Evolution of reproductive strategies in stored product insect species

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Contents

1. General Introduction

1-1. Sexual Selection

Sexual selection is one of the most important evolutionary concepts, same as natural selection, indicated by Charles Darwin. Natural selection favours to the individuals which have traits increasing their viability. On the other hand, sexual selection favours the individuals with a trait increasing their fitness rather than their viability.

Males produce a large number of small gametes, i.e. sperms. In contrast, females produce relatively large gametes, i.e. eggs. Because of the larger gametes, the number of offspring produced by female is limited. Therefore, there is the sex specific difference in the fitness variation. Males have large variation in the fitness because male fitness is decided by the number of mate. On the other hand females depend on the number of egg produced thus female fitness is stabilize rather than male.

In various taxa, it has been known that female gains direct benefit, including fecundity and longevity (Alcock 1994) and/or indirect benefit, e.g. higher offspring viability and son's mating success (e.g. Head et al. 2005, Okada et al. 2013), from mating with higher quality male. However, many studies also reported that females suffer the cost such a decrease of longevity and fecundity by mating and often reject additional mating (Arnqvist and Rowe 2005). Therefore, males often compete among males over female and mating opportunity. Consequently, differences in size and the number of gamete between sexes promote sexual selection and cause evolution of female preference (female choice) and attractive or competitive trait (male-male competition).

1-2. Sperm Competition and Male Reproductive Strategies

The concept of sperm competition was expected by Parker (1970) for the first time; the competition among sperm from more than two males over fertilization of ova into female. When female mated more than two males, sperm competition occurs. In many taxa, including Insecta, female mated more than two males may have offspring which are sired by several males (e.g. Wedell et al. 2002, Harano et al. 2006). In this situation, males attempt to avoid the sperm competition and/or compete with rivals. Several traits to avoid sperm competition such as mating plug (Eberhard 1996, Baer et al. 2000), female guard after mating (Alcock 1994, Rowe 1992), nuptial gift (Vahed 1998) and accessory gland and seminal fluid to decrease the female receptivity for the mating with other males (Yamane et al. 2008, Yamane and Miyatake 2010) are known. In particular, quantity and/or quality of seminal fluid including accessory gland play a key role to determine a paternity because seminal fluid includes sperm. It has been expected that male ejaculate volume depends on risk and intensity of sperm competition. Sperm competition risk is possibility that mated-female mates with other male. On other hand, intensity of sperm competition is how many male (sperm) compete into female reproductive tract (Simmons 2001). In fact, numerous empirical and theoretical studies has shown that male varies the volume of seminal fluid and the number of sperm on one mating depending on the risk and/or intensity of sperm competition (e.g. Parker 1998, Ball and Parker 2006, Wedell and Cook 1999, Yamane and Miyatake 2008). In raffle principle, male ejaculated larger is able to get higher paternity than other male with smaller ejaculation (Parker 1998). However, male has to adjust the volume of ejaculation depending on the situation; e.g., female mating status, own age and resource condition, because present reproductive investments influence future mating event.

Sperm competition is often confused with cryptic female choice. Cryptic female choice is that females choose the sperm derived from more attractive male into female reproductive organ (Eberhard 1996). Sperm competition and cryptic female choice are not mutually exclusive and then their effects are hard to be separated.

1-3. Sexual Conflict

Sexual conflict is defined as a conflict between evolutionary interests of individuals of the two sexes (Parker 1979). In males, the number of offspring increases with the number of mate but not in females (Bateman 1948). Because mating is often cost for female (e.g. Yanagi and Miyatake 2003), female often rejects additional mating (Arnqvist and Rowe 2005). This difference of optimal mating frequencies between sexes causes the conflict; one sex accomplishes the optimal trait value but not for other sex. To acquire the optimal trait value, antagonistic sexual selection occurs.

Sexual conflict is divided into two: inter- and intra-locus sexual conflicts. Inter-locus sexual conflict is the conflict over optimal trait value in alleles at different loci (traits). In contrast, intra-locus sexual conflict is in one locus expressed in both sexes, i.e. same trait in both sexes (Parker and Partridge 1998). Regardless of inter- or intra-locus sexual conflicts, male and female compete with each other to gain an optimal trait value and increase fitness. This conflict would promote not only the reproductive strategies of both sexes but also the life-history strategies.

1-4. Scope and Aim

Mating success in male directly increases the fitness. To mate with female, male would adopt optimal mating strategies, depending on the mating situation and the amount of resource acquired, i.e. condition. Therefore, male has to invest for reproductive traits to ensure and increase his paternity with considering the investment for other traits. However, mating situation and condition of both sexes are environmentally and genetically not equilibrium.

Environmental factors are important for individual fitness, for example, temperature affects individual metabolic and behavior activity (Matthews and Matthews 1978, May 1979, Chapman 1998). Similarly, resource quality and quantity affect fitness and decide the amount of resource for traits, including body size and reproduction, and influence individual survival, developmental period, longevity and so on (reviewed in Reznick et al. 2000). Therefore, these environmental factors influence individual condition. When environmental factors influence the amount of resource acquired or affect male reproductive trait values selected for, not only reproductive traits but also other life-history traits would be also influenced because of trade-off between traits (Roff 2002).

Although studies about influence of environmental factors were reported, most studies reported the effect of environmental factors though life-history (e.g., Matthews and Matthews 1978, May 1979, Chapman 1998). However, individuals would be influenced by environmental factors even if a part of life-history, i.e., only developmental period and reproductive period. However, it is paid less attention to separate the effects between developmental and reproductive periods. Moreover, females are also influenced directly by the environmental factor and indirectly through male. Therefore, female activity and receptivity for matings would be the selective pressure for male reproductive traits. To consider the environmental factors, the effects of interaction between sexes and trade-off between traits is needed to understand the evolution of life-history and male reproductive strategies.

The stored product insects have been used in the studies of sexual selection and sexual conflict (e.g. Fricke et al. 2009, Michalczyk et al. 2011) because these insects have high plasticity for environmental factors and availability of resource. Moreover, they are easy to rear in laboratory and, therefore, useful for artificial selection and operational experiment. In my studies, three stored product insect species, which are bean beetle *Callasobruchus chinensis*, *Callasobruchus maculatus* and broad-horned flour beetle *Gnatocerus cornutus* were used, depending on the experiment design.

In this study, I investigated the impacts of environmental factors, condition and interaction between sexes for the male reproductive investments by using the stored product insects. First, I investigated the effect of ambient temperature on male reproductive investment and female remating receptivity (Chapter 2). Next, I focused on the effect of developmental period, especially resource acquisition pattern (Chapter 3). Moreover, I researched the resource allocation between reproductive and other traits in males, and male alternative mating strategies (Chapter 4). Chapters 1-4 were focused on only male. Chapters 5 and 6 were focused on the interaction and difference of male and female. I examined the relationship between intra-locus sexual conflict and offspring sex ratio in chapter 5 and the difference for the impact of diet quality on life-history and reproductive traits in male and female in chapter 6.

2. Effects of temperature on mating duration, sperm transfer and remating frequency in *Callosobruchus chinensis*

2-1. Introduction

Insects are generally ectothermic, meaning that their body temperature is a function decided by ambient temperature. Therefore, most biochemical and physiological processes underlying behaviour patterns are temperature dependent (Matthews and Matthews, 1978, May 1979, Chapman 1998). The effect of ambient temperature on a variety of insect behaviours has been studied, for example activity (Mellanby 1939), foraging (Guarneri et al. 2003), flight (Taylor 1963), territorial behaviour (Wickman 1985), death-feigning (Miyatake et al. 2008), and courtship calling power (Sueur and Sanborn 2003).

Mating duration is also affected by ambient temperature. In the Japanese beetle *Popillia japonica*, the duration of mate guarding by males is shorter at higher temperature (Saeki et al. 2005). Similarly in three species of Anthocoridae, mating duration is longer at lower (15 ^{o}C) than higher (25 ^{o}C) temperature (Horton et al. 2002). These studies suggest a negative correlation between temperature and mating duration, possibly mediated by the effect of temperature on activity.

Males transfer substances to females during mating, including sperm and seminal fluid, which often have the function of reducing the frequency of female remating (e.g., Eberhard 1996, Wolfner 1997, Chapman et al. 1998, Miyatake et al. 1999). If mating duration is prolonged, the amount of material ejaculated into the female spermatheca may increase. Mating duration is dependent on temperature, and therefore temperature should influence on sperm transfer and female remating frequency. However to my knowledge, the effect of temperature on sperm transfer and female remating frequency has not been studied.

Female remating frequency in the adzuki bean beetle *Callosobruchus chinensis* changes in relation to environmental and genetic factors (Harano and Miyatake 2005, Harano et al. 2006), and is reduced by the male's transfer of ejaculated materials into the female (Yamane et al. 2008). Miyatake and Matsumura (2004) showed that mating duration affected female remating frequency: mating with a shorter duration led to a higher frequency of females remating. Here, I investigate mating frequency, mating duration, duration of sperm transfer and the estimated number of sperm ejaculated into the spermatheca of females mated at three different temperatures, 17 ^{o}C, 25 ^{o}C and 33 ^{o}C, in *C. chinensis*. The relationship between temperature at first mating and female remating frequency at 25 °C is also examined.

2-2. Materials and Methods

2-2-1. Insects and breeding

In the adzuki bean beetle *C. chinensis*, many strains exhibiting variation in female remating frequency have been established (Miyatake and Matsumura 2004, Harano and Miyatake 2005). In the present study, I utilized the isC strain which exhibits one of the highest level of female remating frequency, ca. 40% (Harano and Miyatake 2005). This strain was collected in 1997 at Ishigaki Island, and has since then been maintained in the laboratory (Yanagi and Miyatake 2003). Before my experiments, the population had been maintained on adzuki beans at 25 °C, 60% RH, and 16L:8D.

All beetles used in the present study were derived from parents collected from this stock culture. I reared them on adzuki bean *Vigna angularis* ‘Dainagon’. *C. chinensis* lay their eggs on the surface of adzuki beans. To minimize variation in body size, females were allowed to lay one egg per bean; excess eggs were scraped off manually. I provided adults with food (sugar and yeast = 1:2) and water only during remating experiments in order to maintain a high level of activity.

2-2-2. Mating frequency, mating duration and the number of estimated sperm

Mating was observed for 1 h at each temperature – 17 ^{o}C, 25 ^{o}C and 33 ^{o}C – into a controlled temperature chamber (240 cm width, 240 cm depth, 240 cm height: CC-T2000, Sanyo Electric Co, Osaka). All beetles tested were virgins, between 24 h and 48 h old (and their sample sizes at each temperature were as follows; n = 110 at 17 ^{o}C, n = 116 at 25 ^{o}C, and n = 125 at 33 ^{o}C). Mating observations began 15 min after placed in the chamber. The experiments were conducted between 13:00 and 19:00. I placed a male and a female into a glass vial (1.4 cm diameter, 4 cm height) and observed whether each pair mated. When a female mated, I also recorded the duration of mating.

I estimated the number of sperm ejaculated into the spermatheca of females at each temperature. After copulation, the female was dissected, and her spermatheca removed under a binocular microscope using fine forceps. The spermatheca was placed into a drop of deionized water (20 ml) on a glass slide, and then crushed and cut into small pieces using fine forceps. Another drop of deionized water was added and stirred with forceps for approximately 1 min to distribute the sperm evenly throughout the water. The sperm suspension (about 10 mm diameter) was airdried on the glass slide. The average density of sperm within a fixed area (0.7 × 0.7 = 0.5 mm^2) was estimated (15 samples for each female) under an optical microscope (×200 magnification). By multiplying the area of water on the slide by the average density of sperm, the total number of sperm ejaculated by a male into the spermatheca was estimated. Sample sizes at each temperature were as follows; n = 18 at 17 ^{o}C, n = 18 at 25 ^{o}C, and n = 14 at 33 ^{o}C.

2-2-3. The median time and estimated duration of sperm transfer

I investigated the time at which sperm was transferred at the three temperatures by separating the mating insects at various times after mating began. Mating pairs were separated with tweezers by tugging the male genitalia at 40 s (n = 14), 50 s (n = 7) and 60 s (n = 9) at 17 ^{o}C, 10 s (n = 3), 20 s (n = 5) and 30 s (n = 8) at 25 ^{o}C, and 5 s (n = 4), 10 s (n = 8) and 20 s (n = 8) at 33 ^{o}C from the beginning of mating. The spermatheca of each female was dissected out and the presence of sperm into spermatheca was investigated. The number of females that had received sperm was recorded in each case.

2-2-4. Remating frequency

I also measured female remating frequency at each temperature. Females from each treatment were allowed to mate in a glass vial (1.4 cm diameter, 4 cm height). The male was then removed, and the female was provided with water and feed at 25 ^{o}C. In order to investigate her willingness to remate, three days after her first mating, each female was paired with a different, virgin male, from the same strain, and observed for remating for 1 h at 25 ^{o}C. Sample sizes were as follows; n = 52 at 17 ^{o}C, n = 57 at 25 ^{o}C and n = 64 at 33 ^{o}C.

2-2-5. Statistics

Logistic regression analysis was used to examine the relationship between temperature and the frequencies of mating and remating, with temperature as the independent variable and frequency of mating (mating = 1, non-mating = 0) and remating (remating = 1, non-remating = 0) as dependent variables. The sequential Bonferroni method (Rice, 1989) was applied following logistic regression analyses. Tukey–Kramer’s Honest Significant Differences test was used for the relationship between temperature and mating duration and the number of estimated sperm.

JMP6 (SAS Institute 2005) was used for all the statistical analyses.

The time from the beginning of copulation when half of the females had received sperm into their spermatheca was estimated by logistic regression, and the 95% upper and lower confidence limits of the times were also estimated. Logistic regression analysis was used for the relationship between mating duration and the rate of females which had sperm into their spermatheca, with mating duration as independent variables and the rate of females which had sperm into their spermatheca as dependent variables. Each treatment division was added by 0.5 because treatments with 0% or 100% existed. The time at which 50% of females had sperm transferred into their spermatheca was defined as the "median time of sperm transfer". "Estimated duration of sperm transfer" was defined as the difference between the median time of copulation duration and the median time of sperm transfer.

2-3. Results

2-3-1. Mating frequency, mating duration and the number of estimated sperm

The percentages of females mating ("mating frequency") were 79.3%, 94.6%, and 95.7%, at 17 ^{o}C (n = 110), 25 ^{o}C (n = 116), and 33 ^{o}C (n = 117), respectively

(Fig. 2-1A). Mating frequency at 17 °C was significantly lower than those under other temperatures (25 °C and 33 °C) (Fig. 2-1A; between 17 °C and 25 °C, $\chi^2 = 12.14$, $P < 0.001$; 33 °C, $\chi^2 = 15.50$, $P < 0.001$; logistic regression revised by sequential Bonferroni). No significant difference was found in mating frequency between 25 °C and 33 °C.

Mating duration was significantly different at each temperature; the longest was at 17 °C (mean ± SE = 79 3.1 s; n = 92), the mid was at 25 °C (43.8 ± 1.7 s; n = 104), and the shortest was at 33 °C (25.4 ± 0.9 s; n = 112) (Fig. 2-1B; $P < 0.001$ Tukey HSD method). The estimated number of sperm deposited into the spermatheca was 1516.3 ± 290.2 (mean ± SE), 937.0 ± 145.8, and 681.9 ± 147.0 at 17 °C, 25 °C, and 33 °C, respectively (Fig. 2-1C). The estimated number of sperm at 17 °C was significantly higher than the number at 33 °C (Tukey HSD method $P < 0.05$), but there was no significant difference between 17 °C and 25 °C, nor between 25 °C and 33 °C ($P > 0.05$).

2-3-2. The median time and estimated duration of sperm transfer

Fig. 2-2 shows the relationships at different temperatures between mating duration and the percentage (“frequency”) of females that received sperm in their spermathecae. The median times at which sperm was transferred were 51.1 s, 23.7 s, and 11.7 s, at 17 ^{o}C, 25 ^{o}C, and 33 ^{o}C, respectively. The estimated durations of sperm transfer were 23.8 s, 17.1 s, and 11.7 s, at 17 ^{o}C, 25 ^{o}C, and 33 ^{o}C, respectively. The time of sperm transfer at 17 ^{o}C was significantly later than at 25 ^{o}C and 33 ^{o}C. Thus, there was an inverse relationship between temperature and mating duration, the frequency of sperm transfer, the duration of transfer, and the time at which it occurred.

2-3-3. Remating frequency

Female remating frequency was significantly lower at 17 ^{o}C compared to 25 ^{o}C and 33 ^{o}C (Fig. 2-3; logistic regression revised by sequential Bonferroni; $P < 0.001$); 9.6% (n = 52) at 17 ^{o}C, 39.1% (n = 57) at 25 ^{o}C, and 38.6% (n = 64) at 33 ^{o}C.

2-4. Discussion

Mating duration in *C. chinensis* increased at lower temperatures. In addition, the durations of both pre-sperm transfer and of sperm transfer were longer at lower temperatures. A negative relationship between ambient temperature and mating duration has been reported in other insects (Horton et al. 2002). These authors suggested that it is physically difficult for males to transfer sperm when they mate at low ambient temperatures.

Longer mating duration of *C. chinensis* at lower ambient temperature caused an increased number of sperm to be transferred into the spermatheca of the mated female, and this reduced the frequency of female remating. This suggests that at lower temperatures, the transfer of larger quantities of sperm into the spermatheca reduces the female's willingness to remate. In this species, inhibition of female mating receptivity has previously been shown to be caused by the transfer of ejaculate substances derived from the testis and the accessory gland (Yamane et al. 2008). However my study suggests that the quantity of sperm transferred may also affect the female's willingness to remate in *C. chinensis*.

In *Callosobruchus maculatus*, a species closely related to *C. chinensis*, female remating frequency increases when small quantities of ejaculate substances are transferred to females (Savalli and Fox 1999). Two different types of stimuli may mediate such an effect. In the tsetse fly *Glossina morsitans* a chemical stimulus from the male accessory gland substances has been reported to reduce female receptivity (Gillott and Langley 1981). In the case of the Hessian fly, *Mayetiola* *destructor*, it is the physical act of copulation that reduces female receptivity (Bergh et al. 1992). Therefore, prolonged copulation might cause the reduced female remating frequency in *C. chinensis* via physical stimulation and/or via chemical stimuli, present in sperm and accessory gland substances. It would be intriguing to investigate which factors influence on female remating under different temperature conditions.

Another explanation for the inhibition of female remating at low temperature conditions relates to female activity levels. In the present study, I kept the insects at the same temperature at all times, except during their first mating. The number of sperm ejaculated was significantly lower and the mating duration was significantly shorter at 33 °C compared to 17 °C. The difference in sperm number and mating duration between different temperature conditions might be explained by the different intensity of female refusal behaviour that is directed against mated males. In *C. chinensis*, females kick their mate with her hind legs during mating (M. Katsuki personal observation). This also occurs in *C. maculatus* (Crudgington and Siva-Jothy 2000). In *C. maculatus*, female kicking during mating relieves injuries by male genital spines (Crudgington and Siva-Jothy 2000) and/or seminal fluid transfer (Eady et al. 2007), resulting in prolonged female lifespan (Crudgington and

Siva-Jothy 2000) and increased female lifetime fecundity (Edvardsson and Tregenza 2005). Harano et al. (2006) have shown that female remating is costly for females (i.e., it reduces fecundity) in *C. chinensis*. Moreover, Yanagi and Miyatake (2003) showed that mating itself is costly for female survival of *C. chinensis*, as has also been shown for *C. maculatus* (Crudgington and Siva-Jothy 2000). Therefore, because longer mating duration may result in more damage to females, it would be expected that lower frequency of female remating would be caused at lower temperature.

Ambient temperature during mating could influence the intensity of female's kicking behaviour, although this requires further investigation. I would predict that female kicking behaviour should increase in intensity at higher temperature, because insect activity is generally activated at higher temperatures (e.g., Mellanby 1939). In contrast, it is also possible that females kick more to resist at lower temperatures than at other temperatures because duration of mating is longer and females could receive more damage at lower temperatures. In either case, it would be interesting to measure kicking intensity at different temperatures, and to test the hypothesis that mating duration is influenced by females, rather than by males.

In future, I intend to investigate the relationship between temperature and female reproductive traits, including female activity levels, mating duration and survival rate in *Callosobruchus* beetles. I would predict that at lower temperatures, females cannot kick as frequently as at high temperatures, because female activity reduces at lower temperatures, and that females consequently suffer more damage during mating at lower temperatures.

3. Larval competition causes divergence of male ejaculate expenditure in *Callosobruchus maculatus*

3-1. Introduction

When a female copulates with two or more males, their sperms compete in the female reproductive tract over fertilization (Parker 1970). Under the raffle principle of sperm competition, males that transfer more sperm to the female spermatheca or reproductive duct are predicted to have increased paternity (Parker 1990, Parker and Pizzari 2010, Kelly and Jennions 2011). Furthermore, in some species the male ejaculate includes seminal substances that restrict female receptivity, thereby delaying female remating with other males (Simmons 2001, Arnqvist and Rowe 2005, McNamara et al. 2009). This delay of female remating allows males to avoid sperm competition altogether. Although this investment in reproductive traits enhances male paternity, the production of sperm or seminal fluid is often costly (Dewsbury 1982, Paukku and Kotiaho 2005, Brown et al. 2009, Lewis et al. 2011). There is a trade-off between the investment in energetically expensive traits and that in other traits related to fitness (e.g. Martin and Hosken 2004, Oliver and Cordero 2009, Yamane et al. 2010). Therefore, males may be selected to maximize their fitness by optimizing the balance between investment in mating and in other traits, responding to the risk and/or intensity of sperm competition the male faces (Simmons 2001). Indeed, there is abundant evidence of associations between the intensity of sperm competition and ejaculatory characters in relation to testis and accessory gland size in various taxa (Hunter and Birkhead 2002, Gay et al. 2009, Crudgington et al. 2009).

Under limited environmental conditions in the developmental stage, competition over a resource, i.e., larval competition, often occurs. The degree of this competition shows tremendous variability both between and within species (reviewed in Mideo 2009) and reflects the ability to compete for resources and individual aggressiveness (Toquenaga and Fujii 1990a, b, Toquenaga 1993, Mano 2008). As two extreme degrees of competition, contest competition and scramble competition were characterized by Nicholson's dichotomy (Nicholson 1954, Łomnicki 1988) as follows. In contest competition, the resource intake of an individual and the number of survivors remain constant, irrespective of the increase in the number of competitors, due to resource monopolization. In contrast, in scramble competition, individuals share the available resources, and thus the number of competitors increases. As a result, the degree of competition would cause a difference in the number of rival males, i.e., higher intensity of sperm competition. Theoretically, differences in sperm competition intensity should cause different selective pressures on ejaculate expenditure and testis size (Parker and Ball 2005). Furthermore, while the possibility that variation in the degree of competition is related to reproductive traits was suggested in a review by Isbell (1991), there is little experimental evidence for this. Therefore, I hypothesized that scramble males invest in more in reproduction than contest males, and that there is a negative association between degrees of larval competition and male reproductive investment.

The seed beetle *Callosobruchus maculatus* has a geographic variation in the degree of resource competition in the larval stage, and shows scramble and contest competition types as two extreme examples (Takano et al. 2001). The scramble individual shares resources; in contrast, the contest individual eliminates others and occupies the resource himself. The intensity of aggressiveness differs between geographic strains (Takano et al. 2001). Further, in this species, the amount of investment in sperm competition, especially the ejaculate expenditure in one mating is considerably associated with male fitness. A larger ejaculate from a male inhibits female remating (Eady 1995, Savalli and Fox 1999). In contrast, a female who receives a small ejaculate tends to remate. Female remating directly affects male paternity because the second male that mates with a female achieves a higher paternity than the first male (Eady 1994). Therefore, the ejaculate expenditure of a *C. maculatus* male plays an important role on his fitness in sperm competition.

Here, I examined whether variation in ejaculatory expenditure and variation in the degree of resource competition are negatively correlated. To test this hypothesis using geographic strains of the seed beetle *C. maculatus*, I assessed the degree of larval competition and male ejaculate expenditure of each geographical strain and investigate whether the degree of competition was associated with male reproductive investment.

3-2. Materials and Methods

3-2-1. Insects

C. maculatus females lay eggs on the surface of a bean. Hatched larvae enter and grow in the bean. Emerged adults mate outside of the bean. I used seven strains of *C. maculatus* originally derived from a range of different countries (see Takano et al. 2001): iQ (South India), tQ (Tel Aviv, Israel), yQ (Yemen), a^{o}Q (USA), a^{o}Q2 (USA), hQblk (a black phenotypic mutant of the hQ strain that was originally collected in

Japan from beans imported from New Zealand), and wQ (Japan). These strains were obtained from Tsukuba University, Japan, and subsequently maintained at Okayama University, Japan. Takano et al. (2001) showed genetic differences in the frequencies of larval competition types across these strains. The strains were maintained under constant conditions of 25°C and a 16L8D photoperiod. I used individuals with 2 to 5 days old that emerged from *Vigna angularis* beans that were infested by a single larva to exclude the effects of larval competition.

3-2-2. Estimating C-value of strains

To evaluate the degree of contest type competition across the strains, I used the C-value, which indicates the proportion of the contest type in a population (Takano et al. 2001). The C-value model is based on the following assumptions. Suppose that two eggs are oviposited by females of the same strain on a bean that is large enough for two larvae to complete their development to full size. When both larvae are of the scramble type, two adults emerge. If one of them is of the contest type, only one adult emerges because the contest larva kills the other. Even if both larvae are contest types, I assumed that only one of them emerges. I also assumed that natural mortality occurs before larvae start competition. Thus, a single bean can be assigned to one of the following six emergence patterns: none (N), only a male (M), only a female (F), two males (MM), two females (FF), or one male and one female (MF). I assumed that male and female have the same pre-competition mortality, μ. The proportion of males is m, and the C-values of males and females are p and q, respectively. Each female is assumed to lay eggs randomly on beans. I further assumed that male and female have the same probability of winning the contest when both are contest types.

Summing up the obtained probabilities gives me the expected frequencies of the six emergence patterns as follows:

$N = \mu^2$

$MM = (1-\mu)^2 m^2 (1-p)^2$

$FF = (1-\mu)^2 (1-m)^2 (1- q)^2$

$MF= 2(1-\mu)^2 m (1-m)(1-p)(1-q)$

$M = (1-\mu)^2 mp(2-q-mp+mq)+2\mu(1-\mu)m$

$F = (1-\mu)^2 (1-m)q(2-q-mp+mq)+2\mu(1-\mu)(1-m)$

Note that $N+ MM+ FF+ MF+M+ F=1$.

The natural mortality can be calculated as the proportion of adults that emerge from beans each of which had a single hatched egg (control treatment). The values from the control data were used as the external hypothesis, and the other three parameters (m, p, and q) were estimated by a maximum likelihood method. If the natural mortality before battles between the two individuals is different from that without any competition, then the μ value must be estimated as well. p and q values are not necessarily different, and the two values can be assumed to be equal.

The hypotheses just described are not hierarchical, and I used Akaike's information criterion (AIC) to compare the competing models. There were four types of models:

1. Estimating μ, m, p, and q
2. Estimating μ, m, and p, assuming $p = q$
3. Estimating m, p, and q, using a fixed μ estimated from the control data
4. Estimating m and p assuming $p = q$ with fixed μ estimated from the control data.

AIC calculation and parameter estimation were conducted with an R script using the optim function in R ver. 2. 13. 0 (R Development Core Team 2011). The C-value ranges from zero to one. A value closer to one indicates a high proportion of the contest type, while a value closer to zero indicates a high proportion of the scramble type. The R script for the C-value calculation is available from the authors.

3-2-3. Measurement of ejaculate expenditure

Prior to mating, the weight of each virgin male was measured to the nearest 1/100 mg using an electric balance (Excellence Plus XP, Mettler-Toledo, Columbus, OH). A male was then placed in a vial (diameter × height = 1.4 cm × 4 cm) with a virgin female of the same strain, and permitted to mate. After mating, the male's weight was measured again. I took the difference in male weight before and after mating as an indicator of male reproductive investment, i.e., ejaculate expenditure.

3-2-4. Statistics

In *C. maculatus,* there is a positive correlation between ejaculate size and male body size (Savalli and Fox 1998). Therefore, I analyzed ejaculate expenditure using a general linear model (GLM) with male body mass as a covariate. The adjusted ejaculate expenditure for each strain was calculated using the least square mean from the GLM. To examine whether the mean relative ejaculate expenditure is correlated with the C-value, I used Pearson's product moment correlation. Because it is a proportion, the C-value was arcsine transformed. JMP version 7 was used for all analyses (SAS Institute 2007).

3-3. Results

3-3-1. C-value

There was a variation in degree of competitiveness, i.e., polymorphic competition types, in *C. maculatus*. C-values ranged from zero to one among geographic strains (Fig. 3-1). The iQ strain showed complete contest competition (C-value = 1.0). In contrast, $a^{o}Q2$ and wQ strains showed complete scramble competition (C-value = 0). The other three strains, $a^{o}Q$, tQ, and yQ, had C-values less than 0.2; their C-values were 0.034, 0.081, and 0.104, respectively. The hQ-blk strain had a medium value (C-value = 0.323). The $a^{o}Q$ strain was best fitted to model 4, but the other strains were all best fitted to model 2.

3-3-2. Ejaculate expenditure

The means ± SE of male ejaculate expenditures on the first mating were $a^{o}Q$: 0.21 ± 0.017 mg (n = 26), tQ: 0.18 ± 0.015 mg (n = 19), $a^{o}Q2$: 0.18 ± 0.011 mg (n = 32), hQbl: 0.22 ± 0.018 mg (n = 30), iQ: 0.16 ± 0.021 mg (n = 29), yQ: 0.23 ± 0.013 mg (n = 30), and wQ: 0.23 ± 0.014 mg (n = 30). I found a significant difference in male ejaculate expenditure on the first mating across strains (GLM; strain: $F_{6,203} = 4.7269$, $P<0.01$, male weight: $F_{1,203} = 4.6932$, $P = 0.03$). In addition, the C-values were strongly and negatively correlated with adjusted ejaculate expenditure across the strains (Fig. 3-1, $r = -0.8154$, $P = 0.0254$). Thus, the higher the proportion of scramble resource competition within a strain, the larger ejaculate expenditure by males.

3-4. Discussion

Geographic variation in degree of larval competition was associated with ejaculatory expenditure in male *C. maculatus*: males derived from strains exhibiting scramble type competition invested more in reproduction than males from strains with more contest type competition. Males should be selected to maximize fitness by optimizing the balance between investment in reproduction and other fitness-related traits in response to the intensity and/or risk of sperm competition in which they surfaced (e.g., Parker 1990, 1998). Intensities of sperm competition are likely to differ between larval competition types: under contest competition, due to the elimination of rivals, the number of adults remains constant. However, with scramble competition, high larval density results in high numbers of adults. When adult density is high, the frequency of encounters between the sexes is also high (Gage 1995), and females mate more frequently compared to those at low densities (Gage 1995, Martin and Hosken 2003); thus, the frequency of female mating is liable to be higher under scramble competition. In *C. maculatus,* testis size decreases in males who are reared under an experimental evolution that prevents sperm competition (Gay et al. 2009), suggesting that selective pressures imposed by sperm competition act on investment in ejaculation. Therefore, male ejaculate expenditure may be affected by the intensification of sperm competition in relation to the degree of larval competition.

When a female receives a small ejaculate from a male, the female tends to remate (Eady 1995, Savalli and Fox 1999). In *C. maculatus*, the P2 value (i.e., the proportion of eggs that the second male to mate with a female fertilizes) is relatively high; for example, when the interval between mating is 24 h, P2 = 0.84 (Eady 1994). Therefore, when a male mates with a non-virgin female, the male can sire more offspring by transferring a larger ejaculate to the female (Eady 1995). A positive correlation between the intensity of sperm competition and testis size has been documented in various taxa (Hosken and Ward 2001, Firman and Simmons 2008, Dziminski et al. 2010). In polyandrous species with sperm competition, the survival rate of sperm is higher than in monogamous species (Hunter and Birkhead 2002). Furthermore, males exhibit decreased testis size (Gay et al. 2009) and accessory gland size (Crudgington et al. 2009) when the level of sperm competition is decreased by experimental manipulation. In this species, males transfer seminal substances derived from the accessory gland into females to decrease female receptivity (Yamane et al. 2008). Females also absorb nutrition (Fox and Moya-Larano 2009) and water (Edvardsson 2007, Ursprung et al. 2009) from the seminal fluid. A male could prevent female remating by making up for a nutritional deficit in the female. Intensification of sperm competition can select for increased ejaculate expenditure in this male, resulting in the negative correlation between ejaculate expenditure and larval competition degree.

An alternative explanation for the correlation between larval competition and ejaculatory expenditure is thought to be resource allocation to larval competition and reproduction. The degree of larval competition is likely to reflect the investment in the larval competition, such as attack and defense behavior against competitors (Toquenaga and Fujii 1990a, Mano 2008). Previous work on this species suggests that male reproductive efforts lead to costs in male life history traits (Paukku and Kotiaho 2005, Brown et al. 2009). Thus, investments in larval competition and reproduction are thought to be constrained by the respective allocation of resources to each. To date, less attention has been paid to trade-offs between different developmental stages, larva and adult. Regardless of the mechanisms, the patterns of the evolutionary change seen in male reproduction and the competition-related traits seem to make sense, even if between different stages.

In the present study, I found a correlation between male ejaculate expenditure and degree of larval competition. This relationship is thought to reflect a difference in the intensity of sperm competition during adulthood. In *C. maculatus*, male ejaculatory expenditure is exposed to stronger selective pressures from sperm competition during adulthood under low larval resource competition; in contrast, the evolution of larval competition may relax sexual selection through sperm competition. This beetle provides an excellent model to examine the relationships among larval resource competition, ejaculate expenditure, sperm competition, and sexual conflict.

4. Alternative strategies of dispersion and ejaculation in male seed beetle

4-1. Introduction

Individual fitness strongly depends on successful allocation of energy resources to competing demands, and these allocation decisions are thought to underlie trade-offs (Roff 2002). Under such trade-offs, once a resource has been allocated to one activity, it cannot be allocated to another. However, when the total resource pool of a certain individual is larger, it can allocate more energy to all activities (Getty 1998, Tomkins et al. 2004), and this occasionally complicates the simple concept of a trade-off. Nevertheless, it has shown that individuals from different populations can differ in their resource allocations to fitness-related traits and that inter-population variations sometimes indicate genetic trade-offs between them (Lynch and Walsh 1998, Reznick et al. 2000). Indeed, numerous studies have reported trade-offs between reproductive and life-history traits (reviewed in Roff 2002, Guerra 2011). Life-history evolution can thus be constrained by trade-offs between various traits that contribute to fitness (Miyatake 1997, Hunt et al. 2004, Tomkins et al. 2004).

Males adopt several behavioural strategies in order to mate with females, such as fighting, dispersing, and sneaking (Gross 1996). Since males must allocate available resources to characters that contribute to their mating success, including weapons, wings and testes, competition between the growth trajectories of these characters is predicted. Although less attention has been paid to potential trade-offs between different efforts in reproduction, it has been shown in several animals (Simmons and Emlen 2006, Pitcher et al. 2009, Yamane et al. 2010). Many studies report the trade-off between reproductive and life-history traits in males (reviewed in Kotiaho 2001, Simmons 2001), and it has been suggested that reproductive and life-history strategies are evolutionarily linked with each other in relation to resource allocation trade-offs (Hunt et al. 2006, Okada et al. 2011). The male mating strategy is thus expected to be associated with variations of resource allocation.

I tested this assumption by comparison of populations of the adzuki bean beetle *Callosobruchus chinensis* collected from different geographical regions. Because females copulate with two or more males (Yamane and Miyatake 2005, 2008), the resulting sperm competition should select for reproductive characteristics, such as testis size and the number of sperm transferred to the female (Parker 1990). In general, wing muscles and wings contribute to the ability to fly and are important to obtain not only food but also territory and mates (Gross 1996, Yamane et al. 2010, Okada et al. 2007). Because the male of this species also disperses among hosts to find mates (Shinoda and Yoshida 1984), the investment in searching for mate is thought to be related to fitness in the males of this species. In this study, I first investigated the relationship between population means of testes and hindwing sizes using the geographic strains. Previous studies of this species demonstrated that the number of sperm transferred to females and flight distances differed among the populations (Yamane and Miyatake 2005, 2008, Ohno and Miyatake 2007); therefore, I examined the association between these behavioural characters. Lastly, I discuss the male mating strategy in association with allocation trade-offs.

4-2. Materials and Methods

4-2-1. Insects and breeding

Females of the adzuki bean beetle, *C. chinensis*, lay eggs on the surface of the bean. After it hatches, the larva burrows into and develops in the bean. The adult emerges from the bean and mates. I used eight geographical strains: isC (collected in 1997 on Ishigaki Island, Japan), jCs (collected in 1936 in Kyoto, Japan), jCF (collected in

1936 in Kyoto, Japan, and maintained at Tsukuba University), akC (collected in 2002 in Sanyou Ward, Akaiwa City, Okayama Prefecture, Japan), yoC (collected in 2002 in Yoshii Ward, Akaiwa City, Okayama Prefecture, Japan), SmC (collected in 2002 in Izumo City, Shimane Prefecture, Japan), kiC (collected in 2007 in the Kako district, Hyogo Prefecture, Japan) and mC (collected in the 1960s in Morioka City, Iwate Prefecture, Japan) that have since been maintained in the laboratory. See Yanagi and Miyatake (2003) and Ohno and Miyatake (2007) for details of the strains. The stock cultures of each population had been maintained on adzuki beans *Vigna angularis* 'Dainagon' at 25 ± 2 ^{o}C, 60% RH, and 16L:8D. To minimize environmental fluctuations, females were allowed to lay one egg per bean, then the beans were separated and maintained in separate wells of 48-well plates. Experiments were conducted using virgin adults within 24 h after emergence.

4-2-2. Morphology in flying and reproductive traits

Ten males of each strain were randomly chosen for measurement. The male was anesthetized by ice, and then the right and left testes were removed from the body using a fine forceps and carefully separated from the surrounding tissue in deionized water. The total area was used as the testis size. Subsequently, I gripped the wing axillaries with the forceps and carefully removed the right wing from the body. I put each tissue on a glass slide and traced the area (± 0.01 mm^2) using a dissecting microscope monitoring system (VM-60, Olympus, Tokyo, Japan).

4-2-3. Flying ability and reproductive investment

Ten males of each strain were randomly chosen for measurement. I measured male flying ability and the number of sperm ejaculated into the female spermatheca on first mating. To measure flight ability, the male was dropped into a box (height x length x width = 50 cm x 50 cm x 30 cm) and the distance from center of box to the point at which the male landed was recorded. Because the measurement was limited to 25 cm, a nonparametric method as ‘a score of 25’ (Sokal and Rohlf 1995) was applied to flights over a distance of 25 cm. For a more detailed description of the methods, see Ohno and Miyatake (2007). After this experiment, the males were maintained one day in a 1.5 ml tube until measurement of the number of sperm transferred.

In accordance with Yamane and Miyatake (2005, 2008), I estimated the number of sperm ejaculated into the spermatheca of females. Copulation took place in glass vials (diameter x height = 1.7 cm x 4.0 cm) during the normal light phase. After mating, the female was immediately removed from the glass vial to prevent additional matings and kept 24 h in a chamber, and then were placed in a freezer. As described in chapter 2, the female was dissected and thus the total number of sperm ejaculated by a male into the spermatheca was estimated.

4-2-4. Comparison of male reproductive success

I used males from two strains, one strain was well-frying and the other was well-reproductive invested strain, which were investigated in experiment ‘flying ability and reproductive investment’. To compare with male reproductive success between each strain, I set two experimental treatments; high density and small scale (easy detecting female treatment) and low density and large scale (difficult detecting female treatment). As easy detecting treatment, I used small acryl box (10cm on a side) with two sticky sheets (a square 1cm on a side) on each side at equal interval. On the other hand, in difficult detecting treatment, I set female on the pole located at the center of box to increase the searching cost. The difficult detecting treatment was used large acryl box (30cm on a side) and six plastic dishes (about 6cm diameters) with two sticky sheets (a square 1cm on a side) on each side. Plastic dishes were set in the pale at equal interval and were covered with one-third area from upper dish then the pole was settled in a center of box. Female was placed in a sticky sheet to restrict their movement, respectively. Two males and six males were released per edge of box in small and large box, respectively. The cubic volume of large box ($27000cm^3$) is twenty-seven times compared with small box ($1000cm^3$) but male density was approximately 0.11 times; easy detecting treatment was eight male sixteen individuals in $1000cm^3$ and difficult detecting treatment was twenty-four in $27000cm^3$. The mating rate of virgin females is over 90% (Katsuki and Miyatake 2009), so that I defined that male attached the sticky sheet success mating. All were observed for one hours and the number of male attached on the sticky sheet was recorded.

4-2-5. Statistical analysis

All analysis was conducted with JMP 9 (SAS Institute 2010). Spearman's rank correlation coefficient was conducted in relationship between hindwing and testes area, and flying ability and the number of sperm into female spermathecae on population (strain) means. GLM with a logit link was used in comparison of male reproductive success.

4-3. Results

There were negative correlations between testes and hindwing size (ρ= -0.988, P<0.0001; Fig. 4-1a), and between the number of sperm transferred to the female spermatheca and male flying distance (ρ=-0.779, P=0.0229; Fig. 4-1b) among all geographic strains of *Callosobruchus chinensis*. In comparison of male reproductive success, there was significantly difference between strains (χ^2=24.851, P<0.0001; Fig. 4-2) and between treatments (χ^2=82.556, P<0.0001; Fig. 4-2), respectively. Moreover, there was a significant interaction between strain and treatment (χ^2=5.645, P=0.0175; Fig. 4-2). This means that male reproductive success differs in the environmental situation, i.e. the difficulty of encounter with mates.

4-4. Discussion

My results showed a negative correlation between testes and hindwing size among all geographic strains of *Callosobruchus chinensis* (Fig. 4-1a). This suggests evolutionary trade-offs between investments in testes and wings in this male. Similar allocation trade-offs among reproductive efforts have been reported in other insects (e.g., Saglam et al. 2008, Yamane et al. 2010, Brown et al. 2010). Similar to morphological associations, there was a negative correlation between the number of sperm transferred to the female spermatheca and male flying distance (Fig. 4-1b). That is, a male which has larger testes can transfer more sperm into the female spermatheca but has less flying ability, while a male who flies well cannot allocate as much resource to spermatogenesis because of the allocation trade-offs. This indicates that the ejaculatory and dispersal strategies are evolutionarily linked with each other.

An optimal investment rate in characters may be influenced by ecological factors such as population density. A high-density population will experience more intraspecific competition such as male fighting and sperm competition (Kokko and Rankin 2006). Females are likely to encounter and mate with many males when the population density is high, leading to high risk and intensity of sperm competition (Kokko and Rankin 2006, Pomfret and Knell 2008). Males probably compete by scramble competitions for mates, and high fitness should be related to the ability to succeed in sperm competition (Parker and Ball 2005). Indeed, there is experimental evidence for evolution of an ejaculatory character that is associated with the risk and intensity of sperm competition (e.g., Hosken and Ward 2001, Gay et al. 2009). Additionally, a previous study on *C. chinensis* showed that males from populations with higher risk of sperm competition invest in more ejaculation, whereas males in low risk populations invest in less ejaculation (Yamane and Miyatake 2012). Thus, an evolutionary change in the ejaculatory character may also affect the dispersal strategy in *C. chinensis*. Under such trade-offs, males are forced to make alternative choices from several strategies according to environmental conditions.

Alternatively, Wickman and Rutowski (1999) suggested that high insect density favors males searching for mates because males obtain the most benefit from multiple mating (Hammerstein and Parker 1987, Wickman and Rutowski 1999). In fact, in some species, it is reported that males invest more in dispersion characters to search for mates under high population density than under low population density (Alexander 1975, Alcock 1994). In either evolutionary scenario, the changes in ejaculation and dispersion are likely to be associated with each other. Nevertheless, further studies are required to determine how environmental conditions including population density affect male fitness in *C. chinensis*.

In conclusion, this study indicated an evolutionary trade-off between testes and hindwing size. Males will have alternative mating strategies based on the allocation trade-offs, and the trade-off is between investment in searching for mate or spermatogenesis in the case of *C. chinensis*. Therefore, in order to understand how males adopt their mating tactics, it is necessary to explore how characters that contribute to male fitness are genetically associated.

5. Intralocus sexual conflict and offspring sex ratio

5-1. Introduction

Although there is substantial evidence for the adaptive adjustment of offspring sex ratio by mothers (West and Sheldon 2002), exactly how females should bias sex ratio to maximize fitness is not entirely clear (Trivers and Willard 1973, West and Sheldon 2002, Blackburn et al. 2010, Cox and Calsbeek 2010). Classically it was suggested that high quality females should produce more sons. This is because mothers in good condition have the ability to provision more and provide sons with a competitive advantage, and thus by overproducing sons these high quality females garner higher fitness (Trivers and Willard 1973). Conversely, low condition mothers should invest in producing more daughters because although the poorly provisioned sons produced by them would have little chance of reproducing, their daughters will find a mate regardless of daughter quality (Trivers and Willard 1973). Sex ratio biasing in this way, known as the Trivers–Willard effect, was originally formulated with mammals in mind. However, the argument holds more generally, although precise predictions can vary (West and Sheldon 2002) and depend on a range of factors including population density, social rank and mate competition (Blackburn et al. 2010). Nevertheless, there are good examples of adaptive sex-ratio biasing (Komdeur et al. 1997, Badyaev et al. 2002, West and Sheldon 2002), especially in haplodiploids where the sex determining mechanism makes adjustment of offspring sex simple: fertilized eggs are female, and unfertilized eggs are male (West and Sheldon 2002).

Trivers–Willard predictions are to some extent predicated on gene effects being even in the sexes, such that alleles with positive fitness effects in females also have positive effects in males. However, the genes that make good females do not always make good males, a situation called intralocus sexual conflict (Rice and Chippindale 2001, Arnqvist and Rowe 2005, Hosken et al. 2009). This complicates classical sex-ratio predictions because intralocus conflict can cause high-fitness parents to produce low-fitness offspring of the opposite sex (e.g. Fedorka and Mousseau 2004, Pischedda and Chippindale 2006, Foerster et al. 2007). Intralocus sexual conflict is widespread (Rice and Chippindale 2001, Fedorka and Mousseau 2004, Pischedda and Chippindale 2006, Foerster et al. 2007, Blackburn et al. 2010) and when present, predictions are that offspring sex ratios should be biased in the opposite direction of classical Trivers–Willard predictions, such that low quality mothers should produce an excess of (high quality) sons (Alonzo and Sinervo 2007, Blackburn et al. 2010, Roulin et al. 2010). However, there is little experimental support for the conflict driven sex-ratio adjustment (Cox and Calsbeek 2010). Furthermore, it is not clear if females should bias offspring sex ratio based on their own quality or that of their mates, although theory suggests either assessment would be effective (Blackburn et al. 2010).

I tested whether or not offspring sex ratios were biased as predicted by conflict theory in the broad-horned flour beetle *Gnatocerus cornutus*. I have previously shown that high quality males sire low quality daughters and that selecting for increased (or decreased) male quality results in decreased (or increased) female quality (Okada and Miyatake 2009, Harano et al. 2010). Thus there is the negative intersexual correlation for fitness that is the unequivocal indicator of intralocus sexual conflict (Rice and Chippindale 2001, Arnqvist and Rowe 2005) in this beetle. Here I conducted sex-ratio assays using animals from an unselected stock population and from populations subjected to artificial selection for high and low male fitness (plus control populations). Selecting on male fitness was effective (male fitness increases or decreases in the direction of selection), but selecting for more (less) fit males also results in the production of low (high) fitness females (Okada and Miyatake 2009, Harano et al. 2010). Because of this negative inter-sexual fitness correlation, if there were the adaptive offspring sex-ratio biasing expected under intralocus conflict, an excess of sons should be produced in populations selected for high male fitness, because females in these populations are low in fitness, and an excess of females should be produced in low male-fitness populations for the opposite reasons. I also tested to see if potential biasing was due to a female's quality or because of the quality of her mate.

5-2. Materials and Methods

5-2-1. Stock culture

The *G. cornutus* beetle culture originated from adults collected in Miyazaki City (31° 54', 131 ° 25'), Japan, and has been maintained in the laboratory of the National Food Research Institute, Japan, for about 50 years on whole meal enriched with yeast as food. These beetles are stored product pest and so the laboratory conditions very closely mimic natural conditions. I performed all rearing and experiments in a chamber maintained at 25 ^{o}C, 60% relative humidity, and with a photoperiod cycle of 16 : 8 h light/dark conditions.

For the stock culture assessment, I collected a total of ca. 50 one-week-old virgin males (sires) and females (dams) and paired them in plastic cups with an excess of the food (20 g). Pairs were housed this way for 2 months. I subsequently randomly collected one daughter per sire and these were paired with a virgin male (mate) from the stock culture. Again all beetles were less than a week post-emergence, and were housed as above. To measure the lifetime reproductive success of each daughter, I counted final instar larvae that emerge in the third month after pairing. To examine the sex ratio of these offspring, a sample of 24 larvae were housed in a 24-well tissue culture plate for pupation, and on emergence these were sexed. Females were weighed to the nearest 0.01 mg on an electronic balance (Mettler-Toledo AG, Laboratory & Weighing Technologies, CH-8606, Greifensee, Switzerland). Following Okada and Miyatake (2009), male mandible length was measured using a dissecting microscope monitoring system (VM- 60; Olympus, Tokyo, Japan). All analyses were conducted on offspring sex ratio (=male／male and female) of daughters using general linear models (GLM) with daughter LRS (lifetime reproductive success), the mandible size of their mates and daughters body size as fixed effects, or with sire mandible size, mandible size of daughters mates and daughter body size as fixed effects, using JMP for Windows version 6 (SAS Institute 2007). Sex ratio data were arcsine square-root transformed (Sokal and Rohlf 1995), and all model assumptions were met. I used backward elimination to remove non-significant interaction terms from the full model (Grafen and Hails 2002).

5-2-2. Selection experiment

The artificial selection protocol was described in detail by Okada and Miyatake (2009). Briefly, I established populations selected for high and low male fitness (two replicates／treatments of long and short mandibles respectively), while also maintaining unselected control populations (two replicates). At each generation, 12 males with the shortest and longest mandibles were selected from a random collection of 100 males in each population of long- and short-mandible selection regimes, respectively, and twelve males were randomly selected in each control population. Mandible length responded to selection in both directions and clearly diverged between selection regimes after 10 generations of selection (Okada and Miyatake 2009). The following experiments were performed on generation 16 individuals. I first established that mandible size differed as previously reported, and found that it did [using a General Linear Model with Treatment (selection regime: L = selection for larger mandibles, C = control (no artificial selection on mandibles), S = selection for smaller mandibles) as a fixed effect, there was a significant effect of selection on mandible size (using population as the replicate): Mean mandible length (mm ± SE) L = 0.44 ± .0007; C = 0.36 ± .004; S = 0.25 ± .002. $F_{2,3} = 1264.82$; $P < 0.0001$; Studentt-tests with the sequential Bonferroni method showed L > C > S (all $P < 0.0002$)]. This is consistent with previous assessment 4 and 6 generations earlier (Okada and Miyatake 2009, Harano et al. 2010).

I assessed number and sex ratio of offspring in 30 females collected from each population following the procedure outlined above. All females were paired with males from the corresponding control line (assigned a priori following the propagation regime) to control for male effects. I also assessed whether number and sex ratio of progeny was affected by the males of the experimental populations, by randomly pairing 30 males from each line to the corresponding control line to control for female effects. Again this was done as mentioned previously. I note that mortalities from egg to pupa of all populations were 0–5% and selection regime had no significant effect on the survival from egg to pupa at generation 11 (Okada et al. 2011). However, male survival during mandible development does differ across treatments (Okada et al. 2011), but does so in a way that strengthens my conclusions (see below). Females were weighed as above and all analyses were conducted on population means using general linear models (GLM) using JMP for Windows version 6 (SAS Institute 2007), with selection regime and female body mass as fixed effects.

5-3. Results

I first assessed the offspring sex ratios using stock population beetles (i.e. beetles that had not been subject to artificial selection). I paired virgin males (sires) and females (dams) from the stock culture and then randomly collected one daughter per sire from these pairs. Daughters were subsequently paired with a new virgin male (mate) collected from the stock culture and offspring sex-ratios of these daughter-mate pairs were assessed. This design allowed me to assess the impact of daughter quality and the quality of her mate on offspring sex ratio. Mandible size is the primary determinant of male fitness in these beetles: males with larger mandibles have greater mating success and also win more fights with other males (Okada and Miyatake 2009, Harano et al. 2010). I found that daughters sired by high quality, large mandible sires produced fewer offspring, and thus there is evidence that high-fitness males sire low-fitness daughters (regression of daughter lifetime reproductive success (LRS) on sire mandible size: Overall model $F_{1,48}$ =

9.6; *P* = 0.03; father's mandible size ß = -116 ± 37; t = -3.1; *P* = 0.03), consistent with previous reports of negative intersexual fitness associations (Harano et al. 2010). These lower fitness daughters also produced more sons (Fig. 5-1). There was the negative association between daughter quality (LRS) and the proportion of sons they produced (Table 5-1) expected with intralocus sexual conflict. In addition, if I used fathers' mandible size as a surrogate of daughter quality (remembering the two are inversely related), there was a positive association between sire mandible size and the proportion of sons, which again shows low quality females produce more sons (Fig. 5-1; Table 5-1). Therefore, the sex ratio of low quality daughters is biased in the predicted direction as they produce more sons, but this was apparently not a plastic response because sex ratio did not vary with the quality of a daughter's mate (Table 5-1). Furthermore, dams were randomly assigned to sires here, but the sire effect was still significant.

If sex ratio biasing is based on the quality of a female (and her father's quality) as indicated by the previous results, then offspring sex ratio should vary in populations subjected to artificial selection on male quality. I tested this in populations subject to bidirectional artificial selection for high (large mandibles, L) and low (small mandibles, S) male fitness, plus control populations (C) where I did not select on male quality. I previously found that populations selected for high male fitness produce low-fitness females, and vice versa (Harano et al. 2010) and I also find this here (Fig. 5-2). Based on sexual conflict predictions and the findings presented above, low-fitness females from the high-fitness male populations are expected to produce more sons, whereas high-fitness females from the populations selected for low male fitness, should produce more daughters – in the control populations offspring sex ratio should be intermediate because females and males are of intermediate quality. This is precisely what I found. When low and high quality females were mated to intermediate males (from the control lines, C), low quality females (fathered by high-fitness males from the L-populations) produced an excess of sons. In contrast, high quality females (fathered by low-fitness males from the S-populations) produced more daughters (Fig. 5-2). Intermediate quality females (from the control populations where there had been no selection on mandible size) produced intermediate sex ratios (Fig. 5-2). Again this effect was independent of the quality of a females mate and when intermediate quality (control) females were mated to males from the high or low quality populations there was no significant biasing of offspring sex ratio (Fig. 5-3), which confirms this view. Therefore like the unselected stock population, sex ratios were biased based on female quality, which was determined by the quality of a female's fathers, and not on the quality of their mates. Furthermore, previous work conducted five generations earlier has found that in L and C populations, males tend to have lower survival probability during mandible development (pupae to adult), although in S populations they survive as well as females (Okada et al. 2011). Thus if survival differences remained unchanged, the male-biased sex-ratios I detect here at the adult stage will be even more skewed at fertilisation (c. 61% vs. 57%). In sum these data provide compelling evidence for the sex ratio biasing expected under intralocus sexual conflict.

5-4. Discussion

A number of studies show that parents can bias offspring sex ratios (Trivers and Willard 1973, West and Sheldon 2002, Sheldon and West 2004), but sex-ratio biasing has largely been investigated in the context of the Trivers–Willard effect (Trivers and Willard 1973, Komduer and Pen 2002, Sheldon and West 2004). This idea broadly suggests that female condition should influence offspring sex-ratio, as condition determines how much a mother can invest in her young (Trivers and Willard 1973) and predicts that high condition mothers should invest in more sons, while low condition mothers should invest in producing more daughters (Trivers and Willard 1973). Although there is evidence supporting Trivers–Willard (West and Sheldon 2002, Cameron 2004), in many instances theoretical expectations are not met (Komduer and Pen 2002). Intralocus sexual conflict is one potential explanation for this mismatch and in contrast to predictions based on Trivers–Willard, intralocus conflict predicts that high quality females should not produce more sons because these sons will be of low quality due to sexually antagonistic allelic effects. Instead, high quality females should produce more (high quality) daughters and low quality females should produce excess sons, which is precisely what I find here. It should be noted that if high-fecundity females were reducing investment per egg, then perhaps my results could be interpreted as being consistent with Trivers–Willard. However, previous works found egg quality does not seem to vary with female quality (Harano et al. 2010, Okada et al. 2011).

My results therefore provide good support for offspring sex ratio biasing based on intralocus conflict predictions. I have shown that there are strong sexually antagonistic fitness effects associated with a secondary sexual character (the mandibles) in *G. cornutus* (Harano et al. 2010; current study). Thus unlike most studies (Blackburn et al. 2010), I have documented the sex-specific fitness effects that should underpin conflict driven sex-ratio adjustment. By adjusting offspring sex-ratio, the fitness of both sexes can be increased and this will consequently offset at least some of the intralocus conflict costs I find (Harano et al. 2010, current study).

My results are also intriguing because in contrast with some conflict theory suggesting females should alter offspring sex-ratio according to the quality of their mates (Alonzo and Sinervo 2007, Blackburn et al. 2010, Cox and Calsbeek 2010), the adjustments I documented were independent of mate quality. The reason why sex ratio did not vary with mate quality is not presently clear. However, male sexual phenotype is often strongly influenced by the environment (Cotton et al. 2004), and thus there can be a male phenotype-genotype mismatch (Ingleby et al. 2010). Although there is a significant genetic component to male quality in the beetles, male phenotype is also substantially influenced by environmental factors (Okada and Miyatake 2010). Therefore, it could therefore reward females to only pay attention to their own quality. Thus some ambiguity about the genetic quality of a mate, coupled with the ability to accurately assess self-quality, could explain the lack of a mate effect on offspring sex-ratio. This finding is consistent with recent theoretical investigations, which found sex-ratio adjustment can evolve when females are responding to their own quality, although when errors in judging mate quality occur, the evolution of sex-ratio adjustment is constrained (Blackburn et al. 2010). Furthermore, previous work in a Trivers–Willard context has shown that environmental parameters can have a strong impact on sex ratio adjustments (West and Sheldon 2002). It has also been suggested that quantitative genetic variation in the sexually antagonistic character should select for continued sex ratio adjustment (Blackburn et al. 2010), and the biasing I found in the stock population animals is consistent with this (i.e. the effects documented are not just found in the artificial selection populations which I may have driven from fitness peaks).

The reason why sire quality has such a pronounced impact on daughters when dam quality was random with respect to sire quality in the stock population pairings is more equivocal. One possible explanation is some imprinting of the dam genetic contribution to daughters. This remains to be established, as does the precise mechanism by sex ratio is adjusted. Understanding biasing mechanisms is a general issue for most systems with genetic sex determination (Badyaev et al. 2002), but models have shown that sex-ratio biasing is most likely to persist in male heterogametic systems (Blackburn et al. 2010). In birds it has been suggested that maternal steroid levels could influence sex chromosome segregation and hence sex ratio (Badyaev et al. 2002), but again, I have no indication of hormonal or other potential sources of influence impacting sex ratio in *G. cornutus*, although juvenile hormone (JH) is one potential candidate affecter. JH influences the expression of sexually selected traits in many insects, including the beetles (Gotoh et al. 2011), and JH also influences insect sex determination, although this was in haplo-diploids (Hales and Mittler 1987, 1988). In any case, my results provide compelling experimental evidence that sex ratio can be altered adaptively to recoup costs associated with intralocus sexual conflict. I have shown previously (Harano et al. 2010) and here that this sexual antagonism means daughters of high quality males have a relative fitness of about 0.8 compared to daughters of low quality males. By adjusting sex ratio some of this cost can be indirectly offset. Furthermore, although sex ratio deviations can range from the relatively small to almost absolute (Komdeur et al. 1997), the deviations I report are fairly large, suggesting that selection for adjustment is strong, which is consistent with theory (Blackburn et al. 2010), and with the magnitude of the sexual conflict costs.

Overall my findings provide a potential explanation for some of the mismatch between classical predictions and data, because intralocus sexual conflict can select for sex-ratio deviations in the opposite direction to Trivers–Willard predictions, as I find here. Understanding the contrasting selection on sex ratio is a major barrier to a complete appreciation of sex ratio evolution (West and Sheldon 2002), and the realisation, both theoretical and empirical, that intralocus conflict can impact sex ratio has broadened the potential adaptive scenarios under which sex ratios can evolve. My study is also consistent with a growing body of evidence suggesting intralocus conflict is widespread in laboratory and wild animals (Rice and Chippindale 2001, Fedorka and Mousseau 2004, Pischedda and Chippindale 2006, Foerster et al. 2007). Owing to the difficulties in resolving this form of conflict (Day and Bonduriansky 2004, Harano et al. 2010, Hosken 2011) it is likely to be more evolutionarily important than previously appreciated, and as I show here, can even influence sex ratio adjustment.

6. Impacts of diet quality on life-history and reproductive traits in male and female armed beetle, *Gnatocerus cornutus*

6-1. Introduction

The energy available at any time in an organism's life history is usually limited, and must be allocated among competing demands, such as maintenance of the body, energy storage, growth, and reproduction (Tinkle and Hadley 1975, Roff 2002). Therefore, reproductive investments, the major determinant of fitness, are often limited by levels of resource acquisition (i.e. condition-dependence; Rowe and Houle 1996, Hunt et al. 2004a), and subsequent resource allocation is expected to occur under a trade-off between somatic maintenance and reproductive activity (Roff 2002). Variance in condition is suggested to be strongly affected by environmental factors and by interactions between environmental and genetic factors, rather than by genetic variation among individuals per se (Reznick et al. 2000). Environmental heterogeneities (e.g. food availability, nutrition stress) frequently generate remarkable phenotypic variance in reproductive traits regardless of sex (Hunt et al. 2004a, Boggs and Freeman 2005). Detailed investigations of such plasticity of reproductive traits can be an aid in an understanding of reproductive and life-history strategies (Roff 2002, West-Eberhard 2003).

As reproduction can have energetic and physiological costs for a female, females in good condition are assumed to invest their available resources abundantly in reproduction, but those in poor condition are unable to do this (van Noordwijk and de Jong 1986). Indeed, several field and laboratory studies have demonstrated that female reproduction is largely dependent on concurrent environmental conditions (Reznick et al. 2000, Roff 2002). In addition to the environment in the reproductive (i.e. adult) stage, female reproduction is also influenced by the environment in the developmental stage (Denno and McCloud 1985, Ernsting et al. 1992, Sokolovska et al. 2000, Fischer and Fiedler 2001, Awmack and Leather 2002). Further, the effect of condition on reproduction is not limited to offspring number and size. A female adjusts the offspring sex ratio to increase her own fitness depending on her condition (Trivers and Willard 1973, Leimar 1996). Females in good condition have the ability to provision one sex of their offspring better and provide it with a reproductive advantage, and thus by overproducing one sex compared by the other, these high-condition females garner higher fitness. Various empirical studies in a wide range of taxa provide clear support for the sex allocation theory (Hewison and

Gaillard 1999, Komdeur and Pen 2002, West and Sheldon 2002, Sheldon and West 2004, Katsuki et al. 2012).

Like the female, male reproduction-related traits such as primary (e.g. testis and sperm) and secondary sexual traits (e.g. courtship and ornaments) also show condition dependence (Simmons 1993, Delisle and Hardy 1997, Emlen 1997, Droney 1998, Wagner and Hoback 1999, Wilkinson and Taper 1999, Okada and Miyatake 2010a). Because the production and maintenance of these traits can also be costly in males (Kotiaho 2001, Okada et al. 2011), males in good condition have better sexual trait size and quality (Pomiankowski 1987, Iwasa and Pomiankowski 1994). There is abundant empirical evidence for condition-dependent expression of male sexual traits (i.e. the trait values and environmental condition show positive covariance; Andersson 1986, 1994, Cotton et al. 2004, Hunt et al. 2004a, Tomkins et al. 2004, Bonduriansky 2007). However, within the same individual, different sexual traits sometimes show different responses to its condition (Okada and Miyatake 2010a). Therefore, the condition dependence of multiple traits should be carefully investigated to fully understand male life-history strategies.

Although condition dependence of reproductive investments is common to both sexes, the environment sometimes affects the sexes differently, and the responses of reproductive traits to environmental fluctuation can differ between the sexes (Hunt et al. 2004b). Such sexual differences in plasticity may generate integrated suites of sex-specific morphology, life history, and reproduction (West-Eberhard 2003), resulting in considerably dynamic and sex-specific trait responses to life-history strategy. However, there have been few direct experimental studies that have investigated the condition dependence of reproductive traits in both sexes (e.g. Hunt et al. 2004b, Bonduriansky 2007). Additional experimental studies are needed to directly assess the impact of environmental fluctuation on the reproductive characters of both sexes.

Here I examine the condition dependence of reproductive traits in male and female armed beetle, *Gnatocerus cornutus*. In *G. cornutus*, larvae and adults feed on a variety of grains, flours, yeasts, and dry animal products (Zakladnoi and Ratanova 1987). A difference in nutrients between foods often reflects a difference in resources acquired by individuals (i.e. condition; David et al. 1998, Hunt et al. 2004b, Bonduriansky 2007, Okada and Miyatake 2010a). Although condition is in general largely dependent on the larval environment in metamorphic insects (Emlen 1997, David et al. 1998, Cotton et al. 2004, Bonduriansky 2007, Okada and Miyatake 2010a), it is also affected by the adult environment (e.g. Droney 1998, Kotiaho et al. 2001, Kotiaho 2002). Therefore, condition is thought to be influenced by diet environments in larval and adult stages. In *G. cornutus*, males frequently fight for a territory using an enlarged mandible, which is a secondary sexual trait specific to males (Okada and Miyatake 2010b). In addition, because females copulate with two or more males, the resulting sperm competition can select an ejaculatory character (i.e. testes) as a primary sexual trait (Yamane et al. 2010). Because investment in these sexually selected traits can be costly for males (Yamane et al. 2010, Okada et al. 2011), condition dependent expressions of these traits are expected (also see Okada and Miyatake 2010a). On the other hand, females lack enlarged mandibles completely (Okada and Miyatake 2010a). Nevertheless, it is expected that female reproduction is also influenced by the female's condition because previous studies revealed that female fecundity (Harano et al. 2010) and offspring sex allocation (Katsuki et al. 2012) depend on genetic condition. However, it is unknown whether female condition is affected by environmental factors. In the present study, I investigated the plasticity (i.e. condition dependence) of reproductive and life-history traits in male and female *G. cornutus* by manipulating larval and adult diet quality. Subsequently, I demonstrate that these traits exhibit sexually different responses to environmental fluctuation.

On the basis of these results, I discuss the mating and life-history strategies associated with condition dependence in *G. cornutus*.

6-2. Methods

6-2-1. Stock culture

The stock population of *G. cornutus* was the same as that used by Okada and Miyatake (2010a,b). I described in chapter 5 in detail.

6-2-2. Larval performance and longevity

In all experiments, individuals were reared separately in glass vials (15 mm diameter, 40 mm high). Eggs were collected from the stock culture and transferred to filter paper in a plastic petri dish (1.3 cm high, 3.0 cm diameter). Because whole meal contains more amino acids than wheat bran and is superior in terms of nutrients (Sikka et al. 1975, Peterson et al. 1986), I used whole meal and wheat bran for the control and poor-quality diet, respectively. Thus, by manipulating larval and adult diets, I established three feeding treatments. As a control, newly hatched larvae were reared in glass vials supplied with whole meal (4 g). Then, following Tsuda and Yoshida (1984), each individual was reared in each glass vial with whole meal (4 g) to assess longevity. For the ‘poor larval quality diet treatment’, wheat bran (4 g) and whole meal (4 g) were used for larval and adult diets, respectively, whereas the ‘poor adult quality diet treatment’ was whole meal (4 g) for larvae and wheat bran (4 g) for adults. Because discrimination of larval sex is impossible, I could not distinguish the sex of larvae that died during the experiment. Therefore, I pooled survivors from egg to adult of both sexes. Larval survival was assessed daily and adult survival was assessed weekly. I established 70, 80, and 70 vials for control, poor larval diet, and poor adult diet treatments, respectively. Just after emergence, adults were weighed to the nearest 0.01 mg on an electronic balance (Mettler-Toledo AG, Laboratory and Weighing Technologies, Greifensee, Switzerland).

6-2-3. Testis and secondary sexual selected traits

Eggs were collected from the stock culture and transferred to filter paper in a plastic petri dish (1.3 cm high, 3.0 cm diameter). Newly hatched larvae were reared in glass vials with food (4 g) corresponding to the three treatments, control, poor larval diet, and poor adult diet. I collected approximately 30 males from the three treatments for measurements. At 1 week post-emergence, the males are fertile enough to transfer their sperms into females (Tsuda and Yoshida 1984, K. Okada unpublished). I thus kept each adult male for 2 weeks before dissections because they take a week to reach full sexual maturity. Subsequently, the right and left testes were removed from the body using a fine forceps and carefully separated from the surrounding tissue in deionised water following Yamane et al. (2010). The length (L) and width (W) of both testes were measured to ± 0.01 mm with the dissecting microscope monitoring system, and testis volume (mm^3) was calculated on the assumption that the testis was oval, using the formula $V = (\pi L W^2)/6$. The total volume was used for the testis size. Mandible length of each male (± 0.01 mm) was measured 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. Each length and width was measured as a straight-line distance (see Okada and Miyatake 2010a for landmarks). Measurement error is negligible in this method and the measurements show high repeatability, although this was used for another beetle (Okada et al. 2008).

6-2-4. Male fighting

Contestant males were reared as described above and placed in one well of a 24-well tissue culture plate with food (1 g) corresponding to previous diet quality until the following experiment. To investigate whether adult and larval diet quality are associated with success in male fighting, I staged fights of control male versus poor larval diet male (n = 24) and control male versus poor adult diet male (n = 24) as fallows. To observe male–male interactions, I placed a paper filter (17 mm diameter) in a plastic container (17 mm diameter, 20 mm high) for the fighting site. Two males were simultaneously introduced into the plastic container, and their behavior was observed for 30 min. The male that pushed his opponent and chased him out of the fighting site was denoted the winner. The loser was the male that retreated from the fight site. For a more detailed description of the methods, see Okada and Miyatake (2010b). To control for the effect of body size on fighting success, males were selected so that the difference in body size between contestants was less than 0.01 mm (total range of body width variation of males was less than 2%; Okada and Miyatake 2010b). As shown in the present study (see Table 1) and Okada and Miyatake (2010a), diet quality did not affect the body size of males.

6-2-5. Lifetime reproductive success and offspring sex ratio

I collected 50 virgin females from each of the three treatments (control, poor larval diet, and poor adult diet). Just after emergence, the females were weighed to the nearest 0.01 mg on the electronic balance. I paired each female with a virgin male, both of which were less than a week post-emergence, and placed the pair in a plastic cup (70 mm diameter, 25 mm high) containing an excess of food (20 g) corresponding to the same diet quality treatment. I randomly collected 150 males as mates from the stock culture. Each pair was maintained for 2 months with the male able to mate with the female, and the female allowed to lay eggs. This schedule was chosen because a peak in the numbers of eggs laid by females was observed 2 months after mating (Tsuda and Yoshida 1984), and thus this is considered as the index of lifetime reproductive success (LRS) (Katsuki et al. 2012). To measure the LRS of each female, I counted all larvae that emerged in the third month after pairing. To examine the sex ratio of the offspring of each female, larvae were placed on to a well in 24-well tissue culture plates for pupation.

6-2-6. Statistical analyses

Logistic regression analysis with the survival rate (1 or 0) as the dependent variable and diet treatment as the independent variable was used for comparison of the survival rates at emergence. Development period, adult longevity, and body mass were analysed using generalized linear models with sex and diet treatment as fixed effects. I estimated the adjusted mean of each diet treatment as the mean of sex with leastmean squares to conduct multiple comparisons (Snedecor and Cochran 1980). Mandible length, testis size, and LRS were analyzed using generalized linear models with diet treatment as a fixed effect. As a post-hoc test, Student's *t* -test was used to compare the means, correcting the significance level for multiple comparisons by the sequential Bonferroni method (Rice 1989). The offspring sex ratio (sons/all offspring) was analyzed by generalized linear models (the error distribution was binomial with a logit link) with diet treatment as a fixed effect. 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).

6-3. Results

Of 70, 80, and 70 larvae reared in control, poor larval, and poor adult diet treatments, 65, 67, and 63 larvae, respectively, successfully emerged. There was no significant difference in larval survival rates among the diet treatments (χ^2_2= 3.2395, *P* = 0.1979). The 65, 67, and 63 eclosed adults consisted of 32 males and 33 females, 35 males and 32 females, and 33 males and 30 females, respectively. I used the developmental periods from egg to adult of individuals that successfully emerged for the following analyses.

Table 6-1 shows development periods from egg to adult, adult body mass, and longevity for each treatment. For the three traits, the full model showed a non-significant interaction between sex and treatment (Table 6-2). Thus, the diet manipulation produced no sex-specific response in these characters. Sex and treatment had significant effects on the developmental period and longevity in the reduced model (Table 6-2). Multiple comparisons showed that the adjusted developmental period mean was significantly longer in the poor larval diet treatment than in the poor adult diet treatment and control (Fig. 6-1a, b, Table 6-1), whereas longevity was significantly shorter in the poor adult diet treatment than in the poor larval diet treatment and control (Fig. 6-1c, d, Table 6-1). The sex and treatment had no significant effect on the adult mass. For males, the treatment had a significant effect on mandible length, but not on testis size (Table 6-2). The mandible length was significantly shorter in the poor larval diet treatment than in the poor adult diet treatment and control (Table 6-1, Fig. 6-1e). In control male versus poor larval diet male contests, control males were the winners significantly more frequently than poor larval diet males (control versus larval diet: 17 versus 7 winners, $\chi^2_1 = 4.1667$, $P = 0.0412$). In contrast, no difference was found between the number of control and poor adult diet males winning in the male contests (control versus adult diet: 11 versus 13 winners, $\chi^2_1 = 0.1667$, $P = 0.6831$). Treatment had a significant effect on offspring number laid by each female, i.e. LRS (Table 6-2), which was significantly smaller in the poor adult diet treatment than in the poor larval diet treatment and control (Table 6-1, Fig. 6-1f). On the other hand, the model with treatment as a fixed effect showed no significant effect of treatment on offspring sex ratio (Table 6-1, $\chi^2_2 = 0.4065$, $P = 0.8161$).

6-4. Discussion

The quality of the larval diet environment affected the larval developmental period of both sexes but not larval survival (i.e. the developmental periods were significantly longer with the poor-quality larval diet than with the control diet) (Table 6-1, Fig. 6-1a, b). In contrast, larval diets had no effect on adult body mass and longevity in either sex (Table 6-1, Fig. 6-1c, d), and this finding in body size is consistent with previous work on this population (Okada and Miyatake 2010a). Because food availability can directly affect individual condition (van Noordwijk and de Jong 1986, Reznick et al. 2000), reduction in larval condition may affect their growth rate, resulting in developmental delay. However, there was no difference in numbers of offspring produced by the females (i.e. LRS). A longer developmental period can be potentially beneficial because late emerging females have an advantage in acquisition of resources during the developmental stage (Metcalfe and Monaghan 2001). Thus, the longer developmental period in females may compensate for low food availability. As a result, larval diet quality is unlikely to affect LRS. The male also may be compensated by longer developmental periods. In males, poor larval diet did not reduce the size of any trait except the mandible. Thus, it is also possible that a prolonged developmental period may compensate for male body size and testis size.

In males, mandible size, a secondary sexual trait, was significantly smaller in the poor larval diet treatment than in the control (Table 6-1, Fig. 6-1e). This suggests that the male condition is strongly affected by larval nutritional environments and indicates the condition dependence of the male mandible. In metamorphic insects with exaggerated secondary sexual traits, there is evidence of male-specific costs of secondary sexual traits based on resource allocation; i.e. larvae accumulate resources during development and subsequently allocate them to exaggerated structures, some of which are costly for males to produce during pre-pupal and pupal stages (Nijhout and Emlen 1998, Emlen 2001, Okada et al. 2011). Thus, my findings provide further evidence that male secondary sexual traits can be condition-dependent (also see Emlen 1997, David et al. 1998, Wilkinson and Taper 1999, Cotton et al. 2004, Bonduriansky 2007, Emlen et al. 2007, Okada and Miyatake 2010a). Together with the fact that male fighting success was affected by larval diet quality, larval nutritional environments strongly affect pre-copulatory sexual selection. I found that primary and secondary sexual traits exhibit different responses to environmental fluctuation. These different responses suggest that *G. cornutus* males are selected for investing excess resources in exaggerated secondary sexual traits more than in primary sexual traits. An alternative explanation is a developmental constraint. Primary sexual traits, including testes, often show nutrition-insensitive growth patterns and relatively low phenotypic variance due to developmental canalisation (e.g. Eberhard et al. 1998, Simmons and Emlen 2006, Emlen et al. 2007), and compensation due to the longer development that was mentioned above may contribute in part to developmental canalisation. Thus, the primary sexual traits may not respond to the environmental condition in this *G. cornutus* population.

Adult diet quality affected longevity in both sexes, and longevity was significantly shorter with the poor adult diet than the control treatment (Table 6-1, Fig. 6-1c, d). The adult diet manipulation is likely to affect resource acquisition of both males and females. It has a similar tendency to affect female LRS (Table 6-1, Fig. 6-1f). Generally, there are positive associations between body size and LRS and between body size and longevity (Roff 2002, Chown and Gaston 2010). On the other hand, I found no difference in body size between poor adult diet and control treatments. Thus, the difference in LRS may reflect the difference in adult condition. In contrast to females, the male primary sexual trait (i.e. testes) in *G. cornutus* was not affected by adult diet environments. Furthermore, male fighting success was not affected by adult diet quality. These results suggest that adult nutrition has stronger impacts on the life-history strategy in females. Dietary restriction often extends the life span and reduces fecundity in diverse organisms (Partridge et al. 2005). It is widely thought that optimal reallocation of nutrient intake away from reproduction and towards somatic maintenance can aid survival during a food shortage (reviewed in Holliday 1989, Mair and Dillin 2008). On the other hand, my results were inconsistent with such an effect of dietary restriction. In my experiment, the nutritional environment of the ‘poor diet’ might have been extremely inadequate, resulting in the decrease of longevity (e.g., van Noordwijk and de Jong 1986, Reznick et al. 2000, Roff 2002). In addition to the number of eggs per se, theoretically, females adjust the offspring sex ratio when the fitness returns from one sex are higher than those from the other (Trivers and Willard 1973, Leimar 1996). In particular, it is known that maternal body conditions influence offspring sex ratio in several insects (West and Sheldon 2002). In my previous study, females of *G. cornutus* were able to adaptively bias the sex ratio depending on their genetic condition (Katsuki et al. 2012); higher condition females produce more daughters with a genetically high reproductive advantage, and they thus garner higher fitness by overproducing daughters. On the other hand, my results showed that a biased sex ratio is not associated with any maternal environmental condition (Table 6-1). Based on these facts, the *G. cornutus* female shows the genetic condition dependence of biased offspring sex ratio.

In conclusion, the traits related to body maintenance and growth, such as longevity, developmental period, and body mass, exhibited similar responses to environmental fluctuation in both sexes. On the other hand, the reproductive traits exhibited different sexual responses. That is, the adult nutrient environment strongly affected female condition, whereas the larval nutrition affected male condition. This suggests that the timings of energetic demands of reproduction differ between sexes. Because reproductive investments are thought to have a trade-off relationship with various other traits that contribute to their fitness, life-history strategies can be affected by the change of their reproductive characters (Roff 2002, Tomkins et al. 2004). Thus, a sexual difference in timings of the energetic demands for reproduction may lead to different selection pressures between the sexes, even if both sexes develop and/or live in the same environment, and may lead to a generation of sex-specific life-history and reproductive strategies.

7. General Discussion

Male mating success directly increases his fitness (Bateman 1984). To mate with female, males would adopt optimal mating strategies, depending on their condition and mating situation. Moreover, male has to invest for reproductive traits to ensure and increase his paternity, with considering the investment for other traits. Moreover, a various environmental and genetic factors would influence male reproductive investments and paternity, resulting in different selective pressures for male reproductive strategy.

Although studies about influence of environmental factors were reported, most studies reported the effect though life-history. However, individuals would be influenced by environmental factors during short term. Therefore, it is need to investigate the effects of environmental factors for male reproductive investment in developmental and reproductive period to understand the evolution of life-history and male reproductive strategy.

To know that environmental factor in only reproductive period affects male reproductive investment and female mating events, I investigated the effect of ambient temperature on male reproductive investment and female remating receptivity (Chapter. 2). The ambient temperature on mating influenced male reproductive investment. Male reproductive investment was allocated to present and future reproduction, based on trade-off. In addition, there was the trade-off between reproduction and other life-history traits. For example, *C. maculatus* males have a negative correlation between mating duration and longevity (Brown et al. 2009). Therefore, the variation of reproductive investment by temperature would influence not only future reproductive event but also other life-history traits. Moreover, male reproductive variation affects female remating receptivity. Female receptivity correlates with female fitness (fecundity and longevity) and male paternity. As the results, ambient temperature would provide the impacts on male and female fitness.

In chapter 2, I investigated the effect of environmental factor, temperature, in reproductive period and thus revealed that environmental factor has strong impact to male reproductive investment. On the other hand, it would be assumed that the effect in developmental period affects male reproductive investment. Therefore, I focused on the effect of developmental period, especially resource acquisition pattern (Cahpter.3). I showed that the differences of larval resource acquisition cause different selective pressure for male reproduction through the difference of adult density; in scramble type, males were selected for investing to ejaculate expenditure. Therefore, the reproductive traits would be selected not only by environmental factors in adult but also by behavior in developmental period. Together with chapter 2, it was showed that male reproductive investment is influenced by genetic factor during developmental period or environmental factor during reproductive period, even if in only mating situation. This indicates that environmental factors even for slightly fluctuation in short term would influence the male reproductive investment and paternity.

In chapter 4, I researched the resource allocation between reproductive and flying traits, and male alternative mating strategies in *C. chinensis*. I found the genetic trade-off between reproductive trait (testes size and the number of sperm transferred into female) and flying trait (hindwing size and flying ability). The amount of reproductive investment in male is selected for depending on the situation that male faced, for example the risk or intensity of sperm competition (Hosken and Ward 2001, Firman and Simmons 2008, Dziminski et al. 2010). In this chapter, it was suggested that higher male reproductive investment increases his paternity, because it increases the advantage in sperm competition and decrease female receptivity. On the other hand, investments for dispersal traits decrease investment for reproductive traits. The trade-off between reproductive investment and other life-history trait has been reported (Roff 2002). The male with high reproductive investment decreases dispersal ability, i.e., the male with high dispersal ability has lower reproductive investment. The male with high dispersion appears to get high paternity, however, well-flying male gets an advantage in term of mate searching. It indicates that *C. chinensis* male has the alternative strategy, increasing a paternity or mate encounter. The presence of male strategy based on trade-offs would keep the variation for male reproductive traits.

I was focused on not only male but also the interaction (Chapter 5) and difference (Chapter 6) between male and female because it is possible that female reaction is one of the important factors in male reproductive investments. In broad-horned flour beetle, *Gnatocerus cornutus*, intra-locus sexual conflict was existed and, females compensated their fitness decrease by adjusting their offspring sex ratio (Chapter 5). I paid attention to biased offspring sex ratio as compensation of female fitness, however, other studies have also indicated females operate and increase their fitness by another manner, e.g. cryptic female choice (Anqvist and Rowe 2005). By female operation and resistance, males would be faced on a various selective pressure, thus be selected. This suggested that female resolves her cost from males, and her counter-adaptation promote antagonistic sexual selection. In addition, the impact of environmental factor was different between female and male (Chapter 6). In females, the number of offspring was influenced by the adult diet. In contrast, in males, secondary sexual trait was influenced by larval diet but not primary sexual trait. Larval diet is important for male reproductive success. However, female fitness is influenced by adult diet. Because female longevity and fertility depend on the adult diet and affect male mating opportunities, male fitness would be changed not only by larval diet but also by adult environment, indirectly. Same as chapter 6, I also showed the possibility that female resistance behavior and receptivity for remating influence the male reproductive investments in chapter2.

Enormous studies have showed the existence of male reproductive strategies, including sperm competition, and of sexual conflict (Simmons 2001, Arnqvist and Rowe 2005). My studies present that male reproductive investment and sexual interactions in both sexes would not cause the simple evolutionary consequence by environmental and developmental factors. Moreover, my studies would add to the fields of sexual selection and sexual conflict that there is a profound background in the occurrence of male reproductive strategy and sexual interaction.

8. Summary

1. In the adzuki bean beetle, *Callosobruchus chinensis*, I found negative relationships between ambient temperature and mating duration, sperm transfer and sperm transfer duration. Female remating frequency at lower temperature (17 ^{o}C) was lower than at other temperatures (25 ^{o}C and 33 ^{o}C). The number of ejaculated sperm was significantly lower at 33 ^{o}C than at 17 ^{o}C; the effect of temperature on sperm transfer is discussed in relation to the intensity of female refusal behavior directed against males.

2. I examined the relationship between larval competition types and male reproductive investment in mating. I assessed the male ejaculate expenditure in a mating across geographic strains of *C. maculatus*. As a result, I found that male investment (ejaculate expenditure) increased with the degree of scramble competition and decreased with the degree of contest competition. I therefore suggest that males experience different selective pressures depending on the type of larval competition: scramble type males are selected for increased reproductive investment.

3. I tested this assumption by comparing different populations using geographical strains of a seed beetle, *Callosobruchus chinensis*. I found a trade-off between testes and hindwing size in males. Likewise, my studies showed a negative association

between the number of sperm transferred into the female spermatheca and flying ability. These results indicate that ejaculatory and dispersal strategies are evolutionarily linked with each other and that males have alternative mating strategies based on resource allocation trade-offs. Exploring how characters that contribute to male fitness are genetically associated can be a powerful aid in understanding alternative mating strategy and how males adopt their mating strategy.

4. I tested for offspring sex-ratio biasing consistent with these predictions in broad-horned flour beetles. I found that in both wild-type beetles and populations subject to artificial selection for high- and low-fitness males, offspring sex ratios were biased in the predicted direction: low-fitness females produced an excess of sons, whereas high-fitness females produced an excess of daughters. Thus, these beetles are able to adaptively bias sex ratio and recoup indirect fitness benefits of mate choice.

5. I investigated the condition dependence of life-history and reproductive traits of males and females in the beetle *Gnatocerus cornutus* Fabricus by manipulating larval and adult diet quality. I found that male and female life-history traits exhibited similar responses to environmental fluctuations. By contrast, the sexes exhibit different patterns of condition dependence in reproductive traits (i.e. the adult

nutritional environment has a strong impact on the female lifetime reproductive success, whereas larval nutritional environment strongly affects the secondary sexual trait in males). This difference in the plasticity of reproductive traits may lead to different selection pressures for each sex, even if both sexes develop and/or live in the same environment.

9. List of Publications

1. Katsuki M and Miyatake T (2009)

   Effects of temperature on mating duration, sperm transfer and remating frequency in *Callosobruchus chinensis*.

   Journal of Insect Physiology 55(2) 112-119.

   doi:10.1016/j.jinsphys.2008.10.012

2. Katsuki M, Harano T, Miyatake T, Okada K and Hosken DJ (2012)

   Intralocus sexual conflict and offspring sex ratio.

   Ecology Letters 15(3)193-197.

   doi: 10.1111/j.1461-0248.2011.01725.x

3. Katsuki M, Okada Y and Okada K (2012)

   Impacts of diet quality on life-history and reproductive traits in male and female armed beetle, *Gnatocerus cornutus*.

   Ecological Entomology 37(6)463-470.

   doi: 10.1111/j.1365-2311.2012.01390.x

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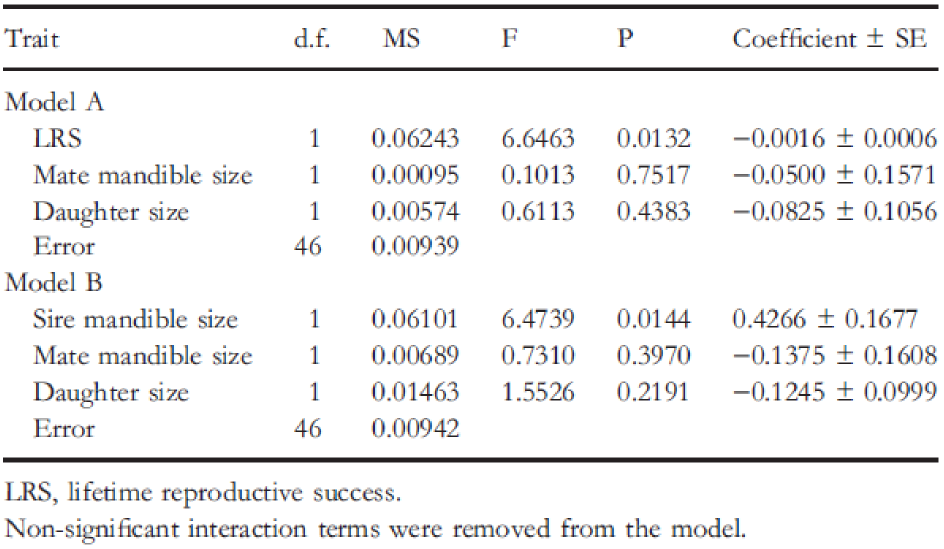
12. Tables

Table5-1 Results of general linear models (GLM) on offspring sex ratio.

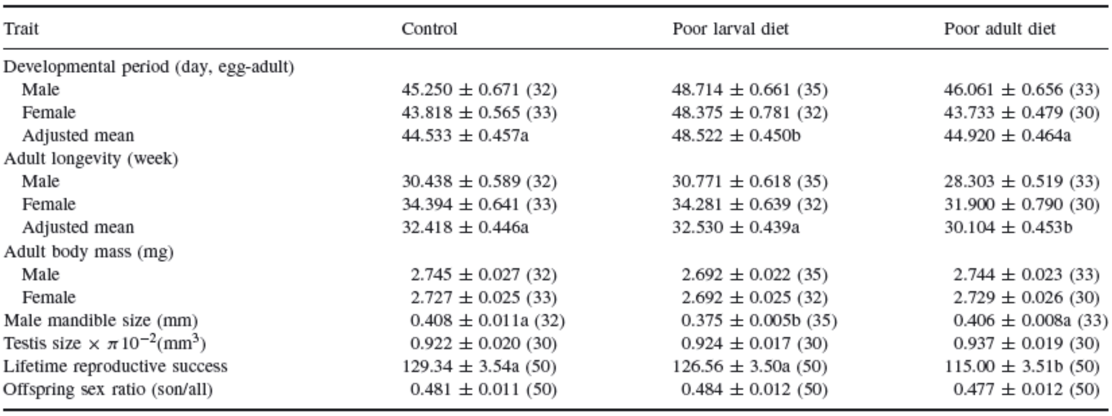


Trait	d.f.	MS	F	P	Coefficient ± SE
Model A					
LRS	1	0.06243	6.6463	0.0132	−0.0016 ± 0.0006
Mate mandible size	1	0.00095	0.1013	0.7517	−0.0500 ± 0.1571
Daughter size	1	0.00574	0.6113	0.4383	−0.0825 ± 0.1056
Error	46	0.00939			
Model B					
Sire mandible size	1	0.06101	6.4739	0.0144	0.4266 ± 0.1677
Mate mandible size	1	0.00689	0.7310	0.3970	−0.1375 ± 0.1608
Daughter size	1	0.01463	1.5526	0.2191	−0.1245 ± 0.0999
Error	46	0.00942			

LRS, lifetime reproductive success.
Non-significant interaction terms were removed from the model.

Results of general linear models of offspring sex ratio in stock (nonartificially selected) beetles. Model A uses daughters' fitness (lifetime reproductive success: LRS) as a measure of female quality, whereas in Model B, the quality of daughters' fathers (the size of sires mandibles) was used as an indicator of female quality. In both cases low quality females produced more sons (Fig. 5-1)

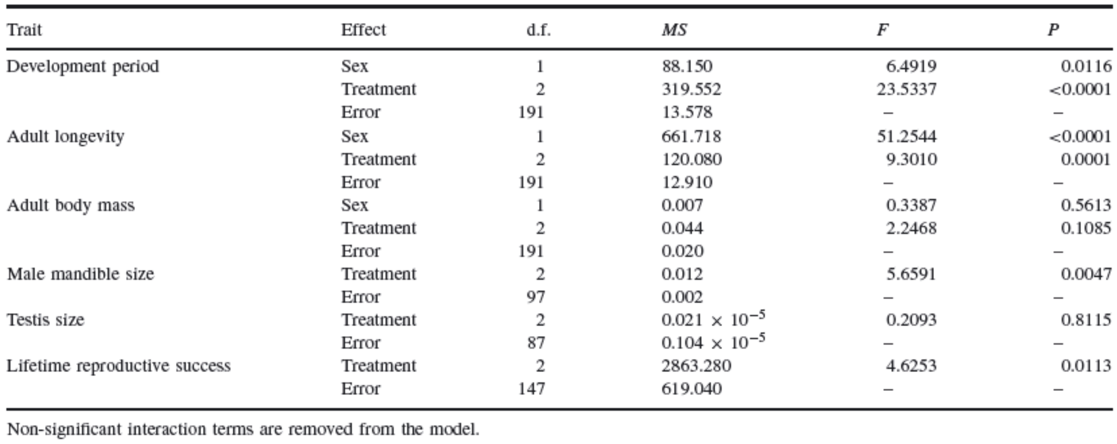
Table 6-1. Actual and adjusted trait mean ± SE in each treatment.



Trait	Control	Poor larval diet	Poor adult diet
Developmental period (day, egg-adult)			
Male	45.250 ± 0.671 (32)	48.714 ± 0.661 (35)	46.061 ± 0.656 (33)
Female	43.818 ± 0.565 (33)	48.375 ± 0.781 (32)	43.733 ± 0.479 (30)
Adjusted mean	44.533 ± 0.457a	48.522 ± 0.450b	44.920 ± 0.464a
Adult longevity (week)			
Male	30.438 ± 0.589 (32)	30.771 ± 0.618 (35)	28.303 ± 0.519 (33)
Female	34.394 ± 0.641 (33)	34.281 ± 0.639 (32)	31.900 ± 0.790 (30)
Adjusted mean	32.418 ± 0.446a	32.530 ± 0.439a	30.104 ± 0.453b
Adult body mass (mg)			
Male	2.745 ± 0.027 (32)	2.692 ± 0.022 (35)	2.744 ± 0.023 (33)
Female	2.727 ± 0.025 (33)	2.692 ± 0.025 (32)	2.729 ± 0.026 (30)
Male mandible size (mm)	0.408 ± 0.011a (32)	0.375 ± 0.005b (35)	0.406 ± 0.008a (33)
Testis size × $\pi 10^{-2}$(mm^3)	0.922 ± 0.020 (30)	0.924 ± 0.017 (30)	0.937 ± 0.019 (30)
Lifetime reproductive success	129.34 ± 3.54a (50)	126.56 ± 3.50a (50)	115.00 ± 3.51b (50)
Offspring sex ratio (son/all)	0.481 ± 0.011 (50)	0.484 ± 0.012 (50)	0.477 ± 0.012 (50)

Adjusted mean is estimated from a general linear model with least-mean square (SAS Institute, 2005). Different letters indicate a significant difference at $P < 0.05$ by Student’s t -test with the sequential Bonferroni method (Rice, 1989). Values in parentheses are numbers of individuals.

Table 6-2. Results of a general linear model of the effects of sex and treatment on each trait.



Trait	Effect	d.f.	*MS*	*F*	*P*
Development period	Sex	1	88.150	6.4919	0.0116
	Treatment	2	319.552	23.5337	<0.0001
	Error	191	13.578	–	–
Adult longevity	Sex	1	661.718	51.2544	<0.0001
	Treatment	2	120.080	9.3010	0.0001
	Error	191	12.910	–	–
Adult body mass	Sex	1	0.007	0.3387	0.5613
	Treatment	2	0.044	2.2468	0.1085
	Error	191	0.020	–	–
Male mandible size	Treatment	2	0.012	5.6591	0.0047
	Error	97	0.002	–	–
Testis size	Treatment	2	0.021×10^{-5}	0.2093	0.8115
	Error	87	0.104×10^{-5}	–	–
Lifetime reproductive success	Treatment	2	2863.280	4.6253	0.0113
	Error	147	619.040	–	–

Non-significant interaction terms are removed from the model.

13.Figures

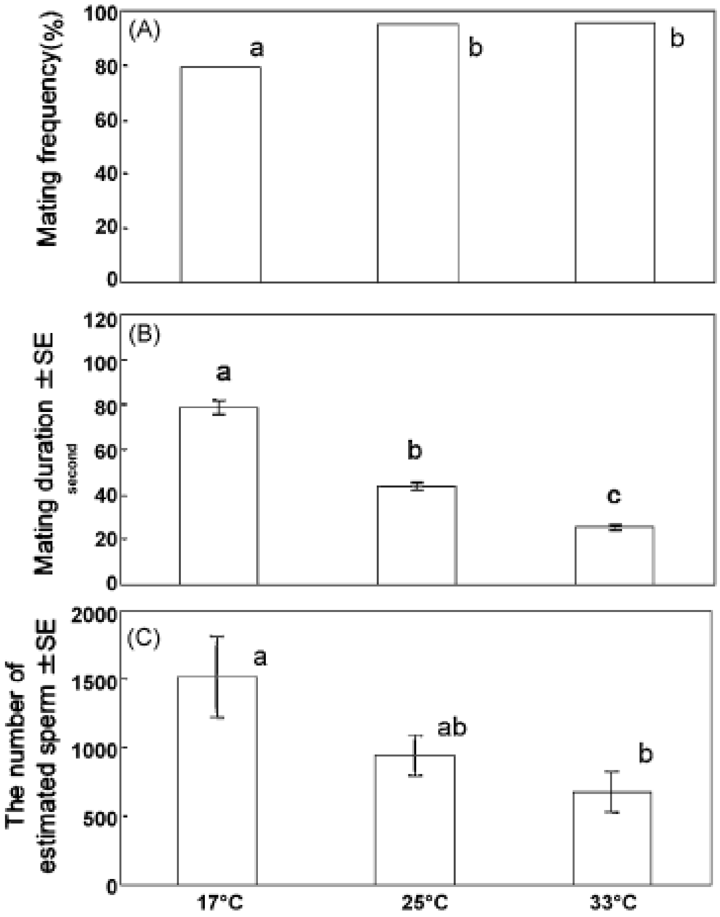


Figure 2-1. Relationships between ambient temperatures and mating frequency (A), mating duration (B) and the number of estimated sperm in spermathecae (C). The same alphabetic letters show no significant difference from each other at $P < 0.001$ by sequential Bonferroni tests (A) and at $P < 0.05$ by Tukey HSD method (B and C). SE is showed by bar in figures B and C.

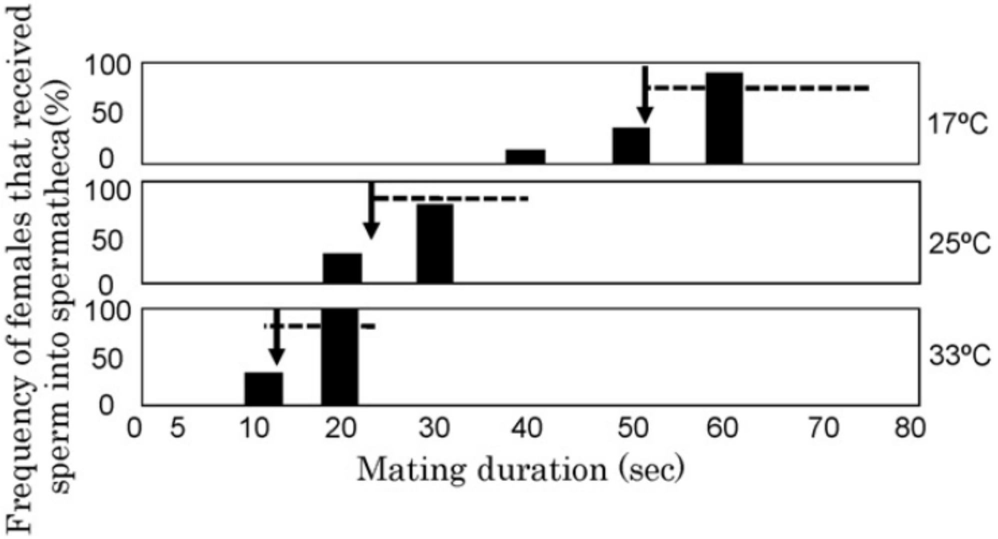


Fig. 2-2. Relationships between mating duration and the frequency of female that received sperm into spermatheca. The arrows in each figure show median time of mating duration. Dotted lines show the estimated duration of sperm transfer.

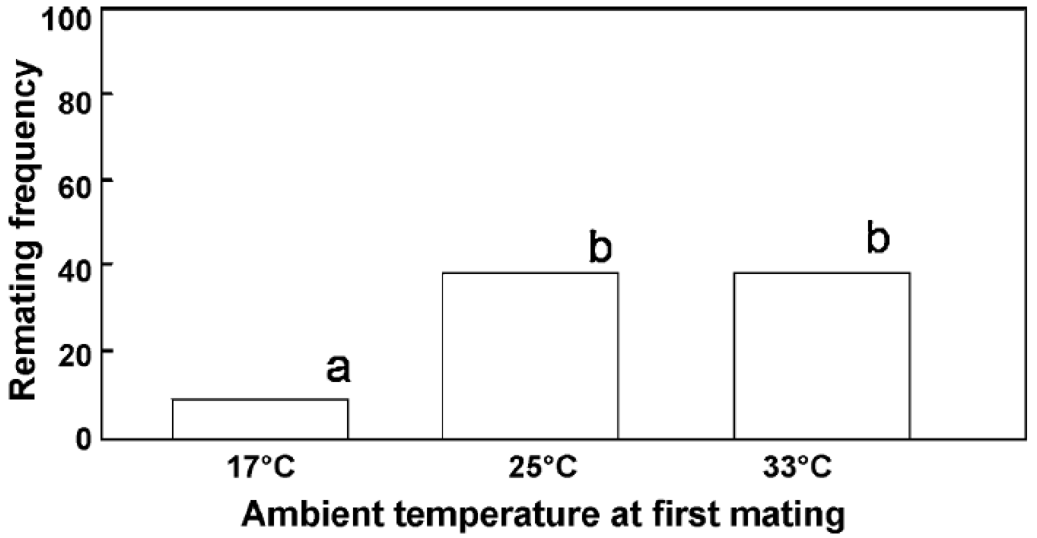


Fig. 2-3. Relationship between ambient temperatures during first mating and remating frequency. The same alphabetic letters show no significant difference from each other at $P < 0.001$ by sequential Bonferroni tests.

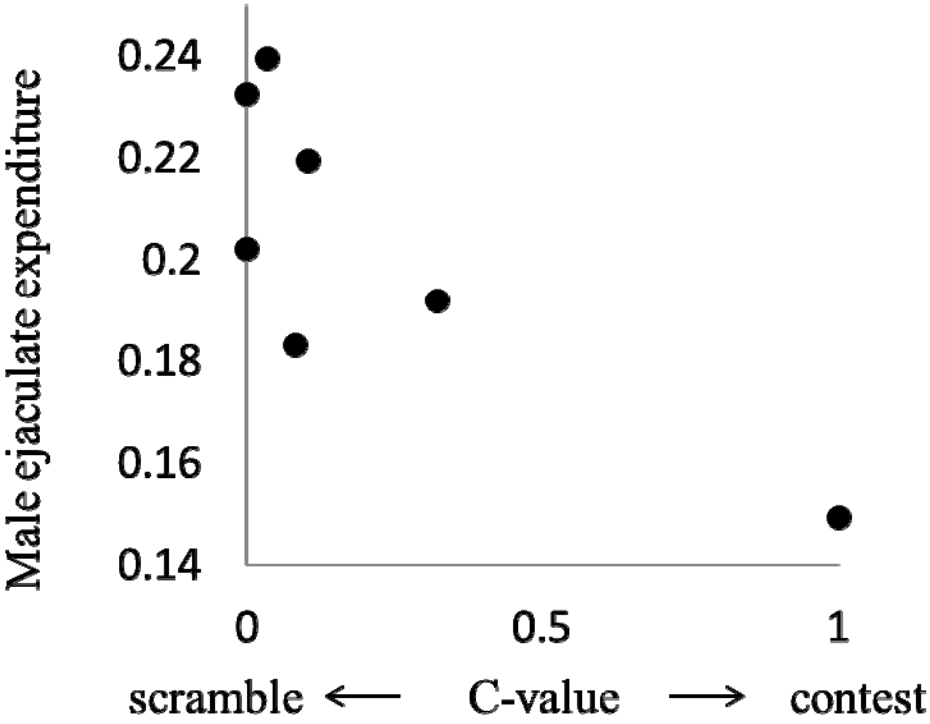


Fig. 3-1. Across geographic strains of *C. maculatus*, there is a negative correlation between the C-value (index of the proportion of contest competition type individuals) and adjusted ejaculate expenditure.

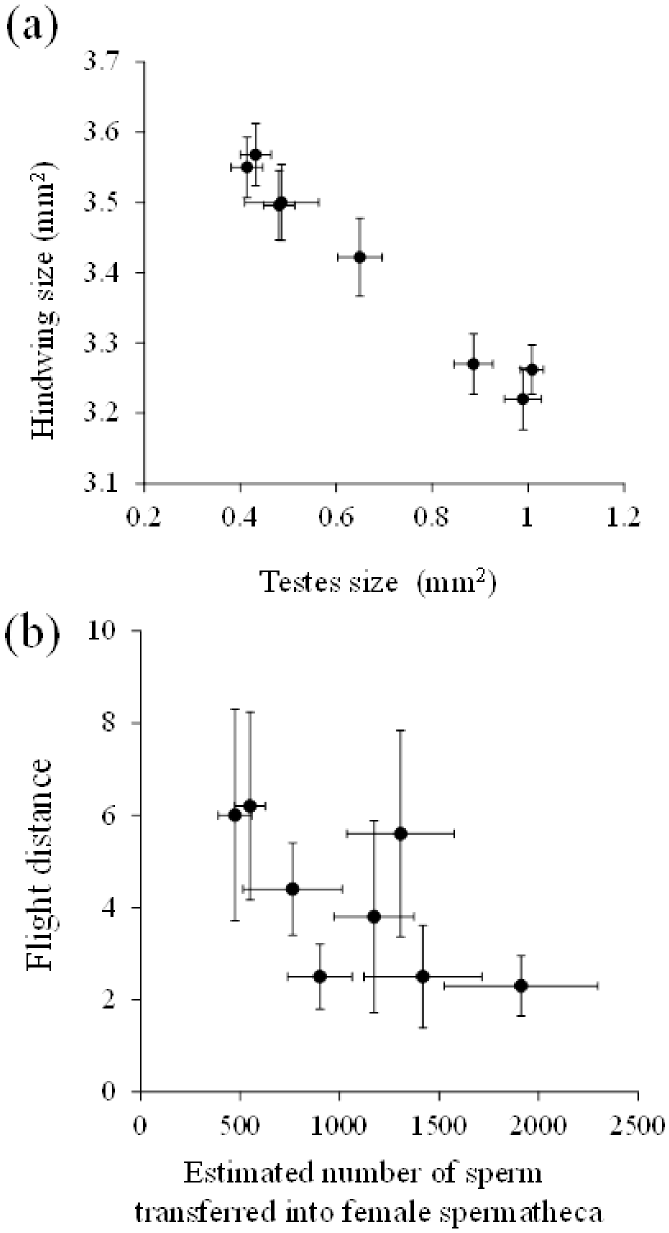


Fig. 4-1. (a) shows an association between testis and hindwing size. (b) shows an association between estimated the number of sperm transferred into female on a mating and male flight ability (cm). Each dot and bar shows population mean and standard error of strain.

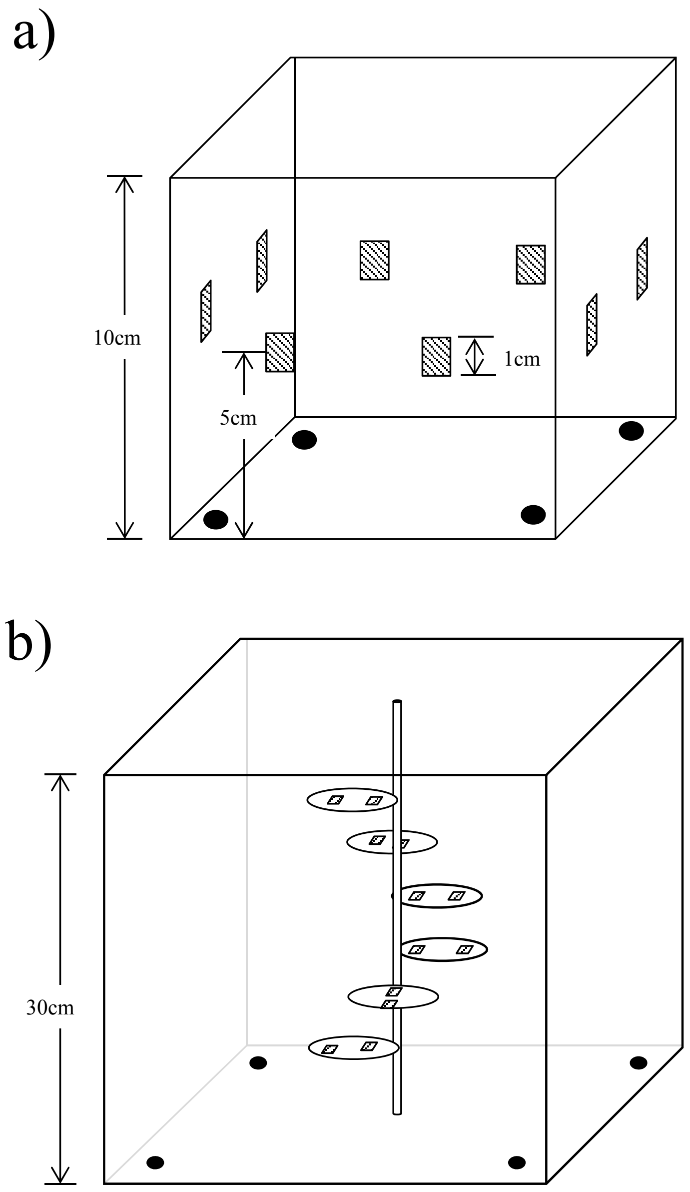


Fig. 4-2 The schematic drawing in experiment compared with male mating success between well-flying strain and well-reproductive investment strain. a) and b) show the situation that male detect easily female (small scale, high density) and that of difficulty detection (large scale, low density), respectively.

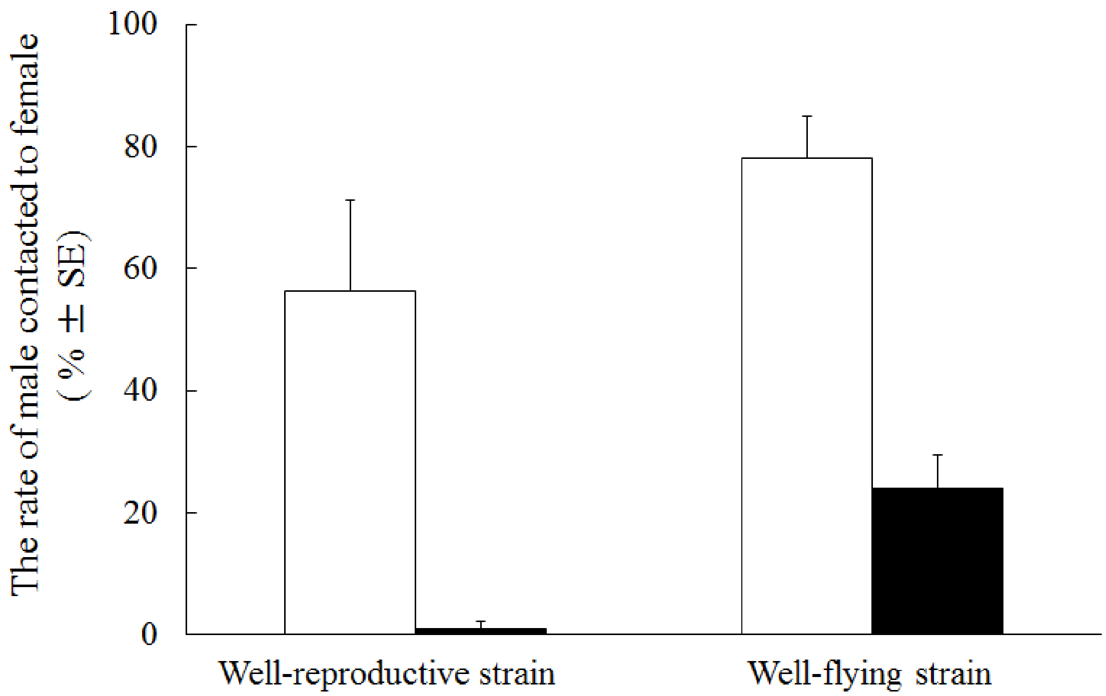


Fig. 4-3. The rate of males that were able to contact female in each strain. White bars and black bars show the rate in small and large spatial scale. Data are mean ± SE.

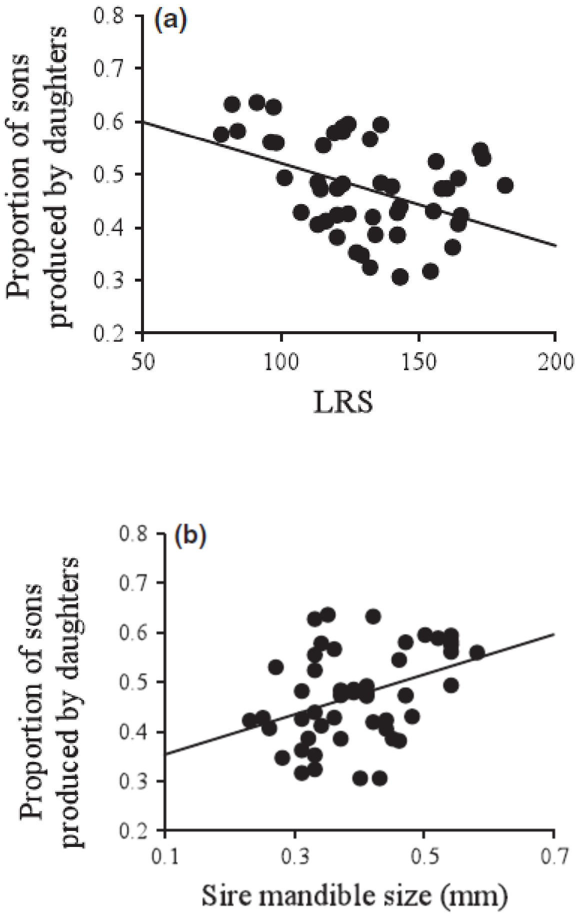


Fig. 5-1. The associations between proportion of sons produced by daughters and daughter quality (lifetime reproductive success: LRS) (a) and sire mandible size (b). There was a significant negative association between daughter quality and proportion of sons produced by daughters (Table 1) and a significant positive association proportion of sons produced and the size of the mandibles of daughtersfathers (a more indirect measure of daughter quality) (Table 1). Note that daughter quality is inversely related to sire quality, and hence a positive slope was predicted for B, and that these results relate to beetles that had not been subject to artificial selection on male quality.

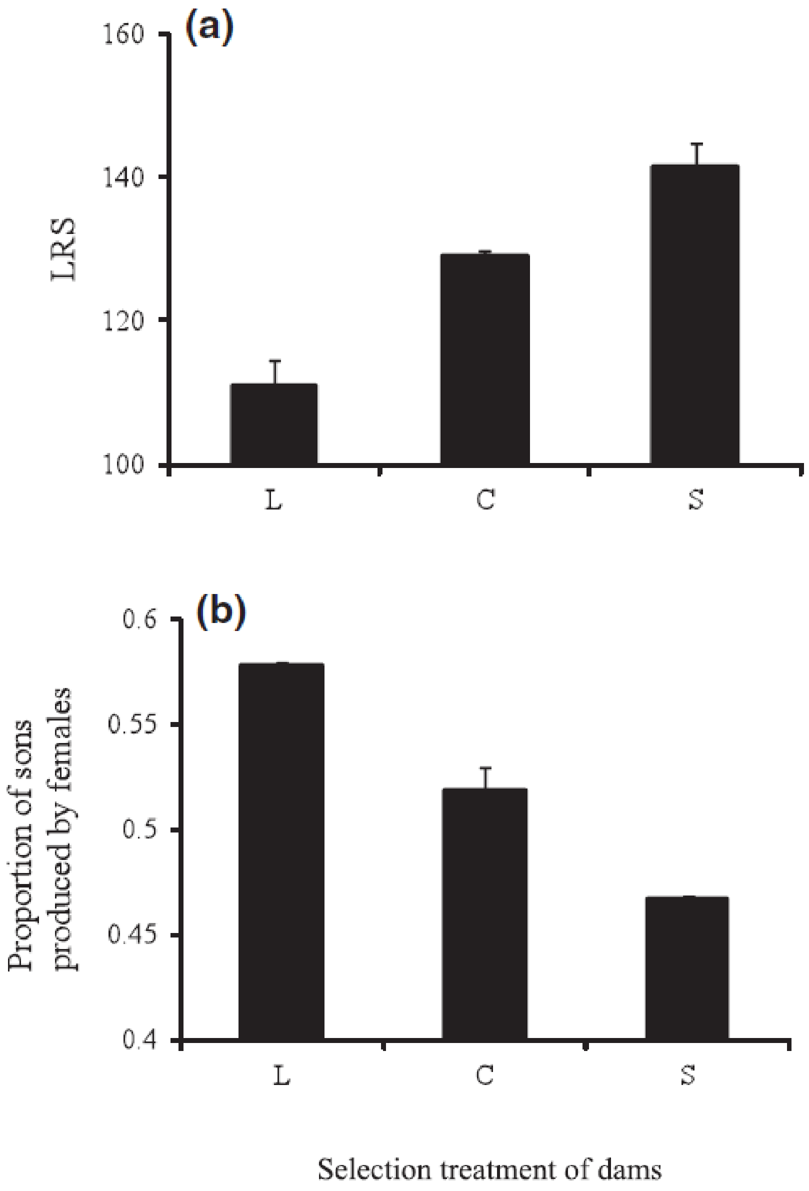

(a)
160
140
120
100
LRS
L
C
S
(b)
0.6
0.55
0.5
0.45
0.4
Proportion of sons produced by females
L
C
S
Selection treatment of dams

Fig. 5-2. Female quality (lifetime reproductive success: LRS) (a) and proportion of sons produced (b) from populations subjected to selection on male quality (mean ± SE). Selecting on male quality (treatment) affected female quality (a) (LRS) ($F_{2,2} = 38.19$; $P = 0.026$) and (b) the proportion of sons produced ($F_{2,2} = 301.95$; $P < 0.004$). Female body size had no effect (LRS: $F_{1,2} = 0.025$; $P = 0.89$. Sex ratio: $F_{1,2} = 4.24$; $P = 0.18$). Removal of female size, did not change these results (selection history influenced LRS [$F_{2,3} = 60.82$; $P < 0.004$; t-tests with sequential Bonferroni adjustment L < C < S (all $P < 0.02$)] and sex ratio [$F_{2,3} = 157.92$; $P < 0.001$; t-tests with sequential Bonferroni adjustment L > C > S (all $P < 0.004$)]. Note that all females in this comparison had been mated to control population males.

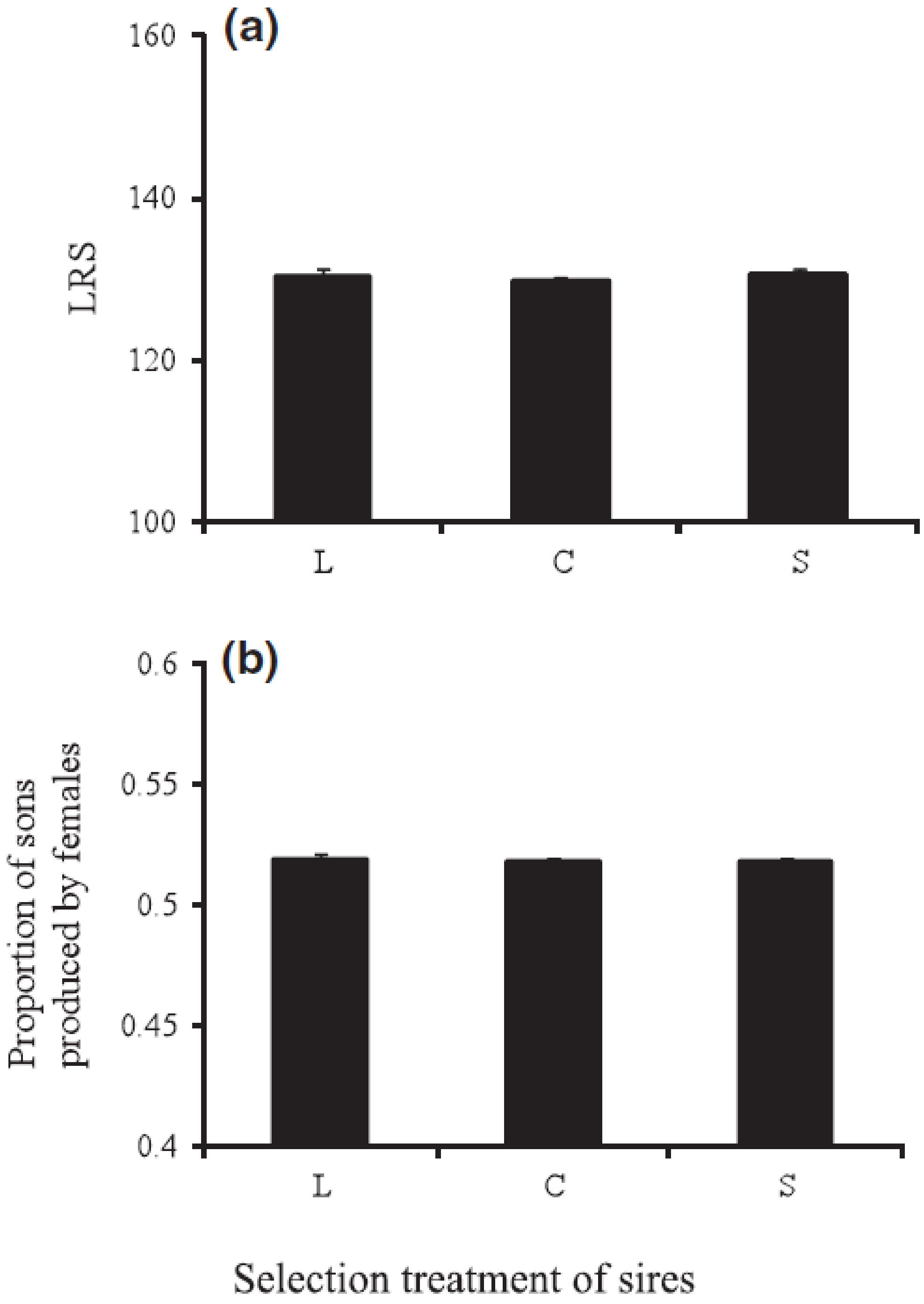

(a)
160
140
120
100
LRS
L
C
S
(b)
0.6
0.55
0.5
0.45
0.4
Proportion of sons produced by females
L
C
S
Selection treatment of sires

Fig. 5-3. Lifetime reproductive success (LRS) (a) and proportion of sons produced (b) in control population females paired with males of the experimental populations (L = high quality males; C = intermediate males; S = low quality males). Data are mean ± SE. There were no significant effects of male selection history (treatment) on LRS (treatment, $F_{2,2} = 0.16$; $P = 0.86$) or offspring sex ratio (treatment, $F_{2,2} = 1.08$; $P = 0.488$). Female body size also had no effect on these measures (LRS: $F_{1,2} = 2.61$; $P = 0.25$. Sex ratio: $F_{1,2} = 2.63$; $P = 0.25$). Results did not change when female body size was removed from the model (all $F_{2,3} < 0.34$; $P > 0.74$).

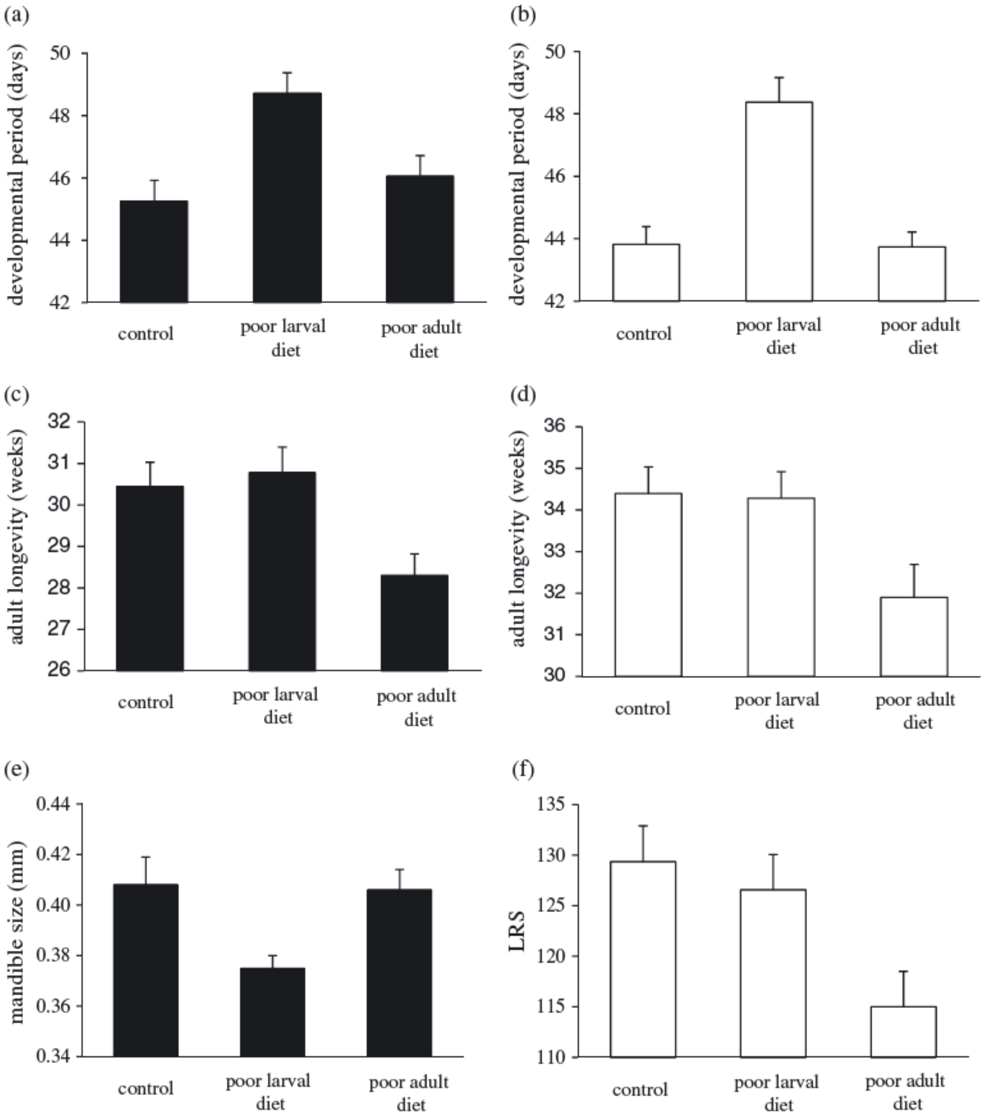


Fig. 6-1. Character means of males (black bars) and females (white bars). (a,b) Developmental period. (c,d) Adult longevity. (e) Male mandible size. (f) Female lifetime reproductive success (LRS). Data are mean ± SE.