and 19 days only 32 and 33 males, respectively, provided signals. For each male I averaged parameter values from 3 signals obtained at each age class and used these mean values in subsequent analyses.

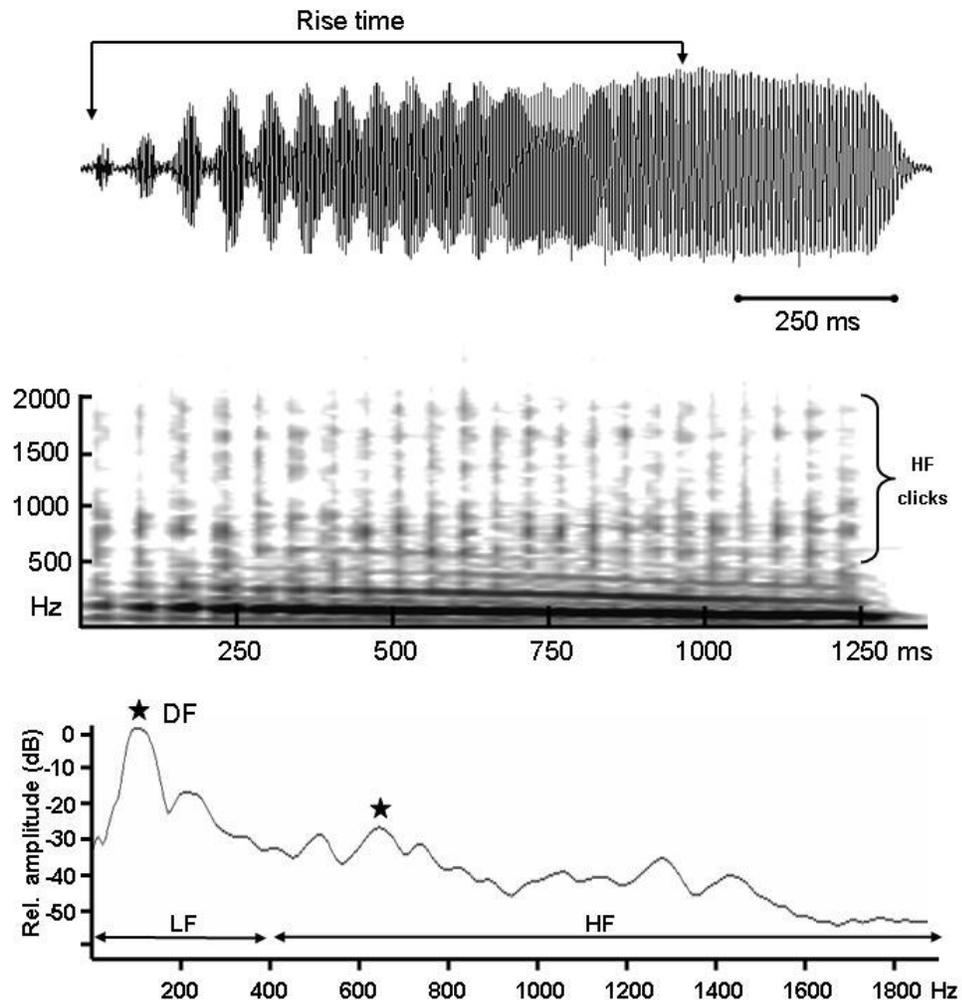


Figure 1. Illustration of a typical male *U. crassicornis* vibrational signal. Top: Waveform showing the low frequency (LF) component. Amplitude modulation (AM) occurs at the beginning of a signal. Middle: Frequency spectrogram. The LF component is composed of a downward frequency sweep from 200-130 Hz, while the high frequency (HF) component is made up of a series of broadband clicks from 500-2000 Hz. Bottom: Amplitude spectrum. Arrows indicate the frequency range of the LF and HF components, and stars mark the frequencies of peak amplitude. DF = dominant frequency.

Table 1. Description of vibratory signal parameters measured in this study. For additional information see Figure 1

Parameter	Description
Temporal	
Duration	Length of signal, measured in seconds
Rise time	Time from start to maximum amplitude, expressed as a % of the total duration
Proportion of signals with amplitude modulation (AM)	Proportion of signals (out of 3) that contain AM within one signaling bout
High frequency (HF) click rate	Number of HF clicks per second in one signal
Spectral	
Dominant frequency (DF)	Frequency with the greatest relative energy in a signal
DF at beginning of signal	Frequency with the greatest relative energy during the first 200 ms
DF at middle of signal	Frequency with the greatest relative energy during the middle 200 ms
DF at end of signal	Frequency with the greatest relative energy during the final 200 ms
LF-HF amplitude difference	Difference in relative maximum amplitude between the low frequency (LF) and high frequency (HF) signal components, measured in decibels (dB)
Energy	
Peak velocity	Peak amplitude of a signal, expressed in dB rel. 0.1 mm/s

Discriminant function analysis of age-related signal variation

I used discriminant function analysis (DFA) in JMP IN 5.1 (SAS Institute Inc., 2003) to classify signals according to age, following the procedure outlined by Blumstein and Munos (2005). A total of 237 cases was used. Each case represents the measurements from one male at one age class. Thus, in 2003 there were 104 cases (32+31+25+16), and in 2005 there were 133 cases (32+33+34+34). Each case in a DFA requires a full data matrix (i.e., a measurement for each variable); therefore peak amplitude was not included here because I did not have values for this variable for males from 2003. The effect of age on peak amplitude was analyzed separately (see below). First, I examined whether there were significant differences in signals obtained between the recording years (i.e., 2003 vs. 2005), and then, I examined whether signals could be reliably grouped according to male age (i.e., 12, 19, 25, 33 days).

The DFA also provided an assessment of how distinct signals from the different age groups were by calculating a percentage score for each group that indicated how many observations were correctly classified. Scores were calculated by tabulating the number of correct assignments when observations from two different groups were compared against each other. These pair-wise comparisons were performed for all possible pairs of groups. In this case, there were four age groups and thus, 6 possible pair-wise comparisons. If observations from 2 groups are not significantly different, then the null expectation is that each observation has a 50% chance of falling into either group, yielding an overall classification score of 50% for each group (Blumstein and Munos, 2005).

Peak amplitude

To assess the effect of age on peak amplitude I used a partially nested repeated-measures ANOVA in JMP IN 5.1 (SAS Institute Inc., 2003). I log-transformed values to achieve normality (Shapiro-Wilk $W = 0.982$, $p = 0.08$), and homogeneity of variance (Bartlett test, $p = 0.06$), before proceeding (Zar, 1984). Factors in the model included male family, male identity (nested within family), male age, and a family \times age interaction term. Family and male were entered as random effects and age as a fixed effect. If a factor was significant at the $p = 0.05$ level I used Student's t post-hoc comparisons to evaluate which factor levels were significantly different from one another.

Design of stimuli for the playback experiment

Rather than using pre-recorded natural male signals spanning the range of age-based variation to test female preferences, I constructed synthetic signals to serve as my playback stimuli. This was done to simplify the playback design since using natural signals would require a large number of exemplars from each age class to adequately sample among-male variation (Kroodsma et al., 2001; McGregor et al., 1992). I synthesized signals with a custom-written program in Matlab v. 6.5. Mean values for parameters measured from 2005 were used to construct stimuli. Four stimuli were made that each represented an average signal from one of the four age classes described above (Fig. 3). Some LF spectral parameters (i.e., beginning, middle, and ending frequency) did not vary significantly with age (see Results); therefore the same values were used in all four stimuli. A preliminary experiment testing the effectiveness of synthetic stimuli revealed no significant difference in response compared to natural signals (proportion of

females responding: natural = 0.82, synthetic = 0.91; $\chi^2 = 0.679$, $p = 0.41$, $N = 11$

females). Each stimulus had the typical *U. crassicornis* bout structure of three signals of equal amplitude separated by an interval of 2 s between signals. Prior to the playback experiment I digitally filtered the stimuli at a distance of 5 cm from the transducer (see below) to compensate for the differential filtering of their frequency components from propagation along a plant substrate. For a detailed description of the compensation procedure see Cocroft and Rodríguez (2005).

Playback procedure

I performed the experiment from April-May 2007 in a climate controlled room adjacent to the greenhouse where the *U. crassicornis* colony was kept. I used females that were second-generation offspring of individuals collected from Florida in 2006, and so they were not related to adults from whom vibrational signals were characterized in 2003 and 2005. Females were unmated and between 3-5 weeks old post adult eclosion, at the peak of their receptivity. I used a potted *A. julibrissin* plant (height = 50 cm) to play back stimuli to females. I attached a magnet to the stem of the plant with wax (Endevco, San Juan Capistrano, CA) and placed an electromagnet (Edmund Scientifics, Tonawanda, NY) about 5 mm from it. The electromagnet received signals from a Dell PC notebook which were amplified with a RadioShack MPA-250 amplifier. Stimulus presentation was controlled with a custom-written program in Matlab. To minimize noise generated by building vibrations, the plant rested on a flat piece of iron (50 x 50 x 2 cm) that was placed upon an inflated bicycle tire. I isolated the plant further from the table by placing it on a thin sheet of shock-absorbing sorbothane (Edmund Scientifics, Tonawanda, NY).

I recorded the playbacks (and female responses) using an accelerometer (Endevco 256-100 connected to an Endevco 133 signal conditioner), that was attached to the plant stem (diameter = 3 mm) with wax 5 cm below the magnet. For testing, I placed one female at a time on the plant ≤ 5 cm from the magnet. Recordings were high-pass filtered at 60 Hz with a Krohn-Hite 3202 filter and low-pass filtered at 10 kHz with the Endevco signal conditioner. The filter output was acquired with a Marantz PMD-670 solid state recorder (Marantz America, Inc. Mahwah, NJ), at a sampling rate of 44.1 kHz. I monitored the playbacks with headphones connected to the Marantz, and visually with a Tektronix 2225 oscilloscope (Tektronix, Inc., Richardson, TX).

I adjusted the amplitude of the stimuli at the point females were placed on the stem. I used a peak amplitude (0.18 mm/s) corresponding to the average amplitude of a male signaling about 5 cm away, on the basis of measurements from 34 males. I allowed females to settle for a few minutes after being placed on the plant, and they tended to remain in the same place during the playback. If a female moved I terminated the trial, and tested the female again later. The temperature of the room was maintained at $25 \pm 1^\circ\text{C}$.

Female response analysis

Every female received four signal bouts, each one representing one of the four age classes. I randomized the order of the stimuli for each female, and each stimulus was separated by an interval of 10 s. I tested a total of 50 females (10 females from each of 5 different families). I analyzed the recordings with Raven v. 1.2 and measured whether females gave reply calls to the different stimuli. My experimental unit is an individual female. I included female family in the statistical model with female identity nested

within family. I used logistic regression in JMP v. 5.1 (SAS Institute Inc., 2003) to evaluate differences among females in their response to signals from the different age classes. I analyzed female responses in two ways: first, whether females replied or not to a particular stimulus, and second, the number of responses (0 - 3) to each stimulus.

Results

General patterns of age-based signal variation

There were significant age-based differences in several of the signal parameters measured (Fig. 2). As males aged, they gave longer signals with shorter rise-times, and a greater proportion of signals within a bout contained AM. In general, most of the spectral features varied less with age than did temporal features.

Discriminant function analysis

There were some differences between signals recorded in 2003 vs. 2005. Results showed that 84% of the 237 cases could be correctly assigned to their proper year. The first discriminant function (DF1) explained 100% of the variance (eigenvalue = 1.231, canonical correlation = 0.743). Dominant frequency had the highest loading, indicating that this parameter was the greatest source of signal discrimination between years. Based on the high percentage of cases being correctly classified to year, I analyzed signals obtained in the different years separately to avoid confounding age-related variation with other potential sources of variation resulting from the recording year.

For signals obtained in 2003, 65% of the total cases were correctly assigned to age class. DF1 explained 66% of the variance (eigenvalue = 1.277, canonical correlation

Table 2. Loadings from a discriminant function analysis assessing whether signals could be reliably grouped according to age. Signals recorded in different years were analyzed separately. Numbers in bold denote the parameter with the highest loading with the first canonical discriminant function. For both years temporal parameters had higher loadings than most spectral parameters

Parameter	2003	2005
Temporal		
Duration	0.386	0.611
Rise-time	-0.319	-0.471
Proportion of calls with AM	0.744	0.412
HF click rate	0.438	0.499
Spectral		
Beginning frequency	-0.141	0.102
Middle frequency	-0.372	-0.029
Ending frequency	0.046	-0.262
Dominant frequency	0.077	0.248
LF-HF amplitude difference	0.655	0.789

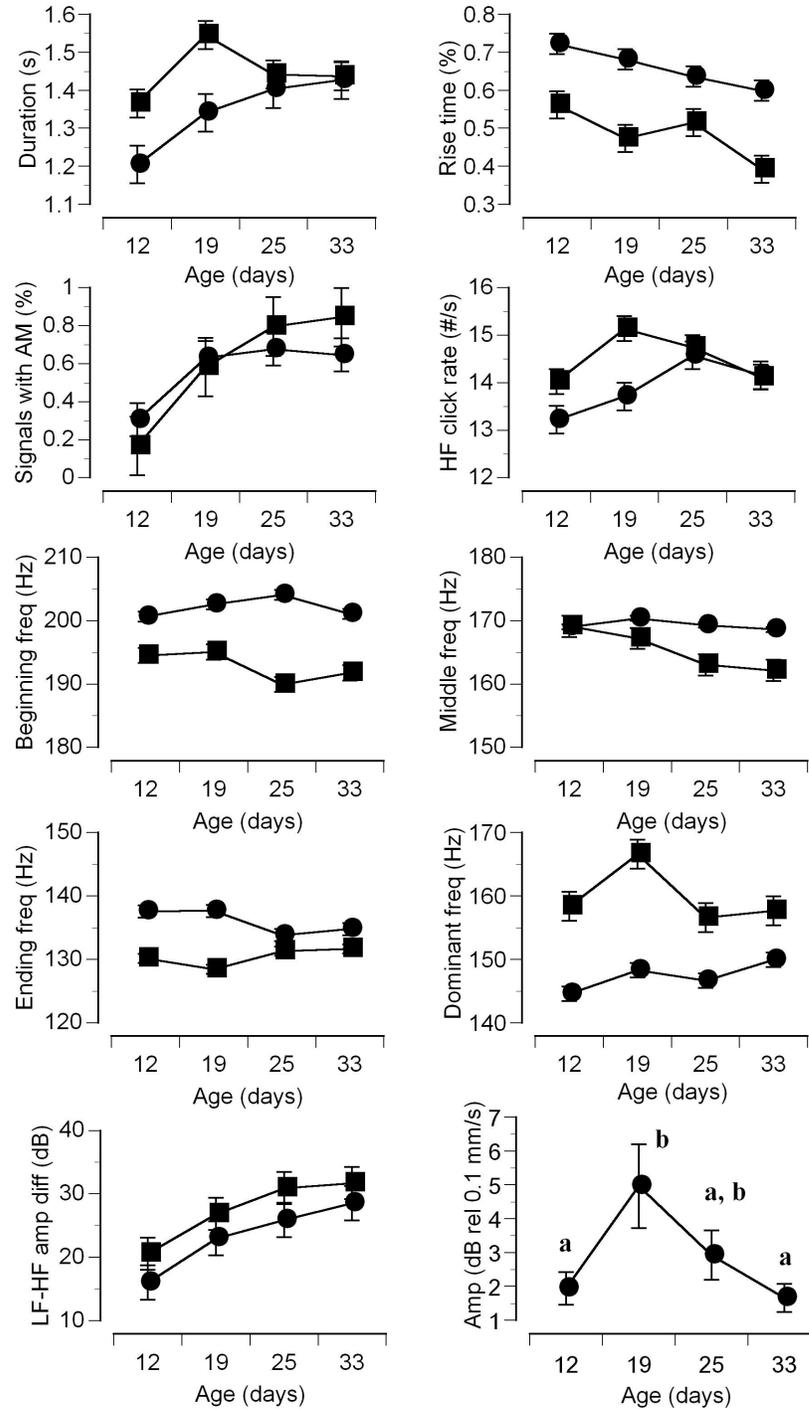


Figure 2. Change with age in the values of signal parameters. For each graph data points represent the mean (\pm SE). Squares = signals from 2003 ($N = 32$ males); circles = signals from 2005 ($N = 34$ males). For peak amplitude (bottom right), letters indicate age classes that are significantly different using a Student's *t* post-hoc test.

= 0.748). In general, temporal parameters had higher loadings (Table 2), and thus provided greater levels of discrimination with age than spectral parameters (except for the LF-HF amplitude difference). The proportion of signals containing AM had the highest loading with DF1.

For signals obtained in 2005, 54% of the total cases were correctly assigned to age class. DF1 explained 90% of the variance (eigenvalue = 1.12, canonical correlation = 0.727). As with signals from 2003, temporal parameters had higher loadings (Table 2), although the rank order was slightly different. In 2005, the LF-HF amplitude difference had the highest loading with DF1.

Classification scores were all greater than the random expectation of 50%, indicating that observations from different age classes were, for the most part, distinct from each other (Table 3). Signals from the youngest age class were most distinct from those of the 3 older age classes, with classification success ranging from 72 – 100%. Signals of 25 vs. 33 day old males had the lowest scores and thus were most similar, but they were still grouped correctly about 60% of the time. Classification scores for 2003 were similar to those for 2005, indicating that although signals from different years were distinct according to the DFA, the magnitude of the differences separating signals by age was comparable between years.

Peak amplitude

Peak amplitude varied significantly with male age (Fig. 2, bottom right). Signals with the highest intensity were produced by 19 day old males, while signals with the lowest intensity were produced by 12 and 33 day old males. The average difference in peak amplitude between these age classes was about 3 dB, which translates into a factor

Table 3. Pair-wise comparisons of the percentage of cases correctly assigned. Although signals recorded in 2003 and 2005 were analyzed separately, they showed similar patterns of correct groupings. All scores were larger than the random expectation of 50%, indicating that signals from different age classes were mostly distinct from one another

Age comparison (days)	2003	2005
12 vs. 19	83	72
12 vs. 25	100	95
12 vs. 33	98	93
19 vs. 25	74	67
19 vs. 33	88	82
25 vs. 33	66	62

Table 4. Repeated-measures ANOVA assessing the effect of various factors on the peak amplitude (velocity) of vibrational signals

Source	df	<i>F</i>	<i>p</i>
Male (Family)	30	2.04	0.006
Family	3	10.11	0.001
Age	3	4.54	0.005
Family x age	9	1.16	0.329

of about 1.4. There were also significant differences in peak amplitude among males within a family, and between families (Table 4). There was no family \times age interaction, indicating that peak amplitude changed over time in a similar way across the different families.

Female response to age-related signal variation

The probability of a female responding was not significantly influenced by the age class of the stimulus (log-likelihood ratio (LLR) $\chi^2 = 3.16, p = 0.37$). The proportion of females that responded to each stimulus ranged between 90-96%. Neither the family of origin, nor individual identity of a female, influenced the likelihood of response (family: LLR $\chi^2 = -0.00013$; female: LLR $\chi^2 = 59.77, p = 0.07$). Note that the negative χ^2 value for family was probably due to a rounding error, and no associated p -value was provided in the statistical output.

The number of responses given by females was not influenced by the age class of the stimulus (LLR $\chi^2 = 6.29, p = 0.1$). The average number of responses per female for all stimuli ranged from 2 - 3 (Fig. 4). Family identity did not influence the number of responses given to a particular stimulus (family: LLR $\chi^2 = 0.00076, p = 1.0$). However, there was a significant effect of female identity, indicating individual-level differences in the number of responses given to the different stimuli (LLR $\chi^2 = 171.79, p < 0.0001$).

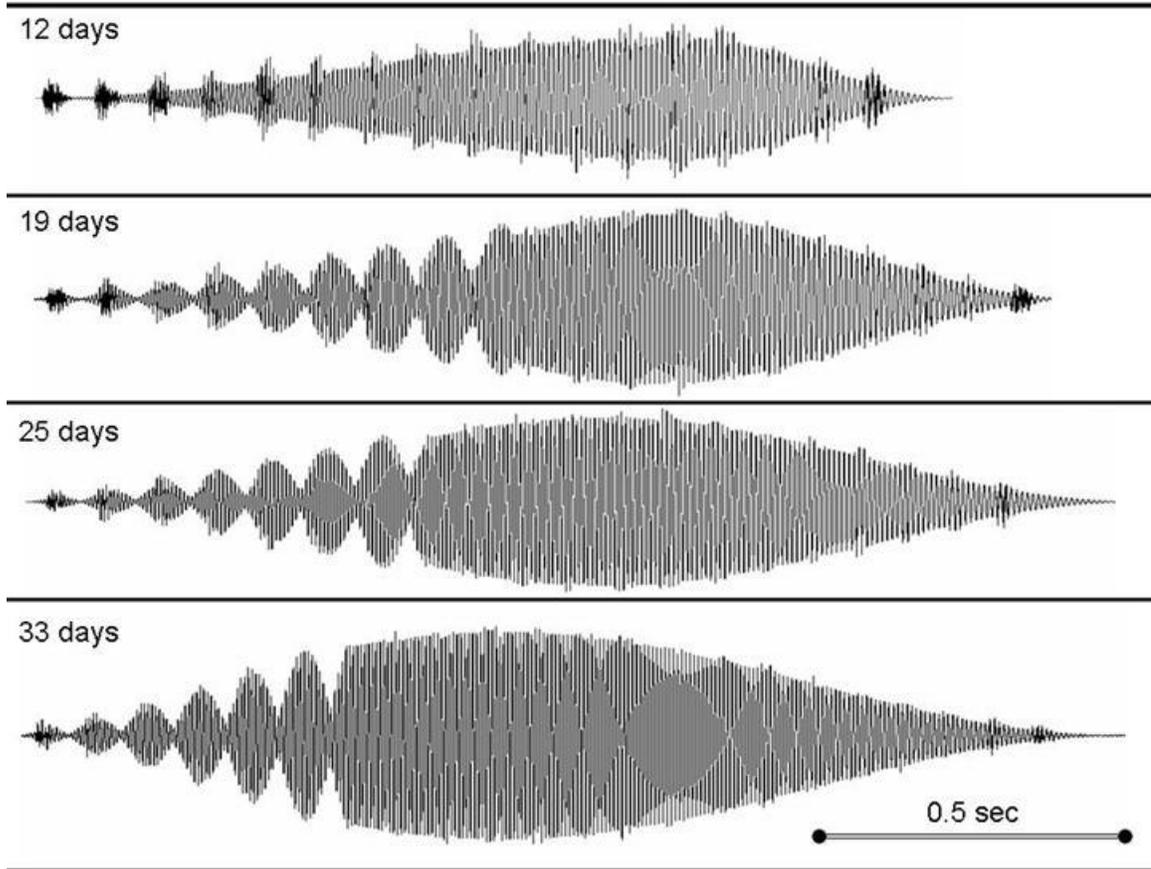


Figure 3. Waveforms of synthetic stimuli used in the playback experiment. Stimuli were constructed using parameter mean values from 2005. See text for additional details.

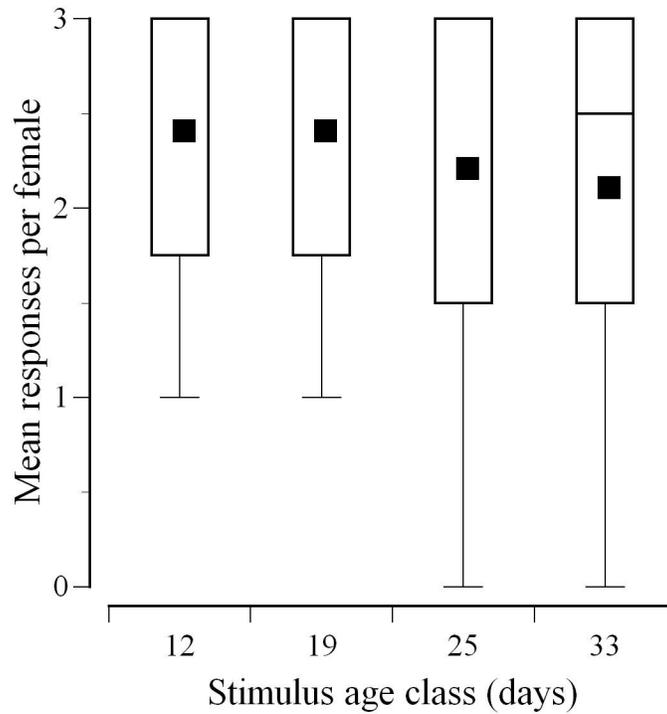


Figure 4. Box-plots showing the mean number of responses per female (black squares) to signals from different age classes. Each female received all 4 stimuli ($N = 50$ females). Rectangles: 75th and 25th percentiles; bottom whiskers: 10th percentile. There was no significant difference in the number of responses given to signals from different age classes.

Discussion

The vibrational signals of *U. crassicornis* show a level of plasticity not usually associated with insect mating signals. This study is the first to show complex age-based variation in multiple features of a vibrational mating signal. Male signals contain several age-dependent features that could potentially provide females with reliable age cues. However, the playback experiment revealed no significant effect of this variation on female preferences. Thus, females do not appear to use mate-advertisement signals to choose older males. Nonetheless, these results offer some insight into potential factors that may affect age-related changes in signal expression.

The ontogeny of signaling in *U. crassicornis* is analogous to the pattern of song maturity in birds, in that a lengthy period of time is required for male songs to develop (Garamszegi et al., 2007; Gil et al., 2001; O'Loughlen and Rothstein, 2003; Poesel et al., 2006). In fact, the onset of signaling in *U. crassicornis* is atypical for insects, beginning about 10 days following adult eclosion. In other species, males usually begin calling as early as 1 - 4 days following adult eclosion (Cade and Wyatt, 1984; Ciceran et al., 1994; Kumar and Saxena, 1985; Souroukis et al., 1992; Walker and Cade, 2003; Zeigler and Stewart, 1985). The fact that signaling takes a relatively long time to commence in *U. crassicornis* suggests that the underlying mechanisms controlling signal production require an extensive period of development and coordination, and thus may reflect a male's physical condition following adult eclosion (Elias et al., 2006; Holzer et al., 2003; Scheuber et al., 2003a). Variation in the onset of signaling may also be linked with the timing of other behaviors such as dispersal, and this could have implications for male

mating success by affecting how soon males are able to begin mate searching following adult eclosion (Alexander and Stewart, 1996).

Females in duetting species are expected to be highly selective when replying to male signals in order to avoid the increased risks and energy costs associated with attracting a large number of males (Bailey, 2003; Greenfield, 2002; Jennions and Petrie, 1997). In this study, however, females showed a wide preference for signals, which suggests that under natural conditions they may respond to a range of males when soliciting potential mates. In fact, the mating ecology of *U. crassicornis* often includes females interacting with several males at once during courtship (Wood, 1974; Wood and Dowell, 1985), which is supported by the playback results. This has implications for the conditions under which female mating decisions might be made in this species. Since female *U. crassicornis* mate only once (Wood, 1974), responding to a wide range of signals could be adaptive by allowing females the opportunity to evaluate several males up close before making an important mating decision (Alexander et al., 1997; Bateson and Healy, 2005; Janetos, 1980). Attracting several males at once may also facilitate mate choice through male competition (Wong and Candolin, 2005). By instigating agonistic interactions between rivals during courtship, females might be attempting to increase their chances of mating with a dominant high quality male (McGhee et al., 2007; Parri et al., 1998).

The difference in peak amplitude between the vibrational signals of older (33 day) and younger (19 day) males might be related to changes in other aspects of mate acquisition behavior occurring with age. Male *U. crassicornis* begin dispersing from their natal site when they are 15-20 days old (Wood and Dowell, 1985). Recently dispersed,

sexually active males might therefore be at the peak of their physical condition, and thus able to produce higher intensity signals when advertising for mates. As males get older, the decrease in the amplitude of their signals may be the result of declines in condition, or changes in the investment of signaling towards other aspects of mate acquisition, such as mate searching, courtship, or signaling effort. Age-based changes between different components of male reproductive behavior have been documented in many species (Hunt et al., 2006; Marden and Wagge, 1990; Moreira, 1993; Otronen, 1995; Petersson, 1989; Plaistow and Siva-Jothy, 1996; Saino et al., 2002; Zeigler and Stewart, 1985), and will be the subject of investigation in Chapter 4.

Upon adult eclosion *U. crassicornis* individuals are unable to fly for about 10-15 days (Wood, 1974), which suggests that their flight musculature requires a period of maturation before it is able to function. Similar patterns have been described in other insect taxa. For example, in honeybees age-related changes in flight muscle biochemistry occurring 14-21 days following adult eclosion prepare hive workers to conduct long-distance foraging trips (Roberts and Elekonich, 2005). In many species of Diptera and Odonata, age-related changes in flight muscle physiology occur 5-15 days following adult eclosion to facilitate dispersal, mate-searching, and territory patrol (Collatz and Wilps, 1986; Dudley, 2000; Kutsch, 1989; Marden, 1989, 2000; Zera and Denno, 1997).

The close synchrony in the onset of flight and signaling in *U. crassicornis* suggests that these behaviors may share a common physiological basis. Although the putative muscles responsible for producing the LF component have not been identified in *U. crassicornis*, one possible source is the thoracic muscles that control flight. The frequency range of the LF component is within the range of wing beat frequencies

reported for similarly-sized insects (Casey, 1989). Also, bi-functional flight muscles are used by males in many insect groups to control the production of acoustic or vibrational mating signals (e.g., Hemiptera: Gogala, 2006; Orthoptera: Hennig, 1990; Hymenoptera: Hrnčir et al., 2006; Diptera: Kanmiya, 2006). In this study, the fact that temporal parameters varied more with age than most of the spectral features suggests that males were improving their fine control of signal production as they got older, possibly in conjunction with the development of their flight motor pattern. At present this is just speculation, and thus must await experimental confirmation.

Most of the spectral parameters of *U. crassicornis* signals did not vary significantly among males or age classes. Low within-population variation in spectral parameters tends to occur when they are used for species recognition (Claridge, 1985b; Price and Lanyon, 2004; Rodríguez et al., 2004; Schul and Patterson, 2003). In some species of acoustical insects and anurans the dominant frequency of male signals is correlated with body size, and females may use it to discriminate in favor of larger males (Bailey et al., 1990; Bennet-Clark, 1998; Gerhardt and Huber, 2002; Jacot et al., 2007; Simmons, 1995; Wedell and Sandberg, 1994). However, a previous study with *U. crassicornis* found no significant correlation between male body size and dominant frequency (Cocroft and De Luca, 2006). In this study, dominant frequency was also the major source of signal discrimination between years, which likely resulted from the use of different *A. julibrissin* plants as recording substrates. This finding is in agreement with other studies that show dominant frequency to be highly substrate dependent (Cocroft et al., 2006; Cökl and Virant-Doberlet, 2003; Michelsen et al., 1982).

The only spectral parameter that varied significantly with age was the LF-HF amplitude difference. In some species of Orthoptera females use the relative amplitude difference between low and high spectral song components to determine the distance to a signaling male (Bailey et al., 1990; Keuper and Kuhne, 1983; Latimer and Sippel, 1987; Morris et al., 1978; Römer and Lewald, 1992; Schul and Patterson, 2003). However, unlike orthopteran insects where females typically localize to singing males, female *U. crassicornis* remain stationary and wait for males to come to them. Thus, the potential for LF-HF amplitude difference to facilitate localization in this kind of system requires further study, especially since the playback results showed that female preferences were unaffected by variation in this parameter.

The increase in the LF-HF amplitude difference with age may reflect changes in the way males stressed the two spectral components during signaling. Some theoretical models predict that males should increase signaling effort as they get older (Candolin, 2000; Kokko, 1997; Polak and Starmer, 1998; Proulx et al., 2002), and since most of the energy in an *U. crassicornis* male signal comes from the LF component, older males may be emphasizing it more, possibly to increase overall signal attractiveness (Brooks and Couldridge, 1999; Elias et al., 2005; Miller and Brooks, 2005; Ryan and Keddy-Hector, 1992). Alternatively, age-associated wear of the tymbal used to generate the HF component may have resulted in a decrease in the amplitude with which the clicks were produced relative to the LF component. Although few studies have investigated age-based wear of signal production mechanisms in arthropods (Hartley and Stephen, 1989; Ritchie et al., 1995), it could be one reason why the HF clicks are less pronounced in the vibrational signals of older males.

Our study has some implications for the role of age as a source of variation. First, although more studies in recent years have begun to investigate age effects on mating signals in invertebrates like arthropods, most have focused on a single insect group (Orthoptera). Additional taxa need to be examined, as well as other signaling modalities (e.g., Kaltenpoth and Strohm, 2006), in order to determine the generality of age as source of variation. For example, it might be interesting to determine if the expression of bioluminescent mate attraction signals (e.g., fireflies) are also affected by age. Second, some cross-sectional studies that report age-related signal variability only measured males once, and even when multiple measurements were made, the exact ages were unknown (e.g., Ciceran et al., 1994). These analyses may not correctly estimate the magnitude of within-male variation, which could alter the inferences researchers make regarding age as a source of signal variation. And third, future studies are encouraged to include playback experiments that measure the extent to which age-related signal variation matches female preferences. At present, only one other study in invertebrates has done so (Ritchie et al., 1995). The usefulness of including playbacks is that they allow researchers to estimate the relative contribution of ultimate (female preferences) vs. proximate (ageing) processes. Furthermore, results from playbacks can also provide a springboard from which to develop new hypotheses regarding signal function. In short, by integrating both proximate and ultimate analyses, our understanding of the various factors that maintain variation in mating signals within a population can be greatly increased.

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Chapter 4

Age effects on mate acquisition behavior in *U. crassicornis*

Abstract

A male's success at obtaining a mate depends upon many factors, including his mate searching ability, long-range advertisement signals, and courtship behavior. These aspects of mate acquisition often vary with age as a result of changes in a male's condition, social environment, or mating experience. Age-related variation in male behavior can affect the choices made by females when selecting mates. In the treehopper *Umbovia crassicornis*, females mate more often with older males, but the factors that provide older males with a mating advantage are unclear. Mate searching in this species involves the use of a call-fly/walk strategy, and my goal in this study was to determine if age-related variation in the ability of males to find and solicit matings from females explained the mating advantage of older males. I performed an experiment that monitored age-based changes in patterns of flight, within-plant movement, courtship duration, and signaling effort. Results showed that as males aged, decreases in flight activity were accompanied by more within-plant searching behavior. Older males required more time to find females on a host plant; however, once located they remained in courtship for longer periods compared to younger males. Signaling effort increased as males dispersed and began mate searching, but then decreased as males continued to age. Taken together, these results suggest that *U. crassicornis* males adopt different mate searching tactics as they age. These alternative tactics may also be influenced by the spatial distribution of females. For example, younger males may be better at finding solitary dispersed females,

while more sedentary older males may be better at soliciting matings from aggregations of pre-dispersal females.

Introduction

A male's success at obtaining a mate depends upon many factors, including his mate-searching ability, long-range advertisement signals, and courtship behavior. These aspects of mate acquisition often vary over time because of age-associated changes in a male's condition, social environment, or mating experience (Bertram, 2000; Kemp, 2002b; Kemp et al., 2006; Marden and Rollins, 1994; Miller and Brooks, 2005; O'Loughlen and Rothstein, 2003; Otronen, 1995; Petersson, 1989; Vanhooydonck et al., 2005). Age-based behavioral variation can take one of two forms. In the first, discontinuous variation occurring with age results in discrete groups using alternative tactics. For example, in some species where males fight for access to females, younger males do not engage in physical combat with experienced older males, but instead employ sneaky or satellite behavior in order to mate (Andersson, 1994). In the second, age-based behavioral variation exhibits a continuous gradation over time (Caro and Bateson, 1986). In these cases, it is the intensity, rate, or duration of a behavior that varies with age, rather than the type. Irrespective of whether age-related variation results from qualitative or quantitative differences, an important consequence is that males often behave differently at different ages, and this can affect the choices made by females when selecting a mate. Thus, understanding how different aspects of male mating behavior change over time can provide insight into the factors affecting patterns of age-based mate choice.

In the treehopper *Umbonia crassicornis* (Hemiptera: Membracidae) females mate more often with older males (Chapter 2). However, the proximate mechanisms that provide older males with a mating advantage are unclear. Males advertise for females using substrate-borne vibrational signals, and female replies elicit searching behavior from males (Cocroft and McNett, 2006). In Chapter 3 I showed that male vibrational signals contain potentially useful age cues; however, a subsequent playback experiment revealed that females do not prefer the signals of older males. Nevertheless, other aspects of signaling behavior might be important in affecting the outcome of mate choice in *U. crassicornis*. One such factor is signaling effort (i.e., number of signals given per unit time). This is often used as a predictor of male mating success in many species because it reflects the energy devoted to signal production (Gerhardt and Huber, 2002; Hedrick, 1986; Hunt et al., 2004a; Jang and Greenfield, 1998; Ryan and Keddy-Hector, 1992). Males are predicted to increase their signaling effort with age as their residual reproductive value declines (Bentsen et al., 2006; Jacot et al., 2007; Kokko, 1997; Stearns, 1992), and since duetting facilitates pair formation in *U. crassicornis*, increased signaling may give older males an advantage during mate searching. Males that signal more might receive more replies, which could improve their ability to locate receptive females (Bailey, 2003; Greenfield, 2002).

In addition to signaling effort, another aspect of mate acquisition behavior that may contribute to male mating success in *U. crassicornis* is courtship. After locating a female, males initiate courtship by climbing onto one side of her body (Wood, 1974), and producing a series of vibrational signals while attempting genital contact (unpublished data). Only about one-third of courtships result in mating (Chapter 2), which suggests

that variation among males in courtship ability may affect mating success. One aspect that might be relevant is the amount of time a male spends courting, as this affects the probability of mating in many species (Conner, 1989; Demary et al., 2006; Felton et al., 2006; Kotiaho, 2002; Kotiaho et al., 1996; Petersson, 1989; Tallamy et al., 2003; Woodhead, 1986).

Mate searching behavior is another component of mate acquisition that may influence male mating success in *U. crassicornis*. As males reach sexual maturity they disperse from their natal aggregation and begin searching for females (Wood and Dowell, 1985). As in many species of insects, *U. crassicornis* males employ a call-fly/walk strategy when searching for mates. Flying males will land on a plant, signal for a short while, and if no female replies are received they may search briefly within the plant or fly to another (Cocroft and McNett, 2006). In Chapter 2 I found that older males performed significantly more courtships than younger males, and one possible explanation for this is that older males are more successful in finding females during mate searching.

The role of flight in mate searching has typically been studied in species where males compete for territories or breeding sites by engaging in aerial fights with other males (Kemp, 2000; Marden and Wagge, 1990). In these studies, both age and condition affect the outcome of contests. Older males tend to be in poorer condition as a result of their longer participation in activities related to mate acquisition (Otronen, 1995; Petersson, 1989). However, their experience in fighting or holding a territory often offsets their physical disadvantage (Kemp, 2002a; Marden and Rollins, 1994; Martin et al., 2003; Plaistow and Siva-Jothy, 1996). For insects like *U. crassicornis* where males use a call-fly/walk strategy rather than engage in aerial combats with rivals, the role of

age or condition in affecting flight behavior has not been addressed. Flight activity typically decreases with age in insects (Petersson, 1989; Sohal, 1976), which could affect mate finding by altering patterns of search behavior over time. One potential consequence of this is that as males age, they may change their mate searching tactics to compensate for a reduction in their flight ability.

The goals of this study are to assess age-based changes in different aspects of mate acquisition in *U. crassicornis*. I use a longitudinal (within-male) approach to monitor the ontogeny of several behaviors over the first month of adult male's life. This time frame reflects the relevant period of male sexual activity in this species. Specific mate searching behaviors that I will examine include flight activity and within-plant movement rates. I will also examine changes in courtship duration and signaling effort. In addition, I will measure the extent to which changes in a male's physical condition over time correlate with variation in these different aspects of mate acquisition.

Methods

Insect maintenance

Individuals used in this study were first-generation descendents of insects collected in Miami, FL, USA in 2006. I collected individuals as late-instar nymphs or teneral adults that were still aggregated in their family group, and collected a total of 9 families (each comprising 20-40 individuals). I kept the families in a greenhouse at the University of Missouri-Columbia and maintained them on their own potted *Albizia julibrissin* (Mimosaceae) host plant isolated within a screen cage. Approximately one week after adult eclosion I separated the sexes in each family to prevent sibling mating. I

produced the families used in this study through selective mating of unrelated individuals.

Study design

I monitored mate acquisition behaviors once per week over 4 consecutive weeks beginning at the onset of sexual maturity (~12 days following adult eclosion). A total of 12 families of males was examined. My experimental unit for statistical analyses was a group of males from a single family. I conducted the experiment between November 2006 and January 2007, in a temperature and humidity-controlled room. Illumination was provided by 4 34 Watt flat spectrum bulbs positioned 20 cm above each cage (see below). Temperature was maintained at $25 \pm 2^\circ\text{C}$, and the humidity ranged between 40-50%.

I used 2 cages (145 x 68 x 92 cm) that were monitored simultaneously. Within each cage I placed 2 potted *A. julibrissin* plants (approximate height of each = 80 cm), set at opposite ends. I marked each *A. julibrissin* plant with colored nail polish in 5 cm intervals along its stem, branches and petioles. The top, bottom, and sides of each cage were also marked in 5 cm intervals along the wood frame, so that if any insects moved off the plants I could still record their location.

I randomly selected individuals from their respective family groups for use in this study. I placed 6 adult sibling females into each cage (3 per plant), releasing them near the tops of the plants. The females were between 10-15 days old post-eclosion and so were not yet sexually mature (Chapter 2). After the females were released I then placed 6 sibling males into the cage (3 per plant), but these were placed near the base of the plants. Males were not related to females within the same cage. I marked each male with a numbered plastic tag ($\sim 3 \text{ mm}^2$) glued to the left side of the pronotum, and then weighed

them individually on an electronic balance (Ohaus AS64) to the nearest 0.1 mg prior to being placed into the cage. The identification tag did not significantly add to the weight of a male (average mass of a male \approx 50 mg; average mass of a tag \approx 0.6 mg). Insects were allowed to settle for 0.5-1 hr before observations began. Mortality reduced the number of males in some families over the course of the experiment, therefore, in order to maintain the initial 1:1 sex ratio throughout the study the number of females placed into a cage always equaled the number of males.

When males were not being observed they were maintained with their brothers on a potted *A. julibrissin* plant enclosed within a screen cage. During this time males were kept with an equal number of females from a different family. I did this to provide males the opportunity to interact with females as they would in nature, so that during the observation periods the behaviors I observed would be as natural as possible, and not the result of sexually active males being isolated from females for extended periods of time.

Mate searching behavior

I examined age-based changes in two aspects of mate searching behavior: within-plant movement and flight activity. Each week I conducted observations of both cages every 15 minutes for 3 hours. This protocol enabled me to determine which movements were due to males walking or flying from one location to another.

I assessed within-plant movement by calculating how far each male moved over the 3 hour period. I only included movements that males made when walking from one location to another within a plant. For each male I calculated an average movement rate (cm moved/15 min), and then averaged the data for all males within the cage to obtain a family mean.

I assessed flight activity for each male by calculating how often a male initiated a flight event over the 3 hour period. I divided the number of flights a male performed by the total amount of time he spent on the plants (# flights/min). I only included flights that were initiated from the plants and ignored those that were made from the top, bottom, and sides of the cage. I then averaged the data for all males within the cage to obtain a family mean.

Courtship behavior

In *U. crassicornis*, males will attempt to court both receptive and unreceptive females (Chapter 2). However, female response to courtship is dependent upon whether they are sexually mature. Unreceptive females allow males to court freely without interruption, whereas receptive females will often reject some courting males by buzzing their wings repeatedly to get them off (personal observation). This difference in response to courtship may be because receptive females are assessing a potential mate, whereas unreceptive females are not, and therefore do not react to the male's advances. I therefore decided to use unreceptive females in order to eliminate female receptivity as a potential confounding source of variation on the amount of time a male spent in courtship. Thus, any variation in courtship duration I observed would not be influenced by cues received from females, but rather on age-based differences occurring within (or among) males. When a courtship was observed I recorded the identity of the male, and measured the duration (in minutes) from the time the male climbed onto the female to when he dismounted.

Signaling effort

I monitored signaling effort by taking 4 minute recordings every 30 minutes during each 3 hour observation period. It was not possible to obtain individual rates of signaling because the recording procedure did not enable me to distinguish which males were producing signals. However, I was able to obtain family-based rates of signaling. I attached a pickup transducer (Signal Flex SF-30) to each plant to detect vibrational signals produced by males. Signals from both pickups were amplified with a mini-amplifier (Johnson JA-004) and recorded simultaneously onto a Marantz PMD670 solid state recorder using a sampling rate of 44.1 kHz. During recording I monitored the signals visually on an oscilloscope (Tektronix 2225), and acoustically with headphones. Sound files were examined using Audacity v. 1.2.6 analysis software. I counted the total number of signals produced on each plant in the 4 minute period and divided this by the number of males present on each plant when the recording was made. I then averaged the data from both plants to obtain an index of male signaling effort (average number of signals/4 minutes/male) for each family.

Male condition

I calculated a condition index for each male at each age class by taking the ratio of body mass divided by volume (Cotton et al., 2004). Three measures of the pronotum (length, width, and height) were multiplied to estimate body volume. I measured each male twice to the nearest ± 0.01 mm with Manostat calipers, and used the average value in the analysis.

Statistical analysis

Statistical analyses were conducted using JMP IN v.5.1 (SAS Institute Inc., 2003). I examined the effect of age on within-plant movement rates using a repeated-measures

ANOVA with age (fixed effect) and family identity (random effect) as factors. Within-plant movement rate was log transformed to achieve normality (Shapiro-Wilk $W = 0.981$, $p = 0.62$), and homogeneity of variance (Bartlett test, $p = 0.68$), before proceeding.

I examined the effect of age on the average flight rate for each family using a non-parametric Quade test. This test is an extension of the Wilcoxon rank test that allows for post-hoc comparisons between treatment levels (Conover, 1980).

For courtship duration, males in some families courted at only one age, and males in other families never courted at all. This made the data unbalanced due to the presence of several missing cells (i.e., family means) both within, and between age-classes. Because of these limitations, I used a non-parametric Durbin test to evaluate the effect of age on courtship duration. This test is similar to the Quade test, but is appropriate when there are missing cells in the data (Conover, 1980).

I also examined whether age-based differences occurred in the time it took males to locate females and initiate their first courtship. For each male that courted, I calculated the elapsed time from the start of the observation period to when he was observed courting for the first time. As with courtship duration, the data were unbalanced due to some missing family means and so I also used a Durbin test to evaluate the effect of age.

I examined the effect of age (fixed effect) and family identity (random effect) on calling effort with a repeated-measures ANOVA. Values were square-root transformed to achieve normality (Shapiro-Wilk $W = 0.965$, $p = 0.22$), and homogeneity of variance (Bartlett test, $p = 0.08$) before proceeding.

I evaluated changes in male condition using a repeated-measures ANOVA. Condition values were log-transformed to achieve normality (Shapiro-Wilk $W = 0.992$, p

= 0.26), and homogeneity of variance (Bartlett test, $p = 0.82$) before proceeding. Factors in the model included age, family, male (nested within family), and an age x family interaction term. I also assessed whether condition was correlated with variation in the different behaviors that I monitored throughout the study. I calculated separate Pearson correlation coefficients between condition and within-plant movement rate, flight rate, courtship duration, time to first courtship, and signaling effort. Correlations were calculated using family means. Control of the false discovery rate resulting from multiple comparisons was done using the Benjamini and Hochberg procedure (Verhoeven et al., 2005).

For all parametric analyses if a factor was found to be significant we used a Student's *t* post-hoc comparison to evaluate differences between factor levels.

Results

Within-plant movement rates varied significantly with age ($F_{3,33} = 3.83$, $p = 0.018$). Males aged 12 days had the lowest mean rate, while males aged 25 days had the highest mean rate (Fig. 1A). Within-plant movement rates did not differ significantly between males aged 19 to 33 days, however, there was a trend for 33 day old males to move less often. There was no significant effect of family identity on within-plant movement rates ($F_{11,33} = 1.12$, $p = 0.372$).

As males aged, their flight rates decreased significantly (Quade test: $N = 12$ families, $T = 4.62$, $p < 0.05$; Fig. 1B). Pair-wise multiple comparisons revealed

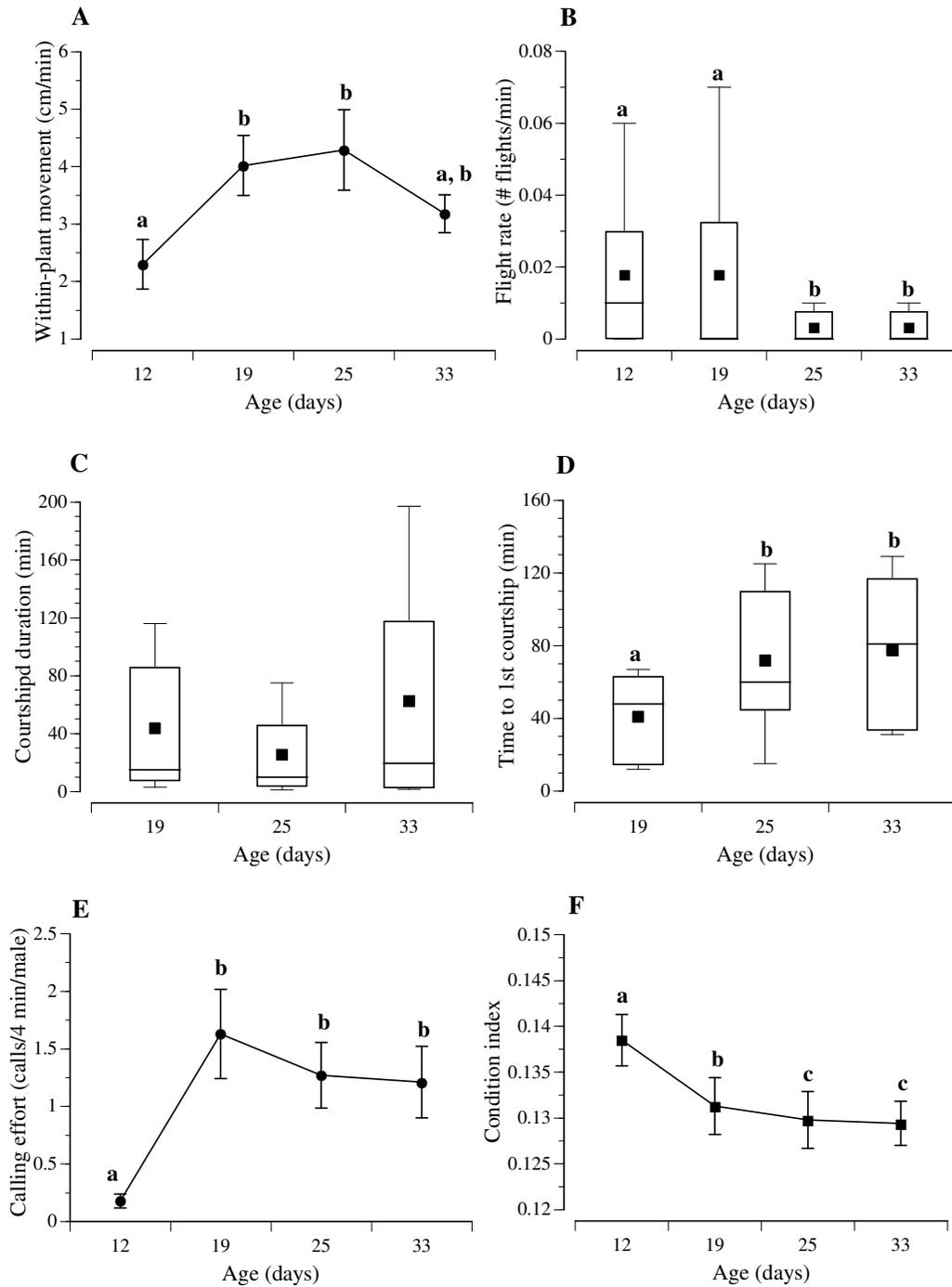


Figure 1. The effect of age on various aspects of mate acquisition. (A) Within-plant movement rate. (B) Flight activity. (C) Duration of time spent in courtship. (D) Time required to initiate courtship. (E) Signaling effort. (F) Physical condition. Graphs A, E, and F show the mean \pm SE. Box-plots B, C, and D show the 90th, 75th, median, 25th, and 10th percentiles, with black squares representing the mean. For all graphs letters indicate which age groups were significantly different following post-hoc tests.

significant differences between 12 and 25, 12 and 33, 19 and 25, and 19 and 33 day old males only ($\alpha = 0.05$, critical $t = 82.01$ for multiple comparisons: 12 vs. 25 days, $t = 116.5$; 12 vs. 33 days, $t = 97.5$; 19 vs. 25 days, $t = 114.5$; 19 vs. 33 days, $t = 99.5$; all $p < 0.05$).

A total of 88 courtships was observed. Males from the youngest age class were never observed courting. For the three older age classes (in increasing order), a total of 33, 41, and 14 courtships were observed, respectively. The shortest courtship lasted only 1 minute, while the longest lasted over 3 hours (Fig. 1C). There was no significant difference in courtship duration between age-classes (Durbin test: $N = 8$ families, $T = 4$, $p = 0.13$), although there was a trend for 33 day old males to court the longest.

The time it took males to initiate their first courtship increased significantly with age (Durbin test: $N = 8$ families, $T = 6.67$, $p = 0.03$, Fig. 1D). Pair-wise multiple comparisons revealed significant differences between 19 and 25, and 19 and 33 day old males only ($\alpha = 0.05$, critical $t = 3.43$ for multiple comparisons: 19 vs. 25 days, $t = 4$, $p < 0.05$; 19 vs. 33 days, $t = 6$, $p < 0.05$).

There was a significant effect of age on signaling effort ($F_{3,28} = 5.18$, $p = 0.006$, Fig. 1E). The youngest males (12 days) produced very few vibrational signals; however, signaling increased significantly when males reached 19 days. Signaling effort then declined with age after 19 days but the differences were not significant. There was a significant effect of family identity ($F_{11,33} = 2.16$, $p = 0.048$).

Physical condition declined significantly with age ($F_{3,108} = 59.71$, $p < 0.0001$, Fig. 1F). There were also significant effects of family ($F_{11,108} = 107.74$, $p < 0.0001$), and male

identity ($F_{60,108} = 20.59, p < 0.001$). A significant age x family interaction also occurred ($F_{33,108} = 3.18, p < 0.001$).

Correlations between condition and different aspects of male behavior are listed in Table 1. The only noteworthy correlation was in the time to first courtship. Males in better condition required less time to initiate their first courtship than males in poorer condition, although the relationship was marginally significant (Pearson $r = -0.419, p = 0.05, N = 22$).

Table 1. Pearson correlations (r) between physical condition and different aspects of mate acquisition behavior. The analysis between condition and signaling effort excluded data from 12 day old males due to the extremely low level of signaling for this age class. The correlation between condition and time to first courtship remained marginally significant following the Benjamini and Hocheberg procedure for multiple comparisons

Behavior	r	F	p	N
Within-plant movement rate	0.008	0.003	0.96	48
Flight rate	0.036	0.061	0.81	48
Courtship duration	0.126	0.339	0.57	22
Time to first courtship	-0.419	4.26	0.05	22
Signaling effort	0.029	0.029	0.87	36

Discussion

Age influenced multiple aspects of mate acquisition, although the patterns varied according to the specific behavior. In general, younger males (12 and 19 days) had significantly higher levels of flight activity compared to older males (25 and 33 days). Within-plant movement rates increased steadily from 12 to 25 days, but then declined for 33 day old males. Males required more time to find females on a plant as they got older, however, once located 33 day old males tended to remain in courtship for longer periods. Taken together, these results suggest that *U. crassicornis* males may be adopting different mate searching tactics as they age.

The high level of flight activity for 12 day old males combined with low within-plant movement rates are consistent with the start of dispersal that occurs at this age in the field for *U. crassicornis* (Masters, 1997; Wood and Dowell, 1985). In addition, the low signaling rates and complete lack of courtship behavior, indicate that 12 day old males have not yet begun to conduct intensive mate searching. Therefore, in my discussion below I will limit my focus to the three older age classes, since it is within this age range that the complete repertoire of reproductive behavior is expressed, and thus can be fully compared.

Recently dispersed, sexually active *U. crassicornis* males are in better condition than older males, which is reflected in their higher levels of flight activity. Mate searching involves a significant amount of flying in this species (Cocroft and McNett, 2006), which suggests physical condition may be important. The fact that condition was not significantly correlated with variation in flight activity may be a result of the index used to estimate condition. The ratio of mass/volume may be too simplistic, as it neglects

other physiological factors such as lipid or carbohydrate levels that may be better predictors of a male's energetic ability (Cotton et al., 2004; Marden and Wagge, 1990; Matsubara et al., 2005; Otronen, 1995). Since linear measures of the pronotum were used to calculate volume, they were fixed upon adult eclosion. Thus, the decline in condition with age was due to males losing mass, which may have resulted from age-related declines in stored fuel reserves (Kemp, 2002a; Marden and Rollins, 1994; Sartori et al., 1992; Yuval et al., 1994).

Mate searching in many species of insects includes the use of a call-fly/walk strategy (Claridge, 1985a; Claridge and de Vrijer, 1994; Cocroft, 2003; Demary et al., 2006; Hunt and Nault, 1991). However, the effect of intrinsic processes like condition and age in influencing the expression of a call-fly/walk strategy within a population of males has not been explored. In this study, both age and condition represent important factors affecting flight and movement activity, which provides some insight into the proximate mechanisms that may cause *U. crassicornis* males to change their mate searching tactics over time. Although younger males in good condition are able to search larger areas for females, this may come at the cost of increasing their risk of predation (Gwynne, 1987). As males age, decreases in their flight activity indicate that the call-fly strategy is replaced by more sedentary behavior, which includes increasing the time spent in courtship with females.

Males aged 19 days required about half the time to initiate their first courtship opportunity compared to 33 day old males, probably because their higher rates of within-plant movement enabled them to locate females faster. Since within-plant mate searching involves a great deal of walking, being able to cover a large area in a relatively short

amount of time may increase the probability of finding a female. Recall that females in this study were unreceptive, and therefore did not provide reply calls to signaling males. However, the ability of younger males to still locate females more quickly than older males even without female assistance reveals a significant difference in the activity levels of younger vs. older males. This difference is surely an asset for younger males when females are receptive and provide reply calls, as one major risk in duetting systems is eavesdropping by other males (Greenfield, 2002). Since *U. crassicornis* females mate only once, locating a responding female as quickly as possible is probably important in order to prevent the potential loss of a mating opportunity to an interloping male.

Older males tended to court for longer periods, although the wide range of values within each age class indicates a substantial amount of individual-level variation. Since females were unreceptive, they were passive participants and did not influence the duration of time males spent in courtship. Variation in the time devoted to courtship therefore reflects decisions by males that may have been based on factors such as female encounter rates, physical condition, or the presence of other males (Conner, 1989; Demary et al., 2006; Gray and Eckhardt, 2001; Parri et al., 1998; Wong and Candolin, 2005; Woodhead, 1986). The low number of courtships from older males in this study contrast with results from Chapter 2 that showed older males performing significantly more courtships than younger males. One factor that might explain this discrepancy is the difference in the spatial distribution of females between the two studies. In the previous study, female densities were much higher (10 females per plant) and females were also receptive. Dense aggregations of receptive females usually result in high levels of male competition (Hu and Morse, 2004; Jones et al., 2000; LeBas et al., 2004; Petersson, 1989;

Wong and Candolin, 2005), which may favor older males in *U. crassicornis*. In this study, by contrast, female densities were much lower (3 females per plant), and females were unreceptive. In this kind of social environment, younger males may opt to spend less time in courtship with unresponsive females, and instead continue mate searching elsewhere.

The low level of signaling behavior by 12 day old males is in agreement with results from Chapter 3 that showed this age to be approximately when males reach sexual maturity and begin producing advertisement signals. By the time males are about 19 days old, most have dispersed from their natal site, and thus the large increase in signaling effort is consistent with the onset of intensive mate searching activity. Although there was a non-significant trend for signaling effort to decline with age after 19 days, this finding should be interpreted with some caution. When monitoring signaling behavior I was unable to differentiate between signals produced during mate-advertisement, and those produced during courtship, as both contexts occurred simultaneously within a cage. Also, there were no obvious differences in the structure of these signals that enabled them to be distinguished during the analysis. As a result, values for signaling effort were based on signals produced in two different social contexts, and thus the lack of a significant difference after males reached 19 days may not be an accurate reflection of the true pattern. Age-dependent differences in signaling rates during mate-advertisement and courtship occur in a number of species, and are often associated with changes in other aspects of mate searching behavior (Bertram, 2000; Felton et al., 2006; Hunt et al., 2004a; Jacot et al., 2007; Miller and Brooks, 2005; Moreira, 1993; Simmons, 1995; Woodhead, 1986; Zeigler and Stewart, 1985). In this study, higher levels of flight activity

by younger males suggest that they may be signaling more during mate-advertisement as a result of their frequent plant to plant searching behavior. In contrast, longer courtship times by older males might be associated with higher rates of courtship signaling. Additional studies need to be conducted in order to investigate these possibilities.

One implication of this study is that the spatial distribution of females may have significant effects on male mate searching behavior. In their natural habitat, the distribution of *U. crassicornis* individuals is variable even within the same general area. It can range from a few females scattered among several plants, to extremely dense aggregations that sometimes reach hundreds of individuals on one plant (Masters, 1997; Wood, 1974; Wood and Dowell, 1985). Low vagility by females also contributes to the maintenance of persistently high populations on some plants (Wood and Dowell, 1984). As a result, males are likely to encounter different social environments within a single habitat, and this may favor the use of alternative mate searching tactics (Partridge and Halliday, 1984). However, the expression of a particular tactic in *U. crassicornis* appears to be age-dependent as a result of constraints resulting from a male's condition or mating experience. Thus, sparse distributions of dispersed females may require frequent plant to plant searching and signaling, which favors younger males in good condition. In contrast, aggregations of pre-dispersal females may give less active older males an advantage through competition or courtship behavior. Variation in the spatial distribution of females occurring over time may also contribute to the maintenance of age-based behavioral tactics by allowing males to exploit mating opportunities at different periods in their life (Emlen and Oring, 1977; Shuster and Wade, 2003). In conclusion, variation in lifetime mating success in *U. crassicornis* may depend upon a male's ability to adjust his mate

acquisition behavior in an age-dependent manner in response to changes in intrinsic (e.g., condition) and extrinsic (e.g., female distribution) factors.

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Chapter 5

General Discussion

The experiments conducted in this dissertation yield three main results regarding the effect of male age on the mating behavior of *U. crassicornis*. First, mating with older males has the beneficial consequence of promoting inbreeding avoidance. Second, male vibrational mate-advertisement signals change significantly in structure with age. However, females do not prefer the signals of older males. Nonetheless, age-based changes in signal features provide some insight into the potential factors affecting age-dependent signal expression in this species. Third, significant age-related changes in the behaviors used to find and solicit matings from females suggest that different mating tactics are used by older and younger *U. crassicornis* males.

Age-based mate choice and inbreeding avoidance are two important themes in behavioral ecology that have not previously been examined in a single study. Although Wood and Dowell (1985) first proposed the hypothesis that mating with older males could promote inbreeding avoidance, their evidence in *U. crassicornis* was only suggestive, as they had no data on female mating patterns. The main thrust of the argument for age-based mate choice is that females derive some sort of mating benefit by choosing males of a particular age (Brooks and Kemp, 2001). This dissertation shows that female *U. crassicornis* mate more often with older males, which lends support to the Wood and Dowell (1985) hypothesis. Inbreeding avoidance is thus an important benefit of mating with older males in *U. crassicornis*, especially since females do not appear to discriminate siblings from non-siblings when choosing mates. However, age-based

mating is not the only mechanism that reduces inbreeding risk in *U. crassicornis*. Pre-breeding dispersal from the natal site also reduces the frequency of sibling encounters. Although the effect of dispersal on inbreeding risk was not explicitly tested in this dissertation, results from a field study by Wood and Dowell (1985) showed that females are less likely to encounter siblings the farther they move from their natal site. Thus, pre-breeding dispersal and mating with older males are two mechanisms that both provide *U. crassicornis* females with increased protection from inbreeding.

One mechanism that that may have enabled females to identify older males in my experiment was age-based variation in mate-advertisement signals. Since mate attraction represents the first stage in the mating sequence (Alexander et al., 1997), mating signals were a likely source of age information for females. While there were significant age-based changes in several temporal and spectral signal features, a playback experiment showed that females did not favor the signals of older males. At present, only one other study in arthropods has conducted a playback experiment examining female choice for age-based signal variation (Ritchie et al., 1995). In that study, female katydids (*Ephippiger ephippiger*) preferred the signals of younger males and avoided those of older males. However, the authors also showed that older male song was still preferred over an allopatric song type, indicating that the former was still recognized by females as conspecific. In *U. crassicornis*, the fact that females preferred signals across a wide range of male ages suggests that long-range advertisement signals likely function in species identity, and possibly to facilitate mate localization, rather than in discriminating among individual males (Bailey, 2003; Gerhardt, 1991; Schul, 1998).

The finding of substantial age-based plasticity in the vibrational signals of male *U. crassicornis* has important implications. First, given the relatively few studies that have examined age effects on vibrational signals, and the fact that vibrational communication in arthropods is extremely widespread taxonomically (Cocroft and Rodríguez, 2005), there are likely to be many more examples awaiting discovery. Second, the significant effect of age reveals that even for short-lived, determinate growth species like *U. crassicornis*, age is a significant source of signal variation. The fact that males do not begin signaling for several days following adult eclosion is also atypical for insects, and suggests that the underlying production mechanisms require a long period of development. Even after calling begins, male signals continue to change significantly in structure for nearly two more weeks, and do not begin to stabilize until males are about 25 days old. Advertisement signals are thus changing significantly through about half of a male's adult lifespan. A third implication regarding the effect of age on signal structure is that researchers may wish to consider using longitudinal studies when evaluating potential causes of signal variation within a population (Blumstein and Munos, 2005; Miller and Brooks, 2005; Slabbekoorn and Smith, 2002). Male age can be a significant source of signal variation, and this knowledge may be useful when planning additional studies.

Age-based behavioral strategies are expected to evolve when environmental or phenotypic factors that affect a male's ability to procure matings change within his lifetime (Caro and Bateson, 1986; Partridge and Halliday, 1984; Shuster and Wade, 2003). In *U. crassicornis*, age-related changes in within-plant movement rates and flight activity, as well as in the amount of time males spend in courtship, have implications for

the ways in which younger and older males find and solicit mating from females. Results from the experiments conducted in Chapters 2 and 4 suggest that one environmental factor, i.e., the spatial distribution of females, may have an important influence on variation in male mate searching tactics, although additional experiments are needed to formally test this idea. Age appears to also influence the use of a given tactic, possibly resulting from changes in a male's physical condition. Thus, younger males in better physical condition may outperform older males when plant to plant searching and signaling is required to locate females that may be sparsely distributed throughout the habitat. Conversely, dense aggregations of females may benefit more sedentary older males, as they might be better competitors in agonistic encounters with rivals, or when it comes to using courtship to procure matings from females. Although one might expect males in better condition to always win in agonistic encounters, I found that older males out-compete younger males under high density conditions. It is possible then that younger and older *U. crassicornis* males may actually have the same overall level of mating success, but within each age class there likely will be substantial mating variance arising from differential success of males in their ability to search and compete for mates.

In their extensive re-classification of animal mating systems Shuster and Wade (2003) described one mating scheme that matches very closely to that of *U. crassicornis*: *cursorial polygyny*. Here, semelparous females are moderately asynchronous in their receptivity, and are also moderately clumped in their spatial distribution. These factors result in a large opportunity for sexual selection on males, which results in fairly high levels of competition between males during mate searching. As a result, males in a cursorial polygyny mating system are expected to be highly mobile in order to search for

clusters of females that may be scattered throughout the habitat. When aggregations of females are found, males may compete with each other for control of this resource, and thus behavioral variation in the way males defend females, or usurp other males from females is expected to occur.

The natural history of *U. crassicornis* is characterized by several ecological and social factors that lead to this type of mating system. Recall that siblings mature at about the same rate, and thus a group of sisters would be receptive at approximately the same time (Wood, 1974). Female receptivity is therefore moderately synchronous at a local scale. Also, since female *U. crassicornis* are philopatric, groups of sisters that mate at the same time can usually be found rearing their young within close proximity to each other (Wood and Dowell, 1985). However, some females disperse a significant distance from their natal site and are scattered singly throughout the habitat (Masters, 1997). Populations are thus moderately clumped; with female densities being very high where aggregations occur, and becoming increasingly sparse the farther one moves away from these concentrated areas. Mate searching *U. crassicornis* males will thus encounter variable female densities within a single habitat, ranging from large clusters of females at one extreme, to single females scattered throughout the habitat at the other extreme.

Shuster and Wade (2003) argue that these aspects of female life history favor the use of alternative behavioral strategies among males, which may include body size trade offs to cope with different scenarios. For example, they suggest that larger males may have an advantage during agonistic encounters with rivals when females are clumped in space, whereas smaller, more agile males might be better at conducting extensive mate searching when females are sparse. Although the authors did not discuss male age, their

scenario could also apply to older vs. younger males in *U. crassicornis*, rather than larger vs. smaller males. For example, results from Chapter 2 showed older males were more active in courtship, while in Chapter 4 younger males were more active in mate searching. These differences were observed under varying levels of female density (high in Chapter 2, low in Chapter 4), and thus it is tempting to conclude that variable female spatial patterns directly influences the expression of age-based mating tactics in *U. crassicornis*. However, other factors also varied between these studies (e.g., female receptivity, operational sex ratio, densities of competing males), and thus one cannot say with certainty that variation in female spatial patterns is the causal factor affecting the expression of a given tactic. Furthermore, since condition declines with age in *U. crassicornis* (Chapter 4), it is not entirely clear why older males were able to outperform younger males during courtship and mating in Chapter 2, regardless of the temporal and spatial distributions of females.

One possibility that might explain the older male courtship and mating advantage observed in Chapter 2 is that although physical condition declines with age as a whole, variation among some older males in their ability to maintain their physical condition might enable them to be successful during courtship and mating. In fact, 57% of older males obtained at least one mating in the study conducted in Chapter 2, compared to 41% of younger males. Thus, a high proportion of older males may have been in good physical condition in that study, and therefore able to successfully compete for matings. Another possibility is that older *U. crassicornis* males cannot compete at mate searching due to reductions in their flight and movement activity (Chapter 4), and so remaining at aggregations of females and courting for longer periods may allow them to make the

most of their current situation (Andersson, 1994; Gross, 1996). A similar pattern is found in horseshoe crabs where older males tend to be in poorer condition than younger males. As a result, older males are unable to directly compete for females against their younger rivals. To compensate, older males adopt a satellite tactic that enables them to fertilize about 40% of a female's eggs, whereas younger males fertilize the remaining 60% (Brockmann et al., 1994). For older *U. crassicornis* males, the best option might be to remain at an aggregation of females and attempt to obtain at least one mating. However, potential disadvantages of this tactic include wasted time courting unreceptive females, and having females disperse prior to mating. In contrast, younger males in better condition can afford to move elsewhere if a particular aggregation of females does not yield decent mating opportunities.

What are some directions for future research with *U. crassicornis*? First, the fact that females did not discriminate among signals representing males of different ages suggests that they may use other mechanisms for assessing age. One possible source of age cues may lie in courtship interactions. Courtship involves the use of vibrational signals, as well as tactile behaviors performed by males while in physical contact. These behaviors appear to vary among males (personal observation), and thus investigating whether they show age-dependent expression may help to reveal whether females use cues obtained during close range interactions to identify older males.

Second, determining if male age *per se* confers reproductive benefits to females independent of outbreeding would provide strong empirical support that mating with older males in *U. crassicornis* is a result of selection for age-based mating benefits. However, since females mate only once in this species, a careful breeding design is

needed in order to account for potential confounding sources of variation in offspring fitness, such as maternal effects (Shuster and Wade, 2003).

A third potential research direction is to examine female choice for variation in individual signal traits. Although females did not show a preference for signals reflecting differently aged males, this does not preclude the possibility that female choice is an important factor shaping signal evolution in *U. crassicornis*. The stimuli that were used in the playback experiment each varied simultaneously in multiple features, and thus all may have been equally chosen because the different combinations of parameter values were equally attractive (Brooks et al., 2005). Univariate playbacks that vary individual parameters one at a time are thus required to determine which specific signal features represent potential targets for female choice in *U. crassicornis*.

Finally, studies are needed to identify the mechanisms by which vibrational signals are produced in *U. crassicornis*. Recall that male signals contain two distinct elements that are produced simultaneously and in different frequency ranges: a tonal low frequency component (100-200 Hz), and a series of high frequency clicks (500-2000 Hz). The high frequency clicks are likely produced with a frequency-multiplier device, such as a tymbal, which is common throughout the Auchenorrhyncha (Ossiannilsson, 1949). However, the putative source for generating the low frequency component remains somewhat more speculative. Results from this dissertation, along with data from other insect species, provide suggestive evidence that the thoracic flight muscles may be involved in producing the tonal low frequency component. Bi-functional flight muscles would also offer a parsimonious explanation for the temporal synchrony in the onset of flight and signaling behavior in *U. crassicornis*. In addition, this would provide an

exciting avenue for future research in this species, especially regarding potential physiological trade-offs that may occur in the allocation of resources between flight and signaling behavior, and its possible effect on male mating success. Recent studies in crickets have shown that signaling and flight behavior share a common resource pool, and both positive and negative relationships between these traits have been identified in different species (Bertram, 2000, 2007; Roff and Gelinas, 2003). It would be interesting to investigate whether similar relationships also occur in other insect species such as *U. crassicornis*, where signaling and flight constitute important components of male sexual behavior.

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