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Flavonoids from cabbage are feeding stimulants for diamondback moth larvae additional to glucosinolates: Chemoreception and behaviour

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Abstract

In caterpillars two styloconic contact chemoreceptors on the maxillary galea are assumed to contain the main taste receptors involved in host plant selection. The diamondback moth, *Plutella xylostella* L. is a specialist feeder of plants in the Brassicaceae, a plant family characterized by the biosynthesis of glucosinolates. We used pea (*Pisum sativum* L., Leguminosae) as a neutral non-host for a dual-choice leaf disc assay to quantify feeding stimulation by glucosinolates and flavonoids. Increasing concentrations of sinigrin resulted in significant preferences for sinigrin-treated leaf discs, with a threshold between 1 and 3 μM. Millimolar concentrations of four of the five flavonol triglucosides likewise elicited a significant preference for flavonoid-treated leaf discs. A mixture of four flavonoids and sinigrin was significantly preferred over sinigrin-treated leaf discs alone. Vigorous unicellular electrophysiological responses of medial maxillary styloconic taste sensilla were observed in response to five glucosinolates (glucocapparin, sinigrin, glucobrassicin, glucoiberin, and gluconasturtiin). This medial taste neuron responded in a dose-dependent manner to a concentration series of sinigrin, with a threshold of response of ca. 1 μM. The lateral sensillum styloconicum contained a neuron sensitive to sucrose, glucose, and fructose. However, no responses in the two types of maxillary styloconic sensilla to the phagostimulatory flavonoids could be detected, suggesting that other taste organs mediate chemoreception of flavonoids. We conclude that diamondback moth larvae employ a combination of biosynthetically distinct categories of feeding stimulants which allows for a higher degree of discriminatory ability than when this would be based on glucosinolates alone.

Introduction

The diamondback moth *Plutella xylostella* L. (Lepidoptera: Yponomeutidae) is an oligophagous species that accepts over 40 plant species in the Brassicaceae (Thorsteinson, 1953; Talekar & Shelton, 1993). The chemical basis for the oligophagy of *P. xylostella* larvae has historically been one of the first investigated. Analogous to the study by Verschaffelt (1910) on *Pieris* species, Thorsteinson (1953) tested the response of larval *P. xylostella* to three glucosinolates (sinigrin, sinalbin, and glucocheirolin), secondary plant metabolites characteristic for the Brassicaceae plant family. Glucosinolates strongly stimulated feeding by *P. xylostella* fourth instar larvae on artificial diets and 18 completely unacceptable non-host plants could be made acceptable by coating leaf material with these compounds (Thorsteinson, 1953; Gupta & Thorsteinson, 1960; Nayar & Thorsteinson, 1963). The application of such analogous reasoning implicitly assumes that when a compound acts as a token stimulus for an oligophagous herbivore species, it will act as a token stimulus for other species that are oligophagous on that plant family. However, such an approach-by-analogy allows no definite conclusion on the exclusive role of glucosinolates in

host-plant specialisation as no exhaustive bioassay-guided phytochemical analysis procedure has been employed. In investigations on the phytochemical basis of host-plant specificity of two flea beetle species, *Phyllotreta armoraciae* (Koch) and *Ph. nemorum L.* (Coleoptera: Chrysomelidae: Alticinae), a possible role of flavonoids as host-specific feeding stimulants in addition to glucosinolates has been suggested (Larsen et al., 1992; Nielsen, unpubl.). Here we report behavioural responses of *P. xylostella* caterpillars to sinigrin, five flavonoids, and their mixture. Contact-chemoreceptors innervating two maxillary styloconic pegs on the galea are known to act as the major chemosensory mediators of host plant discrimination behaviour of caterpillars (Schoonhoven, 1987; Schoonhoven & van Loon, 2002). Here we document an initial analysis of the electrophysiological responses of these taste receptors to both groups of secondary plant substances and sugars.

Materials and methods

Insects. *Plutella xylostella* were reared on greenhouse grown Brussels sprouts plants *B. oleracea* L. var. *gemmifera* cv Icarus (Sluis & Groot, Enkhuizen, The Netherlands) under a L15:D9 photoperiod, at 23–25 °C, and 60–70% r.h.

Chemicals. Sinigrin was purchased from Janssen Chemica (Beerse, Belgium), glucocapparin and glucoiberin from Carl Roth (Karlsruhe, Germany). Gluconasturtiin and glucobrassicin were kindly provided by Dr. J.A.A. Renwick (Boyce Thompson Institute for Plant Research, Ithaca, U.S.A.). Sucrose and Tween 80 were obtained from Merck, D-(+)-glucose from Sigma, D(-)-fructose from Fluka. Purity of all chemicals was >99%. Flavonol glucosides have been isolated from *B. oleracea* L. convar. capitata var. alba cv 'Zefa Wädenswiler' and purified and identified as described by Nielsen et al. (1993, 1998). Purity was >95% as determined by HPLC with UV-detection at 330 nm. The compounds studied and their abbreviations as used in the following were: kaempferol-3-O-β-D-sophoroside-7-O-β-D-glucoside (K3), acylated at C-2′ with caffeic acid (K3-caf) or sinapic acid (K3-sin), and quercetin-3-O-β-D-sophoroside-7-O-β-D-glucoside, acylated at C-2′ with caffeic acid (Q3-caf). The fifth compound tested was kaempferol-3-O-β-D-[β-D-glucopyranosyl(1→2) glucopyranoside]-7-O-β-D-[β-D-glucopyranosyl(1→4) glucopyranoside, acylated at C-2′ with ferulic acid (K4-fer).

Behavioural bioassays. A dual-choice leaf disc assay employing four week old, greenhouse grown pea plants (*Pisum sativum* L., cv. Kelvedon Wonder) was used to quantify the behavioural responses of *P. xylostella* larvae to glucosinolates and flavonoids. Five μl of the solvent (2% Tween 80 in distilled water; control discs) and sinigrin solutions (0.00002, 0.0002, 0.002, 0.02, 0.2, or 2 mM in 2% Tween 80 in distilled water; treated discs) were evenly distributed on the upper surface of freshly punched pea leaf discs (diameter 1.6 cm, fresh weight ca. 15 mg) by means of a paint brush. Flavonoids were presolubilized in a small amount of methanol and then diluted with 2% Tween-80 in water to a solution with a final methanol concentration of 2%. The solution painted on the control discs in the flavonoid bioassays likewise contained 2% methanol. Calculated concentrations of compounds encountered by the caterpillars were assumed to be three times diluted relative to that applied on the surface (5 μl added to 15 mg leaf fresh weight after water was allowed to evaporate) and the latter values have been graphed. In addition, a mixture of four flavonoids (K3, K3-sin, K3-caf, and K4-fer, total concentration 2.65 mM) and sinigrin (1 mM) was tested against sinigrin (1 mM) alone. Control (C) and treated (T) discs were placed in CTCT fashion around the circumference of 5 cm diameter Petri dishes lined with a circular filter paper disc (Schleicher & Schull) wetted with 100 μl tap water to maintain humidity in the dish. Three caterpillars with a body weight of 2.2 ± 0.2 mg (day 1 in the final stadium) were deprived of food for 6 h, and were subsequently placed in Petri dishes for each treatment for 12 h in an illuminated climate cabinet at 25 °C. At the termination of the test the remaining leaf disc areas were scanned with a Hewlett Packard 4300 series scanner and quantified with Scion Image software. Intact leaf discs kept under similar conditions during the bioassay were scanned at the start and the end of the bioassay to determine initial surface area and to account for possible shrinkage during the assay, which turned out to be negligible. A preference index was calculated as the consumed area of C discs divided by the sum of the consumed area of C and T discs. Wilcoxon's matched pair signed rank test was used for detecting differences between leaf areas consumed of control and treated discs.

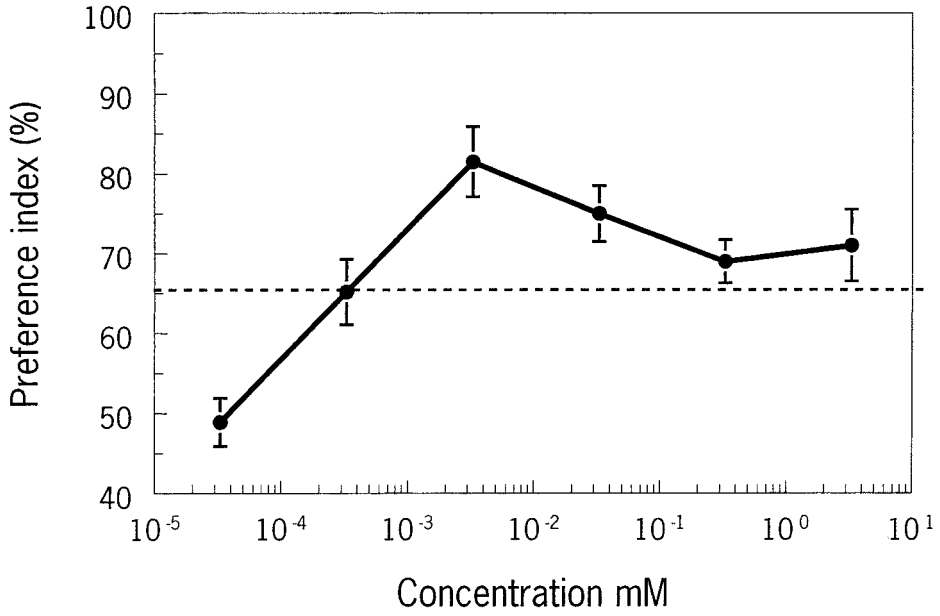

Figure 1. Preference index (mean ±SEM) as measured in a dual-choice leaf disc bioassay on fourth *instar P. xylostella* larvae as a function of sinigrin concentration applied on pea (*Pisum sativum* L.) leaf discs. Mean values of the preference index higher than the dashed line are significantly different from 50% ($P < 0.05$; Wilcoxon's matched pair signed rank test). Each point is based on 15 replicates.

Electrophysiology. A tip recording technique (van Loon, 1990) was used to record action potentials from the lateral and medial sensilla styloconica on the maxillary galea. Because pilot experiments showed that 5–10 mM KCl solution in distilled water, which we previously have used routinely as the solvent for contact-chemosensory studies (van Loon, 1990; van Loon & Schoonhoven, 1999), elicited considerable responses from the galeal styloconic taste sensilla, distilled water served as control stimulus for the glucosinolate and sugar solutions (van Drongelen, 1979) whereas 2% methanol in distilled water was the control stimulus for the flavonoid solutions. Chemicals were tested in random order and each stimulus has been tested on both lateral and medial sensilla. Distilled water and three to six stimuli were tested on one individual insect. Experiments were carried out with larvae which were 1–2 days into their final stadium and starved for 0.5–1 h. Electrophysiological responses were quantified using the number of spikes in the first second after the start of stimulation. Neural activity was sampled with an Intel Pentium based personal computer equipped with a Metrabyte DAS16 A/D conversion board. An interface was used (GO-box) for signal conditioning. This involved a second order band pass filter (−3 dB frequencies: 180 and 1700 Hz). Digitized traces were analyzed by means of Sapid Tools v.16 software (Smith et al., 1990). ANOVA and Duncan's multiple comparison test were used for detecting differences in electrophysiological response strength.

Results

Preference behaviour. Sinigrin had a significantly stimulatory effect on fourth instar *P. xylostella* caterpillars, showing dose-dependency (Figure 1). The preference index stabilised above 3 μM at values ranging from 69–77% (Figure 1).

Each of the four acylated flavonoids tested in pure form induced a significant preference in the pea leaf disc bioassay at 1 mM or higher concentrations whereas the unacylated K3 did not (Figure 2). The mixture of four flavonoids (K3, K3-sin, K3-caf, and K4-fer, total concentration 2.65 mM) and sinigrin (1 mM) elicited a significant preference for the mixture over sinigrin alone (preference index 68%; $P < 0.05$, Wilcoxon's matched pairs signed rank test; $n = 20$).

Electrophysiological responses of sensilla styloconica on the maxillary galea. Only the medial sensilla styloconica exhibited a vigorous unicellular response to all five glucosinolates tested (Table 1). Glucoiberin

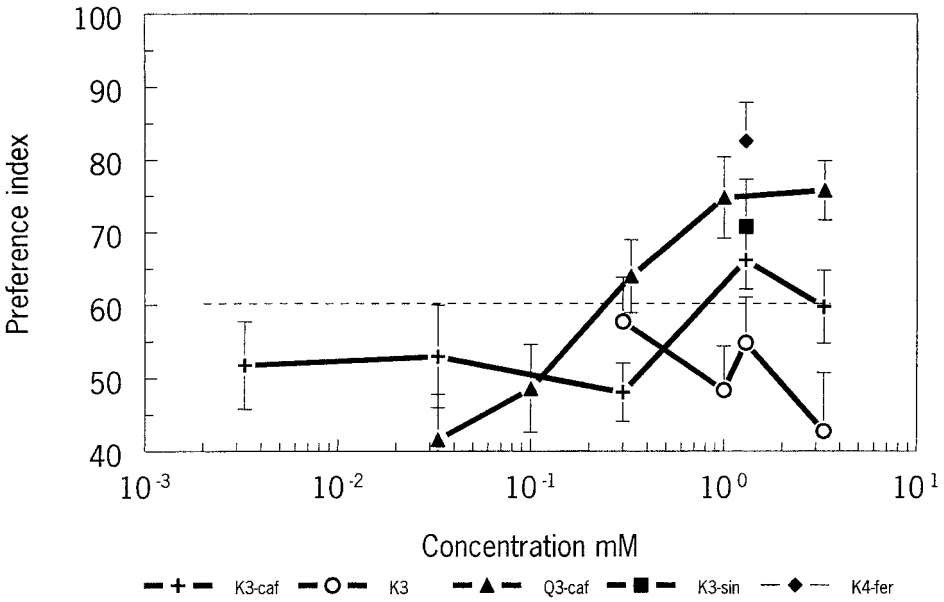

Figure 2. Preference index (mean ± SEM) as measured in a dual-choice leaf disc bioassay on fourth instar *P. xylostella* larvae as a function of concentrations of five flavonol glucosides applied on pea (*Pisum sativum* L.) leaf discs. Mean values of the preference index higher than the dashed line are significantly different from 50% ($P < 0.05$; Wilcoxon's matched pair signed rank test). Each point is based on 18–20 replicates, errors bar are SEM.

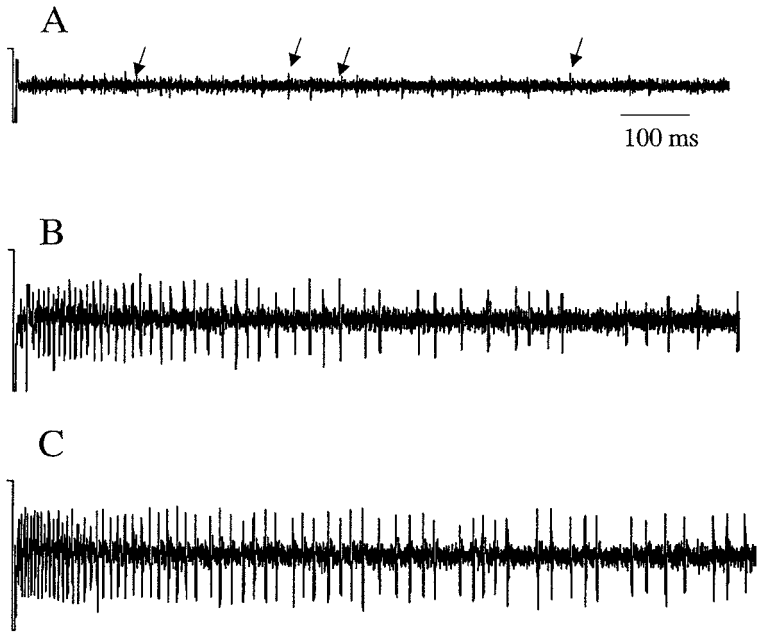

Figure 3. Electrophysiological recordings obtained by tip-recording from a neuron innervating the medial sensillum styloconicum on the maxillary galea of *P. xylostella* fourth instar larvae. in response to A: distilled water; B: 0.001 mM sinigrin; C: 1 mM sinigrin. Arrows in A indicate examples of spikes with a small amplitude and an average frequency below 10 spikes/s (see Tables 1 and 2).

Table 1. Electrophysiological responses of the two lateral and medial sensilla styloconica on the maxilla of *Plutella xylostella* final instar larvae to 0.3 mm or 2 mm of five glucosinolates

	Lateral sensilla		Medial sensilla	
Stimulus	N	Mean ± SE	N	Mean ±SE
Control (distilled water)	16	32.0 ±3.1 a	16	8.3±2.2 a
Glucobrassicin 0.3 mM	16	29.9 ±3.2 a	16	70.0±6.4 b
Glucoiberin 0.3 mM	16	32.2±2.5 a	16	89.6±5.3 c
Glucocapparin 0.3 mM	16	36.4±2.7 a	16	85.0±3.8 bc
Gluconasturtiin 0.3 mM	12	30.4±2.0 a	16	74.9±5.3 bc
Gluconasturtiin 2 mM	18	30.0± 5.8 a	10	70.6±6.3 bc
Sinigrin 0.3 mM	16	30.8±3.3 a	16	79.6±5.5 bc
Sinigrin 2 mM	18	45.4±3.7 b	16	86.3±4.6bc

Values are action potentials occurring in the first 1000 ms of response. N refers to the number of insects tested. Values having no letter in common differ significantly (One-way ANOVA followed by Duncan's multiple comparison test).

Table 2. Electrophysiological responses of the two sensilla styloconica on the maxillary galea of *Plutella xylostella* final instar larvae to three sugars at 10 mM

	Lateral sensilla		Medial sensilla	
	N	Mean ±SE	N	Mean ±SE
Control (distilled water)	16	18.9 ±3.8 a	16	2.1 ±1.1 a
Fructose	16	25.5±7.0 ab	16	5.2 ±1.7 a
Glucose	16	15.8 ±4.9 a	16	7.4 ±2.6 a
Sucrose	16	135.6 ±9.1 c	16	5.3 ±2.1 a

Values are action potentials occurring in the first 1000 ms of response. N refers to the number of insects tested. Values having no letter in common differ significantly (One-way ANOVA followed by Duncan's multiple comparison test).

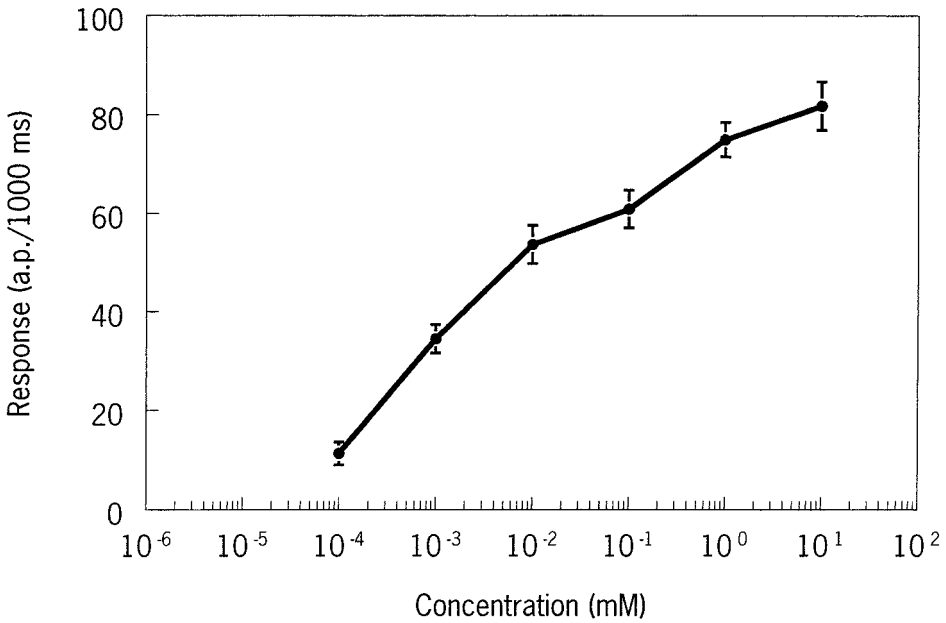

Figure 4. Dose-response relationship for the intensity of excitation (action potentials (a.p.) per 1000 ms; mean ± SEM) of a single neuron innervating the medial sensillum styloconicum on the maxillary galea of *P. xylostella* in response to concentrations of sinigrin.

was significantly more stimulatory than the other four compounds. The response in the medial sensillum to a concentration series of sinigrin was dose-dependent (Figures 3 and 4). In the lateral sensillum a response to sinigrin was found only at a 2 mM concentration, whereas gluconasturtiin did not elicit a response at this concentration (Table 1). A correlation emerges between the strength of behavioural preference and the intensity of electrophysiological responses to sinigrin (cf. Figures 1 and 4). In contrast, no significant electrophysiological response to any of the four behaviourally active flavonoid glucosides could be demonstrated at concentrations that induced preference behaviour. The lateral sensillum styloconicum contained a neuron that responded in a dose-dependent way to sucrose, the dominant foliar sugar, with a threshold of response between 0.01 and 0.1 mM (Figure 5). At 10 mM, a concentration at the low end of the range found for the most common leaf sugars (Schoonhoven et al., 1998), no significant responses to glucose or fructose were noted (Table 2).

Discussion

Behavioural responses to sinigrin. The behavioural responses to a concentration series of sinigrin reported here are similar to those reported by Thorsteinson (1953) but differ from those reported by Nayar & Thorsteinson (1963). In the first paper final instar diamondback moth caterpillars were given agar-cellulose based artificial diets supplemented with pea leaf powder in a no-choice assay, in the latter paper glucose was offered as the only feeding stimulant in the agar-cellulose substrate. Both methods yielded contradictory results as the latter resulted in a negative relationship between sinigrin dose and feeding intensity. Our feeding stimulant assays were of the dual-choice type, by which we established a threshold between 0.3 and 1 μM for preference behaviour, a concentration five times lower as that estimated by Thorsteinson (1953).

Behavioural responses to flavonoids and sinigrin-flavonoid mixtures. The kaempferol and quercetin tri- and tetraglucosides acylated with sinapic, caffeic, and ferulic aromatic acids induced significant preference behaviour in *P. xylostella* final instars in the absence of any glucosinolate. Interestingly, preference has also been found for the pea leaf discs treated with a mixture of flavonoids (K3, K3-sin, K3-caf, and K4-fer, total concentration 2.65 mM) and 1 mM sinigrin over the respective sinigrin concentration alone. Total glucosinolate concentrations in *Brassica* species on average amount up to 1–5 mM (Fenwick et al., 1983; Bodnaryk, 1994). These findings supplement our understanding of the oligophagy of diamondback moth larvae in an unexpected way. It demonstrates that it is not adequate to assign an exclusive role to glucosinolates as token stimuli for this species. A similar reasoning has been employed to explain the preference of the specialist *P. armoraciae* (Koch) for certain brassicaceous species (Larsen et al., 1982) but the relative contribution of glucosinolates and flavonoids was not tested. The flavonoids studied here as well as structurally similar compounds occur in different *Brassica* species (Nielsen et al., 1993). The strongest stimulant was K4-fer, which warrants extended research into flavonol tetraglucosides. These flavonoids have not been documented for plant species outside of the Brassicaceae thus far and can therefore be considered as novel candidate token stimuli for specialist herbivores of Brassicaceae. Total concentrations of flavonoid glucosides in *Brassica* species range from 0.3–9 mM (assuming a leaf dry matter content of 15%; Larsen et al., 1982).

Behaviour and electrophysiology. Behavioural and electrophysiological responses to sinigrin were found to correlate well, which corroborates the importance of the styloconic taste receptors in signalling acceptable food in a quantitative manner, a role which has been well established for several other caterpillar species (van Loon & Schoonhoven, 1999; Schoonhoven & van Loon, 2002). Similar to the specialist *P. brassicae* L. (Schoonhoven, 1967), diamondback moth larvae seem to possess a glucosinolate sensitive neuron in both the lateral and medial sensillum styloconicum, the lateral neuron being less sensitive. In *P. brassicae* one neuron responds to all of seven glucosinolates offered whereas the other neuron responds only to aromatic glucosinolates. The putatively analogous neuron in *P. xylostella* responds stronger to the aliphatic sinigrin and is insensitive to the aromatic gluconasturtiin. The initial analysis of the galeal taste receptors of *P. xylostella* reported here shows that the lateral sensillum is innervated by a sucrose sensitive neuron and a sinigrin sensitive neuron. In addition, in this sensillum an inositol sensitive neuron was found (J.J.A. van Loon, unpubl.). The medial sensillum is innervated by a glucosinolate sensitive neuron and a deterrent neuron (J.J.A. van Loon, unpubl. results). This gustatory complement fits the general pattern ob-

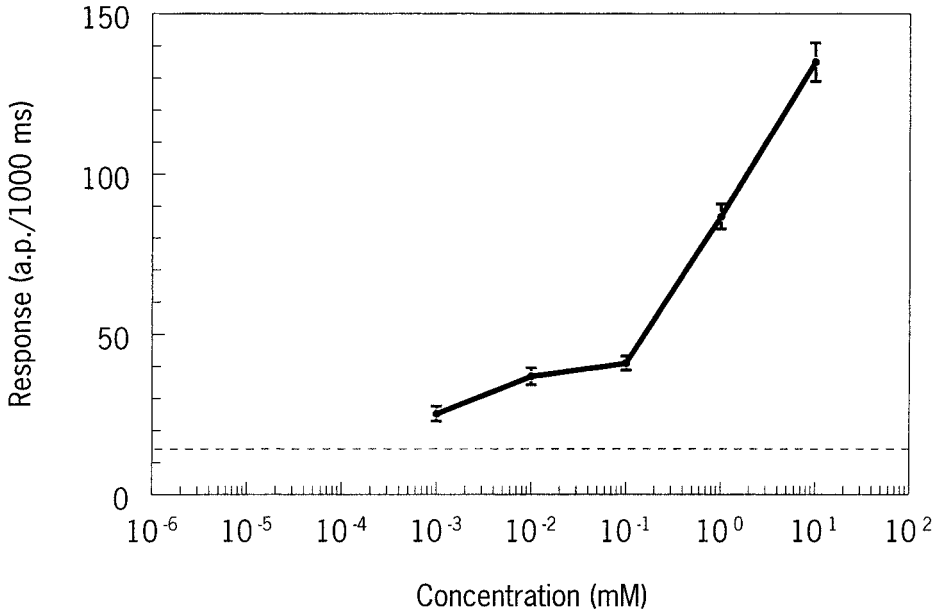

Figure 5. Dose-response relationship for the intensity of excitation (action potentials (a.p.) per 1000 ms; mean ±SEM) of a single neuron innervating the lateral sensillum styloconicum on the maxillary galea of *P. xylostella* in response to concentrations of sucrose in distilled water. The dashed line indicates the average action potential frequency recorded in response to distilled water alone.

served in lepidopterous larvae (Schoonhoven & van Loon, 2002). Additional electrophysiological studies are required to reveal both the details of specificity of these neuron types and the specificity spectrum of the remaining three galeal taste neurons. The threshold value for the sucrose sensitive neuron (between 0.01 and 01. mM) is located at the low end of the range reported for other caterpillar species and similar to those reported for other yponomeutid species (van Drongelen, 1979). The threshold for the medial glucosinolate sensitive neuron is comparable to that found for the glucosinolate neurons of *P. brassicae* final instar larvae (Schoonhoven, 1967).

Different sensory inputs. The behaviourally active flavonol glucosides did not excite taste neurons on the maxillary galea at concentrations which stimulate feeding. One explanation for this lack of correlation between behaviour and taste physiology might be that other gustatory neurons mediate perception of flavonol glucosides. In addition to the eight taste neurons innervating the styloconic sensilla on the maxillary galea, caterpillars possess taste neurons in basiconic sensilla located on the distal end of the maxillary palpus (eight of such sensilla are found in *Yponomeuta* species) or in epipharyngeal sensilla (van Drongelen, 1979). We observed one bilateral pair to be present on the interior surface of the labrum of *P. xylostella* using phase-contrast microscopy (J.J.A. van Loon, unpubl. results). Ablation experiments on caterpillars to reveal the role of the taste neurons present on the maxillary palp tip have yielded variable results, ranging from no demonstrable effect to a clear effect (de Boer, 1993; Schoonhoven & van Loon, 2002). Thus far, *P. xylostella* is the smallest caterpillar studied electrophysiologically and not particularly amenable to ablation experiments necessary to study the respective roles of the three major groups of gustatory sensilla in caterpillars (Schoonhoven & van Loon, 2002). Likewise, tip-recording of individual basiconic sensilla of the maxillary palp tip and the epipharyngeal sensillum (when this would have a gustatory function) is technically difficult. Nevertheless, the results reported here suggest that such studies are necessary to analyse the sensory basis of larval host-plant selection in this species.

This study shows that the approach-by-analogy originally taken in studying the phytochemical basis of oligophagy in *P. xylostella* larvae (Thorsteinson, 1953) has left the role of flavonol glucosides uncovered for 48 years. We conclude that diamondback moth larvae employ a combination of biosynthetically distinct categories of feeding stimulants which is likely to allow a higher degree of discriminatory ability between dif-

ferent brassicaceous species than when this would be based on glucosinolates alone.

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