

Kontyû, 47(2): 176-184. June 25, 1979

Preliminary Experiments on the Crossing between Two Puzzling Phytophagous Ladybirds, *Henosepilachna vigintioctomaculata* and *H. pustulosa* (Coleoptera: Coccinellidae)*

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Synopsis The sexual isolation between *Henosepilachna vigintioctomaculata* (Hv) and *H. pustulosa* (Hp), both belonging to *Henosepilachna vigintioctomaculata* complex, were studied with an allopatric combination (V-I=Honshû form of Hv and P-II=Typical form of Hp) and a sympatric combination (V-II=Hokkaido form of Hv and P-III=Sapporo form of Hp) by male multiple choice method. Almost all V-I males mated with homogamic females but P-II males mated at random with both V-I and P-II females. Males of both V-II and P-III mated more homogamicly than heterogamicly. In eggs laid by adults not mated before experiment, hatching ratio was distinctly lower in eggs from heterogamic pairs than those from homogamic pairs. Hatching ratio of eggs laid by females presumably already mated were intermediate between those of homo- and heterogamic pairs of newly mated adults.

Henosepilachna vigintioctomaculata (MOTSCHULSKY) and *H. pustulosa* (KÔNO)** (henceforth abbreviated as Hv and Hp, respectively) are the two major "forms" of *Henosepilachna vigintioctomaculata* complex (Hv-complex) consisting of several "forms", the taxonomic relation of which is fairly complicated. According to YASUTOMI (1966) Hv and Hp could successfully produce fertile F₁ hybrids and the F₂ normally grew under laboratory conditions as far as the studied local populations were concerned. TAKENOUCHI (1955) observed no cytological anomalies in the meiotic process of the F₁ between some local populations of Hv and Hp. Nevertheless, two forms keep both morphological and biological differences in natural conditions despite they are sympatric over a wide range. The co-existence of the adults of two forms is sometimes found at potato fields (habitat of Hv) adjacent to thistle stands (habitat of Hp) but the hybrid-like specimens are very rare (KATAKURA, unpubl.). Since biological differences so far observed on food habits and phenology are insufficient to explain the isolation of two forms in natural conditions (KATAKURA 1976), much studies are needed to clarify the isolating mechanisms between Hv and Hp.

As the seventh report of our serial papers on Hv-complex, the present paper deals with some new items of information on the isolating mechanisms based on the preliminary crossing experiments between Hv and Hp. Further, the available

* Contributions to the knowledge of *Henosepilachna vigintioctomaculata* complex. VII.

** Tentative usage of these scientific names is explained in KATAKURA (1974).

knowledge concerning the reproductive isolation in a sympatric combination, Hokkaido form of Hv and Sapporo form of Hp, is briefly summarized and discussed.

Before going further, we wish to express our sincere gratitude to Professor Shôichi F. SAKAGAMI of Hokkaido University for his pertinent guidance through the present study and critical reading of the manuscript. Cordial thanks are also due to Messrs. Hitoshi HINOMIZU, Kazuo HOSHIKAWA and Toshihiro KIMURA for their valuable suggestions in the course of the present study.

Materials and Method

Crossing experiments were undertaken between Honshû form of Hv (V-I) and Typical form of Hp (P-II), that is an allopatric combination, in 1976, and between Hokkaido form of Hv (V-II) and Sapporo form of Hp (P-III), a sympatric combination, in 1977 by means of male multiple choice method. In both combinations, experiments were repeated with post hibernating adults (HA) collected from fields (considered already mated) and newly emerged pre-mating adults (NA) obtained by rearing.

Materials. Provenance of HA is given in Table 1. Before the experiment, males and females of HA were kept separately for seven to ten days with sufficient fresh food (potato leaves for Hv, thistle leaves for Hp). NA was got in 1976 from eggs laid by HA before the experimental period and in 1977 by rearing mature larvae taken in late July at the localities where the respective HA were collected. Experiment with NA started more than two weeks after the emergence in order to get sexually matured individuals.

Table 1. Provenance of the materials used.

Year	Form	Locality	Collected	
			from	on
1976	V-I	Tsukuda, Gumma Pref., Honshû	potato	May 22
	P-II	Kamuikotan, central Hokkaido	thistle	May 14, 21
1977	V-II	Hiroshima-chô, near Sapporo	potato	June 13
	P-III	Nakanosawa, Sapporo	thistle	June 13

Method. One male of either Hv or Hp and one female of Hv and Hp each, in total three individuals all so far kept isolately, were put together in a transparent plastic case (5 cm ϕ \times 8.5 cm in 1976, 6 cm \times 5.5 cm in 1977). Thus, two types of trio were made, namely, A) Hv δ \times (Hv ϕ + Hp ϕ) and B) Hp δ \times (Hv ϕ + Hp ϕ). The number of trios used for each experiment was:

1976, HA: A (24), B (23); NA: A (20), B (24).

1977, HA: A (25), B (25); NA: A (15), B (15).

Mating behavior was recorded by direct observation for a given time (60 min in 1976, 90 min in 1977). When a pair was still in mating at the end of observation time, observation was continued until the removal of the male from the female (1976) or, until 60 min since the beginning of mating (1977). If neither mating behavior (1976) nor actual mating (cf. below, 1977) was observed within the observation time, each specimen was again kept separately with sufficient food, and the experiment was repeated on other days, but not more than thrice for the same trio.

In 1976 HA and NA and 1977 HA, only the pairs made heterogamic mating (or mating behavior) in the experiment were reared each with potato. The hatching ratio and growth of their progeny were examined. In 1977 NA, all mated pairs of both homo- and heterogamic pairs, together with some artificially made heterogamic pairs, were reared separately with potato and/or thistle in order to know the hatching ratio in detail. Some of the obtained hybrid offspring were

also reared with potato to know the mortality during the growth.

Experiments and rearing were made under room conditions during early June to late September in 1976, while made during mid June to mid September under the constant temperature (23°C) and day length (16L8D) in 1977.

Results

1. *Mating behavior.* As far as our observations go, mating behavior of both Hv and Hp was simple, not accompanied with complicated behavioral sequence known in many insects. Directed approach to a female by male was seldom observed except for a very few cases. When a male meets a female, he immediately mounts on her and extends his abdomen and projects the penis toward her ventral side. The female either accepts or rejects the male. Accepted mating was characterized by a long mating time. Outwardly, we could not confirm any particular behavior suggesting the sperm ejection by the male. The male removed himself from the female a few hours after the beginning of the mating. Weak rejecting acts by the female were sometimes observed before the removal of the male. The types of rejection were lifting the abdomen dorso-posteriorly, kicking by the hind legs, blocking by crossing both hind legs, walking about, falling down, body shaking, and extending the elytra, of which the first four acts were frequent. Since the rejected male generally tries to mate again often immediately, repeated trials by the same

Table 2. Results of crossing experiments between V-I and P-II (1976) and between V-II and P-III (1977).

Combination and crosses		Mating behavior observed				Actual mating ³⁾		First trial by male ³⁾	
		HO ²⁾	HT	HO+HT	not	HO	HT	HO	HT
1976									
V _I ♂/V _I ♀P _{II} ♀	HA ¹⁾	19	0	1	4	14**	0	19**	1
	NA	7	1	1	11	6*	0	7	2
	T	26	1	2	15	20**	0	26**	3
P _{II} ♂/V _I ♀P _{II} ♀	HA	7	4	8	4	6	3	9	10
	NA	4	6	1	13	2	4	5	6
	T	11	10	9	17	8	7	14	16
1977									
V _{II} ♂/V _{II} ♀P _{III} ♀	HA	12	8	5	0	13	8	14	11
	NA	12	2	1	0	9	3	13**	2
	T	24	10	6	0	22	11	27*	13
P _{III} ♂/V _{II} ♀P _{III} ♀	HA	14	3	8	0	10	5	18*	7
	NA	7	3	5	0	10	5	11	4
	T	21	6	13	0	20	10	29**	11

1) HA: post hibernating adult, NA: newly emerged adult.

2) HO: homogamic, HT: heterogamic.

3) * and **: the difference between HO and HT is statistically significant at 5% and 1% levels, respectively.

Table 3. Acceptance (a) and rejection (r) by females at the first trial of males (*cf.* Table 2, right column). Columns by Gothic numerals: positive choice of female is statistically significant at 5% level by FISHER's exact probability test.

Trial by	V _I -P _{II}		P _{II} ♀		V _{II} -P _{III}		P _{III} ♀		
	a	r	a	r	a	r	a	r	
HA	Homogamic male	12	7	2	7	9	5	8	10
	Heterogamic male	1	9	0	1	3	4	5	6
NA	Homogamic male	5	2	2	3	7	6	5	6
	Heterogamic male	3	3	0	2	1	3	2	0
HA+NA	Homogamic male	17	9	4	10	16	11	13	16
	Heterogamic male	4	12	0	3	4	7	7	6

male is not rare. Females once rejected the males accepted them at the second or third trial. In 1977, mating lasting more than 60 min. without rejection was regarded as "actual mating". In 1976, however, mating continued more than 60 min. was regarded as actual mating since some rejecting acts by the female were failed to observe.

2. *Sexual isolation.* Results of crossing experiments between V-I and P-II and between V-II and P-III are summarized in Table 2. In 1976NA, mating was not observed in many trios, probably because adults became inactive due to uncontrolled high room temperature. Repeated trials by the male resulted in the occurrence of both homo- and heterogamic matings in a same trio in every combination. However, the frequency of mating types showed: 1) strong positive isolation in V-I ♂ × (V-I ♀ + P-II ♀), 2) virtually random mating in P-II ♂ × (V-I ♀ + P-II ♀), and 3) positive but moderate isolation in V-II ♂ or P-III ♂ × (V-II ♀ + P-III ♀). Frequency of the number of trios which made actual mating also showed quite similar trends. No significant difference was observed between HA and NA in all cases except V-II ♂ × (V-II ♀ + P-III ♀). In V-II ♂ × (V-II ♀ + P-III ♀), mating seemed at random in HA while positive isolation was observed in NA. In order to elucidate the role of sexes in the sexual isolation, choice by each sex was examined on the first trial by the male and subsequent rejection by the female. As obvious from Table 2 (right column), choice by the male at the first trial strongly correlated with the trends of sexual isolation mentioned. On the other hand, positive choice by females was statistically significant only in HA of V-I (Table 3). Selective acceptance by the female of other forms was not observed. The results are not yet conclusive but the choice by the male might be more responsible for the sexual isolation than the choice by the female.

3. *Hatching ratio (Table 4).* Hatching ratio was studied only with heterogamic pairs except NA in V-II × P-III, in which both hetero- and homogamic pairs were examined. In V-I × P-II, only the eggs produced by P-II ♂ × V-I ♀ were examined since the eggs by the reversed combination, V-I ♂ × P-II ♀, could not be obtained.

Table 4. Hatching ratio of eggs by homo- and heterogamic pairs of NA (newly emerged adult) between V-II and P-III and that of heterogamic of HA (post hibernating adult) between V-II and P-III and between V-I and P-II.

Combination (mean %)	Pair code											T
	1	2	3	4	5	6	7	8	9	10	11	
Homogamic												
$V_{II}\sigma \times V_{II}\phi$												
NA	371	197	359	337	458	402	308	55	236			2723
(71.6)	316	112	302	224	349	260	248	31	174			2016
	85.2	56.9	84.1	66.5	76.2	64.7	80.5	56.4	73.7			74.3
$P_{III}\sigma \times P_{III}\phi$												
NA	88	65	90	64	57	61	124	51	80	147		827
(74.2)	54	37	61	43	45	44	99	41	73	126		623
	61.4	56.9	67.8	67.2	78.9	72.1	79.8	80.4	91.3	85.7		75.3
Heterogamic												
$V_{II}\sigma \times P_{III}\phi$												
NA	89	77	59	206	196	157	164					948
(4.0)	0	0	0	3	0	15	28					46
	0	0	0	1.5	0	9.6	17.1					4.9
$P_{III}\sigma \times V_{II}\phi$												
NA	254	280	192	340	130	262	270	313	287	299		2627
(4.0)	8	0	0	3	0	92	1	0	0	0		104
	3.1	0	0	0.9	0	35.1	0.4	0	0	0		4.0
$V_{II}\sigma \times P_{III}\phi$												
HA	9	30	24	90								153
(26.3)	5	5	1	26								37
	55.5	16.7	4.2	28.9								24.2
$P_{III}\sigma \times V_{II}\phi$												
HA	173	79	72	245	298	90	551	190	82	185	150	2115
(31.6)	80	9	51	85	57	1	185	60	48	2	59	637
	46.2	11.4	70.8	34.7	19.1	1.1	33.6	31.6	58.5	1.1	39.3	30.1
$P_{II}\sigma \times V_{I}\phi$												
HA	45	54	110	55	96	52	20	20	12			464
(40.0)	38	24	28	20	73	35	6	0	0			224
	84.4	44.4	25.5	36.4	76.0	67.3	30.0	0	0			48.3

a: number of eggs laid, b: number of eggs hatched, c: percentage hatching.

V-II × *P-III*. NA: Hatching ratios of homogamic pairs of both *V-II* and *P-III* are similar with each other, ranging approximately 50~90% (\bar{x} about 70% or more). While those of heterogamic pairs were remarkably low with the means only a few percents in either combination, being 0% in many pairs with the maximum 17% in *V-II* ♂ × *P-III* ♀ and 35% in *P-III* ♂ × *V-II* ♀. HA: Hatching ratio is variable, ranging 4~56% (\bar{x} =26%) in *V-II* ♂ × *P-III* ♀, and 1~71% (\bar{x} =32%) in *P-III* ♂ × *V-II* ♀.

P-II ♂ × *V-I* ♀. NA: Only one pair laid three egg masses in total, each consisting of 3, 20 and 12 eggs. Among them, one egg mass (20 eggs) produced 7 larvae. Total hatching ratio is 20% (7/35). HA: Hatching ratio for the eggs produced by each pair fluctuated from 0 to 84% (\bar{x} =40%).

4. *Growth*. Table 5 shows the growth of some hybrid immatures obtained by the experiment with NA. Larval mortality and larval period of the hybrids were, except for the mortality of *V-II* ♂ × *P-III* ♀, normal in comparison with those of homogamic mating so far reported (WATANABE and SUZUKI 1965, HINOMIZU 1976).

Table 5. Growth of some hybrid larvae obtained by the crossing of newly emerged adults (NA). Reared with potato at 23°C.

Combination of parents, codes of parents and egg mass	No. of individuals reached each stage ¹⁾						Larval period ²⁾ (days)	
	L ₁	L ₂	L ₃	L ₄	P	A(♂/♀)		
<i>P_{III}</i> ♂ × <i>V_I</i> ♀	1a	7	6	6	6	6	6 (4/2)	28-34 ³⁾
	%	100	86	86	86	86	86	\bar{x} =29.0
<i>P_{III}</i> ♂ × <i>V_{II}</i> ♀	1a	3	3	3	3	3	3 (2/1)	24
	4a	2	2	2	2	2	2 (1/1)	22-23
	4b	1	1	1	1	1	1 (0/1)	21
	6a	11	11	11	11	11	11 (6/5)	23-25
	6b	2	2	2	2	2	2 (2/0)	22-23
	6c	19	17	16	16	12	12 (5/7)	22-25
	6d	6	6	6	6	6	6 (2/4)	21-26
	6e	20	19	19	19	19	19 (11/8)	21-25
	6f	21	21	21	20	19	19 (11/8)	23-26
	7a	1	1	0	0	0	0	
	Total	86	83	81	80	75	75 (40/35)	
%	100	94	92	91	87	87	\bar{x} =23.3	
<i>V_{II}</i> ♂ × <i>P_{III}</i> ♀	5a	3	1	1	1	1	1 (1/0)	24
	7a	3	0	0	0	0	0	
	7b	6	4	3	3	3	3 (1/2)	22-29
	7c	5	4	2	2	0	0	
	8a	21	18	18	17	10	10 (2/8)	22-25
	8b	6	1	1	0	0	0	
	Total	44	28	25	23	14	14 (4/10)	
	%	100	64	57	52	32	32	\bar{x} =23.4

1) L₁~L₄: 1st to 4th instar larvae, P: pupae, A: adults.

2) Days from hatching to emergence.

3) Under uncontrolled room temperature.

Although the larval mortality in V-II ♂ × P-III ♀ seems fairly high, the result is still inconclusive. On the other hand, by rearing offspring of heterogamic pairs of HA (cf. Table 4), we got 64 males and 83 females (offspring of seven pairs combined) from P-II ♂ × V-I ♀, 6 males and 10 females (single pair) from V-II ♂ × P-III ♀ and 78 males and 82 females (nine pairs) from P-III ♂ × V-II ♀. Among them only one male from P-III ♂ × V-II ♀ was regarded as the hybrid offspring. All others evidently conspecific with the mother as far as external morphological features were compared with the hybrid specimens obtained by the experiment with NA. Probably they were produced from eggs inseminated by homogamic sperms received before the experiment. The morphological features of hybrids showed the states intermediate of the parents. Their detailed morphology will be reported elsewhere together with that of other available specimens.

Discussion

As mentioned above, an obvious positive sexual isolation was observed in V-I × P-II and V-II × P-III. As to the allopatric combination, V-I and P-II, however, it is uncertain whether a similar result can be obtained or not with respective sympatric counterparts. Choice seemed to be made mainly by the male, but, by what kind of information the male recognizes the conspecific female is yet unknown. Remarkable low hatching ratio and intermediate hatching ratio of eggs laid respectively by the heterogamic mating in NA and HA experiments are noticeable. Low viability in hybrid embryos and gametic isolation are suspected as the causes of low hatching ratio in NA. Since the hybrid immatures grew normally, the low viability in hybrid embryos may be not crucial, even if not excluded completely. Then, gametic isolation is considered as a more important factor being responsible for the low hatching ratio in heterogamic pairs of NA. Although the mechanism of gametic isolation is unknown, the intermediate hatching ratio in heterogamic pairs of HA may be explained as follows:

Most HA females are considered already mated with homogamic males before collected. Therefore, a female mated with a heterogamic male in the experiment probably received the sperms of both species. Provided that equal amount of sperms of both species are preserved in the spermatheca and that sperms of both "forms" are equally accepted by the eggs, the hatching ratio of the eggs laid by the female can be calculated from the results with NA in V-II and P-III (cf. Table 4) as:

$$\text{V-II♀: } 0.5 \times 0.72 + 0.5 \times 0.04 = 0.38$$

$$\text{P-III♀: } 0.5 \times 0.74 + 0.5 \times 0.04 = 0.39$$

The more increases the ratio of heterogamic sperms, the more decreases the hatching ratio. Then the fluctuation of actual hatching ratio among the pairs of heterogamic HA given in Table 4 is explained by the different ratio of heterogamic sperms possessed by each female. If such assumption is valid, however, we should find more hybrid specimens than actually obtained. As mentioned earlier, we got

only one individual out of 323 by rearing larvae of heterogamic HA. Since a large proportion of the larvae of heterogamic HA died by unfavorable rearing conditions, three possibilities can be pointed out: A) the hybrid larvae were less tolerable than the normal larvae under unfavorable rearing conditions, so that much more hybrid larvae were eliminated, B) the hybrid larvae were eliminated by competition with the normal larvae, or C) the activity of heterogamic sperms in the spermatheca was inferior than that of homogamic ones. Apparently there may be other likely explanations. The final solution may be obtained by further studies on the mechanism of gametic isolation.

Finally we summarize the present knowledge of isolating mechanisms between V-II and P-III as an example of sympatric Hv and Hp. In a previous paper, one of us (KATAKURA 1976) discussed the role of some ecological factors as mechanisms isolating V-II and P-III:

Difference of food habits: V-II virtually depends on potato in field and *S. bryoniaefolius* in forest, while P-III on thistle (*Cirsium kamtschaticum*) and/or *Caulophyllum robustum*. V-II can not complete its growth with the host plants of P-III, namely thistle and *C. robustum*. However, P-III can complete its growth with potato or *S. bryoniaefolius* (KUROSAWA 1953, WATANABE and SUZUKI 1965, HINOMIZU 1976, KIMURA unpubl.). Further, according to HOSHIKAWA (pers. comm.), P-III prefers *S. bryoniaefolius* as equal as thistle or *C. robustum*, though potato is less preferred than these three plants.* Field observation revealed the invasion of some P-III into potato fields adjacent to its habitat. P-III also sometimes fed nearby *S. bryoniaefolius* especially in the fall (KATAKURA 1975, 1976).

Phenological difference: The appearance of post hibernating adults of P-III is about four weeks earlier than that of V-II. Phenology of P-III or V-II and respective host plants are well coincided with each other. Consequently, potato and *S. bryoniaefolius* become available for P-III three or four weeks after the appearance of the latter, while thistle and *C. robustum* precede the appearance of V-II.

Phenological difference of V-II and P-III indubitably reduce the chance of encounters between them. Further, the entry of V-II into the habitat of P-III is nearly completely unsuccessful by the host plant specificity alone. The reversed entry by P-III may be restricted at least partly by the phenological difference between P-III and the host plants of V-II. Therefore, the combination of these two types of isolation must fairly reduce the chance of encounters between P-III and V-II. Moreover, as reported in the present paper, two other types of isolation mechanisms may act even if two forms encountered and mated. The sexual isolation decreases the frequency of heterogamic mating at casual encounters. And the low hatching

* Based on the information available at that time, KATAKURA (1976) considered that P-III could not complete its growth with *S. bryoniaefolius* under natural condition. Therefore, he regarded that V-II and P-III were separated with each other by the host plant specificity as far as thistle and *S. bryoniaefolius* were considered respectively as host plant of P-III and V-II. This explanation was now invalidated since subsequent studies (KIMURA unpubl., HOSHIKAWA unpubl.) revealed the normal growth with, and high preference for *S. bryoniaefolius* of P-III.

ratio of hybrids minimizes the occurrence of natural hybrids. Thus, the rare occurrence of the hybrids in natural condition is explainable by the combination of these isolating mechanisms, though any of which alone can not isolate V-II and P-III completely, and some other unknown mechanisms may strengthen the isolation.

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