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Citation	Zoological Science, 3(2): 315-322
Issue Date	1986-04
Type	article
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## 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

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**ABSTRACT**—To know the cause of low hatchability by the interspecific mating in a pair of closely related ladybirds, *Henosepilachna vigintioctomaculata* and *H. pustulosa*, the embryonic development of eggs laid by interspecifically mated females was examined. In the crossing *vigintioctomaculata* ♀ × *pustulosa* ♂, most eggs were fertilized but died before the hatching, whereas in the reverse combination, *pustulosa* ♀ × *vigintioctomaculata* ♂, approximately a half of unhatched eggs were not fertilized and the other half died. The results given in this and a previous paper strongly suggest that both the failure of fertilization and the death of embryos were resulted from the interspecific incompatibility between the sperm and the female genital tract.

### INTRODUCTION

It is well known that in certain species of the genus *Drosophila*, alien sperm received by interspecific matings are incapacitated and killed in the female genital tracts [1, 2]. As a result, the fertilization of eggs with the alien sperm was effectively prevented in these species. A similar phenomenon was recently reported on a pair of closely related phytophagous ladybirds, *Henosepilachna vigintioctomaculata* (Motschulsky) and *H. pustulosa* (Kôno) [3]. In this sympatric species pair, the sperm ejaculated in the body of a heterospecific female were considered incapacitated during the migration from the bursa copulatrix to the sperm reservoir due to the incompatibility with the female reproductive tract. Consequently, only a part of them were able to reach the sperm reservoir and to be preserved there. These preserved sperm were motile but probably more or less incapacitated. Katakura [3] explained the low hatchability of eggs laid by the interspecific mating between these two species as the unfertilization due to the reduced fertility of the heterospecific

sperm, or the death of zygotes during embryonic stages because of the fertilization with the incapacitated anomalous sperm. However, whether the low hatchability is caused by the unfertilization or by the death of embryos is yet not confirmed. In the present paper, fertilization ratios and embryonic mortality of eggs laid by conspecifically or interspecifically mated females of these two species are reported on the basis of the crossing experiments, which were made in 1984 in order to solve this problem.

### MATERIALS AND METHODS

Eggs and larvae of the so-called Hokkaido form (form V-II [4, 5]) of *H. vigintioctomaculata* were collected from the potato plant *Solanum tuberosum* L. at Fujino and those of the Sapporo form (P-III) of *H. pustulosa* from a thistle species, *Cirsium kamschaticum* Ledeb., at Ichinosawa, both localities in the vicinity of Sapporo. Reared during the larval stage on the foliage of potato plants planted in flower pots placed in a glass-room, they were sexed soon after the emergence and afterward kept each sex separately with fresh potato leaves as food. After assuring the sexual maturity, the following two rearing series, each

including all four types of reciprocal crosses, were prepared:

**Single-rearing series:** Virgin females were put together with males and allowed to mate once. After the copulation, the females were reared singly.

**Pair-rearing series:** Like the single-rearing series, unmated females were allowed to mate once with males. After a female mated successfully with a male, the female and the male were reared in a pair afterward. When the male had died, a new male was added to the female.

All the rearing after the emergence was made under a constant temperature and daylength (23°C, 16L8D). Eggs that were laid in mass were collected once everyday. About a half of egg masses laid by each female were used for the check of the hatching ratio. The other half of egg masses were used for examining the fertilization ratio. The numbers of eggs examined for the estimation of hatching ratios or fertilization ratios were variable individually (Fig. 1), and were not specifically mentioned in the results given below. On the average, approximately 210 eggs for the hatching ratio and 260 eggs for the fertilization ratio were examined for each female of *H. vigintioctomaculata*, and 160 eggs for the hatching and 130 eggs for the fertilization were examined for that of *H. pustulosa* (Fig. 1). Further, the number of sperm kept by each female was counted by the method described in a previous paper [3] for

the pair-rearing series when the female was still alive after a sufficient number of eggs were obtained. Since the females used in the single-rearing series were subsequently used for other experiments, the results of which will be reported elsewhere, the number of sperm kept by them was not examined.

The fertilization ratio was examined by the following procedure: Egg masses were fixed with Carnoy's solution four days after the collection. This is because the eggs usually hatch 5 to 6 days after the collection under the present rearing conditions. After removal of the chorion, eggs were stained with carbol-thionin, dehydrated with alcohol, cleared with benzene and mounted with cedar oil. Then, the numbers of fertilized and unfertilized eggs were counted for each female. Eggs without any trace of embryo were regarded as unfertilized in the present study. Since zygotes died in the very early stages of embryonic development could not be detected by this method, the fertilization ratio given in the present study may be underestimated.

## RESULTS

### Fertilization ratio

Fertilization ratios of various combinations of crosses examined in the present study are given in Table 1. The fertilization ratios of eggs laid by conspecifically mated females were somewhat lower in *H. pustulosa* than in *H. vigintioctomaculata*. By both conspecific and heterospecific sperm, almost all eggs of *H. vigintioctomaculata* were fertilized. Although the fertilization ratios of eggs of *H. vigintioctomaculata* by the heterospecific sperm appear to be slightly lower than those by the conspecific sperm, the difference in the fertilization ratios between the con- and the heterospecific sperm was not significant. On the other hand, the difference in the fertilization ratios between the con- and the interspecific matings was statistically significant in *H. pustulosa*, in which more than 40% of eggs laid by the interspecific matings were unfertilized.

Successive changes of the fertilization ratio per egg mass by interspecifically mated females of both

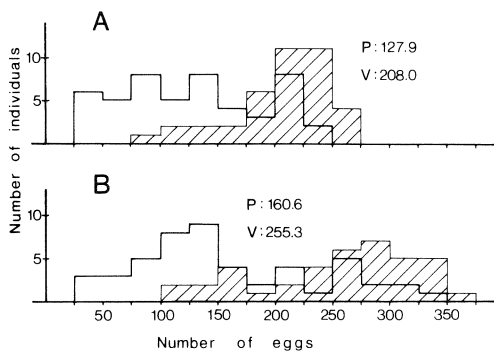


FIG. 1. Numbers of eggs examined to assess fertilization ratios (A) and hatching ratios (B). Hatched (V), *H. vigintioctomaculata* (N=39); open (P), *H. pustulosa* (N=49). The mean values were given in the figure.

TABLE 1. Fertilization ratios (%) of eggs laid by conspecific and interspecific matings of *H. vigintioctomaculata* and *H. pustulosa*

Species of female and series	N	Fertilization ratio (%)		Difference*	
		Conspecific mating Mean ± SD (range)	Interspecific mating Mean ± SD (range)		
<i>vigintioctomaculata</i>					
Single	4	97.4 ± 0.8 (96.4–98.3)	8	90.2 ± 14.8 (54.3–100)	NS
Pair	13	95.1 ± 3.0 (89.4–98.2)	14	93.7 ± 3.4 (86.8–97.8)	NS
<i>pustulosa</i>					
Single	8	94.3 ± 4.9 (85.9–98.7)	10	53.5 ± 38.5 (0–95.7)	++
Pair	16	87.7 ± 12.4 (50.7–98.1)	15	57.6 ± 23.6 (11.0–96.1)	+++

Results of single-rearing and pair-rearing series are separately given.

\* Mann-Whitney U-test: NS, not significant ( $P \geq 0.1$ ); ++,  $0.01 \leq P < 0.05$ ; +++,  $P < 0.01$ .

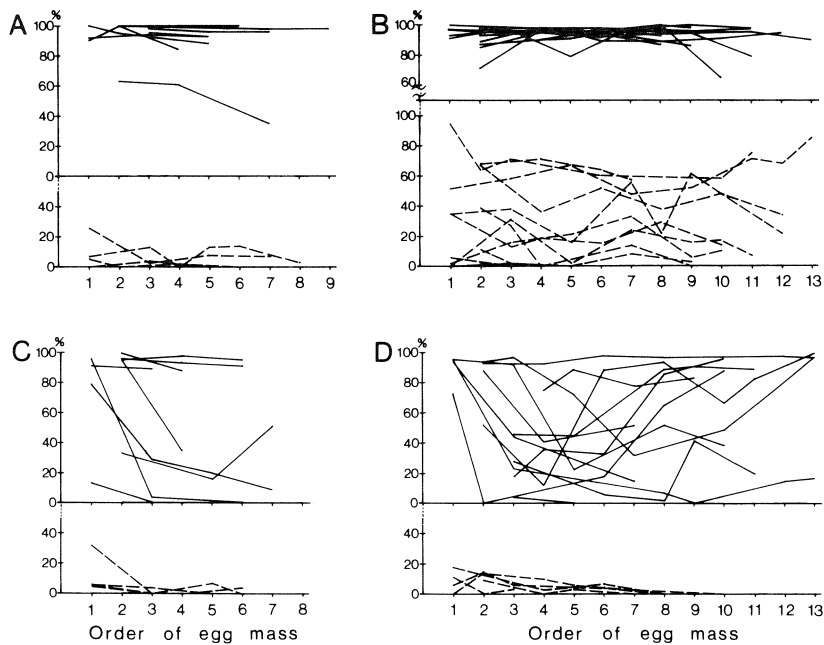


FIG. 2. Successive changes of fertilization ratios (solid lines) and hatching ratios (broken lines) of egg masses in the interspecific matings between *H. vigintioctomaculata* and *H. pustulosa*. The results of single-rearing and pair-rearing series are separately shown. A, *vigintioctomaculata* ♀ × *pustulosa* ♂, single-rearing; B, ditto, pair-rearing; C, *pustulosa* ♀ × *vigintioctomaculata* ♂, single-rearing; D, ditto, pair-rearing.

*H. vigintioctomaculata* and *H. pustulosa* are given in Figure 2, together with those of the hatching ratio. The fertilization ratios of the eggs of *H. vigintioctomaculata* by the heterospecific sperm were consistently high and no remarkable change with respect to the order of oviposition was noticed (Fig. 2 A, B). On the other hand, the fertilization

ratios of the eggs of *H. pustulosa* by the sperm of *H. vigintioctomaculata* remarkably changed successively; in some cases in the single-rearing series, the fertilization ratios apparently decreased with the oviposited order of the egg mass became later (Fig. 2C), whereas in the pair-rearing series, the fertilization ratios did not gradually decrease but

considerably fluctuated (Fig. 2D).

#### Hatching ratio

We previously reported that the hatching ratios by the interspecific matings between *H. vigintioctomaculata* and *H. pustulosa* were definitely lower than those by the conspecific matings [3, 6–8]. In the present study, too, the hatching ratios by the interspecific matings were significantly lower (Mann-Whitney U-test,  $P < 0.01$ ) than those by the conspecific matings in all experimental series (Table 2). In the interspecific matings between females of *H. vigintioctomaculata* and males of *H. pustulosa*, the hatching ratios were considerably higher in the pair-rearing series than in the single-rearing series. The reason of this difference is not known, but may be a sampling error or may be related in some ways to the repeated supply of fresh sperm in the pair-rearing series. The hatch-

ing ratios of *vigintioctomaculata* ♀ × *pustulosa* ♂ in the pair-rearing series were also remarkably higher than those we already obtained [3, 6–8]. Since the provenance of the material was different between the present and the previous studies, this discrepancy indicates that the hatchability by the interspecific matings between *H. vigintioctomaculata* and *H. pustulosa* may be variable according to the combination of populations used in experiments.

Successive changes in the hatching ratios of egg masses laid by interspecifically mated females were different between two reciprocal combinations (Fig. 2). In the egg masses of *H. vigintioctomaculata*, the hatching ratios considerably fluctuated but no clear trend could be detected in the successive change (Fig. 2A, B), whereas in those of *H. pustulosa*, the hatching ratios appeared to decrease, especially in the pair-rearing series, as the order of oviposition became later (Fig. 2C, D).

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

Species of female and series	N	Hatching ratio (%)		Difference*
		Conspecific mating Mean ± SD (range)	Interspecific mating N Mean ± SD (range)	
<i>vigintioctomaculata</i>				
Single	4	89.0 ± 4.6 (83.7–93.8)	8 2.7 ± 4.0 (0–10.1)	+++
Pair	13	82.3 ± 10.4 (63.4–94.3)	14 24.3 ± 25.3 (0–67.0)	+++
<i>pustulosa</i>				
Single	8	75.9 ± 12.9 (55.0–91.2)	10 1.4 ± 2.6 (0–8.3)	+++
Pair	16	66.7 ± 20.5 (10.6–96.0)	15 1.3 ± 1.9 (0–5.6)	+++

Results of single-rearing and pair-rearing series are separately given.

\* Mann-Whitney U-test: ++,  $P < 0.01$ .

TABLE 3. Embryonic viability (=percentage of fertilized eggs hatched) in conspecific and heterospecific matings of *H. vigintioctomaculata* and *H. pustulosa*

Species of female and series	N	Embryonic viability (%)		Difference*
		Conspecific mating Mean ± SD (range)	Interspecific mating N Mean ± SD (range)	
<i>vigintioctomaculata</i>				
Single	4	91.3 ± 4.8 (85.2–95.9)	8 2.8 ± 4.0 (0–12.8)	+++
Pair	13	86.6 ± 11.7 (63.0–99.5)	14 26.3 ± 27.6 (0–71.0)	+++
<i>pustulosa</i>				
Single	8	81.0 ± 15.9 (55.7–102.0)	9 2.6 ± 4.6 (0–12.8)	+++
Pair	16	75.3 ± 19.6 (14.9–97.2)	15 3.1 ± 6.2 (0–24.0)	+++

\* Mann-Whitney U-test: ++,  $P < 0.01$ .

*Embryonic viability*

The difference between the fertilization ratio and the hatching ratio of eggs laid by each female is attributed to the death of embryos. Then, the embryonic viability, here defined as the percentage

of fertilized eggs hatched, was calculated for each female by dividing the hatching ratio by the fertilization ratio (Table 3). In the conspecific matings, the embryonic viability was high, and on the average 75–90% of fertilized eggs hatched, though it was somewhat higher in *H. vigintiocto-*

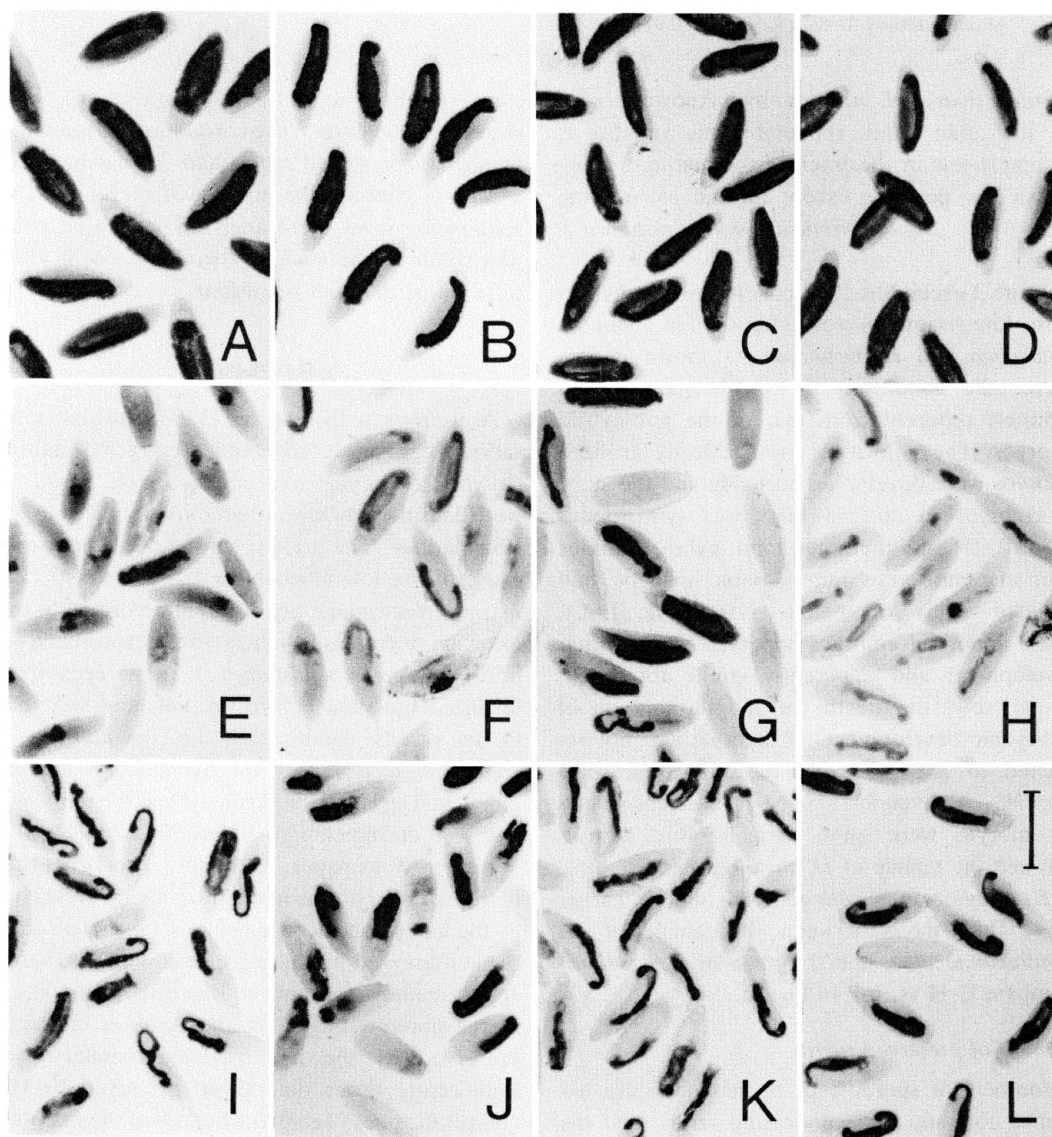


FIG. 3. Conditions of eggs (approximately 4 days old) laid by four reciprocal crosses between *H. vigintioctomaculata* and *H. pustulosa*, showing variation in embryonic development within egg masses. A, B, *pustulosa* ♀ × *pustulosa* ♂; C, D, *vigintioctomaculata* ♀ × *vigintioctomaculata* ♂; E–H, *pustulosa* ♀ × *vigintioctomaculata* ♂; I–L, *vigintioctomaculata* ♀ × *pustulosa* ♂. Unfertilized eggs and various shapes of embryos were seen in E–L. Scale bar: 1 mm.

TABLE 4. Estimated numbers of sperm of *H. vigintioctomaculata* and *H. pustulosa* preserved by conspecific and heterospecific females in the pair-rearing series

Species of sperm	N	Estimated number of sperm ( $\times 10^4$ ) preserved by		Difference*
		Conspecific female Mean $\pm$ SD (range)	Heterospecific female Mean $\pm$ SD (range)	
<i>vigintioctomaculata</i>	13	65.1 $\pm$ 30.0 (28.3–134.5)	6 15.8 $\pm$ 8.4 (5.8–25.3)	++
<i>pustulosa</i>	5	53.2 $\pm$ 42.1 (19.3–124.9)	14 8.1 $\pm$ 3.2 (2.6–13.6)	++

\* Mann-Whitney U-test: ++,  $P < 0.01$ .

*maculata* than in *H. pustulosa* by unknown reason. On the other hand, the embryonic viability is extremely low in the interspecific matings, being only a few percents except for the pair-rearing series of *vigintioctomaculata* ♀  $\times$  *pustulosa* ♂ (26%).

Figure 3 exemplified the conditions of eggs laid by the four reciprocal crosses between *H. vigintioctomaculata* and *H. pustulosa*. As shown in this figure, the condition of hybrid embryos was definitely different from that of the non-hybrid embryos. The majority of conspecifically fertilized embryos were developing normally and the stage of embryos in an egg mass was synchronous (Fig. 3A–D). On the other hand, hybrid embryos in an egg mass were quite variable in shape, and included many anomalous ones (Fig. 3E–L). These abnormal embryos appeared to stop their development and apparently could not hatch. Remarkably, the death (or the termination of embryonic development) of hybrids was not restricted to any particular developmental stage (Fig. 3E–L). Even in a single egg mass, the shapes of embryos were quite variable. But, hybrids between the female of *H. pustulosa* and the male of *H. vigintioctomaculata* generally died in earlier stages than those between the female of *H. vigintioctomaculata* and the male of *H. pustulosa* (compare E–H vs. I–L in Fig. 3).

#### Number of preserved sperm

Numbers of sperm kept by females were examined for only the pair-rearing series, and the results were summarized in Table 4. As in the previous study [3], the numbers of sperm kept by the conspecific females were significantly larger than those by the heterospecific females. The former was approximately four (*H. vigintioctoma-*

*culata* sperm) or seven (*H. pustulosa* sperm) times larger than the latter. The correlation between the number of preserved sperm and the hatchability, as well as between the number of sperm and the fertilization ratio, were analysed, but in all pairwise combinations studied, correlation coefficients were statistically not significant.

## DISCUSSION

As we repeatedly reported [3, 6–8], the hatching ratios of eggs produced by the interspecific matings between *H. vigintioctomaculata* and *H. pustulosa* were considerably lower than those by the conspecific matings. The present study revealed that the cause of the low hatchability by the interspecific matings was remarkably different according to the direction of crosses. Namely, in the crossing *vigintioctomaculata* ♀  $\times$  *pustulosa* ♂, most eggs were fertilized but died before the hatching, whereas in the reverse combination, i.e., *pustulosa* ♀  $\times$  *vigintioctomaculata* ♂, approximately a half of unhatched eggs were unfertilized and the remained half died during embryonic development (Fig. 4).

In a previous paper, Katakura [3] reported that in this species pair the numbers of sperm preserved by the heterospecific females were far smaller than those preserved by the conspecific females, being approximately one tenth to one fifth of the latter. In the present study, too, the numbers of sperm preserved by the heterospecific females were significantly fewer than those preserved by the conspecific ones (Table 4). In both of the previous and the present case, preserved alien sperm were motile, but were assumed to be more or less incapacitated. Although no correlation between the number of preserved sperm and the hatchability could be detected, Katakura [3] attributed the

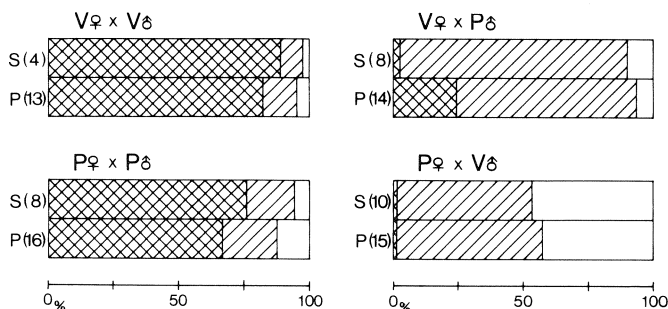


FIG. 4. The proportion of eggs hatched (crosshatch), died during embryonic stages (hatch), and unfertilized (open) in four reciprocal combinations of crosses between *H. vigintioctomaculata* and *H. pustulosa*. Averages for single-rearing (S) and pair-rearing (P) series are separately shown. Numerals in parentheses indicate the numbers of individuals examined.

low hatchability of eggs by the interbreeding between *H. vigintioctomaculata* and *H. pustulosa* to the incapacitation of alien sperm. That is, he explained the low hatchability of eggs as resultant from the unfertilization due to the reduced fertility of preserved heterospecific sperm, or from the death of zygotes during embryonic development because of the fertilization with incapacitated anomalous (but not necessarily heterospecific) sperm.

The results obtained by the present study seem to support this interpretation. The failure of fertilization observed in the eggs laid by interspecifically mated females of *H. pustulosa* can be explained by the reduced fertility of *H. vigintioctomaculata* sperm in the sperm reservoir of *H. pustulosa* female, though "the failure of fertilization" in the present study might involve the death of zygotes in the very early stage. On the other hand, death of hybrids during embryonic development (a case of hybrid inviability) is usually explained as the result of the disharmonious interaction between two different gene systems coexisted in the hybrid individual, a set of genes from the mother and the other from the father [2, 9]. In the present case, too, the substantially high mortality of hybrid embryos might have involved the death due to such direct interaction of incompatible gene systems. However, judging from the results given above and those in the previous paper [3], most, if not all, cases of the death of embryos in the present study are more reasonably interpreted as

the result of the incapacitation of alien sperm, i.e., the death due to the fertilization of eggs with the sperm that were weakened in the genital tract of the heterospecific female [3].

Katakura [3] postulated that in this species pair the alien sperm were incapacitated in the body of the female during the migration from the bursa copulatrix to the sperm reservoir. However, the incapacitation of alien sperm may have proceeded even after the sperm had been preserved in the sperm reservoir, since the fertilization ratios of eggs laid by some single-reared females of *H. pustulosa* apparently decreased successively (Fig. 2). If so, the remarkably fluctuated fertilization ratio in the pair-rearing series of interspecifically mated *H. pustulosa* (Fig. 2) may be interpreted as resulted from this gradual decrease of fertility in preserved older sperm and the repeated supply of fresh and more fertile sperm. On the other hand, the fertility of *H. pustulosa* sperm kept by *H. vigintioctomaculata* females was consistently high (Fig. 2). This indicates that the incompatibility between the sperm and the female genital tract may be stronger between sperm of *H. vigintioctomaculata* and females of *H. pustulosa* than the reverse combination.

Thus, the present study suggested that not only the unfertilization, but also the major part of embryonic mortality in the crossing between *H. vigintioctomaculata* and *H. pustulosa* was resulted from the incompatibility between the sperm and the female genital tract. As mentioned above,



hybrid inviability has been often ascribed to the incompatibility of parental gene systems. However, caution must be paid in determining whether the hybrid mortality is really caused by the incompatibility of parental gene systems, because such cases might involve the death of hybrids due to other factors as demonstrated in the present paper.

#### ACKNOWLEDGMENTS

We would like to express our heartiest thanks to Dr. Morihisa Kurihara, Iwate University, who kindly taught us the techniques for the observation of ladybird embryos. All the rearing and experiments were made at Center for Experimental Plants and Animals, Hokkaido University, and the food plants were cultured at Agricultural Experiment Farm, Faculty of Agriculture, Hokkaido University.

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