

# Sexual selection and evolution of sexual traits in heteropteran species

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## **Table of contents**

<b>Chapter 1. General introduction</b>	4
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<b>Chapter 2. Male Aggressive Behavior and Exaggerated Hindlegs of the Bean Bug</b>	
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<i>Riptortus pedestris</i>	11
2-1. Introduction	11
2-2. Materials and Methods	13
2-3. Results	16
2-4. Discussion	19

<b>Chapter 3. Effect of weapon-supportive traits on fighting success in armed bean</b>	
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<b>bug</b>	23
3-1. Introduction	23
3-2. Materials and Methods	26
3-3. Results	29
3-4. Discussion	30

<b>Chapter 4. Male courtship behavior and weapon trait as indicators of indirect benefit in the bean bug, <i>Riptortus pedestris</i></b>	34
4-1. Introduction	34
4-2. Materials and Methods	38
4-3. Results	42
4-4. Discussion	43
<b>Chapter 5. Testing for adaptive explanations of bimodal genital insertion duration in the stalk-eyed seed bug</b>	50
5-1. Introduction	50
5-2. Materials and Methods	54
5-3. Results	61
5-4. Discussion	64
<b>Chapter 6. General Discussion</b>	70
<b>Summary</b>	73

<b>List of publications</b>	76
<b>Acknowledgement</b>	77
<b>Reference</b>	78
<b>Tables</b>	113
<b>Figure legends</b>	119
<b>Figures</b>	122

## **Chapter 1. General Introduction**

### *Sexual selection*

Natural selection theory supposed that traits of organisms evolved via struggle for survival (Darwin 1859). This theory has explained evolution of various organism's traits such as bill of Darwin's finch (Darwin 1859). However, there are many phenomena which cannot be explained according to natural selection theory. For example, in many animals, only males have exaggerated ornament such as feather of peacock and antler of deer (Andersson 1994). Generally, expression and maintenance of these ornaments are costly because of increasing physiological expenditure and/or predation risk (Andersson 1994). Since natural selection theory assumed that selection pressure prefer traits which improve viability and act on both sex equally, evolution of male ornament cannot be expounded by this theory.

Accordingly, Darwin (1871) proposed sexual selection theory which argued that male ornaments evolved to enhance their ability to access prospective mates. Generally, males produce smaller gametes than females, indicating that investment for reproduction may differ between sexes. Because of the smaller investment for reproduction in males compared with that in females suggested by the smaller gamete size, males can increase the reproductive success by increasing the number of mating with little cost (Bateman 1948). In contrast, since females produce gamates with larger

size and smaller number (i.e., ova), they have scarcity value in reproduction and males will compete over females (Bateman 1948). Consequently, exaggerated ornaments evolved in males rather than in females.

#### *Male-male competition*

Process of sexual selection can be divided into two major mechanisms: male-male competition and female mate choice (Darwin 1871; Andersson 1994). Male-male competition is defined as competition among males for prospective mates (Darwin 1871; Andersson 1994). This process favors traits which enhance male's competitive ability such as body size and weapon traits and causes evolutionary exaggeration of those characters (Andersson 1994). For example, it is suggested that mandibles of stag beetle and eyestalks of stalk-eyed fly evolved via male-male competition.

#### *Female mate choice*

Female mate choice is another major component of sexual selection (Darwin 1871; Andersson 1994). Here, females choose their mates based on direct and/or indirect benefit from mating with prospective mates (Andersson 1994). To judge the benefit from mating, females use male characters that they believe to reflect male quality as

indicators of fitness benefit (Andersson 1994). Traits such as bright coloration and vigorous courtship display are suggested to be evolved via female mate choice and have been regarded as cues of fitness benefit (Andersson 1994). Direct benefits increase female productivity and/or longevity as represented by nuptial gift, parental care and monopolizing resource of male's territory (Andersson 1994; Simmons 2001). Conversely, indirect benefits increase fitness of their offspring (Andersson 1994). There are two main types of indirect benefits: Fisherian runaway process and good genes (Fisher 1930). Fisherian runaway process assumed that when male attractiveness is inherited to their sons, females who mated with attractive males will be able to increase their fitness via their son's mating success (Fisher 1930). Good gene hypothesis supposes that male attractiveness reflects male genetic quality, and females increase their fitness throughout their offspring's viable success by choosing attractive males.

### *Postcopulatory sexual selection*

In traditional sexual selection theory, it is assumed that sexual selection prefers male traits which enhance access to females. Recently, however, Parker (1970a) proposed a new sexual selection theory, called sperm competition theory. In the theory, they argued that when females mate with more than two males, there occurs competition over ova

fertilization between sperms from different males. In fact, there are many cases where females choose sperm from preferred males for fertilization and/or eject sperm from unpreferred males (reviewed in Eberhard 1996). Therefore, various male strategies can evolve via postcopulatory sexual selection as well as precopulatory sexual selection (Eberhard 1996; Simmons 2001).

### *Sexual conflict*

Classic female mate choice theory assumes that females choose their mates based on fitness benefit and cost (Andersson 1994). Practically, however, there are a lot of cases where females suffer decreasing fitness by mating with successful males (Arnqvist and Rowe 2005). In this situation, females are expected to evolve resistance to male sexual harassment (Holland and Rice 1998) and if female resistance can reduce adaptability of male harassment, it is predicted that antagonistic coevolution between sexes occurs (Parker 1979; Rice and Holland 1997).

### *Total sexual selection*

Although I have mentioned various processes of sexual selection separately, these processes usually interact with each other (Qvarnström and Forsgren 1998; Hunt et al.

2009). For example, in the stalk-eyed fly *Teleopsis dalmanni*, both male-male competition and female mate choice favor larger eye span (Rogers et al. 2008; Small et al. 2009). In the carrion fly *Prochyliza xanthostoma*, males with elongated head are at a disadvantageous in male-male competition, whereas they are favored in female mate choice because they transfer more nutritious ejaculation when mating with females (Bonduriansky and Rowe 2003; Bonduriansky et al. 2005). Furthermore, each process often prefers different traits, as a consequence, males are expected to evolve multiple sexual traits (Candolin 2003, Wong and Candolin 2005). Therefore, to understand sexual selection in comprehensive manner, it is necessary to investigate how individual process of sexual selection interact with each other and what male sexual traits they favor.

### *Study species*

In this study, I investigate how male sexual traits evolved through sexual selection pressure using two heteropteran species, *Reptortus pedestris* and *Chauliops fallax*. Heteroptera is one of the major taxa of insects and approximately 35,000 species have been described around the world (Schu and Slater 1995). In Japan, 1,000 species of heteroptera have been described (Yasunaga 2003). Heteropteran species have been

pupolar study materials in the evolutionary biology because they live in wide range of environments (Yasunaga 2003), from alpine regions to pelagic zone. Therefore, their biology is expected to evolve to be diverse. For example, prolonged copulation is one of characteristic of heteropteran species and it functions as postcopulatory mate guarding (e.g., Sillén-Tullberg 1981; Carroll 1991; Hosokawa and Suzuki 2001). Antagonistic coevolution between sexes also has been reported especially in water strider and bedbug (Arnqvist and Rowe 2005). Furthermore, in some of Coreidae species, males have exaggerated weapon traits and they compete for territory, where males attract females, using those traits (e.g., Miyatake 1997; Eberhard 1998; Miller and Emlen 2010).

However, there still remain some issues to be resolved. For instance, in some species, prolonged copulation does not serve as postcopulatory mate guarding (e.g., García-González and Gomendio 2004; Wong et al. 2008). Although exaggerated hind legs of males are reported in various Coreidae, few studies have investigated their role in sexual selection. Furthermore, precopulatory female mate choice has been largely unknown in heteroptera as compared with other insect taxa.

#### *Aim of this study*

Here, to reveal how male traits and mating behavior evolved through sexual selection, I investigated precopulatory sexual selection in the armed bean bug *Riptortus pedestris* (Heteroptera: Alydidae) and postcopulatory sexual selection in the stalk-eyed seed bug *Chauliops fallax* (Heteroptera: Malcidae) respectively. Because males of *R. pedestris* have exaggerated hind legs and exhibit typical precopulatory courtship behavior, they are suitable materials for the study of precopulatory sexual selection. On the other hand, males of *C. fallax* show an unique mating: males mount a female and insert his genitalia repeatedly and intermittently. Furthermore, genital insertion duration exhibits bimodal distribution. Thus, the mating behavior of *C. fallax* can be an useful material for the study of postcopulatory sexual selection. Finally, using the two species, I provided more evidence of sexual selection and deepened the discussion about evolution of male sexual traits.

## **Chapter 2. Male Aggressive Behavior and Exaggerated Hindlegs of the Bean Bug**

*Riptortus pedestris*

### **2-1. Introduction**

Male-male competition is a powerful force influencing the evolution of morphological traits (Andersson 1994). Weapons associated with male-male competition are conspicuous examples of the exaggerated traits produced by sexual selection (Andersson 1994). Obvious examples include the horns of beetles and forceps of earwigs (Eberhard 1979; Thornhill and Alcock, 1983; Emlen and Nijhout 2000; Okada and Miyatake 2010). Scaling relationships between weapon and body sizes, i.e., allometry, sometimes depart from linearity in these insects (Eberhard and Gutierrez 1991; Emlen and Nijhout 2000; Tomkins et al. 2005; Okada et al. 2007, 2008) and have high allometric values (slopes of  $> 1.0$  in log-log regressions on indicators of body size, ‘positive allometry’ (sensu Gould 1966)). Because these relationships have frequently attracted attention in studies of morphology, weapon structures have been statistically analyzed using several methods in a range of taxa (reviewed in Knell 2009).

However, the subjects of these studies seem located within a taxa-based perspective, for example, forficulid earwigs and lucanid and scarabaeid beetles (reviewed in Emlen

and Nijhout 2000; Knell 2009). It is thus of general interest to conduct morphological analyses of weapon structures in other insect groups (e.g., Eberhard 1998; Emlen and Nijhout 2000). Additionally, among the numerous morphological analyses (Eberhard and Gutierrez 1991; Emlen and Nijhout 2000; Knell 2009), relatively few studies have observed detailed intraspecific interactions between males.

Although the sequence and outcomes of aggressive behavior are also key components of the evolution of weapons (Emlen 2008; Okada and Miyatake 2009), they have been investigated in relatively few species (e.g., Moczek and Emlen 2000; Hongo 2003; Pomfret and Knell 2006).

Many bugs have modified hindlegs that are thought to function in male-male interactions (Schuh and Slater 1995; Eberhard 1998; Emlen 2008). The most typical modification is thickened hindfemora adorned with spines (Miyatake 1997; Eberhard 1998; Miller and Emlen 2010). However, only a few studies have conducted morphological analyses of modified hindlegs (*Leptoglossus australis*: Miyatake 1997; *Acanthocephala declivis*: Eberhard 1998; *Leptoscelis tricolor*: Miller and Emlen 2010).

In addition, the sequence or outcome of aggressive behavior has been investigated in relatively few species (*Acanthocephala femorata*: Mitchell 1980; *Acanthocoris sordidus*: Fujisaki 1981; *L. australis*: Miyatake 1993; *A. declivis*: Eberhard 1998;

*Notobitus meleagris*: Miyatake 2002). Although these studies provided correlative evidence that fighting success is positively impacted by weapon size, there have been very few direct experimental studies of the effect of the weapon on fighting success (e.g., Eberhard 1998). In addition, because the subjects of these studies are located within the Coreidae, it is also necessary to conduct morphological analyses and behavioral observation of other taxa.

The bean bug, *Riptortus pedestris* (Fabricius) (Heteroptera: Alydidae), has modified hindlegs (Natuvara 1985), but no morphological analysis of the weapon in *R. pedestris* has been reported. Additionally, no study has investigated the effect of the weapon size on fighting success. Here I conducted a morphological analysis of the hindlegs of *R. pedestris*. I also investigated the relationship between weapon size and the outcome of fights. This study is, to my knowledge, the first report on sexual dimorphism and male fighting in the Alydidae.

## 2-2. Materials and Methods

### *Stock culture*

The stock population of *R. pedestris* was founded with 10 adults (five males and five females) collected from soybean fields in Okayama City (31°41'N, 131°54'E), Japan, in

2009. Insects were reared on soybean seeds, red clover (*Trifolium pratense*) seeds, and water containing ascorbic acid (0.05%) (Kamano 1991) in a chamber (2400 × 2400 × 2400 cm; CC-T2000, Sanyo, Osaka, Japan) kept at 25°C and 60% relative humidity under a photoperiod of 16:8 h light:dark. Each nymph was maintained in a plastic cup (100 mm diameter, 40 mm high) with a density of between 10 and 20 individuals and was provided with an excess of the medium. Each emerging adult was housed in a separate petri dish until the following experiment.

#### *Morphological analysis*

The width (thorax, abdomen, and hindfemur) and length (thorax, abdomen, hindfemur, and hindtibia) were measured ( $\pm 0.01$  mm) as straight-line distances between two points using a dissecting microscope monitoring system (Fig. 2-1; VM-60, Olympus, Tokyo, Japan). Each specimen was carefully positioned so its longitudinal and dorsoventral axes were parallel to the visual axes of the microscope eyepiece. The thorax width was used as an index of body size (Eberhard 1998; Miller and Emlen 2010). However, unlike coreid bugs (e.g., Miyatake 1997; Eberhard 1998), the hindtibia width and spine are too small to measure. Therefore, we did not measure these traits in the present study. One hundred males and eighty females were randomly chosen from the stock culture for

measurement. The actual value for each trait was log 10 transformed for normal distribution and homogeneity of variance prior to analyses. The sexual difference was tested using analysis of variance (ANOVA) with sex as a fixed effect or analysis of covariance (ANCOVA) with sex as a fixed effect and the body size as a covariate. I used a reduced model that removed non-significant interaction terms from the full model (Grafen and Hails 2002). All analyses were performed using JMP 6.0 for windows (SAS Institute 2005). I adopted a power function (Gould 1966) to investigate whether each trait of *R. pedestris* showed positive allometry. The power function is:  $y = mx^\alpha$  where  $y$  is the actual measurement value for each trait size,  $x$  is the actual measurement value for body size (thorax width), and  $\alpha$  and  $m$  are the regression coefficients. When  $\alpha$  for a particular character is  $> 1$ , that character is disproportionately large in large animals and shows positive allometry (Tomkins et al. 2005; Kodric-Brown et al. 2006).

#### *Observation of fighting behavior*

A filter paper (90 mm diameter) placed on a plastic container (90 mm diameter, 15 mm high) was used as the combat arena. The observations were made during the photophase in a laboratory kept at 25°C and 60% RH. Two males, randomly picked from the stock culture, were simultaneously introduced into the plastic container described above, and

their interactions were observed for three hours by the naked eye. The trials were replicated 46 times. After observation, all bugs were preserved for measurement. To determine whether body and weapon sizes were associated with fighting success, I used a generalized liner model with binomial errors and a logit link (Hardy and Field 1998; Pomfret and Knell 2006), with dependent variable (1 = won, 0 = lost) and body size (thorax width) and weapon size (hindfemur length) as predictor variables.

### **2-3. Results**

#### *Morphology*

There was no significant difference in mean thorax width (body size index), thorax length, or abdomen width between the sexes (Table 2-1). In contrast, the means of the lengths of the abdomen, hindfemur, and hindtibia and width of the hindfemur were significantly larger in the males compared to the females (Table 2-1). Figure 2-2 shows the relationship between body size and the lengths of the hind femur, thorax, abdomen, and hindtibia and the width of the hindfemur. For the hindfemur length, the full model showed significant effects of sex and body size, while a significant interaction between these effects was detected (Table 2-2). This indicates that the allometric intercept and slope of the hindfemur length were significantly larger in the males than in the females

(Fig. 2-2E).

For the lengths of the thorax, abdomen, and hindtibia and widths of the abdomen and hindfemur, the full model showed a non-significant interaction between sex and body size (Table 2-2). Sex and body size had significant effects on the lengths of the thorax, abdomen, and hindtibia and width of the hindfemur in the reduced model (Table 2-2). Thus, the allometric intercept of these traits was significantly larger in the males than in the females (Fig. 2-2A, C, D, F), but the slope did not differ between the sexes. Sex had no significant effect on the abdomen width (Table 2-2, Fig. 2-2B). For the lengths of the thorax, abdomen, hindfemur, and hindtibia and widths of the abdomen and hindfemur, the power function test for both sexes yielded a significant allometric value  $\alpha$  (Table 2-3). The coefficient  $\alpha$  values were significantly larger than 1 for the hindfemur length of males but not for other male traits and either of the female traits (Table 2-3). Therefore, the relationship between the male hindfemur length and body size shows positive allometry.

#### *Fighting behavior*

*R. pedestris* males frequently fight each other with their hindlegs. In the laboratory, I observed fighting behaviors as follows. Often, either or both males lifted their

abdomens with their backs to the opponent and flapped their wings (Fig. 2-3A). This behavior may be a display against the opponent. In actual fighting, one male kicks his opponent with one or both of his hindlegs, but the kicked male shows little response (Fig. 2-3B); two males kick each other with their legs (Fig. 2-3C); one male raises his hindlegs to grasp and squeeze the opponent's body; or two males squeeze each other (Fig. 2-3D). The winner is the male that tries to push his opponent out of the arena and to chase him. The loser is the male that tries to retreat from the arena. I found that no loser resumed a fight against the winner, even though observations were continued for one hour after the outcome of the contest was decided.

Of 46 observations, 33 encounters resulted in male-male interactions, but no interaction was observed in the remaining 13 cases. These 13 cases were excluded from the following analyses. The reduced model showed that both hindfemur length and body size were significantly positively associated with fighting success (hindfemur length,  $57.906 \pm 25.537$  (coefficient  $\pm$  s.e.),  $\chi^2_1 = 6.008$ ,  $P = 0.0142$ ; body size,  $71.352 \pm 35.750$ ,  $\chi^2_1 = 5.060$ ,  $P = 0.0245$ ). Additionally, even if we use paired t-tests to more conservatively assess differences in hindfemur length and body size between winner and loser males, my conclusions remain unchanged (paired comparison of hindfemur length: winner,  $8.002$  (mm)  $\pm 0.045$  (mean  $\pm$  s.e.), loser,  $7.514$  (mm)  $\pm 0.051$ :  $df = 32$ ,

paired  $t = -6.898$ ,  $P < 0.0001$ ; body size: winner, 3.235 (mm)  $\pm$  0.017, loser, 3.045 (mm)  $\pm$  0.027;  $df = 32$ , paired  $t = -6.165$ ,  $P < 0.0001$ ).

## 2-4. Discussion

I found sexual dimorphism in the hindlegs of *R. pedestris*; that is, the width and length of the hindfemur and length of the hindtibia were larger relative to body size in males than in females (Table 2-2, Fig. 2-2E, F). This is similar to the results reported for several bugs: *L. australis* (hindfemur and hindtibia; Miyatake 1997), *A. declivis guatemalana* (hindfemur; Eberhard 1998), and *L. tricolor* (hindfemur; Miller and Emlen 2010). All these insects are members of the Coreidae, and hence this study is the first report on sexual dimorphism and male fighting in the Alydidae. The allometric slope of the hindfemur length was also larger in males than females, similar to findings for two coreid bugs (Miyatake 1997; Miller and Emlen 2010). These results suggest that directional selection favors larger hindlegs in this male and that the hindfemora are under stronger selection in larger males than in smaller males. Consistent with this, the male hindfemora show positive allometry (Table 2-3), indicating that the femora of larger males are relatively larger than those of smaller males. Similar findings are reported for *L. australis* (Miyatake 1997) and *A. declivis guatemalana* (Eberhard 1998).

Exaggerated male weapons are common in a range of taxa and are the result of male-male competition as a key component of sexual selection (Andersson 1994; Shuster and Wade 2003). The hindlegs of male *R. pedestris* are frequently used in male fights (Fig. 3). The sequence of aggressive behavior is consistent with previous work on this bug (Natuvara 1985) and is similar to those reported for several coreid bugs (Fujisaki 1981; Miyatake 1993; Eberhard 1998). Additionally, I found that not only a larger body but also larger hindlegs increase male fighting success in *R. pedestris*. This suggests that males with relatively larger hindlegs have the advantage in male fights. Thus, the hindfemora are thought to be under stronger directional selection in the male than in the female, and this corresponds to sexual dimorphism in the hindlegs. Combining my morphometric and behavioral data, sexual selection may favor larger hindlegs in male *R. pedestris* through male fighting, as observed in a number of studies on male exaggerated weapons (reviewed in Andersson 1994; Emlen and Nijhout 2000; Shuster and Wade, 2003). Alternatively, the exaggerated hindleg may be a result of mate choice by females (e.g., peacocks' tails). However, a detail observation showed that the male hindlegs were unlikely to be used for courtship of a mate (Numata et al. 1986). Instead, a chemical cue facilitated by a male aggregation pheromone is likely to play an essential role in courtship behavior in this bug (Numata et al. 1986).

In addition, the thorax and abdomen lengths were larger relative to body size in the male than in the female (Table 2-2, Fig. 2-2A, C). The sexual dimorphism corresponds to the behavioral observation that the males often lifted their abdomens with their backs to the opponent for displays against an opponent (Fig. 2-3A). As a result, the thorax and abdomen lengths are thought to be under stronger selection in the male than in the female, and like the exaggerated hindlegs, the thorax and abdomen lengths may thus show sexual dimorphism.

Unfortunately, my results showed no reason why the hindfemora are under stronger selection in larger males than in smaller males. Positive allometries between weapons and body size have frequently attracted attention in studies of alternative phenotypes, and males can be divided into two types, larger and smaller (Eberhard and Gutierrez 1991), which adopt strikingly different reproductive tactics due to their status, which is usually indicated by body size (Thornhill and Alcock 1983; Tomkins et al. 2005; Okada et al. 2007). Generally, larger males use their enlarged weapons in fights to gain access to females (Gross 1996; Emlen and Nijhout 2000; Moczek and Emlen 2000; Shuster and Wade 2003; Tomkins et al. 2005), whereas smaller males have a rudimentary or no weapon and adopt alternative tactics, such as being a satellite, sneak, or female mimic (Gross 1996; Moczek and Emlen 2000; Shuster and Wade 2003; Yamane et al. 2010).

Theoretical (Gross 1996; Shuster and Wade 2003) and empirical (Hunt and Simmons 2001; Forslund 2003) studies suggest that positive allometry has been maintained under a status-dependent selection in which the optimal tactic for an individual changes at a switchpoint of status. Thus, it will be intriguing to examine whether mating tactics of large and small males differ in this bug.

In summary, I found a remarkable sexual dimorphism in the hindlegs of *R. pedestris*, and the male hindfemur showed positive allometry. Furthermore, I established a method for observation of male fights between *R. pedestris* individuals in a container, although quantitative observation of male fights is sometimes difficult in other animals (Eberhard 1979; Okada and Miyatake 2004). The observation that larger relative femur size increased male fighting success is consistent with the sexual dimorphism of the hindlegs. Moreover, *R. pedestris* is easy to rear during successive generations (see Kamano 1991), and hence I hope that this study will improve the insect's status as a model animal for evolutionary studies on weapons.

## **Chapter 3. Effect of weapon-supportive traits on fighting success in armed bean bug**

### **3-1. Introduction**

Sexual selection can result in the exaggeration of morphological traits, such as the ornaments used for attracting mates (e.g. elongated tails of birds and the eyestalks of stalk-eyed flies) and weapons associated with male-male competition (e.g. the horns and mandibles of beetles; Darwin 1871; Andersson 1994; Shuster and Wade 2003; Emlen 2008). Enlargements in nontarget traits may accompany those in the primary targets of exaggeration (as reviewed in Husak and Swallow 2011), leading to coupling of exaggerated traits and correlated modifications in other somatic characters (Pigliucci and Preston 2004).

A well-known example is secondary sexual trait compensation. The production and maintenance of an exaggerated trait can be costly, in terms of predation and/or parasitism risk and energetic and physiological expenditure (Kotiaho 2001; Cotton et al. 2004; Okada et al. 2011a). These potential costs can be offset by the changes in somatic traits that compensate for the cost of exaggeration (Tomkins et al. 2005). Compensation has been suggested from morphological data in several species (Hedenstrom and Møller

1992; Andersson and Andersson 1994; Balmford et al. 1994; Tomkins et al. 2005; Husak et al. 2011). For example, in male barn swallows, *Hirundo rustica*, and stalk-eyed flies, male wings are enlarged in addition to primary (exaggerated) ornaments, probably to offset the cost of carrying them (e.g. tail streamers and eyestalks, respectively; Møller et al. 1995; Husak et al. 2011).

Similar findings in nontarget traits have also been reported in several armed insects (Tomkins et al. 2005; Okada and Miyatake 2009): males with larger weapons have been found to possess larger traits (hereafter referred to as ‘supportive traits’) that help males to use their enlarged weapons. For example, a larger head, prothorax and forelegs (Okada and Miyatake 2004; Tatsuta et al. 2004; Tomkins et al. 2005; Okada et al. 2007) may act as supportive traits for male beetles that use their horns and mandibles to fight with rival males over access to females (Eberhard 1979; Otte and Stayman 1979; Emlen and Nijhout 2000). Supportive traits are also thought to be important structures in male combat (Otte and Stayman 1979), independent of the primary exaggerated traits. As such, they are predicted to increase male fighting success (Tomkins et al. 2005; Okada and Miyatake 2009). Weapon-supportive traits can also be compensatory traits during nonantagonistic situations, such as carrying large weapons during walking and thus compensation and support are not always mutually exclusive (see Tomkins et al. 2005).

Nevertheless, in this study, we refer to these traits as supportive because I focus on the weapon-supportive function of nontarget traits during fighting, rather than cost compensation. The supportive traits should be selected concurrently with exaggerated weapon traits, and selection should favor a mechanism that provides suitable combinations of multiple traits in relation to fighting behavior (Pigliucci and Preston 2004; Irschick et al. 2007, 2008). Surprisingly, no studies to date have empirically tested the possibility that supportive traits influence fighting success. Therefore, I developed the current study to test this assumption in the bean bug, *Riptortus pedestris*. In this species, males use enlarged weapons (modified hindlegs) to fight for mates, with fighting success positively influenced by weapon size. Since these structures are limited to males, and body sizes and these weapons have positive allometric relationships, they are good examples of exaggerated morphologies (Okada et al. 2011b). Male bean bugs use their hindlegs to kick, grasp and squeeze rival males (Okada et al. 2011b).

To identify potential supportive traits, I used principal components analyses (PCA) and generalized linear models (GLMs) to find phenotypic correlations between weapons and somatic traits (see also Tomkins et al. 2005). Informative principal components (PCs) from the PCA were then used to assess whether fighting success is improved by possession of enlarged supportive characters.

### **3-2. Materials and Methods**

#### *Culturing and morphological measurements*

The stock population of *R. pedestris* originated from adults collected in 2009 from soya bean, *Glycine max*, fields in Okayama City, Japan (31°41'N, 131°54'E). Males compete for territories on the leaves and stems of their host plant, where they mate and reproduce (Natahara 1985). Bugs were reared on an excess of medium containing seeds of soya bean and red clover, *Trifolium pratense*, and water containing ascorbic acid (0.05%). They were housed in a chamber measuring 2400 × 2400 mm and 2400 mm high (CC-T2000, Sanyo, Osaka, Japan) kept at 25°C and 60% relative humidity. Nymphs were maintained in plastic cups (diameter: 100 mm; height: 40 mm) at densities of 10-20 individuals. Each emerging adult was housed in a separate petri dish until the fighting experiment (see below). Further details on housing are discussed in Okada et al. (2011b).

I randomly selected 120 males from the stock to measure eight body parts: head width, thorax width, thorax length, abdomen width, abdomen length, forefemur length, midfemur length and hindfemur length (Fig. 3-1), employing the same system used for the beetles. Because I matched the size of paired males (see below), I used only 30 of 120 males measured for the following experiment.

### *Fighting success*

I staged 15 contests between males 15-20 days after emergence, using methods previously described elsewhere (Okada et al. 2011b). Briefly, the arena consisted of a plastic container (diameter: 90 mm; height: 15 mm) lined with filter paper (90 mm diameter). Introduction of two males into a small arena can induce male combat because of the males' territorial habits. For each contest, two males were simultaneously introduced into the arena and their behavior was observed for 30 min. The winner was the male that pushed his opponent and tried to chase him out of the arena. The loser was the one that retreated from the opponent and never resumed a fight against the winner (Okada et al. 2011b).

To control for an effect of hindfemur size on fighting success, I ensured that the difference in hindfemur length between contestants was <0.04 mm. The difference between contestants was less than 1.6% of the range of hindfemur length (Table 3-1). Size differences in other traits were 10-30% of trait size ranges (Table 3-1).

### *Statistical Analysis*

Since all the traits were highly correlated and multiple regression was unsuitable

because of the high multicollinearity, I used a PCA to partition the variance attributable to the various traits using a correlation matrix (Tomkins et al. 2005). This allowed me to examine developmental integration of the measured phenotypic traits. In general, PCs can explain overall variation until the cumulative proportion of variation becomes 80-90% (Jolliffe 2002). In this study, PCs were regarded as informative until their cumulative contribution exceeded 85%. To determine which traits interacted with the males' primary weapons, I constructed a GLM, using normal error distributions and an identity link. Weapon size (hindfemur length) was entered as the dependent variable and informative PCs were included as predictor variables. Because the relationship between weapon and body sizes departs from linearity (Okada et al. 2011b) and shows 'positive allometry' (sensu Gould 1966), actual weapon size was log transformed prior to inclusion in the GLM (Tomkins et al. 2005; Okada and Miyatake 2010b). I used a reduced model that removed nonsignificant interaction terms from the full model (Grafen and Hails 2002). To investigate which PCs were associated with fighting success (a binomial variable, for which 1 = won and 0 = lost), I constructed a GLM with binomial errors and a logit link (Hardy and Field 1998; Pomfret and Knell 2006). Fighting success was entered as the dependent variable, and informative PCs and weapon size (hindfemur length) were included as predictor variables. Additionally, to

investigate whether each trait was associated with fighting success, I constructed a GLM with fighting success as the dependent variable and each nontarget trait as the predictor variable. I adopted a reduced model that removed nonsignificant interaction terms from the full model (Grafen and Hails 2002).

### **3-3. Results**

The PCA generated seven PCs, four of which were informative (Table 3-2). PC1 accounted for overall body size. PC2 loaded positively for thorax, abdomen and midfemur length, but negatively for thorax and abdomen width; thus, this variable describes body shape in that large PC2 indicates a slender body with longer midfemur. PC3 loaded positively for forefemur length, while PC4 loaded negatively for head width (Table 3-2).

The GLM revealed a significant positive relationship between hindfemur length and both PC1 (body size) and PC2 (body shape and midfemur length). However, hindfemur length was not significantly associated with PC3 (forefemur length) or PC4 (head width; Table 3-3). PC1 was included in the final model so that the remaining PC2 effects were on relative weapon size. Therefore, the relative hindfemur length is positively associated with thorax length, abdomen length and midfemur length, but negatively

with thorax width and abdomen width.

Males did not fight in five of 15 observations for unknown reasons; thus my final analysis only included data from 10 aggressive encounters. Male fighting success was significantly positively related to PC2, but was not associated with hindfemur length or PCs 1, 3 or 4 (Table 3-4). When each trait alone was used as a predictor variable in a GLM, I detected a significant positive relationship between fighting success and midfemur length (coefficient  $\pm$  SE =  $9.9788 \pm 4.5481$ ;  $\chi^2_1 = 8.718$ ,  $P = 0.003$ ), and negative relationships between fighting success and thorax and abdomen widths (thorax width:  $-9.6860 \pm 4.3283$ ;  $\chi^2_1 = 8.482$ ,  $P = 0.004$ ; abdomen width:  $-14.9929 \pm 6.9684$ ;  $\chi^2_1 = 11.581$ ,  $P = 0.0007$ ). Other traits did not have significant effects on contest outcome (thorax length:  $\chi^2_1 = 0.014$ ,  $P = 0.906$ ; abdomen length:  $\chi^2_1 = 0.374$ ,  $P = 0.541$ ; head width:  $\chi^2_1 = 0.012$ ,  $P = 0.912$ ; forefemur length:  $\chi^2_1 = 1.582$ ,  $P = 0.209$ ).

### 3-4. Discussion

PCA indicated that several body parts are positively associated with the exaggerated weapon characters in *R. pedestris*. This morphological feature indicates that these body parts act as weapon-supportive traits (Otte and Stayman 1979; Tomkins et al. 2005). Indeed, for bean bugs, fighting success was significantly increased by enhancement of

these traits, suggesting that they act as supportive traits. Thus, I have provided the first empirical evidence, to my knowledge, that supportive traits are beneficial in providing structural support for sexually selected weaponry.

During aggressive *R. pedestris* encounters, males turn their backs to their opponents and kick them with exaggerated hindlegs that are supported by strong midfemora (Okada et al. 2011b). Additionally, males often lift up their abdomens and perform displays towards their opponents (Okada et al. 2011b). Given these behaviors, I was not surprised to find positive associations between hindfemora size and thorax, abdomen and midfemur length; these three characters appear to be supportive traits for the exaggerated hindfemora. These results are similar to those of Tomkins et al. (2005), who detected positive relationships between earwig forceps and hindleg size. As predicted from PC2, midfemur length had a positive effect, and thorax width and abdomen width had negative effects on contest outcome, supporting the hypothesis that a large midfemur contributes to fighting success. In contrast, thorax length and abdomen length had no significant effects. There is a certain combination of nontarget traits that provides a suitable body shape for combat (i.e. slender body with long midfemur), although independent effects of some nontarget traits require further investigation.

Cumulatively, my results indicate that most characters that are positively associated

with weapons in *R. pedestris* can be thought of as supportive traits. The interspecific differences in which body parts are associated with the weapon and how probably reflect differences in the ways in which weapons are used during aggressive encounters. The supportive structures may ultimately emerge through male fighting, since successful males (i.e. those with the better supportive traits) gain access to females, and mate with them (Okada and Miyatake 2009). Over time, this leads to changes in body shape across the population, such that exaggerated weaponry is associated with appropriate supportive structures, generating an overall body shape that is more suitable for fighting (e.g. Otte and Stayman 1979; Emlen 2008).

Fighting success in *R. pedestris* is positively impacted by both larger weapon size (Okada et al. 2011b) and enhanced supportive traits. Since fighting success is a major determinant of male fitness in general (reviewed in Andersson 1994; Shuster and Wade 2003), the weapon and its supportive traits are likely to be under correlational selection.

As a result, they should be genetically correlated and develop in an integrated manner. In horned flour beetle *G. cornutus*, Okada and Miyatake (2009) found positive genetic correlations between the weapon and its supportive traits, suggesting that these characters have undergone correlational selection. This association among the weapon and its supportive traits is a clear example of phenotypic integration, which refers to

coordination among functionally related multiple traits (see details in Olson and Miller 1958; Pigliucci 2003; Pigliucci and Preston 2004; Klingenberg 2008). Phenotypic integration results from correlational selection on a particular trait and other somatic traits, where the variance in the relationship between traits is reduced by selection (Cheverud 1996; Wagner and Altenberg 1996; LeBas et al. 2003; Klingenberg 2004).

In conclusion, I empirically examined whether supportive traits for weapons improved performance in male-male contests. My results indicate the presence of coordination among multiple, functionally related traits (Pigliucci and Preston 2004; Klingenberg 2008). Since supportive structures for exaggerated weapon traits, as well as secondary sexual trait compensation, are thought to be one of the best examples of phenotypic integration (Tomkins et al. 2005), further explorations of integration between exaggerated traits, supportive characters and fighting/display behaviors will increase my understanding of the evolution of complex, exaggerated morphologies.

## **Chapter 4. Male courtship behavior and weapon trait as indicators of indirect benefit in the bean bug, *Riptortus pedestris***

### **4-1. Introduction**

Female mate choice is one of two major mechanisms of sexual selection and is a fundamental and critical force leading to the evolution of behaviors and morphologies (Darwin 1859, 1871; Bakker 1993; Andersson 1994; Andersson and Iwasa 1996; Houde 1997; Hunt et al. 2009). Female mate choice occurs when females are selective in their mating decisions and favor attractive males as prospective mates (Darwin 1871; Fisher 1930; Williams 1966; Bakker 1993; Andersson 1994). Traits such as ornaments for attracting mates are the result of female choice and have been suggested to act as cues to females of direct and indirect benefits (Andersson 1994). Obvious examples of direct benefit include access to territory, nuptial gifts, and conjoined efforts in parental care for offspring (Price et al. 1993; Møller and Jennions 2001). Females can increase their longevity and/or fecundity from the direct benefits (Hoelzer 1989; Andersson 1994).

Indirect benefits also augment female fitness in two general ways (reviewed in Johnstone 1995; Eberhard 1996; Jennions and Petrie 2000; Kokko et al. 2003; Hosken and Stockley 2004). Firstly, attractive males produce sons that inherit their father's

attractiveness and therefore also have increased mating success, and hence female fitness is indirectly increased via their sons' mating success (Fisherian mating advantages: Fisher 1930; Lande 1981; Bakker 1993; Hosken and Stockley 2004). Secondly, attractive males are themselves of high genetic quality and hence viability, and the offspring sired by these males also inherit the "good genes" (Eberhard 1996; Jennions and Petrie 2000; Hosken and Stockley 2004; Andersson 2006). In this case, female fitness is indirectly increased through the production of offspring with high viability (reviewed in Jennions and Petrie 2000; Andersson 2006).

Male-male competition is classified as another mechanism of sexual selection and occurs when males compete for access to prospective mates (Darwin 1859, 1871; Andersson 1994; Andersson and Iwasa 1996). This process favors exclusion of rival males by highly competitive males, resulting in evolutionary exaggerations of weapon characters, such as the antlers of deer and mandibles of beetles (Eberhard 1979; Andersson 1994; Emlen and Nijhout 2000). Indeed, many empirical studies have demonstrated that fighting success is positively impacted by larger weapon size (reviewed in Shuster and Wade 2003; Emlen 2008). Although male-male competition and female mate choice have been the focus of a considerable amount of research separately, both mechanisms of sexual selection rarely act independently (reviewed in

Wong and Candolin 2005).

Current concepts of sexual selection basically assume that competitively superior males confer direct and/or indirect fitness benefits on females (Mead and Arnold 2004; Andersson and Simmons 2006; Kokko et al. 2006). For example, highly competitive males may protect the female during and after mating or provide access to superior resources (Berglund et al. 1996). Also there is an indirect genetic benefit if weapon traits are heritable (Cordero and Eberhard 2003). Under such situations, it is expected that selective forces through male-male competition and female mate choice act together in a reinforcing manner.

However, several recent studies suggest that male competition and female choice sometimes work in opposing or unrelated directions (Boake and Konigsberg 1998; Moore and Moore 1999; Kotiaho 2002; Candolin 2004; Shackleton et al. 2005), and thus the two mechanisms do not always act in a reinforcing manner. Furthermore, each mechanism occasionally selects for different traits, and as a result, multiple sexual traits play different roles in reproductive behavior even in the same individual, for example, one trait is used for courtship behavior and another for male fighting (Andresson et al. 2002; Etges 2002; Bonduriansky and Rowe 2003; Candolin 2003, 2004, 2005; Bretman et al. 2011). So far, relatively few studies have investigated whether competitively

superior males are preferred by females and confer fitness benefits on females (Hunt et al. 2009). Thus, investigations of interaction between male-male competition and female choice are necessary for integrative understanding of sexual selection (i.e., total sexual selection).

Here, I focused on whether higher successful males in male-male competition are preferred by females, and how mating with preferred attractive males confers fitness benefits on females. Males of *Riptortus pedestris* usually fight for territories using their enlarged hind legs (Natuvara 1985), and males with larger weapons tend to win the contest (Okada et al. 2011b, 2012). In contrast, there is little information about male attractiveness and female preference in this species. Numata et al. (1986) suggested that male attractiveness is associated with manners of courtship behavior (e.g., foreleg and/or body rubbing). Similar findings are reported for several insects (Partridge and Fowler 1990; Kotiaho 2002; Fedina and Lewis 2008; Simmons and Holley 2011; Okada et al. 2013). Thus, courtship may also act as an evaluative cue of female choice in *R. pedestris*.

In the present study, I investigated whether male attractiveness (i.e., copulation latency), courtship behavior, and weapon size are heritable and how these characters are genetically correlated using full-sib/half-sib analysis methods. Moreover, I examined

whether mating with attractive males affects female fitness (lifetime reproductive success and longevity).

## 4-2. Materials and Methods

### *Insect culture*

The stock population was cultured from approximately 50 individuals collected in Fukuyama City, Hiroshima, Japan, in late autumn 2006 (Kimura et al. 2008). Insects were reared on soybean seeds, red clover (*Trifolium pratense*) seeds, and water containing ascorbic acid (0.05%) (Kamano 1991). Food and water were replaced once every two weeks. The stock was maintained at 1500-2000 nymphs per generation and kept in plastic cups (diameter 95 mm, height 40 mm) with a standing density of between 10 and 20 individuals per cup. After eclosion, each adult was housed in a separate petri dish (90 mm diameter, 15 mm depth). Thus, adults did not interact with conspecifics until the following experiments. For a more detailed description of the stock culture, see Okada et al. (2011). I performed all rearing and experiments in a chamber maintained at 25°C, 60% relative humidity and with a photoperiod cycle of 16:8 h light:dark.

### *Methods for measurement of copulation latency, courtship rate, and morphology*

A virgin male and a virgin female were chosen randomly and placed in a plastic cup (78 mm diameter, 43 mm height) lined with a paper filter (78 mm diameter). Subsequently, the pair was continuously observed until copulation ended using a digital video camera (Victor GZ-MG880). If I did not observe a successful copulation for two hours, the pair was excluded from the analysis. All observations took place between 1500–2300.

In the laboratory, Numata et al. (1986) observed a highly stereotypical sequence of courtship behaviors as follows. A male first mounts a female's back and subsequently taps her antenna with his foreleg while shaking his body. When a female accepts the male's mating attempt, she opens her ovipositor valves and the male inserts his genitalia. After genital connection, the male turns around and the pair takes the end-to-end position (Numata et al. 1986).

In this study, I used courtship rate (number of such courtship bouts per second) as an indicator of courtship quality (e.g., Kotiaho et al 2001; Kotiaho 2002). Furthermore, copulation always occurs after the courtship behavior in *R. pedestris*. In these insects, because females generally mate sooner with more attractive males, copulation latency (the time from initiation of courtship to commencement of copulation) is often used as an indicator of male attractiveness (also see Shackleton et al. 2005; Taylor et al. 2007; Simmons and Holley 2011; Okada et al. 2013), and I thus measured copulation latency

as male attractiveness. I noted the repeatability of the courtship rate and copulation latency of *R. pedestris* which measured along with the above mentioned methods (courtship rate,  $r = 0.469$ ,  $P = 0.0003$ ,  $N = 52$ ; copulation latency,  $r = 0.648$ ,  $P < 0.0001$ ,  $N = 52$ , Pearson's correlation coefficient).

After mating, each individual was immediately removed from the plastic cup to prevent additional matings. The hind femur length (an estimator of hind leg length) of each male ( $\pm 0.01$  mm) was measured by using a dissecting microscope monitoring system (VM-60; Olympus, Tokyo, Japan). Each specimen was positioned so that its longitudinal and dorsoventral axes were perpendicular to the visual axes of the microscope eyepiece. The length was measured as a straight-line distance (see Okada et al. 2012 for landmarks). Each character was measured twice, and the average value was used in the analyses.

#### *Sib analyses of male attractiveness, courtship rate, and hind leg*

To examine genetic variance and covariance, I conducted the following experiment using a full sib/half sib experimental design. Males (sires) ( $N = 30$ ) were randomly assigned to at least two virgin females (dams) ( $N = 78$ ) and were allowed mate in the manner described above. After mating, each dam was immediately removed from the

plastic container and placed in a petri dish (90 mm diameter, 20 mm deep) containing an excess of food and water. Each female was maintained for four weeks to obtain her offspring. All offspring were reared to adulthood under laboratory conditions identical to the parental generation. A total of 232 sons (mean per dam = 2.97) were assessed for copulation latency, courtship rate, and morphology in the same manner as described above. Females used for this measurement were chosen randomly from the stock culture.

#### *Relationships between female preference and direct benefit*

To examine whether female fitness is affected by male attractiveness, female longevity and lifetime reproductive success (LRS) were measured. In the beginning, the copulation latency, courtship rate, and male hind leg length of 61 pairs were measured using the above described methods. After mating, each female was placed in a petri dish (90 mm diameter, 20 mm high) containing an excess of food and water and 1 cm<sup>3</sup> cotton wool as an oviposition site. Egg number and female survival were assessed weekly. The body size of females was measured after death using prothorax width as a proxy (Okada et al. 2011b).

### *Statistical analysis*

I used a nested model (sire + dam [sire]) for an unbalanced design to estimate the heritability  $\pm$  SE ( $h^2$ ) of each trait (Lynch and Walsh 1998). I estimated the genetic correlations  $\pm$  SE of each trait using the nested model after Falconer and Mackay (1996) and Lynch and Walsh (1998). Z scores were used to test whether  $h^2$  and genetic correlations were significantly different from zero (Rønning et al. 2007; Åkesson et al. 2008).

Because my data showed homogeneity of variances ( $F$  test:  $F_{42} = 1.184$ ,  $P = 0.586$ ) and normal distribution (Lilliefors test: LRS,  $P = 0.20$ ; longevity,  $P = 0.163$ ), female LRS and longevity were analyzed using a multivariate analysis of variance (MANOVA), with copulation latency and female body size as independent variables. Furthermore, to investigate the effects of courtship rate and male hind leg length on female fitness, I also analyzed female LRS and longevity using MANOVA with courtship rate, male weapon size, and female body size as independent variables. I used backward elimination to remove non-significant interaction terms from the full model (Grafen and Hails 2002). All statistical analyses were carried out using JMP 9.0.2 (SAS Institute).

### **4-3. Results**

All traits measured were significantly heritable and showed moderate sire heritabilities (Table 1). Copulation latency had significant negative genetic correlations with courtship rate and hind leg size (Fig. 4-1; Table 4-1). On the other hand, genetic correlations for other pairs were not significant (Table 4-1). Because my results included the one highest point (Fig. 4-1), I reanalyzed the data by excluding the highest point and the statistical significance did not differ from before exclusion (Table 4-1).

Of 61 pairs, successful copulations were observed in 43 pairs. In MANOVA including a copulation latency variable, the reduced model showed that both copulation latency and female body size had non-significant effects (copulation latency,  $F_{1,40} = 0.0106, P = 0.92$ ; female body size,  $F_{1,40} = 0.0012, P = 0.97$ ; Fig. 4-2a, b). In MANOVA including variables of courtship rate and male weapon size, the reduced model also showed that neither courtship rate, male weapon size nor female body size had significant effect on female LRS and longevity (courtship rate,  $F_{1,39} = 0.6914, P = 0.41$ ; Fig. 4-2c, d; male weapon size,  $F_{1,39} = 1.9926, P = 0.17$ ; female body size,  $F_{1,39} = 0.0063, P = 0.94$ ; Fig. 4-2e, f). For each MANOVA, I note that there were non-significant interactions among pairs.

#### 4-4. Discussion

The results showed that males preferred by females tended to have a higher frequency of courtship display and larger hind leg (Table 4-1, Fig. 4-1). Furthermore, male attractiveness and male sexual characters (i.e., courtship display and weapon size) had significant heritability (Table 4-1, Fig. 4-1). However, mating with more attractive males increased neither LRS nor longevity of females (Fig. 4-2).

In many insects, females tend to have increased lifetime fitness because of access to male-derived resources (e.g., sperm, nuptial gifts) that elevate fertility/fecundity (reviewed in Thornhill and Alcock 1983; Simmons 2001). However, males are unlikely to supply nutritional advantages to their mates during mating in *R. pedestris* (Suzaki, unpublished data). Females can also gain direct benefit from resources or territories possessed by males (Hoelzer 1989; Andersson 1994). For example, in red-collared widowbirds, *Euplectes ardens*, a dominant male can monopolize the best territories required by females for breeding (Andersson et al. 2002). However, males of *R. pedestris* fight for soy bean plants as territories, although females do not stay in the territory after copulation (Suzaki, personal observation). Thus, my results suggest that mating with attractive males brings no direct benefit to *R. pedestris* females.

Generally, indirect benefits have a much lower effect than direct benefits (Kirkpatrick and Barton 1997; Møller and Alatalo 1999; Cameron et al. 2003). Nevertheless, when

direct benefits are absent, indirect benefits should be required to maintain preference (Andersson 1994; Kirkpatrick 1996). In this case, females can obtain indirect benefits via their son's mating success or increasing viability of their offspring (e.g., Wedell and Tregenza 1999; Head et al. 2005; Taylor et al. 2007; Simmons and Holley 2011). Because no direct fitness benefit was found in *R. pedestris*, indirect benefits may maintain female preference even if this effect is small (also see Taylor et al. 2008). Indeed, there was a significant heritability in male attractiveness (copulation latency) in this bug and hence the females can produce attractive sons when mated with attractive males. This is consistent with the Fisherian process and contributes to maintenance of female preference (Fisher 1930; Lande 1981; Bakker 1993).

I found a positive genetic association between male attractiveness and courtship rate (Table 4-1, Fig. 4-1). This suggests that the courtship rate acts as an evaluative cue of female choice. These courtship behaviors can also play a critical role in mating success in other insect species (e.g., Barth et al. 1997; Boake and Konigsberg 1998; Ritchie et al. 1999; Kotiaho et al. 2001; Taylor et al. 2007; Omae et al. 2012). By contrast, male courtship displays sometimes negatively impact female fitness as male sexual harassment (Partridge and Fowler 1990; Sakurai and Kasuya 2008). However, mating with males delivering high courtship benefits did not affect female fitness in *R.*

*pedestris* (Fig. 4-2c, d), and thus the female is unlikely to be under such situations. On the other hand, because of a significant heritable variation of the courtship rate, females can sire sons delivering high courtship rates when they mated with attractive males. Production of sons with high courtship rates will contribute indirect benefits to the female.

A similar trend is found in male hind leg size (Table 4-1, Fig. 4-1). Weapon size is positively associated with competitive ability for mates (Okada et al. 2011b, 2012). In this species, males establish their territories on soybean plants (Natuvara 1985) and call over conspecific individuals irrespective of sexes by aggregation pheromone (Numata et al. 1990). When other males encroach, the territory holders fight against intruder, whereas when females are attracted, they court to females and attempt to mate (Natuvara 1985). Because it takes sucking soy bean to secrete aggregation pheromone (Numata et al. 1990), establishment and defense of territory are critical for their mating success. Therefore, the females can also sire sons highly successful in not only female mate choice but also male-male competition when they mate with the attractive males who have larger weapons (also see Berglund et al. 1996; Cordero and Eberhard 2003).

There was no significant genetic correlation between courtship rate and hind leg length (Table 4-1). This suggests that the characters are not functionally linked and act

independently as evaluative cues of male quality or conditions: the courtship behavior and hind leg length reflect abilities to secure more rapid successful copulation and to compete for mates, respectively. Recent studies suggest that females use multiple cues to choose their mates (Møller and Pomiankowski 1993; Etges 2002; Candolin 2003, 2004, 2005; Scheuber et al. 2004). This is because use of multiple cues can reduce assessment errors and cost of choice (reviewed in Candolin 2003). Females of *R. pedestris* may also use multiple cues for mate choice in order to acquire higher quality males who provide larger indirect benefit to their mates.

My results showed that male-male competition and female mate choice facilitate each other, and this is consistent with current concepts of sexual selection (Berglund et al. 1996; Cordero and Eberhard 2003; Mead and Arnold 2004; Andersson and Simmons 2006; Kokko et al. 2006). However, several studies found that males that are successful in competition are not always successful in female choice (Qvarnström and Forsgren 1998; Moore and Moore 1999; Bonduriansky and Rowe 2003; Candolin 2004, 2005; Hunt et al. 2009) because more competitive males frequently impair female fitness (Qvarnström and Forsgren 1998; Wong and Candolin 2005). In this situation, selective forces of male competition and female choice may not be reinforcing (Arnqvist and Rowe 2005). Again, I note that the female of this species is unlikely to be harmed by a

male because they do not stay in the male's territory after copulation. Furthermore, mating with highly competitive males did not affect female fitness (Fig. 4-2e, f). Therefore, the female of *R. pedestris* is unlikely to be under such situations.

Finally, I observed relatively high heritabilities of both sexually selected traits (Table 4-1). Theoretical predictions expected little genetic variance in sexually selected traits because directional selection is assumed to drive beneficial alleles to fixation and therefore a particular genotype should become predominant (Charlesworth 1987; Tomkins et al. 2004; Falconer and Mackay 1996). It is proposed several hypotheses which maintain genetic variance in these fitness-related traits (reviewed in Tomlins et al. 2004). For example, genetic trade-offs between fitness-related traits should act to preserve the genetic variance in these traits (Lynch and Walsh 1998; Roff 2002). Otherwise, condition dependence is predicted to contribute genetic variance of these characters (Rowe and Houle 1996; Tomkins et al. 2004). Indeed, sexually selected traits can be costly for males (Haines and Gould 1994; Emlen 2001; Kotiaho 2001), resulting in genetic trade-off between sexually selected traits and other traits (e.g., Nijhout and Emlen 1998; Lailvaux et al. 2010; Yamane et al. 2010; Okada et al. 2011a). Moreover, many studies demonstrated that sexually selected traits are condition dependent (reviewed in Hunt et al. 2004; Okada et al. 2011a). In future, it is necessary to

investigate genetic trade-offs and condition dependence of traits of male *R. pedestris*, to order to reveal how the genetic variance of sexually selected traits is maintained.

In summary, the courtship rate and hind legs act as an evaluative cue of female choice in *R. pedestris*. A female may obtain indirect benefit from mating with attractive males, but there were no direct benefits for female *R. pedestris* at least as far as I investigated. The indirect benefits in part consist of production of sons with high courtship rates and high competitive ability. The females may use multiple cues to choose their mate in order to maximize the indirect benefits they obtain.

## **Chapter 5. Testing for adaptive explanations of bimodal genital insertion duration in the stalk-eyed seed bug**

### **5-1. Introduction**

Sexual selection leads to the evolution of behavioral, physiological and morphological traits that increase male fitness under male-male competition (Andersson 1994). When females copulate with two or more males and store ejaculates in their reproductive tracts, a specific type of male-male competition, sperm competition, occurs (Parker 1970a). Sperm competition is recognized as a major selection force driving the evolution of various mating behaviors in both males and females (Parker 1970a; Simmons 2001).

When there is a possibility that sperm competition may occur, males use a number of tactics to reduce the risk of it occurring (e.g. mate guarding, Parker 1970b; mating plug, Parker 1970a; sperm displacement, Waage 1979). Bimodal or multimodal distributions of copulation duration are known in some insect species as a result of adaptation to sperm competition risk avoidance (Sillén-Tullberg 1981; Miller 1983; Siva-Jothy 1987; Siva-Jothy and Tsubaki 1989; Wolf et al. 1989; Lorch et al. 1993). In many cases, sperm transfer does not occur during shorter copulations (McLain 1980; Dickinson 1986; Alcock 1988; Lorch et al. 1993; but see Siva-Jothy 1987). Despite this, adaptive

explanations for short copulations have been provided in many cases (e.g. Siva-Jothy 1987; Lorch et al. 1993).

Similarly, seminal fluids are sometimes used to reduce female receptivity and avoid sperm competition (*Callosobruchus chinensis*: Yamane et al. 2008; *Togo hemipterus*: Himuro and Fujisaki 2008). Seminal fluids may also increase female fecundity, so that female, offspring and male fitness increase (e.g. Bonduriansky et al. 2005; McNamara et al. 2008).

Prolonged mating is a well-described phenomenon in many taxa (e.g. mammals, Dewsbury and Estep 1975; birds, Schulze-Hagen et al. 1995; reptiles, Moreira and Birkhead 2004; spiders, Suter and Parkhill 1990; insects, Dickinson 1986), and particularly in the Heteroptera (e.g. Sillén-Tullberg 1981; Carroll 1991; Hosokawa and Suzuki 2001). Prolonged mating behavior in insects is diverse, but typically involves the male inserting his aedeagus into the female once per mating event, with the pair remaining in copula for less than a minute to a number of days, depending on the species. In the stalk-eyed seed bug, *Chauliops fallax* Scott (Heteroptera: Malcidae), however, mating behavior is distinct from other examples of prolonged copulation. When a male encounters a female, he mounts her, then inserts his aedeagus repeatedly and intermittently until the termination of copulation. Compared to other species, the

mounting duration of *C. fallax* is relatively short (mean  $\pm$  SE =  $6.23 \pm 0.76$  h; Y. Suzuki, unpublished data). Female resistance to mating at the initiation of copulation is observed in many animals (e.g. Jivoff and Hines 1998; Filippi et al. 2000; Cuatianquiz and Cordero 2006; Maklakov et al. 2007; Blyth and Gilburn 2011; reviewed in Arnqvist and Rowe 2005), but rarely occurs in *C. fallax*. When female *C. fallax* resist mating, they usually do so in the middle of copulation. Observed female resistance behaviors include the female shaking herself, and kicking and knocking the male on the wall of the petri dish to prevent insertion and to dislodge the male. Moreover, the duration of insertion in *C. fallax* is of two types. Initially, insertions are short, without insemination, and occur from one to three times. Subsequent insertions are long, with insemination, and are performed repeatedly and intermittently in a bout of mating. Preinsemulatory short intromissions have been reported in a number of spiders in the Linyphiidae and Theridiidae (e.g. Willey Robertson and Adler 1994) and several mammals (e.g. Dewsbury 1988). To my knowledge, this is the only known example of bimodal intromission in an insect, where a short intromission consistently precedes a much longer one. In other examples, short and long intromissions are often performed independently or successively only once in a bout of mating (Simmons 2001), or alternatively with a long intromission preceding a shorter intromission (e.g. Eberhard

and Kariko 1996). Investigating the function of complex copulatory behavior may contribute to our understanding of adaptation to sperm competition.

I hypothesized the following regarding the function of short and long insertions in *C. fallax*. (1) Sperm transfer occurs during long insertions, as in other species with a bimodal distribution for copulation duration (reviewed in Simmons 2001). (2) Males reduce female remating receptivity via short and/or long insertions since copulatory mate guarding is short and daily fecundity is low (i.e. one to three eggs). (3) Short insertions function as nuptial gifts; in many species males transfer nuptial gifts to females in the form of prey items or seminal materials before transferring sperm (Simmons 2001; Arnqvist and Rowe 2005).

I investigated genital insertion duration for the first, second and third insertions of each male, plus differences in sperm transfer and hatchability between short and long insertions, to test my first hypothesis. I then examined remating receptivity in females experiencing only short or long insertions, or both types of insertion, to test my second hypothesis. Finally, I tested my third hypothesis by comparing the number of eggs oviposited by females experiencing a long insertion only, with the number of eggs oviposited by females experiencing both types of insertion. If short insertions function as nuptial gifts, females that experience short insertions are expected to lay more eggs

and/or live longer.

## 5-2. Materials and Methods

### *Study species*

*Chauliops fallax* is a bug of approximately 2.5 mm body length at the adult stage, with a unique form of eyestalk (Sweet and Shaefer 1985; Tomokuni et al. 1993). It feeds on leaves of leguminous plants (e.g. *Pueraria lobata* (Willd.), *Amphicarpaea edgeworthii* var. *japonica* and *Desmodium podocarpum* subsp. *oxyphyllum*), and sometimes damages leguminous crops (e.g. soybean, *Glycine max*, adzuki beans, *Vigna angularis*, and kidney beans, *Phaseolus vulgaris*; Sweet and Shaefer 1985; Tomokuni et al. 1993).

In Japan, *C. fallax* has two generations within a year on *P. lobata*; the first generation begins emerging in late July and commences ovipositing in early August, and the second generation emerges in early September and oviposits in late May after overwintering (Tayutivutikul and Yano 1989; Suzuki et al. 2011).

### *Laboratory Rearing*

A laboratory population was cultured from females and nymphs collected from the banks of the Sasagase River, Okayama, western Japan (34°40'N, 133°53'E). Nymphs

were reared in petri dishes (90 mm diameter, 15 mm deep) containing wet filter paper (70 mm diameter) and a small piece of cotton wool, and maintained at a temperature of 25°C with 16:8 h light:dark conditions. Densities were controlled at 10 nymphs per dish. I provided distilled water each day to maintain humidity, and fresh leaves of *P. lobata* every 3-5 days for food. When adults emerged, I separated them according to sex using their genital morphology, and maintained them at four individuals per petri dish. As adults of this bug become sexually mature approximately 15 days after emergence (unpublished data), I used virgin adults that were more than 15 days old for the following experiments.

#### *Copulatory Behavior*

I examined whether insertion duration differs with insertion order. I placed 15-20-day-old virgin males individually with 15-20-day-old virgin females in a petri dish (30 mm diameter, 13 mm height), and then observed mating behavior until either males performed a long insertion (>12 min) or they inserted the aedeagus three times, as they generally performed a short insertion at most three times before a long insertion (see Fig. 5-1a). I defined a long insertion as an insertion that lasted for more than 12 min because the frequency distribution of insertion duration diverged at 12 min (Fig. 5-1a).

The duration of each insertion was recorded with a stopwatch.

#### *Experiment 1: Insemination Success*

I placed 15e30-day-old virgin males individually with 15-30-day-old virgin females in a petri dish. Mating pairs were separated when short insertions (<420 s) were performed once or three times ('S' females,  $N = 15$ ), or when a long insertion occurred after one or two short insertions (>900 s; 'SL' females,  $N = 18$ ). I did not examine the females experiencing only long insertions ('L' females) in this experiment, because all L females received sperm without a short insertion, and thus all were inseminated (see Results of experiment 3). Each separated female was moved immediately from the petri dish to a 1.5 ml Eppendorf tube, and then killed in a freezer at -20°C. The number of sperm in the spermatheca was estimated following the methodology of Yamane and Miyatake (2005). The female was dissected and her spermatheca removed under a binocular microscope (SZX9-3122, OLYMPUS, Tokyo, Japan) using fine forceps. The spermatheca was placed in a drop of distilled water (20 ml) on a glass slide, and then crushed and cut into small pieces using fine forceps. Another drop of distilled water (20 ml) was added and the solution stirred with forceps for approximately 1 min to distribute the sperm evenly throughout the water. This spread the sperm homogeneously across the slide. The sperm

suspension (approximately 10 mm diameter) was air-dried on the glass slide. The average density of sperm within a fixed area ( $0.7 \times 0.7 = 0.5 \text{ mm}^2$ , 15 samples per female) was estimated under an optical microscope ( $\times 200$ ; BX51-33-PH, OLYMPUS, Tokyo, Japan). By multiplying the area of water on the slide by the average density of sperm, I estimated the total number of sperm ejaculated by a male into the spermatheca. If there were few sperm, I counted all the sperm in the sample.

I also investigated whether short insertions can fertilize eggs. I generated S females and SL females in the manner described above (each  $N = 20$ ); however, unlike in the other experiments, 15-day-old individuals were used. These mated females were maintained individually for 35 days from the initiation of the experiment and I counted the eggs laid every day. Females were given fresh leaves of *P. lobata* every 5 days and the old leaves were preserved on wet cotton wool in another petri dish (90 mm diameter, 15 mm height). The number of nymphs that hatched on the leaves for 15 days after their removal was recorded. I counted eggs for 35 days and hatched nymphs for 50 days from the initiation of the experiment.

#### *Experiment 2: Remating Receptivity*

In experiment 2, I examined whether insertion type affects female remating receptivity.

I placed a 20-day-old virgin male individually with a 20-day-old female in a petri dish, and separated them after two short insertions thus generating S females ( $N = 20$ ). The male was then mated to another virgin female. At this point, in many cases, males performed a long insertion *ab initio*, without a short insertion. When this occurred, I separated the pair, generating L females ( $N = 21$ ). In this species, because all males perform short insertions first, I had to use this method to make males carry out long insertions *ab initio*. I also generated SL females in the manner described in experiment 1 ( $N = 26$ ). Females from each treatment were maintained individually until they were tested for remating receptivity.

The day after mating, I placed females from each treatment (S, L or SL) individually with a new male in a petri dish (30 mm diameter, 13 mm height). I observed pairs for 2 h and recorded whether the female resisted the male's mating attempt, and whether the male succeeded in mating. When a mounted female shook her body, kicked the male using her hindlegs, or otherwise attempted to dislodge the male, this was recorded as female resistance to the male's mating attempt. When the female remated, I also recorded female latency and resistance intensity as duration of time from initiation of mounting to first insertion.

In addition, I investigated whether short insertions affected the subsequent male's

success in inducing the female to remate. I generated another batch of SL females ( $N = 24$ ) in the manner described above. The following day, these SL females were placed in a petri dish with a male that had previously carried out two short insertions with a randomly chosen virgin female. The male was permitted to mount the SL female. Thereafter, I observed whether the female resisted the male's mating attempt, and whether the male succeeded in mating within 2 h (SL → L treatment). Data from the SL → L treatment were compared with the data from the SL → SL treatment, that is, the aforementioned SL treatment. As before, when a mounted female shook her body, kicked the male using her hindlegs, or otherwise attempted to dislodge the male, this was considered female resistance. All females that did not resist the male's mating attempt remated (see Results). If a long insertion occurred, this was considered a successful remating; if mounting was terminated before a long insertion occurred, this was considered a failed male mating attempt. The experiment was conducted between 1400 and 1800 hours.

### *Experiment 3: Nuptial Gift*

In experiment 3, I examined whether short insertions function as nuptial gifts. I prepared 15-day-old L females and SL females as above (each  $N = 20$ ). I reared bugs

and counted the eggs they laid and hatched nymphs as in experiment 1. I also noted female longevity, providing the females died during the course of the experiment. As female longevity is approximately 50 days at 25°C (Tayutivutikul and Yano 1989), I recorded female longevity for 35 days from the initiation of the experiment. Note that the data from the SL female treatment were the same as in experiment 1.

#### *Statistical Analysis*

To examine whether insertion duration differed with insertion order, I used a Wilcoxon signed-ranks test following nonparametric Friedman's ANOVAs. To compare the amount of sperm transferred to S females and SL females (experiment 1), I used the Mann-Whitney *U* test. I also used Mann-Whitney *U* tests to compare numbers of eggs laid and egg hatchability. Logistic regression was used to examine the effect of insertion type on female remating receptivity and the Kruskal-Wallis test was adopted for comparing female latency and intensity of resistance (experiment 2). For experiment 3, the Mann-Whitney *U* test was used to compare the number of eggs laid and egg hatchability in L females and SL females. In addition, to examine whether oviposition speed differed between L and SL treatments, the time that elapsed before half the number of eggs were laid during the experimental period was compared using the

Mann-Whitney *U* test. Survival analysis was based on the Kaplan-Meier method, and the log-rank test was used to examine the effects of short insertion on survival time. Where appropriate I corrected the 5% significance level with sequential Bonferroni methods (Rice 1989).

Nonparametric analyses were conducted using SPSS version 11.0.1 (SPSS Inc., Chicago, IL, U.S.A.) and all other analyses were conducted using JMP version 7.0.1 (SAS Institute, Cary, NC, U.S.A.). Data shown are mean  $\pm$  SE.

### **5-3. Results**

#### *Copulatory Behavior*

Friedman's ANOVA showed significant differences in insertion duration with consecutive insertions ( $\chi^2 = 23.520$ ,  $P < 0.05$ ; Fig. 5-1b). The third insertion ( $N = 50$ ) was significantly longer than first and second insertions (each  $N = 71$ ; Wilcoxon signed-ranks test: second versus third:  $P < 0.001$ ; first versus third:  $P < 0.001$ ; Fig. 5-1b). However, no significant difference was detected in duration between the first and second insertions (Wilcoxon signed-ranks test: first versus second;  $P = 0.252$ ; Fig. 5-1b). The first insertion almost always finished within approximately 6 min, generating a bimodal distribution for insertion duration with one peak located at around 6 min and another at

around 30 min representing the second and third insertions (Fig. 5-1a).

#### *Experiment 1: Insemination Success*

SL females received more sperm than S females (SL: sperm number =  $516.30 \pm 58.63$ ,  $N = 18$ ; S: sperm number =  $15.33 \pm 4.91$ ,  $N = 15$ ; Mann-Whitney  $U$  test:  $Z = -4.897$ ,  $P < 0.001$ ). In S females, the number of eggs laid was significantly lower than for SL females (Mann-Whitney  $U$  test:  $Z = -5.413$ ,  $P < 0.001$ ; Table 5-1) and no eggs hatched from S females (Mann-Whitney  $U$  test:  $Z = -5.809$ ,  $P < 0.001$ ; Table 5-1).

#### *Experiment 2: Remating Receptivity*

L females and SL females resisted remating with higher frequency than S females (logistic regression: S versus L:  $\chi^2 = 10.46$ ,  $P = 0.001$ ; S versus SL:  $\chi^2 = 11.34$ ,  $P = 0.008$ ; sequential Bonferroni adjusted:  $P < 0.05$ ; Fig. 5-2a), suggesting that short insertions have no role in the inhibition of female remating. Resistance to remating occurred in L and SL females, while all S females remated (logistic regression: S versus L:  $\chi^2 = 12.56$ ,  $P = 0.0004$ ; S versus SL:  $\chi^2 = 7.532$ ,  $P = 0.006$ ; sequential Bonferroni adjusted:  $P < 0.05$ ; Fig. 5-2a). No significant difference was detected in remating between L females and SL females (logistic regression: resistance:  $\chi^2 = 0.113$ ,  $P =$

0.736; success:  $\chi^2 = 1.25$ ,  $P = 0.264$ ; sequential Bonferroni adjusted:  $P < 0.05$ ; Fig. 5-2a). A Kruskal-Wallis test showed no significant differences in female latency and intensity of resistance across each treatment (time from initiation of mounting to first insertion: S:  $144.27 \pm 17.27$  s; L:  $225.56 \pm 32.63$  s; SL:  $289.08 \pm 57.16$  s;  $\chi^2 = 5.778$ ,  $P = 0.056$ ).

In the SL → L treatment, resistance to remating occurred at a higher frequency than in the SL → SL treatment (logistic regression:  $\chi^2 = 8.602$ ,  $P = 0.003$ ; Fig. 5-2b), but there was no significant difference in the occurrence of remating (logistic regression:  $\chi^2 = 0.025$ ,  $P = 0.874$ ; Fig. 5-2b). In addition, I found no effect of short insertions on the likelihood of remating.

### *Experiment 3: Nuptial Gift*

There were no significant differences in the number of eggs laid or egg hatchability across treatments (L versus SL: Mann-Whitney  $U$  test: eggs laid:  $U = 180.5$ ,  $P = 0.602$ ; hatchability:  $U = 175.5$ ,  $P = 0.512$ ; Table 5-1). In addition I found no significant difference in female longevity across treatments (log-rank test:  $\chi^2 = 0.076$ ,  $P = 0.784$ ; Fig. 5-3). Oviposition speed also did not differ between L and SL treatments (oviposition speed; L:  $17.25 \pm 1.14$  days; SL:  $15.85 \pm 0.78$  days; Mann-Whitney  $U$  test:

$Z = -1.249$ ,  $P = 0.22$ ).

#### 5-4. Discussion

The fact that *C. fallax* exhibits a bimodal distribution of insertion duration and always performs a short insertion first, without insemination, is unique. Besides *C. fallax*, there are several species that exhibit bimodal or multimodal distributions of copulation duration as an adaptation to the risk of sperm competition (Simmons 2001). For example, in the stalk-eyed fly *Cyrtodiopsi whitei*, copulation duration exhibits a bimodal distribution with peaks at 10 and 50 s, and sperm are not transferred during the first 40 s of copulation (Lorch et al. 1993). To avoid sperm wastage caused by first-male sperm precedence, the shorter mating occurs only when a male mates with females that have recently mated (Lorch et al. 1993). The duration of copulation in the provisioning shield bug, *Parastrachia japonensis* is of two types: a widespread short copulation (average 15 s) and a longer copulation (average 23 min) in which sperm transfer usually occurs (Filippi et al. 2000). Filippi et al. (2000) suggested that female *P. japonensis* are imposing selection on males for longer copulations as males must overcome female resistance to insemination. In contrast, *C. fallax* males performed short insertions when they mated successfully with mated females. Furthermore, females rarely resisted male

mating attempts until they had experienced a long insertion, and all males successfully transferred sperm after a long insertion when they mated with virgin females. In addition, although in other species that exhibit a bimodal distribution of copulation duration, sperm transfer does not occur during short matings, a few sperm are transferred during the short insertion in *C. fallax*. However, in the current investigation, females that experienced only short insertions never laid fertilized eggs, and egg laying was not induced. Moreover, it does not seem that males of *C. fallax* manipulate female receptivity or fecundity with short insertions (see below).

The present study showed that males of *C. fallax* not only transferred sperm but also reduced female receptivity with long insertions. In many insect species, males adopt prolonged mating association as a form of postcopulatory mate guarding to reduce the risk of sperm competition (e.g. Sillén-Tullberg 1981; Carroll 1991; Hosokawa and Suzuki 2001; Harari et al. 2003; Knox and Scott 2006; reviewed in Simmons 2001). In these species females lay more eggs in a single batch following postcopulatory mate guarding (Sillén-Tullberg 1981; Harari et al. 2003; Hosokawa et al. 2005; Knox and Scott 2006). However, in species such as *C. fallax* (the present study), *Riptortus pedestris* (Sakurai 1998) and *T. hemipterus* (Himuro and Fujisaki 2008) in which females lay a few eggs continually over a long period, inhibition of female remating is a

better strategy for ensuring paternity than postcopulatory mate guarding. In *R. pedestris*, female receptivity depends on the amount of sperm in the spermatheca (Sakurai 1998), while in *T. hemipterus* male accessory gland substances inhibit female remating (Himuro and Fujisaki 2008). In the present study, however, I did not examine whether *C. fallax* males use sperm and/or accessory gland substances to inhibit remating by females. In mammals, moreover, it is reported that male stimulation by penile spines reduces female receptivity (e.g. Stockley 2002). The mechanism by which males inhibit female remating in *C. fallax* requires further investigation, potentially through micro-injection of the ejaculate components of interest (Himuro and Fujisaki 2008; Yamane et al. 2008) and observation of the morphology of male genitalia.

It seems that short insertions of *C. fallax* play no role in fertilization. In several insect species, the males provide their mates with a food gift during copulation and/or insemination (Thornhill 1976b; Thornhill and Alcock 1983; Simmons and Parker 1989). Nuptial gifts provided by males have been shown to increase the probability of successful copulation (Steele 1986; Sakaluk et al. 1995), as well as the duration of copulation and number of sperm transferred (Thornhill 1976a; Svensson et al. 1990). In the heteropteran *Menida scotti*, males donate secretions to females during copulation which are digested by the females (Koshiyama et al. 1993). However, the seminal fluids

of *C. fallax* males do not appear to increase fecundity, egg hatchability or longevity of females at least until the females are 35 days old; thus short insertions are unlikely to function as a nuptial gift.

I have discussed the possibility that short insertions in *C. fallax* serve to inhibit remating and/or to deliver a nuptial gift through the ejaculate; however, it is necessary to argue other alternative hypotheses. For instance, short insertions may function as copulatory courtship, which may enhance male and/or female fitness via male stimulation during copulation (Eberhard 1996). My results showed that short insertions did not affect female remating receptivity (Fig. 5-2), and fecundity (Table 5-1). However, there still remain other possible functions to be tested including whether short intromissions induce females to reject subsequent male mating attempts, including enhancing the intensity of female resistance behavior, female latency and avoidance of encounter with males, whether they induce females to discard or digest sperm from previous or subsequent males, or whether they stimulate early oviposition (Eberhard 1996; Arnqvist and Rowe 2005).

Although my results showed that short insertions were ineffective in enhancing intensity of female remating rejection, latency and oviposition speed (see Results), they neither support nor refute other hypotheses. In several mammals, repeated,

nonejaculatory intromissions within a pair can trigger ovulation (Jöchle 1975; Dewsbury 1988; Eberhard 1996). However, it seems that this hypothesis is inadequate to explain the function of short insertions in *C. fallax*, as female *C. fallax* were not induced to oviposit by short insertions (experiment 1) and could lay fertilized eggs without them (Table 5-1). Alternatively, preinsemintary insertion may function in species recognition (Gabor and Ryan 2001; Wong et al. 2005; Tanabe and Sota 2008). For example, in the millipede genus *Parafontaria*, males perform preliminary intromission before ejaculation; when males mate with heterogeneous females, they terminate copulation (Tanabe and Sota 2008). However, this does not explain this behavior in *C. fallax*, since it is the only species of *Chauliops* found in Japan (Sweet and Shaefer 1985), and thus discrimination between congenerics is unnecessary. Furthermore, in the bed bug *Cimex lectularius*, males perceive females' mating status via chemoreceptors on the intromittent organ, and thereby adjust ejaculate size (Siva-Jothy and Stutt 2003), and, thus, short insertions could serve as perception of female mating status. However, this was not examined in the present study. Experiments investigating these hypotheses need to be carried out.

In conclusion, insemination in *C. fallax* occurred during long insertions but not during short insertions. Long insertions induced mated females to resist subsequent male

mating attempts, but short insertions did not. In addition, short insertions did not appear to function as nuptial gifts. Although the adaptive significance of the long insertion is clear, that is, sperm transfer and inhibition of female remating, I am no closer to understanding the adaptive function of short insertions in this species. It is necessary to test alternative adaptive explanations for this behavior. Finally, in this study I reused males that had performed short insertions to generate L treatment individuals. Thus, the L treatment may not be comparable to the SL treatment, as the present methods did not exclude the possibility that L males perceived the change in partners and altered their response.

## **Chapter 6. General discussion**

In these studies, I revealed that exaggerated hind legs of males evolved through both of male-male competition and female mate choice in *Reptortus pedestris* (Chapter 2, 4). In male-male competition, males with larger body size and hind legs were more successful (Chapter 2). Furthermore, males with larger hind legs were more preferred by females (Chapter 4). Therefore, it seems that inter- and intrasexual selection facilitate each other in *R. pedestris*. This is why there is sexual dimorphism which only males have enlarged hind legs in this bug (Wong and Candolin 2005).

Furthermore, I detected that sexual selection promotes evolution of not only target traits but also other somatic traits, that is, supportive traits. In *R. pedestris*, males with larger middle leg and more elongated body were more likely to win fights (Chapter 3). It suggests that males have acquired somatotype suitable for fighting via sexual selection (Tomkins et al. 2005).

Females of *R. pedestris* chose their mate based on hind leg size and courtship behavior and obtain indirect benefit which enhances mating success of their sons (Chapter 4). Candolin (2003) argued that because differential type of cues such as vibrational and visual cues reflect differential aspects of male condition, females can maximize their benefit derived from mating with attractive males by using multiple cues. Indeed, there

was significant heritability in male courtship rate and hind leg size, thereby females can obtain sons who may be successful in both intra- and intersexual selection (Chapter 4). Furthermore, since expression of sexual traits is costly, males with larger hind legs and higher courtship rate are assumed to be good condition and to have more acquisition ability (Andersson 1994; Hunt et al. 2004). Therefore, hind leg size and courtship rate also may be indicator of good gene (Jennions and Petrie 2000; Andersson 2006). However, because I did not test this hypothesis in current studies, future study should cope with this subject.

In *Chauliops fallax*, I found that longer genital insertions served as sperm transmission and reducing receptivity of subsequent mating (Chapter 5). Unfortunately, however, I cannot suggest adaptive explanation of shorter genital insertions which occur before longer insertion (Chapter 5). It is necessary for clarification of function of shorter insertions to verify other hypothesis. For example, males of *C. fallax* detect mating status of their mate by shorter insertion and adjust ejaculate volume to reduce resource expenditure (Siva-Jothy Stutt 2003). Moreover, males may manipulate female sperm utilization to his advantage by shorter insertion (Eberhard 1996). Or shorter insertions are probably ancestral adaptation for pre- and/or postcopulatory sexual selection.

My studies revealed various adaptation for pre- and postcopulatory sexual selection in

two heteropteran species. Those studies may be able to contribute to accumulation of evidence for evolution of male sexual traits and deepening of argument about evolution of sexual selection. Since *R. pedestris* are easy to rear, observe behavior and analysis morphology, they are likely to be a new model species for the study of sexual selection. In *R. pedestris*, there still remain some issues to be resolved such as interaction of pre- and postcopulatory sexual selection, genetic correlation of weapon traits between sexes. Furthermore, they exhibit daily rhythms in various behaviors (Kadosawa 1982, 1983; Numata and Matsui 1988; Suzaki and Miyatake in press) and their crock genes were detected (Ikeno et al. 2011). Therefore, using this bug, I may be able to contribute for inclusive understanding of sexual selection. Moreover, since mating behavior of *C. fallax* different from other animal species, investigation of this phenomenon may have an impact on studies of behavioral ecology. In conclusion, I found that diversified mating behavior and sexual traits evolved in Heteroptera, so they are a fascinating subject of evolutionary biology.

## **Summary**

**1.** I conducted morphological analysis and behavioral observations in *R. pedestris*. In this bug, males possess larger hind legs than females. Observations of male-male interactions showed that the enlarged hind legs are used as weapons in male fights, and that males with larger hind legs win fights more frequently. Morphological analysis based on the positive allometry test showed that the femora of larger males are relatively bigger than those of smaller males, but femora of larger females are not relatively larger than those of smaller females. These results suggest that sexual selection in *R. pedestris* favors larger hindlegs for male fighting. In addition, the thorax and abdomen lengths were larger in the male than in the female. The males often lift their abdomen with their back to the opponent for displays against an opponent. As a result, abdominal size may be under stronger selection in the male than in the female, as for the exaggerated hind legs.

**2.** Weapon traits are often coupled with correlated modifications in other somatic traits (hereafter referred to as supportive traits), which are thought to support the weapon functionally. No previous studies have investigated whether supportive traits themselves affect fighting success. I evaluated this possibility in *R. pedestris*. Using a principal components analysis, I identified a subset of supportive traits that were associated with

weapons. Fighting success was higher in males with enhanced supportive traits. It appears that weapon-supportive structures develop as the result of selection that favors coordination among multiple traits associated with fighting behaviour. Thus, my results provide rare evidence of the benefit of a supportive trait that is a clear example of phenotypic integration.

**3.** To integratively understand the evolution of sexually selected traits, I focused on direct and indirect benefits to females from male attractiveness, courtship, and weapon characters in *R. pedestris*. The males use their hind legs to fight other males over territory and perform courtship displays for successful copulation. Females of *R. pedestris* receive no direct benefit from mating with attractive males. On the other hand, I found that male attractiveness, courtship rate, and weapon size were significantly heritable and that male attractiveness had positive genetic covariances with both courtship rate and weapon traits. Thus, females obtain indirect benefits from mating with attractive males by producing sons with high courtship success rates and high competitive ability. Moreover, it is evident that courtship rate and hind leg length act as evaluative cues of female choice. Therefore, female mate choice and male–male competition may facilitate each other in *R. pedestris*. This is consistent with current basic concepts of sexual selection.

4. In many taxa, bimodal distributions of copulation and/or intromission duration are known to be the result of adaptation to sperm competition risk avoidance. However, *C. fallax* exhibits completely different mating behavior to other known examples. The male mounts the female and then inserts his aedeagus repeatedly and intermittently until the termination of copulation. Insertion duration exhibited a bimodal distribution with two peaks: a short insertion within 6 min which occurred one to three times without insemination, followed by long insertions of approximately 30 min with repeated insemination. To examine the adaptive significance of the two types of genital insertion, I investigated whether the amount of sperm transferred, female remating receptivity, number of eggs laid, hatchability and female longevity differed between the two insertion types. Sperm transfer mainly occurred during long insertions but very little occurred during short insertions. Similarly, long insertions induced mated females to resist subsequent mating attempts of other males but short insertions did not. Short insertions did not affect the number of eggs laid, hatchability or female longevity. Although our results showed that *C. fallax* males transferred sperm to females and inhibited female remating with long insertions, I currently cannot explain the adaptive function of short insertions without insemination.

## **List of publications**

- 1.** Okada K, Suzaki Y, Okada Y, Miyatake T (2011) Male aggressive behavior and exaggerated hindlegs of the bean bug *Riptortus pedestris*. *Zoological Science*, 28, 659-663.
- 2.** Suzaki Y, Miyatake T (2011) Testing for adaptive explanation of bimodal genital insertion duration in the stalk-eyed seed bug. *Animal Behaviour*, 82, 1103-1108.
- 3.** Okada Y, Suzaki Y, Miyatake T, Okada K (2012) Effects of weapon-supportive traits on fighting success in armed insects. *Animal Behaviour*, 83, 1001-1006.
- 4.** Suzaki Y, Katsuki M, Miyatake T, Okada Y (2013) Male courthip behavior and weapon traits as indicators of indirect benefit in the bean bug, *Riptortus pedestris*. *PLoS ONE*, 8, e83278.

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## Tables

**Table 2-1.** Means  $\pm$  s.e. and results of ANOVA with sex as a fixed effect in each trait.

Traits (mm)	Male (N = 100)	Female (N = 80)	F <sub>1, 178</sub>	P
Thorax width	3.164 $\pm$ 0.016	3.202 $\pm$ 0.019	2.256	0.1348
Thorax length	5.440 $\pm$ 0.031	5.364 $\pm$ 0.036	2.788	0.0967
Abdomen width	3.810 $\pm$ 0.012	3.804 $\pm$ 0.013	0.093	0.7606
Abdomen length	6.687 $\pm$ 0.022	6.756 $\pm$ 0.026	5.594	0.0191
Hindfemur width	1.237 $\pm$ 0.007	1.184 $\pm$ 0.008	25.541	< 0.0001
Hindfemur length	7.566 $\pm$ 0.054	6.256 $\pm$ 0.033	406.694	< 0.0001
Hindtibia length	6.316 $\pm$ 0.030	5.188 $\pm$ 0.023	848.297	< 0.0001

The actual value was log<sub>10</sub> transformed for ANOVA.

**Table 2-2.** Results of ANCOVA on the effects of each trait.

Trait	Effect	df	MS	F	P
Thorax length	Sex	1	0.00396	9.808	0.002
	Body size	1	0.03794	93.97	< 0.0001
	Error	117	0.0004		
Abdomen width	Sex	1	0.00024	2.174	0.1422
	Body size	1	0.01067	96.391	< 0.0001
	Error	117	0.00011		
Abdomen length	Sex	1	0.00226	17.23	< 0.0001
	Body size	1	0.01436	109.337	< 0.0001
	Error	117	0.00013		
Hindfemur width	Sex	1	0.0215	55.457	< 0.0001
	Body size	1	0.04023	103.757	< 0.0001
	Error	117	0.00039		
Hindfemur length	Sex	1	0.32627	1222.383	< 0.0001
	Body size	1	0.06978	261.434	< 0.0001
	Sex $\times$ Body size	1	0.01146	42.934	< 0.0001
	Error	176	0.00027		
Hindtibia length	Sex	1	0.3418	1591.776	< 0.0001
	Body size	1	0.0299	139.226	< 0.0001
	Error	117	0.00022		

Non-significant interaction terms are removed from the model. Body size: thorax width. The actual value was log 10 transformed for the statistical analysis.

**Table 2-3.** Test for power function yields values of  $\alpha$  in each sex.

Trait	$\alpha \pm SE$	95% confidential interval	t	P
Male				
Thorax length	0.632 $\pm$ 0.092	0.450-0.814	6.9	< 0.0001
Abdomen width	0.365 $\pm$ 0.048	0.269-0.460	7.59	< 0.0001
Abdomen length	0.401 $\pm$ 0.052	0.298-0.504	7.75	< 0.0001
Hindfemur width	0.686 $\pm$ 0.088	0.512-0.861	7.81	< 0.0001
Hindfemur length	1.225 $\pm$ 0.072	1.082-1.367	17.08	< 0.0001
Hindtibia length	0.621 $\pm$ 0.073	0.476-0.767	8.47	< 0.0001
Female				
Thorax length	0.653 $\pm$ 0.097	0.461-0.845	6.76	< 0.0001
Abdomen width	0.314 $\pm$ 0.050	0.214-0.414	6.25	< 0.0001
Abdomen length	0.388 $\pm$ 0.057	0.277-0.499	6.97	< 0.0001
Hindfemur width	0.634 $\pm$ 0.097	0.441-0.827	6.54	< 0.0001
Hindfemur length	0.518 $\pm$ 0.081	0.356-0.680	6.36	< 0.0001
Hindtibia length	0.514 $\pm$ 0.060	0.394-0.634	8.55	< 0.0001

**Table 3-1.** Size, range and between-contestant difference of the morphological traits of *R. pedestris*.

Trait (mm)	Population mean $\pm$ SE	Size range (minimum - emaximum)	Size difference between contestants (mean SE, (% of size range))
Hindfemur length			
Thorax width	7.6656 $\pm$ 0.0478	2.52 (6.38-8.80)	0.0080 $\pm$ 0.0044 (0.32)
Thorax length	3.0863 $\pm$ 0.0174	1.14 (2.40-3.54)	0.2280 $\pm$ 0.0606 (20.00)
Abdomen width	5.3442 $\pm$ 0.0162	0.72 (4.97-5.69)	0.1740 $\pm$ 0.0478 (24.17)
Abdomen length	3.7183 $\pm$ 0.0156	0.84 (3.29-4.13)	0.2300 $\pm$ 0.0458 (27.38)
Head width	6.8369 $\pm$ 0.0205	0.98 (6.28-7.26)	0.1190 $\pm$ 0.0228 (12.14)
Hindfemur length	2.6133 $\pm$ 0.0074	0.38 (2.42-2.80)	0.0430 $\pm$ 0.0068 (11.32)
Forefemur length	4.0678 $\pm$ 0.0161	0.75 (3.73-4.48)	0.1530 $\pm$ 0.0420 (20.40)
Midfemur length	4.2178 $\pm$ 0.0151	0.76 (3.89-4.65)	0.2200 $\pm$ 0.0464 (28.95)

Population mean and size range were calculated from males used for morphological measurement in each trait.

**Table 3-2.** Factor loadings from the principal components analyses of morphological traits in *R. pedestris*

Traits	PC1	PC2	PC3	PC4
Percentage	42.58	26.14	11.39	7.28
Thorax width	<b>0.8224</b>	<b>-0.4566</b>	-0.0983	0.0763
Thorax length	<b>0.7066</b>	<b>0.4431</b>	-0.2813	0.0663
Abdomen width	<b>0.5306</b>	<b>-0.7015</b>	-0.2471	0.2806
Abdomen length	<b>0.7456</b>	<b>0.4489</b>	-0.0705	0.1312
Head width	<b>0.7851</b>	0.0375	0.0015	<b>-0.5863</b>
Forefemur length	<b>0.5316</b>	-0.2661	<b>0.7811</b>	0.0822
Midfemur length	<b>0.2609</b>	<b>0.812</b>	0.1796	0.2307

The highest loading variables for each PC are indicated in bold.

**Table 3-3.** Results of generalized linear models exploring the relationships between weapon size and other somatic characters in *R. pedestris*.

Predictor variables	df	Coefficient ± SE	Mean square	F	P
PC1(body size)	1	0.0322±0.0020	0.3667	269.5337	< 0.0001
PC2 (body shape and midfemur length)	1	0.0126±0.0025	0.0345	25.3661	< 0.0001
PC3 (forefemur length)	1		0.00009	0.0631	0.802
PC4 (head width)	1		0.0017	1.2456	0.267
Error	115		0.1565	0.0014	

Nonsignificant interaction terms have been removed from the model.

**Table 3-4.** Results of the generalized linear models exploring the effects of morphological characters on fighting success in *R. pedestris*.

Predictor variables	df	Coefficient ± SE	$\chi^2$	P
Weapon size (hindfemur length)			1.5715	0.21
PC1(body size)	1		0.4887	0.485
PC2 (body shape and midfemur length)	1	2.8843 ± 1.3364	13.1567	0.0003
PC3 (forefemur length)	1		1.4944	0.222
PC4 (head width)	1		0.1446	0.704

Nonsignificant interaction terms have been removed from the model.

**Table 4-1.** Heritabilities ( $h^2$ ) and genetic correlations  $\pm$  SE in male attractiveness and traits

	Latency	Courtship rate	Hind leg length
Latency	<b>0.822±0.175</b> <b>(0.552±0.116)</b>		
Courtship rate	-0.539±0.105 (-0.693±0.078)	<b>0.681±0.143</b> <b>(0.721±0.153)</b>	
Hind leg length	-0.490±0.114 (-0.561±0.103)	0.005±0.148 (0.005±0.150)	<b>0.742±0.156</b> <b>(0.740±0.157)</b>

Heritabilities ( $h^2$ ) are given on the diagonal and additive genetic correlations below the diagonal. Values in parentheses are estimates re-calculated by excluding the highest point of latency. Estimates significantly different from zero ( $P < 0.05$ ) are shown in bold.

**Table 5-1.** Number of laid eggs and hatchability in groups of S, L and SL females.

Treatment	Egg	Hatchability
S	12.15 ± 1.25	0
L	41.25 ± 3.34	0.74 ± 0.07
SL	41.05 ± 2.22	0.83 ± 0.06

## Figure legends

**Fig. 2-1.** Measured parts of *Riptortus pedestris*. TL: thorax length; TW: thorax width; AL: abdomen length; AW: abdomen width; HFL: hindfemur length; HFW: hindfemur width; HTL: hindtibia length. The left and right panels show a male and a female, respectively.

**Fig. 2-2.** The relationships between thorax width and (A) thorax length, (B) abdomen width, (C) abdomen length, (D) hindfemur width, (E) hindfemur length and (F) hindtibia length. Closed circles indicate males, and open circles indicate females.

**Fig. 2-3.** Male-male aggressive behaviors in *Riptortus pedestris*: (A) two males lift their abdomens with their backs to the opponent each other and flap their wings, (B) one male kicks his opponent with his right hindleg, (C) two males kick each other with both hindlegs, and (D) one male raises his hindlegs to squeeze the opponent's body.

**Fig. 3-1.** Somatic characters of *R. pedestris* that were measured as potential supportive traits. HW: head width; TL: thorax length; TW: thorax width; AL: abdomen length; AW: abdomen width; HFL: hindfemur length.

**Fig. 4-1.** Correlation between male attractiveness and courtship rate and between male attractiveness and hind leg length. The y-axis is the male attractiveness (copulation latency). The x-axis is the courtship rate (**a**) and hind leg length (**b**), respectively. Each circle shows family means for each sire. Increases on the y-axis represent decreased attractiveness.

**Fig. 4-2.** Male attractiveness, courtship rate and hind leg length did not indicate direct benefits on females. Male attractiveness (copulation latency), courtship rate, and male hind leg length were not associated with female lifetime reproductive success and longevity. The y-axis is the female lifetime reproductive success (**a, c, e**) and longevity (**b, d, f**), respectively. The x-axis is the Male attractiveness (**a, b**), courtship rate (**c, d**), and male hind leg length (**e, f**), respectively. In male attractiveness, increases on the x-axis represent decreased attractiveness.

**Fig. 5-1. (a)** Frequency histograms of insertion duration for first to third insertions. **(b)** Mean duration of consecutive insertions until the third insertion.

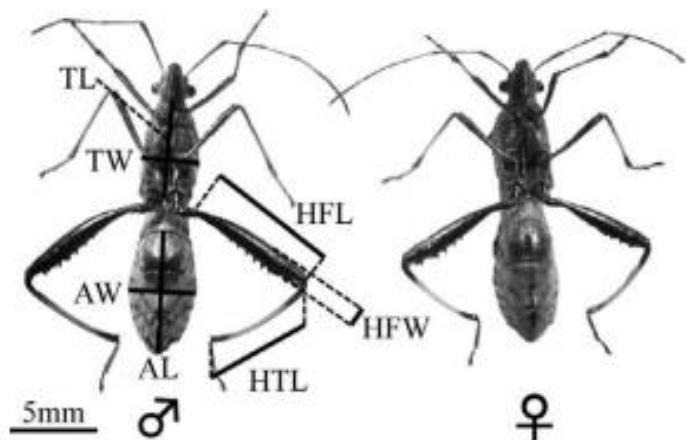
**Fig. 5-2. (a)** Proportion of mated female responses to male mating attempts in each treatment. **(b)** Mated female responses to male mating attempts when they do or do not

involve a short insertion. Sample sizes are shown within each bar.

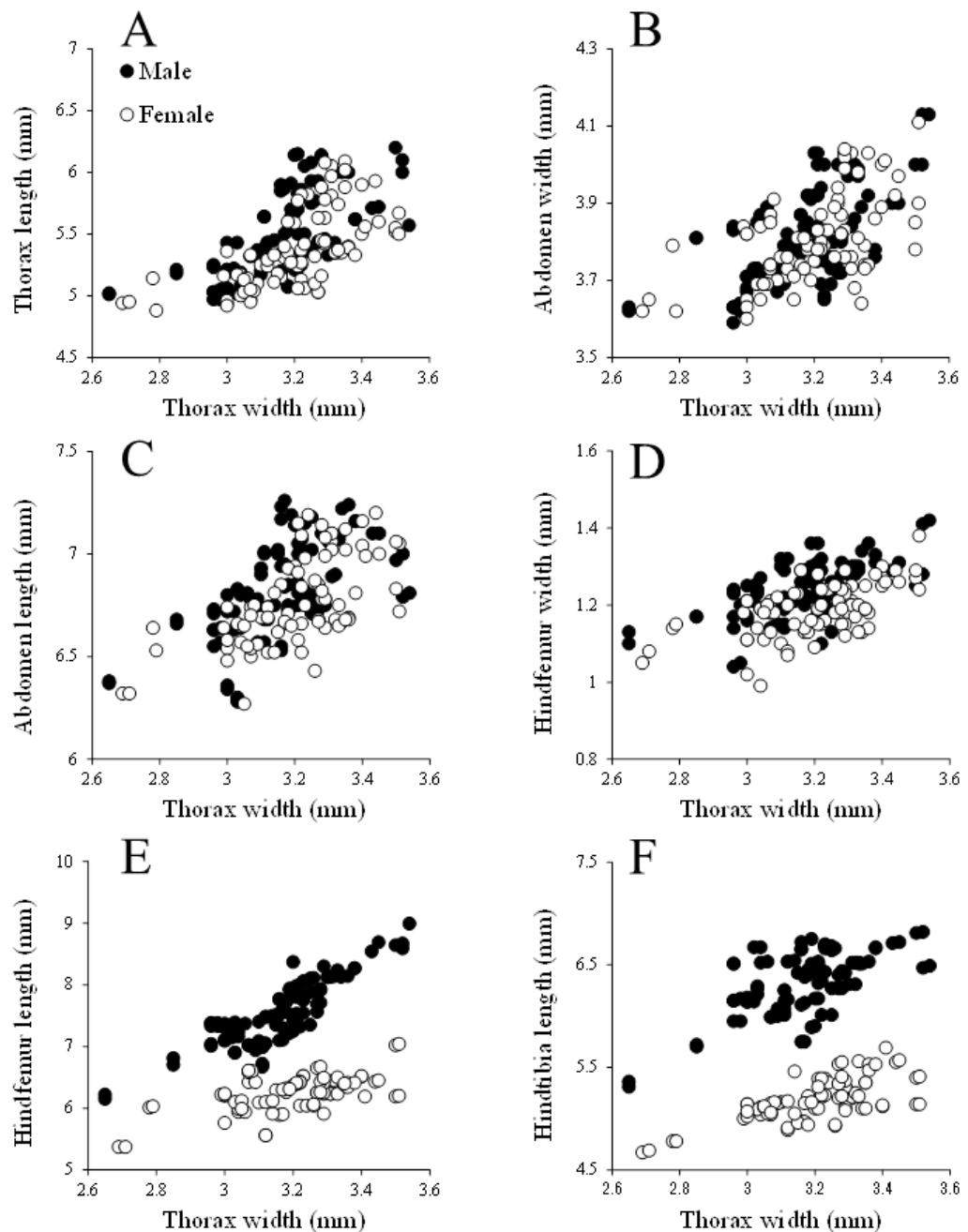
**Fig. 5-3.** Cumulative survival probability against time (d) for L females and SL females.

## Figures

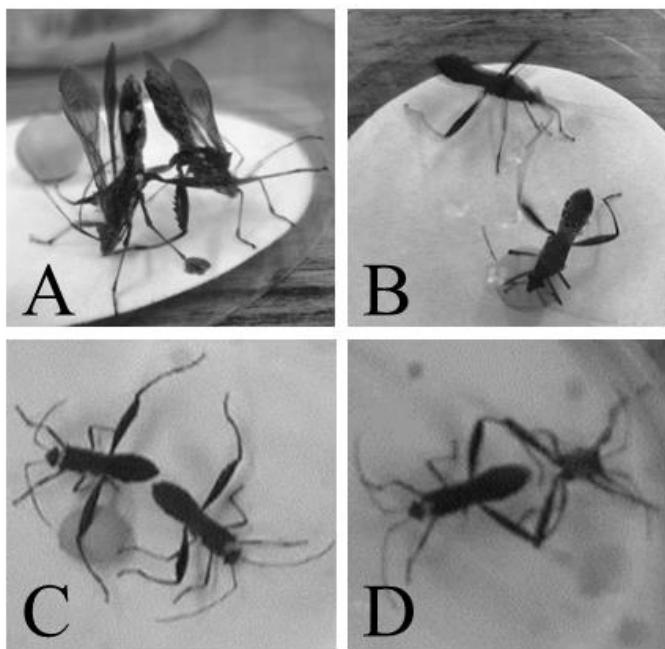
Fig. 2-1.



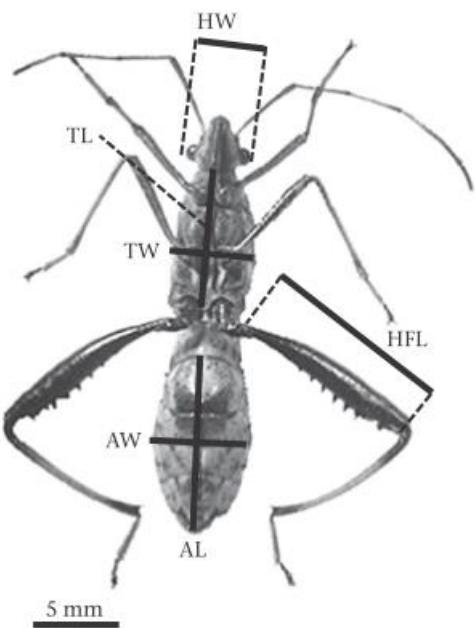
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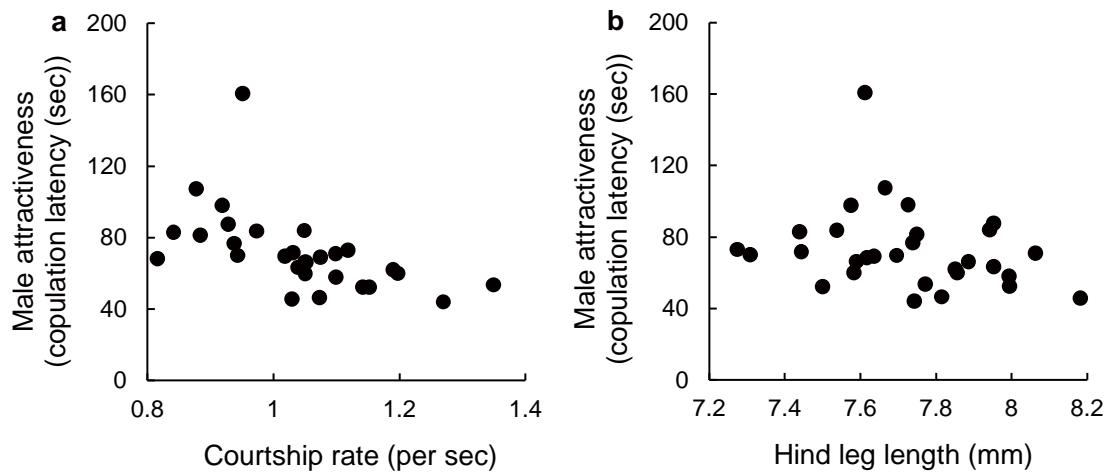
**Fig. 2-3.**



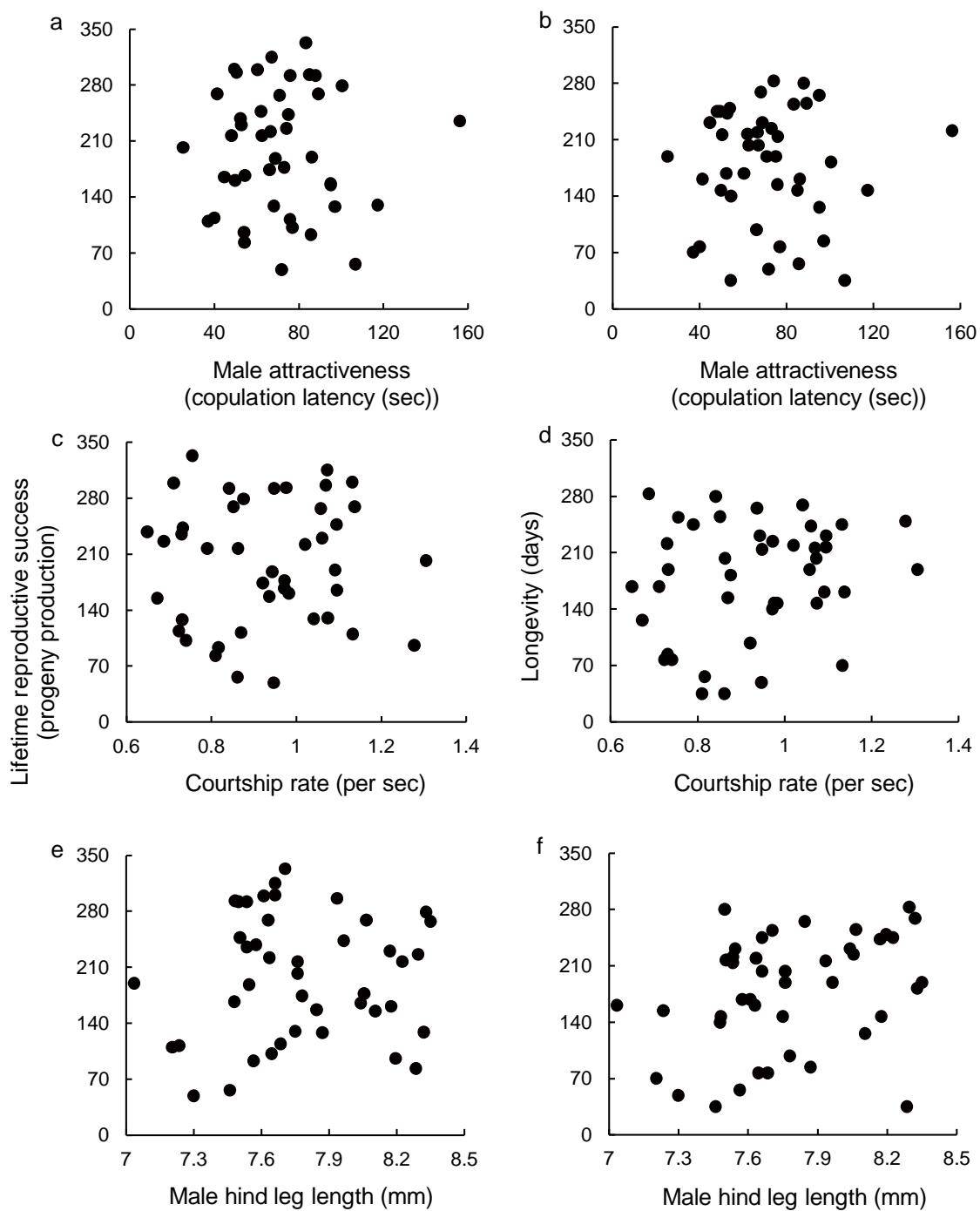
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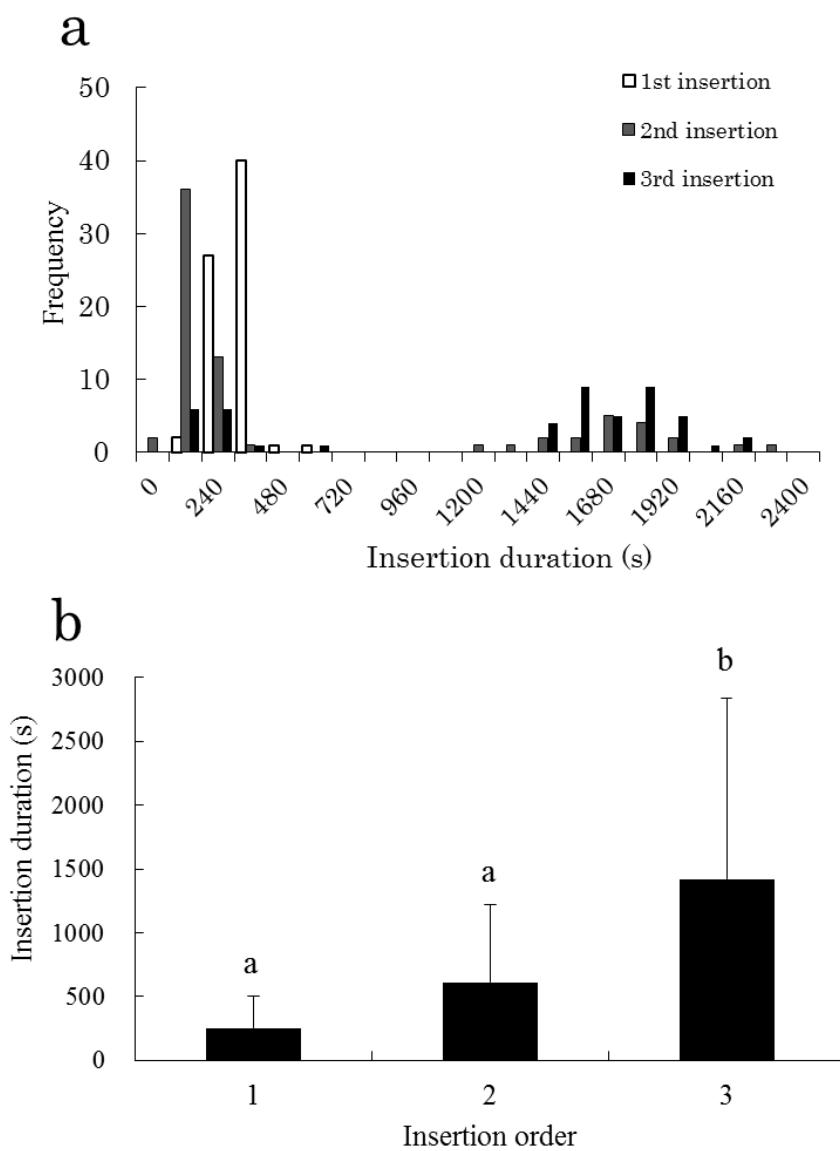
**Fig. 4-1.**



**Fig. 4-2.**

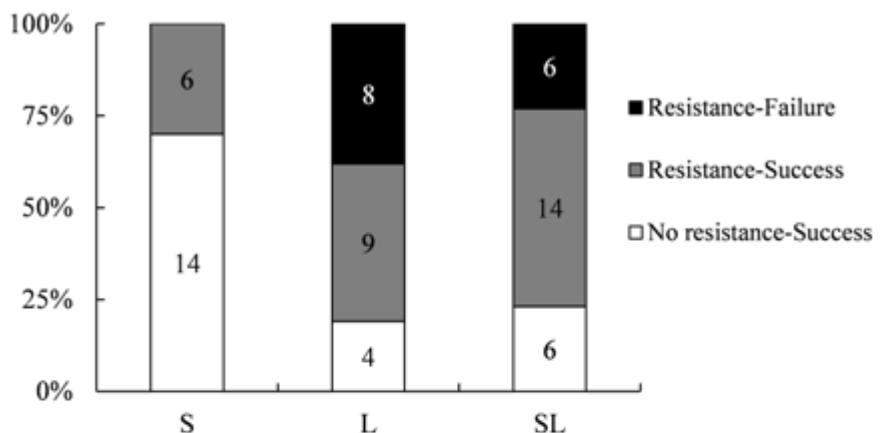


**Fig. 5-1.**

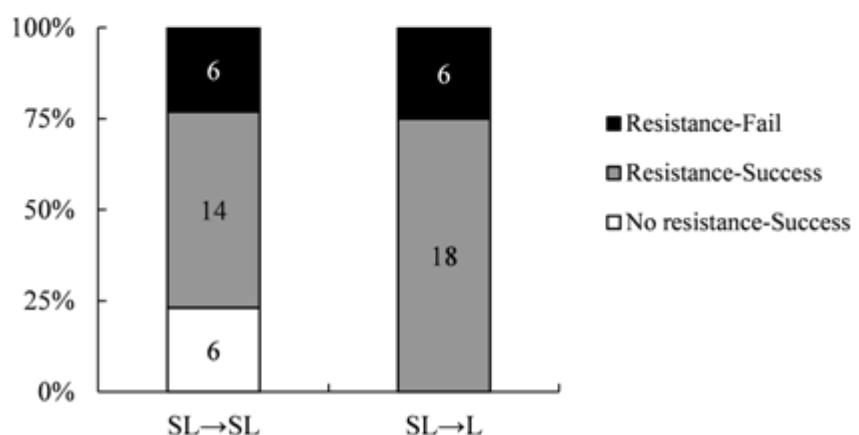


**Fig. 5-2.**

a



b



**Fig. 5-3.**

