



HOKKAIDO UNIVERSITY

Title	Heterospecific sperm reduction in interspecific crosses between two closely related phytophagous ladybird beetles, <i>Henosepilachna vigintioctomaculata</i> and <i>H. pustulosa</i> (Coleoptera: Coccinellidae)
Author(s)	Kohyama, Tetsuo I.; Matsubayashi, Kei W.; Katakura, Haruo
Citation	Entomological science, 19(1), 49-54 https://doi.org/10.1111/ens.12159
Issue Date	2016-01
Doc URL	https://hdl.handle.net/2115/63989
Type	journal article
File Information	ens_12159_authors.pdf, 本文



Heterospecific sperm reduction in interspecific crosses between two closely related phytophagous ladybird beetles, *Henosepilachna vigintioctomaculata* and *H. pustulosa* (Coleoptera: Coccinellidae)

Tetsuo I. KOHYAMA¹, Kei W. MATSUBAYASHI² and Haruo KATAKURA¹

¹*Department of Natural History Sciences, Faculty of Science, Hokkaido University, Sapporo, Japan and* ²*Department of Animal and Plant Sciences, The University of Sheffield. Sheffield S10 2BG, UK*

Present Address: ²*The Faculty of Arts and Science, Kyushu University, Fukuoka, Japan*

Correspondence: Tetsuo I. Kohyama, Department of Natural History Sciences, Faculty of Science, Hokkaido University, N10W8, Kita-ku, Sapporo, Hokkaido 060-0810, Japan.

Email: tetsuo_kohyama@ees.hokudai.ac.jp

Abstract

In interspecific crosses, a mismatch in internal physiological conditions between two species can reduce sperm viability in the interval from insemination to fertilization, leading to gametic isolation. Two closely related Japanese phytophagous ladybird beetles, *Henosepilachna vigintioctomaculata* and *H. pustulosa*, show several isolating barriers, including reduction in the number of heterospecific sperm in the female reproductive tract and low egg-hatching rates in interspecific matings. However, the mechanisms of these two potential isolating barriers and the association between them are unknown. Here we investigated temporal changes in the number of sperm stored in the female reproductive tract and egg-hatching rates in inter- and intraspecific crosses between these species. Although the number of sperm decreased after both inter- and intraspecific crosses, the reduction was more drastic in inter- than in intraspecific crosses for females of both species. Most of the sperm reduction occurred early on, during sperm transfer from the bursa copulatrix to the paired ampullae of the common oviduct (the sperm storage organs). These two species also demonstrated stably low egg-hatching rates in interspecific crosses. Since the degree and timing of the sperm reduction did not correlate with egg-hatching rates, the reduction in heterospecific sperm in interspecific crosses may not directly cause the low hatching rates. These two isolating barriers could be different expressions of the physiological mismatch and/or genetic incompatibility between gametes of these species.

Key words: Epilachninae, gametic isolation, *Henosepilachna vigintioctomaculata* species complex, speciation.

INTRODUCTION

Gametic isolation (i.e. postmating-prezygotic isolation) is an isolating barrier that acts in animals between copulation and fertilization and reduces offspring production in interspecific crosses (Coyne & Orr 2004). This isolating barrier also consistently occurs in plants and other bisexual organisms such as fungi. Gametic isolation can be categorized as competitive or non-competitive (Coyne & Orr 2004). Competitive gametic isolation, also known as conspecific sperm precedence, occurs when females are inseminated by both conspecific and heterospecific sperm, resulting in the reduced production of hybrid offspring compared to conspecific offspring (Howard 1999). In contrast, non-competitive gametic isolation can occur following a single heterospecific sperm insemination and has several causes: (i) a mismatch in physiological recognition between the heterospecific sperm and the ovule (e.g. Palumbi & Metz 1991; Vacquier 1998; Levitan 2002); (ii) increased mortality of heterospecific sperm in the female

reproductive tract (e.g. Gregory & Howard 1994); or (iii) reduced production of eggs due to reduced stimulation from heterospecific seminal fluid (e.g. Fuyama 1983; Gregory & Howard 1993; Price *et al.* 2001; Nosil & Crespi 2006). Detailed physiological studies are thus necessary to understand the mechanism involved in particular cases of gametic isolation.

The two beetles *Henosepilachna vigintioctomaculata* (Motschulsky, 1857) and *H. pustulosa* (Kôno, 1937) exhibit gametic isolation (Katakura 1997). These species belong to the *H. vigintioctomaculata* species complex, a group of closely related phytophagous ladybird beetles (Katakura 1997). *Henosepilachna vigintioctomaculata*, widely distributed in cool-temperate eastern Asia, including all the main islands of Japan, feeds on some crops and weeds in the family Solanaceae (Katakura 1981a), but also occurs on a wild cucurbit, *Schizopepon bryoniaefolius* (Cucurbitaceae) on Hokkaido, the most northern island of Japan (Katakura 1975). In contrast, *H. pustulosa* is endemic to northern Japan (from the northern Oshima Peninsula to central Hokkaido) (Katakura 1981a), feeding on wild thistles (*Cirsium* spp., Asteraceae) and the blue cohosh (*Caulophyllum robustum*, Berberidaceae). The two species occur sympatrically over the whole range of *H. pustulosa*, but utilize different host plants.

Several isolating barriers are known between these species, including host differentiation (i.e. habitat isolation) (Katakura 1997), sexual isolation (Katakura & Nakano 1979), conspecific sperm precedence (Nakano 1985), and low hatching rates of eggs produced by interspecific crosses (Katakura & Nakano 1979; Katakura 1986a; Katakura & Sobu 1986). Nevertheless, none of these barriers alone accomplishes complete reproductive isolation (Katakura 1997). A detailed evaluation of isolating barriers demonstrated that these species are reproductively well isolated by the joint action of multiple isolating barriers, which sequentially preventing gene flow, even between sympatric populations (Matsubayashi & Katakura 2009). In particular, low egg-hatching rates in interspecific crosses are quite effective in the wild, where the two species occasionally encounter one another (Matsubayashi & Katakura 2009). However, details of the genetic and physiological mechanisms resulting in low egg hatchability are unknown.

In addition to these several isolating barriers, a previous study found an obvious reduction in the number of heterospecific sperm in the female reproductive tract (Katakura 1986b), although the number of sperm ejaculated by males of *H. vigintioctomaculata* and *H. pustulosa* into females did not differ between inter- and intraspecific matings. However, it is unclear when, between insemination and fertilization, this sperm reduction occurs. It is also unclear how the reduction in

heterospecific sperm functions as an isolating barrier in these species, although we expect that this reduction could be responsible for the reduced egg-hatching rates in interspecific matings.

In the present study, we examined the timing and degree of sperm reduction in interspecific matings between *H. vigintioctomaculata* and *H. pustulosa*. We then examined the association between the degree of heterospecific sperm reduction and the degree of reduced egg hatchability.

MATERIALS AND METHODS

In late May 2006, we collected overwintered adult beetles of *H. vigintioctomaculata* on *S. bryoniaefolius*, and *H. pustulosa* on *C. kamtschaticum*, at Mt. Sankakuyama (43.06°N, 141.29°E), Sapporo, Japan, where these species occur sympatrically. The field-collected beetles were maintained in the laboratory at 23°C under a 16 h light : 8 h dark (LD 16:8), and were fed fresh leaves of the Japanese nightshade, *Solanum japonense* (Solanaceae), which is commonly used as a food plant for both species under laboratory conditions (Fujiyama & Katakura 2002). After confirmation of intraspecific mating, each female was maintained individually to collect the eggs. Newly hatched larvae from the wild-caught females were reared in a greenhouse on potted *S. japonense* under a LD 14:10. All individuals were reared on a common host to eliminate the potential effects of different foods on larval development. These newly emerged virgin adult beetles comprised our experimental stocks. Laboratory conditions of 23°C and LD 16:8 were maintained throughout the experiments described below.

An adult virgin female and a male of *H. vigintioctomaculata* or *H. pustulosa* were placed together in a plastic case (6 cm × 7 cm × 2 cm) and allowed to copulate freely. We determined whether or not successful copulation occurred by direct observation for 60 min. If no genital contact occurred during this period, we returned the test beetles to their respective stocks and used them in the experiment on another day. We regarded mating as successful when it lasted for more than 30 min, since sperm transfer in these species does not usually occur in copulation lasting less than 30 min (Katakura 1985). Females that copulated successfully were kept individually under the laboratory conditions described above.

In phytophagous ladybird beetles, sperm is first ejaculated into the bursa copulatrix and then transferred to the paired ampullae of the common oviduct (here termed the “seminal ampullae”) within 48 h after copulation (Katakura 1981b, 1985; Katakura *et al.* 1994). We dissected mated females and counted the number of stored spermatozoa within 2 h, 2 days, 4 days, 8 days, 16 days and 32 days after copulation. In

the first observation period (within 2 h after copulation), we removed the whole oviduct, including the seminal ampullae and bursa copulatrix. For later dissections, we first confirmed sperm transfer to the seminal ampullae under a microscope, and then removed the whole oviduct, including the ampullae. Excised oviducts were further dissected with tweezers in 2 ml of a 0.75% NaCl solution in water and stirred with a medicine dropper. We counted the number of spermatozoa in the sperm suspension solution using a hemacytometer (Burker-Turk) under a microscope. Five to ten females were dissected for each experimental period in each cross type, and a total of 172 females were examined.

We also measured egg-hatching rates in inter- and intraspecific crosses. Females that had copulated only once were maintained individually and their laid egg batches were collected daily. In all, 247 egg batches were collected from 51 females. We counted the number of larvae hatched and calculated egg-hatching rates for each egg batch.

The number of spermatozoa in each dissection period and differences between inter- and intraspecific crosses for each female of both species were analyzed by using generalized linear models (GLMs) with a negative binomial error distribution and log-link function. The models included the following parameters as explanatory variables: “interspecific”, a dummy variable that takes the value 1 for interspecific crosses, and 0 for intraspecific crosses; “day”, days after copulation; “transfer”, a dummy variable that takes the value 1 if the sperm transfer from the bursa copulatrix to the seminal ampullae has occurred (2 days after copulation and later), and 0 otherwise; and interactions of “interspecific” × “day” and “interspecific” × “transfer”. Differences in egg-hatching rates between inter- and intraspecific crosses were analyzed for each female of both species by using generalized linear mixed models (GLMMs) with a binomial error distribution and logistic-link function. The models included following parameters as fixed effects: “interspecific”; “day”; and the interaction of “interspecific” × “day”. To take the difference in hatching rate among egg batches collected from different females into account, we also added the parameter “family” as a random effect. We obtained posterior distributions of the parameters in the models based on the Markov chain Monte Carlo (MCMC) method, using the RStan package v2.5.0 (Stan Development Team 2014) in R v3.1.2 (R Core Team 2014). Posterior parameter distributions were summarized by the median and 95% highest posterior density interval (HPDI). If the 95% HPDI of a fixed effect did not include zero, we considered the effect to be significant at $\alpha = 0.05$.

RESULTS AND DISCUSSION

The number of sperm ejaculated by males of *H. vigintioctomaculata* and *H. pustulosa* into conspecific and heterospecific females did not differ significantly (Fig. 1). The number of sperm gradually decreased in the female's internal organs across the experimental periods (Fig. 2), but most of the sperm reduction occurred during the first 2 days after copulation (Fig. 2). Although the number of sperm decreased even in intraspecific crosses during this period, it decreased more drastically in interspecific crosses. Consequently, the number of heterospecific sperm was always much less than that of conspecific sperm, except just after copulation. This result was also supported by the GLM analyses, which detected a significant interaction effect between “interspecific” and “transfer” for both *H. vigintioctomaculata* and *H. pustulosa* females (Table 1). On the other hand, from the interaction between “interspecific” and “day”, there was only a small difference in the degree of sperm reduction after transfer to the seminal ampullae between inter- and intraspecific crosses for *H. vigintioctomaculata* females, but not for *H. pustulosa* females. These results suggest that most heterospecific sperm were lost in transfer from the bursa copulatrix to the seminal ampullae. After the sperm transfer, the number of sperm gradually decreased in the seminal ampullae probably because it is used to fertilize eggs at oviposition. The patterns of heterospecific sperm reduction observed in the present study is consistent with a type of gametic isolation in which there is poor transfer or storage of sperm in the female reproductive tract (Coyne & Orr 2004). This type of gametic isolation has been reported in closely related species of *Drosophila* (Grimaldi *et al.* 1992; Price *et al.* 2001) and in a cabinet beetle, *Trogoderma glabrum* (Vick 1973). Since different species might develop distinct internal physiological environments, this form of gametic isolation can result from a physiological or mechanical mismatch in the process of sperm transfer to a heterospecific female's internal environment.

We also found quite low egg-hatching rates in interspecific compared to intraspecific crosses across experimental periods (Fig. 3). The GLMM analyses revealed that the “interspecific” parameter strongly affected egg-hatching rates for *H. vigintioctomaculata* and *H. pustulosa* females (Table 2). Although the egg-hatching rates in intraspecific crosses slightly but significantly decreased with time after copulation in *H. vigintioctomaculata* females (Table 2), both *H. vigintioctomaculata* and *H. pustulosa* females maintained high egg-hatching rates even up to 1 month after a single insemination (Fig. 3). No significant effect of the interaction of “interspecific” × “day” was detected (Table 2). Thus, we conclude that these beetles demonstrate relatively stable egg-hatching rates in both inter- and intraspecific crosses, while the

number of sperm decreases with time in both types of cross.

Katakura and Sobu (1986) showed that lack of fertilization and the death of hybrid embryos are two major causes of low hatching rates for eggs laid by heterospecifically mated females of *H. vigintioctomaculata* and *H. pustulosa*. In addition, Katakura (1986b) reported an obvious reduction in the number of heterospecific sperm in the female reproductive tract in the interspecific crosses between these species. In the present study, we demonstrated that most of the heterospecific sperm reduction occurred during sperm transfer from the bursa copulatrix to seminal ampullae. Nevertheless, considerable number of sperm was stored in seminal ampullae after the sperm transfer even in the interspecific crosses (e.g. 2 days and 4 days after copulation; Fig. 2). These results suggest that a reduction in the number of heterospecific sperm does not directly affect the reduced egg-hatching rates in the interspecific crosses, since the egg-hatching rates were quite low in interspecific crosses irrespective of number of sperm stored at seminal ampullae (Fig. 3). We also observed slight deformation of some heterospecific sperm but not of conspecific sperm after transfer to the seminal ampulla (Katakura 1986b; TI Kohyama & KW Matsubayashi, per. obs., 2006). Lack of fertilization and the death of hybrid embryos in the interspecific crosses thus might arise from quantitative and qualitative deterioration of heterospecific sperm by physiological mismatch in the female reproductive tract, although we cannot rule out the possibility of the hybrid inviability caused by the difference in karyotypes between *H. vigintioctomaculata* and *H. pustulosa*, as pointed out by Tsurusaki *et al.* (1993).

ACKNOWLEDGMENTS

We thank S Nakano for important information on the heterospecific sperm reduction in these beetles; MH Dick for editing the manuscript; MT Kimura, T Kubo, N Fujiyama, S Kikuta and N Kobayashi for valuable comments; and M Kuwajima and A Togashi for assistance in laboratory rearing. This study was supported by KAKENHI (Nos. 14204081 and 18207005) to HK.

REFERENCES

- Coyne JA, Orr HA (2004) *Speciation*. Sinauer Associates, Sunderland.
- Fujiyama N, Katakura H (2002) Host plant suitability of *Solanum japonense* (Solanaceae) as an alternative larval food for three closely related *Epilachna* ladybird beetles (Coleoptera: Coccinellidae). *Applied Entomology and Zoology* **37**, 551–557.

- Fuyama Y (1983) Species-specificity of paragonial substances as an isolating mechanism in *Drosophila*. *Experientia* **39**, 190–192.
- Gregory PG, Howard DJ (1993) Laboratory hybridization studies of *Allonemobius fasciatus* and *A. socius* (Orthoptera: Gryllidae). *Annals of the Entomological Society of America* **86**, 694–701.
- Gregory PG, Howard DJ (1994) A postinsemination barrier to fertilization isolates two closely related ground crickets. *Evolution* **48**, 705–710.
- Grimaldi D, James AC, Jaenike J (1992) Systematics and modes of reproductive isolation in the holarctic *Drosophila testacea* species group (Diptera: Drosophilidae). *Annals of the Entomological Society of America* **85**, 671–685.
- Howard DJ (1999) Conspecific sperm and pollen precedence and speciation. *Annual Review of Ecology and Systematics* **30**, 109–132.
- Katakura H (1975) *Schizopepon bryoniaefolius* (Cucurbitaceae) as a native host plant for *Henosepilachna vigintioctomaculata* (Coleoptera: Coccinellidae) in Hokkaido. *Applied Entomology and Zoology* **10**, 103–107.
- Katakura H (1981a) Classification and evolution of the phytophagous ladybirds belonging to *H. vigintioctomaculata* species complex (Coleoptera: Coccinellidae). *Journal of the Faculty of Science, Hokkaido University, Series VI, Zoology* **22**, 301–378.
- Katakura H (1981b) Sperm storage place in adult females of the phytophagous ladybirds belonging to *Henosepilachna vigintioctomaculata* complex (Coleoptera, Coccinellidae). *Kontyû* **49**, 477–481.
- Katakura H (1985) Sperm transfer in the potato ladybird *Henosepilachna vigintioctomaculata* (Coleoptera, Coccinellidae, Epilachninae). *Kontyû* **53**, 652–657.
- Katakura H (1986a) A further study on the effect of inter-specific mating on the fitness in a pair of sympatric phytophagous ladybirds. *Kontyû* **54**, 235–242.
- Katakura H (1986b) Evidence for the incapacitation of heterospecific sperm in the female genital tract in a pair of closely related ladybirds (Insecta, Coleoptera, Coccinellidae). *Zoological Science* **3**, 115–121.
- Katakura H (1997) Species of *Epilachna* ladybird beetle. *Zoological Science* **14**, 869–881.
- Katakura H, Nakano S (1979) Preliminary experiments on the crossing between two puzzling phytophagous ladybirds, *Henosepilachna vigintioctomaculata* and *H. pustulosa* (Coleoptera: Coccinellidae). *Kontyû* **47**, 176–184.
- Katakura H, Sobu Y (1986) Cause of low hatchability by the interspecific mating in a

- pair of sympatric ladybirds (Insecta, Coleoptera, Coccinellidae): incapacitation of alien sperm and death of hybrid embryos. *Zoological Science* **3**, 315–322.
- Katakura H, Nakano S, Hosogai T, Kahono S (1994) Female internal reproductive organs, modes of sperm transfer, and phylogeny of Asian Epilachninae (Coleoptera, Coccinellidae). *Journal of Natural History* **28**, 577–583.
- Levitan DR (2002) The relationship between conspecific fertilization success and reproductive isolation among three congeneric sea urchins. *Evolution* **56**, 1599–1689.
- Matsubayashi KW, Katakura H (2009) Contribution of multiple isolating barriers to reproductive isolation between a pair of phytophagous ladybird beetles. *Evolution* **63**, 2563–2580.
- Nakano S (1985) Sperm displacement in *Henosepilachna pustulosa* (Coleoptera, Coccinellidae). *Kontyû* **53**, 516–519.
- Nosil P, Crespi BJ (2006) Ecological divergence promotes the evolution of cryptic reproductive isolation. *Proceedings of the Royal Society B-Biological Sciences* **273**, 991–997.
- Palumbi SR, Metz EC (1991) Strong reproductive isolation between closely related tropical sea urchins (genus *Echinometra*). *Molecular Biology and Evolution* **8**, 227–239.
- Price CSC, Kim CH, Gronlund CJ, Coyne JA (2001) Cryptic reproductive isolation in the *Drosophila simulans* species complex. *Evolution* **55**, 81–92.
- R Core Team (2014) *R: A Language and Environment for Statistical Computing, Version 3.1.2*. R Foundation for Statistical Computing, Vienna. Available from URL: <https://www.R-project.org>
- Stan Development Team (2014) *Stan: A C++ Library for Probability and Sampling, Version 2.5.0*. Available from URL: <http://mc-stan.org/>
- Tsurusaki N, Nakano S, Katakura H (1993) Karyotypic differentiation in the phytophagous ladybird beetles *Epilachna vigintioctomaculata* complex and its possible relevance to the reproductive isolation, with a note on supernumerary Y chromosomes found in *E. pustulosa*. *Zoological Science* **10**, 997–1015.
- Vacquier VD (1998) Evolution of gamete recognition proteins. *Science* **281**, 1995–1998.
- Vick KW (1973) Effects of interspecific matings of *Trogoderma glabrum* and *T. inclusum* on oviposition and remating. *Annals of the Entomological Society of America* **66**, 237–239.

Figure Legends

Figure 1 Box plot of the number of sperm in the female reproductive tract immediately after copulation. Open and filled boxes indicate inter- and intraspecific crosses, respectively. Numbers above boxes indicate the number of females examined. No significant difference was detected between inter- and intraspecific crosses for either *Henosepilachna vigintioctomaculata* (GLM, $\chi^2 = 0.180$, $P = 0.672$) or *H. pustulosa* males (GLM, $\chi^2 = 0.027$, $P = 0.870$). Hv, *H. vigintioctomaculata*; HP, *H. pustulosa*.

Figure 2 Number of sperm per female stored in the reproductive tract after inter- and intraspecific crosses for (A) *Henosepilachna vigintioctomaculata* females and (B) *H. pustulosa* females. Circles and triangles represent intra- and interspecific crosses, respectively. Solid and dashed lines indicate the median sperm number estimated by GLM analysis for intra- and interspecific crosses, respectively. The 95% highest posterior density intervals estimated with the MCMC method are shaded.

Figure 3 Egg-hatching rates after inter- and intraspecific crosses for (A) *Henosepilachna vigintioctomaculata* females and (B) *H. pustulosa* females. Circles and triangles represent intra- and interspecific crosses, respectively. Solid and dashed lines indicate the median sperm number estimated by GLMM analysis for intra- and interspecific crosses, respectively. The 95% highest posterior density intervals estimated with the MCMC method are shaded.