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Evidence for the Incapacitation of Heterospecific Sperm in the Female Genital Tract in a Pair of Closely Related Ladybirds (Insecta, Coleoptera, Coccinellidae)

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ABSTRACT—In the crossing between two closely related phytophagous ladybirds, *Henosepilachna vigintioctomaculata* and *H. pustulosa*, the estimated numbers of sperm preserved by the heterospecific females were approximately one fifth to one tenth of those preserved by the conspecific females. It was supposed that the majority of sperm in the heterospecific females were incapacitated due to the incompatibility with the female reproductive tract and lost during the migration from the bursa copulatrix to the sperm storage place. The sperm preserved by heterospecific females were motile, but may also have incapacitated, and resulted in the low hatchability of eggs by the interspecific mating of these beetles.

INTRODUCTION

In internally fertilizing animals, sperm happened to be ejaculated in the body of a female of foreign species may be inviable or less fertile due to the incompatibility with the genital tract of the female. If such incapacitation of alien sperm does occur, this would result in a hinderance or prevention of the formation of hybrid zygotes and, thus, functions as one type of postmating reproductive isolating mechanism. Patterson and Stone [1] and Dobzhansky [2] reviewed various examples of the incapacitation and resultant gametic isolation in species of the genus *Drosophila*. However, our knowledge on this and related phenomena in other groups of animals is yet poor.

In the course of our studies on the reproductive isolation in a pair of closely related phytophagous ladybirds, *Henosepilachna vigintioctomaculata* (Motschulsky) and *H. pustulosa* (Kôno), we found that the interspecific mating between these two species resulted in the production of eggs with extremely low hatching ratios relative to the conspecific mating [3]. Since the F_1 larvae once hatched grew normally afterward to become fully fertile adults of both sexes [3, 4], we suspected that the gametic isolation, rather than the death of hybrid embryos, might be responsible for the low hatchability [3]. In order to confirm this assumption, the condition and number of sperm kept by conspecific and heterospecific females, and their relation to the hatchability were examined in 1983. Based on the result obtained by this study, I herewith describe the apparent reduction of the number of sperm in the genital tract of foreign female in this species pair and its probable relation to the low hatchability by their interbreeding.

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MATERIALS AND METHODS

The materials used for the present study were the so-called Hokkaido form (Form V–II [5]) of *H. vigintioctomaculata* collected at Kitahiroshima and the Sapporo form (P–III) of *H. pustulosa* at Sankakuyama, both localities situated in the vicinity of Sapporo. The beetles were collected from their respective host plants (*H. vigintioctomaculata*: the potato plant *Solanum tuberosum* L.; *H. pustulosa*: a thistle, *Cirsium kamtschaticum* Ledeb.) as eggs or larvae, and reared on the foliage of the potato plant or a herb, *Solanum megacarpum* Koidz., planted in flower pots placed in a glass-room. They were sexed soon after the emergence

and afterward reared each sex separately with fresh potato leaves until served for experiments. All rearings after the emergence were made under a constant temperature and daylength (23°C, 16L8D). In order to know the condition of preserved sperm, sperm storage organs of some post-copulatory females were fixed, sectioned and stained with Delafield's hematoxylin and eosin. Further, the following three rearing series, each including all four types of reciprocal crosses, were prepared to assess the number of kept sperm and its relation to the hatchability:

Single-mating–single-rearing series (Abbreviated as single-mating series in the following): Virgin females were put together with males and allowed to mate once. After the copulation, the females were reared singly. In this series, each female mated only once before singly reared.

Multiple-mating–single-rearing series (Multiple-mating series): Approximately ten individuals of females were reared together with the same number of males in mass for a week. Then, the females were separated and singly reared. In this series, each female is regarded to have mated a few to several times before singly reared.

Pair-rearing series: Like the single-rearing series, females were allowed to mate once with males. After a female mated successfully with a male, the female and male were reared in a pair afterward. When the male died, a new male was added. Thus, only the females of this series can be supplied fresh sperm repeatedly from the paired males during the experiment.

The numbers of eggs laid during the single- or pair-rearing were counted and the hatching ratio of these eggs were checked for each female or pair. Number of eggs examined for the estimation of hatching ratio was variable individually (Fig. 1). In order to get unbiased estimate, more than 100 eggs were examined whenever possible. On the average, approximately 190 eggs per female were examined in *H. vigintioctomaculata* and 150 eggs were examined for each female of *H. pustulosa* (Fig. 1). After a sufficient number of eggs were obtained, each female was dissected to count the number of preserved sperm with the following procedure: In the present species, sperm received from males are preserved by the females in the

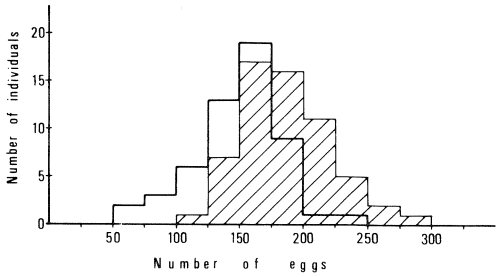

FIG. 1. Numbers of eggs examined to assess hatching ratios. Open: *H. pustulosa*, (N=54, mean=152.2); hatched: *H. vigintioctomaculata*, (N=60, mean=186.8).

seminal node located at the middle part of the common oviduct [6]. The seminal node of intact female was removed, placed in 1.0 ml of saline solution (0.75% NaCl in water), and torn with forceps to make sperm suspension. After violently stirred with the aid of a pippet, the sperm suspension was loaded on a hemocytometer and the number of sperm was counted following the ordinary method for counting leucocytes (cf. [7]) under a phase-contrast microscope. This sperm count was repeated twice for each female and the average was used as the estimate of the number of sperm kept by the female. As exemplified in Figure 2, the results of two sperm counts on the

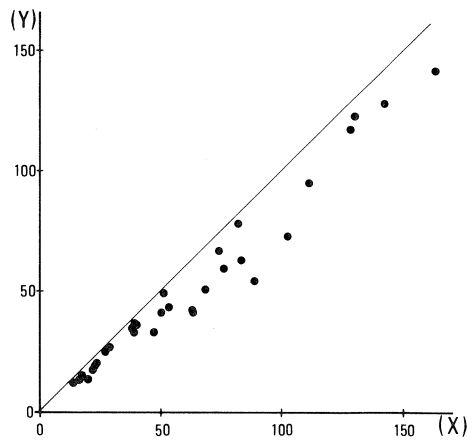

FIG. 2. Relation of two sperm counts on the same females of *H. vigintioctomaculata* mated with conspecific males. Results of single-mating, multiple-mating, and pair-rearing series were combined. Of the results of two sperm counts for each individual, the smaller value (Y) was plotted against the larger value (X). Scale unit: $\times 10^4$.

same specimen were generally not so different, suggesting that the sperm were relatively evenly distributed in the saline. Therefore, the method employed was regarded as appropriate to estimate approximate number of preserved sperm.

RESULTS

Post-coital observations on the sperm in the female genital tracts

Examination of sectioned specimens revealed that the amount of sperm preserved in the node-like sperm storage place of the interspecifically mated female was evidently smaller than that of conspecifically mated one (Fig. 3). The seminal node is composed of two lateral pauches and the median part connecting them. In the conspecifically mated females, sperm filled both the lateral pauches and the median part (Fig. 3a, c), whereas in interspecifically mated females sperm were generally restricted to the lateral pauches alone (Fig. 3b, d). Further, the preserved condition of sperm in heterospecific females often appears to be somewhat anomalous compared with that in conspecific females. However, many, if not most, of the sperm kept by heterospecific females were motile in the saline solution like those by conspecific females.

The results of sperm count were summarized in Table 1. In both conspecific and interspecific matings, the average number of kept sperm was smallest in single-mating series and largest in pair-rearing series. Those of multiple-mating series were intermediate between single-mating and pair-rearing series, but much similar to the former. Since females of single-mating series mated only once, the average value of the sperm number in single-mating series may be close to the minimal level of the number of kept sperm. On the other hand, the distinctly large sperm number in the pair-rearing series, that was apparently resulted from the repeated supply of new sperm from paired males, may be regarded as near the maximum. In the single-mating and multiple-mating series, the relation between the number of sperm and the days from the last mating to the dissection, as well as between the number of sperm and the number of eggs laid, were analysed. But, no clear decrease in the number of preserved sperm was observed with respect to the time from the last mating, or to the increase of the number of eggs laid. Females of *H. vigintioctomaculata* mated with conspecific males possessed on the average 32×10^4 (single-mating series) to 79×10^4 sperm (pair-rearing series), and those of *H. pustulosa*, 11×10^4 (single-mating) to 51×10^4 (pair-rearing). The conspecifically mated females of *H. vigintioctomaculata* appear to have more numerous sperm than those of *H. pustulosa*, suggesting that amount of sperm ejaculated by the male per copula is smaller in the latter species. (This difference was highly significant in both single- and multiple-mating series, but not significant in pair-rearing series as shown in Table 1.) One conspecifically mated female of *H. pustulosa* in each of single-mating and multiple-mating series had no sperm. However, they must have received sperm at the mating since a part of their eggs were hatched. On the other hand, the numbers of sperm kept by interspecifically mated females were fewer, being only one fifth to one tenth of those by

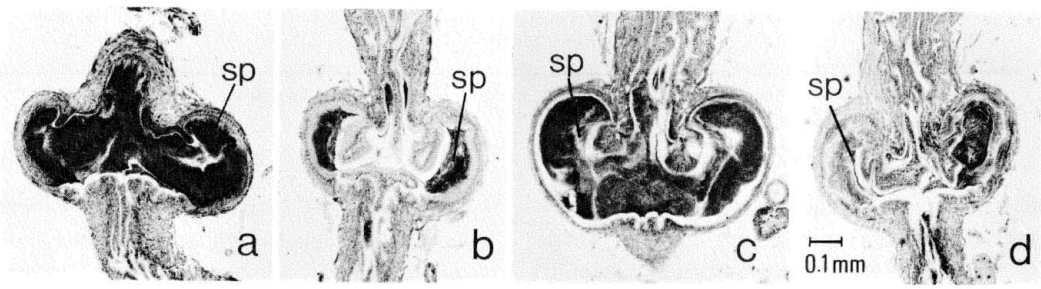

FIG. 3. Female sperm storage organs of *H. vigintioctomaculata* (a, b) and *H. pustulosa* (c, d) with conspecific (a, c) and heterospecific sperm (b, d). sp: sperm.

TABLE 1. Estimated numbers of sperm of *H. vigintioctomaculata* and *H. pustulosa* preserved by conspecific and heterospecific females in three rearing series

Species of sperm and series	Estimated number of sperm ($\times 10^4$) preserved in Conspecific female N	m±SD (range)	Heterospecific female N	m±SD (range)	Difference* (Con. vs Hetero.)
vigintioctomaculata					
Single-mating	10	31.9±18.3 (12.8–70.3)	10	4.8±4.3 (2.1–16.8)	+++
Multiple-mating	7	52.7±39.4 (16.8–126.3)	8	5.1±3.5 (0.8–11.4)	+++
Pair-rearing	12	79.0±41.2 (16.1–152.8)	8	12.5±6.7 (3.4–26.1)	+++
pustulosa					
Single-mating	12	11.1±11.7 (0–39.3)	10	2.3±2.0 (0.1–5.8)	++
Multiple-mating	8	12.5±10.0 (0–33.1)	10	3.3±3.0 (0.9–11.0)	++
Pair-rearing	8	51.0±30.4 (10.3–97.8)	11	11.7±5.1 (4.9–22.4)	+++
Difference* (*viginti.* vs *pustu.*)					
Single-mating		+++		+	
Multiple-mating		+++		NS	
Pair-rearing		NS		NS	

N, number of females examined; m, mean.
* Mann-Whitney U-test: NS, not significant ($P \geq 0.1$); +, $0.05 \leq P < 0.1$; ++, $0.01 \leq P < 0.05$; +++, $P < 0.01$.

TABLE 2. Hatching ratios of eggs laid by various combinations of crosses between *H. vigintioctomaculata* and *H. pustulosa*

Species of female and series	Hatching ratio (%) Conspecific mating N	m±SD (range)	Interspecific mating N	m±SD (range)	Difference*
vigintioctomaculata					
Single-mating	10	42.8±28.7 (1.5–81.4)	10	4.5±10.3 (0–33.1)	+++
Multiple-mating	7	38.7±17.4 (14.6–71.4)	10	0.4± 1.4 (0–4.3)	+++
Pair-rearing	12	50.9±18.6 (8.9–67.1)	11	5.3± 9.8 (0–32.6)	+++
pustulosa					
Single-mating	12	52.4±27.0 (0.8–85.4)	10	0.2± 0.3 (0–0.7)	+++
Multiple-mating	8	33.6±31.2 (7.2–70.8)	8	5.0±11.3 (0–32.6)	+++
Pair-rearing	8	60.0±18.3 (38.9–91.9)	8	0.6± 0.9 (0–2.5)	+++

The results of three rearing series are separately given. N, number of females examined; m, mean.
* Mann-Whitney U-test: +++, $P < 0.01$.

conspecific mating. The average number of *H. vigintioctomaculata* sperm kept by the female *H. pustulosa* was 5×10^4 (single-mating) to 13×10^4 (pair-rearing), and that of *H. pustulosa* sperm in the female *H. vigintioctomaculata* was 2×10^4 (single) to 12×10^4 (pair). There was no marked difference between *H. vigintioctomaculata* and *H. pustulosa* in the number of sperm preserved by heterospecific females (Table 1). In all experimental series here examined, the numerical

difference between the sperm preserved by the heterospecific females and those by the conspecific females was statistically significant (Mann-Whitney U-test, cf. Table 1).

Correlation between preserved sperm numbers and hatching ratios

The hatching ratios of various combinations of crosses examined in the present study were given in Table 2. Like the previously reported cases [3, 4], the hatching ratios by interspecific matings were significantly lower (Mann-Whitney U-test, $P<0.01$) than those by conspecific matings in all experimental series (Table 2). However, the averages by conspecific matings in the present study were very low compared with those previously reported. This unusually low hatching ratios might have resulted from inadequate rearing conditions though exact reason was not clear.

The correlations between the sperm number and

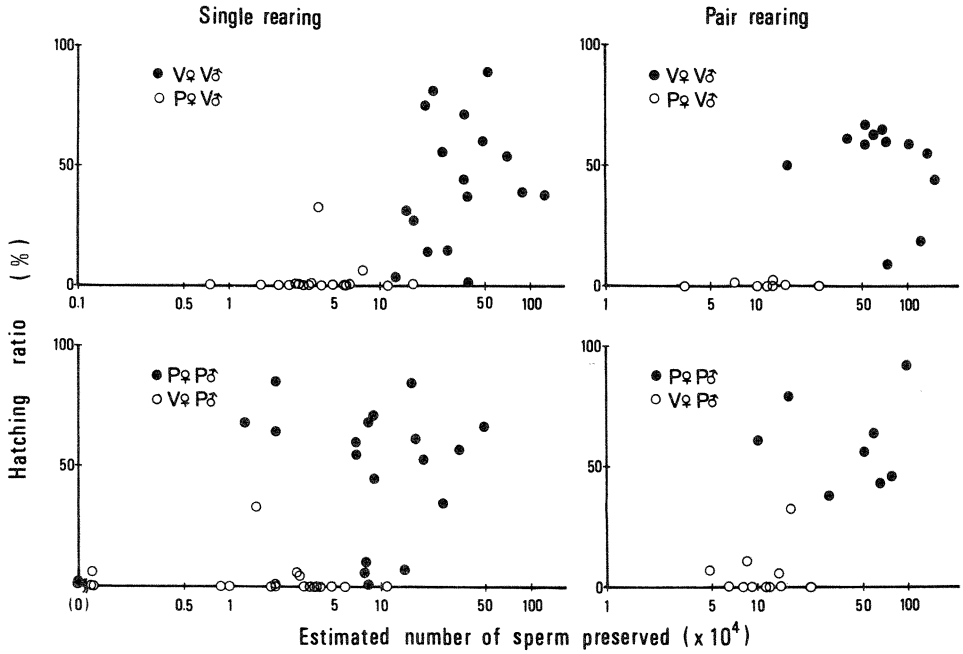

FIG. 4. Relation between the number of preserved sperm and hatching ratio by the four reciprocal crosses between *H. vigintioctomaculata* and *H. pustulosa*. Females mated with conspecific males were denoted by solid circles, and those with heterospecific females by open circles. Results of single-mating and multiple-mating series combined (left column), and those of pair-rearing series (right column) are separately shown.

TABLE 3. Correlation coefficients (*r*s) between the numbers of preserved sperm and the hatching ratios in various combinations of crosses between *H. vigintioctomaculata* (V) and *H. pustulosa* (P)

Series	Combination of crossing							
	V♀ × V♂		V♀ × P♂		P♀ × P♂		P♀ × V♂	
	N	*r*	N	*r*	N	*r*	N	*r*
Single + Multi	17	0.091	20	−0.163	20	0.205	18	−0.028
Pair	12	−0.323	11	0.210	8	0.175	8	−0.109

The data of single-mating and multiple-mating series were combined. Correlation coefficients were not significant in all cases ($P>0.1$). N, number of females examined.

hatching ratio were given in Figure 4. Both the sperm number and the hatching ratio were quite variable individually. But, the correlation coefficient (r) between the sperm number and hatching ratio was statistically not significant ($P>0.1$) not only in the conspecific mating but also in the interspecific mating (Table 3). Further, the difference of sperm numbers kept in conspecific and heterospecific females could not explain the difference of hatching ratios of eggs laid by these females. As shown in Figure 4, although the sperm kept by heterospecific females were in average fewer, the numbers themselves are in many cases enough for keeping high hatching ratio in conspecific females.

DISCUSSION

In the conspecific mating of these beetles, sperm are ejected into the bursa copulatrix and later transferred along the common oviduct to the sperm storage place (Katakura, unpublished). The migration of sperm in the heterospecific females basically followed this process, and as far as judging from the observation of sectioned specimens, there was no marked difference in the amount of semen ejaculated in the body of conspecific and heterospecific females (Katakura, unpublished). Nevertheless, the sperm eventually preserved in the sperm storage place of heterospecific females were difinitely fewer than those in conspecific females as mentioned above. Evidently, a large proportion of sperm ejaculated in the body of heterospecific females were lost in the course of sperm migration from the bursa copulatrix to the sperm storage place. Although no direct evidence was obtained, it is reasonable to regard that they were incapacitated due to the incompatibility with the female reproductive tract, and failed to reach the sperm storage place.

Since the sperm kept by the heterospecific females were fewer than those by the conspecific females, it is assumed that this few number of heterospecific sperm affects, either directly or indirectly, the low hatchability of eggs laid by the interspecific mating. Although the present study showed that the number of preserved sperm is independent from the difference in the hatchability between con- and interspecific matings (Table 3), this fact does not necessarily mean that the nature of heterospecific sperm in the female reproductive tract is also independent of the hatching ratios. Rather, the present result strongly suggests that the incompatibility between the sperm and female reproductive tract is the main factor that causes the low hatching ratios by interbreeding between *H. vigintioctomaculata* and *H. pustulosa*. As discussed above, the fewer number of sperm in females of the foreign species is almost certainly resulted from the incompatibility between the sperm and female reproductive tract. Consequently, it is reasonable to assume that the sperm preserved in the seminal node of heterospecific females were, though motile, also more or less incapacitated. Namely, they may have suffered reduction of fertility which resulted in the unfertilization of eggs (gametic isolation) as we previously postulated [3], or else, they may have retained fertility but became physiologically or genetically anomalous so that eggs fertilized by them died during embryonic development (embryonic mortality).

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