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Running title: Taste sense in adult butterfly proboscis

Tolerance to fermentation products in sugar reception: Gustatory adaptation of adult butterfly proboscis for feeding on rotting foods

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Abstract Adult *Vanessa indica* and *Argyreus hyperbius* frequently forage on flower nectar, but the former also utilize tree sap and rotting fruits. Compared to flower nectar, these rotting foods are characterized by low sugar concentrations and the presence of fermentation products (ethanol and acetic acid). We suspected that gustatory responses by the receptors on the proboscis might differ in these species. Among the three sugars tested, sucrose elicited the greatest probing (behavioral) responses and was followed by fructose and glucose. *A. hyperbius* showed higher sugar sensitivity than *V. indica* in probing responsiveness. In electrophysiological responses of the proboscis sensilla, *V. indica* was slightly more sensitive than *A. hyperbius* to glucose and lower concentrations of the other sugars. The sugar reception in *A. hyperbius* was strongly inhibited by fermentation products, particularly acetic acid at natural concentrations. In contrast, *V. indica* was noticeably less susceptible to them than *A. hyperbius*, and its behavioral and sensory responses to sucrose were enhanced by 5–20% (w/v) ethanol. Thus, *V. indica* not only possesses tolerance to fermentation products but may perceive them as synergists for sugar reception. To utilize rotting foods, such tolerance might be more necessary than high sugar sensitivity.

Keywords Nymphalid butterfly, Proboscis sensilla styloconica, Taste, Rotting foods, Chemoreception

Introduction

The morphology of the mouthparts of insects has evolved concomitantly with their feeding habits and food types (Krenn et al. 2005). With the exception of the three

most basal taxa, Micropterigidae, Agathiphagidae, and Heterobathmiidae, all adult Lepidoptera possess a proboscis consisting of two elongated galeae, which originate from the basal maxillary structures used for sucking fluids (Kristensen 2003). This structure, which is elastic and is capable of being extended and recoiled, has evolved to facilitate nectar intake and flower handling in these organisms. Indeed, nectar-feeding behavior is reported in approximately 98% lepidopteran species, indicating that flower nectar is their primary food source (Pellmyr 1992; Kristensen 2003).

Despite their strong dependence on flower nectar, a considerable proportion of lepidopteran adults forage on various non-nectar foods such as pollen, fruit, honeydew, tree sap, mud, carrion, and dung. These species have significant morphological variation in their proboscis, particularly in the tip region and sensilla. This variation appears to be related to their specialization and adaptation with respect to the physical properties of non-nectar foods (e.g., Guyenot 1912; Paulus and Krenn 1996; Krenn 1998; Krenn et al. 2001; Petr and Stewart 2004; Molleman et al. 2005). In addition, non-nectar foods are significantly different from flower nectar in their chemical properties, suggesting the presence of variation in the taste sense of these species depending on their feeding habits.

When the proboscides of lepidopteran adults are brought into contact with sugar solutions, the insects display feeding behavior (Frings and Frings 1949, 1956; Hodgson 1958; Adler 1989; Lopez et al. 1995). This phenomenon demonstrates that the proboscis acts as a gustatory sense organ. In many species, the tip of the proboscis has sensilla styloconica with a terminal pore and a small number of sensory neurons inside (Städler et al. 1974; Altner and Altner 1986; Krenn 1998; Walter et al. 1998). Electrophysiological studies have revealed that these sensilla respond to sugars, salts,

and amino acids, and provide critical input to induce feeding acceptance (Städler and Seabrook 1975; Altner and Altner 1986; Blany and Simmonds 1988). However, such physiological studies on the taste sense have been conducted using a limited number of nectar-feeding species, and little knowledge is available on those feeding on non-nectar foods.

Here, we describe the proboscis gustatory responses of adult butterflies feeding on exuded tree sap and rotting fruits. The sugar chemistry of these foods is distinctive from that of flower nectar in which fructose and glucose are the dominant sugars. Further, the total sugar concentration (average, 3% w/w) of tree sap and rotting fruits is noticeably lower than that of flower nectar from most plant species (Ômura and Honda 2003). Moreover, these foods contain various fermentation products, the major constituents of which are ethanol (approximately, 1% w/w) and acetic acid (approximately, 0.5% w/w), which are absent in flower nectar (Ômura et al. 2000, 2001; Ômura and Honda 2003). Accordingly, species capable of utilizing rotting foods are likely to possess a characteristic taste sense, i.e., high sugar sensitivity and/or adaptation to fermentation products. In the present study, we examined the behavioral and electrophysiological responses of two nymphalid adults, *Vanessa indica* and *Argyreus hyperbius*, to proboscis stimulation by sugars and fermentation products. *V. indica* inhabits grasslands on the edges of forests, shows frequent flower visiting, and is sometimes observed foraging on rotting foods, while *A. hyperbius* also inhabits grasslands but feeds only on flower nectar (Kawazoe and Wakabayasi 1976). It is feasible that the taste sense in the proboscis plays a major role in their different feeding habits. We examined whether proboscis gustatory neurons of *V. indica* are adapted to feeding on rotting foods.

Materials and methods

Insects

Adult butterflies were obtained from stock cultures in our laboratory or by rearing wild larvae collected in Higashihiroshima city (Hiroshima prefecture, Japan). *V. indica* and *A. hyperbius* were reared on *Boehmeria nipononivea* (Urticaceae) and *Viola* spp. (Violaceae), respectively, at 25 °C under a 16L:8D photoperiod. From two days after emergence, the adults were individually maintained in cylindrical plastic chambers (75 mm height, 80 mm internal diameter) and fed daily with a 10% (w/w) aqueous sucrose solution; access to natural foods was denied.

Microscopic observation

The tip region of the proboscis was subjected to binocular microscopic observation using a Wild M32 stereomicroscope (Wild Heerbrugg Ltd., Switzerland). When an adult butterfly extended its proboscis to forage on a droplet of aqueous sucrose solution on a glass slide, the distal end was fixed unbent by covering with another glass slide and excised. This proboscis preparation was used for observation within 30 min of excision.

Scanning electron microscopic observation was carried out at 5 kV using a JSM-6301F (JEOL Ltd., Japan) scanning electron microscope. The proboscis was excised at its proximal part from a living adult butterfly, and individual galeae were separated. Each coiled galea was mounted on a stage with Dotite paste (Fujikurakasei Co. Ltd., Japan) and sputter-coated with gold.

Behavioral experiments

In our previous study (Ômura and Honda 2003), we examined feeding responses to proboscis stimulation with sugars using 2-day-old naïve adult butterflies conditioned by at least 20-h starvation and 2-h free flight. However, several butterflies continued proboscis extension reflex after stimulation, suggesting that their responsiveness was artificially reinforced by the pre-test conditioning. In addition, most butterflies were shown to probe test solutions with their proboscis tip before feeding behavior. This behavioral sequence suggests that probing behavior is elicited by lower sugar concentrations than feeding behavior. Therefore, the present study was designed to investigate probing responses to three sugars (sucrose, fructose, and glucose), within the concentration range of 0.005 to 2 M, using 40 individuals (20 males and 20 females) of each butterfly. After being fed with a 10% (w/v) aqueous sucrose solution ad libitum, the individuals to be tested were maintained for 2 days at 25 °C in a dark place. Prior to performing the bioassays, it was confirmed that the butterflies did not show positive probing or drinking behavior in response to proboscis stimulation with a droplet of distilled water: those that showed positive responses were discarded. Strips of paper towel (2 × 2 cm, Oji Nepia Co. Ltd., Japan) were placed in transparent plastic dishes (10 cm diameter) and soaked with 0.5 ml of the aqueous test solution. Each butterfly was gently picked up by its wings and its proboscis was uncoiled with a forceps and brought into contact with the paper towel. Probing responsiveness of the butterfly was then evaluated as follows: (i) acceptance: the butterfly continued probing the test solution with the tip of its proboscis for at least 1 s or (ii) rejection: the butterfly coiled its proboscis immediately after contact with the test solution or probed for less than 1 s. The butterflies were not released during the experiments and were offered each type of sugar at increasing concentrations in the test solutions. The probing-stimulatory effect

of sugar at a given concentration was represented as the percentage of individuals showing acceptance in each species. EC_{50} (effective concentration) was defined as the concentration accepted by 50% of individuals; this value was evaluated for each sugar by probit analysis.

We examined the potential effects of fermentation products, ethanol and acetic acid, on the probing response to sucrose, which was the most active among the three sugars, using 40 individuals (20 males and 20 females) of each butterfly. Prior to the bioassays, the individuals were conditioned and confirmed that they did not show positive responses to distilled water in the same manner as above. The test solutions comprised binary mixtures of sucrose at the EC_{50} for probing responses (70 mM for *V. indica* and 50 mM for *A. hyperbius*) and either ethanol or acetic acid in a series of concentrations (0.01%, 0.05%, 0.1%, 0.5%, 1%, 5%, and 10% w/v). Probing performance of the butterflies was examined as described earlier. A series of test solutions was examined in the order of increasing concentrations of ethanol or acetic acid. The average response to each test solution was represented as the percentage of individuals showing acceptance in each species. The IEC_{50} (inhibitory effective concentration) was defined as the concentration rejected by 50% of the individuals that responded to sucrose alone; this value was evaluated for each fermentation product by probit analysis.

Electrophysiological recordings

Electrophysiological recordings from the sensilla styloconica on the galeae of adult butterflies were conducted with a TastePROBE amplifier (Syntech, Hilversum, The Netherlands) and a Syntech IDAC-2 A/D converter by the tip recording technique

(Marion-Poll and van der Pers, 1996). The proboscides were excised at the proximal part from 2- to 14-day-old insects, and the two galeae were separated. Each galea was fixed by adhesive tape onto the stage to expose the sensilla styloconica and subjected to the recording procedure within 2 h of dissection. The indifferent electrode, a glass microcapillary filled with an insect Ringer, was inserted into the proximal cut end of the galea. The recording electrode, a glass microcapillary containing stimulant solution, capped the tip of the sensillum to record gustatory responses.

First, we examined the gustatory responses to three sugars (sucrose, fructose, and glucose) at five concentrations (0.98, 3.91, 15.6, 62.5, and 250 mM), ethanol at eight concentrations (10^{-4}%, 10^{-3}%, 10^{-2}%, 10^{-1}%, 1%, 10%, 20%, and 50% w/v), and acetic acid at five concentrations (10^{-4}%, 10^{-3}%, 10^{-2}%, 10^{-1}%, and 1% w/v). Second, we investigated whether three sugars were responded by the same neuron using binary mixtures of the sugars at the concentration of 15.6 mM. At this concentration, each plain sugar elicited a small number of spikes. Third, we tested the potential effects of fermentation products on sugar reception in the sensilla using binary mixtures of sucrose (31.3 mM) and either ethanol at eight concentrations (10^{-4}%, 10^{-3}%, 10^{-2}%, 10^{-1}%, 1%, 10%, 20%, and 50% w/v) or acetic acid at five concentrations (10^{-4}%, 10^{-3}%, 10^{-2}%, 10^{-1}%, and 1% w/v). Since both species constantly showed intermediate responses to 31.3 mM of sucrose, it was used as a control stimulus. All stimulant solutions contained 20 mM NaCl as an electrolyte. Each series of test solutions was applied to the same sensillum in the order of increasing concentration.

Before recording the responses to stimulants, 20 mM NaCl was applied to the sensillum to examine the responses to water and NaCl. Subsequently, 31.3 mM sucrose was applied to the sensillum to determine the presence of sugar reception.

This sucrose solution was applied as a control stimulant after every two stimulations to check the stability of the sugar responsiveness. Just prior to stimulation, tissue paper was gently applied to the tip of the recording electrode to absorb the test solution and avoid changes in the concentration due to evaporation. To avoid the possible effects of adaptation to the previous stimulation, a stimulation-free interval of at least 3 min was allowed. The number of spikes from 20 ms to 1 s after contact of the recording electrode with the sensillum was recorded as the gustatory response. Within 20 ms after coming into contact with the recording electrode, most of the sensilla generated an artifact signal that was too large to quantify gustatory signals. Action potentials (spikes) were categorized based on their regular and different patterns of firing and by differences in the spike height. Two to four sensilla were randomly selected on each galea of different individuals and subjected to the abovementioned electrophysiological recording. Each stimulant was applied to more than 20 sensilla from at least six different individuals of each sex. Sensory response to each stimulant was expressed as the mean number of spikes in the recording period of 980 ms. Responses to the binary mixtures were expressed as the percentage of the response to plain sucrose solution.

Results

Proboscis sensilla

In *V. indica* and *A. hyperbius*, the sensilla styloconica were arranged in a single row in the distal lateral region of each galea (Fig. 1 A1, B1). In both species, the sensillum consisted of a smooth flattened style and a sensory cone (Fig. 1 A2, B2; arrowhead). There was a pore opening at the tip of the cone (Fig. 1 A3, B3; arrow). However, the distal structure of the style differed between species; in *V. indica*, the sensory cone was

surrounded by several apical cuticular spines (Fig. 1 A3; asterisk), while *A. hyperbius* did not possess these spines. In *V. indica*, the number of sensilla per galea was 61 ± 5 (mean ± SD) in males (N = 25) and 60 ± 4 in females (N = 24). *A. hyperbius* males (N = 32) and females (N = 34) possessed 33 ± 3 and 34 ± 3 sensilla per galea, respectively. The sex difference in the number of sensilla of each species was not significant (Mann-Whitney U test; P = 0.418 for *V. indica*; P = 0.433 for *A. hyperbius*).

Probing performance with sugars

The probing responses of adult butterflies increased with the increase of the sugar concentration (Fig. 2). Since the sex difference in the responses to each sugar was not significant, the results of both sexes were pooled for each species. Among the three sugars tested, sucrose was the most active in terms of stimulating probing, followed by fructose and glucose. The EC_{50} values of sucrose, fructose, and glucose were 68.0 mM, 200.9 mM, and 613.2 mM for *V. indica* and 47.5 mM, 73.5 mM, and 366.8 mM for *A. hyperbius*, respectively; viz. EC_{50} of *V. indica* was 1.43- to 2.73-fold larger than that of *A. hyperbius* for each sugar.

Electrophysiological responses to sugars

In most electrophysiological recordings from the sensilla styloconica, only one type of spike was elicited by the sugars, and the number of spikes was dependent on the sugar concentration (Fig. 3). The results indicated that spikes were derived from sugar receptor cells. Sometimes two types of spikes with different amplitudes were observed (Fig. 5B, trace F_1). Larger spikes were observed in the response to 20 mM NaCl alone and the number of spikes was almost constant, irrespective of the sugar concentration,

237 whereas the number of smaller spikes increased along with the increment of the sugar
238 concentration. In this case, neurons with a small spike amplitude were regarded as
239 sugar responsive. Among the three sugars tested, sucrose was the most active in
240 stimulating sensillum responses in both species. In *V. indica* (Fig. 4 upper), males
241 showed a significantly larger number of spikes to sucrose than fructose at the same
242 concentration (Mann-Whitney *U* test; $P < 0.05$, $P < 0.05$, $P < 0.01$, $P < 0.05$, and $P <$
243 0.01 for the concentration of 0.98 mM, 3.91 mM, 15.6 mM, 62.5 mM, and 250 mM,
244 respectively), while females did at concentrations of 3.91 mM, 15.6 mM, and 62.5 mM
245 (Mann-Whitney *U* test; $P < 0.01$). *A. hyperbius* (Fig. 4 lower) males exerted
246 significantly greater responses to sucrose than fructose at the concentration of 15.6 mM
247 (Mann-Whitney *U* test; $P < 0.05$), while females did at concentrations of 15.6 mM and
248 250 mM (Mann-Whitney *U* test; $P < 0.05$). Glucose was conspicuously less active
249 than sucrose and fructose. *V. indica* responded weakly to glucose in a dose-dependent
250 manner, while *A. hyperbius* showed little response within the range of concentrations
251 tested. Although *A. hyperbius* females showed significantly more frequent spikes than
252 males in response to 62.5 mM and 250 mM sucrose and 15.6 mM glucose (Fig. 4 lower:
253 Mann-Whitney *U* test; $P < 0.01$, $P < 0.05$, and $P < 0.01$, respectively), the sex
254 difference in sugar responsiveness was not significant in other cases in both species.
255 The binary mixtures of sugars, as well as plain sugars, elicited one type of spike in
256 both species (Fig. 5B). Although *V. indica* males showed significantly larger
257 responses to the mixture of sucrose and fructose than plain sucrose (Mann-Whitney *U*
258 test; $P < 0.001$), the binary mixtures did not show an increment of the relative number
259 of spikes in most cases (Fig. 5A). The mixture of sucrose and glucose was
260 significantly less active than plain sucrose in *A. hyperbius* (Mann-Whitney *U* test; $P <$

261 0.05 for males and $P < 0.001$ for females). Although sucrose consists of fructose and
262 glucose units, both sexes of each butterfly showed significantly higher responses to
263 plain sucrose than the mixture of fructose and glucose (Mann-Whitney *U* test; $P < 0.01$).
264
265 Probing performance with binary mixtures of sucrose and fermentation products
266 *V. indica* showed nearly 50% of probing response to 70 mM sucrose (Fig. 6A).
267 Probing performance with the binary mixtures was approximately constant within the
268 concentration range 0.01% to 1% (w/v) of either fermentation product; however, acetic
269 acid suppressed probing responses at the concentration of more than 5% (w/v), whereas
270 ethanol enhanced at the same concentration. The IEC_{50} of acetic acid was found to be
271 11.43% (w/v) for *V. indica*. *A. hyperbius* showed more than 50% responses to 50 mM
272 sucrose (57.5% in the test for acetic acid and 70% in that for ethanol) (Fig. 6B).
273 Probing responses were suppressed as the concentration of fermentation products in the
274 binary mixture solutions increased. Since the IEC_{50} value was 0.275% (w/v) for acetic
275 acid and 3.481% (w/v) for ethanol, acetic acid had significantly greater activity to
276 suppress probing than ethanol.
277
278 Electrophysiological responses to fermentation products and their binary mixtures with
279 sucrose
280 Within the range of concentrations tested, neither ethanol nor acetic acid elicited distinct
281 and countable spikes from the sensilla styloconica. High concentrations of either
282 substance occasionally induced burst responses with irregular spikes or delayed
283 responses with an initial unresponsive period (Fig. 5B, traces Ac).
284 When ethanol was mixed with 31.3 mM sucrose, the two nymphalid butterflies

differed in electrophysiological response (Fig. 7). In *A. hyperbius*, the number of spikes in response to sucrose decreased concentration-dependently on ethanol (Fig. 7B right), and the original response (0% ethanol) was remarkably suppressed at less than 60% by 20% and 50% (w/v) ethanol (Fig. 7A pale column). In contrast, the number of spikes in *V. indica* was maintained at more than 80% of the original response (0% ethanol) within the range of ethanol concentrations tested (Fig. 7B left) and was significantly enhanced to 122% and 116% by 10% and 20% (w/v) ethanol, respectively (Fig. 7A dark column). The sucrose responsiveness (number of spikes) of both species also decreased as the concentration of acetic acid increased (Fig. 8B); however, the two species showed different susceptibility to acetic acid; that of *V. indica* was inhibited 60% by 1% (w/v) acetic acid (Fig. 8A dark column), while that of *A. hyperbius* was strongly suppressed to approximately 50% and 20% by 0.1% and 1% (w/v) acetic acid, respectively (Fig. 8A pale column).

Discussion

Morphology of proboscis sensilla

Adult *V. indica* and *A. hyperbius* possess a proboscis with a brush-like tip, and a row of sensilla styloconica is present on the lateral side of each galea. The sensillum has been categorized as the platyform type, and it consists of a smooth flattened style and a uniporous sensory cone (Petr and Stewart 2004); however, the distal end of the style differs to some extent in the two species; apical cuticular spines were present only in *V. indica*. These structures may protect the sensory cones from mechanical abrasion or may anchor the proboscis tip to the rough surface of foods. In addition, *V. indica* has approximately twice the number of sensilla as *A. hyperbius*. In nymphalid butterflies,

309 species foraging on non-nectar foods are known to possess numerous number of sensilla
310 styloconica in the proboscis (Krenn et al. 2001). Such morphological traits may
311 increase gustatory and/or tactile sensitivity in their feeding on non-nectar foods.
312
313 Sugar reception
314 Among the three sugars tested, sucrose was the most effective in eliciting probing
315 responses to proboscis stimulation. Fructose was slightly less active than sucrose,
316 while glucose showed significantly lower activity than fructose. Sucrose was also the
317 most active in the excitation of sensory responses from the proboscis sensilla, followed
318 by fructose and glucose. These results suggest that sugar reception by the sensilla
319 styloconica acts as a trigger of probing behavior. In electrophysiological
320 measurements, sugar responsiveness greatly differed even among the sensilla located on
321 the same proboscis. This indicates that the proboscis sensilla have different sugar
322 sensitivity, although the present results might be influenced the possible effects of
323 different age and physiological conditions (e.g., the degree of hunger) among the
324 individuals tested. In *V. indica*, sucrose sensitivity of the proboscis sensilla was
325 similar to that of the tarsal sensilla trichodea, which responded to a threshold
326 concentration of 7.8 mM sucrose and fired up to 80 spikes per second (Takeda 1961).
327 *V. indica* had slightly higher sensitivity than *A. hyperbius* in electrophysiological
328 responses to lower concentrations (0.98 mM and 3.91 mM) of sucrose and fructose and
329 all concentrations of glucose. The proboscis sensilla of *V. indica* may be adapted to
330 detect sugar at low concentrations; however, *V. indica* showed lower probing
331 performance (higher EC_{50} values) than *A. hyperbius* in response to the three sugars.
332 Since probing (behavioral) responses are released through CNS processing of gustatory

signals from the proboscis, it is feasible that sensory sensitivity to sugars does not directly correspond to behavioral sensitivity.

Binary mixtures of sugars elicited one type of spike in the proboscis sensilla, suggesting that the three sugars excited the same neuron. Interestingly, the combinations of two different sugars hardly increased the number of spikes. The mixture of fructose and glucose was significantly less active than sucrose, although both stimuli contained the same number of fructose and glucose units. These results suggest that sugar receptive neurons can discriminate the three sugars based on the whole molecular structure. Similar results have been described for sugar receptor neurons of blowfly (Omand and Dethier, 1969).

Sugar reception in the proboscis has so far been investigated using several lepidopteran adults. In the swallowtail butterfly, *Papilio xuthus*, 50 mM sucrose was the threshold concentration for eliciting feeding behavior, and <5 mM sucrose was adequate to stimulate sugar receptor cells in the food-canal sensilla (Ozaki and Tominaga 1999; Inoue et al., unpublished). The sensitivity of *P. xuthus* to sucrose was almost the same as those of *V. indica* and *A. hyperbius* in the present study. The feeding responses of noctuid moth *Spodoptera littoralis* are stimulated by proboscis stimulation with 9 mM sucrose (Salama et al. 1984). The proboscis sensilla styloconica of *Choristoneura fumiferana* (Tortricidae) produced 38–132.3 spikes/s in response to 20 mM sucrose (Städler and Seabrook 1975), while those of three noctuid moths, *S. littoralis*, *Heliothis* (*Helicoverpa*) *virescens*, and *H. armigera*, produced 40–178 spikes/s in response to less than 50 mM sucrose (Blaney and Simmonds 1988).

Exuded tree sap and rotting fruits contain fructose and glucose as the main sugars. Our previous study revealed that sugar concentrations greatly differ with food type and

357 collection date, e.g. the quantitative variations of fructose were 0–2.24% (w/w) for tree
358 sap and 0.84–8.02% (w/w) for rotting fruits (Ômura and Honda 2003). The average
359 concentration was approximately 2% (w/w) for fructose and 1% (w/w) for glucose
360 (Ômura and Honda 2003), corresponding to 111 mM of fructose and 56 mM of glucose,
361 respectively. It is evident that the fructose concentration in these foods is sufficiently
362 high to induce sensory excitation in both species.
363
364 Responsiveness to fermentation products
365 Ethanol and acetic acid did not elicit distinct and reproducible spikes from the proboscis
366 sensilla styloconica of *V. indica* and *A. hyperbius*. Plain ethanol or acetic acid could
367 not elicit feeding responses from three sap-feeding nymphalid butterflies, including *V.*
368 *indica* (Ômura and Honda 2003). It is considered that nerve cells that showed
369 concentration-dependent responses to these fermentation products were absent in the
370 sensilla styloconica; however, high doses of ethanol and acetic acid elicited burst or
371 delayed responses from roughly 10 % of the sensilla. Similar responses to ethanol or
372 acetic acid have been reported in the tarsal sensilla of the blowfly *Phormia regina*
373 (McCutchan 1969) and the antennal sensilla of the American cockroach *Periplaneta*
374 *americana* (Rüth 1976). These irregular responses would to be elicited from the nerve
375 cells, possibly sugar-receptive ones, subjected to chemical damage by fermentation
376 products (Schoonhoven 1982; Schoonhoven et al. 1992).
377
378 Influence on sugar reception by fermentation products
379 Ethanol and acetic acid have been described to inhibit sugar feeding in the blowfly *P.*
380 *regina* (Chadwick and Dethier 1947; Dethier and Chadwick 1947) and the noctuid moth

381 *S. littoralis* (Salama et al. 1984). In agreement with these reports, probing responses
382 of *V. indica* and *A. hyperbius* were inhibited by high concentrations of ethanol and/or
383 acetic acid. These substances also suppressed their electrophysiological responses to
384 sucrose at similar concentrations to enable such behavioral inhibition. Acetic acid
385 served as a stronger deterrent than ethanol; however, *V. indica* was significantly less
386 susceptible to fermentation products than *A. hyperbius* in both probing and
387 electrophysiological responses. In our preliminary examination, *V. indica* could show
388 feeding responses to the mixture of 292 mM sucrose and 60% (w/v) ethanol (Ômura et
389 al. unpublished). Two possible mechanisms are proposed to explain peripheral
390 interaction between phagostimulants and deterrents (Ramaswamy et al. 1992;
391 Schoonhoven et al. 1992; Chapman 2003): firing suppression in sugar receptor cells, as
392 reported for several alkaloids, organic acids, and azadirachtin (e.g., Morita 1959;
393 Mitchell 1987; van Loon 1990, 1996; Bernays et al. 1998), and disruption of sugar
394 receptor cells by the induction of irregular firing, found in alkaloids and aristolochic
395 acid (Schoonhoven et al. 1992; Chapman 2003). It is feasible that both mechanisms
396 are involved in the present results since fermentation products suppressed sugar
397 responses from lower concentrations and sometimes elicited burst responses only at
398 high concentrations.

399 Interestingly, fermentation products are found to not only suppress but also enhance
400 the behavioral and sensory responses of adult butterflies. In the present results for *V.*
401 *indica*, probing responses to 70 mM sucrose were strongly enhanced by 5% or 10%
402 (w/v) ethanol, while electrophysiological responses to 31.3 mM sucrose were also
403 increased by 10 or 20% (w/v) ethanol. Our previous study revealed that ethanol and
404 acetic acid, when mixed with sugars at their natural concentrations, enhance feeding

responses of *V. indica* (Ômura and Honda 2003), suggesting that certain concentrations of fermentation products have potential synergistic effects on sugar reception of *V. indica*.

We have described that rotting foods contain approximately 1% (w/w) of ethanol and 0.5% (w/w) of acetic acid, though these substances, as well as sugars, show large quantitative variations in the samples collected (Ômura and Honda 2003). In terms of their natural abundance, ethanol would slightly suppress probing and sensory responses of *A. hyperbius*, while acetic acid could induce critical inhibition of both responses. Compared with *A. hyperbius*, *V. indica* was less susceptible to the natural level of fermentation products in probing and sensory responses, which demonstrates that the sugar receptive neurons possess some tolerance to these substances. Such a physiological trait is probably characteristic of the butterflies feeding on rotting foods. We have revealed that peripheral gustatory reception in proboscis sensilla styloconica, especially for interaction between sugars and fermentation products, plays a key role in determining the feeding behavior of adult butterflies.

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526 Figure legends

527 **Fig. 1.** Proboscis sensilla styloconica of *V. indica* (**A**) and *A. hyperbius* (**B**) on light
528 microscopy and scanning electron microscopy. (**A1**, **B1**) Proboscis tip regions of *V.*
529 *indica* and *A. hyperbius*. (**A2**, **B2**) Sensilla styloconica of *V. indica* and *A. hyperbius*.
530 Arrowheads indicate sensory cones. (**A3**, **B3**) Sensory cones of *V. indica* and *A.*
531 *hyperbius*, each with a terminal pore (arrow). That of *V. indica* is surrounded by apical
532 cuticular spines (asterisk).

533

534 **Fig. 2.** Probing responses of *V. indica* (**A**) and *A. hyperbius* (**B**) adults to proboscis
535 stimulation by three plain sugar solutions. The points on the concentration-response
536 curves for each species represent average responses from 20 males and 20 females.
537 The horizontal broken line represents the 50% level of the response.

538

539 **Fig. 3.** Typical tip-recording traces of *A. hyperbius* in response to three plain sugars
540 (**A**: sucrose, **B**: fructose, and **C**: glucose) dissolved in 20 mM NaCl. All traces were
541 obtained from the same sensillium. Labels of each trace represent concentrations of
542 sugars tested (mM). Vertical scale bar = 1 mV; horizontal scale bar = 100 ms.

543

544 **Fig. 4.** Electrophysiological responses of proboscis sensilla styloconica of *V. indica*
545 and *A. hyperbius* to three plain sugar solutions. Each stimulus was dissolved in 20
546 mM NaCl. Number of spikes was counted from 20 ms to 1 s after contact with the
547 recording electrode. Mean responses to each stimulus were obtained from 20–25
548 sensilla of each sex. Significant sex difference in the number of spikes is represented
549 by an asterisk (Mann-Whitney *U* test: * $P < 0.05$; and ** $P < 0.01$).

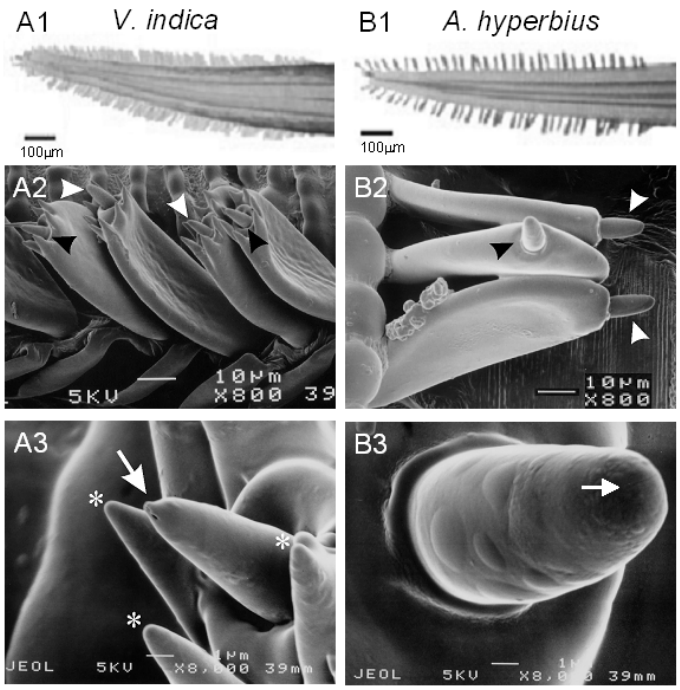
Fig. 5. Electrophysiological responses of proboscis sensilla styloconica of *V. indica* and *A. hyperbius* to 15.6 mM of three plain sugars and its binary mixtures. (**A**) Relative number of spikes from 20 ms to 1 s after contact with the recording electrode. S, F, G, SF, SG, and FG denote sucrose, fructose, glucose, a mixture of sucrose and fructose, that of sucrose and glucose, and that of fructose and glucose. Each stimulus was dissolved in 20 mM NaCl. Mean responses to each stimulus were obtained from 40–53 sensilla and expressed as percentages of the response to S. (**B**) Tip-recording traces from sensilla of *A. hyperbius*. Traces Na (20 mM NaCl alone), S, F, G, SF, SG, and FG were obtained from the same sensillum. Trace F_1 (response to F) contained two different spikes and larger spikes (arrowhead) were regarded as a response to NaCl. Traces Ac were the responses of two different sensilla to 1% (w/v) acetic acid dissolved in 20 mM NaCl. Vertical scale bar = 1 mV; horizontal scale bar = 100 ms.

Fig. 6. Probing responses of *V. indica* (**A**) and *A. hyperbius* (**B**) adults to proboscis stimulation by binary mixtures of sucrose and increasing concentrations of ethanol and acetic acid. The concentration of sucrose was constant 70 mM for *V. indica* and 50 mM for *A. hyperbius*, which were estimated to elicit probing from 50% individuals. Data points on the concentration-response curves for each species represent average responses from 20 males and 20 females.

Fig. 7. Electrophysiological responses of proboscis sensilla styloconica of *V. indica* and *A. hyperbius* to the binary mixtures of 31.3 mM sucrose and increasing concentrations of ethanol. (**A**) Relative number of spikes from 20 ms to 1 s after

574 contact with the recording electrode. Each stimulus was dissolved in 20 mM NaCl.
575 Mean responses to each stimulus were obtained from 42–55 sensilla and expressed as
576 percentages of the response to 31.3 mM sucrose. Different letters indicate significant
577 differences among relative responses (Steel-Dwass multiple comparison of means; $P <$
578 0.05, roman type for *V. indica* and italic type for *A. hyperbius*). (**B**) Tip-recording
579 traces from the same sensilla of *V. indica* and *A. hyperbius*. Labels of each trace
580 represent ethanol concentrations in the mixtures. Vertical scale bar = 1 mV; horizontal
581 scale bar = 100 ms.
582
583 **Fig. 8.** Electrophysiological responses of proboscis sensilla styloconica of *V. indica*
584 and *A. hyperbius* to the binary mixtures of 31.3 mM sucrose and increasing
585 concentrations of acetic acid. (**A**) Relative number of spikes from 20 ms to 1 s after
586 contact with the recording electrode. Each stimulus was dissolved in 20 mM NaCl.
587 Mean responses to each stimulus were obtained from 42-54 sensilla and expressed as
588 percentages of the response to 31.3 mM sucrose. Different letters indicate significant
589 differences among relative responses (Steel-Dwass multiple comparison of means; $P <$
590 0.05, roman type for *V. indica* and italic type for *A. hyperbius*). (**B**) Tip-recording
591 traces from the same sensilla of *V. indica* and *A. hyperbius*. Labels of each trace
592 represent acetic acid concentrations in the mixtures. Vertical scale bar = 1 mV;
593 horizontal scale bar = 100 ms.

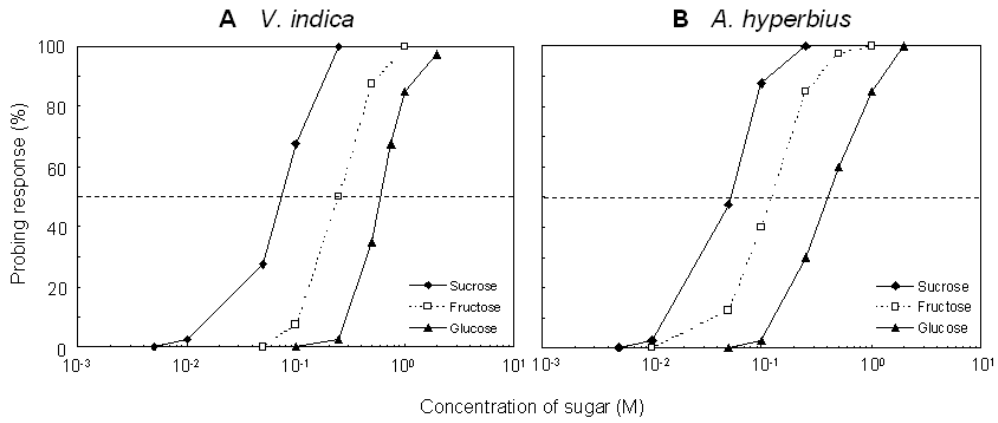
594 Fig. 1


A1
V. indica
B1
A. hyperbius
100µm
100µm
A2
B2
A3
B3
5KU
10µm
X800
JEOL
5KU
39mm

595

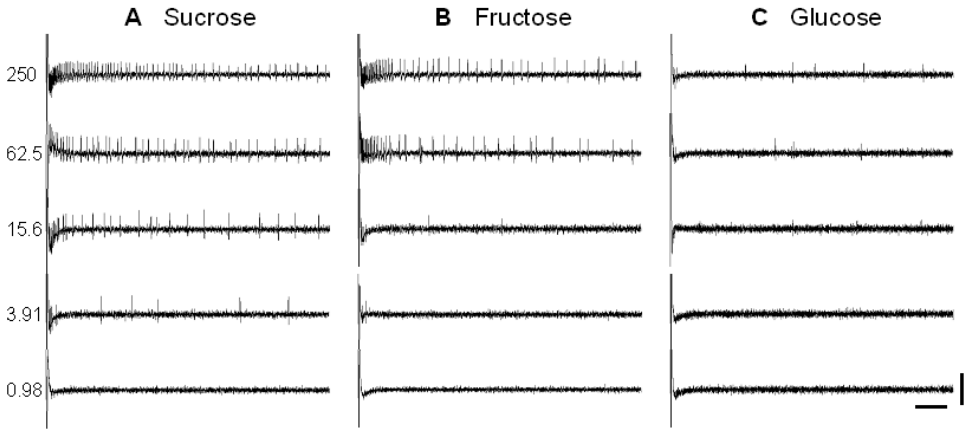
596

597 Fig. 2



598

599 Fig. 3



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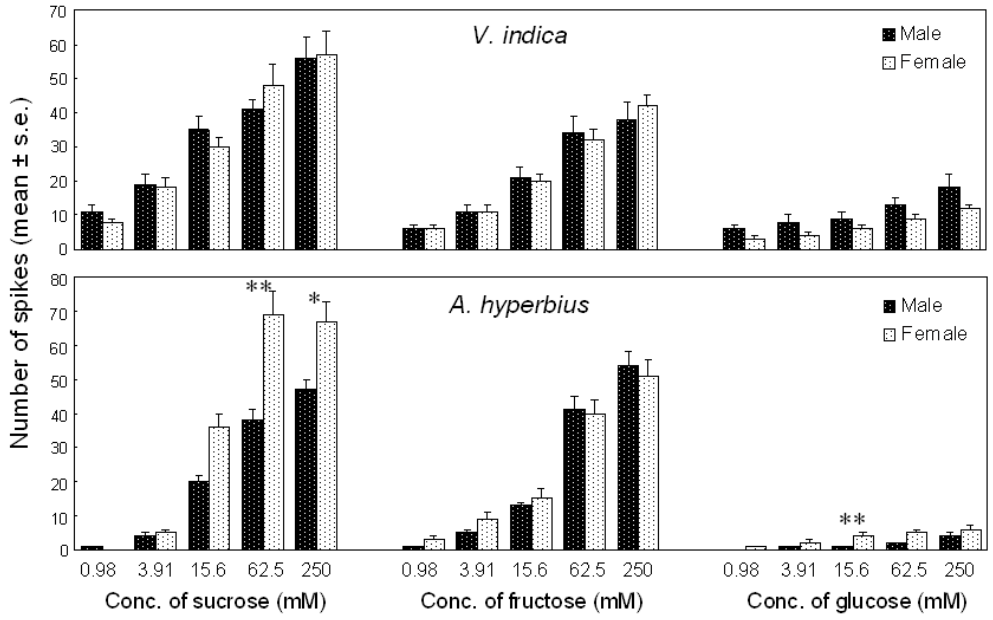
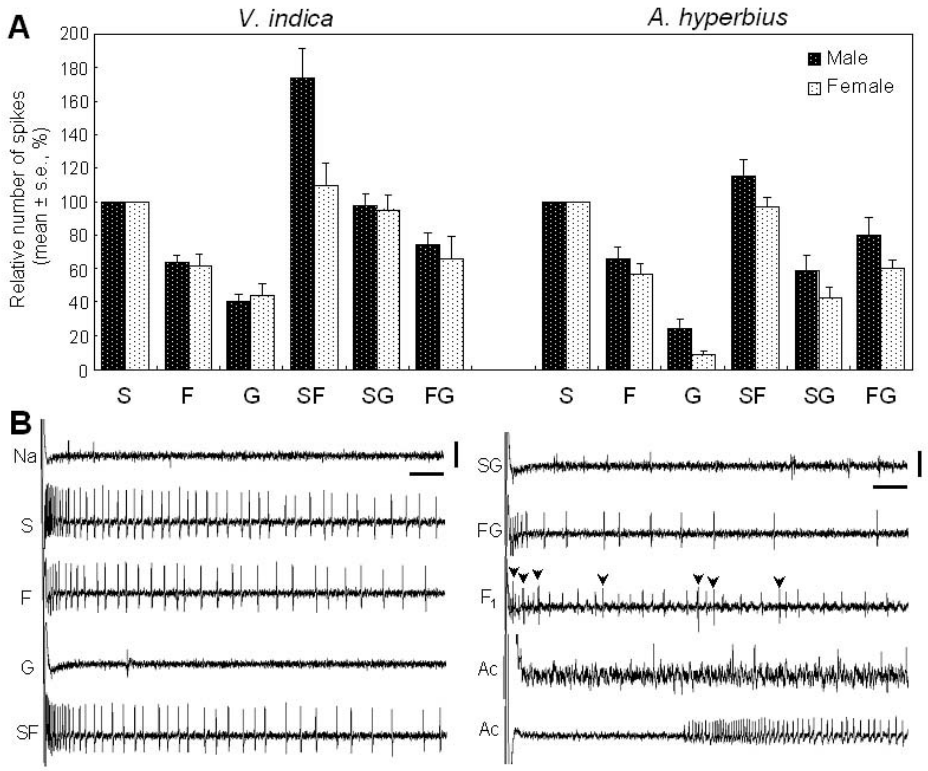
601 Fig. 4


V. indica
A. hyperbius
Male
Female
Number of spikes (mean ± s.e.)
**
*
**
0.98
3.91
15.6
62.5
250
Conc. of sucrose (mM)
Conc. of fructose (mM)
Conc. of glucose (mM)

602

603 Fig. 5



604

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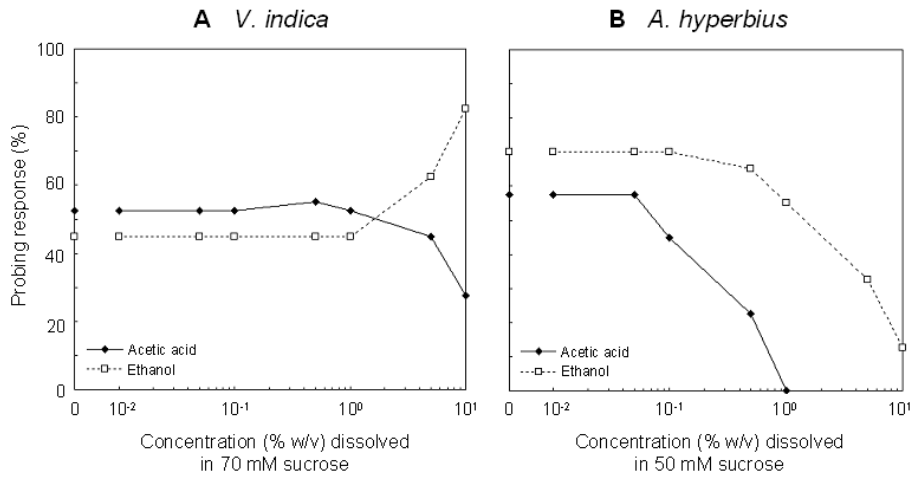
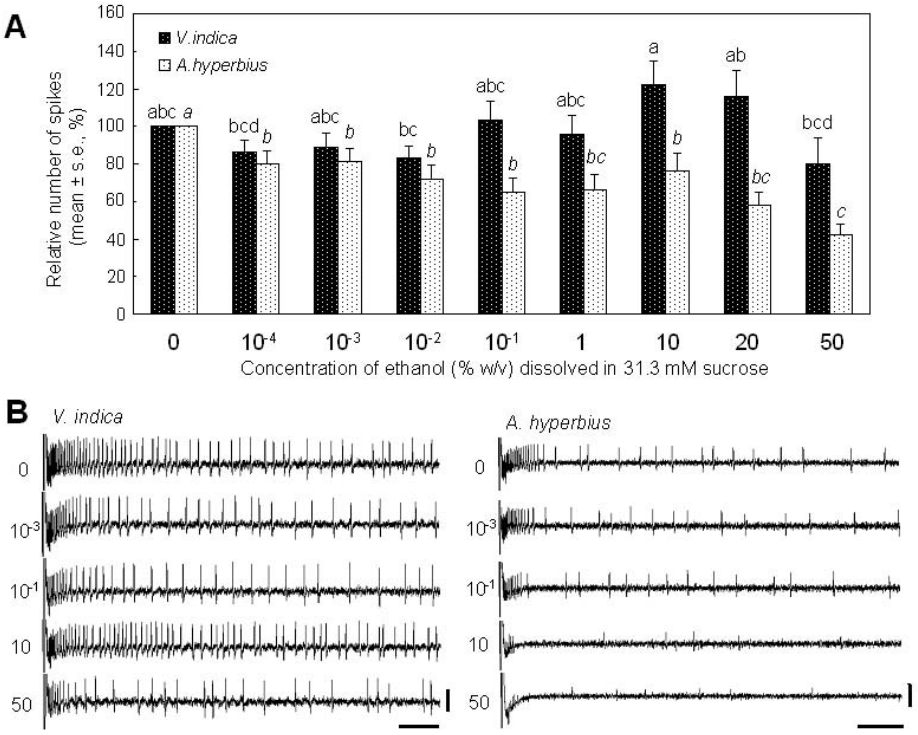
606 Fig. 6


A V. indica
B A. hyperbius
Probing response (%)
100
80
60
40
20
0
Acetic acid
Ethanol
0
10-2
10-1
100
101
Concentration (% w/v) dissolved in 70 mM sucrose
Concentration (% w/v) dissolved in 50 mM sucrose

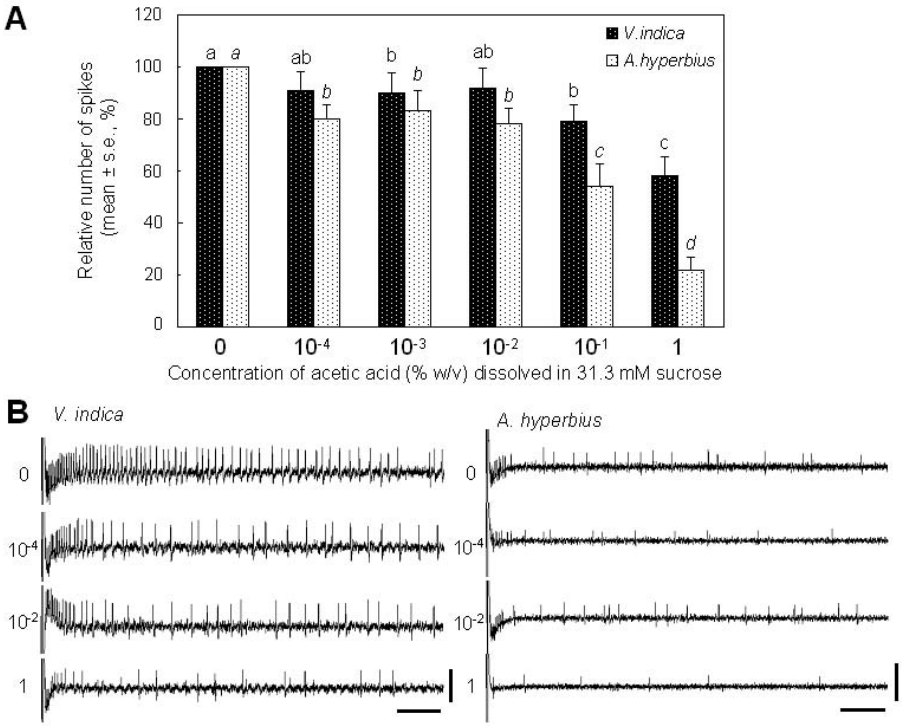
607

608 Fig. 7


A
V.indica
A.hyperbius
Relative number of spikes (mean ± s.e., %)
160
140
120
100
80
60
40
20
0
abc a
bcd b
abc b
bc b
abc b
abc bc
a b
ab bc
bcd c
0
10-4
10-3
10-2
10-1
1
10
20
50
Concentration of ethanol (% w/v) dissolved in 31.3 mM sucrose
B
V. indica
A. hyperbius
0
10-3
10-1
10
50

609

610 Fig. 8


A
Relative number of spikes (mean ± s.e., %)
V.indica
A.hyperbius
a a
ab b
b b
ab b
b c
c d
0 10-4 10-3 10-2 10-1 1
Concentration of acetic acid (% w/v) dissolved in 31.3 mM sucrose
B
V. indica
A. hyperbius
0
10-4
10-2
1

611