

Serotonin-induced mate rejection in the female cabbage butterfly, *Pieris rapae crucivora*

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Abstract Virgin female cabbage butterflies, *Pieris rapae crucivora*, accept and mate with courting males, whereas mated females reject them and assume the “mate refusal posture”. This study tested whether the biogenic amines, serotonin (5HT), dopamine (DA), and octopamine (OA), were responsible for this change in behavior. The results showed that 2–3-day-old virgin females fed with 5HT rejected courting males significantly more frequently compared with controls fed on sucrose. In contrast, the proportions of courting males rejected by virgin females fed with either DA or OA did not differ from sucrose-fed controls. Oral application of each amine resulted in significantly increased levels of the amine applied (or its metabolite) in the brain. The results strongly suggest that 5HT or a 5HT metabolite may be responsible for the post-mating change in behavioral response of 2–3-day-old virgin females to courting males. Similar effects of 5HT treatment were observed in 6–8-day-old virgin females, but in this case the results were only marginally different from the controls, suggesting that the effect may decline with increasing female age.

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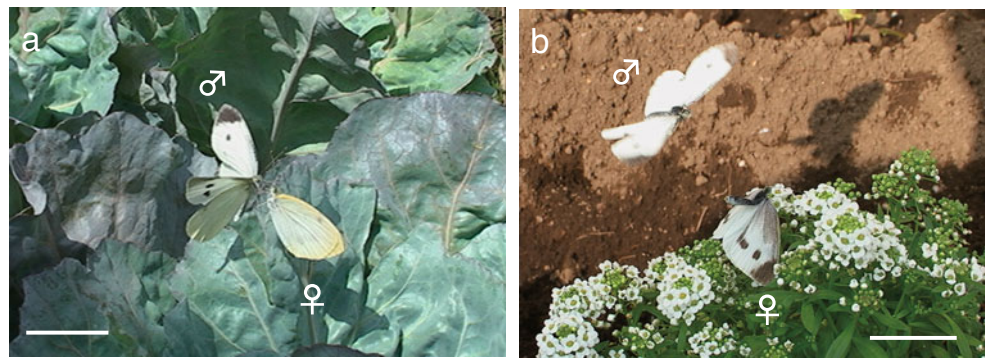
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Introduction

Individual animals often change their behavioral repertoire as a reproductive or survival tactic. These behavioral transitions may be caused by humoral factors, such as neuromodulators and neurohormones. The presence of neuroactive substances can result in a specific behavioral state which may persist for hours or even days. Biogenic amines act in this way in the peripheral and central nervous systems of insects, as well as influencing many other physiological processes (Evans 1980; Roeder 2005; Scheiner et al. 2006).

Female Japanese cabbage butterflies, *Pieris rapae crucivora*, exhibit a dramatic behavioral change toward conspecific courting males following copulation (Obara 1964). Before copulation, virgin females respond to courting males by assuming the resting posture with the wings closed, thereby accepting males for copulation (Fig. 1a). In contrast, mated females respond to courting males by assuming the “mate refusal posture”, rejecting copulation by sustained wing depression and abdomen erection (Obara 1984; Itoh and Obara 1994) (Fig. 1b). This change in female response to males has been attributed to the inflation stimulus of the bursa copulatrix by male ejaculates transferred during copulation (Obara et al. 1975; Sugawara 1979). It is suggested that nerve impulses from mechanical stimuli during copulation pass along the ventral cord from the last abdominal ganglion to at least the first abdominal ganglion (Obara et al. 1975), perhaps inducing release of a “mate refusal hormone” that causes mated females to become unreceptive and assume the mate refusal posture (Obara et al. 1975; Obara 1982).

Fig. 1 Behavioral response of a virgin (a) and a mated female (b) to a courting male, shown assuming a rest posture and a mate refusal posture, respectively. Scale bars, 25 mm



However, the substances involved in the mate refusal hormone have yet to be determined. The present investigation consisted of three experiments (experiment 1–experiment 3) which were designed to test the possibility that one of the three biogenic amines, serotonin (5HT), dopamine (DA), and octopamine (OA), may be involved with the mate refusal hormone.

Materials and methods

Experiment 1: effect of biogenic amines on the behavioral responses of younger virgin females

Experiment 1 comprised two parts (experiment 1a and b). Females used in experiment 1a were derived from larvae reared on potted cabbage plants in the laboratory under a 15:9 h light/dark regime at 25°C. Freshly emerged females were introduced into a dark room maintained at 5°C and remained kept there for 2 days without food. This starvation treatment was performed to facilitate their subsequent feeding on the biogenic amines used in the experiment. Two days after emergence, the females were moved in the evening from the cold room to a laboratory room at 25°C and randomly divided into four groups. Individual 2–3-day-old virgin females in the four groups were then allowed to feed freely on one of the three biogenic amines (5HT, DA, and OA) or sucrose as a control. A 10% sucrose solution containing 2 mg ml⁻¹ of dissolved amine was used, following previous studies where significant behavioral and physiological effects in adult insects were observed using similar concentrations (Scheiner et al. 2002; Sasaki et al. 2009). The next day, the females were introduced into an outdoor cage measuring 9×9×2 m, containing over 200 virgin males.

Each female was placed on top of some cabbage leaves and her behavioral response to courting males was observed. Each behavioral assay is referred to as a trial, and each female was subjected to a maximum of five trials. If a female accepted a courting male during the first trial (by assuming the rest posture and mating), its response was judged as acceptance (Fig. 1a). If a female responded to a

courting male by assuming the mate refusal posture, she was tested for a maximum of four further trials. This was because some females adopted the mate refusal posture and rejected courting males in the first few trials but accepted males in subsequent trials. Females that responded with the mate refusal posture in all five trials were judged as rejection (Fig. 1b), while those that accepted a courting male before the fifth trial were categorized as ambiguous acceptance/rejection.

In experiment 1b, 2–3-day-old virgin females were developed from larvae collected in cabbage fields in Natori City, north Japan, during June 2010, and reared outdoors on potted cabbages. Emerging females were released into a 30×30×45 cm cage that was covered with black cloth to keep them still. Cages were maintained without food in a room at temperatures ranging from 18°C to 27°C for 2 days. As in experiment 1a, this starvation treatment was performed to facilitate the intake of amines. The females were fed with amines when they were 2 days old, following the same protocol as experiment 1a. The next day, when they were 2–3 days old, they were introduced into an experimental cage, 40×48×54 cm, containing doubled number of virgin experimental females and mating was observed. Mated females were removed with their male mates. This experiment was performed for each of the three amines and the sucrose control. Total of 25 females from each amine and sucrose group were subjected to the copulation experiment. Each experiment lasted for 1.5 h.

Experiment 2: effect of biogenic amines on the behavioral responses of older virgin females

In experiment 2, 6–8-day-old virgin females were tested for their rejection response. These females were treated in exactly the same way as those in experiment 1a, except that they were fed with sucrose every day from emergence until they were 3–5 days old. They were then starved until they were 5–7 days old, before being allowed to feed freely on amine or sucrose solutions in the evening. The following day, when they were 6–8 days old, they were tested for their mate rejection/acceptance response.

Experiment 3: measurements of brain levels of biogenic amines

Measurements of brain biogenic amine levels confirmed their uptake to the central nervous system following feeding with amine solution. The female butterflies were fed amines or sucrose (control) using the same procedure as in experiments 1 and 2, and were then euthanized using liquid nitrogen. The brain of each subject was dissected out and homogenized in a micro-glass homogenizer in 50 μl of 0.1 M perchloric acid containing 12.5 ng/ml 3,4-dihydroxybenzylamine as the internal standard and then centrifuged at 20,000 $\times g$ for 30 min at 4°C. Supernatants were analyzed by HPLC-ECD to quantify the biogenic amines present, following Sasaki et al. (2009).

Results

Experiment 1

Experiment 1a showed that feeding on 5HT by 2–3-day-old virgin females resulted in significantly greater rejection of males compared with the control (Fisher's exact test, 3 \times 2 tables; 5HT vs. control, $P < 0.001$, Fig. 2a-1). The effects of DA and OA intake were not significantly different from the control (DA vs. control, $P = 0.199$; OA vs. control, $P = 0.463$; Fig. 2a-1).

The results of experiment 1b are shown in Fig. 2a-2. Ingestion of 5HT resulted in significantly fewer copulations of treated females compared with the sucrose control. The effects of DA and OA were not significantly different from the sucrose control (Fisher's exact test, 2 \times 2 tables; 5HT vs. control, $P < 0.001$; DA vs. control, $P = 1.0$; OA vs. control, $P = 1.0$; Fig. 2a-2).

Experiment 2

The effect of 5HT on 6–8-day-old virgin females was similar to that on 2–3-day-old females but was not quite significant (Fisher's exact test, 3 \times 2 tables; 5HT vs. control, $P = 0.087$; DA vs. control, $P = 0.809$; OA vs. control, $P = 0.903$; Fig. 2b).

Experiment 3

In 2–3-day-old virgin females, brain levels of 5HT and its metabolite (*N*-acetylserotonin, N5HT) were significantly higher in 5HT-fed individuals than in control (Kruskal–Wallis test; 5HT: $H = 16.61$, $P < 0.01$; N5HT: $H = 12.05$, $P < 0.01$; Steel–Dwass test, $P < 0.05$, Fig. 3a, b). Brain levels of DA were not significantly different between DA-fed and

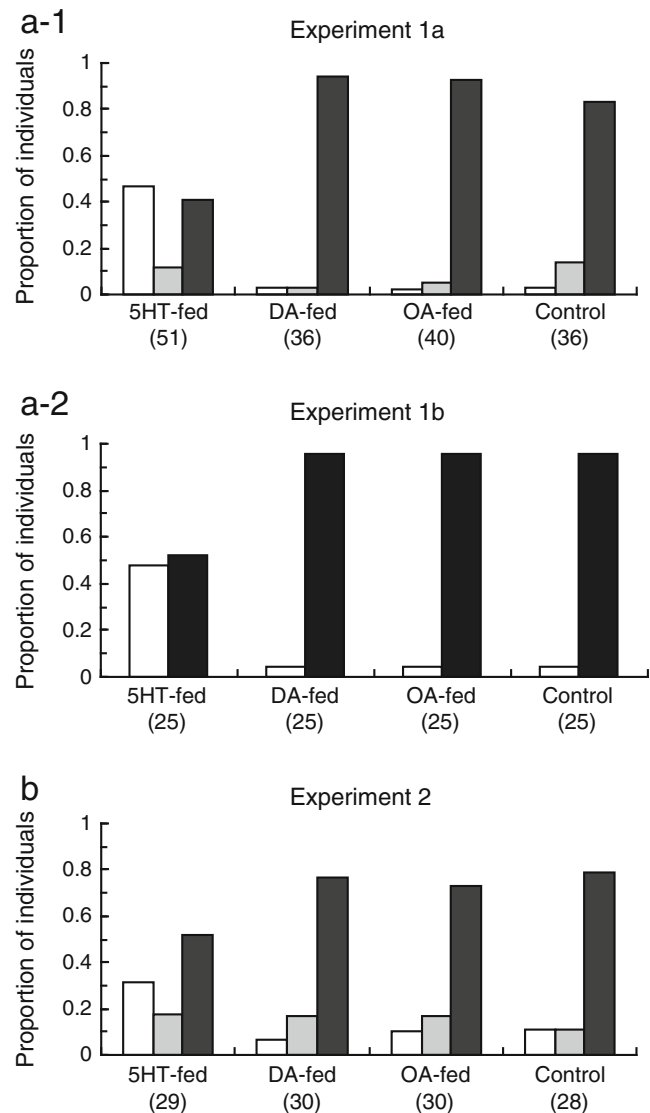
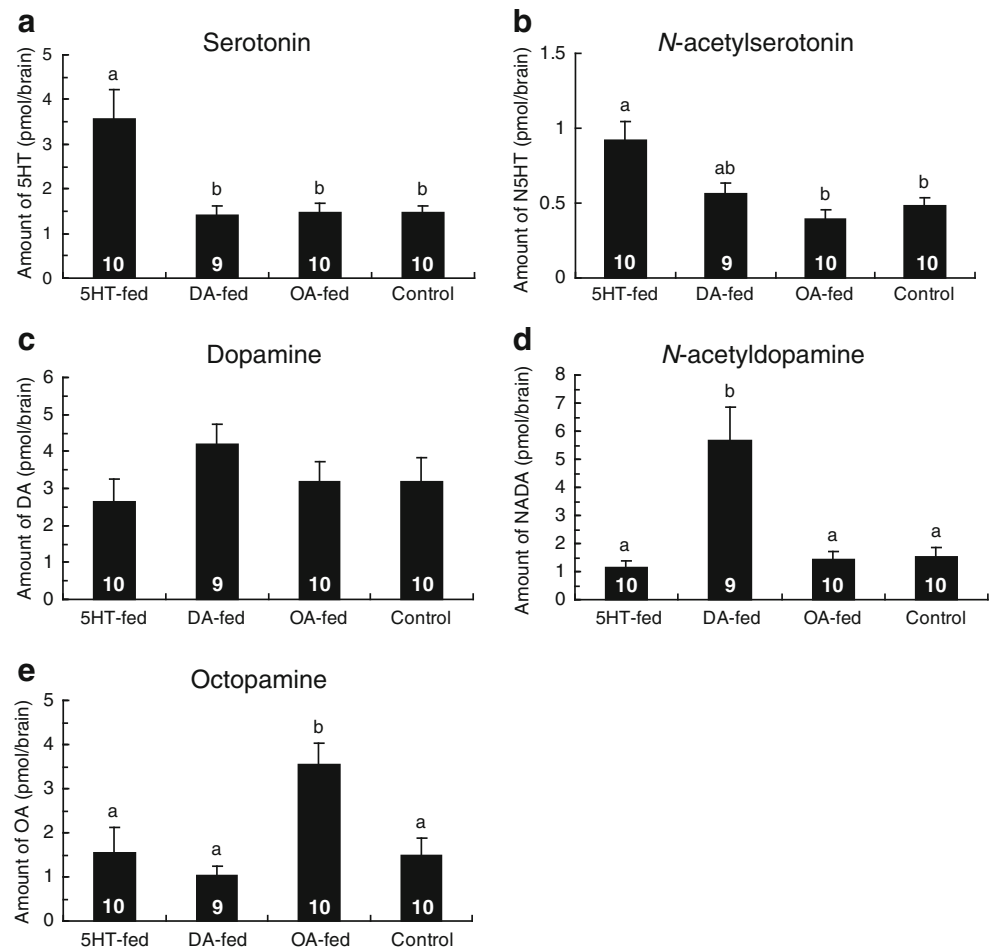


Fig. 2 Behavioral response of 2–3-day-old (a) or 6–8-day-old (b) virgin females to courting males. The effect of biogenic amines on the induction of mate rejection in 2–3-day-old virgin females was assessed by the “mate refusal posture” (a-1) and mating rate (a-2). a-1, b Open-, gray-, and filled columns indicate rejection, ambiguous rejection/acceptance, and acceptance responses, respectively. a-2 Open- and filled columns indicate rejection and copulation responses, respectively. Numbers in parenthesis on the x-axis indicate the number of female tested per treatment

control individuals ($H = 4.50$, $P = 0.21$, Fig. 3c), but brain levels of DA metabolite (*N*-acetyldopamine, NADA) were significantly higher in DA-fed individuals than in others ($H = 16.18$, $P < 0.01$, Fig. 3d). Brain levels of OA were significantly higher in OA-fed individuals than in others ($H = 14.60$, $P < 0.01$, Fig. 3e).

In 6-day-old virgin females, brain levels of 5HT and N5HT were significantly higher in 5HT-fed individuals ($n = 5$) than in the controls ($n = 4$) (Mann–Whitney U test,

Fig. 3 Brain levels (mean±SE) of serotonin (a), *N*-acetylserotonin (b), dopamine (c), *N*-acetyldopamine (d), and octopamine (e) in 2–3-day-old virgin females fed each with amine or sucrose (control). Statistically significant differences by Steel–Dwass ($P<0.05$) are indicated by different letters above bars. Numbers within bars give sample sizes



5HT: $Z=-2.45$, $P<0.05$; N5HT: $Z=-2.45$, $P<0.05$). Brain levels of DA, NADA, and OA were not significantly different between the two groups (DA: $Z=-0.49$, $P=0.62$; NADA: $Z=-0.25$, $P=0.81$; OA: $Z=-1.47$, $P=0.14$).

Discussion

A previous study suggested that a humoral factor, tentatively named “mate refusal hormone”, caused mated female cabbage butterflies to adopt a “mate refusal posture” when encountering courting males (Obara 1982). Experiment 1a (Fig. 2a-1) showed that 5HT-treated 2–3-day-old females assumed the mate refusal posture in response to courting males, suggesting that 5HT is a candidate substance for mate refusal hormone, a conjecture supported by the alternative method used in experiment 1b (Fig. 2a-2). Accumulation of each amine in the central nervous system following ingestion were confirmed by enhanced brain levels of the amine or its metabolite, strongly suggesting 5HT or its metabolite as a candidate mate refusal hormone in 2–3-day-old females.

Compared with the above mentioned effect of 5HT in younger, 2–3-day-old females, the effect of 5HT in older, 6–8-day-old females was weaker and not statistically different from controls (Fig. 2b). However, the effect is close to statistical significance, and the observed trend is consistent with that observed in our previous experiment, suggesting that the effect is still present but weaker. This implies that the 5HT effect may decline gradually not abruptly with the female adult age. If so, why is the effect of 5HT weaker in older females? The reason for this cannot be determined in the present study, but it may be related to the mating strategy of aging females. In some butterflies in which females face scarce host plants to lay eggs on and therefore have to secure the time to seek for them, females should finish mating as early as possible and avoid useless remating. In *Pieris rapae rapae*, it is reported that females lay as little as 10% of their daily complement of eggs because they are unable to find enough host plants (Jones et al. 1980). To avoid dying with remaining eggs within the ovaries, females should mate early. Hence virgin, older females should mate as soon as possible to maximize reproductive success. This seems true for older, 6–8 day-old

females of *P. rapae crucivora* because their longevity is about 2 weeks which is nearly the same as that of the related *P. rapae rapae* with the longevity of 16 days (Jones et al. 1980). It is therefore likely that the lower effect of 5HT on the induction of mate rejection in older virgin females may be the result of the ecological requirements imposed on them.

Little is known regarding the physiological mechanisms by which mate rejection is induced and controlled by 5HT. Based on the results of nerve bundle severance experiments, it was suggested that mechanical or inflation stimuli of the bursa copulatrix by ejaculates transferred from males during copulation may trigger secretion of the mate refusal hormone in some region of the central nervous system anterior to the first abdominal ganglion (Obara et al. 1975; Sugawara 1979). In insects, 5HT-immunoreactive neurons, including intersegmental interneurons, afferent neurons, and efferent neurons, are distributed between the brain and the terminal abdominal ganglion (Taghert and Goodman 1984; Tyrer et al. 1984). A similar distribution of serotonergic neurons in *Pieris rapae* would imply that 5HT could be released from the brain and/or other ganglia anterior to the first abdominal ganglion. However, the details of any mechanism underlying such a pathway, and the target site for induction of the mate refusal posture, remain unknown.

In summary, the present study showed that 5HT or its metabolite is a possible candidate substance for inducing the mate refusal posture. It would be of interest to investigate whether a similar mechanism exists in related pierid species, such as *Pieris melete*, in which females show homologous postures in the presence of courting males (Obara and Majerus 2009). However, it should first be demonstrated that butterflies treated with serotonin blockers do not show the mate rejection behavior described here.

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