

Asymmetric reproductive interference between specialist and generalist predatory ladybirds

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Summary

1. Closely related species often differ greatly in the quality and breadth of resources exploited, but the actual mechanisms causing these differences are poorly understood. Because in the laboratory specialized species often survive and perform as well or better on host species that are never utilized in nature, negative ecological interactions restricting host range must exist. Here, we focused on reproductive interference, which has been theoretically predicted to drive niche separation between closely related species with overlapping mating signals.
2. We examined the interspecific sexual interactions in relation to ecological specialization and generalization in two sibling ladybird species, *Harmonia yedoensis* and *Harmonia axyridis*. *Harmonia yedoensis* is a specialist predator that preys only on pine aphids, which are highly elusive prey for ladybird hatchlings, whereas *H. axyridis* is a generalist predator with a broad prey and habitat range.
3. We experimentally showed that conspecific sperm fertilized the vast majority of eggs regardless of mating order (i.e. conspecific sperm precedence) when a female of *H. yedoensis* or *H. axyridis* mated with both a conspecific and a heterospecific male. Moreover, we demonstrated that mating opportunities of *H. yedoensis* females strongly decreased as heterospecific density increased relative to conspecific density. In contrast, in *H. axyridis*, female mating success was high regardless of conspecific or heterospecific density.
4. Our results suggest that the generalist *H. axyridis* should be dominant to the specialist *H. yedoensis* in terms of reproductive interference. Our results support the hypothesis that asymmetric reproductive interference from the dominant species may force the non-dominant species to become a specialist predator that exclusively utilizes less preferred prey in nature.

Key-words: host specialization, interspecific competition, mating preference, species coexistence, species recognition

Introduction

Understanding how ecological specialization and generalization occur and are maintained is a crucial issue in evolutionary ecology, because it is closely associated with mechanisms of species coexistence and phenotypic divergence with regard to resource use (Futuyma 2001; Mayhew 2006). Generally, genetically based trade-offs in performance among different resources have been considered to promote host specialization, under the assumption of strict co-evolution of a host and its consumer (Ehrlich & Raven 1964). However, in the laboratory, animals often survive and perform as well or better on host species that are never utilized in nature, an

observation that offers little support to the trade-off hypothesis (Jaenike 1990). Therefore, elucidation of the role of ecological interactions sufficient to restrict the 'fundamental niche' of a species to a smaller 'realized niche' (cf. Hutchinson 1957) is essential to understand interspecific differences in the breadth of resources used in nature.

Reproductive interference refers to any kind of interspecific sexual interaction that is caused by imperfect species recognition and that reduces the fitness of individuals (Gröning & Hochkirch 2008). Because the ecological significance and the generality of interspecific exploitative competition for shared resources have been ignored for decades, especially in communities of phytophagous insects but also in many predator communities (e.g. Strong, Lawton & Southwood 1984; Schluter 2000; Okuzaki, Takami & Sota 2010), reproductive

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interference has been proposed as an alternative driver of host specialization and subsequent phenotypic divergence (Schluter 2000). The most conspicuous effect of reproductive interference on population dynamics is its self-reinforcing mechanism through a positive feedback: the relatively more abundant species interferes with its competitor more frequently, amplifying the disproportionate abundance from generation to generation (Kuno 1992; Yoshimura & Clark 1994). Moreover, the costs of sexual interactions are often asymmetric, resulting in local extinction of the less dominant species by the dominant species (e.g. Dame & Petren 2006; Liu *et al.* 2007; Kishi, Nishida & Tsubaki 2009; Crowder, Sitvarin & Carrière 2010a). This asymmetric property is probably due to ecological traits such as reproductive periods and dispersal abilities, as well as differences in the species recognition abilities of the other species involved (Wirtz 1999; Gröning & Hochkirch 2008). Thus, reproductive interference is one of the most promising prospective interspecific interactions that may promote host specialization.

One frequently documented ecological consequence of reproductive interference is geographical separation of the species. For example, the maintenance of parapatry in the absence of a significant environmental gradient by symmetric reproductive interference has been demonstrated [e.g. copepods (*Skistodiaptomus*), Thum 2007], and in diverse animal and plant taxa, native species have been rapidly displaced by invasive congeneric species through asymmetric reproductive interference [house geckoes (*Hemidactylus*), Dame & Petren 2006; whiteflies (*Bemisia*), Liu *et al.* 2007; Crowder *et al.* 2010b; dandelions (*Taraxacum*), Takakura *et al.* 2009; Matsumoto, Takakura & Nishida 2010]. However, empirical studies of reproductive interference resulting in ecological specialization and generalization, which occurs at smaller spatial scales among closely related, regionally sympatric species (i.e. allotropy), are comparatively scarce (but see Gröning *et al.* 2007; Hochkirch, Gröning & Bücken 2007; Friberg 2009). This is probably because the ongoing evolutionary process of host specialization is difficult to observe, as the competing species are now partitioning their food resources and habitats after possible negative interactions in the past (i.e. the ghost of competition past, Connell 1980). Here, we focused on the asymmetric property of reproductive interference, which might select for niche divergence between two sibling predatory ladybirds.

The specialist predator *Harmonia yedoensis* Takizawa and the generalist predator *Harmonia axyridis* Pallas are regarded as sibling species with sympatric distributions in central Japan (Sasaji 1998). Adults of the two species are morphologically very similar, resulting in imperfect species recognition and interspecific mating behaviour (Okada, Nijima & Toriumi 1978). In the field, *H. yedoensis* preys mostly upon the giant pine aphid *Cinara pini* Linné, which has both a large body size and a high walking ability (Noriyuki, Osawa & Nishida 2011); this ladybird species has never been found on any vegetation other than pine trees in central Japan (Sasaji 1998). To hunt the elusive prey efficiently, *H. yedoensis* hatchlings are provided with a large amount of maternal

resources through egg size and sibling cannibalism in a clutch (i.e. trophic egg consumption, Osawa & Ohashi 2008). In addition, *H. yedoensis* hatchlings have a larger head capsule and longer legs than *H. axyridis* hatchlings (Noriyuki, Osawa & Nishida 2011). It is interesting to note that *C. pini* is also nutritionally unfavourable for the development of the ladybird larva; the growth rate in *H. yedoensis* and *H. axyridis* is higher when experimentally fed with various kinds of aphids than when fed with *C. pini* (S. Noriyuki & N. Osawa unpublished data). Thus, the host specialization in *H. yedoensis* is not explicable solely by trade-offs in performance against different aphid species in various habitats. Rather, because *H. yedoensis* has sacrificed a larger clutch size to specialize to elusive prey (Osawa & Ohashi 2008), it is plausible that its host range is restricted by some unavoidable negative interaction in nature. In contrast, *H. axyridis* is a polyphagous predator that utilizes a broad range of aphid species on various deciduous trees as well as on pine trees (Osawa 2000; Noriyuki, Osawa & Nishida 2011). Because *H. axyridis* hatchlings can rarely capture *C. pini* because of the lack of the specialized foraging morphology, the pine trees may be sub-optimal breeding habitat for *H. axyridis* (Noriyuki, Osawa & Nishida 2011). However, *H. axyridis* adults can utilize multiple food patches, where the quality and quantity of aphid colonies are highly heterogeneous in space and time, and then maintain a stable population (Osawa 1992a, 2000). Therefore, as the benefits of ecological generalization would be obvious from a fitness perspective (e.g. Courtney & Forsberg 1988; Futuyama 2001; Wiklund & Friberg 2009), the problem then is why specialist species apparently forsake such benefits in resource use.

In this study, we tested whether reproductive success is dependent on the relative abundances of sexually interacting sibling species and whether the impact of costly interaction might be asymmetric between specialist and generalist species. First, we examined the mating preference of each ladybird species to determine the completeness of their species recognition system, because an incomplete system is a necessary condition for reproductive interference. Second, we examined the rate of utilization of sperm from conspecific males for fertilization when both conspecific and heterospecific males have inseminated a female (i.e. conspecific sperm precedence, Howard 1999) to determine the cost of interspecific insemination to reproductive success. Finally, we conducted an experiment to determine the effects of heterospecific abundance on the mating opportunities of individuals. On the basis of our results, we discuss the possibility that asymmetric reproductive interference between closely related species may contribute to specialization by one species in terms of habitat and food source.

Materials and methods

STUDY ORGANISMS

Adults of the specialist predator *H. yedoensis* and the generalist predator *H. axyridis* are difficult to distinguish because of their similar body size and morphology, but larvae, especially the third and fourth

instars, show distinctive morphological differences (Sasaji 1998). Under laboratory conditions, heterospecific mating occurs and females that have been inseminated solely by heterospecific males may lay many clutches, but F1 hybrids never hatch (Okada, Nijima & Toriumi 1978; Sasaji 1998). We used laboratory-reared virgin adults of both species from several populations for our experiments because (i) there were not enough virgin *H. yedoensis* adults in the original field collection for a statistical analysis and (ii) the identification of the two species could be confirmed by using larval morphology. Many virgin adults were obtained in 2009 and 2010 by the following procedures.

In April 2009, adults of *H. yedoensis* and *H. axyridis* were collected at Iwakura, Kyoto (135°79'E, 35°09'N), central Japan. In the laboratory, seven *H. yedoensis* females and 20 *H. axyridis* females were individually maintained in plastic Petri dishes (9 cm in diameter by 1.5 cm high) at 25 °C, with a 16:8-hour light/dark cycle and c. 70% relative humidity. They were provided each day with a surplus of frozen *Ephesia kuehniella* Zeller eggs (Beneficial Insectary®, Redding, CA, USA). From the seven *H. yedoensis* and from 14 of the *H. axyridis* females, many eggs were obtained, and the offspring were reared in plastic Petri dishes to the adult stage under the same laboratory and dietary conditions. Because it takes c. 1 month for most individuals of both *H. yedoensis* and *H. axyridis* to mature sexually after adult emergence (Okada, Nijima & Toriumi 1978), virgin adults were reared individually in plastic Petri dishes for at least 30 days before the mating experiments, while being provided with frozen *E. kuehniella* eggs every other day.

Six females of *H. yedoensis* and 16 females of *H. axyridis* were also collected at Hiedaira, Shiga (135°83'E, 35°02'N), in May 2010, and at Takano, Kyoto (135°78'E, 35°05'N), on 28 April 2010, respectively. The Takano and the Iwakura populations are geographically very close to each other, and both are about 7 km from the Hiedaira population. In the laboratory, many clutches were obtained from five *H. yedoensis* females and 12 *H. axyridis* females by the same procedure as that used in 2009. In addition, 70 and 14 egg clusters of *H. yedoensis* and *H. axyridis*, respectively, oviposited on the fresh and dead leaves of Japanese red pine (*Pinus densiflora* Sieb. et Zucc.) were collected at Hiedaira. From the Hiedaira and Takano clutches, many sexually mature virgin adults were obtained by the same procedures as were used in 2009.

We took into account the fact that *H. yedoensis* (N. Osawa, unpublished data) and *H. axyridis* females (Majerus *et al.* 1998; Nakamura, Ueno & Miura 2005) can carry male-killing bacteria that are transmitted from mother to daughter and that killed male embryos look like infertile eggs. To avoid any confounding effects of male-killing bacteria on the mating behaviour (see Majerus 2003) or the degree of conspecific sperm precedence in ladybirds, *H. yedoensis* and *H. axyridis* adult females that were derived from clutches with more than 40% non-developing eggs were excluded from the analysis, whether the clutches had been produced by wild-caught mothers (Iwakura population, $N_{\text{yedoenis}} =$ four females, $N_{\text{axyridis}} =$ three females) or collected in the field (Hiedaira population, $N_{\text{yedoenis}} =$ 26 clutches, $N_{\text{axyridis}} =$ two clutches). The threshold of 40% was chosen as a conservative criterion for exclusion (Perry & Roitberg 2005).

MATING PREFERENCE

To determine whether *H. yedoensis* and *H. axyridis* would attempt mating with one another, two different mating experiments were conducted: a choice experiment and a no-choice experiment. In the choice experiment, one male (*H. yedoensis* or *H. axyridis*) and two

females (one *H. yedoensis* and one *H. axyridis*) from the Iwakura population were kept together. In the no-choice experiment, one male (*H. yedoensis* or *H. axyridis*) and one female (*H. yedoensis* or *H. axyridis*) originating from field-collected clutches of the Hiedaira population were kept together. In both cases, the experimental arena was a Petri dish, the bottom of which was covered fully with filter paper, on a laboratory bench at room temperature (25 °C) under constant fluorescent lighting. Sexually mature individuals were introduced into the arena with a soft brush and then allowed to acclimate for 1 min. In all mating experiments in this study, females were never placed with sibling males to preclude any effects of inbreeding avoidance on mating behaviour. We recorded whether a male mating attempt, female rejection behaviour or successful copulation occurred in an experimental session, which consisted of 15 min of continuous observation of mating activities. The presence of male genital extrudate was used to indicate a male mating attempt. Obata (1987) classified behavioural sequences prior to copulation in *H. axyridis* into five stages: the male (i) turns and approaches the female; (ii) watches her at a close distance; (iii) palpates her elytra with his antennae and forelegs; (iv) mounts on her back; and (v) extrudes his genitalia. However, the first to fourth steps are not always discrete, and the watching and palpation steps are often skipped (Obata 1987). Our preliminary observation confirmed that the behavioural sequences for mating are not different between *H. axyridis* and *H. yedoensis*. Male mating attempts are sometimes rebuffed by the female retracting her abdomen to prevent genital contact (Su *et al.* 2009). This retraction when continued for more than 1 min was regarded as female rejection behaviour. Mating was considered successful when abdominal shaking by the male, a reliable indication of insemination (Obata 1987), was observed. Sometimes, rejecting females later acquiesced to the male mating attempt and genital coupling occurred.

To analyse male mate preference in the choice experiment, the proportion of mating attempts towards conspecific and heterospecific females were compared between species by using Fisher's exact test. Data were included only if a male mating attempt occurred ($N_{\text{yedoenis}} =$ 18, $N_{\text{axyridis}} =$ 33). Similarly, in the no-choice experiment, the proportion of male mating attempts in each ladybird species was compared between the conspecific and heterospecific female treatments by using Fisher's exact test. The female mate preference in each species was examined by calculating the proportion of conspecific and heterospecific male mating attempts that elicited rejection behaviour in the choice (sample sizes as above) and the no-choice ($N_{\text{yedoenis}} =$ 43, $N_{\text{axyridis}} =$ 51) experiments. The duration of copulation (from insertion of genitalia to separation of male and female) of the four species combinations in the no-choice experiment (i.e. one male of *H. yedoensis* or *H. axyridis* and one female of *H. yedoensis* or *H. axyridis*) was also recorded and compared among the treatments by one-way analysis of variance (one-way ANOVA) with Tukey's HSD test for multiple comparisons.

CONSPECIFIC SPERM PRECEDENCE

To determine the effects of interspecific mating on female fertility, the degree of conspecific sperm precedence (Howard 1999) in both ladybird species was examined. Females singly mated with a conspecific or heterospecific male in the mating preference experiment in either 2009 or 2010 were randomly assigned to one of the two following treatments: no second mating or a second mating with a virgin male of a different species from the first mating. Second mating trials were conducted the next day after the first mating under the same experimental protocol. Females that did not remate after 15 min of observation were eliminated from the analysis. Moreover, copulations

without abdominal shaking by the male (which happened only a few times) were also eliminated because they can result in abnormal insemination (Obata 1987). Females with a single or a double successful mating were kept individually in a Petri dish at 25 °C, with a 16:8-h light/dark cycle, and provided with a surplus of pea aphids *Acyrtosiphon pisum* Harris every day to obtain clutches for the determination of female fertility. The first five clutches laid by each female were used for the data analysis, or all clutches when fewer than five clutches were laid. The number of eggs was counted on the day of the oviposition. In aphidophagous ladybirds, newly hatched offspring may eat any undeveloped sibling eggs or developed eggs with delayed hatching in the clutch, both of which function as trophic eggs for the offspring (Kawai 1978; Osawa 1992b; Perry & Roitberg 2005). Therefore, each clutch was maintained in a plastic Petri dish at 25 °C, and the number of hatchlings after sibling cannibalism was counted. A clutch was regarded as being unfertilized when no offspring hatched within 7 days after the oviposition. Then, the hatching rate, defined as the number of hatched offspring divided by the number of eggs in the clutch, was calculated. See the number of replicates of each treatment in Fig. 3.

The hatching rate was analysed by using a generalized linear mixed effects model (GLMM; Schall 1991) using the `glmmPQL` function of the MASS library (Venables & Ripley 2002) of the R software package (version 2.10.1, R Foundation for Statistical Computing, Vienna, Austria), which can represent both fixed and random components. GLMMs are the best tool for analysing non-normal data that involve random effects (Bolker *et al.* 2008). The identity of the mothers of the clutches and the experiment year were represented as random terms, which consider repeated sampling within the same focal mothers or the same experimental year (Schall 1991). The hatching rate was arcsine transformed prior to analysis, and a Gaussian error distribution was modelled with `glmmPQL`. Tukey's HSD test was used for multiple comparisons.

MATING OPPORTUNITY

To determine whether the presence of heterospecific individuals affected female mating opportunity with conspecific males, a laboratory experiment was conducted in plastic cages (21 cm long, 13 cm wide, 13 cm high). The cage was covered with a piece of small-mesh nylon netting, allowing light penetration, and held at 25 °C with a 16:8-h light (0700–2300)/dark cycle. Approximately 10 days prior to the experiment, the cage was filled with a mixture of culture and loamy soil to a height of 3 cm, and 20 seeds of the broad bean *Vicia faba* L. were buried in the soil mixture. The day before the experiment, 12 shoots of successfully germinated broad bean plants were selected, and the others were removed from the soil; then, an excess of pea aphids from the stock culture was attached to the leaves and stems of the broad bean plants. The proportion of successfully fertilized females was compared among three different treatments. In the first treatment, only a single conspecific couple was released into the cage (number of conspecific pairs/number of heterospecific pairs = 1:0). In the second treatment, three pairs of each species were released into the cage (3:3). In the last treatment, five males and five females of one species, and one male and one female of the other species were released into the cage (5:1 and 1:5); this treatment represents a situation with a biased species ratio, but with the same density as the second treatment. These experimental designs allowed us to disentangle the effects of both absolute conspecific density and relative species density (number of conspecific ladybirds divided by total number of con- and heterospecific ladybirds) on the mating opportunity of females during a trial (Friberg 2009). Each trial started in the middle

of the day with the release of unmated and sexually mature *H. yedoensis* stock from the Hiedaira population and *H. axyridis* stock from the Takano population into the cage. The ladybird species were discriminated by the elytral colour or by slight differences in spot size on the pronotum and elytra between individuals. At 24 h after the release, we recorded whether con- and heterospecific copulation was occurring or recaptured all individuals in the cage. Females that were copulating with a conspecific male at the end of the trial were regarded as being successfully inseminated. In contrast, females that were not copulating with conspecific males were kept individually in the Petri dish under laboratory conditions and provided with a surplus of pea aphids everyday to obtain clutches by which female fertility could be judged. A few clutches from each female were isolated and kept in a Petri dish under laboratory conditions, and the female was regarded as having been inseminated if offspring hatched from their clutches. This judgement should be valid because conspecific sperm precedence was found in both species (see Results), indicating that if hatchlings were obtained successful fertilization of females had taken place. Ten replicates of each treatment were carried out.

Individual female mating success of each species was analysed by using GLMM with a binomial error structure that indicated whether each individual female was successfully fertilized (no fertilization = 0, fertilization = 1). Absolute species density (number of conspecific pairs/cage) and relative species density (number of conspecific pairs/total number of conspecific and heterospecific ladybirds in cage) were represented as fixed factors of continuous predictors, and each trial was represented as a random term.

Results

MATING PREFERENCE

The direction of male mating attempts in the choice experiment differed significantly between species (Fig. 1, Fisher's exact test, d.f. = 1, $\chi^2 = 6.18$, $P = 0.03$). *Harmonia yedoensis* males directed mating attempts towards both conspecific and heterospecific females in a similar proportion, whereas *H. axyridis* males preferably attempted to copulate

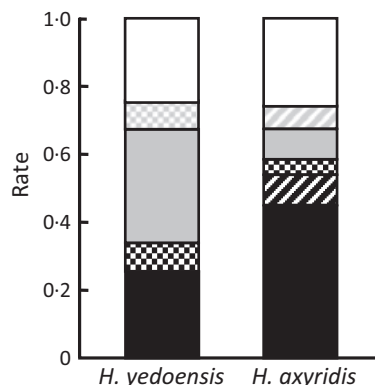


Fig. 1. Proportions of conspecific (black) and heterospecific (grey) females exhibiting mating behaviour with males of *Harmonia yedoensis* and *Harmonia axyridis* in the choice experiment. Filled segments, mating attempts resulting in copulation without female rejection behaviour; hatched segments, mating attempts resulting in copulation after female rejection; checked segments, mating attempts ending in copulation failure; white segments, no mating attempts observed. *Harmonia yedoensis*, $N = 24$; *H. axyridis*, $N = 45$.

with females of their own species. Interspecific copulation was observed in all four of the species combinations in the experiments. The proportion of conspecific and heterospecific male mating attempts that elicited female rejection behaviour differed not significantly between the species (Fig. 1, Fisher's exact test, *H. yedoensis*, $\chi^2 = 0.64$, d.f. = 1, $P = 1.00$; *H. axyridis*, $\chi^2 = 1.09$, d.f. = 1, $P = 0.35$).

In the no-choice experiment, the proportion of mating attempts by *H. yedoensis* males was not significantly different between conspecific and heterospecific female trials (Fisher's exact test, $\chi^2 = 3.07$, d.f. = 1, $P = 0.08$), whereas *H. axyridis* males discriminated against heterospecific females ($\chi^2 = 47.31$, d.f. = 1, $P < 0.001$, Fig. 2). The proportion of conspecific and heterospecific male mating attempts that elicited female rejection behaviour was not significantly different between the two species (Fisher's exact test, *H. yedoensis*, $\chi^2 = 0.32$, d.f. = 1, $P = 0.70$; *H. axyridis*, $\chi^2 = 0.27$, d.f. = 1, $P = 0.78$).

Duration of copulation was significantly longer in combinations including an *H. yedoensis* male than in those including an *H. axyridis* male (ANOVA, $F = 26.20$, d.f. = 3, $P < 0.0001$; Tukey's HSD test, $P > 0.05$: *H. yedoensis* male \times *H. yedoensis* female, mean \pm SE = 228 \pm 8.29 min, $N = 21$; *H. yedoensis* male \times *H. axyridis* female, 193 \pm 16.48 min, $N = 13$; *H. axyridis* male \times *H. axyridis*

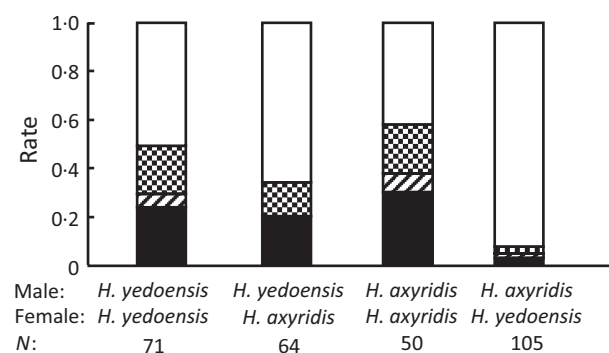
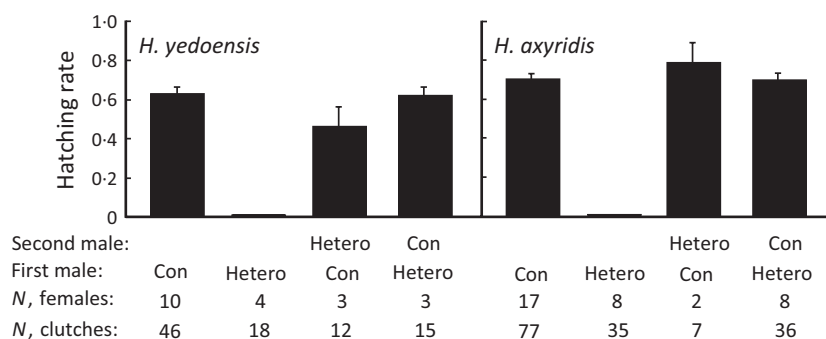


Fig. 2. Proportions of mating attempts with conspecific and heterospecific females by males of *Harmonia yedoensis* and *Harmonia axyridis* in the no-choice experiment. Black segments, mating attempts resulting in copulation without female rejection; hatched segments, mating attempts resulting in copulation after female rejection; checked segments, mating attempts ending in copulation failure; white segments, no mating attempts observed.

Fig. 3. Hatching rate (mean \pm SE) of each clutch produced by *Harmonia yedoensis* and *Harmonia axyridis* females. Con and Hetero refer to conspecific male and heterospecific male, respectively. The rate was not significantly different among treatments in each species, except in the treatment in which a female copulated only with a heterospecific male.



female, 124 \pm 4.51 min, $N = 19$; *H. axyridis* male \times *H. yedoensis* female, 127 \pm 17.34 min, $N = 6$).

CONSPECIFIC SPERM PRECEDENCE

Figure 3 shows the proportion of hatching eggs for all copulation procedures performed in the experiment. Not all eggs from females who mated once with a conspecific male hatched in either *H. yedoensis* (average 63%) or *H. axyridis* (average 70%). Females of both species who mated once with a heterospecific male never produced viable offspring although they laid egg clutches. However, when females of both species mated twice, once with a conspecific and once with a heterospecific male, the hatching rate of the eggs was as high as that from females who mated once with a conspecific male, regardless of the mating order (GLMM, $P > 0.05$ after Tukey's HSD test). The parameter estimates for fixed effects and the predictions for the random effect in each GLMM analysis are shown in Table 1.

MATING OPPORTUNITY

The mating rate of *H. yedoensis* females was significantly affected by the relative species density in a given treatment, but was unaffected by the absolute species density in the cage (Fig. 4a, GLMM, fixed effects, relative density, the parameter estimate \pm SE = 3.06 \pm 1.18, d.f. = 36, $t = 2.59$, $P < 0.05$; absolute density, 0.15 \pm 0.19, d.f. = 36, $t = 0.80$, $P = 0.43$; random effects, SD = 0.35). In fact, mating success was relatively high in the 1:0 treatment but was lowest in the 1:5 treatment. By contrast, *H. axyridis* female mating success was not affected by the relative or absolute density of ladybirds and was consistently high irrespective of the treatment (Fig. 4b, fixed effects, relative density, the parameter value \pm SE = -1.55 \pm 2.03, d.f. = 37, $t = -0.78$, $P = 0.44$; absolute density, 0.36 \pm 0.36, d.f. = 37, $t = 1.03$, $P = 0.30$; random effects, SD = 2.69).

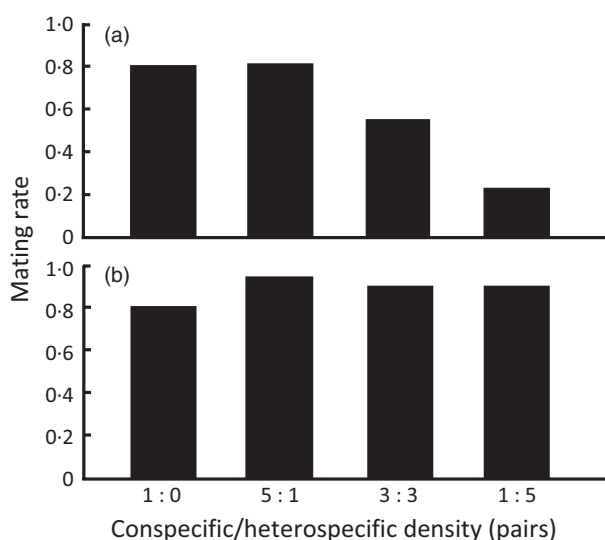
Discussion

The choice and no-choice mating experiments revealed that species discrimination skills of males and females of *H. yedoensis* and *H. axyridis* were imperfect and resulted in interspecific mating behaviours (Figs 1 and 2). Moreover, the

Table 1. Summary of GLMM of the factors affecting the hatching rate in *Harmonia yedoensis* and *Harmonia axyridis* females. Con and Hetero refer to conspecific male and heterospecific male, respectively

Treatment			Parameter estimate				
Female	First mating	Second mating	Value	SE	d.f.	<i>t</i>	<i>P</i>
<i>H. yedoensis</i>	Con (Intercept)		50.48	5.93	71	8.51	< 0.0001
	Hetero		-50.66	6.93	15	-7.31	< 0.0001
	Con	Hetero	-10.38	8.04	15	-1.29	0.22
	Hetero	Con	0.11	7.41	15	0.01	0.99
<i>H. axyridis</i>	Con (Intercept)		58.70	2.71	120	21.69	< 0.0001
	Hetero		-58.70	4.82	30	-12.17	< 0.0001
	Con	Hetero	3.93	8.77	30	0.45	0.66
	Hetero	Con	0.32	4.81	30	0.07	0.95

Prediction of random terms: *H. yedoensis*, mother, SD = 9.42; year, SD = 5.95; *H. axyridis*, mother, SD = 8.98; year, SD = 1.39×10^{-3} .

**Fig. 4.** Mating rate in relation to relative local heterospecific density in (a) *Harmonia yedoensis* and (b) *Harmonia axyridis* females.

proportion of *H. yedoensis* females that successfully copulated with a conspecific male was significantly dependent on the species ratio in each trial (Fig. 4a). Importantly, the absolute conspecific density in the cage did not affect the mating success in *H. yedoensis* females (Fig. 4a). In contrast, *H. axyridis* female mating success was quite high regardless of the conspecific-to-heterospecific density ratio (Fig. 4b). Although females of both species could utilize the sperm from the conspecific males for fertilization when both conspecific and heterospecific males had inseminated the female (Fig. 3), the results suggest that in the presence of sibling species, the loss of mating opportunity would cause a significant fitness reduction in *H. yedoensis* individuals. We concluded therefore that the generalist *H. axyridis* should be dominant to the specialist *H. yedoensis* in terms of reproductive interference. The results presented here support the hypothesis that reproductive interference can promote host specialization in *H. yedoensis*. Because the giant pine aphid is very difficult to capture especially for *H. axyridis* hatchlings (Noriyuki, Osawa & Nishida 2011), the habitats on a pine tree may function

as a refuge for *H. yedoensis* to mitigate the costs of reproductive interference from *H. axyridis*, namely the asymmetric reproductive interference from the dominant species might force the non-dominant species to become a specialist predator that exclusively utilizes less preferred prey in nature.

BEHAVIOURAL MECHANISMS OF REPRODUCTIVE INTERFERENCE

The interspecific difference in female mating success may be a consequence of the mating preference of the males of the two species. The choice and the no-choice experiments consistently showed that *H. yedoensis* males attempted to mate with conspecific and heterospecific females promiscuously, whereas *H. axyridis* males performed more mating attempts with conspecific females than with heterospecific females (Figs 1 and 2). Moreover, female mate recognition seems to be equally imperfect in *H. yedoensis* and *H. axyridis*, because the proportions of conspecific and heterospecific male mating attempts eliciting female rejection behaviour did not differ significantly between *H. yedoensis* and *H. axyridis* (Figs 1 and 2). Furthermore, the duration of copulation was long in these ladybirds, especially when the copulation was with an *H. yedoensis* male (ranging from 2 h up to more than 5 h), suggesting that interspecific mating can lead to a loss of opportunities for intraspecific mating. These results suggest that when *H. yedoensis* is less abundant, *H. yedoensis* males are likely to attempt mating with *H. axyridis* females because the probability of their encountering them is high; thus, *H. yedoensis* females are likely to lose opportunities to mate with conspecific males. Both *H. yedoensis* males and *H. axyridis* males insert spermatophores into a female with which they copulate (Sasaji 1998). Because spermatophore production by males takes energy and time (e.g. Wedell 1994), investing spermatophores in a female of the other species may reduce mating frequency, especially of males. In contrast, *H. axyridis* females are likely to copulate with conspecific males because of the male propensity for assortative mating in this species. These mating behaviours may be responsible for the asymmetric outcomes in mating success in this two-ladybird system.

In this study, some possible factors affecting the asymmetric male mating preference could not be tested owing to the sample size restriction. Specifically, the effects of morphological appearance in terms of elytral colour on the differences in mate and species recognition skills between *H. yedoensis* and *H. axyridis* were not explicitly examined. Indeed, in *H. axyridis*, elytral colour has been reported to be an important cue inducing mating behaviour (Osawa & Nishida 1992; Su *et al.* 2009). Therefore, it is possible that elytral colour may partly be responsible for the asymmetric outcomes of male mating preference in *H. yedoensis* and *H. axyridis* (Figs 1 and 2). Moreover, *H. axyridis* must also have chemically and physiologically based attractiveness that is independent of elytral colour (e.g. Osawa & Nishida 1992). In fact, in the two-spot ladybird *Adalia bipunctata* L., cuticular hydrocarbons are needed to trigger mating behaviour (Hemptinne, Lognay & Dixon 1998). In sexual interactions between *H. yedoensis* and *H. axyridis*, the behavioural and physiological basis of reproductive interference appears to be fairly complex. Therefore, elytral colours, chemical signals and their interactions may be involved in the determination of the asymmetric species recognition systems that result in reproductive interference.

We showed that when a female mated with both a conspecific and a heterospecific male, the conspecific sperm fertilized the vast majority of the eggs, regardless of the order of mating (Fig. 3), and this conspecific sperm precedence was quite strong in both species. Our result that reciprocal conspecific sperm precedence occurred in two closely related species is consistent with previous findings in phytophagous ladybirds (Nakano 1985), flour beetles (Wade *et al.* 1994), grasshoppers (Hewitt, Mason & Nichols 1989) and crickets (Gregory & Howard 1994). Complete conspecific sperm precedence means that the negative impact of a heterospecific mating can be largely eliminated when females mate at least once with a conspecific male before the onset of oviposition (Nakano 1985). Nevertheless, our experiments revealed a significant fitness reduction in terms of mating opportunities in virgin *H. yedoensis* individuals when they were mixed with many *H. axyridis* individuals (Fig. 4). Therefore, our results suggest that the cost involved in precopulatory interactions may determine the reproductive interference winner, even though both species possess the physiological mechanism of conspecific sperm precedence. Previous studies have investigated conspecific sperm precedence from an evolutionary perspective, focusing on it as a mechanism of postcopulatory reproductive isolation that might promote speciation (Howard 1999). Therefore, it would be interesting in future to investigate the ecological significance of conspecific sperm precedence in terms of the spatial distribution and resource use of closely related species.

ECOLOGICAL AND EVOLUTIONARY CONSEQUENCES OF ASYMMETRIC REPRODUCTIVE INTERFERENCE

Our results suggest that the direction of reproductive interference is a possible determinant of ecological generalization or specialization in these two ladybirds. We found that the

mating success of *H. yedoensis* suffered strongly when it was the minority species, whereas *H. axyridis* enjoyed a high fitness advantage independent of heterospecific density (Fig. 4). Thus, *H. yedoensis* adults may not be able to invade multiple habitats colonized by their less elusive prey but occupied by many *H. axyridis* individuals. As demonstrated by studies on sympatric speciation through host-race formation, in insects, hosts (habitats) commonly serve as the point of rendezvous for courtship (e.g. Feder *et al.* 1994). Indeed, *H. axyridis* adults aggressively aggregate and mate on trees where high-quality aphid species are abundant (Osawa 2000; Koch 2003). In contrast, *H. yedoensis* benefits more by utilizing giant pine aphids, which are highly elusive prey for *H. axyridis* larvae (Noriyuki, Osawa & Nishida 2011), resulting in becoming a habitat specialist. The cost of being rare (Fig. 4a) may be mitigated in a core population on pine trees where the relative conspecific density is high. Importantly, *H. yedoensis* females sacrifice having a large number of offspring in favour of investing a large amount of maternal resources in each hatchling, which enables them to utilize their elusive prey efficiently (Osawa & Ohashi 2008). Therefore, we suggest that the habitat selection of *H. yedoensis* may be a consequence of it making 'the best of the bad situation'; although they are obligated to specialize to elusive prey, reproductive interference by *H. axyridis* is thereby mitigated.

Our argument that the host specialization of *H. yedoensis* is interference driven is supported by the fact that the food sources of *H. yedoensis* are not restricted to pine aphids in areas where *H. axyridis* does not occur. On the islands of Okinawa and Amami, southern Japan, where *H. axyridis* does not occur, *H. yedoensis* utilizes the *Leucaena* psyllid *Heterophylla cubana* Crawford, which infests the wild tamarind *Leucaena leucocephala* Lamk, as well as pine aphids (Azuma *et al.* 1996; S. Noriyuki unpublished data). Thus, its release from negative interaction with *H. axyridis* may allow *H. yedoensis* to expand its niche breadth in these islands. A similar geographical variation in niche breadth is also found in the *Leptidea* butterfly system, in which reproductive interference also occurs (Friberg 2009), suggesting that habitat suitability might be affected by the presence or absence of an interfering species, rather than the local resource quality and abiotic factors in these systems.

We should also consider possible alternative forces, other than reproductive interference, that might contribute to host specialization, such as exploitative resource competition and intraguild predation. These alternative hypotheses are not mutually exclusive, and it is likely that host specialization is actually caused by the multiple effects of the different mechanisms. Interspecific resource competition has been proposed to be a major cause of host specialization and phenotypic divergence among species of animals (Schluter 2000). In fact, *H. axyridis* larvae often overexploit aphid colonies, suggesting that interspecific resource competition does occur (Osawa 1992a). However, in many animal taxa, even including predators, interspecific competition for a common resource has seldom been detected (Strong, Lawton & Southwood 1984; Schluter 2000; Kaplan & Denno 2007). Alternatively, a

directly harmful interaction via intraguild predation has been suggested as a mechanism promoting habitat partitioning between species (Polis, Myers & Holt 1989; Holt & Polis 1997). In particular, some laboratory experiments clearly showed that larvae of *H. axyridis* is an aggressive intraguild predator that dominantly attacks larvae of other ladybird species such as *A. bipunctata* and *Propylea japonica* Thunberg (Koch 2003; Yasuda *et al.* 2004; Pell *et al.* 2008; Ware & Majerus 2008). However, *H. axyridis* larvae engage in symmetric intraguild predation with *H. yedoensis* larvae, probably because of their similar body size (H. Yasuda & Y. Yamada, unpublished data). In the literature, reproductive interference has been overlooked as a major mechanism of host specialization in predators as well as in herbivores. Therefore, alternative hypotheses that can reliably and plausibly explain the evolution of host specialization should be carefully tested in the *Harmonia* ladybird system.

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