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Effects of two bitter substances on olfactory conditioning in the moth *Heliothis virescens*

Kari Jørgensen[1,*], Marit Stranden[1], Jean-Christophe Sandoz[2], Randolf Menzel[3] and Hanna Mustaparta[1]

[1]*Neuroscience Unit, Department of Biology, Norwegian University of Science and Technology, Trondheim, Norway,* [2]*Research Center on Animal Cognition, University Paul Sabatier, Toulouse, France and* [3]*Department of Biology, Freie Universität, Berlin, Germany*

*Author for correspondence (e-mail: kari.jorgensen@bio.ntnu.no)

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Summary

In nature, moths encounter nutritious and toxic substances in plants, and thus have to discriminate between a diversity of tastants. Whereas olfactory learning allowing memory of nutritious plants is well demonstrated, little is known about learning and memory of toxic items in adult lepidopterans. Moths may use bitter substances to detect and possibly learn to avoid noxious plants. We have studied the physiological and behavioural effects of two bitter substances, quinine and sinigrin, on the moth *Heliothis virescens*. Electrophysiological recordings showed responses to both compounds in gustatory receptor neurons on the antennae. The response patterns suggested a peripheral discrimination between quinine and sinigrin. We evaluated their putative aversive effect in an appetitive conditioning context where the moths learned to associate an odour with sucrose. We first aimed at enhancing olfactory conditioning of the proboscis extension response by testing the effect of the sucrose concentration on acquisition, retention and extinction. 2 mol l^{-1} and 3 mol l^{-1} sucrose concentration gave similar acquisition, retention and extinction performances. Experiments involving pre-exposure or facilitated extinction with an odour paired with quinine, sinigrin or no tastant showed a latent inhibitory effect, as well as an aversive effect of quinine and, to a lesser extent, of sinigrin. The results suggested that the two tastants may act as negative reinforcers in *H. virescens*.

Key words: aversion, learning, memory, gustation, *Heliothis virescens*.

Introduction

The ability to learn, remember and forget is important for the adaptation of an organism to a changing environment. In food consumption, learning and memory of the taste and smell of nutritious or noxious food is crucial for survival. For example, insects searching for nectar learn to prefer the odour of the favourable flowers. Stimulation with sucrose of the gustatory receptor neurons (GRNs) of contact chemosensilla (insect taste organs) located on different appendages of the insect body, e.g. antennae, mouthparts and tarsi, causes the hungry insect to extend its proboscis in order to feed. This response, the proboscis extension response (PER), has been utilised to study classical conditioning, particularly appetitive olfactory learning in several insect species, including the honeybee *Apis mellifera* (Bitterman et al., 1983; Menzel, 1993; Hammer and Menzel, 1995), the bumblebee *Bombus terrestris* (Laloi et al., 1999), and several moth species (Hartlieb, 1996; Fan et al., 1997; Daly et al., 2004; Skiri et al., 2005). In all these species, including moths, it was demonstrated that the olfactory conditioning of the PER is associative. If an initially neutral odour puff (the conditioned stimulus, CS) is given a few seconds before the sucrose stimulation (the unconditioned stimulus, US), the insects learn to associate the odour with the sucrose reward, and the CS will then trigger a conditioned response (CR), the insects extending the proboscis to the odour. In heliothine moths, previous studies have shown that they will learn to associate odours with an appetitive reward, both in the laboratory and in the field (Cunningham et al., 1999; Hartlieb et al., 1999; Skiri et al., 2005; Cunningham et al., 2006). The olfactory pathways involved in olfactory conditioning have been extensively studied and are well described in several species, including *A. mellifera* and the moth *Heliothis virescens*. The odorants are detected by olfactory receptor neurons located on the antennae, and olfactory information is transmitted *via* synapses within the glomeruli of the antennal lobes to local interneurons that carry out local computation, and to projection neurons (Menzel and Giurfa, 2001; Mustaparta and Stranden, 2005; Rø et al., 2007). Projection neurons further convey odour information *via* the antennocerebral tracts to the calyces of the mushroom bodies and to the lateral horn, a premotor area.

In the gustatory system, the sucrose solution used as US is detected by the GRNs on the antennae and the proboscis, and information is conveyed to the suboesophageal ganglion and the tritocerebrum (Mitchell et al., 1999; Kvello et al., 2006; Jørgensen et al., 2006). In *A. mellifera*, the suboesophageal-calycal tract is comprised of neurons passing on information directly from the

suboesophageal ganglion to a particular area of the calyces of the mushroom bodies that is segregated from the olfactory areas (Schröter and Menzel, 2003). In addition, the ventral unpaired median neuron of the maxillary neuromere 1, VUMmx1, has dendrites converging with the gustatory pathways in the dorsal suboesophageal ganglion and the tritocerebrum and axonal arborisations that converge with the olfactory pathways in the antennal lobes, the mushroom bodies and the lateral horn (Hammer, 1993). The VUMmx1 forms a modulatory connection between the pathways of the conditioned olfactory stimulus and the unconditioned sucrose stimulus. Electrical stimulation of this neuron in association with an odour puff is sufficient to replace sucrose reinforcement (although it does not elicit PER), suggesting that it comprises the neural substrate for sucrose reinforcement in bees. Changes in odour responses in the antennal lobes and the mushroom bodies after olfactory conditioning have been demonstrated in several studies with optical or intracellular recordings (Faber et al., 1999; Faber and Menzel, 2001; Sandoz et al., 2003; Daly et al., 2004; Yu et al., 2004).

Bitter taste, warning against the ingestion of unfavourable food, is important in all organisms. Bitter stimuli constitute the largest and structurally most diverse class of gustatory stimuli, and a wide range of molecules of varying sizes and functional groups are perceived as bitter tasting (Rouseff, 1990). Both in insects and mammals, bitter taste stimuli are detected by many divergent bitter receptor proteins expressed in single GRNs (Adler et al., 2000; Thorne et al., 2004; Wang et al., 2004; Mueller et al., 2005). In the fruitfly *Drosophila* sp., the receptor proteins are co-expressed in subsets of bitter GRNs. If the different subsets of bitter GRNs synapse on different interneurons or motorneurons in the central nervous system (CNS), or if several transduction mechanisms are involved, passing on different information to the downstream neurons, this would provide mechanisms enabling flies to discriminate between bitter tastants. In insects, different bitter stimuli may elicit different behavioural reactions, indicating the presence of a differential coding system (Glendinning and Hills, 1997).

In the present study, two bitter substances that are indiscernible to humans were tested for their aversive value in *H. virescens*. The prototypical bitter compound, quinine, is an alkaloid known to act through blocking of certain K^+ channels in vertebrates or permeate cell membranes directly and activate G-proteins, bypassing the receptor in *in vitro* preparations (Spielman et al., 1992; Naim et al., 1994). We also chose sinigrin (a glucosinolate) because it was previously found to be non-appetitive in *H. virescens* (Blaney and Simmonds, 1988; Jørgensen et al., 2006). Analyses of antennal GRN responses to the two substances were performed and their aversive effects were tested in the appetitive context of olfactory conditioning of PER. Two main protocols were used to study the aversive effect of the two tastants. In the first protocol (pre-exposure), moths were pre-exposed to the odour CS associated with one of the tastants (no tastant as control), and the success of subsequent acquisition of the same CS and sucrose was observed. In the second protocol (facilitated extinction), moths were first subjected to an acquisition phase with CS and sucrose, before being subjected to an extinction phase, where the same CS was associated with one of the tastants (no tastant as control). Possible facilitation of extinction was determined. Such experiments in which a decrease in CRs is expected because of the bitter stimuli, have to rely on high learning rates. A previous study of appetitive conditioning in *H. virescens* analysed the effect of CS quality and concentrations (Skiri et al., 2005). Conditioning with increased CS concentrations increased the learning rate, and odorants activating different receptor neuron types caused different learning performances. *Racemic* linalool induced strong and reliable learning, and was chosen as CS in the present study. However, the effect of sucrose concentration on learning success was unknown. Therefore, we first performed an experiment comparing the effect of two high sucrose concentrations (2 mol l^{-1} and 3 mol l^{-1}) on acquisition of CRs, retention between 15 min and 48 h, and resistance to extinction at the same intervals. This allowed us to choose adequate conditions for the pre-exposure and facilitated extinction experiments with the bitter substances.

Materials and methods

Insects and preparation

Adult *H. virescens* (Fabricius) used in the experiments were received as pupae from Syngenta (Basel, Switzerland). The male and female pupae were sorted and hatched in separate climate chambers (22°C, reversed photoperiod, Refritherm 200; Struers-Kebolab, Albertslund, Denmark). Experiments with males and females were performed in separate groups. Newly hatched insects were placed in containers with free access to 5% (w/v) sucrose solution. After 24 h the insects were immobilised in Plexiglas holders with tape between the head and the thorax, exposing the head with the proboscis and the antennae. The insects were then deprived of food for 48 h in the climate chambers. One hour before the experiments started, the insects were placed in the experiment room for familiarisation to the experimental context.

Test compounds

The odorant used as CS was *racemic* linalool (95% checked in gas chromatograph; Sigma-Aldrich, Steinheim, Switzerland), which was diluted in n-hexane (99%, v/v, 1:100) and stored at –20°C. A dose (100 μl) of this solution was applied to a piece of filter paper (160 mm diameter) from which the n-hexane evaporated before it was placed in a glass cartridge sealed with Teflon caps. Each cartridge was used for 1 h (maximum 124 stimulations), and was made the day of the experiment. The appetitive stimuli were 2 mol l^{-1} or 3 mol l^{-1} sucrose (99.9%, Sigma-Aldrich). The 3 mol l^{-1} solution was put on a stirrer for 4–5 h at room temperature for all the sucrose to dissolve. The putative aversive stimuli were 1 mol l^{-1} sinigrin monohydrate (99%; VWR International, Oslo, Norway) or 0.16 mol l^{-1} quinine hydrochloride dihydrate (98%; VWR International). Because of the low solubility of quinine in water, this was the highest possible molarity without adding acid or alcohol. Quinine (0.01 mmol l^{-1}, 0.1 mmol l^{-1}) and sinigrin (1.0 mmol l^{-1}, 10 mmol l^{-1}, 100 mmol l^{-1}) were solved in the electrolyte 0.01 mol l^{-1} KCl (99.5%, Sigma-Aldrich) for the electrophysiological recordings.

Experiment 1

US concentration, retention and extinction

The experiments were performed in a dimly lit room with a constant temperature of 23°C. One at a time, each moth was

placed in front of a ventilation outlet with a weak suction. Facing the insect at 2 cm distance was a glass tube with a constant air flow (~400 ml min^{-1}). The cartridge containing the CS was inserted into the tube, and the odour stimulus was given as a 5 s puff of ~100 ml min^{-1} flow into the constant air stream. The sucrose US (5 s) was applied with a toothpick 2.5 s after the onset of the odour puff, first to both antennae, and then to the extended proboscis. Because moths tend to be unresponsive at the beginning of conditioning because of low attention, the same method as in previous work was used to ensure learning success (Skiri et al., 2005): if the insect did not extend its proboscis at first encounter with the sucrose, the proboscis was forced out, and the insect was allowed to drink. This was not done in subsequent trials, meaning that the insects that failed to show PER were not rewarded. Each insect was placed in the setup 15 s before CS onset in order to adapt to the air flow, and was removed 10 s after the end of the US. For each insect there were eight conditioning trials, with 15 min inter-trial intervals (ITI). Subsequently, there were eight extinction trials in which the odour was given without reward (15 min ITI). At the end of every experiment, all insects were tested for the unconditioned response (UR) to sucrose. The results were calculated as the percentage of insects that showed CR during each stage of the conditioning trials and the extinction trials. To find out whether US concentration affected acquisition, retention or extinction, 2 mol l^{-1} and 3 mol l^{-1} concentrations were used as US in conditioning experiments in different insects. Each of the two groups were further divided into five retention groups, for which the first extinction trial started after the last acquisition trial at 15 min, 2 h, 8 h, 24 h or 48 h, respectively. All retention periods were tested in each experiment. The different parameters were chosen according to previous conditioning experiments in *H. virescens* (Skiri et al., 2005).

Experiment 2

Antennal gustatory neuron responses to quinine and sinigrin

Electrophysiological recordings from GRNs of sensilla chaetica on the *H. virescens* antennae were performed using a tip recording technique (Hodgson et al., 1955). The recording electrode (thin-walled borosilicate glass capillaries; Harvard Apparatus, Edenbridge, UK) was pulled in a two-step electrode puller (PP-830; Narishige Group, Tokyo, Japan) to a tip diameter of approximately 10–20 μm. To avoid crystallisation and concentration changes at the tip, the electrode was filled with the test substance just a few seconds before the start of the recording. The recording electrode containing the test solution was placed over single sensilla hairs for 5 s, with an inter-stimulus interval of at least 10 min to avoid adaptation. Taste sensilla from all parts of the flagellum were included in the experiments. The recording glass electrode was connected to a TastePROBE amplifier (10×; Syntech, Hilversum, The Netherlands) (Marion-Poll and Van der Peers, 1996) and the signals filtered (low pass 50 Hz and high pass 3000 Hz) using the CyberAmp 320 from Axon Instruments (Burlingame, CA, USA). The reference electrode was a 1 mm AgCl-coated silver wire placed in the moth abdomen. Analysis of the spikes was performed using the software AutoSpike-32 (Syntech). The responses were counted as number of spikes elicited during the 5 s stimulation period, and the temporal patterns were assayed, counting spikes in 0.5 s bins.

Experiment 3

CS pre-exposure associated with putative aversive stimuli

In this experiment we tested whether the bitter compounds sinigrin and quinine could induce aversive effects on the subsequent learning of odour–sucrose associations. The experiment consisted of two phases, a pre-exposure phase and a conditioning phase. In the pre-exposure phase, three groups of insects were pre-exposed to different stimuli eight times (15 min ITI). In the control group each insect was exposed to linalool (5 s) paired with stimulation with a dry toothpick (5 s, no tastant, mechanosensory control) of the antennae 2.5 s after the onset of the linalool stimulus. In the two bitter-treatment groups the insects were exposed to linalool (5 s) paired with 1 mol l^{-1} sinigrin or 0.16 mol l^{-1} quinine stimulation, respectively, applied with a toothpick. Bitter tastant stimulation started 2.5 s after the onset of the linalool stimulus and lasted 5 s. Because the aversive value of the tastants might be mediated by GRNs on the proboscis as well as on the antennae, the stimulation was first applied to the antennae, and then to the proboscis. At the first trial, after antennal stimulation, the proboscis was forced out and the bitter tastant or dry toothpick was shortly applied. In nature, if the insect extends the proboscis to an antennal stimulation, it expects to taste the compound with the proboscis. This process could be necessary for choosing to accept or avoid a given food. For this reason, in subsequent trials, moths that extended the proboscis to the tastant received a stimulation of the proboscis. In our control group, moths received CS presentations without sucrose before the acquisition, which could lead to a so-called latent inhibition effect, i.e. a resistance to acquisition. To test for this effect we included a fourth untreated control group in which the moths were left without pre-exposure. In the conditioning phase (starting 15 min after the end of the pre-exposure phase), all groups were subjected to an identical acquisition procedure, with eight conditioning trials (CS associated with 2 mol l^{-1} sucrose US) with 15 min ITI, as in experiment 1. After 15 min, all moths received a retention test with the CS alone for 5 s.

Experiment 4

Extinction of CR combined with putative aversive stimuli

The goal of this experiment was to evaluate the aversive effects of bitter tastants when applied during extinction. The experiment consisted of two phases, a conditioning phase and an extinction phase. In the conditioning phase, all insects were conditioned to linalool with 2 mol l^{-1} sucrose (described in experiment 1). In the extinction phase (starting 15 min after the end of the conditioning phase) the insects were divided into three groups receiving different types of extinction trials (eight trials, 15 min ITI). The control group was given a dry toothpick (no tastant, mechanosensory control) on the antennae and on the proboscis, when extending the proboscis to the CS. The two treatment groups were given 1 mol l^{-1} sinigrin or 0.16 mol l^{-1} quinine, respectively, with a toothpick on the antennae and on the proboscis, when extending the proboscis to the CS.

Statistics

Behaviour

All insects that failed to show UR three times or more during acquisition or at the end of the experiment were considered unmotivated and excluded from the data analysis. To compare extinction performance independently of different retention levels, only insects showing CR at the first extinction trial were included in the analysis (Experiment 1 and Experiment 4). Comparisons of acquisition or extinction performance among groups were performed on the sum of CRs given by each moth during the respective phase, using Mann–Whitney tests (for n=2 groups) or Kruskal–Wallis tests (for n>2 groups). Performance at individual trials was compared between groups using Fisher's exact tests. Depending on the question addressed in each experiment, either multiple comparisons with threshold corrections (experiment 1) or planned comparisons without threshold correction (experiments 3 and 4) were performed. In experiment 1, we compared extinction at different retention times. After a global Kruskal–Wallis test, we performed multiple comparisons using the Noether method [1976 (in Scherrer, 1984)]. The alpha level was corrected using the Dunn–Sidák threshold correction [$\alpha'=1-(1-\alpha)^{1/k}$, where k is the number of two-by-two comparisons in which each data are used]. The goal of experiments 3 and 4 was to test specifically the effect of bitter compounds in appetitive conditioning situations. Therefore, we only performed a few planned comparisons between performance in the bitter-treated groups and the control group, using Mann–Whitney tests with an alpha level of 0.05 [the number of planned comparisons being always lower than the number of degrees of freedom (n groups–1) of the experiment].

Electrophysiology

To compare the time courses of responses of the receptor neurons to the different concentrations of tastants, two-way tastant×time bin analysis of variance (ANOVA) was performed (with repeated measurements). Two-by-two comparisons of tastant responses were performed with one-way ANOVA, using the Dunn–Sidák threshold correction as above. Comparisons between tastants at individual time bins were done using Scheffé tests for multiple comparisons.

Results

Experiment 1

Out of the 554 moths used in the experiment, 348 (62.8%) were included according to the criteria listed in the Materials and methods.

Effect of sucrose concentration on acquisition

Conditioning with 2 mol l^{-1} and 3 mol l^{-1} sucrose as US induced good acquisition, where the responses to the odour increased with trials, from zero at the first conditioning trial (no spontaneous responses), to 50% and 45% at the eighth conditioning trial for the 2 mol l^{-1} and 3 mol l^{-1} groups, respectively (Fig. 1A). The acquisition curves did not reach asymptotic levels after eight conditioning trials, indicating that more trials might further have enhanced the learning success. Acquisition was similar in the two groups (Mann–Whitney test, Z=0.59, P=0.56).

Effects of time after training and sucrose concentration on retention

Retention time is the period between the last conditioning trial and the first extinction trial. The effect on retention of time elapsed after training was studied by comparing responses of the first extinction trial performed after 15 min, 2 h, 8 h, 24 h and 48 h in different groups of moths (Fig. 1B). Overall, memory decreased with time, being strongest at 15 min and declining gradually to a lower level at 48 h. Retention was highest in the 2 mol l^{-1} reward group tested after 15 min, where the proportion of insects responding was 67%, and lowest (21%) in the 3 mol l^{-1} reward group tested after 48 h. An exception from the gradually declining response with time appeared for the 3 mol l^{-1} group, showing a slightly stronger retention after 24 h than after 8 h. No statistical differences between the two concentrations at any of the retention times were found (Mann–Whitney, 15 min: P=0.473; 2 h: P=1; 8 h: P=0.626; 24 h: P=0.311; 48 h: P=1), so the data of the 2 mol l^{-1} and 3 mol l^{-1} groups were pooled before testing whether the first extinction trial differs between the five retention groups. The 15 min and 2 h groups were significantly different from the other retention groups (Fisher's exact tests, all P<0.01), but not from each other (P=1). The 8 h, 24 h and 48 h groups were not significantly different from each other (Fisher's exact tests, P>0.04) when the α-level was corrected for multiple comparisons (Dunn–Sidák correction, α'=0.0127).

Effect of time on extinction

To compare the strength of the odour–sucrose association at different times after conditioning, we assessed its resistance to extinction during the eight extinction trials (Fig. 1C). To be able to compare extinction between groups, despite the differences observed in absolute retention scores (see above), only moths showing a CR at the first extinction trial were included (Fig. 1D). In all cases the responses decreased with increasing number of extinction trials. The moths tested after 8 h showed the fastest and highest overall extinction, the percentage of responses declining to 4% at the last trial. The 48 h group showed a slower and lower overall extinction than the other groups, 40% of the moths still showing CR at the last trial. There was a significant heterogeneity in overall extinction among the five groups (Kruskal–Wallis, P=0.03). Two-by-two comparisons indicated that extinction in the 48 h group was significantly lower than in the 8 h and the 24 h groups (Noether multiple comparisons with Dunn–Sidák correction, Z=3.11 and Z=2.53, respectively, P<0.0127) and just short of significance compared with 15 min and 2 h groups (Z=2.35 and Z=2.39, respectively, P<0.02). Although retention decreased with the interval between acquisition and extinction, the remaining association was strongest for the 48 h interval.

Experiment 2

Antennal gustatory neuron responses to quinine and sinigrin

When applying different concentrations of sinigrin and quinine to the contact chemosensilla, s. chaetica, on the flagellum of the *H. virescens* antenna, responses to the two substances seemed to be elicited in separate receptor neurons. A bursting firing pattern was elicited in one type of receptor neuron during stimulation with 1 mmol l^{-1} quinine compared

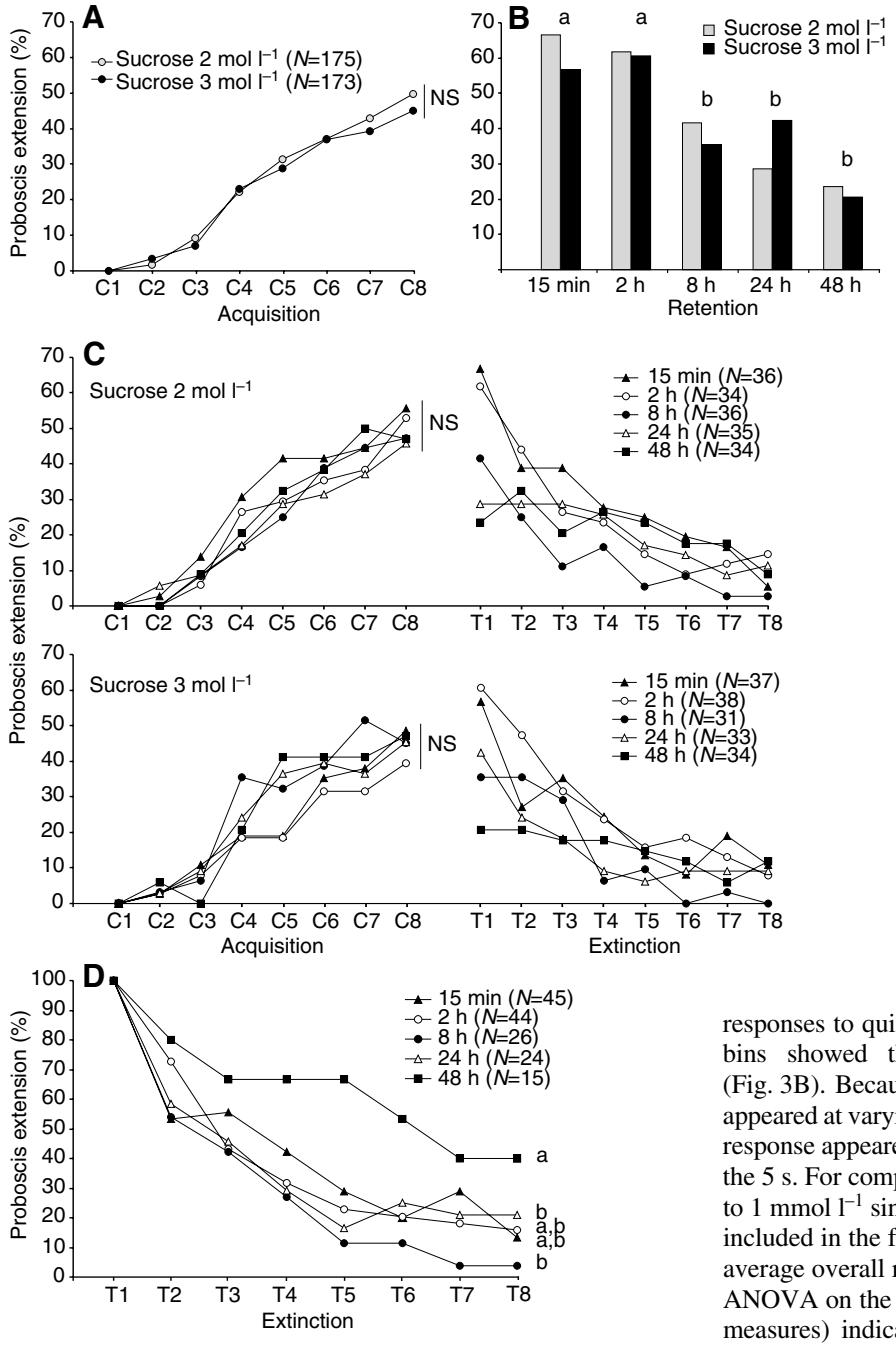

Fig. 1. The effect of US concentration on acquisition, retention and extinction of the conditioned PER, and the effect of time on retention and extinction in *H. virescens*. The proportion (%) of moths showing CR in each of the acquisition, retention and extinction trials is shown. (A) Average acquisition curves obtained in classical conditioning experiments with *racemic* linalool as CS and 2 mol l^{-1} and 3 mol l^{-1} sucrose as US. The letters NS indicate no significant between-group differences (Mann–Whitney test, P<0.05). (B) Retention in moths receiving 2 mol l^{-1} or 3 mol l^{-1} sucrose reward tested at different times after acquisition. Retention decreased significantly from 15 min to 48 h. N>31 in all retention groups. Different letters indicate significant between-group differences (Fisher's exact tests, P<0.0127). (C) Acquisition and extinction curves for the five retention times and the two sucrose concentrations. The extinction curves were obtained by stimulating with CS alone. No significant between-group differences were found, indicated by the letters NS (2 mol l^{-1}: Kruskal–Wallis test, P>0.05; 3 mol l^{-1}: Kruskal–Wallis test, P>0.05). (D) Extinction curves for moths tested after 15 min, 2 h, 8 h, 24 h or 48 h. Only moths showing CR at the first extinction test were included. Extinction was slower in the moths tested after 48 h. Different letters indicate significant between-group differences (Noether tests, P<0.0127).

with no activity when stimulating with the electrolyte KCl (Fig. 2A,B). The GRN responding to quinine often showed a long latency, and the bursts appeared at varying intervals in different recordings. The same concentration of sinigrin induced only a few spikes with smaller amplitude and no bursting activity when recording from the same sensillum (Fig. 2A). When increasing the concentration of sinigrin to 100 mmol l^{-1}, the number of spikes per 5 s was in the same range as that of 1 mmol l^{-1} quinine, enabling comparison of the average temporal firing patterns induced by the two substances (Fig. 2, Fig. 3A). Sinigrin elicited a phasic-tonic firing, and quinine a bursting firing. The bursting response to quinine did not change across recordings, and was similar in sensilla showing responses to quinine alone or both quinine and sinigrin. The mean responses to quinine and sinigrin in 74 sensilla plotted in 0.5 s bins showed the temporal differences in firing patterns (Fig. 3B). Because the bursts of the quinine-responsive GRNs appeared at varying intervals in different recordings, the average response appeared as a sustained high level of firing throughout the 5 s. For comparison, the average temporal response patterns to 1 mmol l^{-1} sinigrin and the electrolyte 10 mmol l^{-1} KCl were included in the figure. There were significant differences in the average overall responses to the different tastants. A two-factor ANOVA on the effects of tastants and time bins (both repeated measures) indicated a significant tastant effect ($F_{3,219}$=15.48, P<0.001), a significant time bin effect ($F_{9,657}$=42.76, P<0.001) and a significant interaction (F_{27}=12.79, P<0.001). In particular, the time courses of spiking activity were significantly different between responses to 1 mmol l^{-1} quinine and 100 mmol l^{-1} sinigrin (tastant×time bin ANOVA, $F_{9,657}$=10.21, P<0.001), although the average response over the 5 s to the two tastants was not different (tastant ANOVA, $F_{1,73}$=3.60, P=0.06). The responses to 10 mmol l^{-1} KCl and 1 mmol l^{-1} sinigrin over the 5 s were not significantly different (tastant ANOVA, $F_{1,73}$=0.42, P=0.51), but the response to both substances differed from the response to 100 mmol l^{-1} sinigrin and 1 mmol l^{-1} quinine (tastant ANOVA, $F_{1,73}$>8.68, P<0.01). During the first 0.5 s (tastant effect: $F_{3,219}$=17.00, P<0.001), the response to 100 mmol l^{-1} sinigrin was significantly higher than that to 1 mmol l^{-1} quinine, indicated with letters in the first dotted area

in Fig. 3B (Scheffé test, P=0.004), but by the third time bin (1–1.5 s, tastant effect: $F_{3,219}$=13.84, P<0.001), the relationship was reversed, the response to 1 mmol l^{-1} quinine being significantly higher than the 100 mmol l^{-1} sinigrin response, indicated with letters in the second dotted area in Fig. 3B (Scheffé test, P=0.0005). A high proportion of the sensilla (93%) had GRNs responding to 1 mmol l^{-1} quinine, whereas 83% of the sensilla had GRNs responding to 100 mmol l^{-1} sinigrin, and 68% to the electrolyte 10 mmol l^{-1} KCl. A few sensilla (5%) had GRNs that responded to 100 mmol l^{-1} sinigrin, but not to 1 mmol l^{-1} quinine, whereas 15% of the sensilla had GRNs responding to quinine, but not to sinigrin. Twenty-one percent of the sensilla had GRNs responding to quinine and sinigrin, but not to KCl. These results suggested that sinigrin and quinine are detected by different GRNs on the moth antennae. The putative aversive effect of the two substances was tested in the following experiments.

Experiment 3

Out of the 338 moths used in the experiment, 230 (68%) were included according to the criteria listed in the Materials and methods section.

Acquisition after CS pre-exposure associated with quinine or sinigrin

During pre-exposure, no insects showed PER to the odorant linalool whereas 3.4% of the insects showed PER to the dry toothpick (mechanosensory control), 3.5% to quinine and 24.6% to sinigrin (Fig. 4A). The quinine group did not differ from the control (Mann–Whitney test, Z=0.052, P=0.958), whereas stimulation with sinigrin elicited significantly more PER than in the control (Mann–Whitney test, Z=3.38, P=0.001).

Acquisition in the control group reached 25% at the end of training, whereas moths treated with CS + quinine reached only 11%, and moths treated with CS + sinigrin reached only 13% (Fig. 4B). However, in untreated moths, not receiving linalool in the first phase, acquisition reached 42%. Acquisition performance was significantly lower in the quinine group compared with the control (Mann–Whitney test, Z=2.28, P=0.023), but not in the sinigrin group (Mann–Whitney test, Z=1.24, P=0.217). Acquisition in untreated moths was significantly higher than in the control group (Mann–Whitney test, Z=1.94, P=0.05), meaning that pre-exposure to the CS and mechanosensory stimulus (no tastant) led to a resistance to acquisition. The treatment with quinine enhanced this effect, leading to significantly higher resistance to acquisition. The differences in acquisition were not because of differences in the appetitive motivation of the moths, because no significant effects of the pre-exposure treatments on subsequent UR to sucrose in the acquisition phase appeared (Mann–Whitney test, control *versus* quinine: Z=0.247, P=0.805; control *versus* sinigrin: Z=0.838, P=0.402; control *versus* untreated moths: Z=1.532, P=0.126).

The results of a retention test 15 min after acquisition showed the same pattern of response for the bitter compounds: retention was significantly lower in the quinine group compared with the control group (Fisher's exact test, P=0.045), but not in the sinigrin group (Fisher's exact test, P=0.21). However, retention in untreated moths was not significantly higher than in controls (Fisher's exact test, P=0.121).

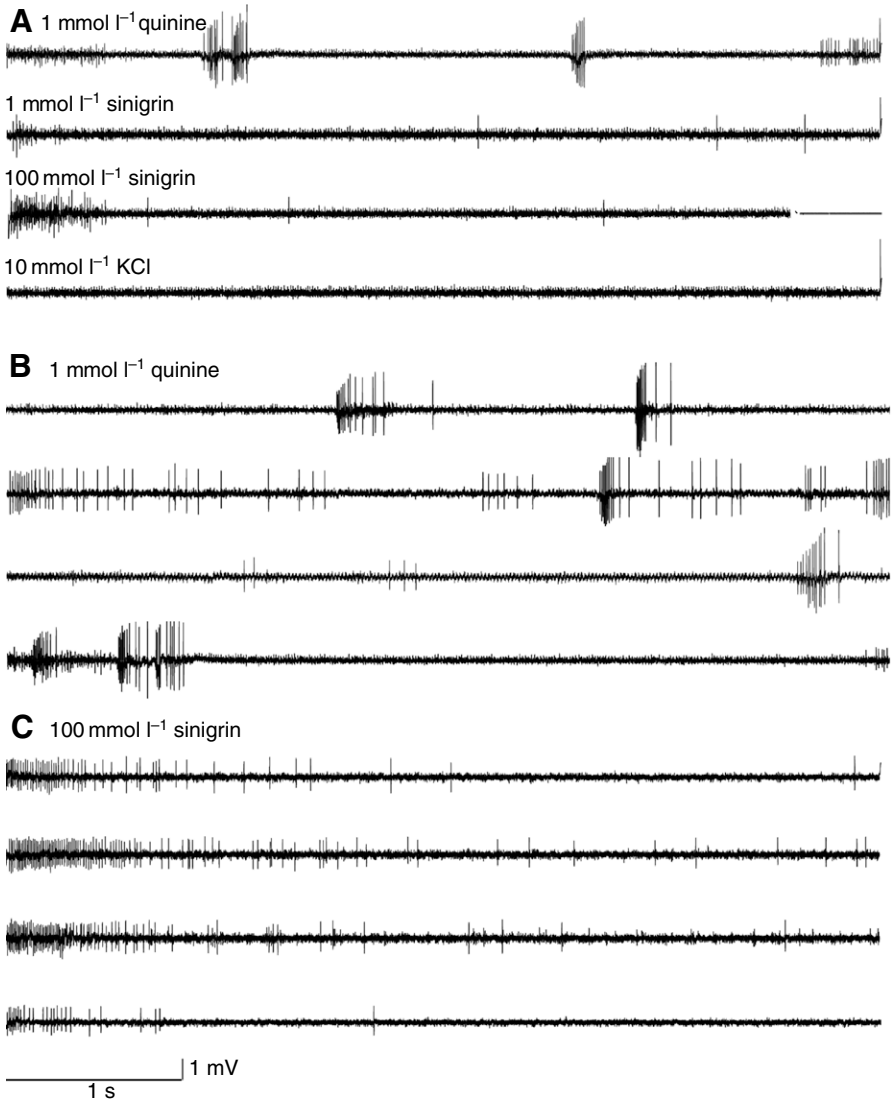

Fig. 2. Typical responses obtained by tip recordings from gustatory receptor neurons in s. chaetica on the flagellum of the *H. virescens* antennae. Stimulation and recording starts simultaneously when the electrode is applied and ends when the electrode is removed, meaning that only the stimulation period is shown. (A) Responses to 1 mmol l^{-1} quinine, 1 mmol l^{-1} sinigrin, 100 mmol l^{-1} sinigrin, and the electrolyte 10 mmol l^{-1} KCl in the same s. chaeticum. (B) Responses to 1 mmol l^{-1} quinine in four different s. chaetica. (C) Responses to 100 mmol l^{-1} sinigrin in four other sensilla.

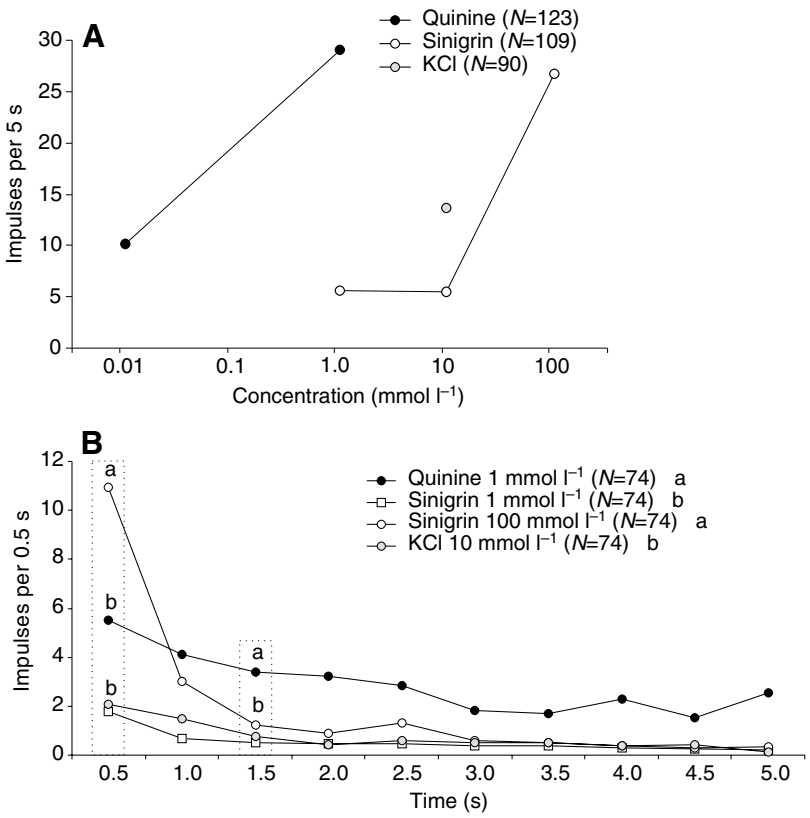

Fig. 3. (A) Average dose–response curves for quinine and sinigrin obtained during 5 s recordings from single s. chaetica. The average response to the electrolyte 0.01 mol l^{-1} KCl is indicated as a reference. (B) Average temporal response patterns for KCl, quinine and two concentrations of sinigrin, counted in 0.5 s bins in 75 s. chaetica during 5 s recordings. Whereas 100 mmol l^{-1} sinigrin elicited a high-response frequency very shortly after application, responses to 1 mmol l^{-1} quinine were bursts of activity distributed over the whole 5 s recordings. Different letters indicate significant between-group differences. The dotted areas show tests within the first and the third time bin, respectively. Letters behind the captions in B indicate differences between the average spiking activity during 5 s (Scheffé tests after ANOVA, $P<0.01$).

This experiment shows a putative aversive effect of quinine on subsequent acquisition. Although sinigrin gave similar results to quinine, no significant difference was found in acquisition between control and sinigrin-treated moths. This experiment also shows that pre-exposure with the CS (here with a mechanosensory stimulation) reduces subsequent acquisition of the CS–sucrose association. This effect suggests the possible existence of a latent inhibition phenomenon in moths. In the following experiment we addressed the putative aversive effects of quinine and sinigrin in a different learning situation.

Experiment 4

Out of the 398 moths used in the experiment, 294 (73.9%) were included according to the criteria listed in the Materials and methods section.

Facilitated extinction of CR combined with quinine or sinigrin

Acquisition was efficient in all groups, reaching 32–34% at the end of training, without any significant difference between treatment and control groups (Fig. 5A, Mann–Whitney, quinine *versus* control, $Z=0.299$, $P=0.77$; sinigrin *versus* control, $Z=0.568$, $P=0.57$). Thirty-nine to forty-three percent of the moths showed CR in the first extinction trial. To compare extinction on an identical basis in the different groups, only these insects were included (Fig. 5B). Extinction was strong in all groups, responses declining with repeated trials, down to 17% in the control group, and 0% and 2% in the quinine- and sinigrin-treated groups, respectively (Fig. 5B). Extinction was significantly stronger both in the quinine group (Mann–Whitney, $Z=2.5$, $P=0.012$) and in the sinigrin group compared with the control group (Mann–Whitney, $Z=2.12$, $P=0.03$).

Discussion

The first part of this study (Fig. 1) was aimed at improving the PER conditioning protocol previously used in heliothine moths (Skiri et al., 2005), as well as investigating the duration of the established memory and the resistance of the CS–US association to contradictory information. All these parameters were crucial for assessing the aversive effects of bitter stimuli. We found similar learning performances when using 2 mol l^{-1} and 3 mol l^{-1} sucrose as rewards. However, in a previous study with the same CS and 1 mol l^{-1} sucrose reinforcement (Skiri et al., 2005), we obtained only 29% CR in the last trial, compared with 45–50% obtained with 2 mol l^{-1} and 3 mol l^{-1} sucrose in the present study. This observation shows that the strength of the US may be important for acquisition in *H. virescens*, as is generally observed in learning studies. The same observation was made in other insects, such as the honeybee and the bumblebee (Bitterman et al., 1983; Loo and Bitterman, 1992; Laloi et al., 1999; Scheiner et al., 1999; Scheiner et al., 2004). In moths, a saturation of the reinforcing effect of sucrose seems to be reached with 2 mol l^{-1} sucrose solution.

Eight spaced conditioning trials were sufficient for the moths to remember the CS–US association for at least 48 h. This implies that moths, although non-social insects with an adult life span of approximately two weeks, can build long memories. In comparison, *A. mellifera* receiving three spaced appetitive learning trials will remember the odour for the rest of their lives (several weeks) (Sandoz et al., 1995; Menzel, 1999), *Drosophila* remember odour–electric shock associations for seven days after 10 spaced aversive conditioning trials (Tully et al., 1994), and memory after four-trial differential conditioning in the crickets lasts one week (Matsumoto and Mizunami, 2002).

The moths tested after 15 min and 2 h showed the highest retention performances. The responses dropped to a lower level after 8 h, suggesting that it is most important for moths to remember an odour within a few hours, and probably less important to remember it for several hours or days. In contrast to honeybees, learning of plant odorants in moths serves only self-consumption and oviposition purposes. A strong memory

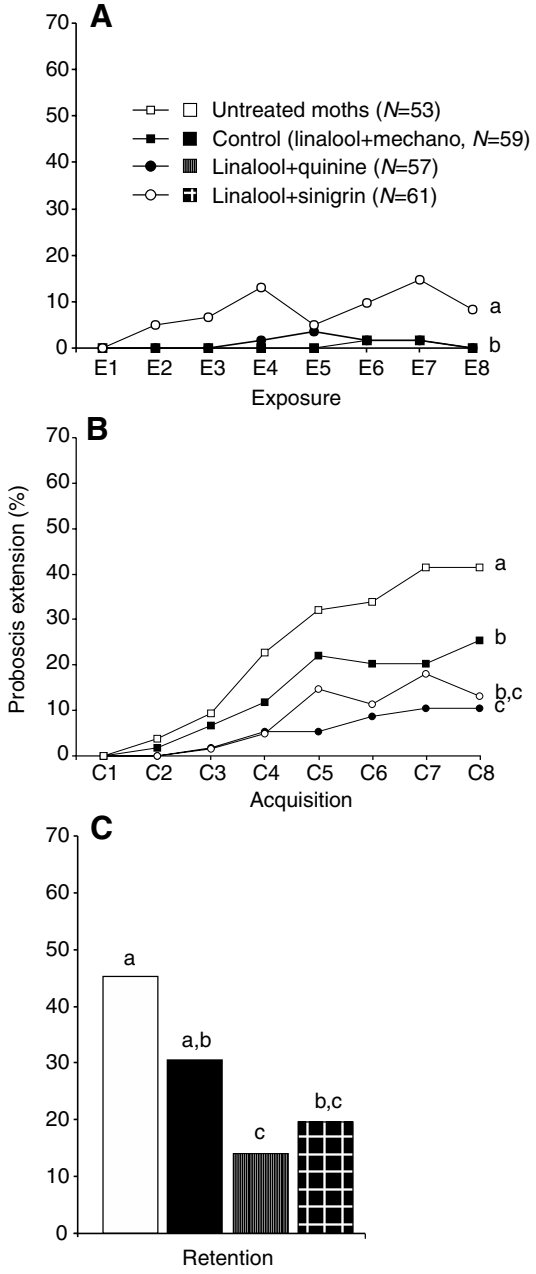

Fig. 4. Inhibitory learning effects of pre-exposure to linalool paired with a mechanosensory control, quinine or sinigrin on acquisition and retention. (A) Responses to the mechanosensory stimulus, quinine and sinigrin during pre-exposure. The odorant linalool alone elicited no responses. Different letters indicate significant between-group differences (Mann–Whitney tests, $P<0.05$). (B) Effect of pre-exposure on acquisition in moths. The group of moths receiving quinine treatment showed lower acquisition than the control group, suggesting an aversive effect of quinine. Such an aversive effect appeared only as a tendency for sinigrin. The untreated group of moths was not pre-exposed. The control group showed reduced acquisition compared with the untreated group, corresponding to a latent inhibition effect. Different letters indicate significant between-group differences (Mann–Whitney tests, $P<0.05$). (C) The control group showed higher retention than the quinine treatment group, but not the sinigrin treatment group. The control group was not different from the untreated group in retention. Different letters indicate significant between-group differences (Fisher's exact tests, $P<0.05$).

shortly after learning may therefore be well adapted to the life of the moth. It is possible that the 15 min and 2 h memories constitute the same forms of memory in the moth, both because of equally high retention and equal resistance to extinction in the two groups, suggesting similar consolidation statuses at the two time intervals. These memories in the moths could be equivalent to the late short-term memory phase described in honeybees, developing over time in the minute range, and used to remember rewards (nectar quality and quantity) between flower patches (Menzel, 1999). In honeybees, this memory stage is transient, and sensitive to retrograde amnesia or additional experience (Erber, 1976; Menzel, 1990). Memory then consolidates to a more stable and amnesia-resistant middle-term memory within approximately 1 h (Menzel, 1990). In *Drosophila* as well, memory is sensitive to cold treatment in the first hour after conditioning (Tully et al., 1994). Experiments using cold treatment after conditioning in moths may help examine amnesia-sensitive and amnesia-resistant memories, providing further insights into memory phases underlying performance. In contrast to honeybees, retention after two hours in the moths declined quickly with time, and was lowest in the group tested after 48 h. In this group, there was a strong resistance to extinction, suggesting that the CS–US association was strong and stable in the moths that remembered the odour. Two different types of stable long-term memory have been described in other insects; one corresponds to the early long-term memory found in honeybees as well as the anaesthesia-resistant memory in *Drosophila*, which are both resistant forms of memory, independent of protein synthesis (Wittstock et al., 1993; Tully et al., 1994; Wüstenberg et al., 1998). The second type is the protein synthesis (transcription)-dependent late long-term memory that is found as early as 5 h after conditioning in crickets (Matsumoto et al., 2003) or as late as 3–4 days in honeybees. Future experiments using protein synthesis inhibitors will reveal which memory phase controls 48 h retention in moths.

The presented electrophysiological recordings show excitatory responses to both quinine and sinigrin in GRNs on the moth antennae. By contrast, one study of the honeybee antennae showed no excitatory responses of GRNs to the bitter substances tested (De Brito Sanchez et al., 2005). In our study, sinigrin and quinine might be detected by two different GRNs (Figs 2–3). This assumption is based on the different temporal firing patterns elicited when stimulating with the two tastants. The bursting firing pattern of the GRNs responding to quinine differs significantly from the phasic-tonic firing pattern elicited in the GRNs responding to sinigrin. Some classes of bitter substances, such as quinine, are known to elicit a bursting firing pattern in GRNs, whereas others are not (Dethier, 1976; Chapman et al., 1991). The observed differences in firing pattern in the present recordings was not because of differences in response intensity, because the temporal firing pattern for sinigrin did not change when the concentration was increased to elicit the same number of spikes as quinine. Moreover, the sensilla with neurons responding to sinigrin, but not to quinine and *vice versa*, further support the assumption of two separate GRNs mediating information about the two tastants. An alternative explanation is that one GRN might respond to both substances, eliciting different temporal firing patterns, where two different receptor types and possibly different excitatory transduction pathways are

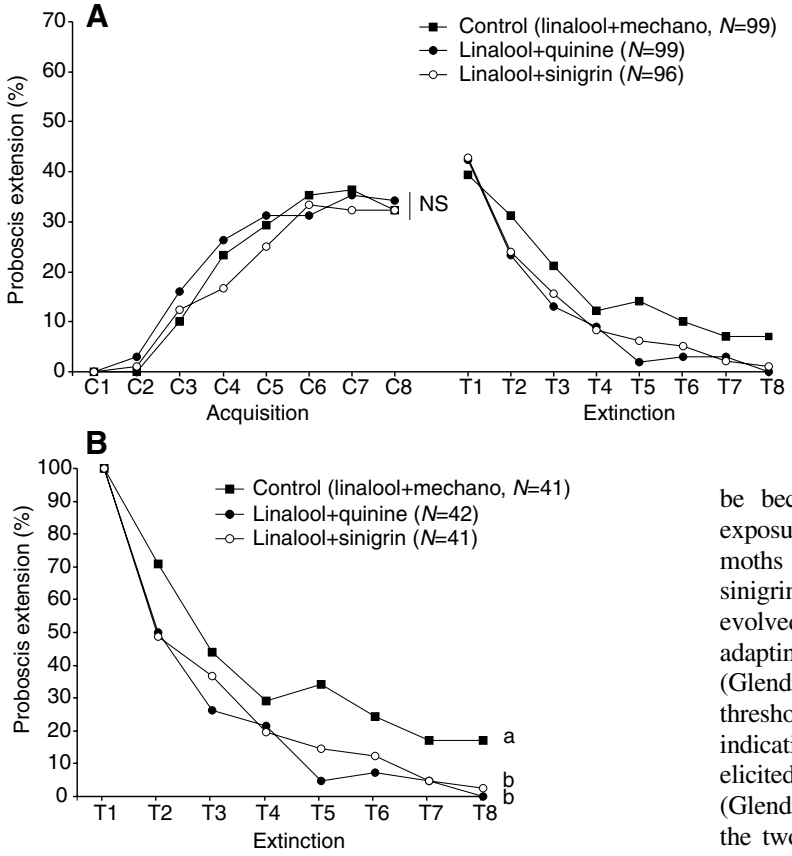

Fig. 5. Acquisition, extinction and facilitated extinction of CRs in moths receiving different treatments during the extinction phase. (A) Acquisition and extinction in moths receiving different extinction treatments. No significant between-group differences were found, indicated by the letters NS (Mann–Whitney tests, $P>0.05$). (B) Extinction curves for moths that have learned the CS. Only moths showing CR at the first extinction test were included. Pairing of linalool with quinine or sinigrin induced a more rapidly decreasing number of responses than the control. Different letters indicate significant between-group differences (Mann–Whitney tests, $P<0.05$).

involved, as suggested in the tobacco hawkmoth *Manduca sexta* larvae (Glendinning and Hills, 1997). Having several receptor proteins for different bitter substances in the same GRN would increase the chances of the insects to detect the components in mixtures of bitter plant substances that are potentially toxic or nutritious. An important presumption for the discrimination mechanism in this case would be that the CNS could differentiate the different spike firing patterns of the same GRNs. Regardless of whether there are one or two GRN types for sinigrin and quinine, our results suggest that the gustatory system of moths is able to discriminate between these two substances.

The putative aversive effects of the two substances were elucidated using pre-exposure (Fig. 4) and facilitated extinction experiments (Fig. 5). In the pre-exposure experiments, only quinine was shown to be significantly aversive, although a clear tendency appeared for sinigrin as well. In the facilitated extinction experiments, both quinine and sinigrin were shown to be aversive. All together, the two experiments showed that both sinigrin and quinine can be aversive to *H. virescens*, with a more consistent effect of quinine relative to sinigrin. Furthermore, during the pre-exposure phase of experiment 3, 24.6% of the insects showed PER to sinigrin stimulation, whereas only 3.5% showed PER to quinine stimulation, supporting the assumption of a stronger aversiveness to quinine. In previous feeding and proboscis extension experiments, sinigrin has been shown to be non-appetitive for *H. virescens* (Blaney and Simmonds, 1988; Jørgensen et al., 2006), but the behavioural effect of quinine has not previously been assayed in this moth. The increasing elicitation of PER to sinigrin during the pre-exposure phase could be because of a familiarity of the substance after several exposures to the moths. Because the substance is not toxic (the moths ingesting it survived), the moths might have learned that sinigrin is harmless in spite of the bitter taste. Insects have evolved a variety of physiological mechanisms for selectively adapting their aversive responses to harmless or toxic substances (Glendinning and Gonzalez, 1995). By contrast, bitter taste thresholds in mammals vary independently of toxicity thresholds, indicating that the bitter rejection response is just as likely to be elicited by a harmless bitter food as it is by a harmful one (Glendinning, 1994). In our experiment, another possibility is that the two-day starvation period before the experiment, which is necessary for PER conditioning in moths, might have caused the insects to elicit PER to substances they would normally avoid.

In the acquisition phase following the pre-exposure phase (experiment 3), we found that previous presentation of linalool (paired with the dry toothpick) caused significantly reduced acquisition performance relative to the untreated group. The dry toothpick elicits a mechanosensory response in the receptor neurons, but presumably this has neither an aversive nor an appetitive influence on the moth. Therefore, it is possible that this group shows a typical latent inhibition phenomenon that has previously been shown in several animals, such as honeybees (Abramson and Bitterman, 1986; Chandra et al., 2001). Whether this is a pure CS pre-exposure effect is not known because there was no control with mechanosensory stimulation alone. During the repeated presentations of CS in the absence of a punishment or a reward, it is believed that the CS is associated with the absence of reinforcement, which leads to a resistance towards re-learning the CS as a predictor for a reward (or punishment) in the subsequent acquisition phase. Other interpretations propose that the CS becomes less and less surprising in the experimental context, and therefore loses meaning throughout the pre-exposure phase (learned inattention) (Lubow, 1997). Most importantly, when the CS was associated with quinine in the pre-exposure phase in our study, the acquisition deficit was significantly increased. In this case, it is possible that the moths built aversive associations between linalool (CS) and quinine as an aversive reinforcer. Thus, at the end of the pre-exposure phase, linalool predicted the presence of a negative stimulus, which had a stronger obstructing effect on acquisition than just an absence of a reward or punishment, as is the case with the mechanosensory treatment.

Quinine has previously been found to have an aversive, but not a reinforcing effect in associative learning in *Drosophila* larvae (Gerber et al., 2004; Hendel et al., 2005). However, conditioned inhibition of the proboscis extension in adult *Drosophila* was observed when the proboscis extension was punished by applying quinine to the foreleg tarsi (DeJianne et al., 1985), supporting that quinine can act as a negative reinforcer. Other experiments on adult *Drosophila* have also shown that quinine supports aversive association with olfactory or other gustatory stimuli (Mery and Kawecki, 2002). In differential conditioning of bumblebees, quinine acted as a negative reinforcer, enabling the insects to discriminate between visual stimuli faster than if the CS was just associated with an absence of reward (Chittka et al., 2003; Dyer and Chittka, 2004). Although our experiments showed that quinine had an aversive effect in moths, a definite proof for a negative reinforcing effect of quinine is still lacking, because we have not controlled for possible non-associative effects of quinine. However, repeated presentations of quinine, sinigrin and the dry toothpick did not seem to reduce the appetitive motivation compared with the untreated control. Future experiments including a pre-exposure phase in which moths receive unpaired presentations of CS and quinine will constitute a control for the formation of aversive CS–quinine associations.

In experiment 3, the group receiving sinigrin treatment showed the same tendency towards reduced acquisition and retention as the quinine group, although its performance was not significantly lower than that of the control group. Possibly, testing an even larger number of animals, or presenting a higher concentration of sinigrin could have yielded a significant difference. To confirm a possible aversive effect of the two tastants, we performed facilitated extinction experiments (Fig. 5), showing that both quinine and sinigrin enhanced extinction, compared with the control. As before, we may explain the results in terms of the formation of aversive associations. Thus, the moths would learn two associations after one another; during acquisition, they would form CS–sucrose associations acting positively on PER, and during the second phase they would form CS–quinine or CS–sinigrin associations, causing a resistance to elicit PER. Responses would thus reflect a balance between the two types of associations, the aversive association progressively overbalancing the appetitive association. In addition, a second type of explanation could apply in the facilitated extinction experiment. Increased extinction with the bitter substances could be a form of operant learning, because the action of PER was punished by providing the bitter substance to the antennae and the proboscis. To test for such effects, adequate controls can be applied, such as the use of omission and yoked groups, in which the bitter reinforcement of the moths would be uncoupled from the PER.

In both the pre-exposure and the facilitated extinction experiments, it was shown that quinine, and to a lesser extent sinigrin, detected by GRNs on the antenna, had aversive effects on the moth behaviour. Although it was not the aim of the present work to study aversive learning in moths, it is possible that the effect found of both impaired acquisition (experiment 3) and facilitated extinction (experiment 4) is caused by the formation of CS–bitter tastant associations. Choice tests could perhaps reveal such associations. For example, in a PER situation, one group of moths could be exposed to an odour combined with quinine or sinigrin, whereas another control group could be exposed to an odour of similar salience combined with no stimulus. If the treated moths in a subsequent choice test actively choose the odour combined with no stimuli, then a formation of CS–bitter tastant association could be proven. Another way of testing this would be to let the same moth receive one odour with quinine or sinigrin and another odour with no other stimulus in a PER situation, and subsequently let the moth choose between odours.

If quinine and sinigrin were negative reinforcers, we would expect that the reinforcement signals triggered by quinine and sinigrin would converge with the olfactory pathway to form associations in the moth, possibly involving a modulatory neuron with opposite effect to the VUMmx1 in honeybees. In honeybees (Vergoz et al., 2007) and in *Drosophila* (Schwaerzel et al., 2003), dopamine has been found to be the neurotransmitter involved in aversive olfactory learning with electric shock as punishment. In crickets (Unoki et al., 2005; Unoki et al., 2006), dopamine was involved in odour– and colour–salt punishment associations. Moreover, in *Drosophila* larvae, activation of dopaminergic neurons in association with an odour stimulus was sufficient to create an aversive olfactory memory (Schroll et al., 2006). All these data point towards a prominent role of dopaminergic modulatory neurons in odour–punishment associations, and in the formation of aversive olfactory memories. The confirmation of the existence of odour–bitter taste associations in moths and their dependency on such dopaminergic reinforcement systems will be the focus of future work.

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