Gustatory Sensing Mechanism Coding for Multiple Oviposition Stimulants in the Swallowtail Butterfly, *Papilio Xuthus*

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The swallowtail butterfly, *Papilio xuthus*, selectively uses a limited number of plants in the Rutaceae family. The butterfly detects oviposition stimulants in leaves through foreleg chemosensilla and requires a specific combination of multiple oviposition stimulants to lay eggs on the leaf of its host plants. In this study, we sought to elucidate the mechanism underlying the regulation of oviposition behavior by multiple oviposition stimulants. We classified chemosensilla on the tarsomere of the foreleg into three types (L1, L2, and S) according to their size and response to oviposition stimulants and general tastants. The L1 was more abundant in females than in males and responded preferentially to oviposition stimulants. Both L2 and S were common to both sexes and responded to general tastants. We found that five oviposition stimulants (synephrine, stachydrine, 5-hydroxy-*N*-methyltryptamine, narirutin, and *chiro*-inositol) elicited spikes from three specific gustatory receptor neurons (GRNs) within L1 sensilla. These three GRNs responded to a mixture of the five stimulants at concentrations equivalent to those found in the whole-leaf extract of citrus, and the mixture induced oviposition at levels comparable to whole-leaf extract. We propose that oviposition is triggered by the firing of three specific GRNs in L1 sensilla that encode the chemical signatures of multiple oviposition stimulants.

Introduction

Host plant selection is a crucial behavior for mono- and oligophagous insects, because it conditions the success of individuals, particularly during larval development and reproduction. In Lepidoptera, choosing the right host plant during oviposition is particularly important, because the small larvae cannot easily forage for alternate host plants. Host plant selection by butterflies depends on several factors, including leaf color, shape, and phytochemical compounds (Rauscher, 1978; Honda, 1995; Kelber, 1999). Among these, the phytochemical compounds are the most important factors for oviposition (Feeny et al., 1983). Females

determine whether a plant is suitable for larval feeding by drumming on the leaf surface and perceiving chemicals through chemosensilla located on the ventral side of their foreleg tarsomeres (Ichinose and Honda, 1978).

Butterflies of the family Papilionidae lay eggs on the leaves of plants in the families Rutaceae, Apiaceae, or Lauraceae (Feeny et al., 1988; Honda, 1990; Carter et al., 1999). Phytochemical compounds acting as oviposition stimulants for the members of the Papilionidae have been identified in plants of the Rutaceae (Honda, 1990; Ohsugi et al., 1991; Ono et al., 2000a, b; Nakayama et al., 2003). Oviposition stimulants are classified as taste stimuli because they are nonvolatile compounds. Interestingly, each butterfly species requires a specific combination of chemicals in their host plants to lay eggs: *Papilio xuthus* requires a mixture of 10 chemicals, *Papilio polytes* 5, and *Papilio protenor* 6; each combination is found in their respective host plants (Honda, 1990; Ohsugi et al., 1991; Murakami, 2003). Notably, each of these chemicals fails to stimulate oviposition when they are used singly in oviposition behavioral assay (Honda, 1990; Ohsugi et al., 1991). Therefore, it is important to elucidate how multiple oviposition stimulants regulate oviposition behavior to understand the mechanism of host plant selection in Papilionidae.

We recently identified the gene *PxutGr1*, which encodes a gustatory receptor specific for synephrine, one of the 10 oviposition stimulants for *P. xuthus* (Ozaki et al., 2011). Given the specificity of this receptor, we hypothesized that the other nine

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Figure 1. Scanning electron micrographs of foreleg tarsus and chemosensilla of the fifth tarsomere of *P. xuthus*. **A**, Ventral side of a female's foreleg tarsus. Scale bar, 300 μm . **B**, Ventral side of a female's fifth tarsomere. Arrows and arrowheads indicate L sensilla and bristles, respectively. Scale bar, 100 μm . **C**, Morphological differences between the L and S sensilla. Scale bar, 40 μm . **D**, L sensillum on fifth tarsomere with single pore at the apex (arrow). Scale bar, 20 μm . **E**, Numbers of sensilla on first to fifth tarsomeres (mean $\pm$ SD, $n = 6$ for first–fourth tarsomere, $n = 21–42$ for fifth tarsomere). * $p < 0.0001$ female versus male (Student's *t* test). The second to fifth tarsomeres housed all three types of sensilla, whereas the first tarsomere had S type sensilla only. Sexual dimorphism was observed only in the number of L1 sensilla on the second to fifth tarsomeres. To count the number of L2 sensilla, 1 mM KCl was applied to all L sensilla. The L sensilla that responded to sugars were L2 sensilla.

chemicals are also detected by specific chemoreceptors, each expressed in specialized taste cells. To test this hypothesis, we performed an extensive electrophysiological analysis of the responses of these taste cells. Because the cellular composition of sensilla in the foreleg tarsus has not been precisely described, we first classified the sensilla by shape and location into several types and recorded their responses to oviposition stimulants as well as to commonly used taste compounds. We also examined the correlation between the response pattern of the taste cells and the oviposition behavior. Our findings indicated that the detection of these oviposition-specific chemicals occurs through a specific neural pathway in Papilionidae.

Materials and Methods

Animals. Adult females of *P. xuthus* were collected at Takatsuki, Osaka, Japan, and allowed to lay eggs on young leaves of *Citrus unshiu* in the laboratory. Larvae were reared on an artificial diet containing Insecta F-II (Nihon Nosan) and dry *Zanthoxylum ailanthoides* (collected at Takat-

suki, Osaka, Japan) leaves reduced to a powder, at $25 \pm 3^\circ\text{C}$ under 16/8 h light/dark conditions.

Chemicals. D,L-Synephrine was purchased from MP Biochemicals. l(–)-Stachydrine and narirutin were obtained from Extrasynthese. 5-Hydroxy-*N*- ω -methyltryptamine, *chiro*-inositol, rutin, hesperidin, adenosine, KCl, NaCl, sucrose, glucose, fructose, dimethyl sulfoxide, and 2-hydroxypropyl- β -cyclodextrin were purchased from Wako Pure Chemical Industries. Caffeine was obtained from Sigma-Aldrich. Quinine hydrochloride was purchased from Tokyo Kasei Chemicals. For electrophysiological recordings, all compounds were dissolved in 1 mM KCl prepared using distilled water and stored at -20°C , and 1 mM KCl was used in all experiments as the electrolyte. In *Drosophila melanogaster* and *Phormia regina*, 1 mM KCl elicits spikes from the water cell but not from other cells (Evans and Mellon, 1962; Hiroi et al., 2002).

Preparation of whole-leaf citrus extract. The whole-leaf citrus extract was prepared as described by Honda (1986). Briefly, young leaves of *Citrus unshiu* were extracted with 100% methanol at $25 \pm 3^\circ\text{C}$ for 1 month. The methanol extract was concentrated *in vacuo* at 50°C , and an aliquot was dissolved in an amount of water equivalent to the wet weight of the leaves. This extract was designated 1.0 $\times$ citrus extract.

Electrophysiological recordings. We used 0–8-d-old adults for the electrophysiological recordings. There are essentially no differences in nature of responses to various stimulants among sensilla prepared from adults of varying in age (data not shown). A foreleg (tarsus, tibia, and femur) was removed from the body using forceps and then fixed on a polystyrene block with fine strips of sticky paper tape. A glass capillary electrode filled with Ringer's solution ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$; 3 mM, KCl; 182 mM, NaCl; 46 mM, Tris base; 10 mM, pH 7.2) was inserted into the leg and connected to the ground. The leg was oriented such that sensilla from the ventral side of the fifth tarsomere were accessible to stimulation by glass capillary electrodes (diameter $\sim 20 \mu\text{m}$) filled with a solution of a given compound. Nerve responses were recorded using the tip-recording method (Hodgson et al., 1955; Calas et al., 2006). The electrical signal

was amplified by a TastePROBE preamplifier DTP-02 (Syntech) (Marion-Poll and van der Pers, 1996) and further amplified and filtered (10–2800 Hz) by a CyberAmp 320 amplifier (Molecular Devices). Data were recorded and stored on a computer with a 16-bit A/D conversion card DT9803 USB A/D (Data Translation) under the control of the custom software dbWave (Marion-Poll, 1995, 1996).

Recordings were analyzed using dbWave (<http://taste.versailles.inra.fr/deterrents/tk/dbwave/>) to detect and sort spikes according to their amplitude and shape. We paid particular attention to correlate our recordings to L1, L2, and S sensilla types. Within the L1 sensilla, we counted the number of responsive and nonresponsive sensilla for a given stimulus. For the “responsive” sensilla, we evaluated the number of active cells based on the spike shape and the presence of doublet spikes, which indicate that at least 2 cells are active (Meunier et al., 2003). All electrophysiological data for L2 and S sensilla were combined, because these sensilla showed almost identical electrophysiological responses to all tested compounds (data not shown).

Assay for oviposition behavior. Females were hand-paired on the day of (or 1 d after) eclosion. For the assay, we used mated 3–6 d-old females,

Figure 2. Typical electrophysiological recordings of L1 sensilla (left column) and L2 or S sensilla (right column) on the fifth tarsomere in response to eight oviposition stimulants during the first 500 ms after stimulation. Vertical bar indicates 1.0 mV. Arrow indicates the onset of stimulation. To the left of each trace, the spikes are superimposed. Open circles, open squares, and triangles indicate the positions of high (1.0–1.5 mV), intermediate (0.6–0.8 mV), and low (0.2–0.4 mV) spikes, respectively. Gray squares indicate spikes elicited by water (0.6–0.8 mV). Recordings show responses to **A**, 2.5 and 10 mM synephrine; **B**, 0.5 and 10 mM 5-hydroxy-*N*-methyltryptamine; **C**, 10 mM stachydrine; **D**, 10 mM *chiro*-inositol; **E**, 5 mM narirutin; **F**, 10 mM adenosine; **G**, 33 μ M hesperidin; and **H**, 55 μ M rutin. **I**, Electrophysiological responses of L1 (left column) and L2 or S sensilla (right column) to increasing concentrations of six oviposition stimulants in females (mean $\pm$ SE spike frequencies; $n = 7$ –88 for synephrine, $n = 5$ –70 for 5-hydroxy-*N*-methyltryptamine, $n = 7$ –80 for stachydrine, $n = 6$ –19 for adenosine, $n = 6$ –53 for *chiro*-inositol, and $n = 5$ –54 for narirutin). Gray boxes indicate the estimated concentrations of oviposition stimulants in intact leaves of *C. unshiu*, as described previously (Nishida et al., 1987; *Figure legend continues*.)

because 0–2 d-old females displayed low (20–50%) oviposition activity with citrus extracts, and some females older than 7 d laid eggs even on artificial green leaves immersed in water alone. One hour before starting the assay, we extended the proboscis of all females with a toothpick and fed them 100 mM sucrose solution in cotton. After feeding, they were placed in a rearing container illuminated by a 12-W fluorescent light. A female was introduced into an assay box (30 × 25 × 25 cm high), illuminated by a 12-W fluorescent light just before the experiment. Artificial plastic green leaves were briefly immersed in plain water or in a solution of the citrus extract, mixtures of oviposition stimulants, or single stimulants just before they were presented to the female. We presented the wetted artificial leaf to each female for 2 min and then examined it for eggs to assess the oviposition responses to each solution.

Scanning-electron microscopy. Tarsi of female butterflies were removed from body by using forceps, dehydrated in an acetone series, dried in air. Mounted legs were sputter-coated with gold and observed by a JSM-5300 LV scanning electron microscope (JEOL).

Statistical analysis. Paired comparison and group comparison were made with the Student's *t* test and the one-way ANOVA followed by Tukey–Kramer *post hoc* test, respectively, using KaleidaGraph Version 4.01 (Synergy Software). A correlation coefficient between the number of spikes and oviposition activities was calculated by Microsoft Excel for Mac, 2011 Version 14.2.4 (Microsoft).

Results

Three types of sensilla are present in the foreleg tarsomeres

We classified sensilla in the first to fifth tarsomere of the *P. xuthus* foreleg on the basis of their position and morphology using scanning electron microscopy (SEM) observations (Fig. 1A–D). Sensilla could be classified by length into two types, short (S, ~50 μm) and long (L, ~100 μm) (Fig. 1C). The L sensilla were further classified into types 1 (L1) and 2 (L2) (Fig. 1E) according to their electrophysiological responses (see Figs. 2, 4). No sex-related differences were noted in the numbers of L2 and S sensilla, but females had more L1 sensilla than males (Student's *t* test; $t_{(10)} = 11.379$, $p < 0.0001$ for second tarsomere; $t_{(10)} = 15.419$, $p < 0.0001$ for third tarsomere, $t_{(10)} = 6.4789$, $p < 0.0001$ for fourth tarsomere, and $t_{(40)} = 32.92$, $p < 0.0001$ for fifth tarsomere). Because L1 sensilla are most abundant in the fifth tarsomere (Tukey–Kramer test; $F_{(3,35)} = 52.82$, $p < 0.0001$ for female, and $F_{(3,35)} = 95.29$, $p < 0.0001$ for male), further electrophysiological analyses were performed with the sensilla in the female fifth tarsomere. Electrophysiological responses by each type of sensilla on other tarsomere are the same as those in the fifth tarsomere (data not shown).

Electrophysiological response of fifth tarsomere chemosensilla to oviposition stimulants

We analyzed the electrophysiological responses to 8 of the 10 identified oviposition stimulants of *P. xuthus* (Fig. 2). Two

←

(Figure legend continued.) Ohsugi et al., 1991). These concentrations were as follows: synephrine: 2.4 mM; 5-hydroxy-*N*-methyltryptamine: 0.04 mM; stachydrine: 5.1 mM; adenosine: 0.02 mM; *chiro*-inositol: 8.3 mM; and narirutin: 0.03 mM. The number of spikes was calculated during the first second. Different letters (a–c) indicate significant differences of water spikes in L2 and S sensilla and low spikes of 5-hydroxy-*N*-methyltryptamine in L1 sensilla among concentrations by the Tukey–Kramer test ($p < 0.05$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$ versus 0.01 mM synephrine, 0.0005 mM 5-hydroxy-*N*-methyltryptamine, 0.01 mM stachydrine, 0.01 mM adenosine, 0.1 mM *chiro*-inositol, and 0.001 mM narirutin, respectively. J, Percentage of L1 sensilla responding to increasing concentrations of five oviposition stimulants (mean ± SE the percentage of responding sensilla; $n = 7$ –14 for synephrine, $n = 3$ –6 for 5-hydroxy-*N*-methyltryptamine, $n = 4$ –18 for stachydrine, $n = 5$ –7 for *chiro*-inositol, and $n = 4$ or 5 for narirutin). Each value of independent recording was the average response of 10–20 sensilla per individual. * $p < 0.05$; ** $p < 0.01$, versus 1 mM synephrine, 0.005 mM 5-hydroxy-*N*-methyltryptamine, 1 mM stachydrine, 2.5 mM *chiro*-inositol, and 0.01 mM narirutin, respectively.

chemicals, vicienin-2 and bufotenine, were not tested because they were not commercially available. The L1 sensilla responded to five stimuli: synephrine, stachydrine, 5-hydroxy-*N*-methyltryptamine, *chiro*-inositol, and narirutin (Fig. 2A–E). Synephrine and 5-hydroxy-*N*-methyltryptamine each elicited 2 types of spikes, high amplitude (1.0–1.5 mV) and low amplitude (0.2–0.4 mV) (Fig. 2A,B). The L1 sensilla responded to synephrine in one of three ways: high-amplitude spikes alone, low-amplitude spikes alone, or a combination of the two. The dose–response curves of L1 sensilla to synephrine show that the gustatory receptor neurons (GRNs) with high-amplitude spikes were more sensitive to synephrine than the GRNs with low-amplitude spikes (Fig. 2I; Student's *t* test; $t_{(16)} = 6.7002$, $p < 0.0001$ for 0.5 mM, $t_{(78)} = 5.7475$, $p < 0.0001$ for 2.5 mM, $t_{(64)} = 4.8299$, $p < 0.0001$ for 5.0 mM, $t_{(173)} = 3.8567$, $p = 0.0001619$ for 10 mM, $t_{(76)} = 3.6836$, $p = 0.0004283$ for 25 mM, and $t_{(46)} = 4.2388$, $p = 0.000107$ for 50 mM). The response of L1 sensilla to 5-hydroxy-*N*-methyltryptamine switched from high- to low-amplitude spikes between 5 and 10 mM (Fig. 2I), indicating that the two GRNs of in L1 sensillum responded separately to different concentrations of 5-hydroxy-*N*-methyltryptamine. Stimulation with stachydrine, *chiro*-inositol, and narirutin each elicited only a single type of spike: stachydrine: 1.0–1.5 mV, *chiro*-inositol and narirutin: 0.6–0.8 mV (Fig. 2C–E). The numbers of spikes elicited by stachydrine, *chiro*-inositol, and narirutin increased with concentration (Fig. 2I; $F_{(8,422)} = 21.55$, $p < 0.0001$ for stachydrine, $F_{(7,264)} = 7.29$, $p < 0.0001$ for *chiro*-inositol, and $F_{(8,217)} = 17.93$, $p < 0.0001$ for narirutin). Adenosine, hesperidin, and rutin did not elicit any spikes in L1 sensilla (Fig. 2F–H). Because hesperidin and rutin dissolve poorly in water and ethanol, we used 10% dimethyl sulfoxide or 20% 2-hydroxypropyl- β -cyclodextrin as a solvent (Calabrò et al., 2005; Majumdar and Srirangam, 2009). No response was found to 1 mM hesperidin or rutin (data not shown). Because estimated concentrations of hesperidin and rutin in intact leaves of *Citrus unshiu* are 0.66 and 0.25 mM, respectively (Nishida et al., 1987), these concentrations may not be sufficient to be detected by L1 sensilla.

In contrast to L1 sensilla, L2 and S sensilla exhibited spikes (0.6–0.8 mV) in response to 8 oviposition stimulants (Fig. 2A–H). These spikes decreased in frequency with increasing stimulant concentration, except for hesperidin and rutin (Fig. 2I; synephrine; $F_{(3,55)} = 26.07$, $p < 0.0001$, 5-hydroxy-*N*-methyltryptamine; $F_{(4,25)} = 24.57$, $p < 0.0001$, stachydrine; $F_{(6,115)} = 32.57$, $p < 0.0001$, adenosine; $F_{(3,43)} = 14.03$, $p < 0.0001$, *chiro*-inositol; $F_{(3,27)} = 3.451$, $p = 0.03036$, and narirutin; $F_{(3,22)} = 5.169$, $p = 0.00513$). These results indicated that these spikes originated from a “water” cell, because water cells function as osmometer and their responses are usually inhibited by increasing concentrations of a given compound (Evans and Mellon, 1962). The L2 and S sensilla were less sensitive to synephrine and 5-hydroxy-*N*-methyltryptamine than L1 sensilla at whole-leaf concentrations (Fig. 2I; Student's *t* test; $t_{(56)} = 7.5563$, $p < 0.0001$ for high spikes of synephrine, $t_{(42)} = 2.5281$, $p = 0.01532$ for low spikes of synephrine, and $t_{(76)} = 2.8164$, $p = 0.006184$ for low spike of 5-hydroxy-*N*-methyltryptamine), but responded to stachydrine comparably to L1 sensilla (Fig. 2I; Student's *t* test; $t_{(87)} = 0.94073$, $p = 0.3494$ for stachydrine). *chiro*-inositol, narirutin, and adenosine elicited water spikes only, indicating that these compounds were not recognized. Spikes elicited by hesperidin and rutin were indistinguishable from water spikes (Fig. 2G,H), because we could not confirm the dose dependency of responses to these stimulants.

We also examined the percentage of L1 sensilla on the fifth tarsomere responding to a given stimulus (Fig. 2J). The percentages of

Figure 3. Responses of L1 sensilla on the fifth tarsomere to mixtures of two oviposition stimulants. **A**, Sample responses of L1 sensilla to 10 mM stachydrine plus 10 mM *chiro*-inositol. Oviposition spike 1 (os1, circles) and os2 (squares) spikes are shown. Arrowheads indicate the doublet spikes. **B**, Total spikes and doublets. Total spikes indicate superimposed spikes of trace A. Doublet spikes 1–5 are shown by arrowheads. **C**, Response to 2.5 mM synephrine and 10 mM *chiro*-inositol. Arrowheads indicate doublets of os2 and os3 (triangles). **D**, Response to 10 mM synephrine and 10 mM *chiro*-inositol. The mixture elicited three types of spikes simultaneously. Arrowheads 1 and 2 indicate doublets consisting of os1 + os2 and os1 + os3, respectively. **E**, Response to 10 mM synephrine and 5 mM narirutin. The mixture elicited three types of spikes. Arrowhead indicates a doublet of os1 and os2. **F**, Response to 10 mM stachydrine and 1.0 mM (Figure legend continues.)

responding sensilla increased with the stimulant concentrations, except for 5-hydroxy-*N*-methyltryptamine (synephrine; $F_{(5,55)} = 6.311$, $p = 0.00011$, stachydrine; $F_{(5,53)} = 6.995$, $p < 0.0001$, *chiro*-inositol; $F_{(4,22)} = 5.227$, $p = 0.00409$, and narirutin; $F_{(4,17)} = 7.769$, $p = 0.00094$). These five stimulants exhibited various success rates of recordings in response to the close concentrations of these compounds found in whole-leaf extracts (the percentage of responding sensilla of 2.5 mM synephrine, 5 mM stachydrine, 0.05 mM 5-hydroxy-*N*-methyltryptamine, 10 mM *chiro*-inositol, and 0.05 mM narirutin; $F_{(4,26)} = 19.39$, $p < 0.0001$). These results suggested that L1 sensilla house different numbers of GRNs for each oviposition stimulant.

Three GRNs respond to five oviposition stimulants

To determine whether spikes elicited by five stimulants arise from discrete or identical GRNs in L1 sensilla on the fifth tarsomere, we examined doublet spikes elicited by mixtures of two oviposition stimulants, synephrine, and 5-hydroxy-*N*-methyltryptamine (Fig. 3). The doublet spikes are known to occur when more than one GRN are activated (Meunier et al., 2003; Hiroi et al., 2004). The mixture of stachydrine and *chiro*-inositol elicited two types of spikes: high (1.0–1.5 mV) and low amplitude (0.6–0.8 mV) (Fig. 3A). Each type contained doublet spikes of the same amplitude and shape as individual spikes elicited by each single stimulant (Fig. 3A,B), indicating that the two stimulants are perceived by discrete GRNs. Likewise, doublet spikes were elicited by mixtures of two stimulants that elicited spikes with different amplitude when used individually (Fig. 3C–F). In contrast, when mixtures of stimulants that elicited spikes of the same amplitude were used, no doublet spikes were observed (Fig. 3G–J). These results suggested that spikes of the same amplitude are elicited by the same GRN, regardless of stimulants. Synephrine and 5-hydroxy-*N*-methyltryptamine each elicited doublet spikes, even when they were used individually (Fig. 3K,L), indicating that two different GRNs responded to these stimulants. These results suggested that L1 sensilla house three GRNs that can respond to five oviposition stimulants. Therefore, we defined the high-amplitude spike elicited by synephrine, stachydrine, and 5-hydroxy-*N*-methyltryptamine as oviposition spike 1 (os1), the intermediate-amplitude spike (by *chiro*-inositol and narirutin) as os2, and the low-amplitude spike (by synephrine and 5-hydroxy-*N*-methyltryptamine) as os3.

Electrophysiological responses to sugar, salt, and bitter compounds

The L1 sensilla appear to be specialized in their responses to oviposition stimulants and differ from L2 and S sensilla. To further examine the differences among sensilla, we studied their responses to sugar, salt, and bitter compounds. Responses to sugar were observed only in L2 and S sensilla (Fig. 4A–C,J). Responses to salt varied with salt concentrations: high-concentration salts (>100 mM KCl and NaCl) elicited spikes (1.5–2.0 mV) in all types of sensilla (Fig. 4E,G,I). In contrast, no

spikes were found in L1 sensilla when low-concentration salts were used (Fig. 4D,F,J). Bitter compounds (caffeine and quinine) elicited spikes in all types of sensilla (Fig. 4H–J).

Relationship between sensilla's response patterns and oviposition-inducing activity

We next examined the correlation between electrophysiological responses and oviposition-inducing activity using whole-leaf citrus extract and mixtures of oviposition stimulants. First, we examined sensilla responses to the whole-leaf extract (1.0× citrus extract), which showed full (100%) oviposition-inducing activity in assays for oviposition behavior in two previous reports (Ohsugi et al., 1991; Ozaki et al., 2011). The 1.0× citrus extract elicited at least 3–4 types of spikes in L1 sensilla, but only one type in L2 and S sensilla (high-amplitude: 1.0–1.5 mV) (Fig. 5A). To examine the dose dependency of these responses by L1 sensilla, the citrus extract was used at four different dilutions from 0.001 to 1.0 (Fig. 5A). A dose-dependent increase in the frequency of all types of spikes was observed (Fig. 5A, Table 1; high spikes; $F_{(3,58)} = 23.95$, $p < 0.0001$, intermediate spikes; $F_{(3,58)} = 9.643$, $p < 0.0001$, and low spikes; $F_{(3,58)} = 26.91$, $p < 0.0001$). We then examined responses of L1 sensilla to several mixtures of oviposition stimulants that were predicted to elicit two or three types of spikes simultaneously, based on the spike patterns of individual stimulants (Fig. 5B). The data indeed showed that each mixture elicited the spike pattern expected from individual patterns.

We asked whether there are correlations between the spiking responses and the oviposition activity of mixtures (Fig. 5C). Mixtures of four or five stimulants (1 + 2 + 3 + 4 + 5, 1 + 2 + 3 + 4), which elicit three types of spikes (Fig. 5B), induced oviposition behaviors comparable to the citrus extract (Fig. 5C; oviposition activities of the citrus extract, 1 + 2 + 3 + 4 + 5, and 1 + 2 + 3 + 4; $F_{(2,21)} = 3.0727$, $p = 0.06753$), while a mixture of four stimulants (2 + 3 + 4 + 5) showed lower activity ($77.4 \pm 4.0\%$) than the citrus extract (oviposition activities of the citrus extract and mixtures of four or five stimulants; $F_{(3,35)} = 5.799$, $p = 0.0025$). Mixtures of three oviposition stimulants showed different oviposition activity according to the number of spike types activated (Fig. 5B,C). Approximately 60% oviposition activity was induced by mixtures eliciting three types of spikes (2 + 3 + 4, 1 + 2 + 4, 1 + 4 + 5), while mixtures eliciting two types of spikes (2 + 4 + 5 and 3 + 4 + 5) showed low induction activity (oviposition activities of five mixtures of three stimulants; $F_{(4,50)} = 25.87$, $p < 0.0001$). Mixtures of only two compounds showed contrasting results (Fig. 5C). The mixtures of synephrine and *chiro*-inositol (1 + 4) and synephrine and narirutin (1 + 5) that elicited three types of spikes (Fig. 3D,E) induced relatively high oviposition behavior ($47.6 \pm 3.0\%$ and $39.4 \pm 3.5\%$, respectively; oviposition activities of eight mixtures of two stimulants; $F_{(7,44)} = 13.37$, $p < 0.0001$), whereas other binary mixtures eliciting only one or two spike types (Fig. 3 and data not shown) barely induced any oviposition behavior. Neither water nor any single oviposition stimulant induced any oviposition (Fig. 5C). These results suggest that combinations of stimulants that elicit all three types of spikes, os1, os2, and os3, are necessary to induce the oviposition behavior.

Oviposition inducing activity by mixtures that elicited three types of spikes varied, suggesting that other factors, such as the frequency of elicited spikes, may affect their potency. A linear correlation was observed between the frequency of the total number of spikes and the oviposition activity elicited by 0.001–0.1× citrus extracts and mixtures of oviposition stimulants eliciting three types of spikes (Fig. 5D, Tables 2, 3; the correlation coefficient of 0.001–0.1× citrus extracts and mixtures of oviposition

(Figure legend continued.) 5-hydroxy-*N*-methyltryptamine. The mixture elicited two types of spikes. Arrowheads indicate four doublets of os1 and os3. **G**, Response to 10 mM stachydrine and 2.5 mM synephrine. **H**, Response to 10 mM stachydrine and 10 mM 5-hydroxy-*N*-methyltryptamine. **I**, Response to 10 mM *chiro*-inositol and 5 mM narirutin. **J**, Response to 0.05 mM 5-hydroxy-*N*-methyltryptamine and 2.5 mM synephrine. Mixtures **G–J** each elicited a single type of spike. **K**, Responses to 25 mM synephrine and **L**, 10 mM 5-hydroxy-*N*-methyltryptamine. Arrowheads indicate doublets of os1 and os3. Arrows indicate the onset of stimulation. Trace **A** lasted 200 ms and traces **C–L** 500 ms.

Figure 4. Typical recordings of L1 (left column) and L2 or S sensilla (right column) in response to **A**, 100 mM sucrose; **B**, 100 mM fructose; **C**, 100 mM glucose; **D**, 1 mM KCl; **E**, 100 mM KCl; **F**, 50 mM NaCl; **G**, 200 mM NaCl; **H**, 10 mM caffeine; and **I**, 0.1 mM quinine. Gray squares, open circles, open squares, closed squares, and triangles indicate water, sugar, salt (low amplitude-spikes), salt (high amplitude-spikes), and bitter responses, respectively. **J**, Dose dependency of responses to sucrose, fructose, glucose, KCl, NaCl, caffeine, and quinine. (mean $\pm$ SD spike frequencies; $n = 5–50$ for sucrose, $n = 6–21$ for fructose, $n = 7–13$ for glucose, $n = 11–60$ for KCl, $n = 6–66$ for NaCl, $n = 5–33$ for caffeine, and $n = 6–15$ for quinine). Different letters (a–d) indicate significant differences of water spikes and salt spikes (low amplitude) in L2 and S sensilla among concentrations (Tukey–Kramer test, $p < 0.05$). * $p < 0.01$, ** $p < 0.001$, *** $p < 0.0001$ versus 0.1 mM sucrose, 1 mM fructose, 1 mM glucose, 0.01 mM KCl, 0.1 mM NaCl, 0.01 mM caffeine, and 0.01 mM quinine, respectively.

Figure 5. Electrophysiological and oviposition responses to whole-leaf citrus extract and mixtures of oviposition stimulants. **A**, Typical response to $1.0 \times$ citrus extract (left). Scale bar, 1.0 mV. Arrows indicate the onset of stimulation. The first trace showed impulses during the first second after stimulation; the 0.5 s trace below it expands the boxed region in the 1.0 s trace. Circles, squares, and triangles indicate high, intermediate, and low spikes, respectively. The 0.1 – $0.001 \times$ citrus extracts also elicited three types of spikes in L1 sensilla during the first second of stimulation (right). **B**, Typical responses of L1 sensilla to mixtures of oviposition stimulants. The mixtures of oviposition stimulants were based on the estimated concentrations of each compound in whole-leaf extracts of *C. unshui* (see Fig. 2). The five oviposition stimulants were numbered as follows: synephrine, 1; stachydrine, 2; 5-hydroxy-*N*-methyltryptamine, 3; *chiro*-inositol, 4; and narirutin, 5. Circles, squares, and triangles indicate oviposition spike 1 (os1), os2, and os3, respectively. **C**, Assay of oviposition behavior (oviposition activities; $n = 9$ for $1.0 \times$ citrus extract, $n = 5$ for (Figure legend continues.)

Table 1. Number of spikes (mean ± SE) elicited by citrus extract in L1 sensilla

	Dilution ratio			
Stimulants	1.0	0.1	0.01	0.001
Total	64.2 ± 2.4 ^a	41.7 ± 1.3 ^b	28.6 ± 1.9 ^c	12.5 ± 1.3 ^d
High	26.9 ± 3.2 ^a	14.5 ± 1.9 ^b	9.5 ± 1.3 ^b	4.8 ± 0.8 ^c
Intermediate	14.1 ± 2.0 ^a	11.8 ± 1.7 ^a	8.4 ± 2.0 ^{a,b}	2.9 ± 0.8 ^b
Low	23.1 ± 2.1 ^a	14.9 ± 1.8 ^b	11.0 ± 1.7 ^{b,c}	4.8 ± 0.6 ^c

^{a,b,c,d}Different letters (a–d) indicate significant differences in total spikes or in each type of spike by Tukey–Kramer test ($p < 0.05$).

Each value represents the mean ± SE in number of spikes ($n = 11$ for $1.0 \times$ citrus extract, $n = 20$ for $0.1 \times$ citrus extract, $n = 15$ for $0.01 \times$ citrus extract, and $n = 19$ for $0.001 \times$ citrus extract).

Table 2. Number of spikes (mean ± SE) elicited by citrus extract and mixtures of oviposition stimulants in L1 sensilla

	Dilution ratio			
Stimulants	1.0	0.1	0.01	0.001
Citrus extract	64.2 ± 2.4 ^a	41.7 ± 1.3 ^b	28.6 ± 1.9 ^c	12.5 ± 1.3 ^{d,e}
1 + 2 + 3 + 4 + 5	41.6 ± 2.9 ^b	27.6 ± 3.2 ^c	10.0 ± 2.3 ^{d,e}	—
1 + 2 + 3 + 4	42 ± 2.5 ^b	24.4 ± 4.9 ^c	5.0 ± 0.9 ^d	—
1 + 2 + 4	22.5 ± 1.5 ^{c,f}	6.0 ± 1.8 ^{d,e}	4.2 ± 1.5 ^d	—
1 + 3 + 4	30.1 ± 3.5 ^c	14.0 ± 3.4 ^{e,f}	2.5 ± 0.5 ^d	—
2 + 3 + 4 + 5	41.0 ± 2.0 ^b	—	—	—
1 + 4 + 5	35.4 ± 2.2 ^{b,c}	—	—	—
1 + 4	14.9 ± 2.2 ^{e,f}	—	—	—
1 + 5	22.3 ± 5.1 ^{c,f}	—	—	—
2 + 4 + 5	31.3 ± 5.8 ^c	—	—	—
3 + 4 + 5	43.0 ± 5.6 ^b	—	—	—

^{a,b,c,d,e,f}Different letters (a–f) indicate significant differences among all results by Tukey–Kramer test ($p < 0.05$).

1, Synephrine; 2, stachydrine; 3, 5-hydroxy-*N*-methyltryptamine; 4, *chiro*-inositol; 5, narirutin. "—" indicates no recordings. Each value represents the mean ± SE in number of spikes ($n = 7–40$).

stimulants eliciting three types of spikes; $r_{(15)} = 0.9325$, $t = 10.0024$, $p = 4.98099 \times 10^{-8}$). On the other hand, mixtures of oviposition stimulants eliciting two types of spikes (2 + 4 + 5 and 3 + 4 + 5) showed low oviposition activities (2 + 4 + 5 and mixtures of $1.0 \times$ mixtures of three stimulants; $F_{(3,39)} = 18.793$, $p < 0.0001$, and 3 + 4 + 5 and mixtures of $1.0 \times$ mixtures of 4 or 5 stimulants; $F_{(3,38)} = 88.124$, $p < 0.0001$), even though they elicited as many spikes as would induce oviposition by some mixtures that elicited three types of spikes (2 + 4 + 5 and $1.0 \times$ mixtures of three stimulants; $F_{(3,93)} = 2.398$, $p = 0.07292$, and

←

(Figure legend continued.) 1 + 2 + 3 + 4 + 5, $n = 10$ for 1 + 2 + 3 + 4, $n = 15$ for 2 + 3 + 4 + 5, $n = 7$ for 2 + 3 + 4, $n = 9$ for 1 + 2 + 4, $n = 12$ for 3 + 4 + 5, $n = 12$ for 2 + 4 + 5, $n = 15$ for 1 + 4 + 5, $n = 12$ for 1 + 5, $n = 20$ for 1 + 4, $n = 3$ for 2 + 3, $n = 3$ for 1 + 3, $n = 3$ for 1 + 2, $n = 4$ for 3 + 4, $n = 4$ for 2 + 4, $n = 5$ for 5, $n = 7$ for 4, $n = 4$ for 3, $n = 4$ for 2, $n = 7$ for 1, and $n = 7$ for water). Each value represents the mean ± SE of 3–20 independent determinations, each of which was the average of 7–10 individuals. The gray, white, and black bars indicate whether three, two, or one GRN was fired by each solution, except for water. * $p < 0.0001$ versus oviposition activity induced by water alone (Tukey–Kramer test; $F_{(22,157)} = 48.947$). **D**, Correlation between number of spikes and oviposition activity. Gray circles, closed circles, squares, triangles, open circles, open square, open diamond, gray square, gray diamond, closed triangle, and closed triangle (downward) indicate citrus extract and mixtures of 1 + 2 + 3 + 4 + 5, 1 + 2 + 3 + 4, 1 + 2 + 4, 1 + 3 + 4, 2 + 3 + 4 + 5, 1 + 4 + 5, 1 + 4, 1 + 5, 2 + 4 + 5, and 3 + 4 + 5, respectively. Each value represents oviposition activity on the vertical axis (mean ± SE $n = 4–11$ for citrus extract, $n = 3–5$ for 1 + 2 + 3 + 4 + 5, $n = 3–10$ for 1 + 2 + 3 + 4, $n = 3–7$ for 1 + 2 + 4, $n = 3–9$ for 1 + 3 + 4, $n = 15$ for 2 + 3 + 4 + 5, $n = 15$ for 1 + 4 + 5, $n = 20$ for 1 + 4, $n = 12$ for 1 + 5, $n = 12$ for 2 + 4 + 5, and $n = 12$ for 3 + 4 + 5) and the number of spikes on the horizontal axis (mean ± SE $n = 12–20$ for citrus extract, $n = 17–30$ for 1 + 2 + 3 + 4 + 5, $n = 7–26$ for 1 + 2 + 3 + 4, $n = 12–21$ for 1 + 2 + 4, $n = 11–28$ for 1 + 3 + 4, $n = 40$ for 2 + 3 + 4 + 5, $n = 10$ for 1 + 4 + 5, $n = 13$ for 1 + 4, $n = 12$ for 1 + 5, $n = 40$ for 2 + 3 + 4, and $n = 7$ for 3 + 4 + 5). The results of *post hoc* multiple comparisons of these data are described in Tables 2 and 3, respectively.

Table 3. Oviposition activities (mean ± SE) elicited by citrus extract and mixtures of oviposition stimulants

	Dilution ratio			
Stimulants	1.0	0.1	0.01	0.001
Citrus extract	97.5 ± 2.5 ^a	97.5 ± 1.7 ^a	54.2 ± 4.2 ^c	9.4 ± 6.0 ^d
1 + 2 + 3 + 4 + 5	95.1 ± 3.0 ^a	52.0 ± 5.9 ^{c,e}	21.5 ± 8.3 ^{d,e}	—
1 + 2 + 3 + 4	86.7 ± 3.9 ^{a,b}	45.5 ± 8.8 ^{c,e}	14.8 ± 5.3 ^{d,e}	—
1 + 2 + 4	61.9 ± 7.2 ^c	15.3 ± 5.2 ^{d,e}	6.5 ± 3.6 ^d	—
1 + 3 + 4	62.0 ± 7.9 ^c	30.5 ± 3.7 ^{d,e}	6.5 ± 3.6 ^d	—
2 + 3 + 4 + 5	77.4 ± 4.0 ^b	—	—	—
1 + 4 + 5	54.6 ± 4.5 ^c	—	—	—
1 + 4	47.6 ± 3.0 ^{c,e}	—	—	—
1 + 5	39.4 ± 3.5 ^{c,e}	—	—	—
2 + 4 + 5	13.9 ± 3.1 ^d	—	—	—
3 + 4 + 5	12.1 ± 3.4 ^d	—	—	—

^{a,b,c,d,e}Different letters (a–e) indicate significant differences among all results by Tukey–Kramer test ($p < 0.05$).

1, Synephrine; 2, stachydrine; 3, 5-hydroxy-*N*-methyltryptamine; 4, *chiro*-inositol; 5, narirutin. "—" indicates no data. Each value represents the mean ± SE of oviposition activity ($n = 3–20$).

Figure 6. Model of the neural pathway modulating oviposition behavior elicited by oviposition stimulants.

3 + 4 + 5 and $1.0 \times$ mixtures of 4 or 5 stimulants; $F_{(3,98)} = 0.0341$, $p = 0.99148$). These results suggest that both quality (three types of spike) and quantity (a critical level of the frequency of three types of spikes) are required to induce oviposition behavior.

Discussion

We found that *P. xuthus* had three types of sensilla—L1, L2, and S—with different sensory functions via a systematic electrophysiological analysis of sensilla on the fifth tarsomere of forelegs (Figs. 1, 2, 4). The L1 sensilla responded preferentially to five oviposition stimulants: synephrine, stachydrine, 5-hydroxy-*N*-methyltryptamine, *chiro*-inositol, and narirutin. Furthermore, the responses in L1 sensilla showed a consistent correlation between the spike patterns elicited by some mixtures of stimulants and the oviposition-inducing activities of these mixtures (Fig. 5D). In contrast, L2 and S sensilla responded to three oviposition stimulants and to general tastants (Figs. 2, 4). Among the three oviposition stimulants, stachydrine and 5-hydroxy-*N*-methyltryptamine elicited spikes at concentrations comparable to those found in *C. unshiu* leaves (Fig. 2I), but the mixture of these stimulants elicited no oviposition activity (Fig. 5C). These results suggest that L2 and S sensilla do not participate directly in eliciting the oviposition behavior, although L2 and S sensilla might have some physiological role because they possess

GRN(s) that respond to stimulants and to the citrus extract (Fig. 5A). We therefore postulate that chemosensory neurons in the L1 sensillum are mainly involved in triggering the oviposition behavior in the females.

In L1 sensilla, five stimulants elicited three types of spikes — os1, os2, and os3 — either alone or as a combination of os1 and os3 (Fig. 2). The os1, os2, and os3 elicited by various mixtures of stimulants showed doublet spikes with the same amplitude and shape as spikes elicited by individual stimulants (Fig. 3). Because these three types of spikes originated from discrete three GRNs and the combinations of oviposition stimulants that elicited the same-amplitude spikes did not elicit the doublet spikes, the oviposition stimulants that elicited spikes with the same amplitude are probably detected by the same GRN. The citrus extract also elicited at least three types of spikes (Fig. 5A), suggesting that the spikes are the same as those elicited by the five oviposition stimulants. These results suggest that specific three GRNs encoding the multiple oviposition stimulants exist in the gustatory sensing system of *P. xuthus*.

In oviposition behavioral assays, the oviposition activities induced by the mixtures of oviposition stimulants that elicited three types of spikes, os1, os2, and os3 were almost comparable to that of tenfold diluted citrus extract (Fig. 5D). Conversely, the mixtures eliciting one or two types of spikes induced low oviposition activities (Fig. 5C,D). Considering the oviposition and the electrophysiological data together, we propose two conditions that must be met for a chemical mixture to induce the oviposition behavior. First, the simultaneous occurrence of multiple spikes — os1, os2, and os3 — in three discrete GRNs of L1 sensilla should be necessary to induce oviposition behavior. For example, mixtures of the two compounds, synephrine and either *chiro*-inositol or narirutin, elicited higher oviposition activity than any other two-compound mixtures (Fig. 5C). Synephrine was the sole stimulant that elicited both os1 and os3 spikes simultaneously in concentrations equivalent to those estimated in whole *C. unshiu* leaves (Fig. 2I). Because *chiro*-inositol and narirutin evoked the os2 spike, the two mixtures elicited the os1, os2, and os3 spikes. Second, the frequency of all three types of spikes should be greater than a certain level to trigger sufficient synergistic effects for oviposition activity, because a clear correlation was observed between the frequency of spikes and oviposition activity elicited only by the mixtures of stimulants elicited three types of spikes (Fig. 5D). We propose that oviposition behavior can be elicited only when the three GRNs in the L1 sensilla responded simultaneously to oviposition stimulants in frequency of stimuli above each of certain levels.

Our data indicated that *P. xuthus* would use three GRNs to encode the chemical signature of multiple oviposition stimulants, although the five oviposition stimulants of *P. xuthus* have little structural similarity. These three neural pathways possibly represent three new modalities for regulating single behavior, oviposition, in addition to the four taste modalities generally described in insects, namely sweet, bitter, salt and water. A classification of GRNs was established in *Drosophila melanogaster* and *Phormia regina*: GRNs have been classified into water, sugar, salt, and bitter cells (Dethier and Goldrich-Rachman, 1976; Rodrigues and Siddiqi, 1978; Fujishiro et al., 1984; Wiczorek and Wolff, 1989; Meunier et al., 2003). Therefore, multiple sugars and bitter compounds seem to be perceived by individual GRNs. Previous reports indicated that Z-7-tricosene, an inhibitory sex pheromone of *Drosophila* males, was recognized by the *Gr66a*-expressing neuron (bitter cells) and induced aversive responses in a feeding test, and showed the same Ca^{2+} -response as bitter com-

pounds in *Gr66a*-expressing neurons of the CNS (Lacaille et al., 2007; Inoshita et al., 2011). These findings indicated that different chemicals processed by the same neural pathway could induce similar behavioral responses.

We found that the electrophysiological response to a given oviposition stimulant varied among L1 sensilla (Fig. 2J). Some L1 sensilla exhibited only one or two types of spikes when stimulated by citrus extract or mixtures of oviposition stimulants that elicited os1, os2, and os3 (data not shown). These results suggest that L1 sensilla house a different number of GRNs that respond to each oviposition stimulant and that oviposition behavior is induced by the total response to different combinations of the three GRNs in a population of L1 sensilla. The different responses to oviposition stimulants among L1 sensilla might also result from distinct expressions of receptors that perceive oviposition stimulants among GRNs, as known in *Drosophila* gustatory receptors (Weiss et al., 2011). To classify sensilla based on response spectra, we have tried to make a distribution map of sensilla by numbering them, but had no success, because the locations of these sensilla on the tarsomere varied among individuals.

We have recently identified a gustatory receptor PxutGr1 that is specific for synephrine and is expressed in the foreleg tarsi of *P. xuthus* (Ozaki et al., 2011). Females injected with PxutGr1-dsRNA exhibited reduced responses to synephrine and reduced oviposition activity induced by a mixture of synephrine and *chiro*-inositol. Because synephrine elicits the os1 and os3 spikes, we believe that the PxutGr1 receptor may be expressed by two GRNs. Based on the present findings, we propose a model for the neural pathway involved in modulation of oviposition behavior by the five oviposition stimulants (Fig. 6). In constructing the model, we assumed that the butterfly *P. xuthus* uses the CNS region to receive and process signals from GRN in contact chemosensilla as reported in Diptera and Lepidoptera (Marella et al., 2006; Thorne et al., 2004; Jørgensen et al., 2006; Kvello et al., 2006; Koganezawa et al., 2010). The PxutGr1 appears to be expressed in GRN1 and GRN3, because synephrine elicits os1 and os3. Likewise, putative receptors for the other four oviposition stimulants may be expressed in three GRNs depending on encoding pattern of them. In other words, PxutGr1 and the putative receptors for 5-hydroxy-*N*-methyltryptamine and stachydrine become the common signature for GRN1, *chiro*-inositol and narirutin are the common signature for GRN2, and synephrine and 5-hydroxy-*N*-methyltryptamine are the signature for GRN3. The three GRNs may project into the CNS. These signals transduced from the 3 GRNs may be then integrated and processed in the CNS to give rise to signals to induce oviposition behavior.

This study showed that *P. xuthus* could use three GRNs that encode the taste signals of multiple oviposition stimulants that regulate the oviposition behavior. Our findings provide a novel mechanism to modulate butterfly oviposition behavior based on multiple taste signals and represent the first step toward elucidating the perception mechanism underlying host plant selection by Papilionidae butterflies.

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