

**Mechanisms of reproductive interference in seed beetles: experimental tests of
alternative hypotheses**

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1. GENERAL INTRODUCTION

Detecting the patterns in biological communities and explaining the causal processes that underlie them are fundamental goals of community ecology. One of such conspicuous patterns in biological communities is the absence of local coexistence among closely related species. For example, a pair of closely related species may be found in different local communities (Diamond 1975; Graves and Gotelli 1993; Cavender-Bares et al. 2006; Swenson et al. 2006) or utilize different microhabitat (Ohsaki and Sato 1994; Noriyuki et al. 2012; Walter et al. 2014). The absence of local coexistence, together with the fact that, in the absence of related species, organisms often thrive in the environment that is not used by them in nature (e.g., Hixon 1980; Ohsaki and Sato 1994; Noriyuki et al. 2011; Noriyuki and Osawa 2012), strongly suggest the presence of antagonistic interaction among closely related species. Based on this notion, Hutchinson (1957) proposed the distinction between fundamental and realized niche. Niche is (in Hutchinson's definition) a conceptual hyper-volume in the multidimensional space of environmental variables, within which a viable population can persist. Fundamental niche is the one in the absence of a focal competitor, and realized niche is that in the presence of the competitor. The requirement of a species for a specific suit of environmental variables, in turn, is expected to affect the availability of such environment (e.g., food item) to other species (MacArthur and Levins 1967), affecting its realized niche and ultimately determining the set of species that can coexist. The concept of niche, with its origin in early 20th century (Grinnell 1917; Elton 1927), has played a central role in the study of interspecific competition (Leibold 1995; Chase & Leibold 2003; McGill *et al.* 2006; Devictor et al. 2010). Yet, despite the conceptual

utility of niche, its empirical applicability has been controversial (Lewin 1983; Hubbell 2001; Chase and Leibold 2003; McGill *et al.* 2006).

Even though some modifications to the theory have been suggested (e.g., Connell 1990; Tokeshi 1999; Chase and Leibold 2003), niche theory has focused on competition over shared resource (Tokeshi 1999; Chase and Leibold 2003). On the other hand, some pioneering theoretical studies predicted that interspecific reproductive interaction can also affect species coexistence (Ribeiro and Spielman 1986; Kuno 1992; Yoshimura and Clark 1994). Interspecific reproductive interaction that affects a fitness component, such as persistent heterospecific courtship and heterospecific mating, is referred to as reproductive interference (Gröning and Hochkirch 2008; Kyogoku *in press*). Though hybridization has been known to affect species coexistence (Gröning and Hochkirch 2008), even interactions without hybridization can affect species coexistence, given fitness reduction. Reproductive interference is more likely to cause extinction of an interacting species than resource competition, as the extent of individual fitness reduction due to reproductive interference depends on relative abundance of the interacting species (e.g., Hettyey and Pearman 2003; Kishi *et al.* 2009; Kyogoku and Nishida 2012; Friberg *et al.* 2013). That is, less abundant species suffers from greater fitness loss, which will cause more pronounced abundance disadvantage in next generation, generating positive feedback toward extinction. Additionally, reproductive interference is expected to be more likely among closely related species, as closely related species are likely to share similar signaling traits. Therefore, reproductive interference is a likely alternative defining force of realized niche. Empirical studies, particularly in recent years, have indeed suggested the causal role of reproductive interference in affecting species coexistence (e.g., Colwell 1986; Fujimoto

et al. 1996; Thum 2007; Okuzaki et al. 2010; Takakura and Fujii 2010; Eaton 2012; Noriyuki et al. 2012; Friberg et al. 2013).

Reproductive interference occurs as behavioral interaction between individuals of different species, and it can eventually affect distribution of organisms through its effect on individual fitness. Here it is useful to distinguish three aspects related to reproductive interference (Kyogoku in press): the occurrence of interspecific reproductive interaction, the reduction of a fitness component due to it (component reproductive interference), and eventual reduction of absolute overall fitness (demographic reproductive interference). The occurrence of interspecific reproductive interaction is necessary for reproductive interference (i.e., a fitness component loss) to occur, but interspecific reproductive interaction is not necessarily costly. For example, as operational sex ratio is often male-biased, allocating a part of male mating effort to heterospecific mating interaction may not affect the male's fitness. Furthermore, if mate identification is error-prone, taking the risk of heterospecific mating attempt can be adaptive rather than strictly avoiding low quality (i.e., heterospecific) mates, particularly for males (Parker 1979, Parker and Partridge 1998; Schmeller et al. 2005; Takakura et al. in press). Even in females, the fitness cost of heterospecific mating without hybridization may depend on the species identity of the male (Shuker et al. in press). Even when interspecific reproductive interaction causes component reproductive interference (i.e., a fitness component loss), it may not translate into demographic reproductive interference. For example, reduced number of juveniles due to component reproductive interference may be compensated for by increased juvenile survival due to reduced competition among the juveniles. Therefore, for interspecific reproductive interaction to affect demography and distribution of organisms, that interspecific

reproductive interaction needs to cause a fitness component reduction, and then the fitness component reduction needs to translate into the change in absolute overall fitness (Kyogoku in press). Most of previous studies have concerned the occurrence of interspecific reproductive interaction and/or component reproductive interference. However, relations among interspecific reproductive interaction, component reproductive interference and demographic reproductive interference have received little attention. Specifically, factors that affect the strength of component reproductive interference (the extent of fitness component reduction) due to a specific form of interspecific reproductive interaction (e.g., interspecific mating) are unknown. Revealing this relation and identifying traits that determines the strength of component reproductive interference will allow us to identify selection pressures that are likely to affect the strength of component reproductive interference. For another, studies that documented demographic reproductive interference are scarce (but see De Barro et al. 2006; Kishi et al. 2009; Crowder et al. 2010; De Barro and Bourne 2010). Precise investigation of each relation will deepen our understanding to reproductive interference.

A pair of seed beetle species, *Callosobruchus chinensis* (Linnaeus) and *Callosobruchus maculatus* (Fabricius), is an exceptional system in which demographic reproductive interference has been documented, and is an ideal model system to investigate the relation between interspecific reproductive interaction, component reproductive interference and demographic reproductive interference. These two species show reproductive interference, which prevents their coexistence at least in laboratory condition (Kishi et al. 2009). Males of both species attempt to mate with females of the other species even in the presence of their conspecific female. Though the interspecific

mating attempt is reciprocal, component reproductive interference due to the interspecific reproductive interaction is almost unilateral, with *C. maculatus* females suffering from greater fecundity loss without hybridization. In the study by Kishi et al. (2009), this component reproductive interference by *C. chinensis* males on *C. maculatus* females translated into demographic reproductive interference, driving *C. maculatus* extinct within several generations when they are housed together despite the superiority of *C. maculatus* during larval resource competition. Even before Kishi et al. (2009), there have been many studies performing such competition experiments between *C. chinensis* and *C. maculatus* (e.g., Utida 1953; Yoshida 1966; Fujii 1965, 1967, 1969; Bellows and Hassell 1984; Ishii and Shimada 2008). Some of those studies reported the extinction of *C. maculatus* (e.g., Yoshida 1966; Bellows and Hassell 1984) as Kishi et al. (2009) did, but the others reported the extinction of *C. chinensis* (Utida 2008; Ishii and Shimada 2008). In addition, the strength of component reproductive interference by *C. chinensis* males on *C. maculatus* females varies depending on the strain of *C. chinensis* (Kishi 2014). Such variations in competition results and the strength of component reproductive interference may offer an opportunity to reveal the relations among interspecific reproductive interaction, component reproductive interference and demographic reproductive interference.

In this study, I attempt to reveal the mechanism of component reproductive interference in these seed beetles in order to identify the trait that affects the strength of reproductive interference and ultimately the underlying selection pressures that affect the strength of reproductive interference. In Chapter 2, I determine whether component reproductive interference by *C. chinensis* males on *C. maculatus* females is due to interspecific copulation or to persistent mating attempts, the former of which turns out

to be the case. In Chapters 3 and 4, I examined the precise mechanism by which interspecific copulation reduces fecundity. Specifically I considered two alternative hypotheses: the effect of heterospecific ejaculate and that of physical injury due to interspecific copulation. The former is addressed in Chapter 3, and the latter is addressed in Chapter 4. In Chapter 5, I briefly discuss the general implications of these studies. Hereafter in this thesis, “reproductive interference” refers to component reproductive interference (note that demographic one is a subset of component one, and any reproductive interference is component one), unless demographic reproductive interference is specified.

2. THE MECHANISM OF THE FECUNDITY REDUCTION IN *CALLOSOBRUCHUS MACULATUS* CAUSED BY *CALLOSOBRUCHUS* *CHINENSIS* MALES.

2.1. Introduction

Male promiscuity sometimes results in interspecific reproductive interaction, also known as reproductive interference (Gröning and Hochkirch 2008), which because of the costs it entails for the individuals involved affects the population growth rate (Ribeiro and Spielman 1986; Kuno 1992) and is an important factor determining community structure (Gröning and Hochkirch 2008). Reproductive interference has been documented in a wide range of taxonomic groups (Andrews et al. 1982; Söderbäck 1994; Hettyey and Pearman 2003; Dame and Petren 2006; Liu et al. 2007; Thum 2007; Valero et al. 2008), but our understanding of the mechanisms generating reproductive interference is still insufficient. In particular, it is still unclear whether one mechanism of reproductive interference is more prevalent than others across taxonomic groups. Because reproductive interference can potentially occur at any stage of mate acquisition (Gröning and Hochkirch 2008), it is difficult to elucidate its precise mechanism. For example, interspecific copulation is usually accompanied by pre-mating heterospecific sexual harassment (Okuzaki et al. 2010). Solely from behavioral observations, it is hard to distinguish whether the actual fitness reduction is due to the sexual harassment or to the interspecific copulation itself. Therefore, when multiple alternatives exist, the fitness cost of each potential mechanism must be examined separately to differentiate the

contribution of each to reductions in reproductive success. Few studies, however, have examined the precise mechanisms of reproductive interference in terms of reproductive success.

Two congeneric bean weevils, *Callosobruchus chinensis* (Linnaeus) and *C. maculatus* (Fabricius), show asymmetric reproductive interference. In a laboratory experiment, Kishi et al. (2009) found that *C. chinensis* males caused a larger fecundity reduction in *C. maculatus* females than *C. maculatus* males, whereas *C. maculatus* males hardly affected the fecundity of *C. chinensis*. Two possible mechanisms, which are not mutually exclusive, might explain the fecundity reduction in *C. maculatus* caused by heterospecific males: heterospecific sexual harassment and interspecific copulation. Males of both species attempt to mate with heterospecific females indiscriminately even in the presence of conspecific females (Kishi et al. 2009). These symmetric interspecific mating attempts might be expected to result in a symmetric fecundity reduction, suggesting that a behavioral difference in heterospecific sexual harassment between the species might be responsible for the observed asymmetry of the fitness cost. On the other hand, *C. chinensis* males and *C. maculatus* females sometimes copulate with each other, but *C. maculatus* males and *C. chinensis* females seldom do (Yamane and Miyatake 2010). To date, no study has compared the effects of these two possible mechanisms of reproductive interference to identify which is responsible for the fecundity reduction in *C. maculatus* by *C. chinensis* males.

Disentangling the costs of sexual harassment and copulation is a challenge when a female is subjected to both by the presence of a male. Several studies have experimentally quantified the effect of sexual harassment by using males that can attempt to mate with a female but cannot actually copulate (Sakurai and Kasuya 2008;

Gay et al. 2009), and the cost of interspecific copulation can be estimated by excluding the cost of heterospecific sexual harassment from the total cost caused by both. In this study, I ablated the genitals of *C. chinensis* males to prevent them from copulating while enabling them to court normally. Thus, a *C. maculatus* female housed with a *C. chinensis* male with ablated genitals experiences persistent heterospecific sexual harassment but no interspecific copulation.

The aim of this study was to elucidate the mechanism of reproductive interference by *C. chinensis* males on *C. maculatus* females by comparing how each mechanism affected lifetime fecundity. First, I differentiated the costs of heterospecific sexual harassment and interspecific copulation: I placed a *C. maculatus* female that had previously mated with a conspecific male together with an ablated or an intact *C. chinensis* male and then allowed her to lay eggs in the presence of the heterospecific male, keeping them together throughout the remainder of their lives. Then, I quantified the effects of this treatment on the fecundity of the *C. maculatus* female. I then conducted two additional experiments to further explore how the number of interspecific copulations affected the fecundity of *C. maculatus* females. In these experiments, I first examined the effect of a single interspecific copulation with a *C. chinensis* male on the fecundity of *C. maculatus* females. I then investigated the fecundity of *C. maculatus* females exposed to various numbers of *C. chinensis* males for 24 h prior to oviposition to estimate the effect of multiple interspecific copulations on the fecundity of *C. maculatus*, under the assumption that a *C. maculatus* female exposed to more *C. chinensis* males would experience more interspecific copulations. Finally, I examined whether the number of *C. chinensis* males housed with a *C. maculatus* female actually altered the number of interspecific copulations that the *C. maculatus* female

experienced.

2.2. Materials and methods

2.2.1. INSECTS

I used the jC-F strain of *C. chinensis* (see Harano and Miyatake 2005) and the hQ strain of *C. maculatus* (see Miyatake and Matsumura 2004), because the same strains were used in the studies that confirmed asymmetric reproductive interference by *C. chinensis* on *C. maculatus* (Kishi et al. 2009; Kyogoku and Nishida 2012). Both strains were derived from strains maintained at Okayama University. Unless they have also copulated with a conspecific male, *C. maculatus* females do not lay eggs after interspecific copulation with a *C. chinensis* male, and no ejaculate is transferred during the interspecific copulation (Yamane and Miyatake 2010). Hatched eggs are readily distinguishable from those that have not hatched because they become opaque. In my experiments, I used only virgin individuals that had emerged within 24 h from the bean in which they developed, and when necessary I allowed them to copulate with a conspecific male before the experiments. In a preliminary analysis, I determined the effect of *C. chinensis* males on the hatching rate of *C. maculatus* eggs to establish how the definition of fecundity (number of eggs or number of hatchlings) might affect the analysis results. For this purpose, I investigated the hatchability of eggs laid by *C. maculatus* females (previously mated with a conspecific male) in the presence or

absence of a *C. chinensis* male (16 replications of each treatment). I analyzed the data using a generalized linear mixed model (GLMM; Wolfinger and O'Connell 1993) with a binomial error structure and a logit link function, incorporating individual *C. maculatus* females as a random effect and calculating the likelihood by Laplace approximation. I found that males of *C. chinensis* had no significant effect on the hatching rate of *C. maculatus* eggs, which was $82.9 \pm 3.5\%$ (mean $\pm$ SE) in the presence of a *C. chinensis* male and $84.2 \pm 3.9\%$ in the absence of a *C. chinensis* male (Wald test: *C. chinensis* male, coefficient = -0.12 ± 0.34 , $z = -0.35$, $P = 0.73$). Therefore, I defined fecundity as the number of hatched eggs, because the number of hatched eggs is a better predictor of the population growth rate than the total number of eggs. I performed all experiments under fixed environmental conditions (30°C, 70% relative humidity, and 16 h: 8 h light: dark).

2.2.2. CONTINUOUS EXPOSURE OF *C. MACULATUS* FEMALES TO A GENITAL ABLATED AND AN INTACT *C. CHINENSIS* MALE

While *C. chinensis* males were copulating, I anesthetized them briefly and used a razor to ablate their genitals (see Sakurai and Kasuya 2008). Neither the number of mating attempts nor longevity differed significantly between males with ablated genitals and non-ablated males. Ablated *C. chinensis* males attempted to mate 5.00 ± 1.34 times within 20 min and lived 10.25 ± 0.50 days, whereas non-ablated males attempted to mate 5.81 ± 0.76 times and lived 11.13 ± 0.24 days (Wilcoxon rank sum test: mating attempts, $W = 152$, $N_1 = N_2 = 16$, $P = 0.37$; longevity, $W = 90.5$, $N_1 = N_2 = 16$, $P = 0.15$).

Therefore, I assumed that genital ablation had no effect on *C. chinensis* males other than preventing them from copulating. In this experiment, I first allowed a *C. maculatus* female to copulate with a conspecific male. Within an hour after the copulation, I housed the mated *C. maculatus* female together with an ablated *C. chinensis* male throughout the remainder of their lives in a plastic Petri dish ($\phi 70$ mm $\times$ 15 mm) and provided 20 adzuki beans, *Vigna angularis* (Willd.) cv. Dainagon, as an oviposition substrate. I counted the number of hatched eggs in the Petri dish 20 days after the introduction of the beetles, by which time all of the beetles, which have a 1–2 week lifespan, had died. I similarly investigated the fecundity of a *C. maculatus* female housed with an intact (i.e., without ablated genitals) *C. chinensis* male or alone (without any *C. chinensis* male) as positive and negative controls, respectively. I performed 16 replications of each treatment. I first used Bartlett test to examine the homogeneity of the variances of the fecundity among treatments. Because the Bartlett test result showed significant heterogeneity of the variances (see Results), I then used the *F* test to determine which pairs of treatments showed significant differences in the variances. I used Welch's *t* test to detect a significant difference in mean fecundity between treatments when I found a significant difference in the variances. If I did not detect a significant difference in variances, I used a pairwise *t* test assuming equal variance to compare fecundity between treatments. I controlled the false discovery rate by the method of Holm (1979). I conducted all statistical analyses with R software version 2.14.1 (R Development Core Team 2011).

2.2.3. SINGLE INTERSPECIFIC COPULATION

First, I allowed a *C. maculatus* female to copulate with a conspecific male. Within an hour after the copulation, I placed the mated *C. maculatus* female with a *C. chinensis* male in a mating arena ($\phi 56 \text{ mm} \times 17 \text{ mm}$) until they copulated with each other. After one interspecific copulation, I transferred the *C. maculatus* female to a plastic Petri dish ($\phi 70 \text{ mm} \times 15 \text{ mm}$) and allowed the female to lay eggs on 20 adzuki beans until she died. I also prepared another female and reversed the order of the conspecific and heterospecific copulations: I allowed a *C. maculatus* female to copulate with a *C. chinensis* male before she copulated with a conspecific male and then after the conspecific copulation, the female was allowed to lay eggs in the same manner. As a control, I allowed a mated *C. maculatus* female that had not copulated with a *C. chinensis* male to lay eggs in a plastic Petri dish. I counted the number of hatched eggs in each Petri dish 20 days after the introduction of the female. I performed 16 replications of each treatment. In the first treatment (interspecific copulation after conspecific mating), I observed no interspecific copulation during one hour of observation in two replications. I therefore excluded these two replications from my analysis, replacing them with two more in which interspecific copulation was observed. I used the Bartlett test to check whether variances were equal among treatments. Then, because the result showed no significant difference in the variances (see Results), I used analysis of variance (ANOVA) to analyze the effect of a single interspecific copulation on the fecundity of *C. maculatus*.

2.2.4. EXPOSURE OF *C. MACULATUS* FEMALES TO *C. CHINENSIS* MALES PRIOR TO OVIPOSITION

I housed a *C. maculatus* female that had mated with a conspecific male within an hour with one, two, or three *C. chinensis* males in a plastic Petri dish ($\phi 70 \text{ mm} \times 15 \text{ mm}$) without any oviposition substrate for 24 h. Then, I removed the *C. chinensis* males, provided the *C. maculatus* female with 20 adzuki beans, and allowed her to lay eggs until she died. I also housed a *C. maculatus* female that had mated with a conspecific male within an hour alone in a plastic Petri dish for 24 h without any oviposition substrate, and then provided her with 20 adzuki beans and allowed her to lay eggs until she died. I performed 16 replications of each treatment. I counted the number of hatched eggs in each Petri dish 20 days after the introduction of the beetles. To analyze the effect of the number of *C. chinensis* males on the fecundity of the *C. maculatus* female, I used a GLMM with a Poisson error structure and a log link function, incorporating the individual females of *C. maculatus* into the model as a random effect and calculating likelihood by Laplace approximation.

2.2.5. INTERSPECIFIC COPULATION RATE

I housed a *C. maculatus* female that had mated with a conspecific male within an hour with one, two, or three *C. chinensis* males in a plastic Petri dish ($\phi 70 \times 15 \text{ mm}$). One minute after the introduction, I started to observe interspecific copulations and I recorded the number that occurred during a 20-min period. I allowed the beetles to acclimate for 1 min before beginning our observations because some beetles feigned death at first. I examined whether the incidence rate of interspecific copulations was proportional to the number of *C. chinensis* males by using a generalized linear model

with a Poisson error structure and a log link function.

2.3. Results

2.3.1. CONTINUOUS EXPOSURE OF *C. MACULATUS* FEMALES TO A GENITAL ABLATED AND AN INTACT *C. CHINENSIS* MALE

The variance of the fecundity of *C. maculatus* differed significantly among treatments (Bartlett test: $\chi^2 = 8.88$, $df = 2$, $P = 0.01$). The fecundity of *C. maculatus* females that laid eggs in the presence of an intact *C. chinensis* male varied significantly more than that of *C. maculatus* females that laid eggs in the presence of an ablated *C. chinensis* male (F test with Holm correction: $F_{15, 15} = 5.16$, $P = 0.009$). I found no significant difference in the other comparisons (F test with Holm correction: negative control versus positive control, $F_{15, 15} = 0.58$, $P = 0.30$; negative control versus genital ablation treatment, $F_{15, 15} = 3.00$, $P = 0.08$). The average fecundity of *C. maculatus* was 38.69 ± 4.33 (mean $\pm$ SE) hatched eggs in the presence of an intact *C. chinensis* male, and 54.19 ± 3.30 hatched eggs in the absence of any *C. chinensis* male (Fig. 2.1), a significant difference (pairwise t test with Holm correction: $t_{30} = 2.85$, $P = 0.02$). The average fecundity of *C. maculatus* in the presence of an ablated *C. chinensis* male was 57.56 ± 1.90 hatched eggs (Fig. 2.1), which was not significantly different from that in the absence of a *C. chinensis* male (pairwise t test with Holm correction: $t_{30} = 0.89$, $P = 0.38$), but which was significantly larger than the average fecundity in the presence of

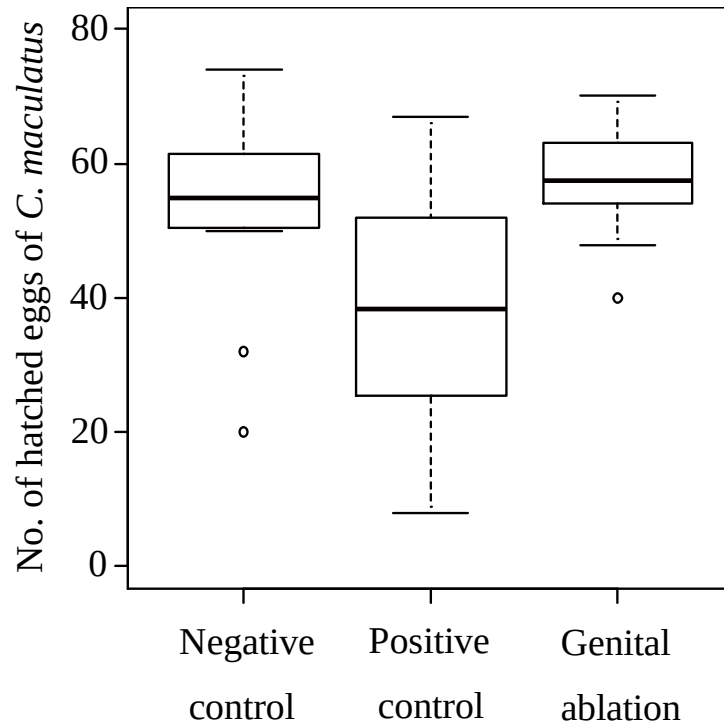


Fig. 2.1 The fecundity (number of hatched eggs) of *C. maculatus* females that were allowed to lay eggs in the presence or absence of a *C. chinensis* male (intact or not): Negative control, a mated *C. maculatus* female alone; Positive control, a mated female in the presence of an intact *C. chinensis* male; and Genital ablation, a mated female in the presence of a *C. chinensis* male with ablated genitals. The bold horizontal line in each box is the median, and the top and bottom of the box are one quartile above and below the median, respectively. The whiskers extend to the most extreme data within a 1.5 interquartile range. Dots are data beyond the whiskers.

an intact *C. chinensis* male (Welch's *t* test with Holm correction: $t_{20.60} = 3.99$, $P = 0.002$).

2.3.2. SINGLE INTERSPECIFIC COPULATION

The variances of fecundity did not differ significantly among treatments (Bartlett test: $\chi^2 = 3.03$, $df = 2$, $P = 0.22$). Fecundity of *C. maculatus* was not significantly affected by a single interspecific copulation with a *C. chinensis* male, regardless of whether the heterospecific or the conspecific copulation occurred first (ANOVA: $F_{2,45} = 1.00$, $P = 0.38$, Fig. 2.2).

2.3.3. EXPOSURE OF *C. MACULATUS* FEMALES TO *C. CHINENSIS* MALES PRIOR TO OVIPOSITION

The average fecundity of *C. maculatus* not exposed to a *C. chinensis* male was 55.94 ± 4.19 hatched eggs (Fig. 2.3). The average fecundity of *C. maculatus* decreased with the number of *C. chinensis* males to which the female was exposed prior to oviposition; average fecundity was 48.44 ± 4.73 , 17.88 ± 5.80 , or 13.00 ± 5.48 hatched eggs after exposure to one, two, or three *C. chinensis* males, respectively (Fig. 2.3). This reduction in fecundity as the number of *C. chinensis* males increased was significant (Wald *z* test: *C. chinensis* males, coefficient = -1.35 ± 0.32 , $z = -4.26$, $P < 0.001$).

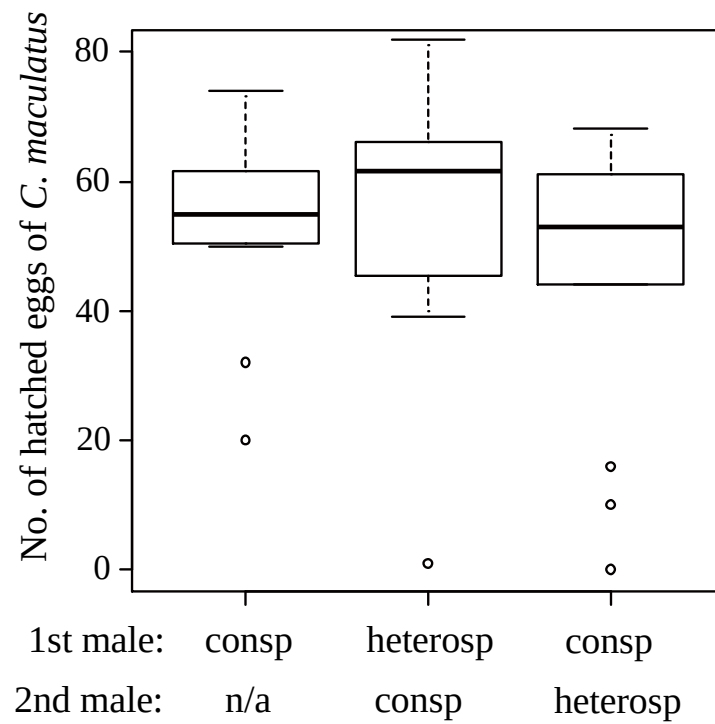


Fig. 2.2 The fecundity (number of hatched eggs) of *C. maculatus* females that copulated with only a *C. maculatus* (conspecific) male, or both a *C. maculatus* male and a *C. chinensis* (heterospecific) male. See the legend of Fig. 2.1 for the explanation of the box and whisker plots.

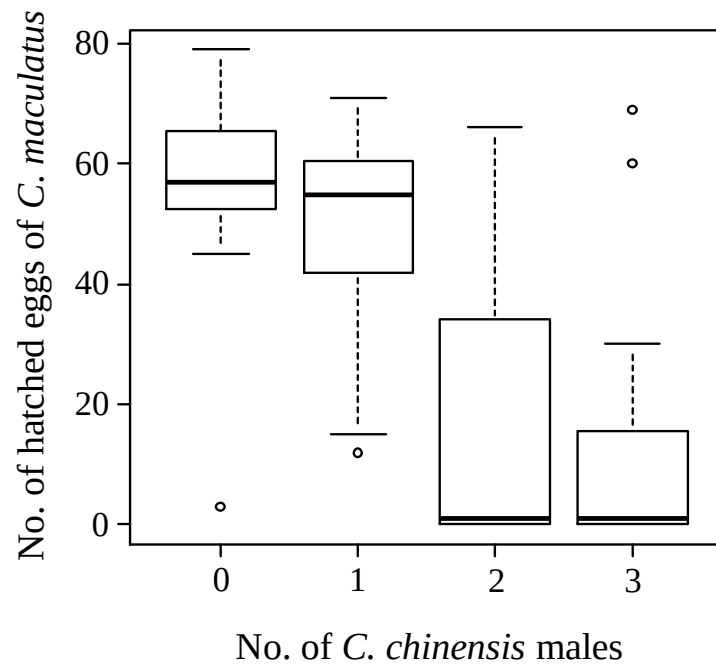


Fig. 2.3 The fecundity (number of hatched eggs) of *C. maculatus* females exposed to different numbers of *C. chinensis* males for 24 h prior to oviposition. See the legend of Fig. 2.1 for the explanation of the box and whisker plot.

2.3.4. INTERSPECIFIC COPULATION RATE

When housed with a single *C. chinensis* male, females of *C. maculatus* experienced on average 0.63 ± 0.18 interspecific copulations in 20 min. When housed with two or three *C. chinensis* males, females of *C. maculatus* experienced on average 1.00 ± 0.24 or 1.75 ± 0.47 interspecific copulations, respectively, in 20 min. The number of interspecific copulations increased significantly with the number of *C. chinensis* males (Wald test: *C. chinensis* males, coefficient = 0.52 ± 0.18 , $z = 2.93$, $P = 0.003$).

2.4. Discussion

Being housed continuously with a *C. chinensis* male reduced the fecundity of *C. maculatus* females but only when the males had intact genitals (Fig. 2.1). These results indicate that interspecific copulation, rather than heterospecific sexual harassment, caused the fecundity reduction in *C. maculatus*. A single interspecific copulation, however, did not significantly affect the fecundity of *C. maculatus*, regardless of the order of the heterospecific and conspecific copulations (Fig. 2.2). On the other hand, the fecundity of *C. maculatus* decreased when females were exposed to *C. chinensis* males for 24 h prior to oviposition, and their fecundity was negatively correlated with the number of *C. chinensis* males to which they were exposed (Fig. 2.3). I also found that the incidence rate of interspecific copulation increased when a *C. maculatus* female was housed with more *C. chinensis* males. These results suggest that the fecundity of *C. maculatus* females decreases depending on the number of interspecific copulations with

C. chinensis males that a female experiences. Taken together, my results lead me to conclude that the fecundity reduction in *C. maculatus* females by *C. chinensis* males most likely resulted from their repeated interspecific copulations.

With regard to the reproductive interference by *C. chinensis* males on *C. maculatus* females, my experiments elucidated the importance of interspecific copulation between *C. chinensis* males and *C. maculatus* females by separating the effects of this mechanism of reproductive interference from the effect of heterospecific sexual harassment and by quantifying lifetime fecundity (Fig. 2.1). Though Kishi et al. (2009) previously reported that *C. chinensis* males persistently harass *C. maculatus* females, the fitness cost of sexual harassment alone was not statistically significant in my experiment (Fig. 2.1). This result indicates that to reveal the behavioral mechanism of reproductive interference, it is not sufficient to document the incidence of interspecific reproductive interaction; the effects of different potential mechanisms of reproductive interference must be distinguished and reproductive success must be quantified. Many previous studies have investigated mechanisms of reproductive interference without quantifying reproductive success (reviewed by Gröning and Hochkirch 2008). Unless reproductive success is quantified, the fitness costs of reproductive interference cannot be exactly known but might be either over- or underestimated. The relative prevalence of different mechanisms of reproductive interference might be revealed by quantifying their effects on reproductive success across various taxonomic groups.

I found that the incidence rate of interspecific copulation increased when *C. maculatus* females were housed with more *C. chinensis* males, which suggests that *C. maculatus* females did not show any refractory period against *C. chinensis* males.

Although the remating receptivity of *C. maculatus* females with conspecific males is reported to be low (Miyatake and Matsumura 2004; Edvardsson and Tregenza 2005), interspecific copulation with a *C. chinensis* male does not induce a refractory period in a *C. maculatus* female (Yamane and Miyatake 2010). However, the frequent incidence of interspecific copulation of *C. maculatus* females with *C. chinensis* males is probably attributable to a behavioral difference between *C. chinensis* and *C. maculatus* males, because in my experiments I used already mated *C. maculatus* females, which should have shown low remating receptivity. For example, mating attempts by *C. chinensis* males may not induce fleeing behavior in *C. maculatus* females.

Several studies have examined the ecological significance of interspecific copulations that do not result in hybrid formation (Vick 1973; Nakano 1985; Takafuji 1986; Collins and Margolies 1991; Ben-David et al. 2009; Yamane and Miyatake 2010). However, when a single interspecific copulation is observed to have only a negligible effect, researchers have rarely considered interspecific copulation to be of potential ecological importance (e.g., Vick 1973; Nakano 1985; but also see Okuzaki et al. 2010), perhaps because they considered only heterospecific fertilization to have ecological significance. Besides sperm transfer, however, copulation sometimes involves the transfer of toxic substances (Chapman et al. 1995; Rice 1996) or causes injury to the genital organs (Crudgington and Siva-Jothy 2000; Rönn et al. 2007), both of which are evolutionary consequences of sexual conflict. Furthermore, a morphological genital mismatch may cause the genitals of both sexes to be destroyed during interspecific copulation (Sota and Kubota 1998). If any of these phenomena quantitatively affect the fitness of the individuals involved, then even apparently harmless interspecific copulation may be costly, especially if it occurs repeatedly. My results indicate that

interspecific copulation can quantitatively affect reproductive success (Fig. 2.3). Therefore, even apparently harmless interspecific copulation can play an ecologically important role in nature. A possible example from nature may be the host plant partitioning that occurs in two sympatric ladybird species, *Henosepilachna vigintioctomaculata* and *Henosepilachna pustulosa* (Katakura 1981), which might be caused by repeated interspecific copulations. Interspecific copulations between these species have been observed when their host plants are adjacent (Nakano 1985). Most eggs from an interspecific copulation do not hatch, but females that mate once with a conspecific and once with a heterospecific male produce as many offspring as females that mate with conspecific males twice (Nakano 1985). Thus, a single interspecific copulation between these species is apparently harmless, as long as the female also copulates with a conspecific male. However, repeated interspecific copulation might result in reproductive interference, that is, a reduction in the fitness of the individuals involved, and thus might contribute to the observed host plant partitioning (Colwell 1986; Kuno 1992).

The results of this study suggest that repeated interspecific copulations, but not a single interspecific copulation or heterospecific sexual harassment, cause reproductive interference by *C. chinensis* males on *C. maculatus* females. Further, apparently harmless interspecific copulations are of potential ecological importance because multiple interspecific copulations can be costly for the individuals involved even if the effect of a single one is negligible. Moreover, it was necessary to quantify lifetime fecundity to show the importance of multiple interspecific copulations as a mechanism of reproductive interference. Such quantification is essential to advance our understanding of the mechanisms of reproductive interference.

3. DOES HETEROSPECIFIC SEMINAL FLUID REDUCE FECUNDITY IN INTERSPECIFIC COPULATION BETWEEN SEED BEETLES?

3.1. Introduction

Closely related species are likely to share similar sexual signaling traits, and males sometimes try to mate with females of other species. Interspecific sexual interactions, such as heterospecific mating attempts and matings, can be costly to the females involved, and are generally referred to as reproductive interference. Reproductive interference, defined as any interspecific sexual interaction that affects the fitness of at least one species involved, has been documented in various taxonomic groups, including insects (Gröning and Hochkirch 2008). Reproductive interference can have ecological and evolutionary consequences, such as the displacement of native species by invasive ones (Liu et al. 2007; Kondo et al. 2009), maintenance of parapatric distribution (Fujimoto et al. 1996; Thum 2007), habitat segregation (Noriyuki et al. 2012; Friberg et al. 2013), and character displacement (Bargielowski et al. 2013). Thus, reproductive interference can be an important interspecific interaction affecting community organization (Gröning and Hochkirch 2008). Reproductive interference occurs through various behavioral mechanisms such as signal jamming, interspecific mating, and hybridization (Gröning and Hochkirch 2008). Of these, interspecific mating has been one of the most frequently reported mechanism (e.g., Takafuji 1988; Söderbäck 1994; Sota and Kubota 1998; Kondo et al. 2009; Tripet et al. 2011). However, little is known about what determines the extent of fitness reduction due to interspecific

mating, especially when no hybrid is produced (e.g., Shuker et al. in press). Identifying the traits affecting the fitness cost of interspecific copulation and reproductive interference in general is critical to understanding the nature of reproductive interference.

I hypothesized that sexual conflict is one of the evolutionary forces that determines the strength of reproductive interference. Sexual conflict is the conflict between the evolutionary interests of males and females within a species (Parker 1979; Arnqvist and Rowe 2005), and it is most likely to occur during reproduction. Males are favored by selection if they can manipulate their mates or outcompete rival males to enhance their own reproductive success (Parker 2006), but a trait that increases the reproductive success of a male can be harmful to the females that he mates with (e.g., Chapman et al. 1995; Stutt and Siva-Jothy 2001; Wigby and Chapman 2005; Hotzy and Arnqvist 2009; Garcia-Gonzalez and Simmons 2010; Hotzy et al. 2012). For example, the seminal fluid of *Drosophila melanogaster* lowers female mating rates, therefore securing paternity, but it is toxic to females and reduces female fitness (Chapman et al. 1995; Wigby and Chapman 2005). The evolution of male harmful trait can cause female counter adaptations, which can in turn modify the selection acting on the male trait. Tension between male and female reproductive strategies can create rapidly changing coevolutionary dynamics of male and female traits (Gavrilets 2000). Thus, sexually selected traits can vary among closely related species or even among populations within a species (Rönn et al. 2007; Hotzy and Arnqvist 2009; Sugano and Akimoto 2011). During secondary contacts between allopatric species, male harmful traits that have evolved due to intraspecific sexual conflict may inflict additional fitness costs of interspecific copulation on heterospecific females and may prevent the coexistence of

species (see also Kawatsu 2013). However, although many studies have examined the role of sexual conflict in the evolution of reproductive isolation and consequent speciation (e.g., Arnqvist et al. 2000; Gavrillets 2000; Gavrillets and Waxman 2002; Martin and Hosken 2003), the role of sexual conflict in the distribution patterns of interacting closely related species has received little attention.

The seed beetle species *Callosobruchus chinensis* (Linnaeus) and *Callosobruchus maculatus* (Fabricius) are an ideal model system for reproductive interference research (Kishi et al. 2009; Yamane and Miyatake 2010; Kyogoku and Nishida 2012, 2013; Kishi and Tsubaki 2014). Males of both species indiscriminately try to mate with females of the other species even when the choice between conspecific and heterospecific females is given (Kishi et al. 2009). However, the occurrence of interspecific copulation is almost unilateral, being mostly between *C. maculatus* females and *C. chinensis* males (Yamane and Miyatake 2010). The interspecific copulation between *C. maculatus* females and *C. chinensis* males does not produce hybrid offspring; instead, it reduces the number of eggs laid per *C. maculatus* female, resulting in almost unilateral reproductive interference from *C. chinensis* to *C. maculatus* (Kishi et al. 2009; Kyogoku and Nishida 2013). This fecundity loss is known to occur as a result of repeated, rather than single, interspecific copulations (Kyogoku and Nishida 2013). However, the mechanism by which repeated interspecific copulation reduces the fecundity of *C. maculatus* is unknown. Furthermore, *C. chinensis* shows intraspecific variation in the strength of reproductive interference; the extent of fitness costs on *C. maculatus* females varies among *C. chinensis* males from different populations (Kishi et al. 2009; Kyogoku and Sota unpublished results). This intraspecific variation in the strength of reproductive interference may result from

variations in some reproductive traits among males, such as those involved in antagonistic coevolution between males and females. *Callosobruchus* beetles have also been a model system for sexual conflict studies (e.g., Crudgington and Siva-Jothy 2000; Edvardsson and Tregenza 2005; Eady et al. 2007; Rönn et al. 2007; Hotzy and Arnqvist 2009; Hotzy et al. 2012; Sakurai et al. 2012; Yamane 2013), and the mechanisms by which male *Callosobruchus* beetles affect conspecific female fitness have been extensively studied. The seminal fluid of *C. chinensis* includes toxic substances that negatively affect female longevity (Yamane 2013). Male *C. chinensis* also have spines on their genitalia (Rönn et al. 2007; Sakurai et al. 2012) that injure female reproductive organs during copulation. These male traits involved in intraspecific sexual conflict may also cause deleterious effects on females of closely related species during interspecific copulation, with phenotypic variation in such traits potentially generating the variation in the strength of reproductive interference.

As a first step to identify the male traits affecting the extent of fecundity reduction, I aimed to elucidate the mechanism by which interspecific copulation reduces fecundity in seed beetles. Specifically, I addressed the effects of interspecific ejaculate transfer on the fitness of *C. maculatus* females and tested the hypothesis that *C. chinensis* ejaculate is the major cause of the fecundity reduction in *C. maculatus* caused by *C. chinensis* males. I first observed mating between a female and a male in all combinations of species in a no-choice experiment by manipulating the female mating experience to confirm the asymmetric and repeated occurrence of interspecific copulation. Previous studies have not systematically examined the effects of the female mating experience on the occurrence of interspecific copulations. Kishi et al. (2009) and Kyogoku and Nishida (2013) used only females that had once mated with a conspecific

male, and Yamane and Miyatake (2010) used only virgin individuals. Second, I examined the incidence of interspecific ejaculate transfer. Yamane and Miyatake (2010) reported the absence of ejaculate transfer from *C. chinensis* males to *C. maculatus* females in a single interspecific copulation. However, the fecundity reduction due to reproductive interference occurs only when *C. maculatus* females have copulated with *C. chinensis* males repeatedly (Kyogoku and Nishida 2013). Thus, it is possible that the probability of ejaculate transfer during a single interspecific copulation event is low and that only repeated copulations result in substantial interspecific ejaculate transfer. Finally, because I found frequent ejaculate transfer from *C. chinensis* males to *C. maculatus* females, I studied the effect of the ejaculate on fecundity by injecting the extract from *C. chinensis* male reproductive organs into *C. maculatus* females, which simulated interspecific ejaculate transfer. Yamane and Miyatake (2010) performed similar injection experiments to examine its effect on female mating propensity, but the effect of the extract injection on fecundity has not been studied.

3.2. Materials and methods

3.2.1. ANIMALS

I used the jC-F strain of *C. chinensis* (Harano and Miyatake 2005) and the hQ strain of *C. maculatus* (Miyatake and Matsumura 2004), both of which were derived from the stocks at Okayama University (provided by Takahisa Miyatake). These two strains have

been used in the previous studies of reproductive interference between *C. chinensis* and *C. maculatus* (Kishi et al. 2009; Kyogoku and Nishida 2012, 2013; Kishi and Tsubaki 2014). Repeated interspecific copulations with *C. chinensis* males reduce the fecundity of *C. maculatus*, but the hatchability of eggs is not affected by interspecific copulations (Kyogoku and Nishida 2013). Females of the strains I used typically do not accept further conspecific mates for at least a day after a single conspecific mating (Miyatake and Matsumura 2004). Adults of both species were reproductively active immediately after emergence. I used virgin individuals within 24 h after emergence. All experiments were performed under laboratory conditions (30°C, RH 70%, 16L8D), unless otherwise noted.

3.2.2. MATING PROPENSITY

I determined the mating propensity of a virgin female and a virgin male for conspecific and heterospecific pairs in the first mating trial (i.e., *C. chinensis* pairs, $n = 61$; *C. maculatus* pairs, $n = 41$; *C. chinensis* female $\times$ *C. maculatus* male pairs, $n = 42$; *C. maculatus* female $\times$ *C. chinensis* male pairs, $n = 63$). Each pair was housed in a plastic Petri dish (56-mm diameter $\times$ 17 mm deep), and beetles were given 1 min to acclimate. I then observed mating behavior for 20 min and recorded whether mating (genital coupling) occurred (i.e., binary data). I also recorded whether males attempted to mate at least once during the observation.

Within 30 min after the first mating trial, I determined the remating propensity of once-mated females. Females that had mated in the first mating trial were housed

with a virgin male of either species, and their mating behavior was observed as in the first mating trial. I prepared all possible conspecific and heterospecific pairs except for pairs including *C. chinensis* female mated with a *C. maculatus* male in the first mating trial, as no mating was observed between them in the first mating trial. Sample sizes for each treatment are shown in Fig. 1. All mating experiments were conducted at room temperatures of 24.4–30.5°C. Because temperature may affect mating and remating propensity (Katsuki and Miyatake 2009), I recorded the room temperature at the time of each trial.

3.2.3. INTERSPECIFIC EJACULATE TRANSFER

To examine the incidence of ejaculate transfer through a single interspecific copulation, I allowed each *C. maculatus* female to mate with a *C. chinensis* male once in a plastic Petri dish (50-mm diameter × 15 mm deep). When beetles did not mate within 30 min, they were discarded. Thus, I discarded seven females to obtain 16 females with a single interspecific copulation. The females were frozen at –20°C within 1 h after mating. Then, I dissected the females under a dissecting microscope (SMZ800, Nikon, Japan) to remove the bursa copulatrix onto a glass slide. The bursa copulatrix was opened, and its content was applied on the glass slide. After dehydration, the sample was stained with Giemsa. I observed the sample with an optical transmission microscope (BX53, Olympus, Japan) at 400 × magnification and recorded the presence or absence of sperm; I inferred the occurrence of ejaculate transfer from the presence of sperm. Because I observed no interspecific mating between *C. chinensis* females and *C. maculatus* males,

this experiment was performed only for *C. maculatus* females.

Next, I examined the incidence of interspecific ejaculate transfer when a female had multiple heterospecific mates, as *C. maculatus* females experience more interspecific copulations when housed with more *C. chinensis* males (Kyogoku and Nishida 2013). I housed a *C. maculatus* female with three *C. chinensis* males in a plastic Petri dish (70-mm diameter × 15 mm deep) for 24 h. Then, I sacrificed the female by freezing and examined the presence or absence of sperm in her bursa copulatrix. I also examined the incidence of ejaculate transfer from *C. maculatus* males to *C. chinensis* females in the same way, as repeated mating attempts by *C. maculatus* males may result in a few successful interspecific copulations and consequent ejaculate transfer despite the low frequency of interspecific mating between *C. chinensis* females and *C. maculatus* males (Yamane and Miyatake 2010). The sample size was 16 replications for both species. In preliminary observations, I confirmed the presence of sperm in the bursa copulatrix 24 h after conspecific mating in both species. Therefore, I assumed that the presence of sperm in the bursa copulatrix indicated the occurrence of interspecific ejaculate transfer. No sperm ejection is known in these beetles.

3.2.4. EXTRACT INJECTION

I injected the extract from the testes and accessory glands of *C. chinensis* males into *C. maculatus* females using the simplified method of Yamane and Miyatake (2010) and examined its effect on the fecundity of *C. maculatus* females. I dissected 50 *C. chinensis* males in 0.9% saline and collected their testes, accessory glands, and

ejaculatory bulbs, which contained seminal fluid. The samples were stored in a microfuge tube (1.5 ml) and kept in a freezer (−20°C) until use. Immediately before the experiment, I added 5.0-μl saline to the sample and homogenized it. After centrifugation at 12,000 rpm for 10 min at 4°C, the supernatant (hereafter “extract”) was collected for use in the injection.

I first examined the effect of injecting the extract on the conspecific mating receptivity of *C. maculatus* females to confirm the physiological activity of the extract because that from the accessory glands and testes of *C. chinensis* males is known to reduce the mating rate of *C. maculatus* females (Yamane and Miyatake 2010). I injected 50 nl of extract (equivalent to the extract from 0.5 individuals) into the abdomen of a virgin *C. maculatus* female using an injector (Nanoject II Auto-Nanoliter Injector, Drummond Scientific, USA). Females were anesthetized by diethyl ether immediately before injection. More than 3 hours after the injection, I housed each female with a conspecific male in a plastic Petri dish (50-mm diameter × 15 mm deep) and recorded the incidences of copulation in the 20 min after 1-min acclimation. As a control, I performed the same experiment using intact *C. maculatus* females and a second control group that was injected with 50-nl saline. Each treatment included 20 females.

I then examined the effect of the extract injection on the fecundity of *C. maculatus* females. I first allowed a *C. maculatus* female to mate once with a conspecific male. After mating, I injected 50 nl of extract into the female. I then housed her in a plastic Petri dish (50-mm diameter × 15 mm deep) with 20 adzuki beans, *Vigna angularis* (Willd.) cv. Dainagon. The female was allowed to lay eggs on the beans until she died. Eighteen days after the injection, after the female had died and all viable eggs had hatched, I counted the number of hatched eggs and used this number as a measure

of fecundity. The fecundity of *C. maculatus* is correlated with female body size (Fox 1993), so I also measured the lengths of both elytra as a proxy of body size using a dissecting microscope (SMZ800, Nikon, Japan) equipped with an eyepiece micrometer (R1000-25, Shibuya Optical, Japan). I similarly quantified the fecundity of females who were intact or who received the saline control. Extract injection, saline injection, and intact control were replicated for 23, 29, and 16 times, respectively.

3.2.5. STATISTICAL ANALYSES

All statistical analyses were performed with R software version 2.15.1 (R Development Core Team 2012). The proportion of mating and remating *C. maculatus* females (i.e., data of first and second mating trials combined) were analyzed by generalized linear mixed models (GLMMs) using the glmer function of the lme4 package. I assumed a binomial error structure for the occurrence of mating, which was binary. Explanatory variables were male species, prior mating experience of the female, their interaction, and temperature. Female identity was incorporated as a random effect. The maximum likelihood was calculated by Gauss–Hermite quadrature. First, I examined the effect of temperature using a likelihood ratio test (LRT) by dropping the temperature term. I then examined the effect of the interaction term by LRT. Whenever the null hypothesis was rejected, including in the analyses below, post hoc tests were conducted using the Holm–Bonferroni method (Holm 1979) to control for false discovery rates. In the post hoc tests, I created three subsets of data, each of which consisted of two categories of prior female mating experience (virgin, mated with a *C. chinensis*, or mated with a *C.*

maculatus), and then the effect of the interaction term was examined by LRT for each data subset. I did not conduct GLMM analysis for the mating propensity of *C. chinensis* females because I observed no mating of *C. chinensis* females with *C. maculatus* males, and appropriate model fitting was difficult due to the null incidence of mating in some categories of the data. I analyzed the data for *C. maculatus* females in isolation from that of *C. chinensis* females for the same reason.

A generalized linear model (GLM) with a binomial error structure was fitted to the data of interspecific ejaculate transfer. I used experimental treatment as the sole explanatory variable, which consisted of three categories: *C. maculatus* female with a single interspecific copulation, *C. maculatus* female exposed to three heterospecific males for 24 h, and *C. chinensis* female exposed to three heterospecific males for 24 h. I used the single treatment term, rather than a combination of two different treatment terms (male species and mating regime), because my experimental design was not orthogonal (i.e., *C. chinensis* female with a single mating was absent) due to the low mating frequency of *C. chinensis* females and *C. maculatus* males. I used LRT to examine the effect of the treatments and conducted post hoc tests for all comparisons, except that between *C. maculatus* female with a single interspecific copulation and *C. chinensis* female with three heterospecific males, which was outside of our interest.

The proportion of receptive *C. maculatus* females after extract injection was described as a function of treatments using a GLM with binomial error structure. The effect of experimental treatment was examined by LRT. I analyzed the fecundity data using an ANCOVA with experimental treatments and elytral length (mean of both elytra) incorporated into the model as explanatory variables.

3.3. Results

3.3.1. MATING PROPENSITY

Regardless of the room temperature or experimental treatment, 85.0% or more males of both species attempted to mate (Table 3.1). Room temperature during mating observations had no significant effect on the mating propensity of *C. maculatus* females (LRT: $\chi^2 = 1.01$, $df = 1$, $P = 0.31$). Thus, I excluded the temperature term from the GLMMs in the following analyses. The interaction between male species and female mating experience had a significant effect on the proportion of mated *C. maculatus* females (LRT: $\chi^2 = 47.70$, $df = 2$, $P < 0.0001$; Fig. 3.1a); the mating propensity of *C. maculatus* female was affected by male species, female mating experience, and their interaction. Mating propensity did not differ significantly between virgin *C. maculatus* females and those mated once with a *C. chinensis* male, but mating with a *C. maculatus* male made the female significantly less likely to remate with a conspecific (*C. maculatus*) male in the second mating trial (LRT with Holm–Bonferroni correction: virgin vs. mated with a *C. chinensis*: $\chi^2 = 3.602$, $df = 1$, $P = 0.058$; virgin vs. mated with a *C. maculatus*: $\chi^2 = 47.00$, $df = 1$, $P < 0.0001$; mated with a *C. chinensis* vs. mated with a *C. maculatus*: $\chi^2 = 12.48$, $df = 1$, $P = 0.0008$; Fig. 3.1a).

No *C. chinensis* female mated with a *C. maculatus* male regardless of her mating experience (Fig. 3.1b), although most *C. maculatus* males tried to mate with *C. chinensis* females (Table 3.1). Conversely, 68.9% of virgin *C. chinensis* females mated

Table 3.1 Proportions of males attempting to mate in each experimental treatment.

Male	Female	Prior mating experience of female	<i>n</i>	Proportion of males attempting to mate (%)
<i>C. chinensis</i>	<i>C. chinensis</i>	virgin	61	93.4
	<i>C. chinensis</i>	Mated with <i>C. chinensis</i>	20	85.0
	<i>C. chinensis</i>	Mated with <i>C. maculatus</i>	NA	NA
	<i>C. maculatus</i>	virgin	63	87.3
	<i>C. maculatus</i>	Mated with <i>C. chinensis</i>	20	95.0
	<i>C. maculatus</i>	Mated with <i>C. maculatus</i>	21	95.2
<i>C. maculatus</i>	<i>C. chinensis</i>	virgin	42	97.6
	<i>C. chinensis</i>	Mated with <i>C. chinensis</i>	20	85.5
	<i>C. chinensis</i>	Mated with <i>C. maculatus</i>	NA	NA
	<i>C. maculatus</i>	virgin	41	100
	<i>C. maculatus</i>	Mated with <i>C. chinensis</i>	21	100
	<i>C. maculatus</i>	Mated with <i>C. maculatus</i>	20	95.0

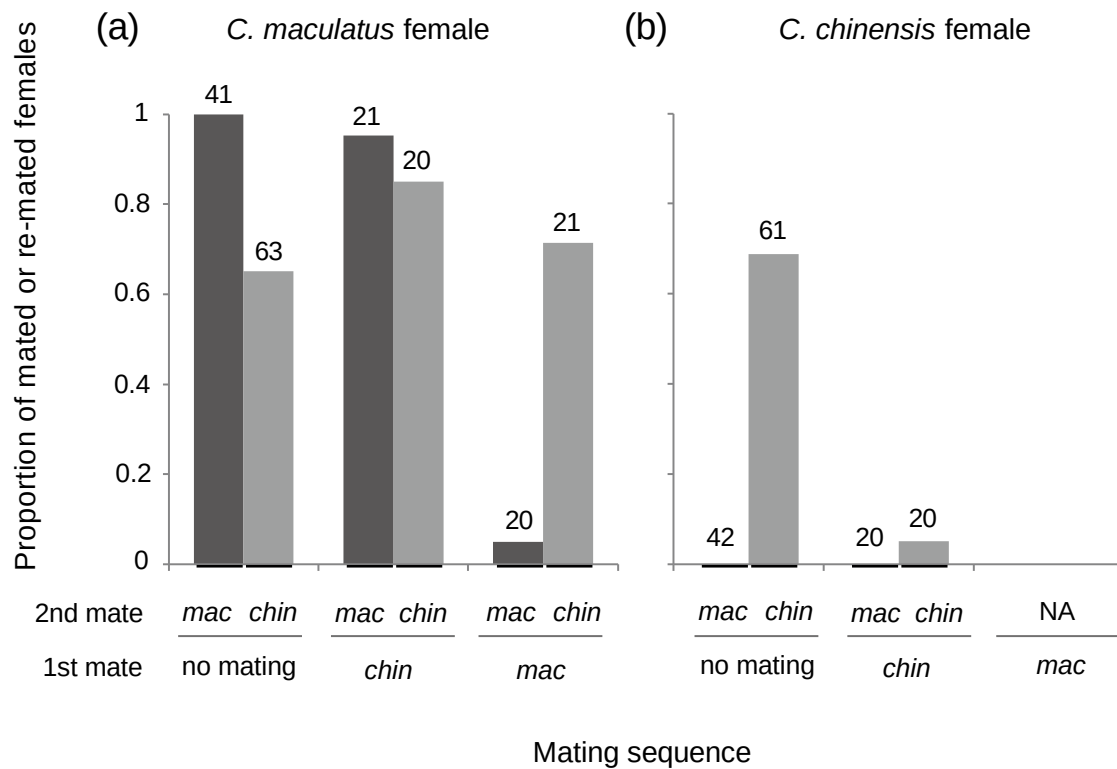


Fig. 3.1 Proportion of mating/remating with a conspecific/heterospecific male for females, which had different previous mating experiences. a: results for *C. maculatus* females. b: results for *C. chinensis* females. No *C. chinensis* female mated with a *C. maculatus* male in the first mating trial, and the data of the second mating trial for this combination are lacking. Numbers above the bars indicate sample sizes. Abbreviations *mac* and *chin* refer to *C. maculatus* and *C. chinensis*, respectively.

with a conspecific male (Fig. 3.1b). Only one of 20 *C. chinensis* females that first mated with a *C. chinensis* male remated with a *C. chinensis* male (Fig. 3.1b).

3.3.2. INTERSPECIFIC EJACULATE TRANSFER

The incidence of ejaculate transfer (as indicated by the presence of sperm) differed significantly among treatments (LRT: $\chi^2 = 17.69$, $df = 2$, $P = 0.0001$; Fig. 3.2).

Callosobruchus maculatus females housed with three *C. chinensis* males for 24 h were more likely to have heterospecific sperm than were *C. maculatus* females with a single interspecific copulation and *C. chinensis* females housed with three *C. maculatus* males for 24 h (LRT with Holm–Bonferroni correction: *C. maculatus* female housed with three *C. chinensis* males for 24 h vs. *C. maculatus* females with a single interspecific copulation: $\chi^2 = 16.09$, $df = 1$, $P = 0.0001$; *C. maculatus* females housed with three *C. chinensis* males for 24 vs. *C. chinensis* females housed with three *C. maculatus* males for 24 h: $\chi^2 = 7.197$, $df = 1$, $P = 0.007$).

3.3.3. EXTRACT INJECTION

The proportion of mated *C. maculatus* females differed significantly among treatments (LRT: $\chi^2 = 24.58$, $df = 2$, $P < 0.0001$; Fig. 3.3). Females with extract injection were significantly less likely to mate with a conspecific male than were females of the other treatments (LRT with Holm–Bonferroni correction: extract vs. intact: $\chi^2 = 19.53$, $df = 1$,

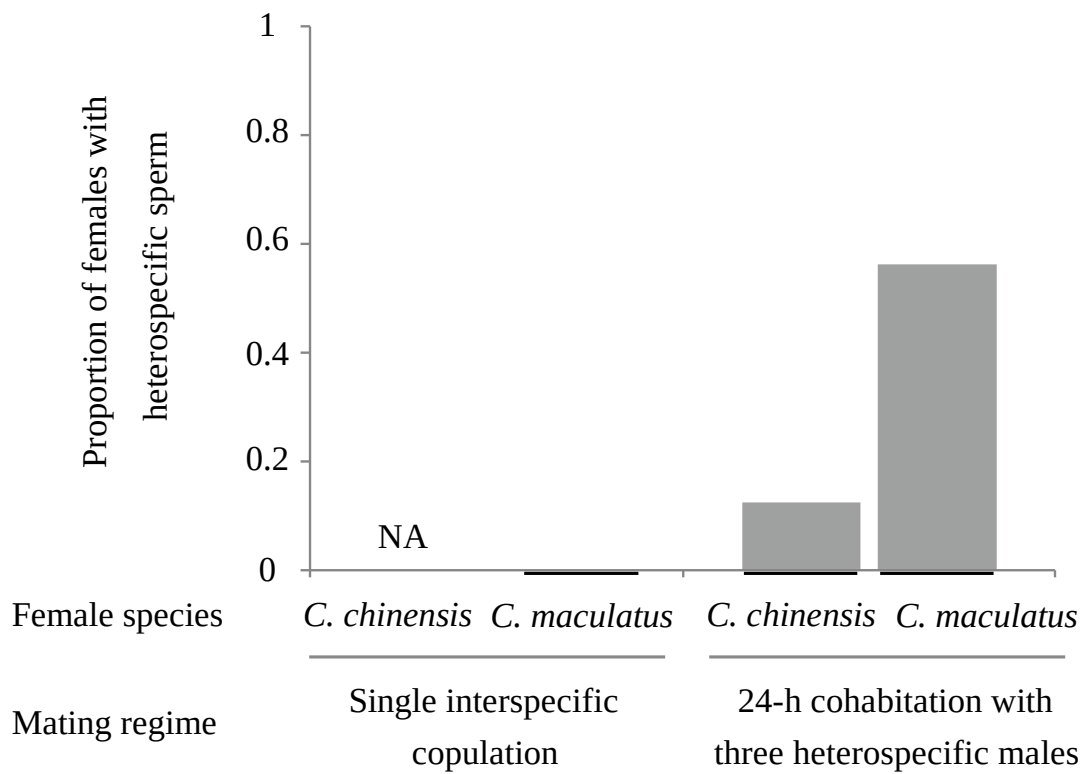


Fig. 3.2 Proportion of females with heterospecific sperm after a single interspecific copulation or 24-h cohabitation with three heterospecific males. The data for *C. chinensis* females with a single interspecific copulation are lacking because no *C. chinensis* female mated with a *C. chinensis* male. $N = 16$ for each treatment. $P < 0.0001$; extract vs. saline: $\chi^2 = 13.40$, $df = 1$, $P = 0.0005$; intact vs. saline: $\chi^2 = 1.412$, $df = 1$, $P = 0.30$).

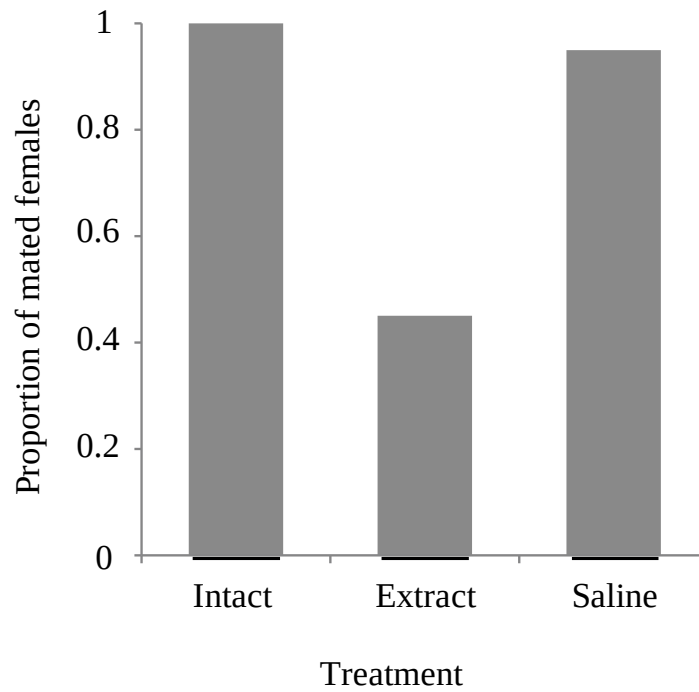


Fig. 3.3 Proportions of mating *C. maculatus* females, which were (1) intact, (2) injected with extract from *C. chinensis* male reproductive organs, or (3) injected with saline. Male species is *C. maculatus* (conspecific). $N = 20$ for each treatment.

Female fecundity differed significantly among treatments after the effect of body size was removed (LRT: treatment: $F = 3.481$, $df = 2$, 64 , $P = 0.037$; elytral length: $F = 0.318$, $df = 1$, 64 , $P = 0.58$; Fig. 3.4). However, post hoc tests showed no significant difference among treatments (LRT with Holm–Bonferroni correction: extract vs. intact: $F = 5.895$, $df = 1$, 36 , $P = 0.061$; extract vs. saline: $F = 1.271$, $df = 1$, 49 , $P = 0.265$; intact vs. saline: $F = 2.754$, $df = 1$, 42 , $P = 0.208$), probably due to outliers. Females who produced three or fewer viable eggs were considered as outliers because all other females produced 20 or more viable eggs (Fig. 3.4). The proportion of outliers did not differ significantly among treatments (LRT: $\chi^2 = 1.75$, $df = 2$, $P = 0.42$). After removing the outliers, female fecundity again differed significantly among treatments (LRT: treatment: $F = 13.47$, $df = 2$, 60 , $P < 0.0001$; elytral length: $F = 2.186$, $df = 1$, 60 , $P = 0.15$). In post hoc comparisons, the fecundity of females injected with either extract or saline was significantly smaller than that of intact females (LRT with Holm–Bonferroni correction: extract vs. intact: $F = 34.76$, $df = 1$, 33 , $P < 0.0001$; saline vs. intact: $F = 15.60$, $df = 1$, 39 , $P = 0.0006$), with extract and saline injection reducing fecundity by 22.94 and 17.91 hatched eggs, respectively, compared with intact females (Fig. 3.4). The fecundity did not differ significantly between extract- and saline-injected females (LRT with Holm–Bonferroni correction: extract vs. saline: $F = 1.738$, $df = 1$, 47 , $P = 0.19$).

3.4. Discussion

My experiments reproduced frequent interspecific copulation between *C. maculatus*

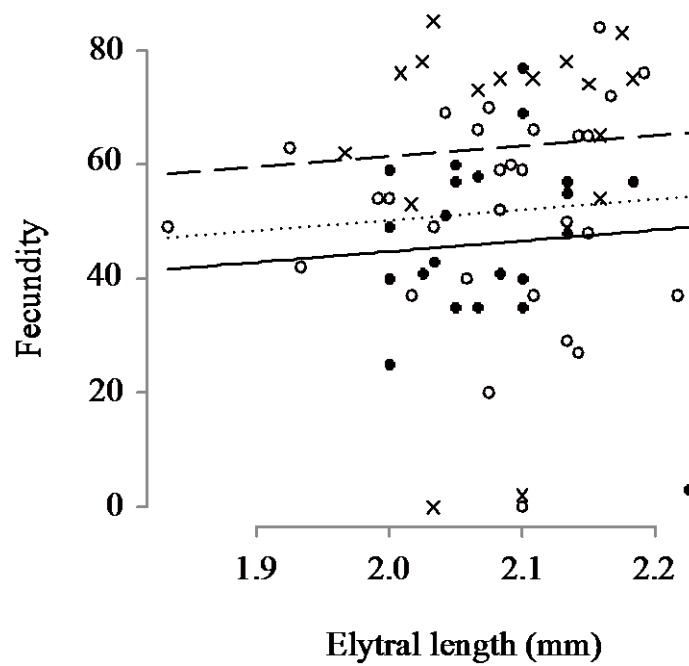


Fig. 3.4 Fecundity of *C. maculatus* females plotted against elytral length. Filled circles and solid line, females injected with the extract from *C. chinensis* reproductive organs; open circles and dotted line, females injected with saline; crosses and broken line, intact females. The lines represent the regressions for each female group.

females and *C. chinensis* males regardless of female mating experience (Fig. 3.1a). Although conspecific mating induced a refractory period in *C. maculatus* females (i.e., females refrained from remating with a conspecific; Fig. 3.1a), *C. maculatus* females in the refractory period were as likely to remate with *C. chinensis* males as were virgin females (Fig. 3.1a), suggesting that *C. maculatus* females were unable to reject mating attempts by *C. chinensis* males. On the other hand, no *C. chinensis* female mated with a *C. maculatus* male (Fig. 3.1b). Although most males of both species attempted to mate across experimental treatments (Table 3.1), male mating behaviors differ between these two species (Kishi et al. 2009). The male *C. maculatus* mounts a female before inserting its aedeagus into the female genitalia. In contrast, a male *C. chinensis* has a long aedeagus and can insert it into the female's genitalia without mounting. This interspecific behavioral difference may make it difficult for *C. maculatus* females to avoid copulation with *C. chinensis* males. The absence of interspecific mating between *C. chinensis* females and *C. maculatus* males may be due to interspecific difference in genital size. The aedeagus and corresponding female reproductive tract of *C. maculatus* are larger than those of *C. chinensis* (Yamane and Miyatake 2010). It may be difficult to insert the thicker aedeagus of *C. maculatus* into the narrower reproductive tract of *C. chinensis*. Alternatively, a *C. chinensis* female may be better at fleeing from a heterospecific mate than a *C. maculatus* female is. I observed that *C. chinensis* females often fled from *C. maculatus* males that attempted to mate, so it is possible that *C. chinensis* female behavior contributed to the absence of interspecific copulation between *C. chinensis* females and *C. maculatus* males.

As expected, the asymmetric and repeated incidence of interspecific mating resulted in asymmetric interspecific ejaculate transfer. While no ejaculate transfer

occurred from *C. chinensis* males to *C. maculatus* females during a single interspecific copulation (Fig. 3.2), more than half of *C. maculatus* females had sperm of *C. chinensis* after 24-h cohabitation with three *C. chinensis* males (Fig. 3.2), during which *C. maculatus* females probably mated with *C. chinensis* males repeatedly and consequently received ejaculate. On the other hand, a relatively small portion of *C. chinensis* females had *C. maculatus* sperm after 24-h cohabitation with three *C. maculatus* males. My results for both female mating propensity and ejaculate transfer were largely consistent with those of Yamane and Miyatake (2010) in that *C. maculatus* females were more prone to interspecific mating than were *C. chinensis* females, and a single interspecific copulation rarely resulted in ejaculate transfer from *C. chinensis* males to *C. maculatus* females. I further demonstrated that frequent interspecific copulation between *C. chinensis* males and *C. maculatus* females translated into more frequent ejaculate transfer from *C. chinensis* males to *C. maculatus* females, suggesting its potential role in the fecundity reduction of *C. maculatus*.

Injection of the extract from *C. chinensis* males made *C. maculatus* females less receptive than saline did (Fig. 3.3), confirming the physiological activity of the extract. That is, the extract induced a refractory period in the injected females (Yamane and Miyatake 2010). However, the effect of extract injection on fecundity did not differ significantly from that of saline injection, although there were significant fecundity differences between intact females and those who underwent saline or extract injection (Fig. 3.4). Specifically, the injection procedure affected *C. maculatus* fecundity, but the extract *per se* did not reduce *C. maculatus* fecundity, suggesting only a negligible role of interspecific ejaculate transfer in reproductive interference in the seed beetles. An alternative interpretation of these results is that *C. chinensis* seminal proteins that

putatively affect *C. maculatus* fitness may need to be delivered directly to the female reproductive tract (Green and Tregenza 2009). I have assumed that the effect of the extract, if any, would result from the direct injection into hemolymph based on Yamane's (2013) experiment on the toxic effect of *C. chinensis* ejaculate on conspecific females performed by direct injection into hemolymph. However, injected seminal proteins may show different effects on conspecific and heterospecific females, and some seminal proteins may show a negative effect on heterospecific females that depends on the site of delivery. I cannot reject this possibility because the physiological mechanism by which ejaculate affects females in *Callosobruchus* beetles is largely unknown.

About 50% of *C. maculatus* females had *C. chinensis* ejaculate after 24-h cohabitation with three *C. chinensis* males (Fig. 3.2). However, this proportion of ejaculate transfer seems to be too small to explain the previously reported 75% fecundity reduction in *C. maculatus* females similarly cohabitating with three *C. chinensis* males for 24 h (Kyogoku and Nishida 2013). Even in an extreme case in which females receiving the ejaculate deposited no egg, the expected fecundity reduction would be 50% if the interspecific ejaculate transfer were the only mechanism by which *C. chinensis* males reduced the fecundity of *C. maculatus*. Moreover, the probability of ejaculate transfer from a *C. chinensis* male to a *C. maculatus* female per mating was very low (Fig. 3.2), and a *C. maculatus* female would have had sufficient time to lay at least some eggs before receiving *C. chinensis* ejaculate, even if the ejaculate had some effect on the fecundity of *C. maculatus* females. Therefore, ejaculate transfer is likely not the mechanism by which fecundity is reduced.

The incidence of interspecific ejaculate transfer during the course of oviposition may prevent females from conspecific remating by inducing a refractory

period (Tripet et al. 2011). den Hollander and Gwynne (2009) reported that 60% of *C. maculatus* females remated with a conspecific male by 24 h after first mating, so their remating rate is not trivial (see also Miyatake and Matsumura 2004). Several studies have reported benefits of conspecific ejaculate for *C. maculatus* females (Fox 1993; Savalli and Fox 1999; Arnqvist et al. 2005; Rönn et al. 2006, but see also Crudgington & Siva-Jothy 2000; Eady et al. 2007; den Hollander & Gwynne 2009). Thus, potential remating prevention due to interspecific ejaculate transfer might be the mechanism of reproductive interference by *C. chinensis* males on *C. maculatus* females. However, this is unlikely for two reasons. First, the previously reported positive effect of remating on *C. maculatus* female is relatively small compared with the effect of reproductive interference by *C. chinensis*. Fox (1993) reported increased fecundity due to remating, but the increase was about 10% at most. On the other hand, cohabitation with a single *C. chinensis* male results in about 30% fecundity loss in *C. maculatus* (Kishi et al. 2009; Kyogoku and Nishida 2013), which is unlikely to be compensated for by conspecific remating. Second, reproductive interference by *C. chinensis* males on *C. maculatus* females occurs even when females are prevented from remating (Kishi et al. 2009; Kyogoku and Nishida 2013). Thus, although a negative effect of remating prevention due to interspecific ejaculate transfer cannot be rejected completely, its effect should be too small to be the major source of fecundity reduction caused by *C. chinensis* males.

The present results call for testing my alternative hypothesis that *C. chinensis* males reduce the fecundity of *C. maculatus* females by injuring the females' reproductive tracts. Male genitalia of *C. chinensis* have spines, and these genital spines may scratch female reproductive organs during interspecific copulation, as in conspecific mating (Rönn et al. 2007). Because the fecundity loss in *C. maculatus*

females occurs when these females copulate with *C. chinensis* males repeatedly (Kyogoku and Nishida 2013), accumulated injury on female reproductive organs may gradually render the female unable to lay eggs. Additionally, *Callosobruchus* beetles show intraspecific variations in male traits that potentially affect female fitness through accumulated genital injury, such as genital spines (Hotzy and Arnqvist 2009) and locomotion (Nakayama and Miyatake 2010). *Callosobruchus chinensis* males with different spine lengths or numbers may inflict different fitness cost on *C. maculatus* females. Also, *C. chinensis* males with differing locomotion may show different encounter rates with *C. maculatus* females, resulting in different interspecific mating rates, which is also likely to generate variation in the fitness cost on *C. maculatus*. Direct examination of the role of a reproductive organ's injury in the reproductive interference in this system may thus allow us to identify the *C. chinensis* male trait that creates the variation in the fitness cost of reproductive interference on *C. maculatus*.

In conclusion, my experiments revealed that interspecific ejaculate transfer is not likely to be the major mechanism of reproductive interference on *C. maculatus* females by *C. chinensis* males. Therefore, it is needed to test my alternative hypothesis, namely that injuries within the reproductive tract due to interspecific copulation are the primary mechanism of reproductive interference in this system. Directly testing this hypothesis is an important step to identifying the trait-level mechanism of reproductive interference in seed beetles and consequently to enhancing our understanding of reproductive interference in general.

4. MALES WITH MORE HARMFUL GENITAL STRUCTURES CAUSE STRONGER REPRODUCTIVE INTERFERENCE THROUGH INTERSPECIFIC COPULATION

4.1. Introduction

The contingency of interspecific competitive interactions on traits of the organisms involved is a fundamental tenet of community ecology (McGill et al. 2006; Devictor et al. 2010). Research in various taxa has shown an association of spatio-temporal community structure with trait differences among species, such as canine teeth size in carnivorous mammals (Dayan et al. 1990), wing length in bird-eating hawks (Schoener 1984), and leaf traits in trees (Cavender-Bares et al. 2006). However, persistent correlations between differences in a given trait and species coexistence do not prove the functional role of that trait (Horn and May 1977). Therefore, an empirical examination of the causal mechanisms by which certain traits affect the strength of species interactions is necessary. Nevertheless, empirical evidence for a mechanistic causation relation between traits and interspecific competitive interactions is amazingly scarce (Grace 1990; Mouquet et al. 2012). Hence, our understanding of the trait contingency of interspecific competitive interaction is greatly limited (Bengtsson et al. 1994; Mouquet et al. 2012). Mechanistic understanding of species interactions is an important prerequisite for predicting the outcome of an interspecific interaction from the traits of the involved organisms and, therefore, for forecasting community dynamics. Improving our understanding of the trait contingency of competitive interspecific

interaction is thus an urgent need in the face of global change.

Ecologists have long focused on ecological traits (i.e., those relevant to resource competition and trophic interactions) as determinants of the extent of interspecific competitive interaction (Chase and Leibold 2003; McGill et al. 2006; Devictor et al. 2010). On the other hand, little is known about the role of reproductive traits in interspecific competitive interactions (but see Sota and Kubota 1998; Okuzaki et al. 2010; Eaton et al. 2012; Nishida et al. 2014). Nevertheless, reproductive interference can be an ecologically important species interaction that affects species coexistence and, thereby, community structure (Gröning and Hochkirch 2008; Kyogoku in press). Reproductive interference is defined as any interspecific reproductive interaction, such as interspecific mating attempts and interspecific copulation, that affects a fitness component of either or both interacting individuals (Gröning and Hochkirch 2008; Kyogoku in press). The extent of fitness loss due to reproductive interference likely depends on the reproductive traits of the species involved; hence, reproductive traits may affect the strength of interspecific competitive interaction.

I previously hypothesized that harmful male traits arising from sexual selection, such as sperm competition, may pleiotropically affect the strength of interspecific competitive interaction by altering the extent of reproductive interference (Kyogoku and Sota 2015). The evolution of harmful male traits due to sexual selection has been documented in the context of sexual conflict. Sexual conflict is a conflict of evolutionary interests between males and females within a species (Parker 1979; Arnqvist and Rowe 2005), and it is especially likely to occur during reproduction. A male can increase its fertilization success by manipulating its mate or outcompeting rival males, even at the cost of the female's fitness (Parker 1979) as is reported in

various taxa (Arnqvist and Rowe 2005; Parker 2006; Madjidian and Lankinen 2009; Lange et al. 2013). For example, the ejaculate of *Drosophila melanogaster* induces a refractory period in females and enhances egg production by them, but it also affects fitness components of the females, such as fecundity and longevity (Wigby and Chapman 2005). During secondary contacts between originally allopatric species, such harmful male traits may reduce the fitness of heterospecific females via reproductive interference, such as by interspecific mating, and may ultimately affect species coexistence, because reproductive interference can cause species exclusion (Ribeiro and Spielman 1986; Kuno 1992; Liu et al. 2007; Kishi et al. 2009).

The seed beetles *Callosobruchus chinensis* (Linnaeus) and *Callosobruchus maculatus* (Fabricius) constitute an ideal model system for testing this hypothesis. This model system has been used already for reproductive interference research (Kishi et al. 2009; Yamane and Miyatake 2010; Kyogoku and Nishida 2012, 2013; Kishi and Tsubaki 2014; Kyogoku and Sota 2015). Males of both species try to mate with females of the other species, even in the presence of conspecific females (Kishi et al. 2009). In contrast, interspecific copulation is mainly unilateral, being more frequent between *C. maculatus* females and *C. chinensis* males than vice versa (Yamane and Miyatake 2010; Kyogoku and Sota 2015). This asymmetric interspecific copulation reduces the number of eggs laid by *C. maculatus* females (Kyogoku and Nishida 2013). Furthermore, *C. chinensis* males from different populations differ in the extent of the fecundity reduction that they induce in *C. maculatus* females (Kishi 2014). Interpopulational differences in some male trait related to sexual conflict may be responsible for this observed variation in the extent of reproductive interference. However, the mechanism by which interspecific copulation reduces the fecundity of *C. maculatus* is not yet fully

understood.

Callosobruchus seed beetles are also a model system for sexual conflict research (e.g., Crudgington and Siva-Jothy 2000; Rönn et al. 2007; Hotzy and Arnqvist 2009; Hotzy et al. 2012; Sakurai et al. 2012; Yamane 2013), and the mechanisms by which male seed beetles affect conspecific female fitness through mating are well studied. Male *Callosobruchus* beetles have spines on their genitalia (Fig. 4.1), which increase the bearer's fertilization success (Hotzy and Arnqvist 2009; Hotzy et al. 2012) but injure the female's reproductive tract (Crudgington and Siva-Jothy 2000; Rönn et al. 2007). Moreover, the ejaculate of *C. chinensis* males is known to be toxic to females, reducing their longevity (Yamane 2013). I previously showed that interspecific ejaculate transfer is not the major mechanism by which *C. chinensis* males reduce *C. maculatus* fecundity, and I hypothesized that physical injury to the female reproductive tract caused by the genital spines is the likely mechanism (Kyogoku and Sota 2015). This hypothesis has not yet been directly tested.

In this study, I examined the role of genital spines in reproductive interference by *C. chinensis* males on *C. maculatus* females. I conducted experiments in which I used *C. chinensis* males from nine separate strains with different origins to exploit the phenotypic variation among them. By housing *C. chinensis* males and *C. maculatus* females together, I first examined whether injury to the female reproductive tract is indeed a mechanism of fecundity reduction. Second, I investigated whether the occurrence of female injury was associated with the morphological dimensions of the male genitalia.

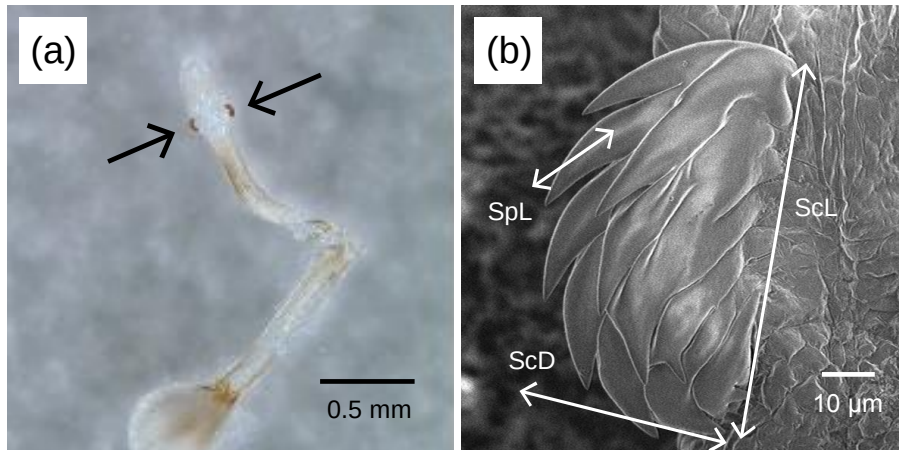


Fig. 4.1 Genital structure of *C. chinensis* male. (a) Genitalia of *C. chinensis* male, with two sclerites (arrows), and (b) a *C. chinensis* male genital sclerite. SpL, spine length; ScD, sclerite depth; ScL, sclerite length. Photographs by D. Kyogoku.

4.2. Materials and methods

4.2.1. STUDY SPECIES

Both *C. chinensis* and *C. maculatus* are postharvest pests of beans of tribe Phaseoleae (Tuda et al. 2006), and their larvae grow inside beans. Hatchlings become adults in about three weeks. Adult beetles can mate and lay eggs soon after their emergence from the bean, and they live for about a week when no food or water is supplied. I used the hQ strain of *C. maculatus* (Miyatake and Matsumura 2004) and the jC-F, isC, mC, smC02, tsC98, mgC98, kiC07, yoC02, and mrC98 strains of *C. chinensis* (Kondo et al. 1999; Harano and Miyatake 2005; 2010) for this study; I used multiple strains of *C. chinensis* to ensure sufficient phenotypic variation. The hQ strain of *C. maculatus* and the jC-F strain of *C. chinensis* used were derived from stocks at Okayama University (provided by Takahisa Miyatake), and the remaining strains were derived from stocks at Kyushu University (provided by Tomohiro Harano, currently at Sokendai University). I used virgin beetles within 24 hours after their emergence. The experiment was performed under laboratory conditions (30 °C; RH, 70%; 16L8D).

4.2.2. OVIPOSITION IN THE PRESENCE OF A HETEROSPECIFIC MALE

I first allowed all of the *C. maculatus* females to mate with a conspecific male once, and then I housed each female with a *C. chinensis* male in a plastic Petri dish (70 mm in

diameter × 15 mm deep). Each Petri dish contained 20 adzuki beans, *Vigna angularis* (Willd.) cv. Dainagon, as an oviposition substrate, and the females were allowed to lay eggs on the beans in the presence of the *C. chinensis* male until they died. I observed the beetles every day, and when either beetle in a Petri dish was found dead or almost dead (i.e., unable to walk), I removed the *C. chinensis* male and preserved its genitalia in 70% ethanol for later morphological observation (see below). About 20 days after the introduction of the beetles to the Petri dishes, by which time all viable eggs had hatched, I counted the number of hatched eggs, which I hereafter refer to as fecundity. I also observed if females had injured genitalia (Fig. 4.2); I recorded the occurrence of an externally identifiable injury as binary data. Because fecundity correlates with female body size (Fox 1993), I also measured female elytral length as a surrogate for body size. This experiment was replicated for 304 times, with 34 or 35 replications for each *C. chinensis* strain.

4.2.3. CALLOSOBRUCHUS CHINENSIS MALE GENITAL MORPHOLOGY

I removed the genitalia from all *C. chinensis* males used in the experiment, together with the ejaculatory bulbs, and put them into distilled water. As the genitalia absorbed water, the internal pressure increased, and the endophallus (intromittent organ) with sclerites eventually was everted. If eversion was not successfully induced in this way within 15 min, I attempted to manually evert the endophallus by using two pairs of fine tweezers. I excluded males for which manual eversion failed from the following analysis. Everted genitalia were put into 70% ethanol until use. Before morphological

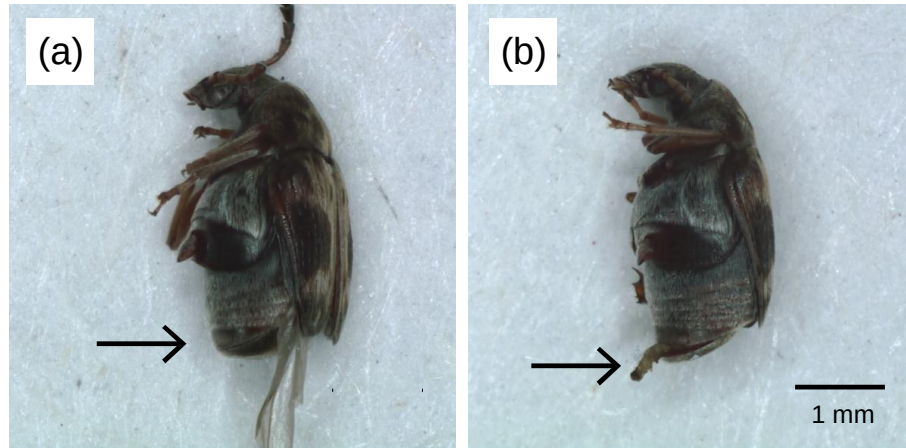


Fig. 4.2 Genital injury in a *C. maculatus* female after lifelong cohabitation with a *C. chinensis* male. (a) A female housed without a *C. chinensis* male without any externally identifiable injury (arrow). (b) In contrast, this *C. maculatus* female housed with a *C. chinensis* male has an externally identifiable injury to her genitalia: a protruding reproductive tract (arrow). Hind legs were removed for observation. Photographs by D. Kyogoku.

observation, the genitalia were transferred to 99% ethanol for dehydration and kept there for at least a day. Male *C. chinensis* genitalia have a lateral sclerite on each side of the endophallus (Fig. 4.1a), and these sclerites are covered with spines (Fig. 4.1b). I photographed the sclerites under a scanning electron microscope (VE-8800, Keyence, Osaka, Japan) and quantified their morphological dimensions on a computer using ImageJ 1.46r. I measured sclerite length and depth and the length of the longest spine, and I counted the number of spines (Fig. 4.1b). I performed all of these measurements on both sclerites of each individual. For sclerite length, sclerite depth, and length of longest spine, the mean values of the two sclerites were used in the following analyses. For spine number, the sum of the number of spines on the two sclerites was used for the analyses. When either sclerite was not available for measurement (e.g., because of damage during processing), the measurements of the intact sclerite were used and its number of spines was doubled.

4.2.4. STATISTICAL ANALYSES

I used R software version 2.15.1 (R Development Core Team 2012) for all statistical analyses and generalized linear mixed models (GLMMs) for all statistical hypothesis testing. The likelihoods of the GLMMs were estimated by adaptive Gauss-Hermite approximation (nAGQ was set to 5 in the glmer function of package lme4). Significance was tested by the Wald z test. I first examined the effect of female genital injury on the fecundity of *C. maculatus*. I assumed a Poisson distribution for the fecundity and incorporated the occurrence of genital injury and female elytral length (mean of both

elytra) into the model as fixed effects. The *C. chinensis* male strain was incorporated into the model as a random effect.

I performed a principal component analysis for *C. chinensis* male genitalia morphological measurements by using a correlation matrix to identify correlations among the measurements. Then I examined the effects of *C. chinensis* male genital morphology on the occurrence of genital injury in *C. maculatus* females. Each male genital morphological dimension was strongly correlated with another dimension (see Results), so the inclusion of all dimensions in a single statistical model was inappropriate. Additionally, the use of principal component scores did not appear to be appropriate for my purpose (see Appendix). Hence, I used GLMMs, each of which included a single genital dimension as a fixed effect, to independently analyze the correlations between each genital dimension and the occurrence of genital injury in *C. maculatus* females.

4.3. Results

As manually everting the endophallus of aged males was technically difficult, I was able to collect everted genitalia of *C. chinensis* males from 186 of the 304 replications. The proportion of males with everted genitalia did not differ significantly among *C. chinensis* strains (likelihood ratio test: $\chi^2 = 12.19$, d.f. = 8, $P = 0.14$). Of the males with successfully everted genitalia, I could not determine all the measurement items for two males. I used the remaining 184 replications in the following analyses.

Female *C. maculatus* with externally identifiable injury had significantly

smaller fecundity than those without any identifiable injury after controlling for female body size (injury: effect size $\pm$ SE = -0.32 ± 0.029 , $z = -10.9$, $P < 0.0001$; elytral length: effect size $\pm$ SE = 1.44 ± 0.18 , $z = 8.14$, $P < 0.0001$; Fig. 4.3). Injured females had on average 27.3% smaller fecundity. The estimated variance of the random effect (i.e., *C. chinensis* strain) was 0.009.

Principal component scores of individual males, plotted against the first two principal components (PC1, PC2; Fig. 4.4) obtained by the principal component analysis show a large variation in the genital morphology of male *C. chinensis* beetles among the nine strains. PC1 accounted for 39.3% of the variance, and PC2 for 34.7% (Table 4.A1). All genital dimensions contributed similarly to PC1, whereas two pairs of dimensions contributed differently to PC2, with similar absolute factor loading values: sclerite length and number of spines contributed positively, and sclerite depth and longest spine length contributed negatively (Fig. 4.4; Table 4.A1), to PC2. The GLMM analysis for the effect of each individual male genital trait on female genital injury showed that *C. chinensis* males with deeper sclerites were significantly more likely to induce genital injury in *C. maculatus* females (effect size $\pm$ SE = 0.20 ± 0.061 , $z = 3.21$, $P = 0.001$, variance of the random effect < 0.0001 ; Fig. 4.5a). In an independent analysis, I also found that *C. chinensis* males with longer spines were significantly more likely to induce genital injury in *C. maculatus* females (effect size $\pm$ SE = 0.24 ± 0.087 , $z = 2.74$, $P = 0.006$, variance of the random effect < 0.0001 ; Fig. 4.5b). On the other hand, the occurrence of genital injury in *C. maculatus* females showed no significant correlation with either sclerite length or number of spines (sclerite length: effect size $\pm$ SE = -0.041 ± 0.045 , $z = -0.91$, $P = 0.36$, variance of the random effect < 0.0001 ; number of spines: effect size $\pm$ SE = 0.088 ± 0.051 , $z = 1.73$, $P = 0.08$, variance of the

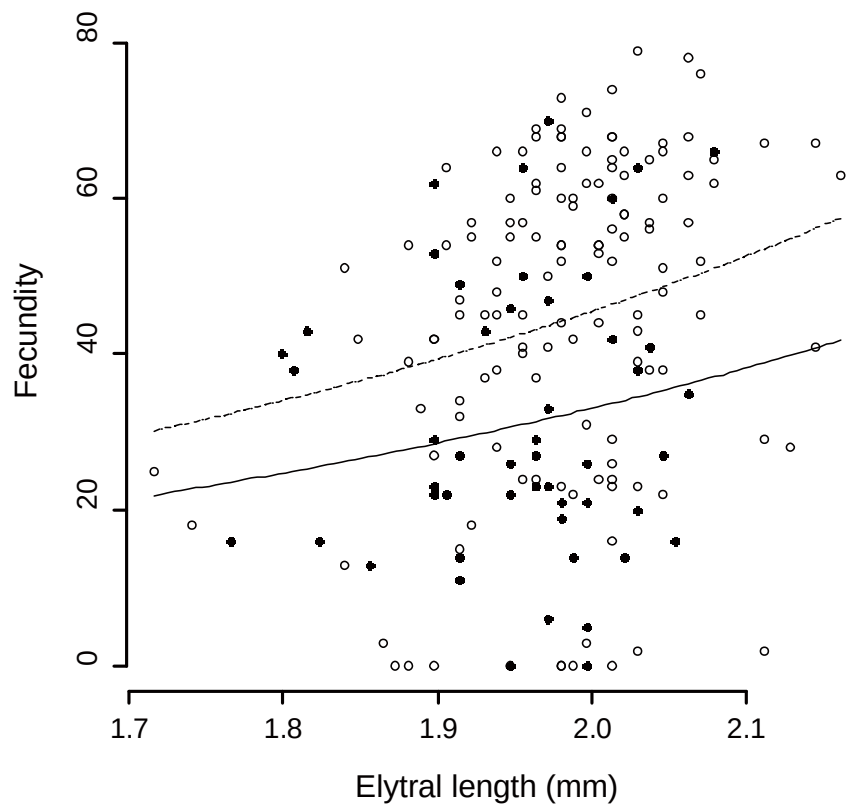


Fig. 4.3 Fecundity of *C. maculatus* females with (filled circles) or without (open circles) identifiable genital injury due to interspecific copulation plotted against elytral length. Solid and dashed curves are estimated regression curves for the fecundity of females with and without any identifiable genital injury, respectively.

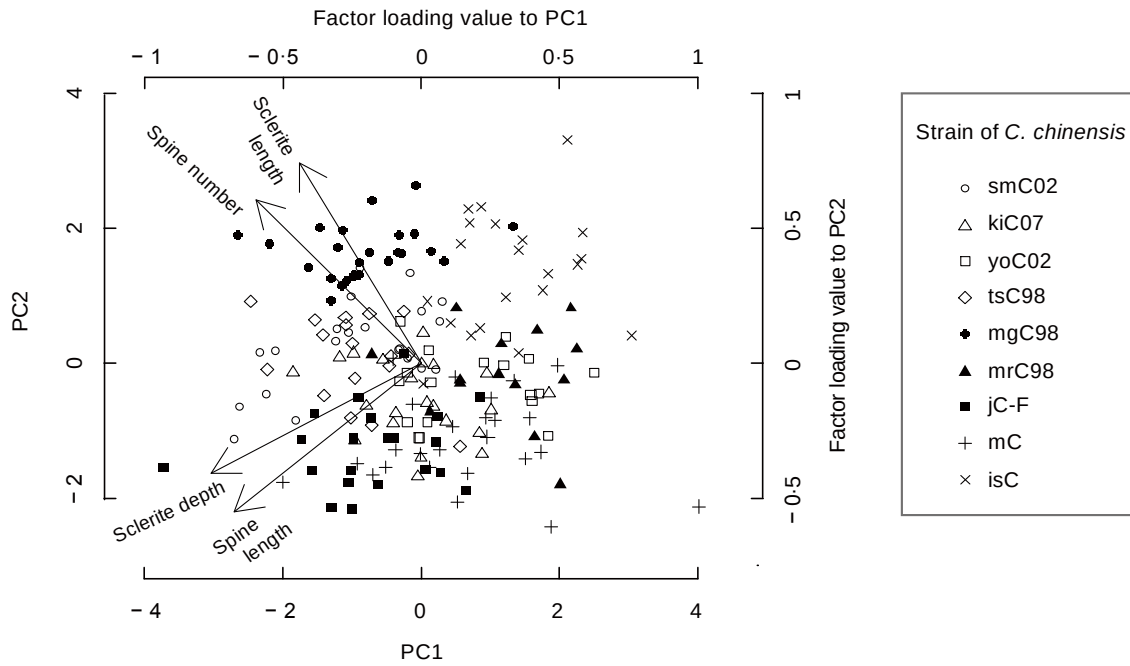


Fig. 4.4 The results of the principal component analysis of *C. chinensis* male genital dimensions. Principal component scores of individual males (left and bottom axes) and vectors (i.e. factor loading values; right and top axes) of each genital morphological dimension are plotted against the first two principal components.

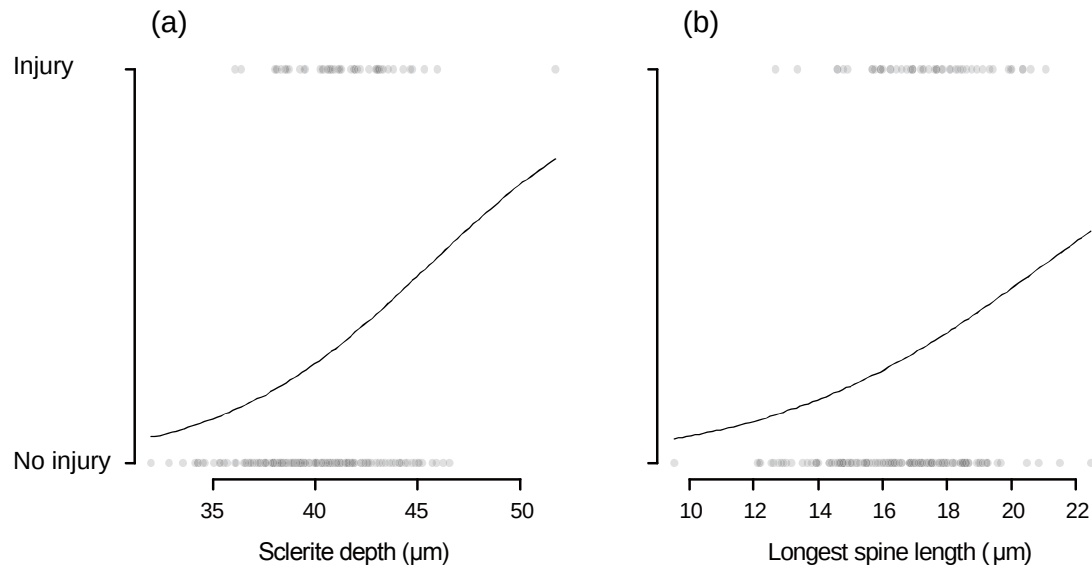


Fig. 4.5 The occurrence of identifiable injury in *C. maculatus* females as a result of interspecific copulation, plotted against (a) sclerite depth and (b) spine length of the *C. chinensis* males with which the *C. maculatus* females were housed. The estimated regression curve for the probability of genital injury occurrence is also shown in each panel.

random effect = 0.15). I also performed similar GLMM analyses incorporating two morphological dimensions that were not strongly correlated with each other, but the results were qualitatively the same (Table 4.A3).

4.4. Discussion

My experimental results are consistent with the hypothesis that genital spines of *C. chinensis* males induce genital injury in *C. maculatus* females during interspecific copulation, thereby affecting the fitness of the *C. maculatus* females. The smaller fecundity of *C. maculatus* females with identifiable injury (Figs 4.2 and 4.3) suggests a causal role of reproductive tract injury in the fecundity reduction. The genital morphology of *C. chinensis* males showed substantial variation among the nine strains (Fig. 4.4), and the reproductive tracts of *C. maculatus* females were more likely to be injured when the females were housed with *C. chinensis* males that had deeper sclerites or longer spines (Fig. 4.5). Sclerite depth and spine length were strongly correlated with each other (Fig. 4.4); hence, I cannot statistically distinguish their relative contributions. Nevertheless, my findings indicate that the more deeply *C. chinensis* genital spines penetrate into *C. maculatus* female tissue, the more likely the females are to incur genital injury during interspecific copulation. Therefore, I suggest that *C. chinensis* males with more exaggerated genital morphology, in terms of either sclerite depth or spine length, inflict a greater fitness cost on *C. maculatus* females through genital injury resulting from interspecific copulation.

One trait, other than genital morphology, that could have potentially affected

my results is the locomotor activity of *C. chinensis* males. Males with different locomotor activity are likely to have different encounter rates with *C. maculatus* females and thus different interspecific copulation rates. The fecundity cost of interspecific copulation to *C. maculatus* females increases with the number of interspecific copulations that a female experiences (Kyogoku and Nishida 2013). Hence, variation in the locomotor activity of *C. chinensis* males could have affected my results. Locomotor activity has, at least in part, a genetic basis in *C. chinensis* (Nakayama and Miyatake 2010). Thus, if substantial variation in locomotor activity existed in the *C. chinensis* males I used, it is likely that interstrain variation in locomotor activity would be observed. Such interstrain variation would have caused substantial variation in the random effect term of the GLMMs in my analysis of genital injury occurrence. However, I found only negligible variation in the random effect term, which suggests that interstrain locomotor variation, if there was any, had little impact on my results.

The evolutionary forces that created the phenotypic variation in *C. chinensis* male genitalia among the strains I used (Fig. 4.4) are unknown. These strains originated from different natural populations (Kondo et al. 1999; Harano and Miyatake 2005, 2010), but it is unknown whether the phenotypic variation observed among them in the present study reflects trait differences among the ancestral populations in nature or whether it resulted from random genetic drift or unintended selection during laboratory rearing. Regardless of the underlying evolutionary history of the strains I used, however, genital spines in *Callosobruchus* beetles are generally thought to have evolved as a consequence of sperm competition. In *C. maculatus*, in which the fitness consequences of genital spines for both males and females have been well studied, males with longer genital spines are more successful in sperm competition (Hotzy and Arnqvist 2009;

Hotzy et al. 2012), and in experimental evolution in this species, genital spines evolved to be more harmful in response to an increased risk of sperm competition (Gay et al. 2011). Therefore, a harmful male trait that has evolved as a consequence of intraspecific sexual selection indeed appears to affect the fitness of heterospecific females through interspecific sexual interactions, potentially causing species exclusion.

Though I did not examine the possible demographic consequence of the variation in the strength of reproductive interference, Kishi et al. (2009) showed that reproductive interference by *C. chinensis* males on *C. maculatus* females leads to the extinction of *C. maculatus* when the two species are kept together for multiple generations, despite the superiority of *C. maculatus* in resource competition. Interestingly, Fujii (1969) reported that which of the two species, *C. chinensis* or *C. maculatus*, becomes extinct in competition experiments depends mainly on the strain of *C. chinensis* and not on that of *C. maculatus*. Fujii (1969) interpreted the result as being caused by the variation in fecundity and adult longevity in *C. chinensis* strains, but the role of reproductive interference was not known in those days. I speculate that, in Fujii's (1969) experiments, the *C. chinensis* strains that drove *C. maculatus* extinct had deeper genital sclerites or longer genital spines, and that the *C. chinensis* strains with less exaggerated genital morphology were competitively excluded by *C. maculatus* through resource competition (see also Kishi 2014). Whether the variation in the strength of reproductive interference due to genital morphology difference translates into different competition outcomes needs to be investigated in future studies.

Sexual conflict is now considered as a universal phenomenon, and harmful male traits have been documented in various organisms (Arnqvist and Rowe 2005; Parker 2006; Fricke et al. 2010). Traumatic insemination, in which mating causes

wounds in females via piercing, has evolved in various animal groups (Lange *et al.* 2013), including *Callosobruchus* beetles. Male ejaculate is toxic to females in some species with internal fertilization (Wigby and Chapman 2005; Garcia-Gonzalez and Simmons 2010; Hoffer *et al.* 2010; Yamane 2013). Aggressive male mating attempts can injure females, thereby increasing female mortality (Réale *et al.* 1996; Le Galliard *et al.* 2005). Even persistent mating attempts by males can interfere with reproduction by females (McLain and Pratt 1999; Gosden and Svensson 2007; Sakurai and Kasuya 2008). Even in plants, interactions between pollen and the recipient flower can increase the reproductive success of one at the expense of the other (Madjidian and Lankinen 2009; Madjidian *et al.* 2012). These harmful male traits may inflict fitness loss on even heterospecific females (or female function) via interspecific reproductive interaction during secondary contacts, thereby potentially preventing such species pair from coexistence (see also Kawatsu 2013; Shuker *et al.* in press).

4.5. Appendix

The result of principal component analysis for *C. chinensis* genital dimensions is summarized in Table 4.A1. I examined the contributions of principal component (PC) scores of *C. chinensis* male genitalia to the occurrence of female genital injury in *C. maculatus* by incorporating PC1 and PC2 scores and their interaction into a GLMM (other model assumptions are identical to those of the analyses in the main text). I found significant correlations with both PC1 and PC2 but not with their interaction (Table 4.A2). To interpret this result, it is convenient to assume that PC1 reflects genital

Table 4.A1 Results of the principle components analysis for genitalia morphological dimensions of *C. chinensis* males belonging to nine strains. PC, principal component.

	PC1	PC2	PC3	PC4
Standard deviation	1.25	1.18	0.75	0.69
Proportion of variance (%)	39.3	34.7	14.0	12.0
Factor loading values				
Sclerite length	-0.437	0.741	-0.438	0.260
Sclerite depth	-0.757	-0.409	-0.299	-0.413
Longest spine length	-0.673	-0.554	0.178	0.456
Number of spines	-0.596	0.602	0.499	-0.181

Table 4.A2 Correlations of the occurrence of genital injury in *C. maculatus* females with principal component scores.

Variable	Estimate $\pm$ SE	z value	P value
PC1	-0.47 $\pm$ 0.15	-3.08	0.002
PC2	-0.35 $\pm$ 0.16	-2.14	0.03
Interaction	-0.093 $\pm$ 0.12	-0.76	0.45

sclerite size and that PC2 reflects its shape. Whereas PC1 was similarly correlated with all measured dimensions, PC2 was differently correlated with two pairs of dimensions (Fig. 4.4). Given the assumption above, the results presented in Table 4.A2 indicate that both the size and shape of male genital sclerites affect the occurrence of female genital injury. The underlying mechanistic cause, however, of the occurrence of a female genital injury such as a protruding reproductive tract is perhaps more likely to be determined by the absolute morphological dimensions (e.g., how deeply sclerites or spines penetrate into female tissue) rather than by independent contributions of sclerite size and shape. Indeed, in the GLMM analyses in which each male genital dimension was considered individually, I found significant correlations of female genital injury only with sclerite depth and spine length (see the main text). Absolute factor loading values of sclerite depth were similar on both PC axes (Table S1), and so were those of spine length. Thus, a decrease in either PC score is associated with an increase in sclerite depth and spine length. The significant correlations found for both PC scores (Table 4.A2) probably reflect the correlations between those PC scores and the two significant morphological dimensions (sclerite depth and spine length). Thus, I suggest that the GLMM analysis carried out with PC scores is not biologically relevant. Further, I incorporated the interaction of PC1 and PC2 into the model expecting to find it to be significant (i.e., that the effect of sclerite size would depend on its shape), but I found no significant effect of the interaction, possibly because of the limited morphological variation in my samples.

I further performed analyses using GLMMs with two raw morphological dimensions to detect potential effects of sclerite length or spine number, whose effects were not significant in the analysis results presented in the main text, on the occurrence

of genital injury in *C. maculatus* females. Because the four morphological measures were separated into two groups within which the variables were strongly correlated with each other (Fig. 4.4), I used one variable from each group to construct a GLMM for each of four possible combinations. Other model assumptions were the same as those described in the main text. These results were consistently qualitatively the same as those reported in the main text (Table 4.A3).

Table 4.A3 Correlations between the occurrence of genital injury in *C. maculatus* females and *C. chinensis* male genitalia morphology analysed by GLMMs for four combinations of morphological measurement pairs.

Variable	Estimate $\pm$ SE	z value	P value
GLMM with sclerite length and sclerite depth			
Sclerite length	-0.055 ± 0.048	-1.14	0.25
Sclerite depth	0.20 ± 0.062	3.27	0.001
GLMM with sclerite length and spine length			
Sclerite length	-0.039 ± 0.048	-0.81	0.42
Spine length	0.24 ± 0.088	2.72	0.007
GLMM with spine number and sclerite depth			
Spine number	0.048 ± 0.045	1.05	0.29
Sclerite depth	0.19 ± 0.062	3.11	0.002
GLMM with spine number and spine length			
Spine number	0.053 ± 0.044	1.20	0.23
Spine length	0.23 ± 0.088	2.68	0.007

5. GENERAL DISCUSSION

With alternative hypotheses being tested, the suit of studies reported here have revealed that (i) reproductive interference by *C. chinensis* males on *C. maculatus* females is caused by repeated interspecific copulations between them, that (ii) the interspecific copulation affect *C. maculatus* fecundity through physical injury to the females rather than physiological effect of heterospecific ejaculate, and that (iii) the extent of the physical injury depends on the extent of the development of *C. chinensis* male genital spines. The unprecedentedly detailed mechanistic understanding of the reproductive interference rendered me to identify the likely selection pressure affecting the strength of reproductive interference: sexual selection. This finding highlights the potential role of sexual selection and resultant harmful male traits in interactions between closely related species and ultimately community organization.

Within a species, the evolution of harmful male trait will cause counter adaptation in females. The evolution of the counter adaptation in turn will modify the selection pressure acting on the harmful male trait, which can create rapid male-female coevolutionary dynamics within a species (Gavrilets 2000). Therefore, the trait relevant to sexual conflict can vary among closely related species or even among populations within a species (Rönn et al. 2007; Hotzy and Arnqvist 2009; Sugano and Akimoto 2011). During secondary contacts between allopatrically formed species, interspecific mating attempts is likely as the two species had experienced no selection against interspecific mating interaction with the other species (Noor 1995). In such interspecific mating interaction, the extent of harmful male trait exaggeration will affect the extent of

fitness loss due to the interspecific mating interaction. That is, population or species with more harmful trait is likely to cause greater fitness loss in the other species. For example, when interspecific copulation takes place, males of a species with less harmful genital structure may have little effect on the fitness of heterospecific females, whereas males of a species with more harmful genital structure may cause great fitness loss.

If such variation in component reproductive interference due to harmful male trait difference translates into demographic reproductive interference, harmful traits males have may in part eventually determine the species pairs that can coexist. As for the likelihood of demographic reproductive interference, it should be noted that harmful male traits may often inflict greater fitness loss on heterospecific females than on conspecific females. Conspecific females would have been exposed to such harmful male traits on evolutionary timescale, and thus they are likely to have evolved counter adaptation to mitigate the negative effect of harmful male traits (e.g., Rice 1996; Rönn et al. 2007). On the other hand, heterospecific females, especially at secondary contact, lack such counter adaptation. In addition, two species may differ in their abundance at the secondary contact, and such difference in relative abundance can make demographic reproductive interference more likely, as fitness loss due to reproductive interference depends on relative abundance of interacting species (e.g., Hettyey and Pearman 2003; Kishi et al. 2009; Kyogoku and Nishida 2012; Friberg et al. 2013).

Previous studies have suggested the ecological significance of harmful male traits in terms of negative effects on conspecific population growth due to female fitness loss (Le Galliard et al. 2005; Rankin and Kokko 2007; Rankin et al. 2011). On the other hand, my study suggests that such harmful male traits can improve the survival of a species in competitive interaction with another species. That is, a species or population

with more exaggerated harmful male traits can more severely suppress the population growth of a different, related species through reproductive interference. Side effects of adaptive evolution by individual selection on higher levels of biological organization, such as populations and ecological communities, are increasingly attracting research attention (e.g., Harmon et al. 2009; Rankin et al. 2011; Carlson et al. 2014; Takahashi et al. 2014). The results of the present study provide additional empirical support for such a phenomenon: sexual selection may affect the assembly of closely related species via the evolution of harmful male traits. It follows that sexual selection can influence the spatio-temporal patterns of ecological communities. Thus, the potential effects of sexual selection and resultant harmful male traits, as well as other reproductive traits such as male interspecific promiscuity (e.g., Schmeller et al. 2005; Hochkirch et al. 2007; Valero et al. 2008), on species interaction should receive more attention.

My findings also have an implication to niche theory. Niche can be classified into either Grinnellian or Eltonian niche, as well as realized or fundamental one (Leibold 1995; Chase and Leibold 2003; Devictor et al. 2010). Grinnell (1917) emphasized, as defining aspect of niche, the requirement of organisms rather than their impact on other environment, as Hutchinson later does. After Grinnell, the environmental requirement of organisms to sustain a viable population is referred to as Grinnellian niche (be they realized or fundamental). On the other hand, Elton (1927) emphasized the impact of organisms on their environment. Eltonian niche thus refers to the impact of organisms on their environment that includes other species. Conventional niche theory, which has focused on resource competition, assumes that the Eltonian niche of one species affects the realized Grinnellian niche of another species through the change in the abundance and thereby the availability of shared resources. When

reproductive interference exerts a dominant effect, the relationship between Eltonian and Grinnellian niche will be different; direct interference between individuals of different species and consequent fitness loss define Eltonian niche, which prevent the coexistence of them and thus differentiate their realized Grinnellian niche. My study implies that Eltonian niche (impact on other species) can be in part determined by harmful male traits, such as genital spines. This is an important conceptual contribution toward rebuilding niche theory by incorporating reproductive interference.

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