Journal of Insect Physiology 55 (2009) 788–792

Contents lists available at ScienceDirect

Journal of Insect Physiology

journal homepage: www.elsevier.com/locate/jinsphys

Chemosensory basis of behavioural plasticity in response to deterrent plant chemicals in the larva of the Small Cabbage White butterfly *Pieris rapae*

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ARTICLE INFO

Article history:
Received 18 January 2009
Received in revised form 6 April 2009
Accepted 22 April 2009

Keywords:
Taste
Cross-habituation
Desensitisation
Deterrent neuron
Food experience
Cross-sensitivity
Gustation

ABSTRACT

Behavioural and electrophysiological responsiveness to three chemically different secondary plant substances was studied in larvae of *Pieris rapae* L. (Lepidoptera: Pieridae). Three groups of caterpillars were studied that during their larval development were exposed to different rearing diets: an artificial diet or one of two host-plants, cabbage, *Brassica oleracea*, or nasturtium, *Tropaeolum majus*. In dual-choice leaf disc assays, caterpillars reared on cabbage were strongly deterred by the phenolic chlorogenic acid, the flavonol glycoside naringin and the alkaloid strychnine. However, behavioural plasticity was found in caterpillars reared on nasturtium or artificial diet in that these did not discriminate against chlorogenic acid. Caterpillars reared on the artificial diet were also significantly less sensitive to naringin and strychnine in the behavioural assay. Electrophysiological studies of the maxillary sensilla styloconica revealed that the deterrent neuron in the medial sensillum, but not in the lateral sensillum, of cabbage-reared caterpillars was more sensitive than the same neuron type of caterpillars reared on nasturtium or artificial diet. We conclude that (1) the diet-induced behavioural habituation to deterrents can at least partly be explained by chemosensory desensitisation of a generalist type of maxillary deterrent neuron; (2) behavioural cross-habituation to the three structurally diverse deterrent compounds can be traced back to cross-sensitivity for these compounds in the same gustatory neuron.

1. Introduction

The contact-chemosensory or gustatory system of insects plays a crucial role in mediating responses to food. Food selection behaviour of herbivorous insects depends primarily on neural input from their taste system. In lepidopteran larvae, taste neurons are located primarily on the ventral side of the labrum, the maxillary palps and galea (Zacharuk and Shields, 1991; Schoonhoven and Van Loon, 2002). Among all the sensilla on the maxillae, neurons innervating the lateral and medial sensilla styloconica on the maxillary galea play a decisive role in food selection behaviour. Each sensillum styloconicum is innervated by five bipolar neurons, one of which has a mechanoreceptive function, the other four function as contact chemoreceptors. The dendrites of the four-chemoreceptor neurons extend into the lumen of the peg and terminate close to the pore at the tip (Dethier and Crnjar, 1982; Schoonhoven and Dethier, 1966). The specificity of taste neurons, observed as a response to a specific class of plant compounds, is genetically determined. Food selection behaviour can be modified by dietary experience (Bernays and Chapman, 1987; Bernays and Singer, 2005; Renwick and Huang, 1995; Renwick and Lopez, 1999). Several studies documented that a change in food selection behaviour is associated with changes in taste neuron sensitivity (Chapman et al., 2003; Del Campo et al., 2001; Miles et al., 2005). Enhanced sensory responsiveness of taste neurons has been reported when insects contacted compounds that serve as token stimuli, recognition cues that have a specific botanical occurrence (Del Campo and Miles, 2003; Del Campo et al., 2001; Miles et al., 2005; Renwick and Lopez, 1999). Reduced sensory responsiveness has also been described in insects that had been exposed to deterrent compounds (Glendinning et al., 2001; Glendinning et al., 2002; Glendinning et al., 1999; Glendinning and Hills, 1997; Simmonds and Blaney, 1983; Van Loon, 1990). Furthermore, dietary exposure of larvae to certain deterrents can profoundly affect taste sensitivity to other, chemically unrelated deterrents. This phenomenon, observed at the behavioural level, has been termed "cross-habituation" (Huang and Renwick, 1997; Renwick and Huang, 1995). The physiological mechanisms underlying diet-induced behavioural plasticity in larval Lepidoptera are poorly understood (Bernays and Weiss, 1996).

Here we report behavioural and electrophysiological responses of caterpillars of the specialist plant-feeding insect *Pieris rapae*

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0022-1910/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jinsphys.2009.04.011

(Lepidoptera: Pieridae) which experienced one of three different diets from the neonate to the fifth instar. We focussed on the electrophysiological sensitivity of maxillary gustatory neurons (1) to assess whether gustatory desensitisation occurred in habituated insects; (2) whether cross-habituation to different deterrent compounds could be due to these compounds exciting the same gustatory neuron.

2. Materials and methods

2.1. Plants and insects

Cabbage (*Brassica oleracea* L. var. *gemmifera* cv. Cyrus) plants or nasturtium (*Tropaeolum majus* L. cv. Glorious Gleam) plants were grown in an air-conditioned greenhouse under long day conditions at 18–22 °C. Laboratory colonies of *P. rapae* L. were reared on cabbage plants in an air-conditioned room at 25 ± 2 °C and 60–70% relative humidity, L:D = 16:8. Larvae for behavioural bioassays testing chlorogenic acid were reared from neonate to fifth instar on cabbage, nasturtium (Renwick and Huang, 1995) or on a wheatgerm-based artificial diet (Webb and Shelton, 1988). Larvae for behavioural bioassays testing naringin and strychnine were reared from neonate to the fifth instar on cabbage or wheatgerm-based artificial diet. Larvae for electrophysiological tests were reared from neonate to fifth instar on cabbage, nasturtium or wheatgerm-based artificial diet. Larvae for behavioural assays and electrophysiological tests had finished ecdysis 24–48 h before testing and had been starved for 2 h (Van Loon and Schoonhoven, 1999).

2.2. Chemicals

Chlorogenic acid was obtained from Fluka, naringin was obtained from Janssen Chimica and strychnine–HCl was obtained from Sigma Chemical Co. Purity of the chemicals was >97%. Due to the limited solubility in water, naringin and chlorogenic acid were first dissolved in methanol and subsequently step-wise diluted in 2% Tween-80 in water for behavioural bioassays or in 1 mmol l^{-1} KCl for electrophysiological tests. The methanol concentration in test solutions was 2%. Strychnine was diluted directly in Tween-80 (for behavioural bioassays) or 1 mmol l^{-1} KCl (for electrophysiological tests). The respective solvents served as controls in the behavioural and electrophysiological tests.

2.3. Behavioural bioassays

Dual-choice leaf disc tests were performed. Five μl of solution of the test compounds were evenly applied on both sides of a cabbage leaf disc (78.5 mm^2). Taking the leaf disc fresh weight into account, the final tested concentration of chlorogenic acid was 1 mmol l^{-1}, of naringin 2.5 mmol l^{-1} and of strychnine 0.2 mmol l^{-1}. Control discs were treated with the solvent (2% MeOH + 2% Tween-80) alone for chlorogenic acid and naringin assays. Control discs were treated with the solvent (2% Tween-80) alone for strychnine assays. After application, leaf discs were left to dry for about 20 min at room temperature. Larvae were placed individually in Petri-dishes lined with a moist filter paper. The experiments were carried out in a climatic chamber at a temperature of 25 °C. Two treated and two control discs were alternately arranged along the circumference of the Petri-dishes. When *ca.* 50% of the control discs had been consumed, the leaf disc remains were digitally scanned using a Hewlett-Packard flatbed scanner (Hewlett Packard Company, Palo Alto, CA, USA). Disc surface area was measured using Scion Image for Windows 4.03 (freeware, Scion Co. http://www.scioncorp.com/). The areas consumed were calculated by subtracting the remaining areas of leaf discs from the average area of three-reference discs which served as shrinkage controls (Messchendorp et al., 1996). A feeding deterrent index (FDI) = $100(C - T)/(C + T)$ was calculated to quantify strength of deterrence, where C represents the leaf disc area consumed from control discs; T represents the leaf disc area consumed from treated discs.

2.4. Electrophysiology

The tip recording technique (Hodgson et al., 1955) was used to record responses to the different stimuli from the sensilla styloconica on the maxillary galea. Excised caterpillar heads were mounted on a silver wire electrode which was connected to the input of a specially designed pre-amplifier (Syntech Taste Probe DTP-1, Hilversum, The Netherlands). Stimulus solutions were filled into glass micropipettes with a tip diameter of *ca.* 30 μm. Amplified signals were digitized by an *A*/*D*-interface (Syntech IDAC-4, Hilversum, The Netherlands) and sampled into an Intel Pentium-based personal computer. Electrophysiological responses were quantified by counting the number of spikes in the first 1000 ms after the start of stimulation. Spikes were classified based on the characteristic large amplitude recorded from deterrent neurons in this species and its tonic temporal firing pattern and counted visually by the experimenter with the aid of Autospike v. 3.7 software (Syntech, Hilversum, The Netherlands).

2.5. Statistical analysis

As the plant-reared caterpillars and artificial diet-reared caterpillars in behavioural tests consumed either treated leaf discs or control leaf discs, paired *t*-tests were performed to analyze all the behavioural data. In electrophysiological tests, plant-reared caterpillars and artificial diet-reared caterpillars are independent samples, thus a two-sample *t*-test was performed to compare electrophysiological responsiveness of insects reared on different diets. All statistical analyses were conducted using SPSS 13.0.

3. Results

3.1. Behavioural assays

Cabbage-reared *P. rapae* was strongly deterred by chlorogenic acid (FDI = 84.9; $P < 0.01$; Fig. 1A). However, *P. rapae* reared on nasturtium or artificial diet did not discriminate between leaf discs treated with chlorogenic acid and related controls (FDI = 12.6 and 10.9, respectively, $P > 0.05$; Fig. 1A). Similarly, cabbage-reared caterpillars were highly sensitive to naringin (FDI = 79.4; $P < 0.01$; Fig. 1B) and strychnine (FDI = 82.8; $P < 0.001$; Fig. 1C). In contrast, for caterpillars reared on the artificial diet, the leaf surface area consumed did not differ significantly between treated and control discs for either naringin (FDI = 2.5; $P > 0.05$; Fig. 1B) or strychnine (FDI = −10; $P > 0.05$; Fig. 1C).

3.2. Electrophysiological responses

Chlorogenic acid and naringin elicited strong responses of similar intensity from the lateral sensilla styloconica of the three food experience-groups (Figs. 2 and 3). The alkaloid strychnine did not elicit any response from the lateral sensilla styloconica of the cabbage-reared and artificial diet-reared caterpillars (Figs. 2 and 3). However, the deterrent neuron in the medial styloconica sensilla of caterpillars reared on cabbage is significantly more sensitive to chlorogenic acid than in caterpillars reared on nasturtium or artificial diet at 1 mmol l^{-1} and 5 mmol l^{-1} ($P < 0.01$ and $P < 0.05$, respectively; Fig. 3). Similarly, *P. rapae* reared on artificial diet produced significantly weaker responses to 1 mmol l^{-1} and 5 mmol l^{-1} naringin than caterpillars reared on

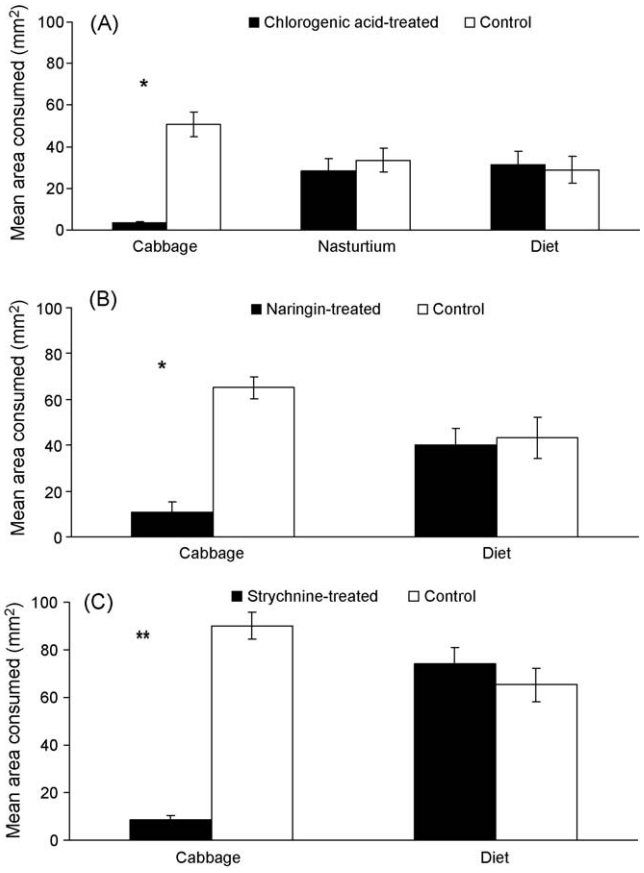

Fig. 1. Feeding preference behaviour of fifth instar *P. rapae* larvae, reared on either cabbage, nasturtium or artificial diet, indicated along horizontal axis, on cabbage leaf discs in choice assays between control and three compounds. (A) chlorogenic acid; (B) naringin; (C) strychnine. Replicated 20 times for (A), (B) and (C). Vertical lines represent standard errors. Asterisks indicate significant differences between treated and control discs according to the paired-samples *t*-test ($^{*}P < 0.01$; $^{**}P < 0.001$).

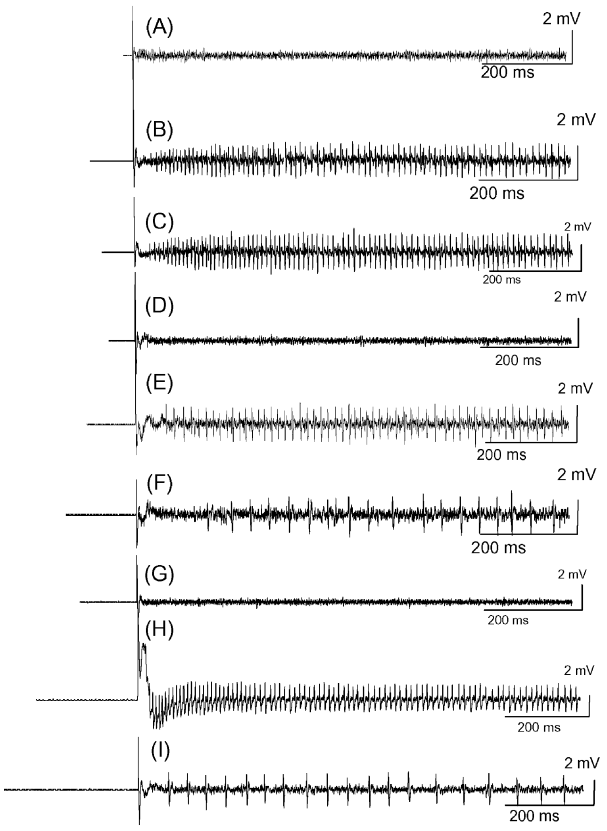

Fig. 2. Exemplary recordings of electrophysiological activity in maxillary taste sensilla of cabbage- or artificial diet-experienced *P. rapae* caterpillars in response to (A) solvent (KCl 1 mmol l^{-1} + MeOH 2%) in the lateral sensillum styloconicum (LSS). (B) Naringin 1 mmol l^{-1} in the LSS of cabbage-reared caterpillars. (C) Naringin 1 mmol l^{-1} in the LSS of artificial diet-reared caterpillars. (D) Solvent (KCl 1 mmol l^{-1} + MeOH 2%) in the medial sensillum styloconicum (MSS). (E) Naringin 1 mmol l^{-1} in the MSS of cabbage-reared caterpillars. (F) Naringin 1 mmol l^{-1} in the MSS of artificial diet-reared caterpillars. (G) Solvent (KCl 1 mmol l^{-1}) in the MSS. (H) Strychnine 1 mmol l^{-1} in the MSS of cabbage-reared caterpillars. (I) Strychnine 1 mmol l^{-1} in the MSS of artificial diet-reared caterpillars. The onset of the stimulations occurred at the beginning of each trace.

cabbage in medial styloconica sensilla ($P < 0.01$; Fig. 3). *P. rapae* reared on artificial diet displayed significantly reduced responses to 0.01–1 mmol l^{-1}, strychnine in medial styloconic sensilla compared with caterpillars reared on cabbage ($P < 0.05$, $P < 0.01$, $P < 0.01$, respectively; Fig. 3);

4. Discussion

A range of factors have been documented to influence food selection behaviour of plant-feeding insects: age (Blaney and Simmonds, 1987), satiety level (Schoonhoven, 1987), dietary self-selection (Abisgold and Simpson, 1988; Cohen et al., 1987; Waldbauer and Friedman, 1991), and dietary experience (Bernays and Chapman, 1987; Bernays and Singer, 2005; Renwick and Huang, 1995; Renwick and Lopez, 1999). Previous studies showed that cabbage-reared *P. rapae* caterpillars refused to feed on nasturtium and this rejection behaviour was ascribed to the presence of the deterrent phenolic chlorogenic acid. However, *P. rapae* caterpillars reared on nasturtium from neonate onward were desensitised to chlorogenic acid (Renwick and Huang, 1995). When *P. rapae* was reared on artificial diet, they also were insensitive to several deterrents belonging to a different class of secondary plant metabolites than chlorogenic acid, the steroid glycosides cymarin, erysimoside (cardenolides) and 2-*O*-/3-D-glucosyl cucurbitacin E (Huang and Renwick, 1995). Our results on a different strain of *P. rapae* confirmed these findings for chlorogenic acid. We expanded on these findings investigating compounds belonging to structurally different classes than Renwick & Huang (1995), and also found "cross-habituation", defined as the phenomenon that dietary exposure of larvae to certain deterrents can profoundly affect behavioural responses to other, chemically unrelated deterrents.

We showed that *P. rapae* caterpillars reared on artificial diet did not discriminate between leaf discs treated with chlorogenic acid or solvent whereas cabbage-reared caterpillars avoided chlorogenic acid-treated leaf material (Fig. 1). The compounds responsible for this behavioural difference have been identified in the wheat germ-fraction of the artificial diet as apigenin (4′,5,7-trihydroxyflavanone)-based flavonoids (Huang and Renwick, 1997; Renwick and Huang, 1995). Furthermore, artificial diet-reared *P. rapae* caterpillars also exhibited habituation to the flavonoid compound naringin and the alkaloid compound strychnine (Fig. 1). Naringin (4′,5,7-trihydroxyflavone) was chosen as it is a flavonoid glycoside structurally similar to the apigenin-based flavonoids in the wheat germ fraction of the artificial diet. Here we show that the desensitisation by dietary flavonoid compounds affect the response of only the medial flavonoid-sensitive deterrent taste neuron. This finding suggests that the expression of gustatory receptor (GR)-proteins tuned to flavonoids were down-regulated in the medial neuron. This generalist neuron responds to a wide range of chemically diverse secondary plant substances including alkaloids (Ma, 1972; Schoonhoven and Blom, 1988; Van Loon,

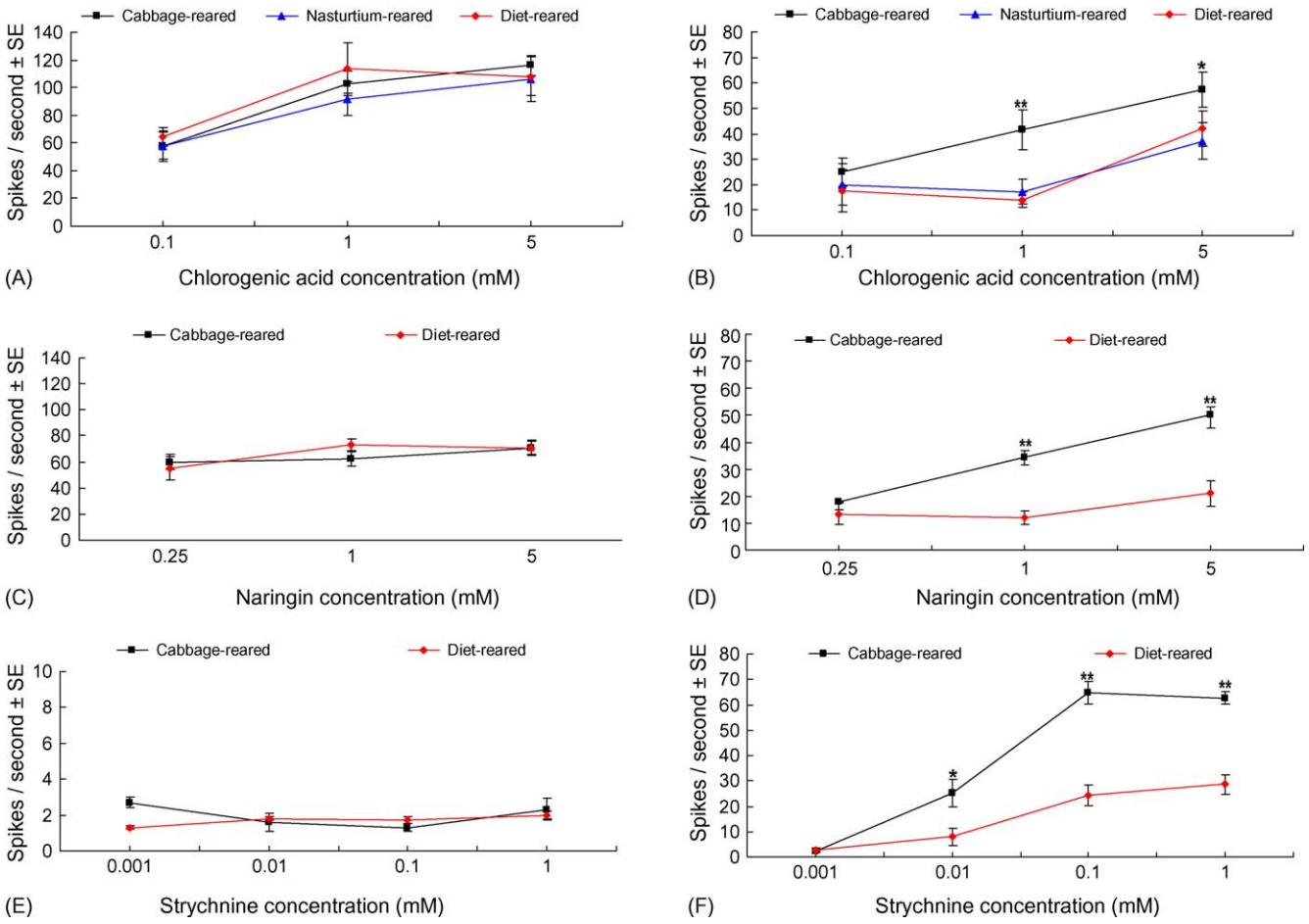

Fig. 3. (A) Dose–response curves for chlorogenic acid; spike frequencies (spikes/s; mean ± SE, $n = 12$) of the deterrent neuron in the lateral styloconic sensillum (LSS) of *P. rapae* caterpillars reared on cabbage leaves, nasturtium leaves or an artificial diet during development to the fifth instar. (B) Dose–response curves for chlorogenic acid in the deterrent neuron of the medial styloconic sensillum (MSS) of *P. rapae* caterpillars reared on cabbage leaves, nasturtium leaves or an artificial diet during development to the fifth instar ($n = 12$). C. Dose–response curves for naringin; spike frequencies (spikes/s; mean ± SE, $n = 12$) of the deterrent neuron in LSS of *P. rapae* caterpillars reared on cabbage leaves or an artificial diet during development to the fifth instar. (D) Dose–response curves for naringin, deterrent neuron in the MSS of *P. rapae* caterpillars reared on cabbage leaves or an artificial diet during development to the fifth instar ($n = 12$). (E) Dose–response curves for strychnine; spike frequencies (spikes/s; mean ± SE, $n = 12$) in LSS of *P. rapae* caterpillars reared on cabbage leaves or an artificial diet during development to the fifth instar. (F) Dose–response curves for strychnine, deterrent neuron in MSS of *P. rapae* caterpillars reared on cabbage leaves or an artificial diet during development to the fifth instar. Vertical lines represent standard errors. Asterisks indicate a significant difference in response frequency between cabbage-experienced caterpillars and nasturtium- or artificial diet-experienced caterpillars (two-sample *t*-test, $^{*}P < 0.05$; $^{**}P < 0.01$).

1990), which corresponds to the recent discovery that insect deterrent neurons co-express several GRs (Cobb et al., 2008). The alkaloid strychnine was chosen as it strongly excites the deterrent neuron in the medial sensillum styloconicum of the closely related *P. brassicae* L. Similar sensitivity to strychnine had previously been found for *P. rapae* (Van Loon, unpubl. results). Differences in ligand specificity and function of the medial and lateral deterrent neurons in response to phenolic, flavonoid, alkaloid and cardenolide deterrents have been demonstrated in the closely related *P. brassicae* (Van Loon and Schoonhoven, 1999). A new finding documented here is that, contrary to the medial deterrent neuron, the lateral deterrent neuron of *P. rapae*, which is also sensitive to chlorogenic acid and naringin did not display any sign of reduced sensitivity to these compounds as a result of dietary experience. This unexpected observation suggests a fundamental difference in the functioning of membrane receptors and/or signal transduction mechanisms between the medial and lateral deterrent neurons.

Chemosensory desensitisation has been reported for several species of lepidopterous larvae. When *Spodoptera littoralis* and *S. exempta* fed on an artificial diet to which azadirachtin was added, the caterpillars showed a lower sensitivity of their deterrent neurons than caterpillars reared on a plain artificial diet (Simmonds and Blaney, 1983). Caterpillars of *P. brassicae* were less sensitive to chlorogenic acid when reared on an artificial diet than cabbage-reared caterpillars (Van Loon, 1990). Tobacco hornworm (*Manduca sexta* L.) larvae fed an artificial diet with either salicin, caffeine or aristolochic acid exhibited desensitisation to salicin (Schoonhoven, 1969) and to caffeine but not to aristolochic acid (Glendinning et al., 1999, 2002; Glendinning and Hills, 1997). Interestingly, in *M. sexta*, desensitisation to the alkaloid caffein generalised to the phenolic acid glucoside salicin but not to the phenanthrene alkaloid aristolochic acid. This was shown to be based on the operation in the same neuron of two different signal transduction pathways for the three compounds (Glendinning and Hills, 1997; Glendinning et al., 1999, 2002). Our results demonstrate that dietary experience affects the chemosensory sensitivity to three chemically distinct classes of deterrents. This generalisation phenomenon suggests that a common step in the signal transduction pathway occurring after GR–ligand interaction is affected.

Desensitisation in theory offers a physiological explanation for habituation to deterrent compounds. Here three constraints to the input–output approach we applied should be noted. First, in the aforementioned studies as in this study, the link between the reduced electrophysiological activity in deterrent neurons and behavioural habituation is correlative. Methods to manipulate the

firing frequency of individual taste neurons, in insects by default contained in multiple innervated sensilla, in order to establish a causal relationship with changes in behaviour are currently not available in lepidopteran larvae. Second, our electrophysiological experiments were confined to the maxillary sensilla styloconica, whereas several caterpillar species harbour deterrent neurons also in epipharyngeal sensilla (Schoonhoven and Van Loon, 2002) and in *M. sexta* the maxillary palp tip was shown to have a function in the detection of deterrents (Glendinning et al., 1998). Third, exposure to specific chemicals for a period of time activates learning processes in the central nervous system (Bernays and Weiss, 1996; Glendinning et al., 1999). Diet-induced behavioural habituation is most likely based on the combined influence of physiological changes in central nervous pathways and peripheral chemosensory desensitisation focused on in this paper. The quantification of the relative contribution of these two mechanisms is complicated. The response of the medial deterrent neuron causes instantaneous inhibition of feeding in *P. brassicae* when strong ligands are offered on leaf discs of a favourite host plant (Luo et al., 1995; Messchendorp et al., 1996). Despite the chemical complexity of leaf discs as testing substrate and the complex chemosensory input generated by leaf saps, the response intensity of the medial deterrent neuron shows a straightforward linear relationship with inhibition of feeding behaviour (Van Loon et al., 2008).

Based on the combination of behavioural and electrophysiological evidence presented, we conclude that (1) the diet-induced behavioural habituation to deterrents can at least partly be explained by chemosensory desensitisation of a generalist type of maxillary deterrent neuron; (2) behavioural cross-habituation to the three structurally diverse deterrent compounds can be traced back to cross-sensitivity for these compounds in the same gustatory neuron. Future work should address to what extent this chemosensory plasticity is due to down-regulation of membrane receptor protein expression and/or modification of signal transduction pathways.

Acknowledgements

This work was funded by grant 05PhD04 of the Royal Dutch Academy of Arts and Sciences (KNAW) to D.Z. and the National Basic Research Program of China (Grant No.2006CB102006). We thank Yu Tong Qiu and Hans M. Smid for technical advice and Leo Koopman, André Gidding and Frans van Aggelen for insect rearing.

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