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Selective adaptation to noxious foods by a herbivorous insect

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Summary

When animals repeatedly sample a noxious food over a period of 1–4 days, they can markedly reduce their aversive behavioral response to the diet's unpleasant taste (e.g. 'bitterness') or toxic effects. This long-term adaptation process is selective, however, permitting insects to adapt physiologically to some but not all noxious foods. We hypothesized (i) that the selective nature of this adaptation process stems from the fact that some unpalatable foods are toxic while others are harmless and (ii) that insects have more difficulty adapting to foods that are both unpalatable and toxic. Our model system consisted of *Manduca sexta* caterpillars and two compounds that taste bitter to humans and elicit an aversive behavioral response in this insect (salicin and aristolochic acid). We found that 2 days of exposure to a salicin diet completely adapted the aversive response of the caterpillars to salicin, but that exposure to an aristolochic acid diet failed to adapt the aversive response to aristolochic acid. We determined that *M. sexta* could not adapt to the aristolochic acid diet because it lacked mechanisms for reducing the compound's toxicity. In contrast, the salicin diet did not produce any apparent toxic effects, and the caterpillars adapted to its aversive taste within 12 h of exposure. We also found that the salicin adaptation phenomenon (i) was mediated by the central gustatory system, (ii) generalized to salicin concentrations that were twice those in the adapting diet and (iii) offset spontaneously when the caterpillar was transferred to a salicin-free diet. We propose that toxicity is a more significant barrier to dietary adaptation than 'bitterness' in this insect.

Key words: bitter taste, toxicity, physiological adaptation, feeding, behaviour, taste cell, *Manduca sexta*.

Introduction

Many plants produce noxious compounds that inhibit feeding by herbivorous insects. Some of these noxious compounds are both unpalatable and toxic, while others are merely unpalatable or toxic (Bernays and Chapman, 1987; Chapman and Sword, 1994; Glendinning, 1996; Harley and Thorsteinson, 1967). Because many plants concentrate noxious compounds in their most nutritious tissues (McKey, 1979), herbivores would benefit from an ability to adapt to foods containing these compounds. Indeed, there are many reports of herbivorous insects adapting to foods containing noxious plant compounds following 1–4 days of chronic exposure (e.g. Blaney and Simmonds, 1987; Blaney et al., 1986; Glendinning and Gonzalez, 1995; Jermy et al., 1982; Raffa and Frazier, 1988; Schoonhoven, 1969; Snyder and Glendinning, 1996; Usher et al., 1988; Glendinning et al., 2001). There are also reports, however, of herbivorous insects failing to adapt to foods containing noxious plant compounds (Yamamoto and Fraenkel, 1960; Chapman and Sword, 1994; Jermy et al., 1987). In some cases, the failure to adapt was so absolute that the insects starved to death rather than eat the noxious food (e.g. Peterson et al., 1993).

Insects have both gustatory and post-ingestive mechanisms for adapting to noxious compounds in foods. Repeated sampling of noxious foods can (i) desensitize the peripheral or central gustatory mechanisms that mediate the aversive response (e.g. Simmonds and Blaney, 1983; Glendinning et al., 1999a; Glendinning et al., 2001), (ii) activate post-ingestive adaptation mechanisms that reduce an insect's toxic sensitivity to the noxious compound (e.g. induction of P_{450} detoxification enzymes in the midgut; Snyder and Glendinning, 1996) or (iii) engage both gustatory and post-ingestive adaptation mechanisms (Szentesi and Bernays, 1984). Given all these adaptation mechanisms, why do insects nevertheless fail to adapt to some noxious plant tissues? We hypothesized that insects have more difficulty adapting to compounds that are both unpalatable (e.g. bitter) and toxic than to compounds that are merely unpalatable. To test this hypothesis, we developed several ways to dissociate the contribution of gustatory *versus* toxicity effects to the physiological adaptation process.

Our model system consisted of the tobacco hornworm caterpillar (*Manduca sexta*; Sphingidae) and two plant compounds, salicin (a phenolic glycoside) and aristolochic acid (an aromatic nitro derivative). Both these compounds taste bitter to humans and inhibit feeding in *M. sexta* through a gustatory mechanism during 2 min feeding tests (Glendinning et al., 1999b). Salicin and aristolochic acid may also inhibit

feeding through a toxicity mechanism during longer-term feeding tests (Lijide et al., 1993; Lindroth, 1989), but this possibility has not been examined in *M. sexta*. There is one report of *M. sexta* partially adapting its aversive behavioral response to salicin after 72 h of dietary exposure to a salicin diet (Schoonhoven, 1969). This behavioral adaptation to salicin was associated with a small but significant desensitization of the salicin-responsive taste cell in the lateral styloconic sensilla, leading Schoonhoven (Schoonhoven, 1969) to conclude that the change in peripheral responsiveness mediated the adapted behavioral response. We have been unable, however, to produce the same change in peripheral responsiveness in *M. sexta* from our colony, despite using similar methodologies (Glendinning et al., 1999a).

We addressed three issues in this study. First, we asked whether *M. sexta* can adapt behaviorally to diets containing an aversive concentration of salicin or aristolochic acid. Second, we determined whether the caterpillar's ability (or inability) to adapt to the salicin or aristolochic acid diet is influenced by toxic effects of either compound. Finally, we explored three aspects of the salicin adaptation process: (i) whether it is mediated by a peripheral gustatory mechanism, (ii) whether it generalizes to all suprathreshold concentrations of salicin and (iii) whether adapted caterpillars spontaneously recover their aversive response to salicin after being transferred to a salicin-free diet.

Materials and methods

Caterpillar rearing

In this and all subsequent experiments, we reared *Manduca sexta* caterpillars from eggs on a wheatgerm-based artificial diet (Bell and Joachim, 1976). We maintained the caterpillars in an environmental chamber on a 16 h:8 h light:dark cycle (27 °C). We began all experiments with caterpillars in the first day of their fifth stadium. All caterpillars were naive to salicin and aristolochic acid prior to testing. To control for any potential differences among caterpillars from different egg batches, we interspersed individuals (in a random manner) from each batch across experimental treatments. We provide the sample sizes for each experiment in the figure legends.

Can Manduca sexta *adapt its aversive behavioral response to salicin or aristolochic acid?*

In this experiment (experiment 1), we tested for self-adaptation to salicin and aristolochic acid. To this end, we used three groups of caterpillars. We exposed the first group to a salicin diet for diet 48 h and then measured their ingestive response to the control diet and then the same salicin diet (see below for a description of the diets). We exposed the second group to an aristolochic acid diet for 48 h and then measured their aversive response to the control diet and then to the same aristolochic acid diet. The third group served as a positive control: we exposed these caterpillars to the control diet for 48 h and then measured their ingestive responses to the control, salicin and aristolochic diets.

Because we wanted to determine whether either exposure diet would adapt the taste-mediated aversive response to salicin or aristolochic acid, we used a brief-access feeding assay that minimizes the potential contribution of post-ingestive factors to the ingestive response. Elsewhere, we have shown that the aversive response to salicin and aristolochic acid (during this 2 min test) is mediated exclusively by sensory input from three bilateral pairs of bitter-sensitive taste cell. These bilateral pairs of bitter-sensitive taste cell occur in the epipharyngeal, lateral styloconic and medial styloconic sensilla, respectively (Glendinning et al., 1999b).

Brief-access feeding test

The feeding test involved five steps. (i) We placed a caterpillar in the 'test arena', which consisted of a clean (inverted) Petri dish covered with a clear plastic cylinder (7.5 cm in diameter and 10 cm tall) and then deprived the caterpillar of food for 30 min to standardize its 'hunger' state. Because the inter-meal interval for *M. sexta* usually ranges between 15 and 30 min (Reynolds et al., 1986), it is unlikely that this food-deprivation period created an extreme state of hunger. (ii) Next, we removed the caterpillar from the test arena, weighed it (to the nearest 0.1 mg), placed a test disk (see below for details) in the arena, and then returned the caterpillar, positioning its head 1–1.5 cm from the disk. (iii) We considered feeding to have begun once the insect made at least two bites on the disk within a 2 s period; at this point, we recorded the timing of all subsequent bites over the next 2 min on a software-based event recorder. Because the test arena was positioned on a turntable-like device, we could rotate the caterpillar slowly and keep its mandibles clearly visible as it fed; the caterpillar's feeding was not disrupted by this rotation. (iv) Immediately after the 2 min feeding test, we reweighed the caterpillar and then offered it the control diet *ad libitum* for the next 30 min; this enabled the caterpillar to overcome any residual effects of the test (e.g. hunger) that may have developed during steps i–iii. (v) Next, we deprived the same caterpillar of food for 30 min, and then ran it through steps i–iv a second time, but with a different diet cake.

We repeated this five-step protocol until the same caterpillar had been tested with all test disks. To ensure that each caterpillar would eat under our testing conditions, we included in our analysis only those caterpillars (in this and all subsequent experiments) that ate 20 mg or more of the control diet; this amounted to more than 95 % of the caterpillars tested in each experiment.

Exposure and test disks

We used a 157 mmol kg^{-1} diet (wet mass) concentration of salicin and 0.38 mmol kg^{-1} diet of aristolochic acid in both the exposure and test diets. We selected these concentrations of each compound because they are the lowest that elicit a robust aversive response in *M. sexta* (Glendinning et al., 1999b). We used the rearing diet as the substrate for both the exposure and test diets. We established the indicated concentration by heating the agar-containing diet to approximately 60 °C,

adding the appropriate quantity of compound, stirring vigorously for 3 min, and then pouring the diet into Plexiglas molds. We prepared the control diet similarly, but neglected to add a 'bitter' compound. Each exposure diet block was 2 cm×3 cm×1.5 cm and could sustain a caterpillar for 24 h. Each test diet disk was 1.5 cm in diameter, 0.4 cm high and had well-defined corners that the caterpillars could grasp securely (with their feet) and bite.

During each exposure period, we placed the caterpillar in a sealed plastic deli-cup (160 ml volume with a vented lid), gave it a block of control, salicin or aristolochic acid diet, and then placed it in the environmental chamber. After 24 h, we gave each caterpillar a fresh block of diet. At the end of the 48 h exposure period, we subjected the caterpillar to the feeding test described above.

Data analysis

We calculated three ingestive parameters across the 2 min feeding test for each caterpillar: the total food ingested (the increase in caterpillar mass over the feeding test; this measure included the mass of any frass produced during the feeding test), the total number of bites taken and average bite size (total intake/total number of bites taken). We made pair-wise comparisons between the response to the control diet and that to each of the 'bitter' diets, separately for each ingestive parameter, using the Wilcoxon matched-pairs signed-rank test. We controlled for the use of multiple paired comparisons on a set of related variables (e.g. the response of the same caterpillars to the control, salicin and aristolochic acid diets) using the Bonferroni correction; this involved dividing the α-level (0.05) by the number of comparisons within each panel. We used non-parametric tests (in this and all subsequent experiments) because the data were not always normally distributed.

What are the patterns of consumption and growth on the salicin- and aristolochic-acid-treated diets?

In this experiment (experiment 2), we determined (i) the pattern of food intake by the caterpillars on the control, salicin (157 mmol kg^{-1}) or aristolochic acid (0.38 mmol kg^{-1}) diet over the 48 h exposure period and (ii) how this pattern of consumption affected growth.

We monitored food consumption indirectly by quantifying the amount of feces (i.e. frass) each caterpillar produced during successive 1 h time intervals. To this end, we used a custom-built automated device to collect all frass pellets produced during each successive 1 h interval. This device housed four caterpillars at a time, each in a separate circular container (7 cm in diameter, 4 cm tall) with a bottom consisting of a 0.6 cm×0.6 cm grid of nylon fishing line; this grid was small enough to prevent escape by the caterpillars but large enough to allow frass pellets to pass through. These containers were located on an elevated platform in a linear array. Approximately 10 cm below the platform, we positioned a polystyrene tray containing a 4×24 grid of wells (each of which was 1.8 cm in diameter and 1.5 cm deep). As each caterpillar defecated, the frass pellet fell through the bottom of the container and was guided by a funnel into the well located directly underneath. We physically coupled the polystyrene tray to a step motor, which caused the tray to slide one step to the left each hour over a period of 24 h. Because one step of the motor corresponded to the distance between each row of wells, we could, in effect, fractionate the total amount of frass produced across a 24 h period by a caterpillar into separate 1 h samples. We assumed that the number of frass pellets produced per hour was proportional to the amount of diet ingested.

At the beginning of an experimental run, we weighed each of the four caterpillars (to the nearest 0.1 mg), placed then in their respective containers, offered them a block of exposure diet (the control, salicin or aristolochic diet), and then turned on the step motor. Twenty-four hours later, we extracted the frass samples from each well and placed them in separate labeled tubes. Next, we reweighed each caterpillar, returned it to its container, gave it a fresh block of exposure diet, and then turned on the step motor. After another 24 h, we removed the caterpillar, weighed it for the third and final time, and then extracted the frass samples from each well and placed them in labeled tubes. To obtain accurate dry mass readings of each frass sample, we put them in a forced-draft oven (set at 60 °C) for 24 h before weighing them (to the nearest 0.1 mg).

Data analysis

To simplify the analysis of the food consumption data, we combined frass samples across four successive samples, yielding a response variable indicating frass production during each 4 h period. We compared the mass of the frass samples during the initial 4 h period between caterpillars on the control diet and those on the salicin or aristolochic acid diet using the Mann–Whitney *U*-test (α=0.05/2). To compare weight gain across the three diets, we determined the percentage mass increase for each caterpillar (relative to the beginning of the experiment), and then made paired comparisons between caterpillars on the control diet and those on the salicin or aristolochic acid diets, separately for masses taken at 24 and then 48 h, using the Mann–Whitney *U*-test (α=0.05/2).

Do post-ingestive effects of salicin or aristolochic acid inhibit consumption during the 48 h exposure period?

In the previous experiment, we could not determine the relative contribution of gustatory *versus* post-ingestive effects of the salicin and aristolochic acid diets on consumption and weight gain. For instance, the caterpillars could have ingested relatively limited quantities of the aristolochic acid diet over the 48 h exposure period because of a failure to adapt to its aversive taste and/or toxic post-ingestive effects. In this experiment (experiment 3), we examined the specific contribution of the post-ingestive effects of salicin and aristolochic acid on weight gain. To this end, we (i) ablated the three bilateral pairs of chemosensilla that underlie the taste-mediated aversive response to salicin and aristolochic acid (the lateral styloconic, medial styloconic and epipharyngeal

sensilla), (ii) exposed the ablated caterpillars to the salicin (157 mmol kg^{-1}) or aristolochic acid (0.38 mmol kg^{-1}) diets or the corresponding control diets (see below) for 48 h, and then (iii) determined their mass at 0, 24 and 48 h. We reasoned that any inhibition of growth in the ablated caterpillars on the salicin or aristolochic acid diets would have to be mediated by a post-ingestive toxicity mechanism.

We performed the ablations using established procedures (de Boer and Hanson, 1987) during the second day of the fourth instar. In brief, we secured each caterpillar's neck with a rubber gasket, inserted it backwards into a water-filled vial (to induce anesthesia), and then ablated the lateral and medial sensilla by snipping them near their base with microdissection scissors. Because epipharyngeal sensilla are extremely small and recessed on the inner side of the labrum, we could not remove them directly. As a result, we removed the entire labrum. Immediately following the ablations, we returned the caterpillar to its holding cage and offered it control diet *ad libitum*. We inspected all caterpillars once they had completed their molt to the fifth instar (usually 3–4 days after the operation). We rejected any caterpillars that seemed feeble, unusually small, had incomplete ablations and/or other signs of surgical complications; this amounted to approximately 6 % of the total.

For each experimental diet compound, we tested a nutrient-balanced control diet that lacked the noxious compound. For instance, the salicin diet contained 6 % salicin (fresh mass), so we made the salicin-control diet contain 6 % alphacel. Alphacel is a non-nutritive form of cellulose (ICN Biomedicals).

We compared percentage of initial mass after 24 and then 48 h between caterpillars reared on (i) the salicin *versus* the salicin control diet or (ii) the aristolochic acid *versus* the aristolochic acid control diet. We made these between-animal comparisons using the Mann–Whitney *U*-test (α=0.05).

How long does it take for the caterpillars to adapt their aversive response to salicin?

In this experiment (experiment 4), we sought to determine how many hours of exposure to the salicin diet were necessary to adapt the aversive response to salicin completely and whether the adaptation process altered both components of the ingestive process (i.e. bite size and biting rate) synchronously. To this end, we subjected each caterpillar to the 2 min feeding test (described above) with the salicin diet (157 mmol kg^{-1}) at the beginning of a trial. If the caterpillar exhibited an aversive response to the salicin diet (i.e. ate <20 mg), we kept it in the experiment; if it failed to exhibit an aversive response, it was removed from the experiment (only 9 % of the caterpillars were removed for this reason). We used this screening criterion to ensure that we included only those caterpillars that actually exhibited an aversive response to the salicin diet. After the feeding test, we offered the caterpillar one of two exposure diets (the salicin or control diet) for one of four exposure periods (6, 12, 24 or 48 h). After the exposure period, the caterpillar was run through the feeding test a second time with the 6 % salicin diet. We expected that the aversive response would persist over time in caterpillars exposed to the control diet but become progressively more adapted over time in caterpillars exposed to the salicin diet.

Data analysis

We determined three ingestive parameters from each 2 min feeding test: total intake, total number of bites and bite size. To determine whether either exposure diet adapted the aversive response, we made pair-wise comparisons between the ingestive response of individual caterpillars before and after each exposure period, separately for each ingestive parameter, exposure diet and exposure duration. We made these within-animal comparisons using the Wilcoxon matched-pairs signed-ranks test (α=0.05)

Does adaptation of the aversive response to salicin reduce behavioral responsiveness to all suprathreshold concentrations of salicin?

In this experiment (experiment 5), we sought to determine whether exposure to the salicin diet simply reduced the responsiveness of caterpillars to salicin (i.e. shifted the concentration/response curve to the right) or whether it eliminated responsiveness to all suprathreshold concentrations of salicin (i.e. made the concentration/response curve flat). To this end, we determined the ingestive response of individual caterpillars to a range of salicin concentrations both before and after exposure to the salicin diet. We used the exposure duration that produced maximal adaptation in the previous experiment (see Results section). Using the 2 min feeding test (described above), we tested caterpillars with five diets before (0, 39, 78, 157 and 236 mmol kg^{-1} salicin) and five diets after (0, 78, 157, 236 and 314 mmol kg^{-1} salicin) the exposure period. We used a higher range of salicin concentrations after the exposure period to increase our chances of detecting a rightward shift in the position of the concentration/response curve.

To control for potential order effects, we randomized the presentation sequence of the different salicin concentrations. To control for any observer bias, we kept the observer blind with respect to the salicin concentration within each diet disk. Finally, to ensure that each caterpillar would eat under our testing conditions, we included in our analysis only those caterpillars that ate 20 mg or more of the control diet; this amounted to more than 96 % of the caterpillars.

Data analysis

We analyzed the results from caterpillars before and after the exposure period separately. In each case, we asked whether the ingestive parameter (total intake, total number of bites and bite size) decreased significantly with increasing concentrations of salicin. To this end, we made pair-wise comparisons between the response to the control diet and that to each of the four salicin concentrations. We performed these within-animal comparisons using the Wilcoxon matched-pairs signed-rank test (α=0.05/4).

Is adaptation of the aversive response to salicin associated with a reduction in peripheral responsiveness to salicin?

In this experiment (experiment 6), we asked whether 24 or 48 h of exposure to the salicin diet reduced the responsiveness of the bitter-sensitive taste cells in the lateral styloconic and epipharyngeal sensilla to 50 mmol l^{-1} salicin, both before and after exposure to either the salicin (157 mmol kg^{-1}) or control diet. We have reported previously (i) that these two bilateral pairs of taste cells are the only ones in *M. sexta* that are sufficient and necessary for mediating the aversive behavioral response to salicin (Glendinning et al., 1999b) and (ii) that 48 h of exposure to the salicin diet does not appear to reduce the responsiveness of the bitter-sensitive taste cell in the lateral sensillum to salicin (Glendinning et al., 1999a).

Experimental protocol

On day 1 of the experiment, we recorded the neural response of a lateral styloconic and an epipharyngeal sensillum (selected randomly) to 50 mmol l^{-1} salicin (from the same caterpillar). After obtaining these recordings (without harming the caterpillar in any way), we removed the caterpillar from the recording apparatus and exposed it to the salicin or control diet for 24 or 48 h. After the exposure period, we re-recorded the response of the same taste sensilla to the 50 mmol l^{-1} salicin solution. We included results only from those caterpillars that yielded clear neural recordings from both sensilla (this amounted to more than 95 % of the total caterpillars tested).

Neural recording technique

We recorded neural responses of individual taste sensilla using a non-invasive extracellular tip-recording technique (Glendinning et al., 1998; Gothilf and Hanson, 1994). In brief, we placed a glass electrode (containing a specific taste stimulus dissolved in 0.1 mol l^{-1} KCl) over the tip of a lateral styloconic sensillum or directly on top of an epipharyngeal sensillum (after bending the labrum back 90 ° from its normal position), and then recorded excitatory responses of taste cells within the sensillum (see de Boer et al., 1977; Glendinning et al., 1999b). We were able to obtain neural responses from both taste sensilla, and then remove the caterpillar unharmed from the recording apparatus, within 20 min. The caterpillars invariably recovered from this procedure and began feeding normally within 45 min.

We recorded alternating current signals from taste sensilla with the Tasteprobe amplifier system (Syntech; Hilversum, The Netherlands; see Marion-Poll and van der Pers, 1996). We preamplified each recording 10×, ran it through a band-pass filter set at 100–1200 Hz, fed it into a computer through a 16-bit analog-to-digital converter board, and then analyzed it off-line with Autospike software (Syntech).

For each neural recording, we stimulated a sensillum for approximately 2000 ms and quantified the number of action potentials generated 0–1000 ms after contact. To minimize the effects of solvent evaporation at the tip of the recording/stimulating electrode, we drew fluid from the tip with a piece of filter paper immediately before each stimulation. We tested only one member of each bilateral pair of gustatory sensilla per caterpillar.

Each taste sensillum contains 3–4 taste cells, and each taste cell within a sensillum exhibits a typical spike amplitude and temporal pattern of firing (for details, see Glendinning et al., 1999b; Glendinning et al., 2000). We used the idiosyncratic response features of each taste cell as a basis for discriminating action potentials from the bitter-sensitive taste cell within each sensillum.

Data analysis

Because we recorded from the same taste cell before and after the exposure period, we used a within-animal data analysis procedure. Our first step was to determine how much the exposure period changed each taste cell's responsiveness. To this end, we divided the taste cell's firing rate (impulses s^{-1}) after the exposure period by that before the exposure period, yielding a response variable called 'percentage of initial response'. Using this response variable, we asked whether 24 or 48 h of exposure to the salicin or control diet altered the responsiveness of the bitter-sensitive taste cells within the lateral or epipharyngeal sensilla. We compared the median of each treatment group with the median expected value (i.e. 100 % of initial response) using a one-sample Wilcoxon matched-pairs signed-rank test (α=0.05). We interpreted any significant departure from the expected value as an effect of dietary treatment.

Does ingestion of a salicin-free diet cause the aversive behavioral response to recover spontaneously in adapted caterpillars?

In the final experiment (experiment 7), we asked whether the caterpillars recovered from the exposure-induced adaptation to salicin. Our general approach involved producing the adaptation phenomenon in a group of caterpillars and then determining whether their aversive response to salicin returns spontaneously after being placed on a salicin-free diet for 24 h.

At the beginning of a trial, we ran all caterpillars through the feeding test described above with the salicin diet, and put those caterpillars that exhibited an aversive response to this diet (i.e. ate less than 20 mg) on the salicin diet for 48 h (we discarded all caterpillars that ate 20 mg or more). At 48 h, we ran all caterpillars through the same feeding test. We discarded all caterpillars that had not adapted to the salicin diet (i.e. ate less than 20 mg), but kept those caterpillars with an adapted aversive response to salicin (i.e. ate 20 mg or more). Our question was whether these adapted caterpillars would recover their aversive response to the salicin diet after being exposed to a salicin-free (i.e. control) diet over a 24 h period (henceforth, the recovery period). To this end, we placed half the adapted caterpillars back on the control diet and the other half on the salicin diet (as a positive control) for 24 h. At the end of the recovery period (i.e. at 72 h), we ran all caterpillars through the third and final feeding test with the salicin diet.

Data analysis

We expected that the caterpillars exposed to the control diet would eat significantly less of the salicin diet at the end of the recovery period (at 72 h) than at the beginning of the recovery period (at 48 h). In contrast, we expected that the caterpillars exposed to the salicin diet would eat similar amounts of the salicin diet at the beginning and end of the recovery period. We made these within-animal comparisons using the Wilcoxon matched-pairs signed-rank test (α=0.05).

Results

Can Manduca sexta *adapt its aversive behavioral response to salicin or aristolochic acid?*

The caterpillars exposed to the control diet exhibited robust aversive responses to the aristolochic acid (0.38 mmol kg^{-1}) and salicin (157 mmol kg^{-1}) diets (Fig. 1A–C). All three ingestive parameters (total intake, total number of bites and bite size) on the aristolochic acid and salicin diets were significantly lower than those on the control diet ($P \leqslant 0.025$). Likewise, the caterpillars exposed to the aristolochic acid diet also exhibited a robust aversive response to the aristolochic acid diet (Fig. 1D–F). The caterpillars exposed to the salicin diet, however, failed to show any evidence of an aversive response to the salicin diet (Fig. 1G–I). These caterpillars not only ate the same quantity of control and salicin diet, but they also fed on both diets in a similar manner (i.e. the number of bites taken and median bite size were statistically indistinguishable). Thus, it appears that the caterpillars adapted to the salicin diet but not to the aristolochic acid diet.

What are the patterns of consumption and growth on the salicin- and aristolochic-acid-treated diets?

The caterpillars on the control and salicin diets produced frass at a similar rate over the entire 48 h exposure period, indicating that they ingested similar quantities of each diet (Fig. 2A). Even though there was a trend for caterpillars on the salicin diet to produce less frass than those on the control diet over most of the exposure period, the difference was relatively small. Median frass production by the two groups was statistically indistinguishable during the first 4 h of the exposure period (P>0.05). The fact that caterpillars consumed similar amounts of control and salicin diet explains why the rates of growth on both diets were so similar (Fig. 2B). We found that caterpillars on the salicin diet grew slightly, but significantly ($P \leqslant 0.05$), more slowly than those on the control diets between 0 and 24 h, but this difference disappeared after 48 h.

In contrast, the caterpillars on the aristolochic acid diet produced substantially less frass than those on the control diet over the 48 h period (Fig. 2A), indicating that they ate substantially less diet. They even produced significantly less frass during the first 4 h of the test than did caterpillars on the control diet ($P \leqslant 0.05$). As a consequence of this low

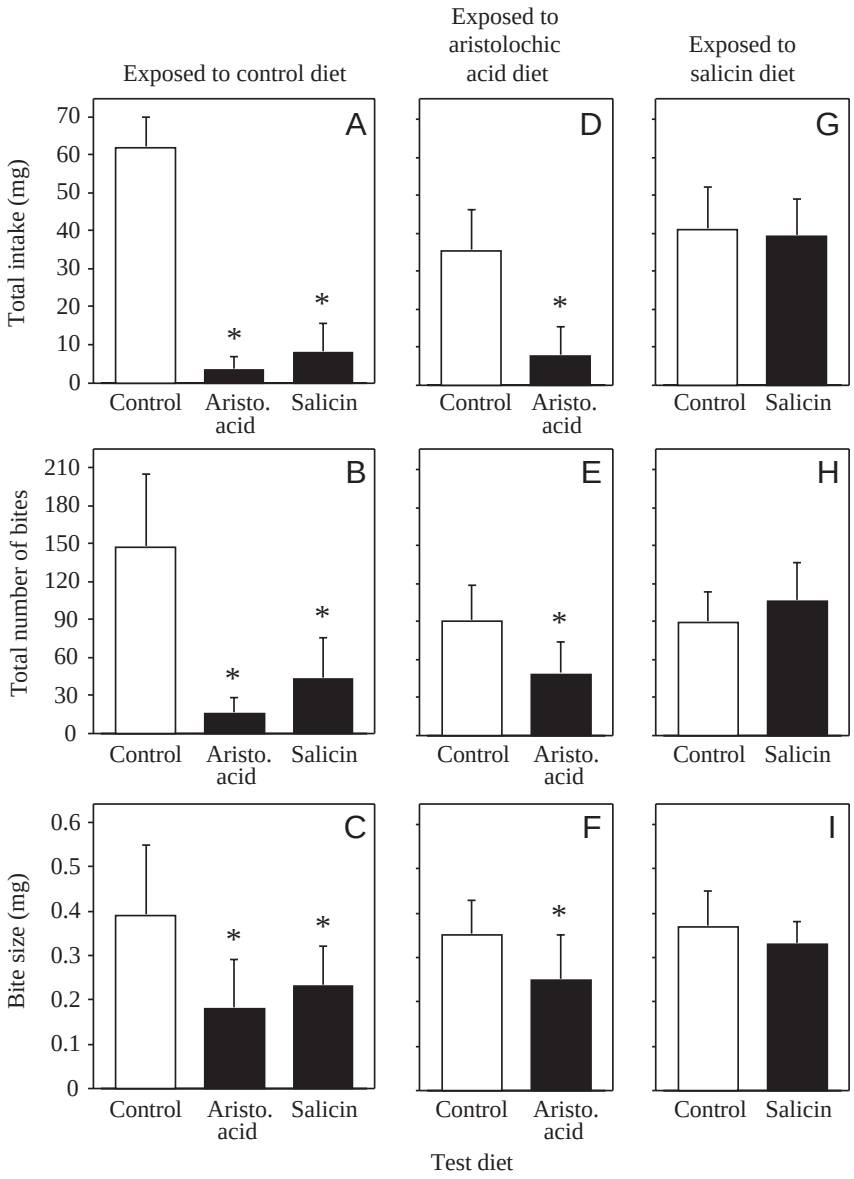

Fig. 1. Ingestive responses of caterpillars: (A–C) to the control, aristolochic acid (0.38 mmol kg^{-1}) and salicin (157 mmol kg^{-1}) diets following 48 h of exposure to the control diet (N=30 caterpillars), (D–F) to the control and aristolochic acid (Aristo. acid) diets following 48 h of exposure to the aristolochic acid diet (N=24) and (G–I) to the control and salicin diets following 48 h of exposure to the salicin diet (N=24). We present three measures of ingestive behavior across the 2 min brief-access biting test: total intake, total number of bites and bite size. We compare the median values in each panel (± median absolute deviation) using the Wilcoxon matched-pairs signed-rank test (*$P \leqslant 0.025$).

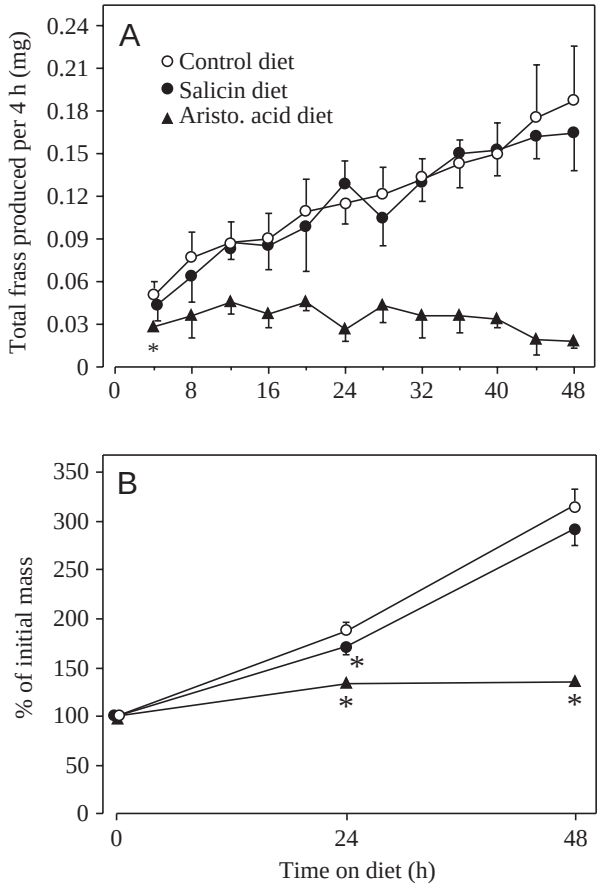

Fig. 2. Food consumption (A) and growth (B) in caterpillars maintained for 48 h on the control (N=18), aristolochic acid (Aristo. acid; N=8) or salicin (N=15) diet. The concentrations of aristolochic acid and salicin in the diets are as in Fig. 1. We monitored food consumption indirectly by measuring the total amount of frass (dry mass) produced during each successive 4 h time interval. We monitored growth directly by determining the percentage increase in mass that occurred over the two successive 24 h exposure periods. In A, we compare frass production during the initial 4 h between caterpillars maintained on the aristolochic acid *versus* control diets (or salicin *versus* control diets) using the Mann–Whitney *U*-test (*$P\leqslant0.05$). In B, we compare the percentage increase in mass between caterpillars maintained on the aristolochic acid *versus* control diets (or salicin *versus* control diets) after 24 and then 48 h of exposure using the Mann–Whitney *U*-test (*$P\leqslant0.025$). All values are medians ± median absolute deviation.

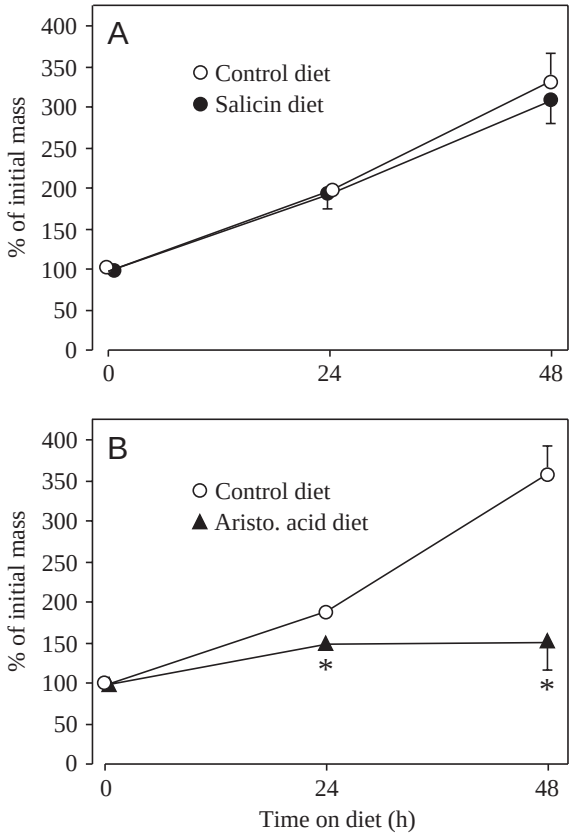

Fig. 3. Growth in caterpillars lacking their lateral, medial and epipharyngeal sensilla on (A) the salicin *versus* salicin control diets or (B) the aristolochic acid (Aristo. acid) *versus* aristolochic acid control diets. We monitored growth directly by determining the percentage increase in mass that occurred over two successive 24 h exposure periods. In each panel, we compare the percentage increase in mass between caterpillars maintained on either diet after 24 and then 48 h of exposure using the Mann–Whitney *U*-test (*$P\leqslant0.025$). All values are median ± median absolute deviation (N=10–11 per treatment group). The concentrations of aristolochic acid and salicin in the diets are as in Fig. 1.

consumption, the caterpillars on the aristolochic acid diet experienced only marginal growth over the 48 h exposure period; after 24 and 48 h, their growth was significantly less than that observed in caterpillars on the control diet ($P\leqslant0.025$).

Do post-ingestive effects of salicin or aristolochic acid inhibit consumption during the 48 h exposure period?

In this experiment, we used caterpillars lacking the three bilateral pairs of taste cells that mediate the pre-ingestive aversive response to salicin and aristolochic acid. We found that the ablated caterpillars exposed to the salicin and salicin control diets grew at a steady and statistically indistinguishable rate ($P>0.05$) after 24 and 48 h of exposure (Fig. 3A). In contrast, the ablated caterpillars on the aristolochic acid diet experienced only marginal growth (Fig. 3B). Their growth was significantly slower than that of caterpillars on the aristolochic acid control diet after 24 and 48 h of exposure ($P\leqslant0.025$). These results indicate that exposure to the aristolochic acid diet, but not to the salicin diet, inhibits growth through a post-ingestive toxicity mechanism.

How long does it take for the caterpillars to adapt their aversive response to salicin?

We found that the caterpillars exposed to the control diet for 6 h experienced a small, but significant ($P\leqslant0.0125$), attenuation of the aversive response to the salicin diet (Fig. 4A–C). That is, they ate significantly more of the salicin diet after the 6 h exposure period than they did before it. This

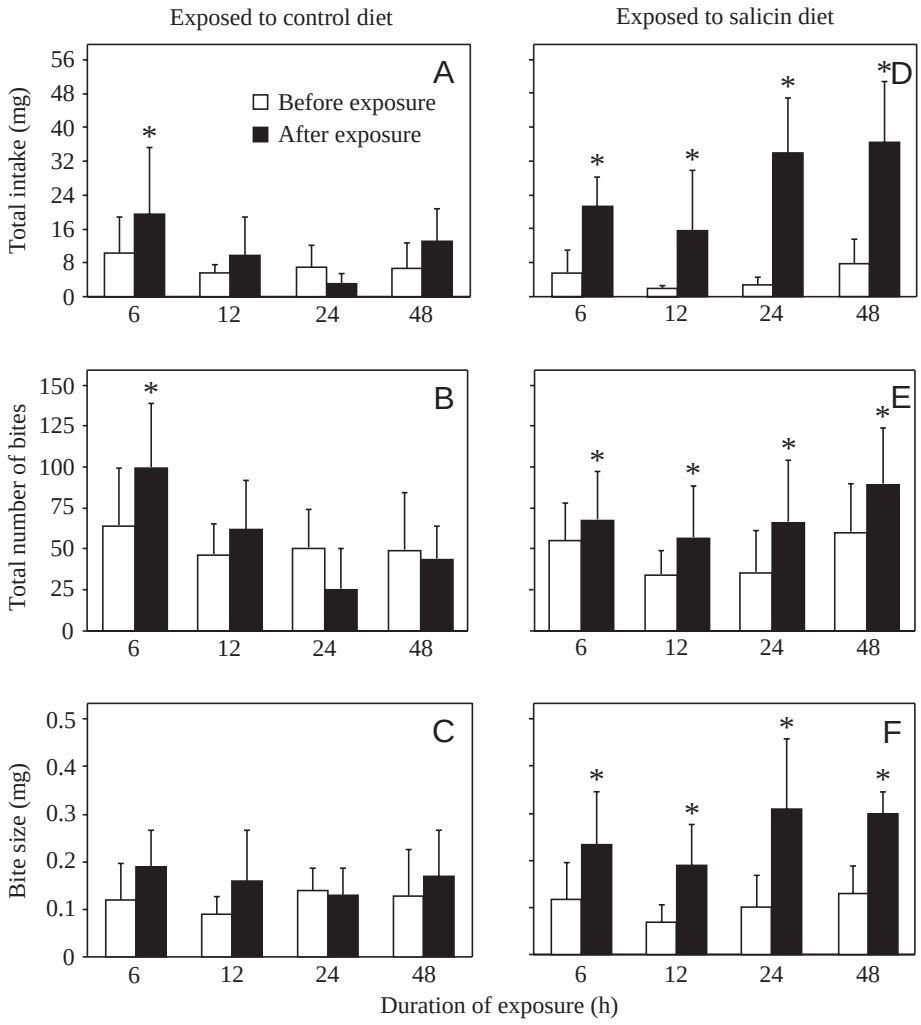

Fig. 4. Effects of different periods of exposure (6, 12, 24 or 48 h) to the control (A–C) or salicin (D–F) diet on the ingestive responses of caterpillars to the salicin diet. We used a 157 mmol kg^{-1} concentration of salicin in both the exposure diet and the diet used to evaluate ingestive responsiveness. We calculated three ingestive parameters across each 2 min biting test: total intake, total number of bites and bite size. We tested all caterpillars both before (open columns) and after (filled columns) exposure to the control or salicin diet. Each caterpillar was exposed either to the control or to the salicin diet over one of the four exposure periods. We made paired comparisons between the ingestive response of caterpillars to the salicin diet before and after an exposure period, separately for each ingestive measure and exposure diet, using the Wilcoxon matched-pairs signed-rank test (*$P\leqslant0.0125$). The number of caterpillars tested at each exposure period ranged between 19 and 23 for those exposed to the control diet and between 18 and 27 for those exposed to the salicin diet. All values are median ± median absolute deviation.

greater consumption stemmed from the caterpillars taking more bites, while leaving bite size unchanged. This attenuation proved to be transient, however, because it was not evident in caterpillars exposed to the control diet for 12, 24 or 48 h.

The results from caterpillars exposed to the salicin diet were quite different. Their aversive response to the salicin diet was significantly attenuated ($P\leqslant0.0125$) following 6, 12, 24 or 48 h of exposure to the salicin diet (Fig. 4D–F). The magnitude of the attenuation increased with the duration of the exposure period, reaching its maximum after 24 h of exposure. The reduction in salicin responsiveness involved a significant increase not only in total intake but also in the total number of bites and bite size. It should be noted that the magnitude of the increase in bite size was consistently greater than that of the increase in total number of bites.

Given that both the control and salicin-exposed caterpillars exhibited a diminished responsiveness to salicin after the 6 h exposure period, we cannot draw any inferences about whether adaptation to salicin occurred during this exposure period. However, following the 12, 24 and 48 h exposure periods, we observed a significant attenuation of the aversive response only in those caterpillars offered the salicin diet. Thus, it appears that the caterpillars adapted their aversive response to salicin within 12 h of chronic exposure to the salicin diet and that the magnitude of this adaptation increased with time, reaching its maximum after 24 h of exposure.

Does adaptation of the aversive response to salicin reduce behavioral responsiveness to all suprathreshold concentrations of salicin?

Before exposure to the salicin diet, the caterpillars ate significantly ($P\leqslant0.0125$) less of the diets containing salicin concentrations $\geqslant$78 mmol kg^{-1} than they did of the control diet (Fig. 5A–C); this reduction in intake stemmed from the caterpillars taking both smaller bites ($P\leqslant0.0125$, at concentrations $\geqslant$78 mmol kg^{-1} salicin) and fewer bites ($P\leqslant0.0125$, at concentrations $\geqslant$157 mmol kg^{-1} salicin). After exposure to the salicin diet, the concentration/response curve for all three ingestive parameters was effectively flat; that is, the caterpillars' ingestive response to all the salicin diets was statistically indistinguishable from that to the control diet, even at salicin concentrations as high as 314 mmol kg^{-1}. Thus, 48 h of exposure to the salicin diet did not simply shift the concentration/response curve for salicin to the right. Instead, it

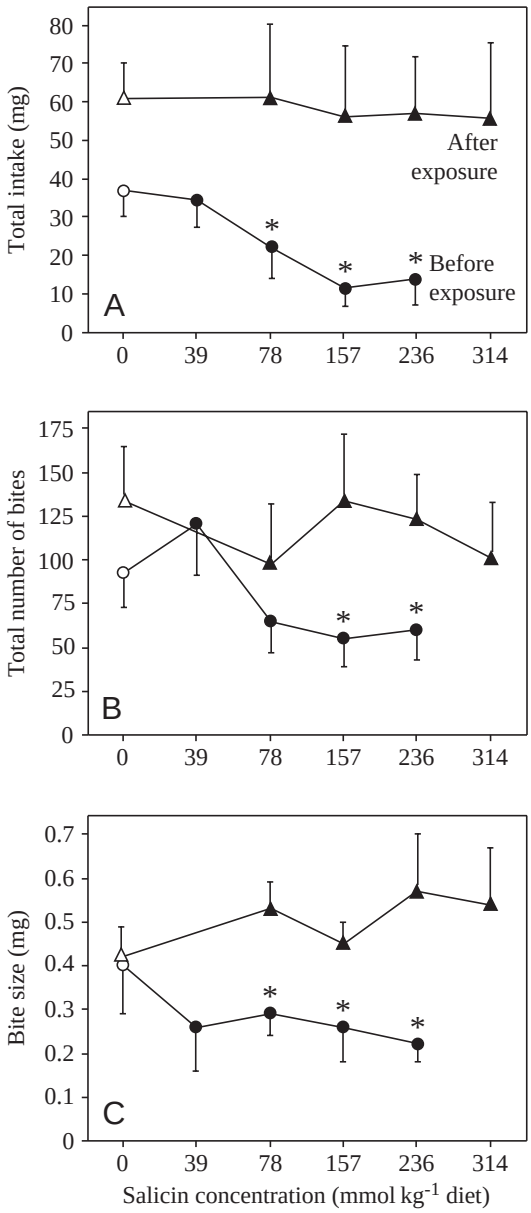

Fig. 5. Ingestive responses of individual caterpillars (N=10) to a range of salicin concentrations, both before and after exposure to the 157 mmol kg^{-1} salicin diet. We calculated three ingestive parameters across each 2 min biting test: total intake, total number of bites and bite size. Within each panel, we determine whether the ingestive responses of the caterpillars decreased significantly with increasing concentration of salicin, both before and then after the exposure period. To this end, we make paired comparisons between the response to the control diet (i.e. 0 mmol kg^{-1} salicin; open symbol) and that to each successively higher concentration of salicin (filled symbols) using the Wilcoxon matched-pairs signed-rank test (*$P\leqslant0.0125$). All values are median ± median absolute deviation.

appeared to eliminate behavioral responsiveness to salicin altogether.

Is adaptation of the aversive response to salicin associated with a reduction in peripheral responsiveness to salicin?

We did not observe any significant exposure-induced changes in responsiveness of the bitter-sensitive taste cells within the epipharyngeal sensilla to 50 mmol l^{-1} salicin, irrespective of whether the caterpillars were placed on the control or salicin diet for 24 or 48 h (Fig. 6A,B). Likewise, there was no significant exposure-induced change in responsiveness of the bitter-sensitive taste cells within the lateral sensilla to 50 mmol l^{-1} salicin in caterpillars following 24 or 48 h of exposure to the salicin diet. There was, however, a small but significant increase in responsiveness of the bitter-sensitive taste cell in the lateral sensillum to 50 mmol l^{-1} salicin following 24 and 48 h of exposure to the control diet (Fig. 6C,D). Representative neural responses are shown in Fig. 6E–H. We reported a similar finding previously and concluded that it reflected a normal developmental process (Glendinning et al., 1999a). Taken together, these findings indicate that the only effect of exposure to the salicin diet is an attenuation of the normal increase in responsiveness of the lateral bitter-sensitive taste cell to salicin. We consider it unlikely, however, that this small effect of salicin exposure could explain the large exposure-induced changes in behavioral responsiveness to salicin.

Does ingestion of a salicin-free diet cause the aversive behavioral response to recover spontaneously in adapted caterpillars?

When salicin-adapted caterpillars were offered the salicin diet over the recovery period (i.e. from 48 to 72 h), they maintained their apparent indifference to the salicin diet (Fig. 7A–C). That is, the three measures of ingestion (total intake, total number of bites and bite size) did not change significantly over the recovery period. In contrast, when salicin-adapted caterpillars were offered the control (i.e. salicin-free) diet over the recovery period, their aversive response to salicin showed significant spontaneous recovery (Fig. 7D–F). That is, two measures of ingestion (total intake and bite size) diminished significantly ($P\leqslant0.05$) over the course of the recovery period. These results reveal the transient nature of the exposure-induced adaptation phenomenon.

Discussion

We found that the caterpillars adapted their aversive response to the salicin diet within 12 h of exposure. This adaptation process enabled caterpillars on the salicin diet to consume large amounts of food over the exposure period and to gain weight at a rate that was statistically equivalent to that of caterpillars on the control diet. Two lines of evidence indicate that this adaptation process was determined principally by a reduction in taste responsiveness to salicin. First, we found no evidence that salicin inhibits feeding through a post-ingestive toxicity mechanism: both intact and ablated caterpillars grew at a similar rate on the salicin diet over the 48 h exposure period. Thus, the adaptation phenomenon could not have been mediated by an exposure-induced reduction in toxic sensitivity to salicin. Second, we

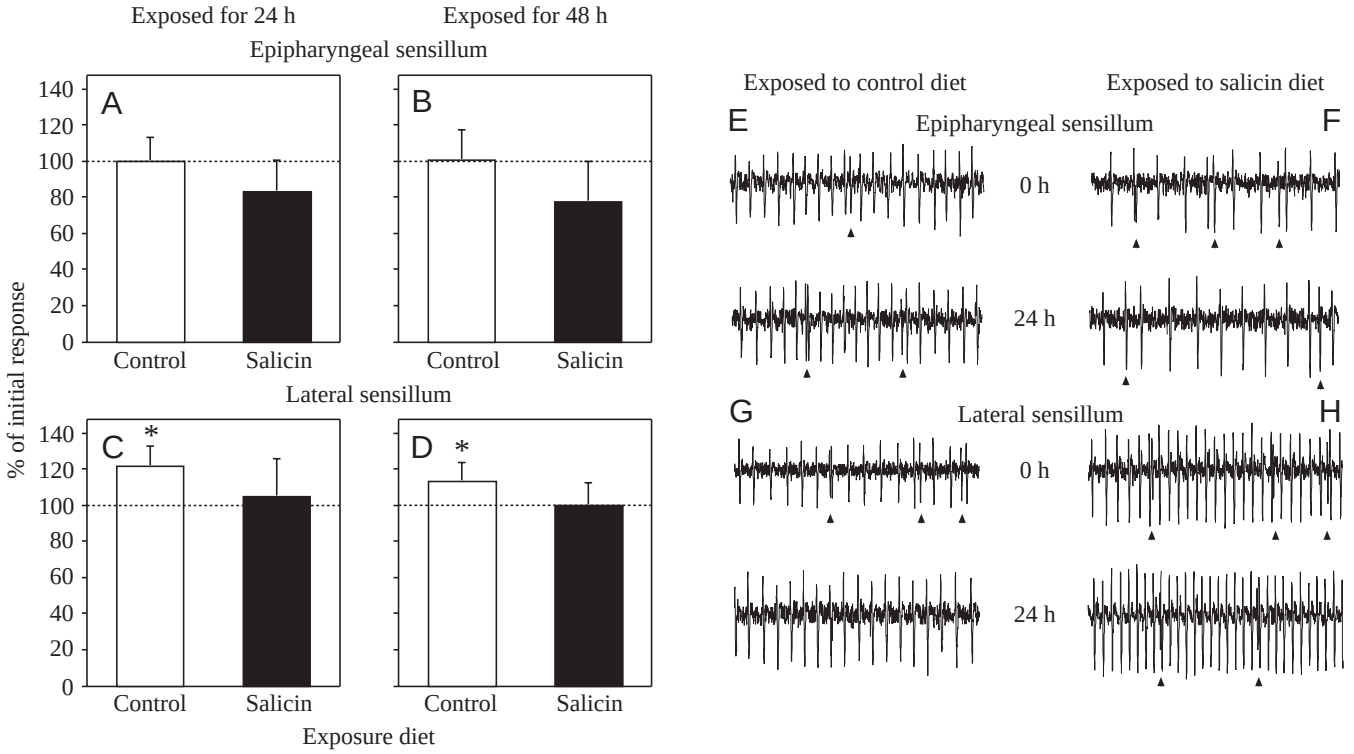

Fig. 6. Responsiveness of the bitter-sensitive taste cell in epipharyngeal and lateral sensilla to 50 mmol l^{-1} salicin, both before (i.e. 0 h) and after (i.e. 24 or 48 h) exposure to the control or 157 mmol kg^{-1} salicin diet. To determine whether the responsiveness of each class of bitter-sensitive taste cell changed significantly over the exposure period, we calculated the percentage of initial response [i.e. (number of spikes s^{-1} after exposure period/number of spikes s^{-1} before exposure period)×100]. These calculations were based on the initial 1 s of the response (median responses ± median absolute deviation). The number of caterpillars tested in each treatment group was as follows: exposed to control diet for 24 h (N=10) or 48 h (N=9); exposed to salicin diet for 24 h (N=10) or 48 h (N=9). For each caterpillar, we recorded from the same lateral and epipharyngeal sensillum both before and after the exposure period. We compared the medians within each panel with an expected null model of 100 % (broken line) using a one-sample Wilcoxon matched-pairs signed-rank test (*P<0.05). We also present representative neural responses of an epipharyngeal (E,F) and lateral (G,H) sensillum to 50 mmol l^{-1} salicin (in 0.1 mol l^{-1} KCl) both before and after 24 h of exposure to the control or salicin diet. In all traces, a single bitter-sensitive taste cell is firing at a consistent and relatively rapid rate, whereas a salt-sensitive taste cell is firing more slowly and irregularly (in response to the electrolyte); action potentials from the latter taste cell are marked with an arrowhead. Each trace displays the initial 250 ms of the neural response.

reported elsewhere (Glendinning et al., 1999b) that the aversive response of *M. sexta* to the salicin diet is mediated exclusively by sensory input from the taste cells. It follows, therefore, that the ability of the salicin-exposed caterpillars (in this study) to ingest the salicin diet reflects a diminished gustatory responsiveness to this compound. Our electrophysiological studies indicated that this diminished gustatory responsiveness was mediated by a mechanism within the central nervous system (see below for details).

We did not expect caterpillars on the control diet to experience a reduction in behavioral responsiveness to salicin across any of the exposure periods. Even though our results generally confirmed this expectation, there was one notable exception: caterpillars exposed to the control diet for 6 h ate significantly more salicin diet at the end of the exposure period than at the beginning. Given that the responsiveness of caterpillar taste cells to salicin and other taste stimuli normally increases over the first half of the instar (Schoonhoven et al., 1991; Glendinning et al., 1999a; see Fig. 6), it is unlikely that this transient change in behavioral responsiveness to salicin was mediated peripherally. Instead, it appears that these caterpillars experienced a centrally mediated reduction in salicin responsiveness during the initial 6–8 h of their fifth instar. We propose that this transient phenomenon facilitated the salicin adaptation process because it made the salicin diet more palatable, enabling the caterpillars to sample the salicin diet more extensively at the beginning of the exposure period (and thereby receive repeated sensory input from it).

The caterpillars did not adapt to the aristolochic acid diet. Their aversive response to this diet persisted across the 48 h exposure period. Furthermore, the caterpillars ingested significantly less of the aristolochic acid diet than of the control diet during the first and all subsequent 4 h time intervals, resulting in only marginal growth. We do not think that this poor growth can be explained by an inability to adapt to the aversive taste properties of the aristolochic acid diet for two reasons. First, both the ablated caterpillars (i.e. those lacking their aristolochic-acid-responsive taste cells) and the non-

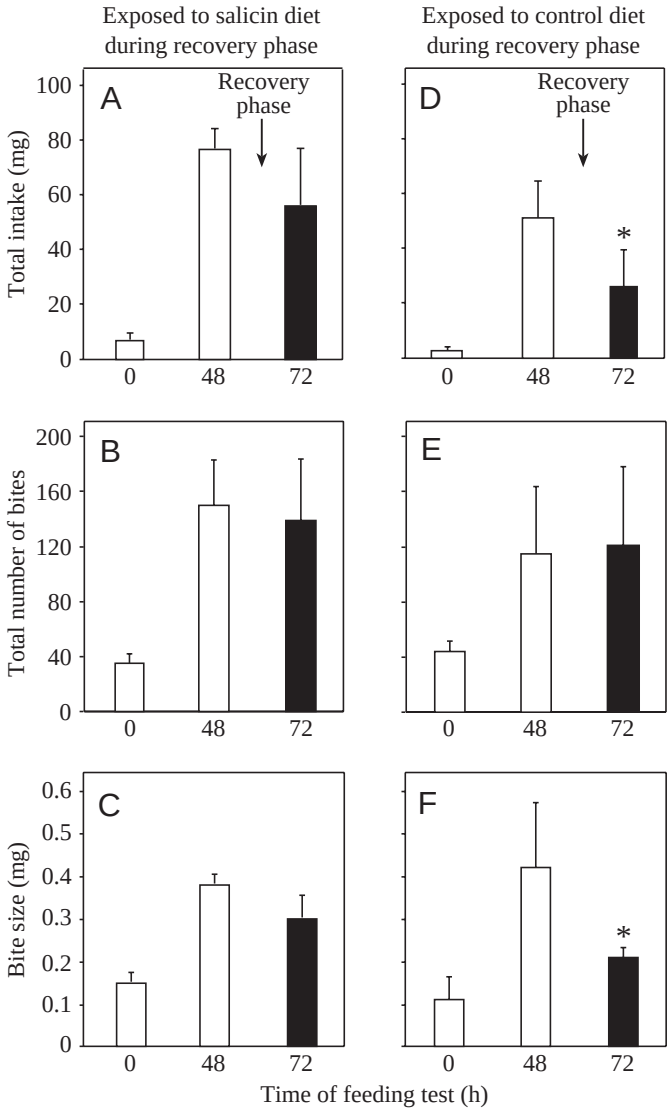

Fig. 7. Recovery from adaptation to salicin. At the beginning of the experiment (i.e. time 0 h), we measured the caterpillars' ingestive response (during the 2 min biting test) to the salicin (157 mmol kg^{-1}) diet. We calculated three ingestive parameters over the 2 min biting test: total intake, total number of bites and bite size. If the caterpillars exhibited an aversive response, we placed them on the salicin diet for 48 h. After this exposure period (i.e. at 48 h), we measured their ingestive response to the salicin diet a second time. If they had adapted to this diet (i.e. did not exhibit an aversive response), we offered them either the salicin diet (A–C) or the control diet (D–F) for an additional 24 h. We called this latter exposure period the 'recovery phase'. Finally, we measured the caterpillars' ingestive response to the salicin diet for a third time (i.e. at 72 h). To determine whether the caterpillars' aversive response to salicin changed during the recovery phase, we made paired comparisons between the ingestive responses at 48 and 72 h using the Wilcoxon matched-pairs signed-rank test (*$P \leq 0.05$).

ablated caterpillars grew poorly on the aristolochic acid diet. Because ablation of the aristolochic-acid-responsive taste cells completely eliminates the aversive response of caterpillars to aristolochic acid during the 2 min feeding test (Glendinning et al., 1999b), it is unlikely that the poor growth of the ablated caterpillars can be explained by a taste-mediated inhibition of feeding. Second, given that animals habituate more readily to weak stimuli (Thompson and Spencer, 1966), it is conceivable that the caterpillars failed to habituate to the aristolochic acid diet because it was more aversive-tasting than the salicin diet. We controlled for this possibility by using concentrations of aristolochic acid and salicin in the exposure diets that are equally aversive to *M. sexta* during 2 min feeding tests (Glendinning et al., 1999b). The most parsimonious explanation for the poor growth on the aristolochic acid diet is an inability to adapt to the toxic post-ingestive effects of aristolochic acid. We propose that, as the caterpillars sampled the aristolochic acid diet, they became progressively more ill; this illness, in turn, inhibited further consumption and, hence, the physiological adaptation process. *Manduca sexta* possesses at least one post-ingestive mechanism that detects ingested poisons and then inhibits subsequent feeding (Glendinning, 1996). A similar post-ingestive response mechanism could have been activated by aristolochic acid.

How did Manduca sexta *adapt to the salicin diet?*

We found that exposure to the salicin diet (for 24 or 48 h) failed to reduce significantly the responsiveness of the bitter-sensitive taste cells in the lateral or epipharyngeal sensilla to salicin. The only significant peripheral effect of salicin exposure was that it attenuated the normal developmental increase in salicin responsiveness that occurs in the bitter-sensitive taste cells within the lateral sensilla. Two lines of evidence indicate that this latter peripheral change cannot explain the large reduction in behavioral responsiveness to salicin that we observed. First, exposure to the control diet for 24 or 48 h did not alter the magnitude of the aversive behavioral response to salicin (Fig. 4A–C). This indicates that the developmental increase in peripheral responsiveness to salicin is not behaviorally significant. Second, previous work has shown that the bitter-sensitive taste cells in the epipharyngeal sensilla are sufficient to mediate an aversive response to salicin (Glendinning et al., 1999b). Because the responsiveness of these taste cells to salicin was not significantly reduced by exposure to the salicin diet, they alone should have been able to mediate the aversive behavioral response to salicin in the salicin-exposed caterpillars.

Our findings may help explain an apparent contradiction between results presented by Schoonhoven (Schoonhoven, 1969) and in the present study. Schoonhoven exposed fifth-instar *M. sexta* caterpillars to a control or salicin diet for 72 h, and then determined the responsiveness of the bitter-sensitive taste cell in the lateral sensilla to salicin. He found that the bitter-sensitive taste cells in caterpillars exposed to the salicin diet responded less vigorously to salicin than did those from caterpillars exposed to the control diet. Because Schoonhoven (Schoonhoven, 1969) did not record from the bitter-sensitive taste cells before and after exposure to the salicin diet, he could not determine whether salicin exposure made the taste cells less responsive to salicin or whether it simply prevented them

from experiencing their normal developmental increase in salicin responsiveness. The results from the present study support the latter explanation.

We believe that our electrophysiological data show that exposure to the salicin diet adapts the aversive behavioral response to salicin principally through a mechanism within the central nervous system. Although we did not determine the nature of this central mechanism, we did identify one of its salient features. Following adaptation to the diet containing 157 mmol kg^{-1} salicin, the caterpillars became totally unresponsive to diets containing salicin concentrations as high as 314 mmol kg^{-1} (Fig. 5). This indicates that the adaptation process effectively uncoupled the normal stimulus/response relationship between excitation of the bitter-sensitive taste cells and activation of the aversive behavioral response. When viewed from this perspective, the adaptation process resembles habituation, which is defined as a progressive loss of responsiveness to a specific stimulus following repeated stimulation (Leibrecht and Askew, 1980). Although we have not determined whether the salicin adaptation process meets all the established criteria for habituation (see Thompson and Spencer, 1966), it does meet at least three of them: (i) it is mediated centrally, (ii) it generalizes to higher concentrations of the habituating stimulus and (ii) it recovers spontaneously over a protracted period. It is notable, however, that the salicin adaptation phenomenon takes substantially longer to onset than other examples of habituation in *M. sexta* (Wiel and Weeks, 1996) and other insects (e.g. May and Hoy, 1991; Braun and Bicker, 1992).

At present, we can only speculate about where the adapted behavioral response is mediated. Several lines of evidence point to the subesophageal ganglion as the most likely site. First, the subesophageal ganglion receives projections (either primary or secondary) from all the taste cells and contains the mouthpart motor neurons (Kent and Hildebrand, 1987; Griss, 1990). Second, a variety of studies indicate that these two classes of neuron have functional connections within the subesophageal ganglion: (i) the gustatory projections intermingle with (or occur in close apposition to) arborizations from mouthpart motor neurons (Altman and Kien, 1987); (ii) some subesophageal ganglion interneurons have branches that occur in neuropil with both sensory and motor terminals (Altman and Kien, 1987); and (iii) stimulating the taste sensilla with plant extracts directly modulates the output of the mouthpart motor neurons (Griss et al., 1991). More work is needed to localize the site of the plasticity phenomenon and, ultimately, to identify the underlying neural circuits.

Concluding remarks

We found that *M. sexta* adapted its aversive behavioral response to a ‘bitter’ and relatively harmless compound, salicin, through a centrally mediated mechanism. Previous studies have shown that *M. sexta* can also adapt its aversive behavioral response to another ‘bitter’ and relatively harmless compound, caffeine, through a peripherally mediated mechanism (Glendinning et al., 1999a; Glendinning et al., 2001). Taken together, these findings show that *M. sexta* has evolved at least two complementary mechanisms for adapting to ‘bitter’ compounds in its diet. We suspect that, even though salicin and caffeine do not occur in the solanaceous host plants of *M. sexta* (Harborne and Baxter, 1993), there may be other ‘bitter’ compounds in solanaceous plants that activate the same gustatory signaling pathways as salicin and caffeine.

Finally, our findings with aristolochic acid show that there are limits to the range of ‘bitter’ compounds to which *M. sexta* can adapt physiologically and that toxicity may be an important factor setting these limits. Future studies are needed with a wider range of ‘bitter’ compounds and species of insect to assess the generality of these findings.

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