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Genital Photoreceptors Have Crucial Role in Oviposition in Japanese Yellow Swallowtail Butterfly, *Papilio xuthus*

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ABSTRACT—Butterflies of both sexes have two pairs of extraocular photoreceptors on the genitalia. Here we demonstrate in female *Papilio xuthus* that a pair of the genital photoreceptors, P1s, is crucial for oviposition. Mated females of *Papilio* lay eggs on citrus leaves. When a female finds a food plant of the larvae, the female lands on its leaf and curls the abdomen often pushing out the ovipositor. As soon as the ovipositor touches the leaf surface, the female deposits an egg and glues it on to the leaf. We observed the oviposition behavior of individuals whose P1s or the mechanoreceptors on the ovipositor were ablated. Females treated in either way could no longer lay eggs, although they actively curled the abdomen and pushed the leaf, often quite strongly, with exposed ovipositor. This indicates that the females first confirm whether the ovipositor is sufficiently pushed out by using the P1 response as the measure, and then they deposit an egg in response to the mechanical stimulation of the ovipositor.

INTRODUCTION

Butterflies of both sexes have extraocular photoreceptors on the genitalia (genital photoreceptors, GP) (Arikawa and Aoki, 1982; Arikawa, *et al*., 1980). Each individual has two pairs of photoreceptor neurons (P1s and P2s), which resemble the phaosome-type photoreceptor cells found in the earthworm skin (Miyako, *et al*., 1993). The GP neuron extends an axon towards the last abdominal ganglion where it terminates and arborizes (Arikawa and Aoki, 1982). The GP neuron produces a train of spikes in response to a light flash in the wavelength region around UV to blue (Arikawa and Aoki, 1982).

What is the biological function of the GPs? We previously demonstrated that the P1s of the male *Papilio xuthus* plays a crucial role in achieving copulation: males cannot mate if the P1 input is ablated. It appeared that males use the P1s to detect the correct coupling with the mate (Arikawa, *et al*., 1996). However, ablation of P1s in females did not interfere with copulation: female P1s are not important for mating (Arikawa, *et al*., 1997).

Then, what is the female P1s for? The P1s of the female exist on both sides of the papilla analis, or the ovipositor (see Figs. 1 and 3). Upon light stimulation of the genitalia, females often curl the abdomen or push out the ovipositor. Such body movements are specifically observed during oviposition, suggesting that the females use the P1s when laying eggs. Therefore, we analyzed the P1 function in oviposition behavior, and found that the photoreceptors are crucial for oviposition.

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MATERIALS AND METHODS

Animals

We used summer-form females of the Japanese yellow swallowtail butterfly, *Papilio xuthus* L. The butterflies were supplied from a laboratory stock culture derived from eggs laid by females caught in the field in Yokohama, Japan. The hatched larvae were fed on fresh citrus leaves under a light regime of 16 hr light : 8 hr dark at 25°C. We mated the newly emerged females with males manually (hand-paring). Experiments were performed on the mated females within 7 days after emergence.

Behavioral experiments

Behavioral experiments were carried out in indoor cages (0.5 × 0.5 × 0.9 m), which were illuminated with fluorescent tubes (5,000–12,000 lux). The room temperature was set at about 28°C. A potted lemon tree was put in each cage.

To test the function of P1s of the female, we ablated the P1s bilaterally either by rubbing the P1 site with a fine heat probe or by painting black mascara over the site. As control treatment, we painted the P1 site with transparent mascara. We also heat-ablated most of the mechanoreceptors on the ovipositor by rubbing the cuticular surface bearing the mechanoreceptive hairs.

A treated female was released in a cage with a potted lemon tree immediately after the treatment. Usually, the females started to lay eggs after a couple of hours after releasing in the cage. When the female started to lay eggs, we carefully observed the behavior and counted the number of eggs laid (N), and divided it by the number of oviposition trials (T, behavioral step 4 = touch the leaf with the exposed ovipositor, see Fig. 1 and Results). We observed about 100 trials for each individual, and calculated the oviposition success rate (OSR) by

$$OSR\ (\%) = (N/T) \times 100$$

for each individual.

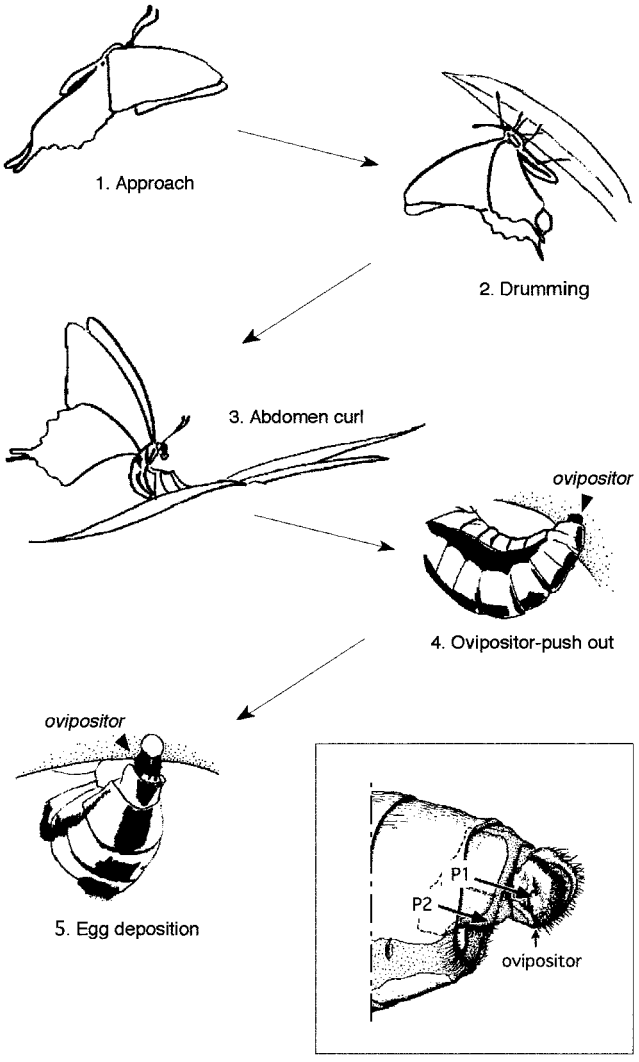

Fig. 1. Sequence of oviposition behavior of *Papilio xuthus*. For steps 4 and 5, only the tip of the abdomen was drawn. Inset; Left side view of the female genitalia, indicating the location of the photoreceptors P1 and P2.

After the behavioral experiments, we confirmed whether the heat-ablation of P1 and mechanoreceptors was successful or not by electrophysiology (see below for methods). It appeared, without exception, that the mechanoreceptors of P1-ablated individuals remained intact, and the P1s remained intact in mechanoreceptor-ablated individuals.

Electrophysiology

The electrophysiological methods were described elsewhere (Arikawa and Miyako-Shimazaki, 1996). Briefly, a female, whose wings and legs were removed, was fixed ventral side up with beeswax on a plastic stage, which was then filled with physiological saline. The ventral cuticle of the abdomen was removed to expose the abdominal nervous system.

Nerve spikes were recorded by using a suction electrode (see Fig. 4 for an example of the response). The numbers of spikes elicited by light stimulation were counted using a spike counter. Stimulus intensity-response functions were determined by counting spikes elicited by a series of 1 s flashes (10 s interval) with intensities varying in a range of 5 log units.

Stimulus light was provided by a 500 W Xenon arc through a set of neutral density filters and a quartz optical fiber. First the stimulus light was directly focused on the P1 photoreceptive site. The best fit of the modified Naka-Rushton equation,

$$F/F_{max} = In / (I^n + K^n),$$

where I = log stimulus intensity, F = spike frequency, F_{max} = maximum spike frequency, K = stimulus intensity eliciting 50% F_{max}, and n = exponent, was then calculated for the set of data using the least squares method in order to determine the response-stimulus intensity characteristics of the female P1s.

For analyzing the difference in the P1 response under various conditions, we used a light-diffusing sphere (9 cm in diameter) whose inner surface was coated with MgO powder. The specimen was mounted around the center of the sphere. The light was guided with an optical fiber whose tip was set to illuminate the inner surface of the sphere.

RESULTS

Oviposition behavior

Fig. 1 summarizes the oviposition behavior of the Japanese yellow swallowtail butterfly, *Papilio xuthus*. Mated females of *Papilio xuthus* search for citrus trees, the host plant of the larvae, on which to lay eggs. In the present study, we released a mated female in a cage with a potted lemon tree inside. The female soon began to lay eggs. Upon oviposition, the female approaches the plant (step 1), lands on a leaf of the plant and tastes the leaf with the forelegs (drumming) to confirm that it is an appropriate food plant (step 2) (Ichinose and Honda, 1978; Ma and Shoonhoven, 1973). She then curls the abdomen towards the leaf (step 3) pushing out the ovipositor (step 4). As soon as the ovipositor touches the leaf, she delivers an egg and attaches it onto the leaf (step 5). Once a female started the drumming behavior, it takes about 5 seconds in average to lay an egg. Once the ovipositor touches the leaf, the female lays an egg at the success rate of about 80% (oviposition success rate, OSR = 79.7%).

Effect of P1 ablation

The P1s exist on the lateral side of the ovipositor (Fig. 1). We ablated the P1 input and measured the OSR. Fig. 2 summarizes the results. The P1 ablated females still tried to lay eggs: they actively curled the abdomen, pushed out the ovipositor, and strongly pressed the ovipositor against the leaf. However, they seldom laid eggs. Calculated OSR for the P1 heat-ablated females was only 2.1%. The OSR of the P1 black-painted females was also significantly small (14.1%). On the other hand, the P1 clear-painted females laid eggs normally (OSR = 81.7%).

Effect of mechanoreceptor ablation for oviposition behavior

In addition to the P1s, the ovipositor bears many mechanoreceptive hairs. Here we observed the oviposition behavior of *Papilio xuthus* after ablating these mechanoreceptors. As in the P1-ablated females, the individuals whose mechanoreceptors were heat-ablated hardly laid eggs (OSR = 9.9%, Fig. 2).

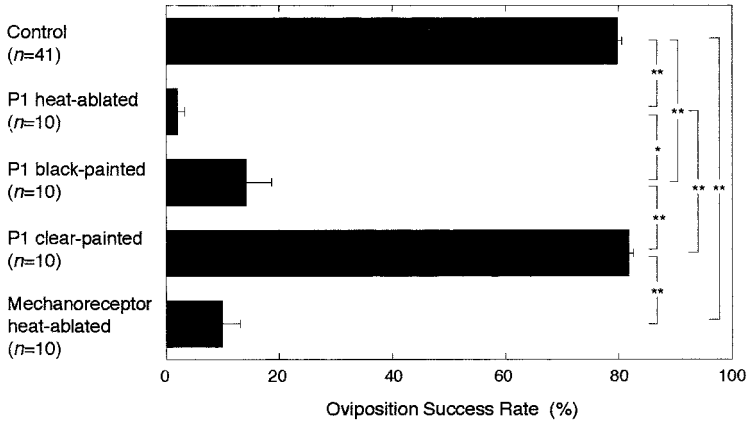

Fig. 2. Effect of ablation of P1 and mechanoreceptive hairs on the ovipositor to the oviposition success rate (OSR, mean±SD). Mann-Whitney *U*-test was used for analyzing statistical difference in OSR between treatments (**, $P < 0001$; *, $P < 0.05$).

Electrophysiological responses of the P1s

The ovipositor is usually covered with hairy scales around the abdominal tip. Fig. 3A shows a feeding female. Apparently, the ovipositor is not visible from outside. Upon oviposition, the ovipositor is pushed out from the tip of the curled abdomen and becomes now clearly visible (Arrowhead in Fig. 3B). When pushed out, the ovipositor must be well exposed to light. To see how the response of P1 to light increases when the ovipositor is pushed out, we measured the P1 response electrophysiologically.

Fig. 4 shows a typical example of P1 spikes and stimulus intensity-response functions of the responses under various stimulus conditions. The inset shows a set of responses recorded by stimulating P1 with focused light. The solid line is

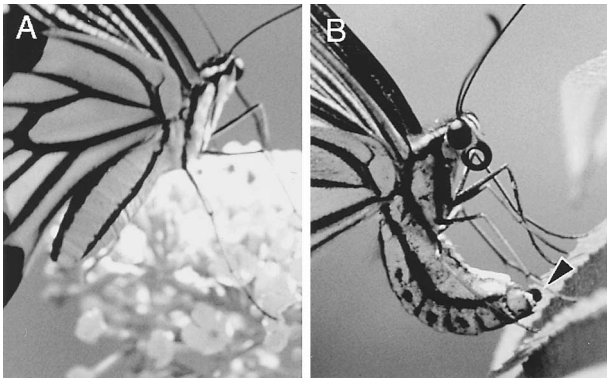

Fig. 3. Tip of the abdomen of feeding (A) and egg-laying (B) female. The ovipositor (arrowhead) is pushed out only upon oviposition.

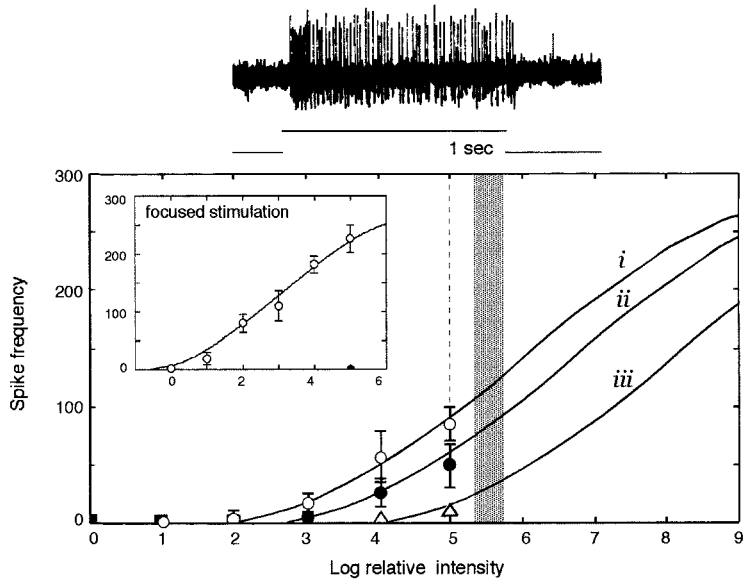

Fig. 4. Stimulus intensity-response curves of female P1s under various stimulus conditions with a typical example of the P1 response. Inset shows a curve recorded by stimulating P1 with focused light. Curves *i–iii* were recorded by using a light-diffusing sphere under different conditions: ovipositor-pushed out (*i*), ovipositor covered by scale, i.e. in the resting position (*ii*), P1 black painted and pushed out (*iii*). Log I=5 corresponds to 2,000 lux (broken line). Shaded area indicates the intensity range under which the behavioral experiments were carried out (5,000−12,000 lux). Each data point is the mean (±SD) of the data from five individuals.

the best fit of the Naka-Rushton equation. The F_{max} of the P1 was estimated as about 280 spikes s^{-1}. The curve was copied and visually fitted to the results obtained when the light-diffusing sphere was used (curves i-iii).

Curves *i-iii* indicate the P1 responses under the following conditions: ovipositor pushed out (*i*), covered with the hairy scales (*ii*), and pushed-out and painted black (*iii*). Heat-treated P1s did not respond even at the highest intensity of the focused stimulation (filled circle in inset). The maximum intensity of light at the P1 site when the sphere was used was about 2,000 lux, which corresponds to relative log intensity 5 (log I = 5) on the abscissa (broken line). The shaded range corresponds to the intensity under which the behavioral experiments were carried out; i.e. 5,000–12,000 lux. This is comparable to the light intensity of cloudy days, which is about one log unit dimmer than that of the direct sunshine, 80,000–120,000 lux. But since *Papilio xuthus* lay eggs both on cloudy and on sunny days, the experimental lighting condition is in the range of actual condition in the field.

DISCUSSION

P1s are crucial for oviposition

The present results clearly indicate that the light stimulation of the P1s is crucial for oviposition. The P1-ablated females behave normally up to step 4, where they curl the abdomen and push out the ovipositor towards the leaves (Fig. 1). However, they had great difficulties in egg-laying: they continued to search for the site, often pushing the leaf strongly with the ovipositor, but this did not result in depositing an egg. Accordingly, the OSR of the P1-ablated females was significantly low (Fig. 2). The crucial role of light was confirmed by painting the P1 site with clear paint: the P1 clear-painted females laid eggs normally (Fig. 2).

Females have another photoreceptor pair, P2 (Arikawa, *et al*., 1980), slightly ventral to the ovipositor. Since the P2s were untreated throughout this study, the function of this pair of photoreceptors still remains unknown.

Mechanoreceptors are also crucial for oviposition

The ovipositor of *Papilio* bears numerous mechanoreceptive hairs as in other lepidopteran species (Arikawa and Aoki, 1982). In the silkmoths, the mechanoreceptors on the ovipositor are important for oviposition: the mechanoreceptor-ablated silkmoth females could not lay eggs in raw as intact females always do (Yamaoka, *et al*., 1971). Here the mechanoreceptors on the ovipositor appeared to be crucial for oviposition also in *Papilio*.

In a preliminary experiments, we carefully observed the behavior of intact egg-laying females under a microscope. The highly motivated females had pushed out the ovipositor even under the microscope. When we stimulate the mechanoreceptors on the ovipositor by touching it with a pair of tweezers, the females did deposit an egg, indicating that the stimulation of the mechanoreceptors directly triggered egg deposition in highly motivated females. In fact, heat-ablation of the mechanoreceptors significantly reduced the OSR (Fig. 2). Such a response of tethered animals would provide an opportunity for analyzing the sensory control of oviposition at neuronal level.

Genital photoreceptors act as optical proprioceptors

How do the light and mechanical inputs control the oviposition behavior? When a female lands on a leaf of food plant, she curls the abdomen and pushes out the ovipositor from the abdominal tip (Fig. 3). The electrophysiological recording of the P1 response (Fig. 4) indicates that the females can detect, by optical means, whether the ovipositor is properly pushed out. Next, the female touches the leaf surface with the ovipositor, which is detected by mechanical means, and then deposits an egg.

For proper oviposition, the ovipositor must be first stimulated optically, and then mechanically. The P1-ablated females cannot be sure whether the ovipositor is sufficiently pushed out even if its position is appropriate. Therefore, the females cannot lay eggs even when the mechanoreceptors are strongly stimulated by pressing the ovipositor against the leaf. On the other hand, the mechanoreceptor-ablated females should know that the ovipositor is pushed out by the remaining photoreceptors, but cannot be informed that the leaf surface is there. In contrast to the case in males, where the P1s are used for detecting correct coupling with the mates (Arikawa, *et al*., 1996), the females use the P1s for detecting the position of the ovipositor with respect to her own abdominal tip. This means that the female photoreceptors most likely function as proprioceptors.

Why do butterflies use the optical system for such a purpose? Monitoring the relative position of the ovipositor can of course be achieved by using a mechanical sense that somehow measures contraction of ovipositor muscles. However, the use of an optical system is probably more reliable. Assume that the tip of the abdomen is covered by some material, such as own excrement or detached scales. In fact, such a situation can be observed in the field sometimes. What might happen then is that the egg cannot be properly delivered and/or attached to the leaf surface. As long as the ovipositor position is monitored mechanically, possible existence of material covering the ovipositor opening cannot be detected. If the ovipositor is exposed to light, there is little chance for the ovipositor to be shielded, then the butterfly can reliably conclude that the ovipositor is surely ready for proper oviposition.

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