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PRE- AND POST-COPULATORY SEXUAL  
SELECTION IN THE TORTOISE BEETLE  
ACROMIS SPARSA (COLEOPTERA:  
CHRYSOMELIDAE)

Paula Alejandra Trillo  
*The University of Montana*

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**PRE- AND POST-COPULATORY SEXUAL SELECTION IN THE TORTOISE  
BEETLE *ACROMIS SPARSA* (COLEOPTERA: CHRYSOMELIDAE)**

by

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B.S., University of California, San Diego, California 1998

Dissertation

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for the degree of

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Pre- and Post-Copulatory Sexual Selection in the Tortoise Beetle *Acromis sparsa*  
(Coleoptera: Chrysomelidae)

Chairperson: Douglas J. Emlen

Females in nearly all sexually reproducing taxa mate with multiple males. In these species, male fitness is dependent on traits used during both the pre-copulatory processes influencing mate acquisition, as well as the post-copulatory processes influencing fertilization. However, few studies have simultaneously examined pre- and post-copulatory sexual selection within a particular species, and we therefore have little understanding of how these processes interact to determine male reproductive success.

The objective of my study was to gain a more comprehensive understanding of the evolution of sexually selected traits by examining the interactions between pre- and post-copulatory processes in the tortoise beetle *Acromis sparsa*.

I examined the relationship between traits involved in pre-copulatory strategies, such as secondary sexual characters, and traits involved in post-copulatory strategies, such as primary sexual characters and copulatory courtship. Primary and secondary sexual characters in *A. sparsa* were not correlated, indicating that males may be able to acquire and develop these traits independently of each other. On the other hand, copulatory courtship behaviors were negatively correlated with secondary sexual characters, suggesting that small males use courtship as a mechanism to compensate for decreased attractiveness to females, or to compensate for decreased access to females as a result of inferior fighting abilities. Thus, males may modulate the quality or intensity of their behaviors based on their pre-copulatory attributes.

I also examined the relative importance of primary and secondary sexual characters as well as copulatory courtship on the reproductive success of *A. sparsa* males using natural insectary experiments, double mating experiments, and paternity analyses. I found that, in natural settings, males with larger testes mated with and fertilized more and larger females, and sired more offspring overall. In double mating experiments, testes mass and copulatory courtship influenced male reproductive success. However, the effects of copulatory courtship were dependent on mating order. Thus, even in species with exaggerated secondary sexual traits, such as *A. sparsa*, under certain conditions, it is post-copulatory sexual traits that make the largest contributions to male fitness. Moreover, the degree to which these traits contribute to reproductive success can vary with mating context.

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## **CHAPTER 1: INTRODUCTION**

Male competition for access to females is ubiquitous in nature (Andersson, 1994). When females mate more than once, male competition continues inside the female reproductive tract. As a result, male reproductive success becomes a function of both the pre- and post-copulatory processes of sexual selection.

The vast majority of studies in sexual selection have focused on pre-copulatory processes. Here, sexual traits operate prior to copulation to determine the number of mates a male will have access to during his lifetime (Andersson, 1994; Shuster & Wade, 2003). Examples of traits involved in pre-copulatory processes include elaborate secondary sexual characters such as the long tails in birds of paradise, antlers in ungulates, and horns in beetles. These characters increase the reproductive success of males through two principle mechanisms: female choice of attractive males (Kirkpatrick & Ryan, 1991; Andersson, 1982; Kirkpatrick, 1982; Moller, 1988), and/or increased fighting success in male-male competitions over females or mating sites (Clutton-Brock et al., 1979; Eberhard, 1982).

A new but intensive program of research suggests that post-copulatory processes can also be major drivers in the evolution of sexual traits (Arnqvist et al., 1997; Danielsson & Askenmo, 1999; House & Simmons, 2002; Simmons, 2003; Simmons & Achmann, 2000; Simmons et al., 1999; Tallamy et al., 2002; Edvardsson & Anqvist, 2000; Hosken & Stockley, 2004; Eberhard, 1985). Here, sexual traits operate during or after copulation, and determine the fertilization success of a male once he has attained a mate. Examples of traits involved in post-copulatory processes include primary sexual

characters such as genitalia and testes, as well as copulatory courtship behaviors. Selection on these traits results from three major non-exclusive mechanisms. First, cryptic female choice includes female processes inside her reproductive tract that affect male fertilization success after the coupling of male and female genitalia (Eberhard, 1996). Second, sperm competition involves the competition among male ejaculates that arises after insemination of a female by more than one male (Parker, 1998; Simmons, 2001a). Finally, sexual conflict models explain the evolution of post-copulatory sexual traits through an antagonistic coevolution, or arms race, between females and males for the control of fertilizations (Arnqvist & Rowe, 2002; Holland & Rice, 1998; Parker, 1979).

With a few exceptions, most studies of pre- and post-copulatory processes have proceeded largely independently of each other. In many species, however, these processes are likely to act concurrently to determine male reproductive success. This is because a males' overall reproductive success depends on both the number of mates he has access to, *as well as* the number of ova he fertilizes per mate. Studies that simultaneously examine the relative importance of traits used in both pre- and post-copulatory processes within a particular species are needed to gain a more comprehensive understanding of the evolution of sexually selected traits and a broader appreciation of sexual selection as an evolutionary process that acts on whole organisms (Simmons, 2001a; Andersson & Simmons, 2006).

The main objective of my research is to determine the interactions between pre- and post-copulatory sexual processes by simultaneously examining the relative

importance of primary sexual characters, secondary sexual characters, and copulatory behavior on the reproductive success of the neotropical tortoise beetle *Acromis sparsa*.

*Acromis sparsa* are especially well-suited for this type of comprehensive analysis. Males congregate around host-plant patches and compete for access to females. Females regularly mate with more than one male and can store sperm for weeks, providing ample opportunities for male competition to continue inside their reproductive tracts. Males of this species are remarkable in that they present multiple exaggerated primary and secondary sexual characters as well as highly stereotyped copulatory behaviors. Their secondary sexual characters are rigid elytral and pronotal projections used in male-male combat for females (Windsor, 1987). Their primary sexual characters include a hardened intromittent organ called the aedeagus, a genitalic whip, or flagellum, that can be longer than the entire body, and testes that occupy roughly 10-15% of the internal cavity (pers. obs.). Finally, males perform stereotyped courtship behaviors during copula, such as palpi vibrations, antennal stroking and foot tapping, that have been shown to influence reproductive success in other insect species (Edvardsson & Anqvist, 2000; Sirot et al., 2007; Tallamy et al., 2002).

Below are the specific objectives I address in my research with *Acromis sparsa* and a brief summary of my findings:

*The relationship between morphological traits involved in pre and post-copulatory processes and the relative importance of these traits for male reproductive success:*

Knowledge of genetic correlations between traits is necessary to make inferences about the evolutionary trajectories of such traits. If traits are genetically uncorrelated,

they will likely respond to different selective pressures independently of each other. If they are positively correlated, an increase in the size of one trait through selection will positively affect the correlated trait. If they are negatively correlated, an increase in the size of one trait through selection will negatively impact the other (Lande & Arnold, 1983; Arnold, 1983). Thus, the evolutionary outcome of selection on these traits will vary depending on the relationships between them and the type of selection they are subjected to. Though measurements of phenotypic correlations may, in some cases mask, genetic correlations, they can still give us information on how different traits relate to each other in nature, and provide some insights into the mechanisms by which these traits evolve (Emlen, 2001; Roff, 1992). One objective of the study presented in Chapter 2 was to determine the general variability and phenotypic correlations between primary and secondary sexual characters as a first attempt to understand the relationships between morphological traits involved in pre- and post-copulatory processes. A quantitative genetic study is currently underway to compare these phenotypic correlations to genetic correlations for the same traits. The results of this study showed that most of the primary and secondary sexual characters measured were not phenotypically correlated with each other. Only aedeagus length showed a positive correlation with weapon size. Thus, males of *A. sparsa* may be able to acquire and develop primary and secondary sexual characters independently of each other.

To understand sexual selection on species that rely on both pre *and* post-copulatory reproductive strategies, it is important to examine the relative importance of sexual traits used during both mate acquisition and offspring production (Andersson & Simmons, 2006). Thus, a second objective of the study presented in Chapter 2 was to

determine the relative importance of primary and secondary sexual characters for male fertilization success in *A. sparsa*, using surveys of natural populations in combination with a natural insectary experiment and paternity analyses. I first developed five species-specific microsatellite markers with high repeatability, high allelic variation, and strong discriminatory power to assign paternity and determine fertilization success in this species. Using these markers, I found that, of all the pre and post-copulatory sexual morphologies measured, testes mass had the largest effect on male reproductive success. Males with larger testes mated with and fertilized more and larger females, as well as sired more offspring overall. This result is interesting because it shows that even in species with exaggerated secondary sexual characters, under some conditions, primary sexual characters can make the largest contributions to male fitness.

*The relationship between behavioral traits involved in post-copulatory processes and morphological traits involved in pre-copulatory processes*

Copulatory courtship behaviors have been found in at least 81% of the species investigated (Eberhard, 1994). These behaviors can play an important role on the success of males during post-copulatory sexual selection. The expression of behavioral traits under post-copulatory sexual selection, such as copulatory courtship, may interact with, and be influenced by traits under pre-copulatory sexual selection. Thus, determining whether individuals base the quality or intensity of their behaviors on their pre-copulatory attributes will help us understand the interactions between pre and post-copulatory processes and the evolution of male reproductive strategies. The objective of the study presented in Chapter 3 was to examine the effects of pre-copulatory traits on the expression of copulatory courtship behavior in *A. sparsa* males. We found that the

intensity of copulatory courtship behavior in *A. sparsa* decreased significantly with both body and weapon size. Thus, small males may use copulatory courtship behaviors as a mechanism to compensate for decreased attractiveness to females, or to compensate for decreased access to females as a result of inferior fighting abilities.

#### *Mechanisms of post-copulatory sexual selection in Acromis sparsa*

The natural setting of the insectary experiment presented in Chapter 2 provided an opportunity to test the overall consequences that variation in primary and secondary sexual characters have on male reproductive success. However, in species with multiply mating females, post-copulatory sexual selection will favor males with traits that can enhance fertilization success with already mated females (male offensive ability), and traits that reduce the fertilization success of males subsequently mating or attempting to mate with the same female (male defensive ability). Genitalia, testes and copulatory courtship may be more relevant for either defensive or offensive mechanisms of fertilization. Thus, a detailed assessment of the effects of these traits across offensive versus defensive roles will provide critical insights about the contexts in which these post-copulatory traits affect male reproductive success. The objective of the study presented in Chapter 4 was to determine the patterns of sperm precedence in *A. sparsa*, and to determine the effects of genitalia, testes and copulatory courtship on fertilization success of first male (defensive role) versus second male (offensive role) strategies. We found that, although there is second male sperm precedence in *A. sparsa*, there was a large amount of variation within first males and second males in the degree to which they successfully fertilized females. High rates of copulatory courtship and large testes mass relative to first males significantly increased the fertilization success of second males.

However, first males did not benefit from high courtship rates. Thus, these findings show that the degree to which some post-copulatory sexual traits contribute to fertilization success in *A. sparsa* is context-dependent and can change across offensive and defensive mating roles.

**CHAPTER 2:**  
**BIGGER WEAPONS OR LARGER TESTES?: THE IMPORTANCE**  
**OF PRIMARY AND SECONDARY SEXUAL CHARACTERS FOR**  
**THE REPRODUCTIVE SUCCESS OF THE TORTOISE BEETLE**  
*ACROMIS SPARSA*

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## 2.1 ABSTRACT

Sexual selection has generated incredibly diverse and exaggerated morphologies. Elaborated plumage in birds and large horns in beetles are well known examples of exaggerated *secondary* sexual morphologies (traits not directly related to reproduction), whereas bizarre-looking genitalia in insects are remarkable examples of exaggerated *primary* sexual morphologies (traits directly related to reproduction). Most work in sexual selection has focused exclusively on either primary *or* secondary sexual characters. This approach may be inadequate to describe how sexual selection operates in many species because a male's overall reproductive success depends on both the number of mates he has access to (through secondary sexual traits during pre-copulatory processes), *as well as* the number of ova he fertilizes per mate (through primary sexual traits during post-copulatory processes). We measured allometries of and phenotypic correlations between primary and secondary sexual characters of *Acromis sparsa*, a neotropical tortoise beetle with exaggerated weapons and testes. We then used behavioral and paternity analyses in an insectary population of *A. sparsa* to simultaneously examine the relative importance of primary and secondary sexual traits for the reproductive success of males. Weapons had the highest positive allometric slope, testes mass had an intermediate positive slope, and genitalic structures had negative slopes. In addition, weapon size and aedeagus length were positively correlated. Testes mass, a primary sexual trait, had the largest effects on male reproductive success. Males with larger testes mated with and fertilized more and larger females, and sired more offspring overall. Thus, our results demonstrate that even in species with exaggerated secondary sexual traits, under some conditions, primary sexual traits can make the largest contributions to male fitness.

## 2.2 INTRODUCTION

Sexual selection has generated an incredible diversity of specialized and exaggerated morphologies (Darwin, 1871; West-Eberhard, 1992; Andersson, 1994). Secondary sexual characters (sexual traits not directly related to reproduction) include familiar examples of extravagant morphologies such as the long tails in birds of paradise, antlers in ungulates and horns in beetles. These characters are thought to increase the reproductive success of males through two principle mechanisms: female choice of attractive, highly ornamented males (Kirkpatrick & Ryan, 1991; Andersson, 1994) and/or increased fighting success in male-male competitions over females or mating sites (Clutton-Brock et al., 1979; Eberhard, 1982). These mechanisms of sexual selection usually operate prior to copulation, and determine the number of mates a male will have access to during his lifetime (Andersson, 1994; Shuster & Wade, 2003).

Primary sexual characters (sexual traits directly related to reproduction) are also diverse and can reach exaggerated proportions (Eberhard, 1985). An intensive program of research suggests that sexual selection can also be a major driver in the evolution of these characters (Arnqvist et al., 1997; Danielsson & Askenmo, 1999; House & Simmons, 2002; Simmons, 2003; Simmons & Achmann, 2000; Simmons et al., 1999; Tallamy et al., 2002; Edvardsson & Anqvist, 2000; Hosken & Stockley, 2004). Selection on primary sexual characters can result from three main non-exclusive mechanisms: First, cryptic female choice includes female processes inside her reproductive tract that affect male fertilization success after the coupling of male and female genitalia (Eberhard, 1996). Second, sperm competition involves the competition among male ejaculates that arises after insemination of a female by more than one male (Parker, 1998; Simmons, 2001a).

Finally, sexual conflict models explain the evolution of primary sexual characters through an antagonistic coevolution, or arms race, between females and males for the control of fertilizations (Arnqvist & Rowe, 2002; Holland & Rice, 1998; Parker, 1979). These mechanisms involved in the evolution of primary sexual characters are thought to operate during or after copulation, and determine the fertilization success of a male once he has attained a mate.

With a few exceptions, most studies of primary and secondary sexual characters have proceeded largely independently of each other, and have followed different trajectories. In many species however, primary and secondary sexual characters are likely to interact with each other. At the developmental level, investment in one sexual character may trade-off with investment on the other (Simmons & Emlen, 2006; Moczek, 2004). At the functional level, we expect to see interactions because a males' overall reproductive success depends on both the number of mates he has access to (pre-copulatory processes), *as well as* the number of ova he fertilizes per mate (post-copulatory processes) (Danielsson, 2001; Preston et al., 2003). How, then, do interactions between primary and secondary sexual characters influence reproductive success in species that commonly rely on *both* pre and post-copulatory reproductive strategies?

One possibility is that pre and post-copulatory processes supplement each other to increase the overall reproductive success of males (Simmons, 2001a; Andersson & Simmons, 2006). This could happen when primary and secondary sexual characters are associated with each other, and males that accrue the most females are also the males that fertilize the most ova per female. Alternatively, primary and secondary sexual characters may interact antagonistically, in cases where acquisition of mates and fertilization of ova

are independent from each other. In these circumstances, the success of some males accrued by pre-copulatory processes may be countered by the success of different males in the post-copulatory processes (Danielsson, 2001; Andersson & Simmons, 2006). Thus, to understand sexual selection on species that rely on both pre *and* post-copulatory reproductive strategies, it is important to examine the relative importance of primary and secondary sexual characters during both mating acquisition and offspring production (Andersson & Simmons, 2006). This explicit consideration of the fitness consequences of simultaneous variation in primary and secondary sexual traits is important because it can provide us with a broader appreciation of sexual selection as an evolutionary process that acts on whole organisms (Simmons, 2001a).

In this study, we used a natural insectary experiment in combination with surveys of natural populations of *Acromis sparsa*, a chrysomelid beetle with exaggerated primary and secondary sexual structures, to quantify the relative importance of these characters for male reproductive success. *A. sparsa* males are remarkable in that they produce multiple exaggerated characters. Their primary sexual characters include a hardened intromittent organ called the aedeagus, a genitalic whip or flagellum that can be longer than the entire body, and testes that occupy roughly 10-15% of the internal cavity (pers. obs.). Their secondary sexual characters are rigid elytral and pronotal projections used in male-male combat (Windsor, 1987). Males vary extensively in the size of the elytral projections, and the smallest individuals resemble females in shape (Chaboo, 2001; Chaboo, 2007). Males engage in aggressive contests over access to females and guard them after mating (Windsor, 1987). Fighting males use their projections as claspers, to lock opponents between the pronotum and elytra. The contests include stereotyped

escalating displays that can terminate in one male clasping another male, flipping it up on its side, and then throwing it off the leaf (Windsor 1987; pers. obs.). An analysis of the scaling relationship between weapon length and measures of body size suggests that males in this species have a weapon dimorphism (see Chapter 3)

*A. sparsa* males may also compete at an internal level, within the reproductive tract of females. Females regularly mate with more than one male and can store sperm for weeks, providing ample opportunities for male competition inside their reproductive tract (pers. obs.). Internal post-copulatory processes have been studied in the related species *Chelymormpha alternans*. In this species, sperm transfer involves sperm migration along the genital flagellum, which is threaded into the spermathecal duct of the female (Rodriguez, 1993; Rodriguez, 1995). Rodriguez (1995) found that males with longer flagella transferred more sperm to the female and he suggested that especially large flagella might be able to transfer sperm directly into the spermathecae. He also found that females copulating with males with longer flagella ejected less sperm from their reproductive tract than when they copulated with males with shorter flagella. In a comparative study, Rodriguez et. al. (2003) found that the size of male and female genitalia were tightly correlated across 57 species of Cassidinae beetles. All these results indicate that the length of the flagellum in tortoise beetles may play an important role in male fertilization success. Finally, testes mass is also correlated with male fertilization success in many insects with multiply mating females (Simmons, 2001a). Thus, the extreme size of testes in *A. sparsa* suggests similar implications for males in this species.

This study simultaneously characterizes the processes of sexual selection on primary and secondary sexual characters in the leaf beetle *A. sparsa* and measures the relative importance of these characters for overall male reproductive success.

## 2.3 METHODS

### 2.3.1 Study Site and Subjects

*A. sparsa* is a neotropical leaf beetle (Chrysomelidae: Cassidinae) that ranges from Mexico to Peru (Blackwelder, 1982; Chaboo, 2007). *A. sparsa* is highly abundant in Panama, Central America, and individuals are easy to find because they feed, mate and oviposit on a single hostplant, *Merremia umbellata* (Convolvulaceae). We studied wild populations of *A. sparsa* from 2003 to 2006 at the Soberania National Park and in the vicinity of Gamboa, Colon Province, Republic of Panamá (9°06' N, 79°41' W). All laboratory and insectary experiments were conducted at the Smithsonian Tropical Research Institute facilities in Gamboa.

### 2.3.2 Field Observations of Mating Behavior:

*A. sparsa* males and females congregate around small patches of the hostplant to feed, mate and lay eggs. Within a patch, females synchronize their oviposition and subsequently exhibit extensive maternal care of their brood from the egg phase to the adult emergence. This female synchronicity makes mating aggregations within a patch short, episodic events that last 3-4 weeks from when females are receptive to when females have already mated and are caring for eggs. To assess the nature of these mating aggregations, we observed wild populations of *A. sparsa* and noted any fighting and mating behavior. We also determined, the average patch size, the ratio of large to small

males per patch and the operational sex ratio of these aggregations. We then used these field observations to set up a mesocosm experiment mimicking a natural mating aggregation where all individuals were marked and male behavior and paternity was fully monitored.

*A. sparsa* mating aggregations were usually distributed in small patches of hostplant that ranged from 2 m<sup>2</sup> to 72 m<sup>2</sup> (Average=18m<sup>2</sup>, SD±24.71, N=10) and held an average of 11.9 ± 5.11 males and 5.87 ± 2.22 active females (females without brood) per m<sup>2</sup>. The operational sex ratio of these aggregations was approximately 65:35 (males to females) and the large to small male ratio was approximately 75:25. In field observations of male fights, winners had significantly larger weapons than losers (Wilcoxon Signed Rank  $Z = -2.389$ ,  $P = 0.017$ ,  $N = 8$ ).

### 2.3.3 Mesocosm Mating Experiment

To assess the effects of primary and secondary sexual characters on male reproductive success, we included males and virgin females in a mesocosm enclosure. Virgin females of *Acromis sparsa* were obtained for this study by collecting first instar larvae during the months of May and June 2006 and rearing them in an outdoor laboratory. Larvae were separated by family and reared under equivalent conditions in small 20x12cm plastic containers until pupation. We obtained fresh *M. umbellata* leaves daily from different vine patches in the area, mixed them and then distributed them randomly to the larval family groups in order to avoid individual plant variation in nutrients. After adult emergence, a single female from each family group was randomly chosen to be used in the experiment. Experimental females were placed in all-female containers for 25-45 days and fed fresh leaves daily, to allow them to become

reproductively active. Each virgin female was individually marked with a small insect tag on her pronotum (Insect Marking Kit, BioQuip, Rancho Domingo, CA). Males were collected from patches of *M. umbellata* in the vicinity of Gamboa. These males were marked in the same manner as the females and then placed in individual containers with fresh leaves for two to three days before the experiment started.

A total of 115 males and females (56 large males, 18 small males, and 41 females) were released into an outdoor insectary mimicking a small hostplant patch (6.25 m<sup>2</sup> footprint x 2.5 m height). The insectary was filled with abundant potted hostplant and placed in an area that had maintained a natural beetle population the year before. We used similar large to small male and male to female ratios as the ones found in the field observations. Beetles started mating soon after their release into the enclosure and they followed a similar mating sequence to that of the natural mating populations previously surveyed. This mesocosm mating aggregation was allowed to run its course for two weeks after the first clutch of eggs was laid (24 days total).

We monitored the mating and reproductive behaviors of males and females in the enclosure daily with scan sampling techniques (Martin & Bateson, 1986). Over the entire experiment, a single observer recorded: the total number matings per male, the number of female mates per male and the number of male mates per female. We also noted any fights, fight winners and other anecdotal observations of the mating behavior of *A. sparsa*.

#### 2.3.4 Morphological Measurements

At the end of the experiment, males, females and their larvae were collected and preserved in 95% ethanol and DMSO for later use in DNA extractions, genotyping and



paternity analyses as well as morphological analyses. Before preserving males, we dissected them to extract and weight their testes. For morphological analyses, we used elytral projection length and pronotum width as indicators of male weapon size and head width and femur length as indicators of male body size (see Figures 2-1 and 2-2 for measurement location). We also measured aedeagus length, flagellum length and testes mass as indicators of male genitalia size. We measured head width and pronotum length as measurements of female body size. Males' body size and weapon size measurements, as well as females' measurements were taken by lining up single individuals at the same angle in graphing paper and taking the measurements with a Mitutoyo digital caliper (nearest 0.01mm). Male genitalia pictures were taken with a digital Hitachi KP-D50 stereoscope camera attached to a Leica MZ6 stereoscope. Scion Image (NIH) software was used to conduct measurements of aedeagus and flagellum length. Testes were weighted to an accuracy of 0.01mg in an A&D Scale (model ER-182A).

### *2.3.5 Microsatellite Development*

Five microsatellites markers were specifically developed for *A. sparsa* in order to assess male paternity. Enriched microsatellite libraries were developed from 30 field collected *A. sparsa* adults by the Genetic Information Services Company (Chatsworth, California) and 68 microsatellites regions were sequenced. Primers for 42 of these microsatellite regions were designed. I tested the primers pairs for 40 of these microsatellite regions on a subset of experimental males to locate highly variable, repeatable and amplifiable markers. Of these 40 microsatellites, I found five primer pairs that produced amplifiable markers with high allelic variation and strong discriminatory power (complete primer sequences for these five primer pairs are in Table 2-1). To

determine the observed heterozygosity for these markers and whether they were in Hardy-Weinberg equilibrium, I combined all experimental adults of this study with those of Chapter 4 and analyzed their allele frequencies (N=184). Analysis was done using the software program Cervus (v.3.0.3, Marshall et al., 1998). Expected heterozygosity ranged from 0.747 to 0.904 and the polymorphic information content (PIC) ranged from 0.634 to 0.894 (Table 2-1). This analysis also showed that one of the five markers (*D3*) was found to be out of Hardy-Weinberg, which indicated a possibility for null alleles ( $\chi^2 = 63.081$ ,  $df = 6$ ,  $P < 0.001$ ,  $N = 180$ ). I subsequently confirmed a high incidence of a null allele for *D3* during the double mating experiment analysis by assessing mismatches between mothers and their larvae. Mothers with null alleles produced a clear signal were a large percentage of larvae from a homozygous mother would be homozygous for a different allele which was also found in one or both of the two candidate fathers. This same method also allowed me to identify a lower frequency null allele for marker *A118*, even though this marker was not out of Hardy-Weinberg in the general analysis.

### 2.3.6 Genetic Analysis

Tissue samples were taken from 100 adult beetles and 932 larvae. Gonads and legs were used for adult DNA extraction whereas the entire larva was used for larval DNA extraction. Before extraction, the tissue was powdered using a 96-well bead mill homogenizer (2000 Geno/Grinder, SPEX CertiPrep, NJ, USA) in conjunction with 2-mm high density zirconium oxide beads (Glen Mills, Clifton, NJ, USA) (Allender et al., 2004). Genomic DNA was extracted following a Phenol-Chloroform extraction protocol (Sambrook et al., 1989) modified for tissue powdering. DNA concentrations were determined by spot-checking the samples with Hoechst 33258 fluorescent dye and a UV

fluorometer (Turner Biosystems TBS-380) and samples were diluted to a standard concentration.

Because *A. sparsa* exhibits maternal care, maternity for all larvae was known with certainty, based on collection of female-larval groups. Three of the microsatellites markers developed allowed for multiplex amplification whereas two were amplified individually. HEX and FAM dyes (Invitrogen Corporation) as well as NED dye (Applied Biosystems) were used to label the primers. The PCR amplification reaction included the following ingredients: 2.0ul 5x GoTAQ Flexi buffer (Promega Corporation), 0.80ul 25mM MgCl<sub>2</sub>, 0.80ul 2.5mM dNTPs, 0.2ul 10x BSA, 0.2ul of each 10uM labeled primer, 0.15ul GoTAQ Flexi (Promega Corporation), and sterile distilled water to a final volume of 10ul. The amplification was completed using the following program: 3 minutes at 94°C, 10 cycles of [30 seconds at 94°C, 30 seconds at 59°C (reduced by 1°C each cycle), 45 seconds at 72°C], 30 cycles of [30 seconds at 94°C, 30 seconds at 49°C, and 45 seconds at 72°C], followed by a final extension for 10 minutes at 72°C. For visualization, 1ul of the PCR product was run through an ABI 3130xl Genetic Analyzer capillary electrophoresis machine (Applied Biosystems, Foster City, California, USA) with Genescan 500 ROX size standard (Applied Biosystems).

We used the Genemapper software package (Applied Biosystems, Forster City, California) to generate genetic profiles of each parent and individual larvae at the five microsatellite loci. These profiles were verified individually by eye and only those samples showing strong and unambiguous peak profiles were used in the final analysis. Error rates were determined by assessing mismatches between mothers and larvae. Paternity was first assigned individually by determining the offsprings' unique paternal

allele combinations and then using a “minimum number of fathers” criterion for each brood. In this criterion, knowledge of the sibling structure was used to assign the minimum number of fathers that had matching alleles at all loci and that could have sired offspring within a family group. Because two of the microsatellite loci we used had null alleles, we modified our exclusion criterion at these markers. In these cases, for every offspring that was homozygous for a maternal allele, we considered as candidate fathers, males that carried the offspring’s matching allele as well as all males that were homozygous at that locus, because they could be potentially carrying a null allele. These males could not be excluded unless they had allele mismatches at other loci. Our paternity assignment was confirmed by using the paternity inference software Cervus 3.0.3 (Marshall et al., 1998), at the 80% confidence level (Pemberton et al., 1999). For all the families that were suspected of carrying null alleles, we run separate analyses in Cervus excluding the loci with null alleles and we used these results to confirm our paternity assignment. For 89% of the paternity assignments we made, the same male we assigned paternity with our criterion was also assigned paternity in Cervus. For all other assignments, the male we assigned as the father was found as one of the three most likely candidate fathers in Cervus.

### *2.3.7 Statistical Analysis*

To investigate the extent of female promiscuity in the experiment, we obtained the total number of male partners for each female during the entire course of the experiment. We also determined the relationship between female size and degree of promiscuity (total number of mates) with a non-parametric correlation.

To examine allometric relationships between weapon, genitalia, testes size and body size we performed simple regressions using the log-transformed values of each measurement. We also determined the relationship between weapons, genitalia and testes size using pair-wise correlations. All analyses using head width and femur length as measurements of body size showed similar results; therefore only the relationships with head width are reported in this study. We used, elytral projection length and pronotum length as measurements of weapon size and aedeagus length, flagellum length and testes mass as measurements of genitalia size.

To assess the relative importance of primary and secondary sexual characters for male mating success, we examined the relationship between a male's probability of being observed mating (number of matings / total number of observations) and our measurements of weapon, body, testes and genitalia sizes. We first conducted univariate analyses to get familiarized with the effect of each single trait on mating success. We then simultaneously analyzed primary and secondary sexual characters by including all variables in a single analysis. We used generalized linear models with a log link function (poisson distribution) for both univariate and multivariate analyses. All statistical analyses in this study were performed with the software program JMP (SAS, Cary, North Carolina).

To assess the relative importance of primary and secondary sexual characters for male fertilization success and overall fitness, we examined the relationship between the total number of offspring sired and measurements of weapon, body, testes and genitalia sizes. We first conducted simple regressions to get familiarized with the effect of each trait on fertilization success. We then analyzed all variables simultaneously in a multiple

regression model. Dependent and independent variables were standardized for the multiple regression following Lande and Arnold (1983). Both univariate and multivariate analyses included linear and quadratic terms to test for directional and non-directional selection. To further explore males' fertilization abilities, we analyzed the effects of primary and secondary sexual characters on the total number of females fertilized per male with a generalized linear model that included a log link function (poisson distribution). This model also included linear and quadratic terms to test for non-linear relationships. All analyses using pronotum and elytral projection length as the correlated measurements of weapon size showed similar results; therefore, in order to minimize the number of variables, we only included elytral projection length in the multivariate analyses.

Finally, to evaluate any type of assortative mating and assortative fertilization occurring during the experiment, we determined whether there were any correlations between female body size and measurements of male weapon, body, testes and genitalia size for all male-female mating pairs and for all fertilization events.

## **2.4 RESULTS**

A total of 66 out of 74 males and 34 out of 41 females were recovered from the enclosure at the end of the experiment and used in the morphological and genetic analyses. Of the 15 individuals that were not retrieved, only one male and one female appeared in the daily scans of mating behavior. This suggests that 13 individuals either died or escaped the enclosure relatively early in the experiment and are unlikely to be biasing estimates of fertilization success. Because these un-retrieved individuals were

random with respect to body size and a 65:35 male to female ratio was maintained, we expect that the initial conditions set up on at the beginning continued throughout the experiment.

#### 2.4.1 Female Promiscuity

*Acromis sparsa*, females mated multiple times before laying eggs in our mesocosm experiment (Average number of partners= 1.74, SD =  $\pm 1.44$ ; range = 0-6). A total of 47% of the females in the experiment were observed mating with more than one male, thus the level of sperm competition risk  $q = 0.47$  was moderate to high (Wedell et al., 2002). Of the 34 females collected, 4 never laid eggs (11%) and 6 were never seen copulating (17.6%). We found a significantly positive relationship between female body size and number of partners, so that promiscuity was more evident on larger females (Spearman  $\rho = 0.453$ ,  $P < 0.05$ ,  $N=30$ ). However, this increase in number of partners did not translate into a larger number of fathers per brood, or a larger number of offspring produced.

#### 2.4.2 Morphological Relationships

Both measurements of weapon size (elytral projection length and pronotal length) and only one measurement of genitalia size (aedeagus size) were significantly dependent on body size (Table 2-2). These three morphological measurements were also significantly correlated with each other (Table 2-3). Both elytral projection length and pronotal length showed highly positive allometries (Table 2-2). Aedeagus on the other hand, showed an allometric slope lower than one (Table 2-2). Flagellum length also showed a negative allometry (slope less than one), and although it was not dependent on body size, it was significantly correlated with aedeagus size (Table 2-3). Finally, we

found a non-significant trend for a positive allometry of testes mass, with a slope higher than one (Table 2-3).

#### *2.4.3 Mating success*

Although males could mate with multiple females (average = 0.91 mates,  $SD = \pm 0.94$ , range 0-3 partners), a high percentage of males did not achieve copulations during our observations (43.9%). Univariate analyses showed no relationship between either of the weapon measurements (elytral projection length and pronotum length) and the probability of observing a male mating (Figure 2-3A and B). Body size (head width), on the other hand, had a marginally significant positive effect on male mating success (Effect = 3.006,  $SE = 1.637$ ,  $P = 0.055$ ,  $N = 64$ ; Figure 2-3C). In terms of primary sexual characters, aedeagus and flagellum length showed no effect on male mating success (Figures 2-3D and E), but males with larger testes were significantly more likely to be observed mating (Effect = 0.873,  $SE = 0.349$ ,  $X^2 = 6.512$ ,  $P = 0.01$ ,  $N=57$ , Figure 3F). The multivariate model showed no relationship between the probability of being observed mating and pronotum, elytral projection, aedeagus or flagellum length. Moreover, the marginal effect of body size was not maintained in this analysis. Only testes mass retained its significant positive effect on mating success and was kept in the minimal relevant model.

#### *2.4.4 Fertilization Success*

Males can also fertilize multiple females, but the average number of female partners decreased slightly from mate acquisition to offspring production (average number of females fertilized = 0.66,  $SD = \pm 0.848$ , range 0-4 partners). A total of 48.5 % of the males had less than 0.25 probability of having sired one single larva from one



single female. Univariate analyses showed no linear or quadratic relationship between elytral projection, pronotum length or head width and the total number of offspring sired (Figure 2-4A,B, and C). In terms of primary sexual characters, we found no linear effect of aedeagus length, flagellum length (Figures 2-4D and E) or testes mass. However, there was a significant negative quadratic relationship between testes mass and total offspring sired (testes mass<sup>2</sup>:  $\gamma = -0.468$ , SE = 0.219,  $P < 0.05$ , N = 57). This effect was maintained in the multiple regression and testes mass<sup>2</sup> was the only significant term in the model (Table 2-4). It is important to note, however, that body size had a marginally significant positive quadratic term in the multiple regression (Table 2-4), although this was not seen in the simple regression. Finally, because the quadratic relationship for testes mass and fertilization success did not seem to increase and decrease symmetrically and because there was a visual linear trend as well, we performed a cubic spline analysis to better assess the shape of this fitness relationship (Schluter, 1988). This cubic spline showed that the total number of offspring sired increased with testes mass at the smaller range of testes values but it then leveled off at larger values (Figure 2-4F). Thus, male fitness increased with larger testes, especially for those males at the lower values.

We also found a significant linear and negative quadratic relationship between testes mass and the total number of females fertilized (Testes mass: Effect = 0.571, SE = 0.294,  $X^2 = 4.57$ ,  $P < 0.05$ ; for Testes mass<sup>2</sup>: Effect = -0.721, SE = 0.259,  $X^2 = 11.84$ ,  $P < 0.001$ , N = 57). Similarly to the number of offspring sired, a cubic spline analysis for this relationship showed that males' ability to fertilize females increased with larger testes, especially for those males at the lower values of testes (Figure 2-5). No other physical

attribute of males included in the analysis contributed to the observed variation in number of females fertilized.

#### 2.4.5 Assortative mating and fertilization

Males and females did not pair randomly during the entire course of our experiment. Males with larger testes mass both mated and fertilized females of larger body sizes (assortative mating: Spearman  $\rho = 0.463$ ,  $P < 0.05$ ,  $N = 29$ ; assortative fertilization: Spearman  $\rho = 0.463$ ,  $P < 0.05$ ,  $N = 24$ ; Figures 6A and B). No other male attribute was correlated with the size of females that males mated with or fertilized.

## 2.5 DISCUSSION

In a large number of species, sexual selection is likely governed by both pre and post-copulatory processes (Andersson & Simmons, 2006; Moller, 1998; Simmons, 2001a). In such cases, selection will act on an integrated sexual phenotype, shaped or determined by its combination of primary and secondary sexual traits. Understanding the evolution of these sexual traits requires approaches that treat the sexual phenotype as an integrated system rather than a set of isolated characters, yet studies that do this are rare (Preston et al., 2003; Andersson & Simmons, 2006). In this study, we used a mesocosm experiment, genetic analyses and multivariate techniques to simultaneously address the effects of primary and secondary sexual characters on the reproductive success of *Acromis sparsa* males.

*A. sparsa* is an excellent species to evaluate the relative importance of primary and secondary sexual characters. Males exhibit exaggerated weapons and genitalia. They use the weapons in contests for access to females and field observations show that males

with larger weapons win more fights. Small males with little or no weapons do not usually fight but they can still gain access to copulations by mating with undefended females. Males also possess elongated genitalia and large testes, which may be important for male competition inside the reproductive tract of multiply mating females.

Our simultaneous analysis of primary and secondary sexual characters in *A. sparsa* showed no strong effects of secondary sexual characters on male reproductive success. Although body size had a weak but potentially interesting effect on fertilization success that warrants further study, we found no relationship between weapon morphology and either mating or fertilization success. On the other hand, primary sexual characters, specifically testes mass, had an important effect on both mating and fertilization success. Furthermore, we found a strong indication of positive assortative mating, where males with larger testes mated with larger females. Thus, for this episodic breeding experiment, primary sexual characters had a much stronger effect on *A. sparsa*'s male fitness than secondary sexual characters.

#### *2.5.1 Female Promiscuity in A. sparsa*

This study corroborated preliminary observations of female promiscuity, with male partners in the experiment ranging from zero to six per female. Many females in the mesocosm experiment mated multiply within small windows of time and were observed in copula during a few consecutive days right before laying eggs (pers. obs.). Larger females mated with more males but this increase in copulations did not translate into an increase in the number of fathers per brood. Thus, females are unlikely to be mating multiply in order to increase the genetic diversity of their brood (Hosken & Stockley, 2004; Simmons, 2001b; Yasui, 1998). There may be some possibility for sperm

limitation in *A. sparsa*; however, more matings did not translate into larger broods and females can lay a full set of eggs after mating with only two males (see Chapter 4). Sperm limitation alone does not explain females that mated three to six times during our experiment.

Though it has not been directly investigated in this species, males of *C. alternans*, a closely related species, have been shown to transfer spermatophores to females during copulation (Rodriguez, 1993). Females of *A. sparsa* may mate multiply to gain access to direct benefits in the form of nutrition from males (Hockham et al., 2004). Finally, if copulations can be used as a direct and reliable mechanism of assessment of male quality, then females, and in particular larger females, may be more selective in their mate choice by sampling males through copulations (Eberhard, 1985; Hosken & Stockley, 2004; Simmons, 2001a).

### 2.5.2 Morphological relationships

As in many other species, secondary sexual characters in *A. sparsa* are also highly influenced by body size (Andersson, 1994). Both measurements of weapon size, pronotum and elytral projection length scaled positively with body size and had highly positive allometries. On the other hand, primary sexual characters followed different scaling patterns. Testes mass showed a marginally significant scaling with body size, with a positive allometric slope that had an intermediate value when compared to genitalia slopes and weapon slopes. This value is within the range of slopes for testes allometries found in other beetles with secondary sexual characters (Simmons et al., 2007). Of the two main traits characterizing *A. sparsa*'s genitalia, only aedeagus length was significantly dependant on body size. Both aedeagus length and flagellum length had

slopes much less than one (0.375 for aedeagus length and 0.110 for flagellum length). Thus, genitalia length increased at a much lower rate than what would be expected if increasing in proportion to body size. A negative allometry, for genitalia has been found in many other insects species (Eberhard et al., 1998; Emlen et al., 2007; Kawano, 2004) and may be due to small males allocating more resources to genitalia than expected based on their body size and/or large males allocating less to genitalia than what is expected based on their body size. This weak relationship between primary sexual characters and body size compared to secondary sexual characters and body size is important because it may confer individuals the ability to invest in primary sexual characters independently of their ability to achieve large body sizes.

### *2.5.3 The importance of secondary sexual characters*

Our univariate analyses for mating success showed that males with larger body sizes had a higher probability of being observed mating. However, this effect was strongly reduced and became non-significant in the multivariate analysis. The importance of large body sizes for mating success can be explained via increased success in male-male competition and/or female choice (Andersson, 1994), but the effect was too weak (and potentially confounded with the effect of testes mass) to assume it played an important role in mate acquisition during our experiment. With respect to fertilization success, although not present in the univariate analysis, we found a positive weak effect for the quadratic term of body size in the multiple regression model, indicating the potential for disruptive selection on body size once the effects of testes mass on male fitness variation were accounted for. Thus, it is possible that, within groups of individuals with similar medium or large sized testes, those males with small and large

(but not medium) body sizes would achieve even higher fertilizations. A larger sample size with a larger variation in body size and testes mass seems necessary to accurately determine the importance of this positive quadratic expression and the potential for disruptive selection to be acting on *A. sparsa*. If an increase in sample size produces a significant quadratic term for body size, this would be an excellent example for a case where the effect of one sexual attribute (in this case body size) on male fitness could only be determined through a simultaneous analysis of primary and secondary sexual traits.

Finally, we found no effect of weapon size on either male mating or fertilization success. In this respect, *A. sparsa* is similar to some recent studies that also measured weak to absent directional sexual selection on exaggerated male weapons (Kelly, 2006). It is important to note, however, that this experiment captured a single reproductive event by replicating one single patch or mating aggregation. These beetles have long-lived adults, and males with larger weapons may achieve higher fertilizations by visiting a larger number of patches or mating aggregations than males with smaller weapons over the course of their lives. In addition, the conditions of population density found in our observation sites and replicated in the experiment need not be the same found in all *A. sparsa* populations. Mating aggregations with different density conditions may be more likely to uncover the effect of weapons on fertilization success (Head et al., 2007; Kokko & Rankin, 2006). Longer and larger scale studies are thus, needed to better address the strength and nature of sexual selection on secondary sexual traits in species with pre and post-copulatory reproductive strategies.

#### 2.5.4 *The importance of primary sexual characters*

In terms of mating success, we found no relationship between aedeagus or flagellum length and the probability of observing a male mating. On the other hand, males with larger testes were observed mating significantly more often than males with small testes. This last result was unexpected given that we assumed primary sexual characters to be only associated with fertilization success. Some possible explanations for this result are the following: First, males with large testes may have a higher sperm production rate and become less depleted than males with small testes (Cornwallis & Birkhead, 2007; Simmons, 2001a). This would therefore, allow for males with larger testes to recycle back to the mating pool faster than males with small testes males and thus achieve a higher number of matings. Alternatively, if females preferentially accept large testes males once they have started copulation (Hockham et al., 2004), these males would be able to stay paired longer than males with small testes. This would translate into an observer seeing more pairings of males with large testes than males with small testes. Finally, male adult condition is known to be an important factor when acquiring mates. Because adult *A. sparsa* are long-lived, morphological structures developed at the imago stage, such as weapons or body size may not be the best predictors of male condition throughout the adult life. Testes, on the other hand, may be able to track changes in nutrition over time, and male differences in testes mass may more accurately reflect differences in condition. Thus, males with larger testes may also mean males in better condition and thus, more likely to acquire mates.

With respect to fertilization success, the only primary sexual character that influenced the total number of offspring sired was testes mass, with a significantly negative quadratic term. A further analysis of the relationship with a cubic spline showed

that the total number of offspring sired increased with testes mass at the smaller range of values but it leveled off at larger values. A very similar result was found when we used the number of fertilized females as the dependent variable for fertilization success. In this case we found a significant effect of both the linear and the quadratic term of testes mass on the number of females fertilized. The cubic spline analysis also showed an increase in females fertilized with an increase of testes mass that was stronger at the lower testes ranges. Thus, male fitness, in terms of number of offspring sired and number of females fertilized, increased with larger testes, especially for those males at the lower values. For males with smaller testes, a small increase in size may reflect an important increase in many attributes associated with testes size. For males with testes sizes that are above average, on the other hand, a small increase in testes size may not reflect a real advantage over slightly smaller but still larger than average males.

For small males, an increase in testes size may reflect an increase in the ability of sperm production, which in turn may increase both absolute quantities and the rate of sperm transfer. If females are sperm limited, a small increase in sperm quantities may avoid the risk of sperm limitation and the subsequent mating of this female with other males (Anderson and Simmons, 2006). Male sperm production may also be important if it reflects an increase in the rate of sperm transfer during copulation. A higher transfer rate may decrease the amount of copulation time and the risk of being dislodged by another male. Finally, females may actively choose to be fertilized by males with higher sperm transfer rate if this translates into her having “sexy sons” with higher sperm transfer rate (Eberhard, 1996; Simmons, 2001a).



An increase in size may also have an advantage in the production of larger or more nutritious spermatophores with which to induce females into laying eggs (Simmons, 2001a). Finally, an increase in testes size may be correlated with an increase in accessory glands' products used to enhance fertilization success.

#### 2.5.5 Assortative mating and fertilization

This study also showed that assortative mating and fertilization occurs in *A. sparsa*, where males with larger testes mate and fertilize larger females. This assortative pairing occurs if females preferentially mate with males that have larger testes and males preferentially mate with larger females. We have already discussed the potential mechanisms by which females may preferentially mate with large testes males, and male choice of larger females can be just as feasible. Rubolini et al. (2006) found that mating males were sensitive to female size and produced larger ejaculates when mating with larger females, which produced more eggs. Thus, female sized-related maternal effects may be an important factor affecting male choice. In *A. sparsa* female body size is highly correlated with brood size in both field and insectary females (see Chapter 4). Moreover, both male and female offspring adult body size is highly dependant on female body size but not on male body size (Trillo, in prep). Finally, although it has not been studied in *A. sparsa*, female body size may also influence offspring viability and adult survival.

To date, models predicting the nature of sexual selection have been developed separately for primary and secondary sexual characters (Danielsson, 2001). This is unfortunate, because pre and post-copulatory processes are not mutually exclusive, and likely interact in many species.

The results of our study suggest an important role of primary sexual characters, especially testes mass, in this species. Males with larger testes in this experiment seem to be achieving the highest reproductive success by mating with more females, fertilizing more females and ensuring higher ovum fertilizations per female. On the other hand, we found little to no effect of secondary sexual characters on the reproductive success of *A. sparsa* males. In this experiment, sexual selection on primary sexual characters through post-copulatory processes seems to have attenuated or dampened sexual selection on secondary sexual characters through pre-copulatory processes. Thus, our results indicate that even in species with exaggerated secondary sexual characters, under certain conditions, it is the primary sexual characters the ones that contribute the most to male fitness. This suggests that the relationship between primary and secondary sexual characters may be dynamic. In other words, the relative importance of these characters on male reproductive success may shift depending on the conditions animals experience. Thus, in order to understand to a full extent how sexual selection acts on whole organisms, with both primary and secondary sexual characters, it is crucial not only to determine the relative importance of these characters for male reproductive success but also to assess how this relative importance changes across different mating conditions, populations and species.

## **2.6 TABLES AND FIGURES**

**Table 2-1:** Locus names, size range, annealing temperature, number of alleles, expected and observed heterozygosities, polymorphic information content, null allele frequency estimates and sample sizes for *Acromis sparsa* microsatellites developed.

Locus	Primer Sequence (5' - 3')	Size Range (bp)	Annealing Temperature ( $^{\circ}\text{C}$ ) <sup>1</sup>	No of alleles	H <sub>O</sub>	H <sub>E</sub>	PIC	Null allele frequency estimate	N
<i>D3</i>	F: TGCCATGTTAATAGCCAACA R: ACCTGCTAGGAGAGTCAACC	280-318	59-49	9	0.517	0.747	0.701	0.1815*	180
<i>A127</i>	F: GTGGCAGAGATTTGTGTTTG R: AGGATCTTGACAGACGAGTTG	282-350	59-49	32	0.901	0.859	0.844	0.0277	182
<i>A115</i>	F: CGAGCCGATGATTGTCTG R: TAATGGCGAACCGCTAAAC	250-290	59-49	27	0.820	0.904	0.894	0.0478	183
<i>A118</i>	F: CGGTAGGATTTTTGATTTTCTG R: TTCAACTTGATTTGGGACAAG	140-152	59-49	6	0.633	0.693	0.634	0.0446	180
<i>D132</i>	F: CACACGATTCTCGGTACTIONG R: GGGTACATTCCCTTGATTTG	244-304	59-49	13	0.734	0.736	0.694	0.0022	177

<sup>1</sup> A range of temperatures indicates a touchdown PCR was used, where the annealing temperature was decreased 1 per cycle for 10 cycles starting at the higher temperature. The remainder of the cycles was performed at the lower annealing temperature. All PCRs were done for a total of 40 cycles.

PIC = Polymorphic information content, gives a measure of the discriminatory power of a marker for a given population. High values indicate high discriminatory power.

\*An asterisk indicates this microsatellite marker that was out of Hardy-Weinberg equilibrium in the analysis, suggesting the presence of null alleles in the sample.

**Table 2-2:** Allometric relationships for *A. sparsa*'s primary and secondary sexual characters ( $\log_{10}$  of weapon size, genitalia size and testes mass regressed on  $\log_{10}$  body size). Asterisks indicate significant regressions.

	<b>N</b>	<b>Slope</b>	<b>Intercept</b>	<b>R<sup>2</sup></b>	<b>F</b>	<b>P</b>
<b>Secondary Sexual Traits</b>						
Pronotum length (mm)	66	2.361	0.458	0.842	341.452	<0.0001*
Elytral projection length (mm)	66	8.240	-1.418	0.872	435.117	<0.0001*
<b>Primary Sexual Traits</b>						
Aedeagus length (mm)	63	0.375	0.357	0.267	22.202	<0.0001*
Flagellum length (mm)	64	0.110	1.068	0.015	0.968	0.328
Testes mass (mg)	57	1.292	-0.093	0.051	2.967	0.091

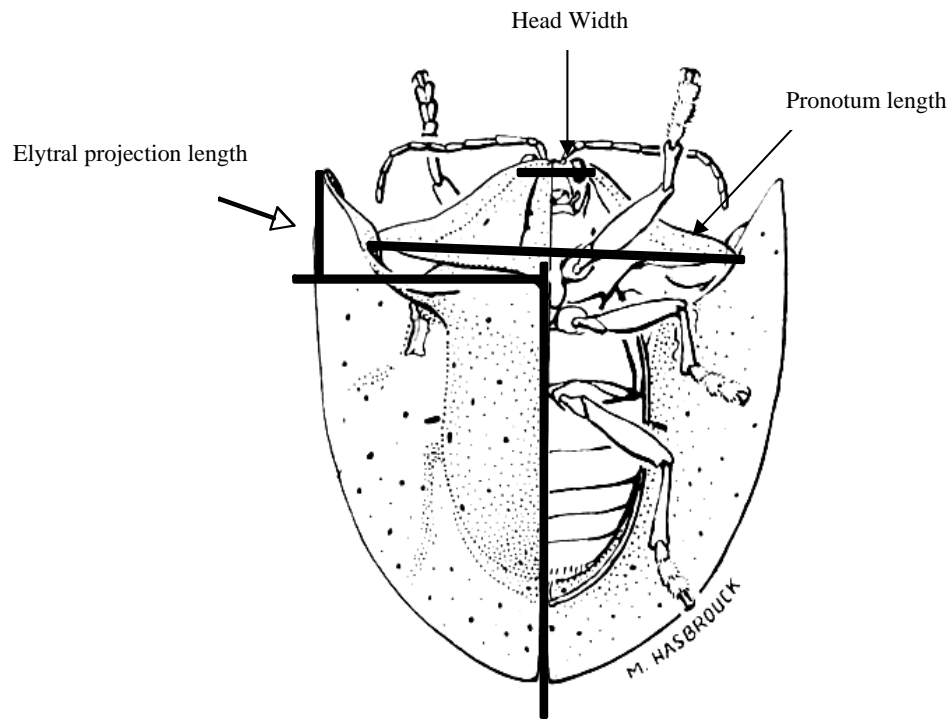
**Table 2-3:** Pearson's correlation coefficients for phenotypic correlations between measurements of *A. sparsa*'s weapons, genitalia and testes.

	Pronotum length	Elytral projection length	Aedeagus length	Flagellum length	Testes mass
<b>Secondary Sexual Traits</b>					
Pronotum length (mm)	—	0.964(66) **	0.536(63)* *	0.221(64)	0.160(57)
Elytral projection length (mm)		—	0.553(63)* *	0.161(64)	0.174(57)
<b>Primary Sexual Traits</b>					
Aedeagus length (mm)			—	0.315(63) *	0.185(57)
Flagellum length (mm)				—	0.103(57)
Testes mass (mg)					—

Values in parenthesis represent sample sizes, \*\* =  $P < 0.0001$ ; \* =  $P < 0.05$

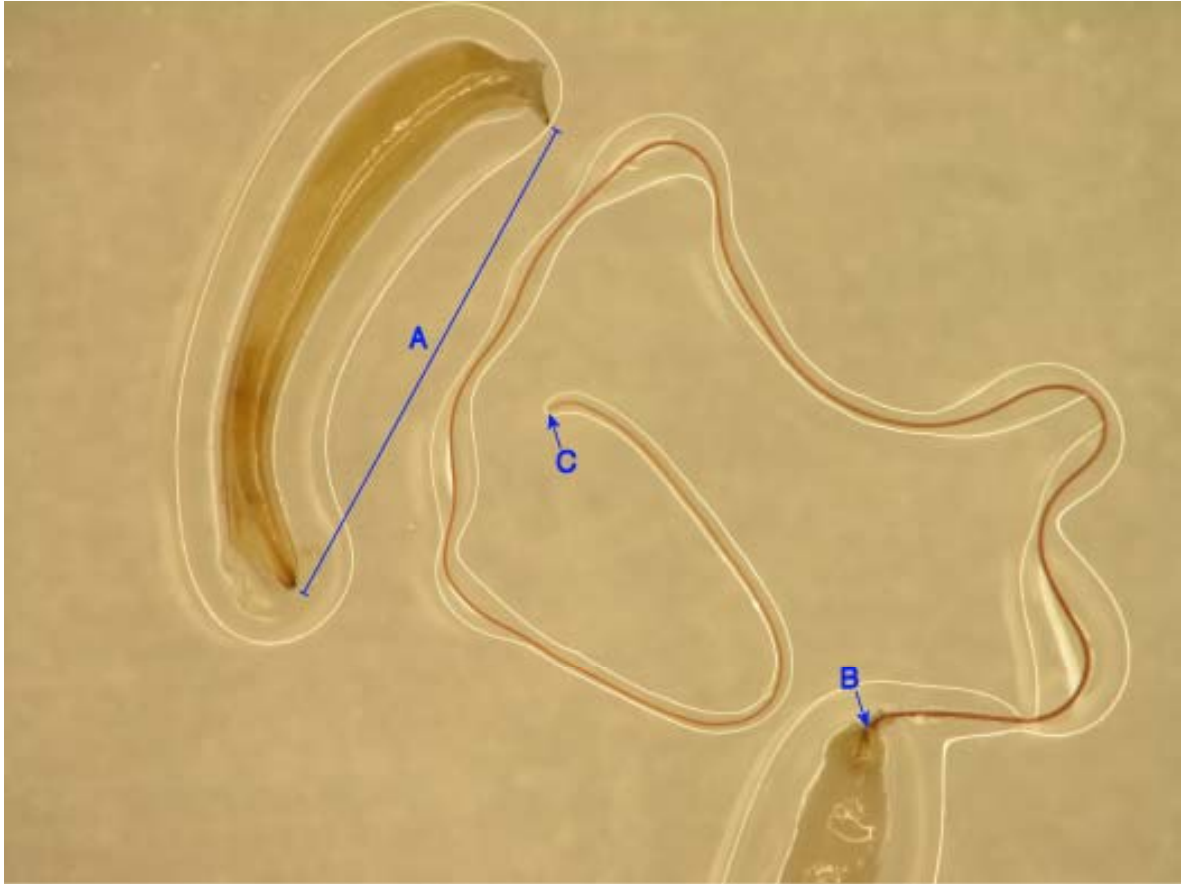
**Table 2-4:** Multiple regression values and selection coefficients for the effects of primary sexual characters, secondary sexual characters and body size on fertilization success. For quadratic regressions, positive coefficients indicate potential disruptive selection, and negative coefficients indicate potential stabilizing selection. Asterisks indicate coefficients of selection significantly different from zero (N = 57).

	<b>Coefficient</b>	<b>SE</b>	<b>T</b>	<b>P</b>
<b>Body Size</b>				
Head width (mm)	$\beta = 0.261$	0.693	0.38	0.708
Head width <sup>2</sup> (mm)	$\gamma = 0.735$	0.375	1.96	0.052
<b>Secondary Sexual Traits</b>				
Elytral projection length (mm)	$\beta = -0.283$	0.655	-0.43	0.668
Elytral projection length <sup>2</sup> (mm)	$\gamma = -0.421$	0.456	-0.92	0.362
<b>Primary Sexual Traits</b>				
Aedeagus length (mm)	$\beta = 0.182$	0.326	0.56	0.579
Aedeagus length <sup>2</sup> (mm)	$\gamma = -0.261$	0.201	-1.30	0.201
Flagellum length (mm)	$\beta = 0.082$	0.295	0.28	0.782
Flagellum length <sup>2</sup> (mm)	$\gamma = -0.352$	0.245	-1.43	0.158
Testes mass (mg)	$\beta = 0.154$	0.268	0.58	0.567
Testes mass <sup>2</sup> (mg)	$\gamma = -0.575$	0.232	-2.48	0.016*

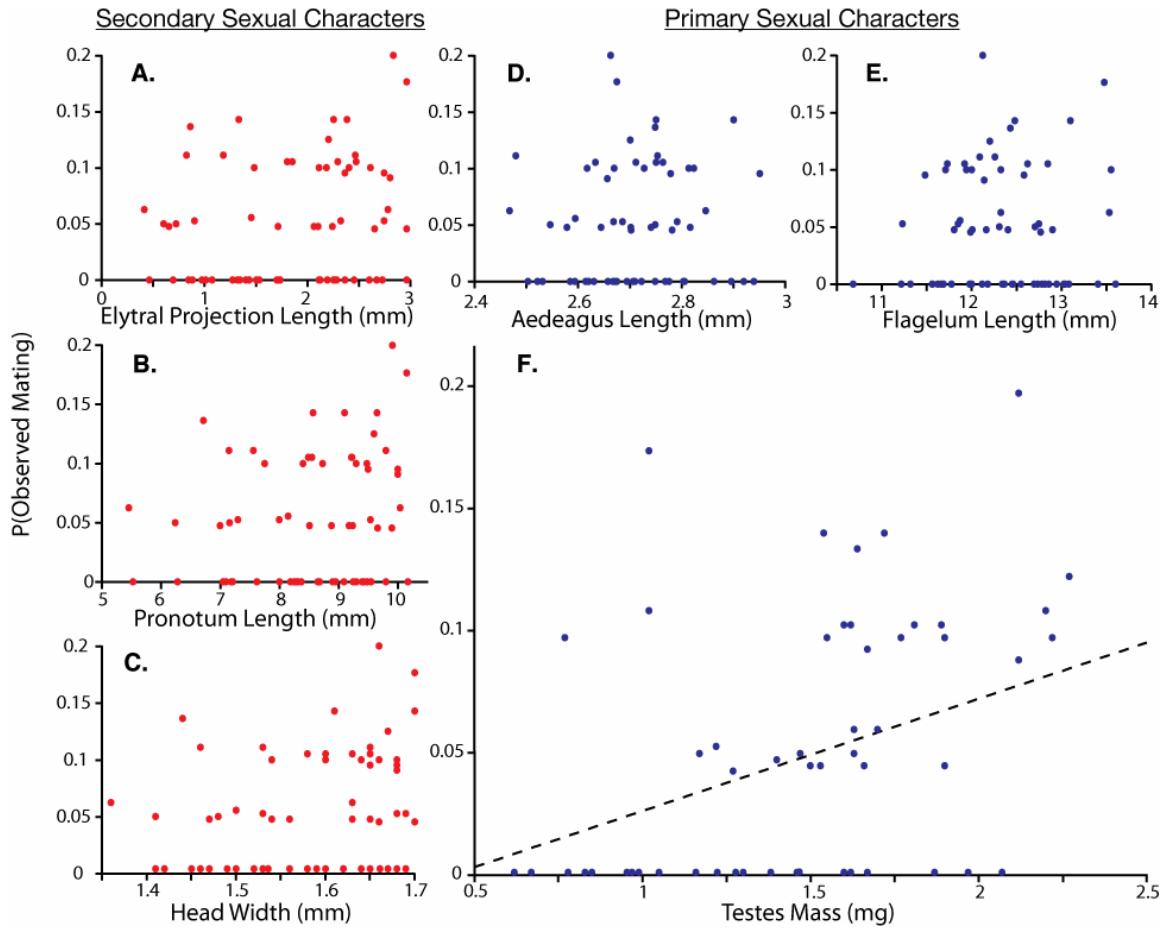


**Figure 2-1:** Location of measurements for weapon size (elytral projection length and pronotum length) and body size (head width) in *A. sparsa* males. Drawing modified from Windsor (1987)

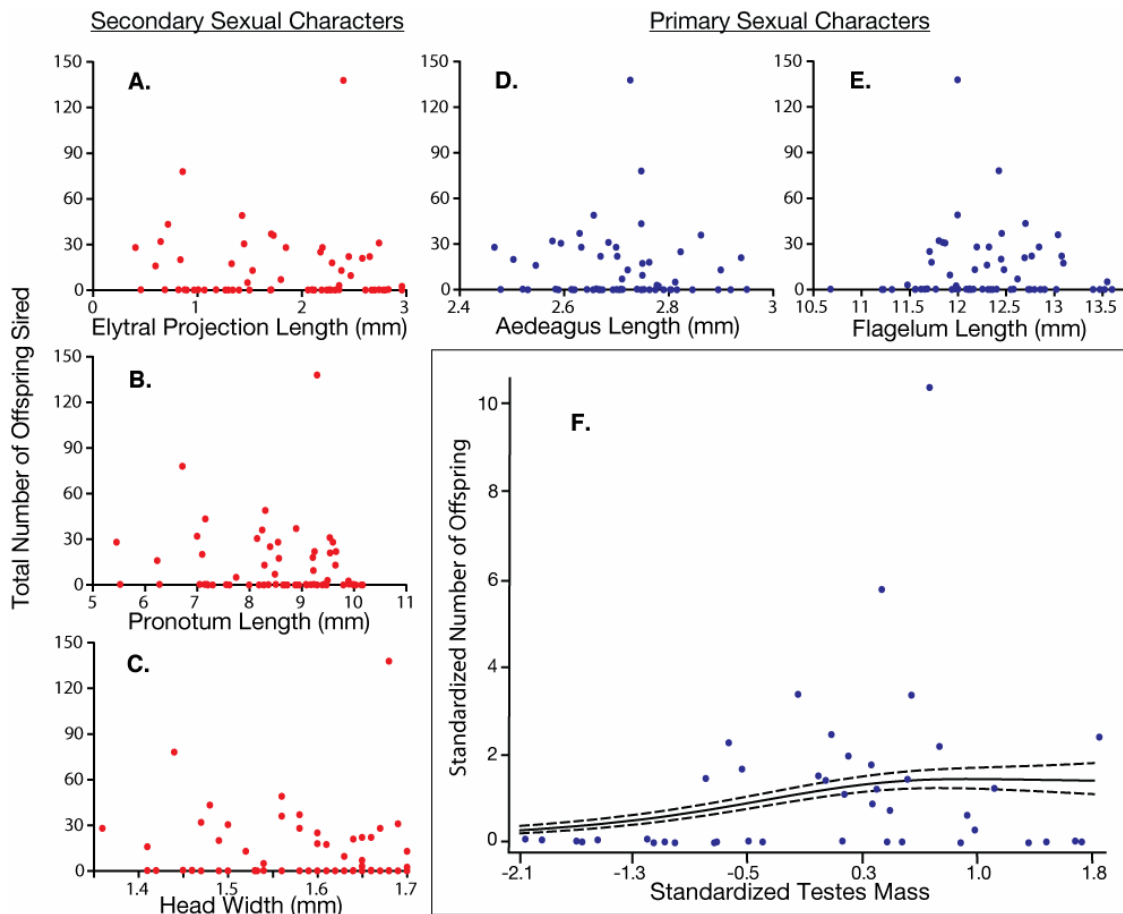




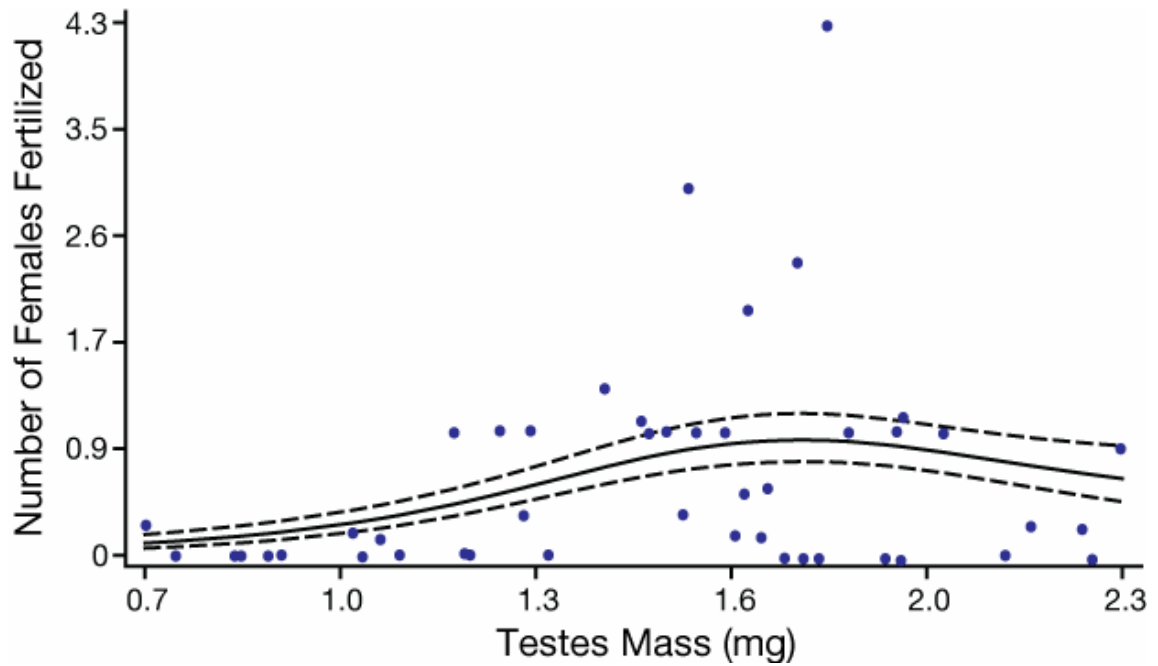
**Figure 2-2:** Location of measurements for genitalia size in *A. sparsa* males. Aedeagus length was measured as the straight line distance between the proximal and distal ventral tip of the aedeagus (A). Flagellum length was measured as the entire length of the sclerotized whip (B - C).



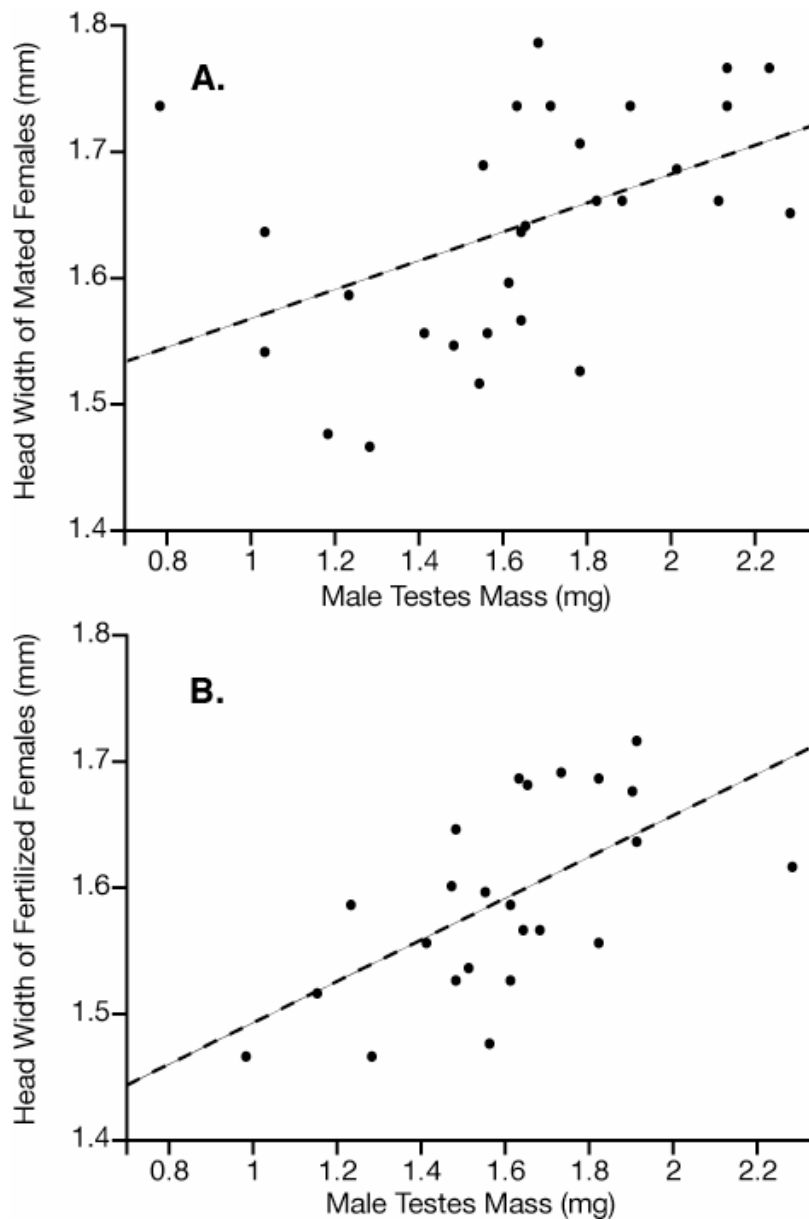
**Figure 2-3:** The individual relationships between primary and secondary sexual characters and male mating success. A male's probability of being observed mating (number of matings / total number of observations) against (A) elytral projection length; (B) pronotum length; (C) head width; (D) aedeagus length; (E) flagellum length and (F) testes mass.



**Figure 2-4:** The individual relationships between primary and secondary sexual characters and male fertilization success. The total number of offspring sired against (A) elytral projection length; (B) pronotum length; (C) head width; (D) aedeagus length and (E) flagellum length. (F) Cubic spline indicating the relationship between male fitness (number of offspring sired) and testes mass. The analysis was performed with  $\lambda = 1.2$  (choice based on minimum cross-validation scores). The dotted lines indicate one standard error of predicted values above and below the fitness function, completed with 50 bootstrap replications.



**Figure 2-5:** Cubic spline indicating the relationship between the total number of females fertilized and testes mass. The analysis was performed with  $\lambda = -2$  (choice based on minimum cross-validation scores). The dotted lines indicate one standard error of predicted values above and below the fitness function completed with 50 bootstrap replications.



**Figure 2-6:** Female body size measured as head width against male testes mass for all pairs where (A) individuals were observed mating; and (B) females were successfully fertilized.

**CHAPTER 3:**  
**BODY AND WEAPON SIZE CORRELATE WITH COPULATORY**  
**COURTSHIP RATE IN THE TROPICAL TORTOISE BEETLE**  
*ACROMIS SPARSA*

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### 3.1 ABSTRACT

In species with promiscuous females, male competition for fertilizations continues inside the female's reproductive tract. The outcome of this competition can be determined directly via sperm competition, whereby traits that ensure dominance over other males' sperm are under selection; and/or indirectly, via cryptic female choice, whereby traits that stimulate females to accept sperm are under selection. Copulatory courtship behaviors are stereotyped movements performed during copulation thought to increase the likelihood that a male's gametes are used by the female. These behaviors have been shown to affect male fertilization success, but little is known about the causes of male variation in copulatory courtship. We investigated whether variation in male copulatory courtship behavior is correlated with traits under pre-copulatory selection such as body or weapon size in the tortoise beetle *Acromis sparsa*. We also examined the relationship between body and weapon size to determine whether a weapon dimorphism exists in *A. sparsa*, and whether the copulatory courtship strategies of major and minor males in this dimorphism differ. The intensity of the courtship behaviors measured (antennal stroke rate, foot tapping rate, and palpi vibration rate) decreased with an increase in body and weapon size. Antennal stroke rate and foot tap rate decreased with time during copulation. Our analysis also indicated a weapon dimorphism in this species. While females were more likely to copulate with major than with minor males during the pairings, minor males expressed higher courtship rates while copulating. Small males may use copulatory courtship behaviors as a mechanism to compensate for decreased attractiveness to females, or to compensate decreased access to females as a result of inferior fighting abilities.

### 3.2 INTRODUCTION

It is widely recognized that females of many taxa copulate with multiple males for each single brood or reproductive event (Cornwallis & Birkhead, 2007). This high level of promiscuity creates a conflict over access to fertilizations, even after insemination, and selection therefore acts upon the ability of males and females to control offspring paternity (Eberhard, 1996). Males may compete over access to the ova inside the female's genital tract directly via sperm competition, whereby traits that have the power to defeat other males' sperm are under selection (Simmons, 2001a); and/or indirectly, via cryptic female choice, whereby traits that enhance the opportunity that a male's sperm is accepted and used by the female are under selection (Eberhard, 1996).

Copulatory courtship behaviors can serve an important role in increasing the likelihood that a female accepts a male's sperm. These behaviors are courtship maneuvers performed in a seemingly ritualized manner by the male during copulation (Tallamy et al., 2002; Eberhard, 1996). Copulatory courtship behaviors may increase the reproductive success of males by stimulating certain female responses during or following copulation that increase a male's chances of fertilizing her ova (Eberhard, 1994). In insects, these behaviors are ubiquitous. Eberhard (1994) found that 81% of the species investigated presented some form of copulatory courtship behavior. Furthermore, recent studies on a wide variety of insect species show a positive relationship between copulatory courtship behavior and fertilization success (Tallamy et al., 2002; Edvardsson & Anqvist, 2000; Otronen, 1997; Sirot et al., 2007).

Insects show many adaptations governed by post-copulatory sexual selection, such as exaggerated and convoluted internal genitalia, external morphologies,



spermatophores, plugs and glandular secretions (Simmons, 2001a; Arnqvist et al., 1997; Danielsson & Askenmo, 1999; Simmons & Achmann, 2000; House & Simmons, 2002), and therefore are an excellent group in which to study cryptic female choice. Despite being an important post-copulatory trait, copulatory courtship behavior has been studied extensively in only a few insect species (Tallamy et al., 2002). Recently, studies have shown that the consequences of variation in copulatory courtship behavior can be far reaching. Sirot et. al (2007) found that copulatory courtship behavior explained most of the variation in male postcopulatory reproductive success of the beetle *D. abbreviatus*. However, not much attention has been given to the causes of this variation among males of a single species. For example, little is known about how males use copulatory courtship behaviors and whether these behaviors can be influenced by other male sexual characteristics, such as weapon and body size.

Copulatory courtship behaviors may be positively correlated to body and weapon size and thus, become a direct signal for male condition and quality. On the other hand, copulatory courtship behaviors may be negatively related to body size, whereby smaller males compensate for their inferior fighting capacities and low opportunities of mating with higher levels of courtship during copula (Simmons et al., 2000). Smaller males could also increase behavior rates to stimulate uninterested females, who might generally prefer large males, into accepting their sperm. Females may be more attracted to males with large bodies or weapons for many reasons: large male body size may be related to higher offspring quality and viability (Sirot et al., 2007), larger males may be more apt to defend females against other-male harassment during copulation or egg laying (Dick &

Elwood, 1996) and, larger males may contribute more to egg production with larger spermatophores or other male-derived products (Sirot et al., 2007).

In this study, we investigated the relationship between body and weapon size and copulatory courtship behaviors of *Acromis sparsa*, a neotropical chrysomelid beetle, highly specialized in a single hostplant vine *Merremia umbellata* (Convolvuleaceae). In *Acromis sparsa*, females can mate multiple times before each single reproductive event. In a large mesocosm experiment, the level of sperm competition risk or fraction of females that mated more than once was moderate to high ( $q = 0.47$ , Wedell et al., 2002) and the number of male partners ranged from zero to six (see Chapter 2). This promiscuity can select for the ability of males to influence paternity via post-copulatory traits such as copulatory behaviors (Eberhard, 1996). *Acromis sparsa* males show a series of behaviors during copula that follow Eberhard (1994)'s criteria for courtship behaviors. Though this study does not link copulatory behaviors with reproductive success, at least one of the behaviors described for *A. sparsa*, antennation rate, was found to affect fertilization success in a different experiment (see Chapter 4). Moreover, similar behaviors to the ones described in this study have also been found in other insect species, where they have been shown to influence male fitness (Sirot et al., 2007; Tallamy et al., 2002; Edvardsson & Anqvist, 2000). Males of *Acromis sparsa* also vary greatly in body size and in the elytral and pronotal projections they use as weapons in contests for females (Windsor, 1987). Fighting males use their projections as claspers, to lock opponents between the pronotum and elytra, flip them on their side, and then throw them off the leaf (Windsor 1987). Here we show that variation in a post-copulatory trait, such

as copulatory courtship behavior is negatively correlated with pre-copulatory traits such as body and weapon size in *A. sparsa*.

### **3.3 METHODS**

#### *3.3.1 Study Site and Subjects*

The study was conducted during 10 August to 30 November 2006 at the Smithsonian Tropical Research Institute facilities in the vicinity of Gamboa, Colon Province, Republic of Panamá (9°06' N, 79°41' W). This area collides with part of the 22,000 hectares that make the Soberanía National Park. All individuals for the experiments came from wild populations of *A. sparsa* monitored from 2003 to 2005 at the Soberania National Park and in Gamboa.

Virgin females of *Acromis sparsa* were obtained for this study by collecting first instar larvae during the months of May and June 2006 and rearing them in an outdoor laboratory. Larvae were separated by family and reared under equivalent conditions in small 20 X12cm plastic containers until pupation. We obtained *M. umbellate* fresh leaves daily from different vine patches in the area, mixed them and then distributed them randomly to the larval family groups in order to avoid individual plant variation in nutrients. After adult emergence, a single female from each family group was randomly chosen. Experimental females were placed in all-females containers for 25-45 days and fed fresh leaves daily, to allow them to become reproductively active. Approximately 24 hours before the experiment started, each female was separated from the group and put in isolation in a small plastic cup. In order to control for the effect of female size, we used only females of average size in the pairings. Males were collected from field patches of

*Merremia umbellata* found around Gamboa. These males were maintained in all-male containers with fresh leaves for one to two days before the assays started.

### 3.3.2 Behavioral sampling

A female was introduced into a mating chamber, which consisted of a 12cm<sup>3</sup> glass box placed upside down with the open side towards the table, and the observer was positioned between 50-100 cm away. This glass container allowed the observer to look at the beetle pairs from all angles without having to approach too closely. First, a female was left alone in the chamber for a total of ten minutes while it acclimated to the area. After these ten minutes, a randomly chosen male was introduced into the chamber. If the pair did not copulate after a maximum of four hours, the male was replaced with a different male. If no copulation occurred the pair was removed from the mating chamber and a new female and male were used. If the male mounted the female, time of mount was recorded and the observer noted if and when the male penis or aedeagus was introduced into the female (hereafter called copulation). At the moment the aedeagus was introduced into the female and copulation was achieved, live behavioral observations began. These consisted of 10-minute periods of focal observation that were conducted every 20 minutes thereafter until minute 130, or until the pair separated. Approximately 63% of the pairs separated at or before minute 130. In another related species, *Chelymorpha alternans*, copulations with successful transfer of sperm, where spermatozooids enter the spermatheca, last a minimum of approximately 20 minutes (Rodriguez, 1993). Thus, only copulations that lasted longer than 20 minutes were considered successful. In order to calculate behavioral rates with more than one single observation period, pairs with more than 20 minutes of copulation time but less than two

focal observation periods were considered as having successful copulations but were still excluded from the behavioral analysis. No differences in the relationship between copulatory courtship behaviors and weapon and body size were found when we excluded single observation period pairs from the analysis. Thus, we used pairs with a minimum number of two focal observation periods (20 minutes of observation total) and a maximum number of five focal observation periods (50 minutes of observation total). All observations were conducted during the day between 0900 and 2100 hours.

### *3.3.3 Copulatory Courtship Behaviors*

During periods of observation, a single observer recorded the numbers of times each of these three male behaviors occurred: (1) Palpi vibrations, which consisted of males touching the females' medial and anterior region of the elytra with vibrating labial and maxillary palps; (2) Antennal strokes, which consisted of males slightly lowering their heads and stroking females one, two or three times at the base of the elytra or pronotum with either the right, left or both antennae. In some occasions, it also included the stroking and rubbing of the lateral edges of the females' antennae; and (3) Foot taps, which consisted of males tapping lightly on the females' elytra with front or mid tarsi. These behaviors were chosen because they followed Eberhard (1994)'s criteria for copulatory courtship behaviors, and because they were easily noticeable so that the observer was able to keep a distance and record the behaviors without disturbing the copulating pair. Behaviors similar to those described above have been suggested to function as copulatory courtship behaviors and influence fertilization success in other insect species (Sirot et al., 2007; Eberhard, 1994; Edvardsson & Anqvist, 2000; Tallamy et al., 2002). The first two behaviors, palpi vibrations and antennal strokes have also been

documented as copulatory courtship behaviors for the related species *C. alternans* (Rodriguez, 1995).

Because the single observer that conducted all focal observations in this study eventually learned the hypothesis being tested, we decided to determine observer bias. On randomly selected days, we brought naïve volunteers to simultaneously conduct observations on the same subjects as the main observer. A comparison of the number and rate of copulatory courtship observations between the main observer and the naïve volunteers showed no significant difference in the number or rate of behaviors observed and no biased directionality for the main observer in either of the three copulatory courtship behaviors examined (Wilcoxon Signed Rank Test: Palpi Vibrations  $Z = -.557$ ,  $N=24$ ,  $P = 0.5774$ ; Antennal strokes  $Z = -.928$ ,  $N=23$ ,  $P = 0.353$ ; Foot taps  $Z = -1.430$ ,  $N=23$ ,  $P = 0.1539$ ).

### *3.3.4 Morphological measurements*

After copulation, pairs were collected and preserved in 70% ethanol for later use in detailed morphological measurements. We measured elytral projection length and pronotum width as indicators of male weapon size and head width as an indicator of male body size. Pictures for all morphological measurements were taken with a digital Hitachi KP-D50 stereoscope camera attached to a Leica MZ6 stereoscope. Scion Image (NIH) software was used to conduct measurements of body and weapon size (see Chapter 2, Figures 2-1 and 2-2 for measurement locations).

### *3.3.5 Statistical Analysis*

To determine if there was a relationship between copulatory behavior and body and weapon size, we analyzed the effects of both morphological traits on the intensity of

courtship measured as the total number of behaviors per minute. For all three behaviors we used generalized linear models with a log link function and a correction for overdispersion (“quasi-poisson” distribution). Simple non-parametric correlation analyses were also performed for the courtship behaviors with body size and weapon size independently to get familiarized with the effect of each trait on copulatory courtship rate. We used a Kruskal-Wallis test to examine any changes in the behavioral rates that might occur through time. We only used the first four observational periods for this test because of a lack of data points for the last observational period. All statistical analyses in this study were performed with the software program JMP (SAS, Cary, North Carolina).

Males of *A. sparsa* have been suggested to have a weapon size dimorphism (Chaboo, 2001). Weapon size dimorphisms are common in insects, where large males produce weapons larger than what would be expected for their body size (majors), and small males produce weapons smaller than expected for their body size (minors). In order to have a better understanding of the biological relationship between copulatory courtship behaviors and body and weapon size, we decided to determine whether a weapon size dimorphism was found in these beetles. For this analysis we used (Eberhard & Gutierrez, 1991)’s model criteria. First, we tested for non-linearity of the scaling relationship between body size and weapon size by performing a partial F-test on a model that included a quadratic coefficient (model 1: Eberhard and Gutierrez (1991)):

$$Y^* = \beta_0 + \beta_1 X^* + \beta_2 X^{*2} + \epsilon \quad (1)$$

where  $Y^* = \ln(\text{weapon size})$ ; and  $X^* = \ln(\text{body size})$ . If the term  $\beta_2$  is significant, the scaling relationship is non linear and further testing is used to determine the type of non-

linear scaling relationship. Thus, we tested for discontinuity and a body size switch-point (model 2: (Eberhard & Gutierrez, 1991)):

$$Y = \beta_0 + \beta_1 X + \beta_2 (X - X^0) D + \beta_3 D + \epsilon \quad (2)$$

in which  $X$  and  $Y$  are in actual measurement units;  $X^0$  is the switch point;  $D = 0$  if  $X < X^0$  and  $D = 1$  in all other cases;  $\beta_i$  are the regression coefficients;  $\epsilon$  is the random component with assumed normal distribution, mean zero, and common variance (Eberhard & Gutierrez, 1991). To determine the body size switch point that explained the greatest proportion of variance, ten different values of  $X^0$  (potential switch points) were substituted in (2) and ten adjusted  $R^2$ s were calculated. The switch point giving the maximum value of adjusted  $R^2$  was visually selected from a fifth degree polynomial plot (Quasi-cubic spline) of the adjusted  $R^2$ s against the potential switch point values. Assumptions of homoscedasticity and normality of errors were checked before the analysis. All analyses were done using head width as a measurement of body size and elytral projection length and pronotum length as measurements of weapon size.

### **3.4 RESULTS**

#### *3.4.1 Mating behavior*

A total of 73 out of 146 pairings resulted in the male mounting the female, introducing the aedeagus, and maintaining the position inside the female for two observation periods or longer. Three other pairings had a successful copulation (more than 20 minutes) but the male dismounted before the end of the second period of observation. In most cases, males approached the female, although in a few instances females were seen approaching the males. Females could stand still and allow the male to



approach and mount or they could walk away. In a typical successful copulation, a male approaches the female from behind and quickly mount her. The pairs usually go through an average 1.6 (SD±1.46) physical encounters before mounting occurs. Although in a few cases females seemed to resist copulation by pressing their abdomen against the flat surface of the container, in most situations, at least partial intromission would occur and the time between mount and copulation was relatively short. During the 73 observed copulations, it took males an average of 185.67 (SD± 305.161) seconds to introduce their aedeagus after mounting the female.

### *3.4.2 Copulatory courtship behaviors versus male body and weapon size*

Male antennal stroke rate ranged from 0.040 to 2.30 strokes per minute (average ± SD =  $0.976 \pm 0.512$  strokes/min; N=73). Palpi vibration rate ranged from 0 to 1.180 strokes per minute ( $0.186 \pm 0.229$ , N=73), and foot taps ranged from 0 to 1.433 taps per minute ( $0.401 \pm 0.399$ ). For all copulatory courtship behaviors included in the study, antennal strokes, palpi vibrations and foot taps, the rate decreased significantly with increasing male body size (Table 3-1, Figures 3-1, 3-2, and 3-3) as well as with increasing weapon size (here we show analyses for one of the two components of male weapons, elytral projection length, since analyses for pronotum length produced very similar results, Table 3-1). Generalized linear models with log link functions for each behavior on both body and weapon size showed significant relationships for the overall regressions, but no significant coefficients of regression for either of the variables alone (Palpi vibrations:  $X^2 = 16.506$ ,  $P < 0.001$ ; antennal strokes:  $X^2 = 18.376$ ,  $P < 0.0001$ ; foot taps:  $X^2 = 8.210$ ,  $P < 0.05$ , N = 73). Thus, the correlation between body size and weapon

size is high enough that either one of these variables is equally successful at explaining a similar amount of variance in the behavioral rates.

Two of the three copulatory courtship behaviors (antennal stroke and foot tap rates) decreased with time. Antennal stroke rate during the first 10-minute observational period was significantly higher than during all other periods (Kruskal-Wallis:  $H=45.131$ ,  $p_{\text{value}} < 0.0001$ ,  $df=3$ ; PostHoc Tukey-Kramer Test: Period 1 significantly different from 2, 3 and 4; Figure 3-4). The rate of foot taps, during the first 10 minutes was not different from the second 10-minute period, but it was significantly higher than the next two periods (Kruskal-Wallis,  $H=19.766$ ,  $p_{\text{value}} < 0.0002$ ,  $df=3$ ; PostHoc Tukey-Kramer Test: Period 1 significantly different from 3 and 4; Figure 3-5)

### 3.4.3 *Dimorphism in weapon size*

The relationship between pronotum length and body size was not significant for (Eberhard & Gutierrez, 1991)'s non-linearity model 1, and we concluded this component trait of the weapon showed no weapon size dimorphism. On the other hand, the scaling relationship between elytral projection length and body size showed a significant  $\beta_2$  coefficient for non-linearity ( $\beta_{2\text{coefficient}} = -13.637$ ,  $t_{71} = -2.7561$ ,  $P < 0.05$ ). A test of (Eberhard & Gutierrez, 1991)'s model 2 for discontinuity showed a significant  $\beta_3$  coefficient, indicating a discontinuity of the scaling relationship with a switch-point at 1.52 head width ( $\beta_{3\text{coefficient}} = 0.503$ ,  $t_{71} = 4.577$ ,  $P < 0.0001$ , Figure 3-6). According to this model, males above or below the 1.52 mm head width switch-point follow a different scaling relationship, potentially resulting in two different types of male morphs. A seemingly bimodal distribution for elytral projection agrees with an intrasexual dimorphism for this component trait of the weapon.

#### 3.4.4 Copulatory courtship behaviors and weapon size dimorphism

We used the body size switch point we found for elytral projection length to divide males into majors and minors and compare copulatory courtship rates between these two categories. Minor males had a significantly higher rate of copulatory courtship behaviors than major males (Palpi vibrations: Mann-Whitney  $U' = 1009$ ,  $P < 0.001$ ,  $N_1 = 36$ ,  $N_2 = 37$ ; Antennal strokes:  $U' = 986$ ,  $N_1 = 36$ ,  $N_2 = 37$ ,  $P < 0.001$ ; and Foot taps: Mann-Whitney  $U' = 865$ ,  $N_1 = 36$ ,  $N_2 = 37$ ,  $P < 0.05$ ). We also used the majors and minor classification to test for an association between male type and pairings that resulted in copulation versus pairings that did not. We found that pairings with major males ended up in copulation significantly more than expected by chance alone, whereas pairings with minor males ended up in no-copulation significantly more than expected by chance ( $X^2 = 4.524$ ,  $P < 0.05$ ,  $N = 108$ ).

### 3.5 DISCUSSION

A new but intensive program of research in sexual selection is focused on determining the consequences of post-copulatory trait variation on male reproductive success (Simmons, 2001a; Arnqvist et al., 1997; Danielsson & Askenmo, 1999; Simmons & Achmann, 2000; House & Simmons, 2002). However, little is known about the causes of variation in these traits, especially in copulatory courtship behaviors. The expression of traits under post-copulatory sexual selection, such as copulatory courtship, may interact with, and be influenced by traits under pre-copulatory sexual selection (Andersson & Simmons, 2006), such as body size and weapon size. Individuals could base the intensity of their copulatory courtship behaviors on their pre-copulatory traits as part of their efforts to maximize their chances of fertilization success. Thus, determining

whether individuals base the quality or intensity of copulatory behaviors on pre-copulatory attributes has important implications for understanding the development of reproductive strategies.

In our study, all copulatory courtship behaviors (palpi vibrations, antennal strokes and foot taps) were negatively correlated with body and weapon size (Figures 3-1, 3-2, and 3-3). Body and weapon size vary greatly among *A. sparsa* males and these characteristics may be associated with variation in copulatory courtship behaviors for two non-mutually exclusive reasons: First, this pattern may arise through male-male competition. Field studies show that in *A. sparsa*, both weapon and body size influence success in pre-copulatory male-male competition for access to females. Fighting success is positively correlated with weapon and body size, and larger males (bigger weapons and body size) win more fights than smaller males (smaller weapons and body size) (see Chapter 2.). For small males, this inferior fighting ability may make actual copulations with females rare or infrequent, causing every single copulation with a female to be a highly valuable event. For each mating opportunity, small males may invest more heavily than larger males on their copulatory courtship behaviors, in an effort to influence females into accepting their sperm and oviposit, once they have succeeded in coupling with them. Sperm competition theory explains that males in disfavored roles with high risk of sperm competition should have a higher ejaculate expenditure (quantity and quality) than males in favored roles with reduced risks of sperm competition (Simmons et al., 1999; Parker, 1990; Wedell et al., 2002; Birkhead & Moller, 1998) Experimental evidence for this has been found in arctic charr, domestic fowl, Norway rats, Atlantic salmon, humans and dung beetles amongst others (Cornwallis & Birkhead, 2007;

Kilgallon & Simmons, 2005; Rudolfson et al., 2006; Simmons et al., 1999; Pound & Gage, 2004; Gage et al., 1995). Males under increased risk of sperm competition can also put more effort into other sexually selected traits such as courtship display, mate guarding and male-male aggressiveness (LeBoef & Peterson, 1969; Mougeot et al., 2001; Gage & Baker, 1991). At least one study has showed that in the Japanese stag beetle *Prosopocoilus inclinatus* the intensity of male courtship during female mounting and immediately preceding copulation was higher in males with smaller weapons and body sizes (Yasukazu & Eisuke, 2005). Thus, the idea of an increase in ejaculate investment when there is a higher risk of sperm competition could extend to other post-copulatory traits that influence female sperm uptake, such as genitalia sizes or copulatory courtship behaviors. In such case, we might predict a negative correlation between copulatory courtship rate and measures of body and weapon size in males.

A second reason for this pattern, which may be working separately or in conjunction with the one described above, involves female choice. Large males may be more attractive to females because larger size may be related to higher quality in the offspring, it may improve a male's ability to defend females against other harassing males, and because large males may make higher contributions to egg production through larger spermatophores and other male-derived products (Sirot et al., 2007). If females are generally more attracted to larger males, then these males may not need the additional enticements of an elaborate copulatory courtship. On the other hand, smaller males may need to increase their behavior rates during copulation to stimulate less receptive, large male-preferring, females into allowing them continue to the subsequent phases of copulation and into accepting their sperm. In insects, the first phase of genital

intromission of the penis or aedeagus into the female's bursa copulatrix does not necessarily guarantee sperm transfer, and where they have been investigated, insect copulations consist of a series of phases that culminate in the deposition of sperm inside the female's genital tract (Tallamy et al., 2002). Once the male's aedeagus is introduced, males may still face internal resistance from females such that they cannot inflate their internal genital sac and properly pass sperm (Eberhard, 1996). The genitalia of *A. sparsa* males also include a long whip-like sclerotized structure that emerges from the internal sac, called the flagellum. In successful copulations of a related species, *C. alternans*, this flagellum was found inside the female genital chamber and in some cases, partially up the spermathecal duct (Rodriguez, 1993). Thus, even after the aedeagus is introduced, *A. sparsa* males may need to use copulatory courtship behaviors to persuade females into permitting them to inflate their genital sac and extend their flagellum inside them.

In our experiment, we found that small males copulated with females less than expected by chance, whereas large males copulated with females more than expected by chance, indicating that females of *A. sparsa* are more likely to accept and copulate with large males. Given the initial preference that females show for large males, small males may experience a higher amount of internal resistance from females than large males. One indirect line of evidence for the idea that copulatory courtship behaviors function to stimulate the female into allowing males to continue to subsequent phases of copulation is that both antennal strokes and foot tap rates decreased over time across the copulation observational periods. It is important to point out that the level of choosiness of the females used in this study was likely influenced by their unmated status, since all females we used were virgin. Future studies should include previously mated females in order to

examine the interactions between re-mating, male pre-copulatory attributes and copulatory courtship behaviors.

An important distinction between pre and postcopulatory courtship may be the ability for males to assess female interest. Pre-copulatory courtship commonly involves unidirectional information transfer from the male to the female. Leks are a familiar example of this, males display, and females use this information to assess male quality (Andersson, 1994). On the other hand, copulation may bring about a degree of interaction between males and females that produces a more readily bidirectional exchange of information. Here, females again have the opportunity to assess male displays, but now males may be better able to assess female receptivity based on internal and external tactile, and chemical cues made available by a more intimate association with their mate. This degree of interaction and intimacy between male and female during copulation increases male assessment capabilities and thus changes the rules that males are subjected to, allowing them to make more tailored choices about how much they should engage in courtship during each copulation event.

Finally, the differences in copulatory courtship behaviors found among males of *A. sparsa* may be related to alternative tactics played by morphologically distinct males. To better understand the relationship between copulatory courtship behaviors and weapon size, we investigated whether males of *A. sparsa* presented a weapon size dimorphism. We found that males were dimorphic for one of two morphologies included in the measurements of weaponry (they were dimorphic for elytral projection but not for pronotum length). An analysis of the scaling relationship between elytral projection and body size showed a discontinuous dimorphism, whereby males below a certain body size

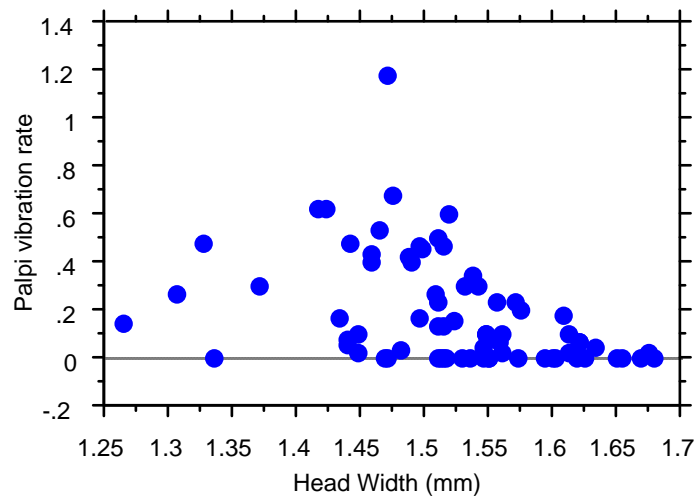
threshold invest less in weaponry than expected for their body size (minors), and males above this threshold body size threshold invest more in weaponry than expected for their body size (majors). When we used the size switch-point to divide major and minors, we found that minors had significantly higher rate of copulatory courtship behaviors than majors per copulation. These differences between the two groups could be interpreted as forming part of a repertoire of traits related to discrete alternative tactics in *A. sparsa* males. It is difficult, however, to interpret the result of the dimorphism test in these beetles, since it was significant for only one of the two morphologies describing the weapon. The discontinuous nature of the scaling relationship is also hard to confirm through visual inspection. Thus, it may be useful to think about this species as occupying an intermediate place along a continuum that moves from non-dimorphic to completely dimorphic species with discrete alternative tactics. Regardless of whether there are two different tactics or not in *A. sparsa*, this study shows that post-copulatory traits, such as copulatory courtship behaviors are negatively correlated with pre-copulatory traits such as male body and weapon size. This explicit consideration of the interaction between both types of sexual traits (pre and post-copulatory) will provide us with a broader appreciation of sexual selection as an evolutionary process that acts, not on single traits, but on whole organisms.



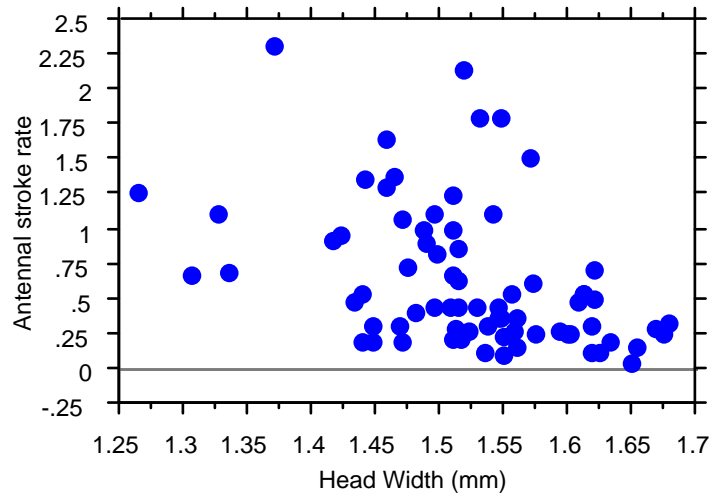
### 3.6 TABLES AND FIGURES

**Table 3-1:** Test statistics for Spearman Rank single correlations between copulatory courtship behaviors (palpi vibrations, antennal strokes and foot taps) and male body and weapon size (N=73).

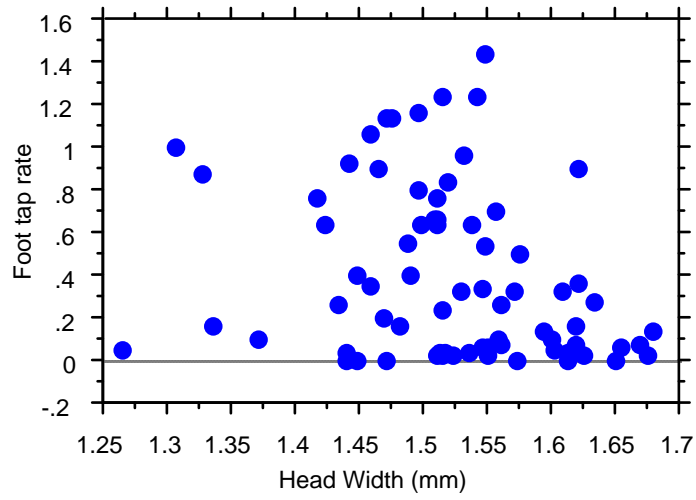
Head width (Body size)				Elytral projection length (Weapon size)			
Behavior	$\rho$	$z_{value}$	$P$	Behavior	$\rho$	$z_{value}$	$P$
Palpi vibration rate	-0.473	-4.016	<.0001	Palpi vibration rate	-0.485	-4.117	<.0001
Antennal stroke rate	-0.465	-3.948	<.0001	Antennal stroke rate	-0.510	-4.324	<.0001
Foot tap rate	-0.286	-2.427	0.0152	Foot tap rate	-0.292	-2.475	0.0133



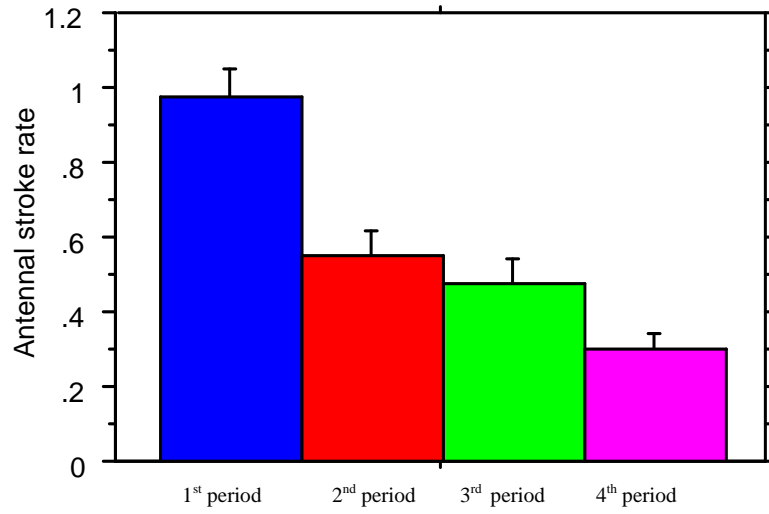
**Figure 3-1:** Rate of palpi vibrations performed during copulation as a function of male body size.



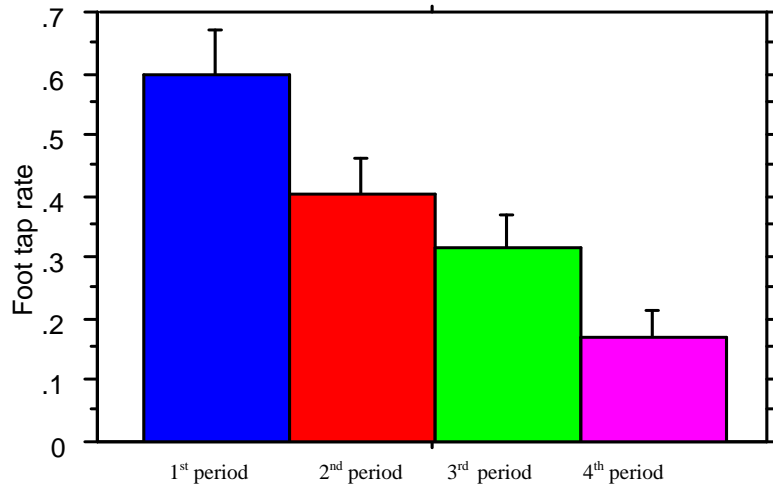
**Figure 3-2:** Rate of antennal strokes performed during copulation as a function of male body size.



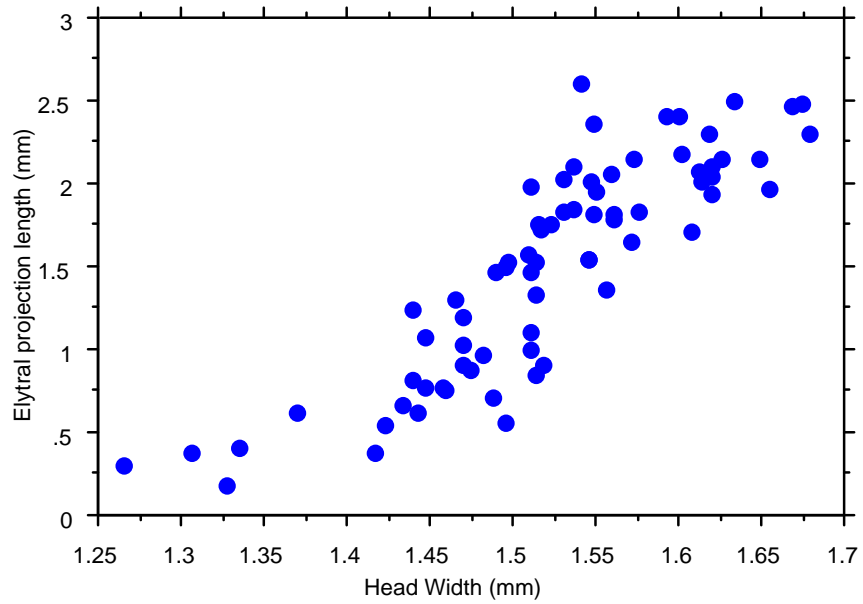
**Figure 3-3:** Rate of foot taps performed during copulation as a function of male body size.



**Figure 3-4:** Changes in mean antennal stroke rate across four subsequent 10-minute observational periods. Bars depict standard error.



**Figure 3-5:** Changes in mean foot tap rate across four subsequent 10-minute observational periods. Bars depict standard error.



**Figure 3-6:** Scaling relationship between head width and elytral projection length. Model 2 indicates a body size switch point at 1.52 head width.

**CHAPTER 4:**  
**SEXUAL MORPHOLOGIES, COPULATORY COURTSHIP, AND**  
**THEIR IMPORTANCE FOR FIRST VERSUS SECOND MALE**  
**MATING ORDER IN THE TORTOISE BEETLE *ACROMIS SPARSA***

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#### 4.1 ABSTRACT

In species with multiply-mating females, males compete for access to fertilizations inside the female reproductive tract. Here, postcopulatory sexual selection will favor males with traits that can enhance fertilization success with already mated females (male offensive ability), and traits that reduce the fertilization success of males subsequently mating or attempting to mate with the same female (male defensive ability). Because different traits may be employed for offensive and defensive male roles, studies measuring variation in male fertilization success for only a single role may be misleading, over- or under-estimating the importance some traits to overall fertilization success. Nevertheless, few studies have compared the contributions of male sexual traits to fertilization success during offensive and defensive roles. Such an approach is important to further our understanding of the mechanisms by which postcopulatory sexual selection affects trait evolution. In this study, I conducted a double mating experiment and paternity analysis with *Acomis sparsa*, a neotropical tortoise beetle, to assess the effects of several sexual traits on the fertilization success of first and second males. I found no effect of genitalic structures, testes mass, body size, or copulatory behaviors on the fertilization success of first males. However, fertilization success was strongly associated with absolute measures of antennal stroke rate and weakly associated with relative testes mass in second males. These findings show that the contribution of some sexual traits to fertilization success in *A. sparsa* is context-dependent, and can vary considerably across offensive and defensive mating roles.

## 4.2 INTRODUCTION

Females of many species copulate with multiple males. This promiscuous behavior allows for male competition for fertilizations to continue inside the female's reproductive tract (Simmons, 2001a; Birkhead & Moller, 1998) and gives females the opportunity to mediate this competition (Eberhard, 1996; Price et al., 1999). One fundamental issue for males engaged in competition inside the female's reproductive tract is that they face two opposing selective pressures (Parker, 1970a). First, males must be successful at resisting displacement of their sperm by the incoming sperm of later mating males. This selects for "defensive" strategies with traits that protect a male's ejaculate from preemption or neutralization. Second, males must be successful at gaining dominance over the sperm that is already inside the female reproductive tract. This selects for "offensive" strategies with traits that are good at overcoming any paternity assurance mechanisms of earlier males (Wigby & Chapman, 2004; House & Simmons, 2006; Parker, 1970b; Parker, 1970a). Thus, for each mating event, males will encounter fundamentally different roles depending on whether they mate with virgin females (first male role) or non-virgin females (second or later male role), and these roles will be affected by the likelihood of the female re-mating afterwards or the sperm competition risk (Wedell et al., 2002) and the type and degree of sperm precedence.

Although many studies have demonstrated clear patterns of either first or second male sperm precedence in insects (Simmons, 2001a), fewer studies have measured which traits or components of a male's sexual phenotype contribute to fertilization success in offensive versus defensive roles (but see Wilkinson & Fry, 2000; House & Simmons, 2006; Clark et al., 1995; Civetta & Clark, 2000; Nilsson et al., 2003; House & Simmons,

2002; Arnqvist & Danielsson, 1999). Moreover, studies that have looked at the importance of sexual traits on first or second male roles have mostly focused on single categories of traits, such as genitalic structures (House & Simmons, 2006; Otronen, 1998; Waage, 1979), seminal fluids (Harshman & Prout, 1994; Price et al., 1999) or copulatory behavior (von Helversen & von Helversen, 1991; Otronen, 1997). However, because first and second males face different contexts, the components of a male's sexual phenotype crucial for fertilization success in each role may differ (House & Simmons, 2006). Alternatively, the same component of a sexual phenotype may be efficient at protecting sperm in the first male role and at gaining dominance over stored sperm in the second male role and therefore, be crucial for fertilization success across both contexts (Price et al., 1999). Comparing the fitness contributions of several sexual traits in first versus second males will highlight contrasts between male sexual strategies in these two roles. Thus, a more comprehensive approach that includes multiple components of the sexual phenotype is needed to gain a better understanding of the mechanisms by which sexual selection affects the evolution of traits through different male mating roles, and how these mechanisms act together to shape the evolution of whole organisms.

In this study, I used double mating experiments that included behavioral observations and paternity analyses with microsatellite markers to assess what components of the sexual phenotype of *Acromis sparsa* (genitalic structures, testes, body size or copulatory courtship behaviors) contributed to the fertilization success of males in first versus second mate roles.

*Acromis sparsa* is an ideal species to determine the importance of different sexual traits on first versus second male mating roles. First, this neotropical tortoise beetle has

multiple exaggerated sexual morphologies and behaviors: They possess secondary sexual characters, which are rigid elytral and pronotal projections used in male-male combat for access to females (Windsor, 1987). Their primary sexual characters include a hardened intromittent organ called the aedeagus, a genitalic whip or flagellum that can be longer than the entire body, and testes that occupy roughly 10-15% of the internal cavity (pers. obs.). They also display copulatory behaviors such as palpi vibrations, antennal stroking and foot tapping, which follow Eberhard (1994)'s criteria for copulatory courtship and are very similar to other behaviors previously described and known to influence male fertilization success in other insect species (Eberhard, 1994; Edvardsson & Anqvist, 2000; Sirot et al., 2007; Tallamy et al., 2002).

Second, females of *A. sparsa* mate multiple times before each reproductive bout and can store sperm for weeks, providing ample opportunities for male competition inside their reproductive tract (pers. obs.). In a large mesocosm experiment, the level of sperm competition risk or fraction of females that mated more than once was moderate to high ( $q = 0.47$ , Wedell et al., 2002) and the number of male partners ranged from zero to six (see Chapter 2)

Finally, microsatellite markers were fully developed for this species in order to accurately assess the fertilization success of males in both the first mate and second mate role. Most studies that include double mating experiments in insects have relied heavily in genetically based morphological markers or irradiated male techniques to determine male reproductive success (but see Simmons & Achmann, 2000; Simmons et al., 2004). However, both of these techniques could be problematic for some species. Techniques using morphological markers may artificially bias estimates of sperm competitive ability

because marker strains with visible mutations generally have lower fitness than the wild-type (Wigby & Chapman, 2004). In the case of irradiation techniques, irradiated sperm may be less successful at attaining “fertilization” of an ovum, which would again bias the estimates towards non-irradiated males. Moreover, even when irradiated sperm is just as competent as non-irradiated sperm at fertilizing an ovum, radiation may affect a male’s copulatory behavior or the female’s perception of the irradiated male, and consequently of the non-irradiated male (Sirot et al., 2007). Thus, the development of microsatellite markers for *A. sparsa* minimized the potential for biased estimates of male competitive ability in the double-mating experiments.

This study simultaneously assessed the contributions of genitalic structures, testes size, body size and copulatory courtship behaviors to the fertilization success of males in first versus second mating roles in *A. sparsa* beetles.

## 4.3 METHODS

### 4.3.1 Study Site and Subjects

*A. sparsa* is a neotropical leaf beetle (Chrysomelidae: Cassidinae) that ranges from Mexico to Peru (Blackwelder, 1982; Chaboo, 2007). *A. sparsa* is highly abundant in Panama, Central America, and individuals are easy to find because they feed, mate and oviposit on a single hostplant, *Merremia umbellata* (Convolvulaceae). All individuals for the experiments came from wild patches of *A. sparsa* monitored from 2003 to 2005 at the Soberania National Park and in the vicinity of Gamboa, Colon Province, Republic of Panamá (9°06' N, 79°41' W). All laboratory rearing and behavioral experiments were conducted at the Smithsonian Tropical Research Institute facilities in Gamboa.

Virgin females of *Acromis sparsa* were obtained for this study by collecting first instar larvae during the months of May and June 2005 and rearing them in an outdoor laboratory. Larvae were separated by family and reared under equivalent conditions in small 20x12cm plastic containers until pupation. We obtained fresh *M. umbellata* leaves daily from different vine patches in the area, mixed them and then distributed them randomly to the larval groups in order to avoid individual plant variation in nutrients. After adult emergence, a single female from each family group was randomly chosen to be used in a double mating experiment. Experimental females were placed in all-female containers for 25-40 days and fed fresh leaves daily, to allow them to become reproductively active. Males were collected in the wild, from patches of *M. umbellata* in the vicinity of Gamboa. These males were placed in individual containers with fresh leaves, for two to three days before the experiment started. We used each male for only one mating in the experiment.

#### *4.3.2 Experimental Design and Behavioral Observations*

To conduct double matings, a female was introduced into a mating chamber, which consisted of a 12cm<sup>3</sup> glass box placed upside down with the open side towards the table, an observer with a video camera was positioned between 50-100 cm away. This glass container allowed the observer to film the beetle pairs from all angles without having to approach too closely. The female was left alone in the chamber for a total of ten minutes while it acclimatized to the area. After these ten minutes, a randomly chosen male was introduced in the chamber. If the pair did not copulate after a maximum of 90 minutes, the male was replaced with a different male. If no copulation occurred this time, the pair was removed from the mating chamber and a new female and male were used. If

the male mounted the female, time of mount was recorded and the observer noted if and when the male penis or aedeagus was introduced into the female (hereafter called copulation). In a related species, *Chelymorpha alternans*, copulations with successful transfer of sperm, where spermatozoids enter the spermatheca, last a minimum of approximately 20 minutes (Rodriguez, 1993). Thus, only copulations that lasted longer than 20 minutes were considered successful and were included in this study. The pairs that surpassed the 20-minute limit were allowed to mate until they separated or for a maximum of 90 minutes. Approximately 27% of the pairs separated before minute 90. After the first pairing, females were isolated for four to six hours before being presented with the second male. For the second mating, females were presented with a randomly chosen male for a total of 90 minutes. If the pair did not copulate, the male was replaced with a different male. Recordings of copulatory behaviors started when the male entered his aedeagus into the female and continued through the entire experimental time. All recordings were done with a SONY TRV350 video camera. Videos of each pair were randomized and later analyzed by a single observer with J-watcher software (v.1.0, Blumstein, D. University of California, Los Angeles). This observer recorded the total number of times each male performed the following two behaviors: (1) Foot taps, which consisted of males tapping lightly on the females' elytra with front or mid tarsi; and (2) Antennal strokes, which consisted of males slightly lowering their heads and stroking females one, two or three times at the base of the elytra or pronotum with the either the right, left or both antennae. In some occasions, it also included the stroking and rubbing of the lateral edges of the females' antennae. In a closely related species, it has been suggested that females can reject sperm by expulsing a white droplet after mating

(Rodriguez, 1995). Thus, females were observed closely after each mating event to check for white droplet expulsion, however, this was not noticed in our study.

After the matings, experimental males were dissected to have their testes extracted and weighed. Immediately after dissections, males and testes were collected and preserved in 95% ethanol and DMSO for later use in DNA extractions, genotyping and morphological analyses. Females were marked with small insect tags on their pronotum (Insect Marking Kit, BioQuip, Rancho Domingo, CA) and placed inside a small insectary (2.25 m<sup>2</sup> footprint x 1.5 m height) with abundant hostplant to encourage oviposition. Because *A. sparsa* exhibits maternal care, females and their second instar larvae broods were collected as distinct groups from the insectary. This allowed me to accurately know maternity for all larvae collected. All females and larvae were also preserved in 95% ethanol and DMSO for later use in analyses.

#### 4.3.3 Morphological Measurements

For morphological analyses, I used head width and femur length as indicators of male body size. I also measured testes mass as an indicator of testes size and aedeagus and flagellum length as indicators of male genitalia size. I measured head width and pronotum length as measurements of female body size. Males and females' body size measurements, were taken by lining up single individuals at the same angle in graphing paper and taking the measurements with a Mitutoyo digital caliper (nearest 0.01mm). Male genitalia pictures were taken with a digital Hitachi KP-D50 stereoscope camera attached to a Leica MZ6 stereoscope. Scion Image (NIH) software was used to conduct measurements of aedeagus and flagellum length (see Chapter 2, Figures 2-1 and 2-2 for



complete description of the measurement location of weapons and genitalia). Testes were weighted to an accuracy of 0.01mg in an A&D Scale (model ER-182A).

#### 4.3.4 Genetic analysis

Tissue samples were taken from 90 adult beetles and 1036 larvae. Gonads and legs were used for adult DNA extraction whereas the entire larva was used for larval DNA extraction. Before extraction, the tissue was powdered using a 96-well bead mill homogenizer (2000 Geno/Grinder, SPEX CertiPrep, NJ, USA) in conjunction with 2-mm high density zirconium oxide beads (Glen Mills, Clifton, NJ, USA) (Allender et al., 2004). Genomic DNA was extracted following a Phenol-Chloroform extraction protocol (Sambrook et al., 1989) modified for tissue powdering. DNA concentrations were determined by spot-checking the samples with Hoechst 33258 fluorescent dye and a UV fluorometer (Turner Biosystems TBS-380) and samples were diluted to a standard concentration.

Because *A. sparsa* exhibits maternal care, maternity for all larvae was known with certainty, based on collection of female-larval groups. Three of the five microsatellite markers I developed for this species were used to assign male paternity in this experiment (*D3*, *A127* and *A115*, see Chapter 2 for a full description of the microsatellite marker development for *Acromis sparsa* and a table with all microsatellite markers). These markers were chosen because they allowed for multiplex amplification. HEX and FAM dyes (Invitrogen Corporation) as well as NED dye (Applied Biosystems) were used to label the primers. The PCR amplification reaction included the following ingredients: 2.0ul 5x GoTAQ Flexi buffer (Promega Corporation), 0.80ul 25mM MgCl<sub>2</sub>, 0.80ul 2.5mM dNTPs, 0.2ul 10x BSA, 0.2ul of each 10uM labeled primer, 0.15ul GoTAQ Flexi

(Promega Corporation), and sterile distilled water to a final volume of 10ul. The amplification was completed using the following program: 3 minutes at 94°C, 10 cycles of [30 seconds at 94°C, 30 seconds at 59°C (reduced by 1°C each cycle), 45 seconds at 72°C], 30 cycles of [30 seconds at 94°C, 30 seconds at 49°C, and 45 seconds at 72°C], followed by a final extension for 10 minutes at 72°C. For visualization, 1ul of the PCR product was run through an ABI 3130xl Genetic Analyzer capillary electrophoresis machine (Applied Biosystems, Foster City, California, USA) with Genescan 500 ROX size standard (Applied Biosystems).

We used the Genemapper software package (Applied Biosystems, Forster City, California) to generate genetic profiles of each parent and individual larvae at the three microsatellite loci. These profiles were verified individually by eye and only those samples showing strong and unambiguous peak profiles were used in the final analysis. Error rates and null alleles were determined by assessing mismatches between mothers and larvae. Paternity was assigned individually by determining the offsprings' unique paternal allele combinations and then matching them against the two candidate fathers. Because one of the microsatellite loci we used had a null allele, we modified our exclusion criterion at this marker. In this case, for every offspring that was homozygous for a maternal allele, we maintained both candidate fathers if they were homozygous at that locus, because they could be potentially carrying a null allele. These males could not be excluded unless they had allele mismatches at all other loci.

#### *4.3.5 Statistical Analysis*

To estimate similarities between female egg laying behavior in the field and in the insectary, I compared the number of eggs laid by experimental females to those of

females found in monitored hostplant patches around Gamboa. I also determined the relationship between female size and egg number in both experimental and field broods.

To establish the degree of first versus second male sperm precedence, I calculated the proportion of offspring sired by males in the second male role ( $P_2$ ) and males in the first male role ( $P_1$ ) (Boorman & Parker, 1976; House & Simmons, 2006; Sirot et al., 2007). ) and determined the effects of mating order on fertilization success.

To determine the contributions of sexual morphologies and courtship behaviors to the fertilization success of first males, I tested for correlations between  $P_1$  and measures of the genitalic structures, testes mass, body size and copulatory courtship behaviors.

To determine the contributions of sexual morphologies to the fertilization success of second males, I tested for correlations between  $P_2$  and measures of the genitalic structures, testes mass, body size and copulatory courtship behaviors. Separate analysis using either aedeagus or flagellum length as the correlated measures of genitalia size showed similar results; therefore in order to minimize the number of variables, I only included flagellum length in the final multivariate models for both first and second male mating order.

To assess whether the differences of first to second male measurements affected fertilization success, I also tested the effect of the relative measures of male traits (ratio of second male measurement/first male measurement) for all morphologies and copulatory behaviors on  $P_2$ . Finally, to test whether female size had an effect on male fertilization success, I determined the relationship between relative male to female body size and  $P_1$  versus  $P_2$ .

Because paternity scores in double mating trials yield proportional data that conform to a binomial, rather than a normal distribution (Zar, 1984), I used generalized linear models with binomial errors, logit link functions and a correction for overdispersion ('quasibinomial distribution', Williams, 1982) in all the analyses. All statistical analyses in this study were performed with the software program JMP (SAS, Cary, North Carolina).

## 4.4 RESULTS

### 4.4.1 Female egg laying behavior

Thirty experimental females with eggs were recovered from the insectary. Experimental females in the insectary laid an average of 39.67 eggs per clutch ( $SD \pm 4.93$ ). There was no significant difference between the number of eggs laid by females in the insectary and those laid by females in the field (Student t-test:  $t_{68} = 1.718$ ,  $P > 0.05$ ). In both, experimental and field females, the number of eggs increased with female body size (Experimental females:  $R^2 = 0.38$ ,  $F_{1,19} = 11.767$ ,  $P < 0.01$ ; Field females:  $R^2 = 0.21$ ,  $F_{1,35} = 9.27$ ,  $P < 0.01$ , Figure 4-1A and B).

### 4.4.2 Sperm precedence

I found a clear signal of last male sperm precedence in *Acromis sparsa*. Second males in the double mating experiment sired significantly more offspring than first males to mate (Effect<sub>(1st male)</sub> = -1.0285, SE = 0.3144,  $X^2 = 12.480$ ,  $P < 0.001$ , N = 29, Figure 4-2). The average proportion of eggs fertilized was 0.739 ( $SD \pm 0.304$ ) for second males to mate and 0.260 ( $SD \pm 0.304$ ) for first males to mate and the proportion of eggs fertilized in both roles ranged from 0 to 1. The coefficients of variation (CV) for the proportion of

offspring sired were 122.31 for first males and 42.29 for second males. Thus, though there is second male sperm precedence in this species, I also found a large amount of variation within first males and second males in the degree to which they successfully fertilized the female.

#### *4.4.3 Effect of sexual morphologies and copulatory behavior on fertilization success in first versus second males*

Although we found a large amount of variation in the proportion of offspring sired for first males ( $P_1$ ), the model including flagellum length, testes mass, body size, antennal stroke rate and foot tap rate showed no relationship between any of these physical and behavioral attributes and fertilization success. Thus, none of the physical and behavioral male attributes measured seem to be able to explain the variation we find in first male reproductive success.

On the other hand, proportion of offspring sired for the second males ( $P_2$ ) was positively associated with antennal stroke rate, and this was the only predictor variable retained in the minimal relevant model (Effect = 1.129, SE = 0.3959,  $X^2 = 10.436$ ,  $P < 0.01$ ,  $N = 29$ , Figure 4-3). None of the other physical or behavioral attributes included in the analysis (flagellum length, testes mass, body size and foot tap rate) contributed to the observed variation in fertilization success of second males.

When comparing the effects of the relative measures of male traits, I found no relationship between second male to first male ratios of sexual morphologies and copulatory behavior and second male fertilization success. However, there was a marginally significant trend towards higher proportion of offspring sired in second males

with an increase in testes mass ratio of second to first males (Effect  $_{2\text{male}/1\text{male}} = 2.71$ , SE = 1.988,  $X^2 = 3.179$ ,  $P = 0.073$ , N = 23).

Finally, I found no effect of relative male to female body size on the fertilization success for either first or second male mating order.

#### 4.5 DISCUSSION

In species where females mate multiply, male competition for fertilizations continues inside the female's reproductive tract (Simmons, 2001a; Simmons, 2003), and males are faced with two strikingly different mating roles depending on their mating order (Parker, 1970a). First males need to ensure it is their sperm and not a later male's sperm that gets utilized in fertilizations, thus following a defensive strategy. On the other hand, second or later males need to secure the dominance of their sperm over sperm from earlier males, thus following an offensive strategy (Parker, 1984). Because offensive and defensive roles are very different contexts of sexual selection, it is important to determine whether the same or different components of a male sexual phenotype contribute to fertilization success in each case (House & Simmons, 2006). This information can give us insights into the mechanisms by which sexual selection acts on traits and how do these traits get integrated in whole organisms.

In this study, I used a double mating experiment, genetic analyses and multivariate techniques to follow different sexual traits and assess their effects on the fertilization success of first and second males roles in *A. sparsa*. I found no effect of genitalic structures, testes mass, body size or copulatory behaviors on the fertilization success of first males. On the other hand, fertilization success was strongly associated

with antennal stroke rate and weakly associated with relative testes mass in second males. Thus, this simultaneous analysis of sexual traits in *A. sparsa* showed that the level of contribution of sexual traits to fertilization success is context-dependent and can change significantly across mating roles.

#### 4.5.1 Female egg laying behavior

Female laying behavior was similar in both insectary and natural settings. I found no difference between the number of eggs laid in the insectary and in the field. Thus, female behavior did not seem to be influenced by the potted plants or confinement in an enclosure. More interestingly, clutch size increased significantly with an increase in female body size both in insectary and in field-monitored females. If female size is an indication of higher fecundity, males may choose to preferentially mate with larger females. Although I found no relationship between female size and male copulatory behaviors in this study, another experiment measuring *Acromis sparsa* mating and fertilization behavior suggests males may prefer to mate with and fertilize larger females (see Chapter 2).

#### 4.5.2 Sperm precedence

The proportion of offspring sired by the second male was greater than the proportion sired by the first male in *A. sparsa*. This pattern of last male sperm precedence has been found in many other insect species (Simmons, 2001a). However, as in many other insects (Lewis & Austad, 1990; Simmons, 2001a), mating order explains only a small part of the variation in fertilization success, which in *A. sparsa* can range from 0% to 100% success in either first or second males. Moreover, for each female, high values of second male sperm precedence can occur through different mechanisms and levels of

sperm competition if, for example, the sperm of different males mixes but the sperm of the second male is superior, versus if the sperm of the first male is lost or ejected from the storage, or if the sperm of two males was stored, but only the sperm of the last one is used (Wigby & Chapman, 2004). Thus, it is important to identify what other causes of variation exist for sperm use patterns in insects.

#### *4.5.3 Effect of sexual morphologies and copulatory behavior on fertilization success in first versus second males*

I found no relationship between any of the measurements of sexual morphologies or copulatory behavior included in this study and fertilization success in first males. First males may be able to assure their paternity using sexual attributes that were not measured due to the nature of this study. In many species an important mechanism for defense strategy is to prevent females from remating. Males in a defensive role can reduce the risk of competition by mate guarding (Parker, 1970b; Alcock, 1994) or by using seminal fluids that suppress female receptivity to remating and stimulate egg laying behavior (Chapman, 2001; Clark et al., 1999). Because males were not allowed to mate guard and females were not allowed to lay eggs until they mated with a second male, traits involved in preventing females from remating were not assessed in this study. Future studies should include single matings or measurements of mate guarding in order to more accurately assess the causes of variation in fertilization success for first males of *A. sparsa*.

This study found that copulatory behavior, specifically antennal stroke rate, was an important factor determining the fertilization success of second males but it did not affect the fertilization success of first males. Antennal strokes or leg strokes have been



shown to affect mating or fertilization success in at least six different insect species: *Diabrotica undecimpunctata* (Tallamy et al., 2002); *Diaprepes abbreviatus* (Sirot et al., 2007); *Ontophagus taurus* (Kotiaho et al., 2001); *Psilothrix viridicoeruleus* (Shuker et al., 2002); *Tribolium castaneum* (Edvardsson & Anqvist, 2000) and *Dryomiza anilis* (Otronen, 1997). However, none of these studies explicitly assessed the effects of antennal or leg strokes in first versus second male roles.

The relationship between antennal stroke rate and fertilization success in second males of *A. sparsa* may be mediated by cryptic female choice. There are many potential mechanisms by which females may select sperm of males with high antennal stroke rates. Females may be less likely to produce resistance, allow more sperm transfer or actively select sperm from those second males with higher antennal stroke rates. Where mechanisms have been studied, there is evidence that females can perceive and make choices of sperm usage based on behaviors such as antennal or leg stroking (Edvardsson & Anqvist, 2000; Tallamy et al., 2002). It is interesting to note that it is the absolute value of antennal stroke rate and not the relative measure that affects fertilization success in second males. If there is cryptic female choice in this species, this indicates that females are not assessing relative values of current to past mates but instead choosing based on the absolute values of the last male they mate with.

Relative measures are important to include because a male's success may be related to his traits relative to the male he is competing against (Sirot et al., 2007). On the other hand, absolute measures will be especially important for traits that are involved in direct removal of sperm or in stimulation of sperm ejection by the female (Cordoba-Aguilar, 1999; Waage, 1979), or whenever it is the total amount of stimulation that

affects the process of sperm usage. Thus, antennal stroke rate would not need to be directly affecting the process of sperm usage, and instead be correlated with traits involved in sperm removal or involved in stimulation of sperm ejection by the female.

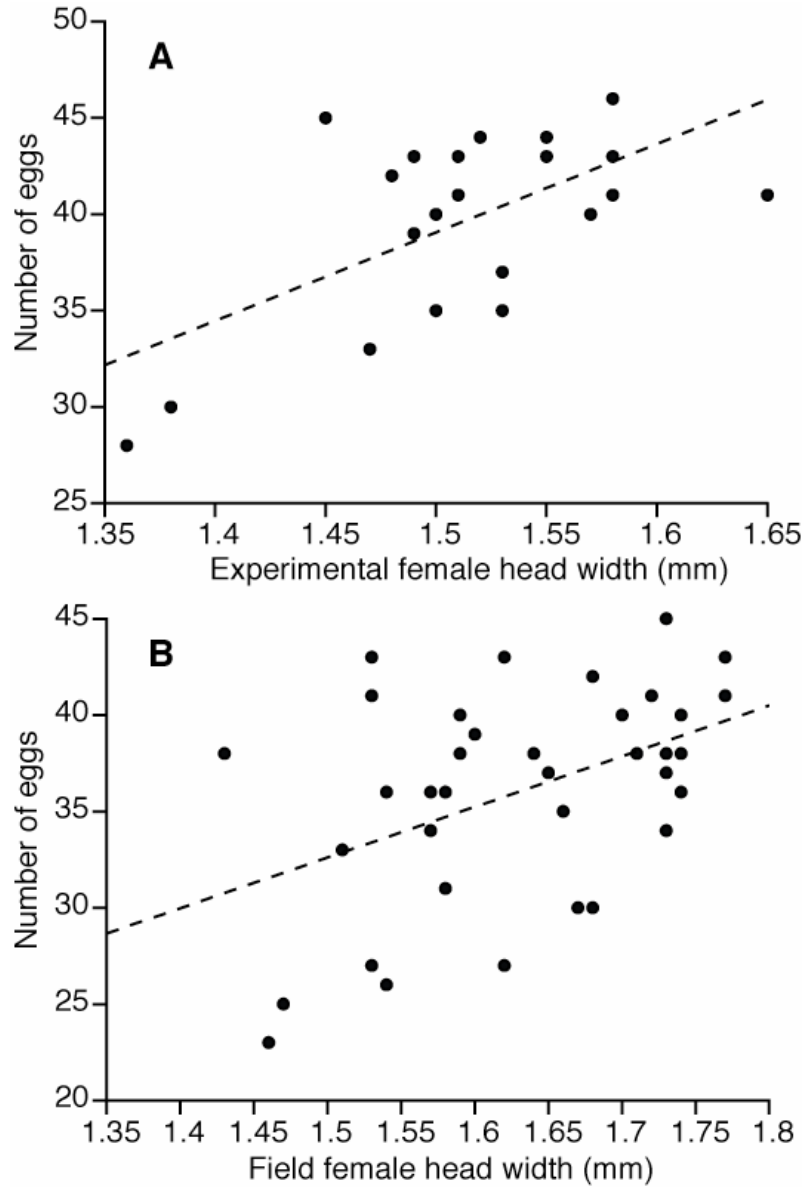
Antennal stroke could also be correlated with the amount of transfer of sperm or seminal fluids (Matthews et al., 1997), or it could be a response to female's level of receptivity or a mechanism by which males transmit pheromones to the female in order to influence receptivity (Sirot & Brockmann, 2001). In these cases, fertilization success in the second male would not be related to antennal stroking itself but to another trait associated with this copulatory behavior. In order to assess whether this copulatory behavior is directly involved in fertilization in second males and whether cryptic female choice mediates the relationship between antennal stroke rate and fertilization success, the specific mechanisms by which females may influence sperm use patterns in *A. sparsa* need to be studied.

I also found a marginally significant relationship between relative testes mass and fertilization success in second males, which may be explained if the amount of sperm transferred to a female by the second male is dependant on how much sperm was transferred by the first male. Testes mass also showed a significant effect on fertilization success for males in a natural insectary experiment, where first and second males were not distinguished (see Chapter 2). Though the relationship between fertilization success and testes in this study is weak, it warrants further study of the specific mechanisms of sperm transfer and uptake in *A. sparsa*.

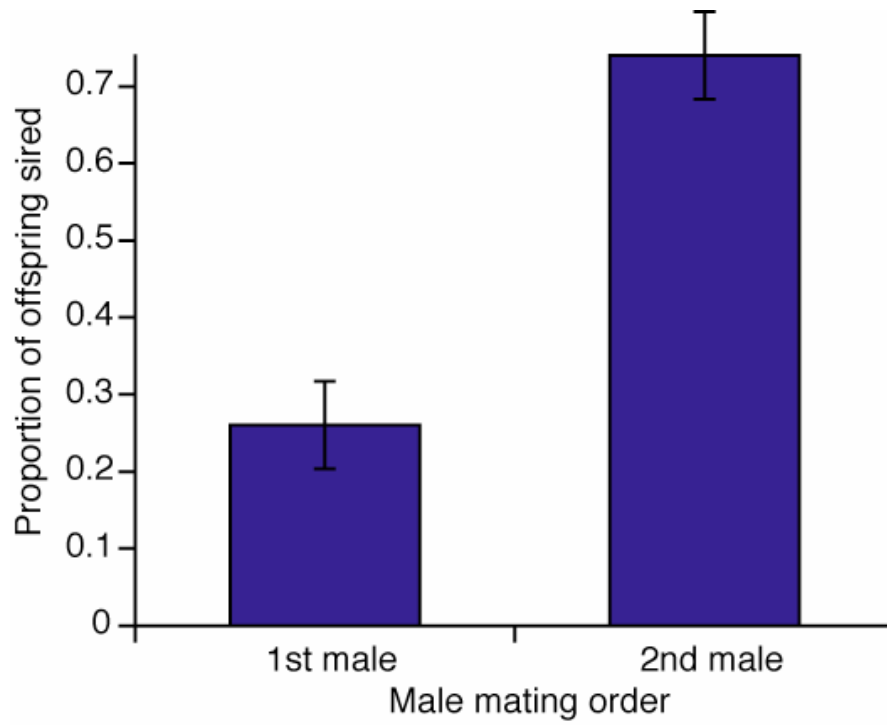
Determining what components of a male sexual phenotype contribute to different mating contexts is critical to understand the effects sexual selection on trait evolution.

This study showed that, in *A. sparsa*, antennal stroke rate changed its level of importance across first and second male mating roles. House and Simmons (2002) found that different types of genitalic sclerites improved fertilization success in first versus second males in dung beetles. Similarly, Arnqvist and Danielsson (1999) found that the dorsal and ventral sclerites of waterstrider genitalia contributed to second male paternity, whereas the lateral sclerites contributed to first male paternity. My study, and these examples show that, as reproductive context changes, the relative contribution of sexual traits to fitness varies. Thus, in order to more accurately link sexual traits with fertilization success for competing males, it is necessary to include a series of contexts for each species (House et al., 2007). By following a range of sexual traits across both first and second male roles, this study gives some insights into how these components function across different contexts, and how they are affected by sexual selection.

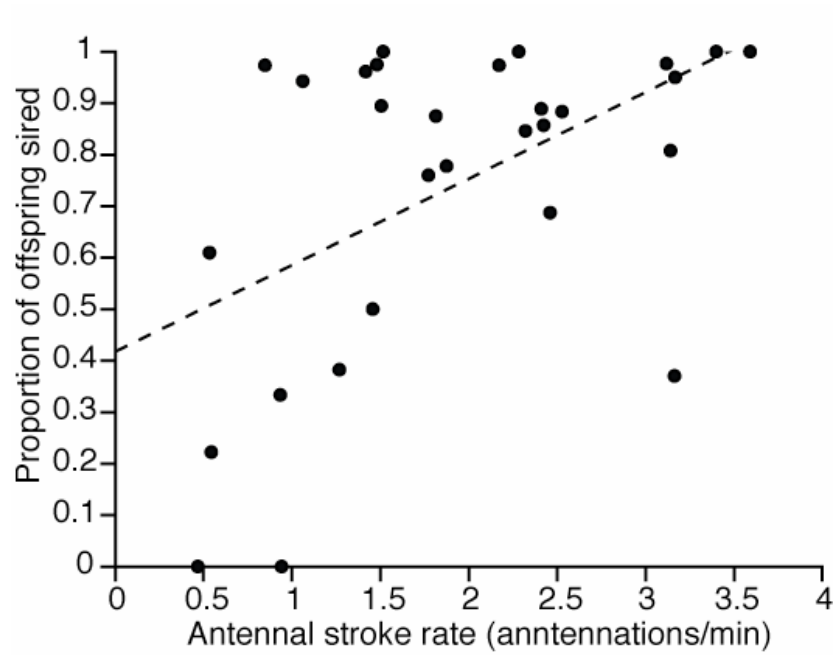
4.6 TABLES AND FIGURES:



**Figure 4-1:** Relationship between female body size, measured as head width, and the total number of eggs laid for (A) Experimental females, and (B) Field monitored females.



**Figure 4-2:** Proportion of offspring sired for first males and for second males. Error bars indicate standard errors.



**Figure 4-3:** Relationship between antennal stroke rate and the proportion of offspring sired ( $P_2$ ) for the second males to mate.

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