Visual Stimuli Eliciting Mate Refusal Posture in the Mated Female of the Cabbage White Butterfly, *Pieris rapae crucivora* (Lepidoptera: Pieridae)

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Visual key stimuli eliciting mate refusal posture (MRP) in the mated female of the cabbage white butterfly, *Pieris rapae crucivora*, were experimentally analysed. Whiteness or luminosity is an essential feature of the visual stimuli and the critical luminosity at which 50% of the experimental females respond falls between N7 and N10 in terms of the Munsell Number. Females responded almost equally (75%) to the models of different sizes ranging from half to quadruple of the normal size and much less (50%) to the models of one fourth. Females responded almost equally to the differently shaped models including butterfly wing shape, rectangle, square, triangle and disk. Movements, or increase and decrease of the stimulative area was found to be another essential feature. The highest percentage of the response was obtained when the models were presented at the revolution rate of 6/s, which is nearly equal to the wing beat frequency of this male butterfly. Low and high critical revolution rates at which 50% of the experimental females responded are ca. 4 and 20–25 per s, respectively. MRP and its key stimuli were discussed in terms of behavioral ecology.

Key words: *Pieris rapae crucivora*, mating behavior, mate refusal posture, visual releaser

INTRODUCTION

It has been repeatedly pointed out that insects are advantageous subjects to study neural mechanisms underlying behavior (HOYLE, 1970; HUBER, 1975; CAMHI, 1980). The reasons for this include availability, simplicity of neural organization, a small number of nerve cells, and relatively steady recording of muscle potentials in unrestrained insects, etc. For the same reasons we have studied neural mechanisms underlying mating-induced behavioral switch in the cabbage white butterfly, *Pieris rapae crucivora*.

A female *Pieris rapae crucivora* changes her responsive manner to the conspecific male's mating activities depending on her life history. A female rejects copulatory attempts by males during ca. 30 min following emergence while her wings are soft (primary rejection) (OBARA, 1987). The primary rejection is performed by a specific mate refusal posture (hereinafter abbreviated as MRP), namely, she depresses the wings and elevates the abdomen when she is approached and attempted to be mated by a male. She remains in this posture as long as the male clings to or stays in the

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vicinity of her in an attempt to mate. Following the primary rejection, she becomes receptive and permits the conspecific male to mate by assuming a rest posture in which she maintains the wings closed and remains motionless. She becomes, however, unreceptive immediately after copulation (secondary rejection) (OBARA, 1964) as do other insects (LOHER and HUBER, 1966; BARTH and LESTER, 1973). The secondary rejection is also performed by MRP. In the secondary rejection period she remains unreceptive for ca. a week, during which she lays substantially all of her eggs (SUZUKI, 1979). Spermatophore counts on wild caught females showed that the average number of matings for females was estimated at 2.84, suggesting that females become receptive again a few days after the secondary rejection (SUZUKI, 1979).

A switch from the rest posture in a virgin female to MRP in a mated female is induced by the stretch stimulation to the bursa copulatrix caused by the spermatophore transferred from a male during copulation (OBARA et al., 1975; SUGAWARA, 1979). It has been suggested that these mechanical stimuli, in turn, induce a "mate refusal hormone" secretion which will provide a humoral background for the occurrence of MRP (OBARA, 1982). Electrophysiological investigation, on the other hand, suggests that MRP may be underlied by slow motor neurons (OBARA, 1994). For further electrophysiological analysis, however, it is necessary to clarify the key stimuli for MRP in order to device a stimulating apparatus by which MRP could be steadily elicited in the female tethered on the electrophysiological setup.

A previous study revealed that visual stimuli are essential in eliciting MRP (OBARA, 1984). In the present study, a series of experiments were carried out to reveal detailed properties of the visual key stimuli.

MATERIALS AND METHODS

(a) *Butterflies*. The butterflies used in the present experiments were raised at a room temperature of 22°C with a 16L-8D photoperiod regime.

Male and female pupae obtained were discriminated by the difference of external reproductive organ origin and were put into separate cages (30×30 cm) for emergence. After emergence they were kept separate according to sex in different cages (30×30×30 cm). The mated females for the experiment were prepared by mating virgin females from the stock with males in a cage for mating (30×30×30 cm) from 10 a.m. until noon on the day of the experiment. The males and females in copulation were separated into smaller cages (15×10×10 cm) and placed in a dark room. By this procedure, the fluttering of the males and females within the cages could be suppressed and we could prevent the mated females from taking MRP before the experiment. We removed the males who completed mating carefully from the smaller cages and thus mated females were prevented from any contact with other butterflies. Thirty minutes before the experiment, the females were moved from their small cages to outdoor cages (4×4×2 m) and allowed to adjust to the surrounding environment.

Females who emerged several days earlier or wild mated females, were used to supplement females for the experiments when they were insufficient in number. However, for most of the experiments, females emerged on the day of experiment were subjected to the experiment. Males used for mating were several days old.

(b) *Stimulating models*. The present investigation consists of seven experiments and one preliminary observation. The method for each is described below.

Experiment 1: Male wings: The following four models were used in the first experiment to examine the role of male wings to elicit MRP (Fig. 1 B.a).

Dorsal surface-dorsal surface model (D-D): the dorsal surface was attached to both sides of paper cut in the shape of the wings so that the model exposes the male wing's dorsal surface only.

Ventral surface-ventral surface model (V-V): the ventral surface was attached to

Fig. 1. Experimental setup and the stimuli used for MRP. Experimental females (F in A) were stimulated through transparent plastic wrap (W in A) by the stimuli including flying male (M in A) and different models (B and C). $A_{\max}$ and $A_{\min}$ in C are, respectively, the maximum and minimum stimulus surface area of the different drum models. For further explanation, see text.

both sides of paper cut in the shape of the wings so that the model exposes the male wing's ventral surface only.

Dorsal surface-ventral surface model (D-V): both dorsal and ventral surfaces of the male wings were attached to paper cut in the shape of the wings so that the model expose both the dorsal and ventral surfaces.

Specimen model (specimen): a living male was pinched in the thorax and killed. Then a pin was placed from the abdominal tip through the male with the wings extended.

For the control experiment, undamaged, healthy "flying" males were used as in the previous paper (OBARA, 1984; Fig. 1 A). Briefly, the "flying" males were prepared first by attaching a 7 to 8 cm string with paraffin to the mesonotum of the male. Then a long, narrow stick ($4 \times 4 \times 200$ mm) was attached to the other end of the string. By lofting the stick, the male could be raised. As soon as the male was raised, he began to flutter his wings and was brought close to the experimental female to stimulate her. This procedure imitates the male as he approaches the female in an attempt to copulate.

Experiment 2: White and black parts of the wings: To reveal the role of the white and black parts of the male wing to elicit MRP, experimental females were stimulated by 5 different black-white models. The models were prepared by cutting off the white and black parts of the wing separately and then attaching them separately to both sides of paper cut in the shape of the wings (Fig. 1 B.a). The 5 models are: the forewing dorsal surface white model (W-FD) to which the white forewing dorsal section was attached to both sides of paper cut in the shape of the wings, the forewing ventral surface white model (W-FV) which had the white forewing ventral section attached, the hindwing dorsal surface white model (W-HD) which had the hindwing dorsal surface white section attached, the hindwing ventral white model (W-HV) which had the hindwing ventral white section attached and the forewing dorsal black model (B-FD) which had the black markings on the apices of the dorsal surface of the forewing attached.

Experiment 3: Color: To reveal the role of color in eliciting MRP, 9 rectangular paper models were prepared (Fig. 1 B.b) using black and white paper, and the following 7 kinds of colored paper which were purchased from Japan Color Science Co. Ltd. The colored paper is standardized by Munsell No. (within parenthesis): white (N10), black (N1), red (5R4/14), yellow-red (5YR/14), yellow (5Y7/14), green (5G5/10), blue-green (5BG4/5), blue (5B5/5) and purple (5P4/12).

The area for each rectangular model (5×2.5 cm) was the same as the wing area of the butterfly.

Experiment 4: Luminosity: To study whether luminosity is involved in eliciting MRP, 3 rectangular paper models were made using grey-colored paper with successive degrees of luminosity. The three types of grey-colored paper were the same standardized type of paper used in the third experiment purchased from Japan Color Science Co. Ltd. They are white (N10), light grey (N7) and dark grey (N4). The models were 5×2.5 cm rectangles (Fig. 1 B.b).

Experiment 5: Shape: To study the role of shape in eliciting MRP, we carried out experiments using the following 5 shapes of white (N10) models: a butterfly shape, a rectangle, a square, a triangle and a disk (Fig. 1 B.a-e). The area of each model was the same as that of the butterfly's wings.

Experiment 6: Size: To study the role of size in eliciting MRP, the 5 following sizes of white (N10) paper models were used. Namely, with a rectangular model of the

natural butterfly size (5×2.5 cm) as 1, models of the same shape at $1/4$, $1/2$, $2 \times$ and $4 \times$ the size were made (Fig. 1 B.b).

Experiment 7: Revolution rate: To study the role of movement in eliciting MRP, we made a white (N10) rectangular (5×2.5 cm) paper model (Fig. 1 B.b) and experimented by revolving the model at 10 different rates; 1, 3, 4, 5, 6, 9, 12, 15, 20 and 25 rev. per s.

Supplement experiment: To reveal what visual stimuli produced by wing beat or model revolution are essential in eliciting MRP, we carried out preliminary experiments supplementing experiment 7. In these experiments, different kinds of drums with the stimulus surface attached around the circumference were presented to a once-mated female while being revolved (Fig. 1 C). When revolved, the spherical drum (Fig. 1 C.a) exhibits no increase and decrease of the stimulus surface area, while the square (Fig. 1 C.b), diamond-shaped (Fig. 1 C.c) and obtuse rectangle drums (Fig. 1 C.d) change their stimulus surface area by 29%, 50% and 75%, respectively.

We attempted to repeat the experiments the same number of times for each model, but in a few cases when the females in the experiment were lost, the experiment ended at that point. Except for the control experiment, all of the models were revolved by motor at the rate of 6 revolutions per second and brought within a distance of 2 to 3 cm of the experimental female. The revolution rate is nearly the same as wing beat frequency of the male (about 7 per s).

For all of the experiments we changed the flying male, the male wings and paper models for new ones with each experiment. In cases when the models were damaged during the experiments, they were exchanged with new ones at that point.

The motor used for the revolving models allowed us to freely select the revolution rate from 0 to 25 rev. per s. There was no more than 3% variation in the revolution rate.

(c) *Procedure.* To exclude all other possible sensory stimulation except for vision such as olfaction, wind and contact, the fluttering male and the various models were presented close to the female through a thin, transparent plastic wrap stretched across a 25×25 cm frame (Fig. 1 A). The distance between the female and the clear wrap, and between the clear wrap and the model was 2 and 3 cm respectively. The model was made to come from behind the female (Fig. 1 A) and move in an area of about 5 cm in diameter slowly to and fro, left to right. The experiment was performed while the female alighted quietly on cabbage or other substrates. Whenever the female was restless, her movement could usually be stopped by interrupting the sunlight with hand or blowing on the butterfly with one's breath.

(d) *Criteria for MRP response.* The responses of once-mated females to each model were classified into 3 categories.

‡: the response was indicated by wings depressed over 90° and abdomen elevated over 60° .

+: the response to the model was clearly indicated, but the degree of wing depression and abdomen elevation were less than ‡.

—: unclear response or no response at all.

We submitted the females to control experiments after each experiment, in which we examined the occurrence of MRP to presentation and contact stimuli of fluttering males. The females that did not show MRP in the previous experiments all showed the typical MRP to the direct stimuli of fluttering males. And the females that did

not show the typical MRP in the experiment (+) showed a typical response without exception in the control experiment. Therefore, the response determined to be (+) can be interpreted as not different from the typical response (⧻). For this reason the response rate of all the experiments was calculated as below.

$$\text{response rate (\%)} = \frac{\text{number of females showing either } (\text{⧻}) \text{ or } (+) \text{ responses}}{\text{number of females experimented}} \times 100$$

Very few females showed no response in the control experiments at all (1%). Those females were excluded from the experiments. Most of those females mated late in the afternoon and remained in copulation with their mates for a long period after that. Those females were found not to have received an adequate spermatophore from the male during copulation.

The experiment was conducted in a metal outdoor cage of 4×4×2 m. In the cage there were a variety of plants including 20 to 30 cabbage plants on which the larva of this butterfly feed. Thus the interior environment of the cage was nearly the same as the natural habitat of this butterfly in wild.

To find out what influence the time of day had on the occurrence of the response, we repeated the experiment every 2 h from 6 a.m. to 5 p.m. but we failed to obtain evidence suggesting a difference in the response rate due to the time of day. The experiments took place twice on the day of copulation from 2 to 4 p.m. and twice the next day from 9 to 11 a.m., for a total of 4 times for each female. The interval between the first and second, and the third and fourth experiments was an hour with each female, but there were 18 h between the second experiment conducted on the day of copulation and the third one conducted on the following morning.

RESULTS

Experiment 1: Male wings

There was no significant difference between the control and the different wing surface models including the ventral-ventral, dorsal-dorsal, ventral-dorsal and specimen (χ^2 -test, $p > 0.19$) (Table 1). The results also showed that the revolving models were as effective as the fluttering male in the control experiment in eliciting MRP. Therefore, the revolving model was used in the following experiments.

Experiment 2: White and black parts of the male wing

The females showed a high rate of response (over 90%) to all of the white models

Table 1. Female mate refusal responses to the different male wing surfaces

Model	N	MRP			%
		⧻	+	—	
D-D	32	23	6	3	90
V-V	32	21	7	4	87
D-V	32	18	4	10	69
Specimen	28	10	11	7	75
Control	34	23	5	6	82

Table 2. Female mate refusal responses to the white and black parts of the male wing

Model	N	MRP			%
		⧻	+	—	
W-FD	23	23	0	0	100
W-FV	27	19	7	1	96
W-HD	26	25	0	1	96
W-HV	27	23	2	2	93
B-FD	27	1	0	26	4

Table 3. Female mate refusal responses to the different colored paper models

Color (Munsell No.)	N	MRP			%
		‡	+	—	
White (N10)	44	29	8	7	84
Black (N1)	20	0	0	20	0
Red (5R4/14)	22	0	0	22	0
Yellow-Red (5YR7/14)	22	0	0	22	0
Yellow (5Y7/14)	22	0	0	22	0
Green (5G5/10)	22	3	4	15	32
Blue-Green (5BG4/5)	22	0	1	21	5
Blue (5B5/5)	22	3	3	16	27
Purpul (5P4/12)	20	0	0	20	0

Table 4. Female mate refusal responses to the neutral paper models with different luminosities

Brightness (Munsell No.)	N	MRP			%
		‡	+	—	
White (N10)	35	15	10	10	71
Light grey (N7)	35	7	3	25	29
Dark grey (4)	33	3	3	27	17

(forewing dorsal surface, forewing ventral surface, hindwing dorsal surface and hindwing ventral surface), whereas negligibly low (4%) to the forewing dorsal black model (χ^2 -test, $p < 0.001$) (Table 2).

Experiment 3: Color

The response rate to the white model was over 80% (Table 3). In contrast, the response rate to other color models was none or significantly lower (χ^2 -test, $p < 0.001$). The green and blue models showed ca. 30% rate of response and the blue-green model showed a response of only 5%. The females did not assume the MRP to the other colored models including black, red, yellow-red, yellow and purple. From these results it is clear that white is an essential factor for the release of MRP; none of the other colors effectively elicit the response.

Experiment 4: Luminosity

There was a clear difference between the response rate of the female for the white model and light- and dark-grey models (χ^2 -test, $p < 0.003$) (Table 4). The more luminosity was, the higher the rate of response was. The critical luminosity at which 50% of the females responded lies between N7 and N10 in Muncell Number.

Experiment 5: Shape

Both the butterfly shape and the rectangular shape gave a response rate of 75% (Table 5). Table 6 shows response rates to the rectangular, square, triangular and disk models. No difference was evident in the response rate due to shape (χ^2 -test, $p > 0.28$).

Table 5. Female mate refusal responses to butterfly-shaped and rectangular models

Form	N	MRP			%
		++	+	—	
Butterfly-shape	16	7	5	4	75
Rectangle	16	6	6	4	75

Table 6. Female mate refusal responses to the differently shaped models

Form	N	MRP			%
		++	+	—	
Rectangle	18	14	2	2	89
Square	18	11	6	1	94
Triangle	18	11	4	3	88
Disk	18	11	5	2	89

Table 7. Female mate refusal responses to the models with different sizes

Size	N	MRP			%
		++	+	—	
1/4	26	7	5	14	46
1/2	22	11	7	4	82
1	24	14	5	5	79
2	24	9	10	5	79
4	24	14	4	5	78

Size 1 is the normal wing size of the average wild male butterfly.

Table 8. Female mate refusal responses to the white paper models with different revolution rates

Revolution/s	N	MRP			%
		++	+	—	
1	22	0	0	22	0
3	22	1	2	19	14
6	22	14	3	5	77
9	22	6	9	7	68
12	22	11	4	7	68
15	22	7	3	12	45
20	22	7	5	10	55
25	22	2	5	15	32

Table 9. Female mate refusal responses to the white paper models with different revolution rates

Revolution/s	N	MRP			%
		++	+	—	
3	29	5	7	17	41
4	29	13	3	13	55
5	29	16	6	7	76
6	29	15	8	6	79

Experiment 6: Size

All models over 1/2 the normal size (1) showed a response rate of ca. 80% (Table 7). In contrast, the model of 1/4 the normal size elicited MRP less frequently than those bigger ones (χ^2 -test, $p < 0.02$).

Experiment 7: Revolution rate

Table 8 indicates that the revolution rate plays an important role in eliciting MRP. The highest response rate was obtained at 6 revolution per s. The response lowered steadily when the revolution rate increased from 6 per s and it lowered sharply when decreased. Assuming the critical rate of revolutions at which 50% of the females responded, the lower critical revolution rate was between 3/s and 6/s and the higher rate between 20/s and 25/s. The results of another experiment with the more precise revolution rate indicated ca. 4 as the lower critical revolution rate (Table 9).

Supplement experiment

The results of the supplemental experiments were suggestive of what parameters produced by revolutions were essential in eliciting MRP. The spherical drum had no efficacy to elicit MRP, whereas the square, diamond shape and obtuse rectangle drums elicited nearly the same degree of response as the control used. No MRP occurred when either the male butterfly with open wings or the male fixed in a rest position, was moved horizontally 6 times per s to left and right, several centimeters above the female.

DISCUSSION

1. Properties of visual key stimuli

a) *Markings and color:* Table 1 shows that both the dorsal and ventral surfaces of the male wings which differ in markings are equally effective in eliciting MRP. This suggests that the markings on the male wing play no essential role in eliciting MRP. This is substantiated by the results of experiment 2 (Table 2) showing that the model consisting of the white section of the male wings with no markings was sufficient to elicit MRP. Table 2 also shows that the black section on the male wing does not perform alone any essential role in eliciting MRP.

Only the white colored paper model induced a response comparable to that of the control experiments (Table 3). This indicates that the elicitation of MRP is fully possible with objects other than the natural butterfly and that whiteness (luminosity) is the essential eliciting factor. No other color is nearly as effective in eliciting MRP. This is not due to the color blindness of this species, since it is capable of perceiving ca. 340 to 650 nm of light (OBARA et al., unpublished). It can, hence, be concluded that the colors would not be stimuli to elicit MRP. It remains to be revealed, however, whether the colors such as blue, blue-green with any efficacy to elicit MRP perform the same function as white or some other function to elicit MRP.

b) *Shape:* Tables 5 and 6 indicate that there seems to be no difference in the efficacy of each of the differently shaped models to elicit MRP. It is therefore unlikely that a mated female recognizes a male willing to mate with her on the basis of the specific shape of the male wing. However, it may be necessary to consider the size of the models used here in connection with the distance between the model and the experimental females. It may be possible that the models used in the present experiment covered the entire visual field of the butterfly because of the relatively close distance of a few centimeters between the model and the experimental females. Therefore, the results might be due to the fact that the entire model was not within the visual field of the butterfly and consequently the entire shape could not have been perceived. For this reason, to reveal the role of shape to elicit MRP, experiments must be conducted which either use smaller models or place the models at a farther distance from the experimental females.

c) *Size:* The results of experiment 7 show that there is no variation among the models of different sizes in their effects to elicit MRP except for 1/4 of the normal size. Consequently it seems likely that the wing size typical of the male of this species is not utilized by a mated female to recognize a male attempting to copulate. However, as for the shape mentioned above in b), the results do not necessarily mean that size plays no role in the elicitation of MRP. If we assume that 1/2 of the normal size

model completely fills the visual field of the butterfly because of the relatively close distance between the model and the experimental butterfly, it follows that models of a larger size would not be expected to increase the response rate. This inference can be confirmed by an experiment which presents the model at a greater distance from the experimental females. By presenting the models used in experiment 6 four times, each at a distance of 20 cm from the females, positive responses to the quadruple model of the normal size were obtained three times, but no response to the other smaller models. It remains to be revealed whether the perception of size was based on either the visual angle or on the absolute size of the model.

d) Movement: The results obtained here (Table 8) show that male wings or effective models failed to elicit MRP as long as they were presented motionless. From this evidence, there is no doubt that movement is essential in eliciting MRP. However, the results of the supplemental experiment for experiment 7 indicate that the spacial displacement of the models which did not accompany the change in the effective stimulus area is not sufficient to elicit MRP. This has been repeatedly supported by our observation that no MRP occurred when the male butterfly with the wings open or the male fixed in a rest position with the wings closed was moved horizontally to left and right at the rate of six times per s, several centimeters above the female.

When we compare the fluttering male with the revolving models, we notice that there is no difference in their efficacy to elicit MRP. This suggests that the movement essential to elicit MRP is not the pattern of the movement itself, but some parameter of the visual stimuli that occurs with the movement. The parameter must be shared by both the fluttering movement and the revolving movement. This leads to the speculation that the parameter may be the increase and decrease of the stimulus surface area or its intermittent exposure as an extreme. In fact the speculation was substantiated by the supplemental experiment for experiment 7. As described above in the results, the spherical drum or male models which exhibited no change in the stimulus area failed to elicit MRP, while those drum models which changed their stimulus surface area when revolved were all effective in eliciting MRP. The results showed that at least a 29% change of the area of the essential stimulus surface was sufficient to elicit MRP as in the case of the square drum.

A slow motion film of a fluttering male (HIDAKA and OBARA, 1968) and electromyograms recorded from the indirect flight muscles of a tethered male (OBARA, 1975) revealed that male wing beat frequency lies around 7/s. When we combine this rough estimate of the male wing beat frequency with the most effective revolution rate (Tables 8 and 9), it can be stated that a female recognizes a male willing to mate on the basis of the most effective and reliable parameter of the wing movement.

2. *Ecological significance of MRP and its key stimuli*

A female of this species receives a sufficient amount of spermatozoa to fertilize substantially all of her eggs by a single copulation (SUZUKI, 1978). It has not been documented that the female fecundity increases by multiple copulations in this species. Therefore it seems beneficial to her that she avoids multiple copulation and thereby reserves time and energy. If so, natural selection will favor a female who rejects repeated copulation and instead starts oviposition as soon as possible because she may thereby succeed in reserving more food plants for her offspring than those females who remate, with delayed oviposition. It is quite likely that her earlier-hatched larvae

may enjoy sufficient amount of leaves to feed, while those of the latter will find scanty leaves left when they hatch, since wild food plants of this species contain only a small amount of leaves. Such poorly fed larvae emerge as small butterflies and in case of females, they will suffer from fecundity loss, since smaller females lay a smaller number of eggs (SUZUKI, 1978).

It may be, however, waste of time and energy if a mated female responds with MRP to males who engage in other activities and are not sexually motivated. It is hence behavior-ecologically important for her to discriminate between males willing to mate and those unwilling to do so. It is reasonable in this respect that a mated female utilizes the two visual features, i.e., whiteness and wing beat to recognize a male willing to mate because they represent a male attempting to mate. The male of this species always approaches a female by flight when he attempts to mate her and thus his fluttering wings produce the essential visual stimuli for MRP. By utilizing the feature of fluttering wings as a cue representing males attempting to mate, she can also avoid useless MRP responses to the resting, roosting or feeding males nearby with the wings unmoved. On the contrary, she may be forced to assume useless MRP in reaction to males who approach a flower to feed or who just pass by, although MRP response elicited in such a situation is observed to appear only momentarily.

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