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20-Hydroxyecdysone Deters Oviposition and Larval Feeding in the European Grapevine Moth, *Lobesia botrana*

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Abstract European grapevine moth females (*Lobesia botrana*, Lepidoptera Tortricidae) select an oviposition site by tasting the host plant surface and then gluing a single egg on berries from grapes or from several other host plant species. In doing so, females should avoid ovipositing on plants that are detrimental to their progeny. Do they sense the same deterrent compounds as larvae, despite the fact that they do not have access to the same compartments of the plants? We tested this hypothesis with 20-hydroxyecdysone (20E), purified from *Leuzea carthamoides*. Phytoecdysteroids are usually found inside plant tissues and accessible to larvae in an aqueous phase, while adults would access them only through the epicuticular wax. We first confirmed that larvae avoid feeding on 20E and that they taste 20E with their lateral sensilla styloconica, at a threshold of 10^{-6} M. Then, we tested whether adult females avoid ovipositing on glass spheres sprayed with 20E. When given a choice, females avoided laying eggs on a treated surface, at a threshold of 8 ng/cm^2. In addition, they deposited significantly fewer eggs in the presence of 20E. Presuming that legs play an important role in assessing the oviposition substrate, we assessed the sensitivity of their taste receptors. In females, 14 taste sensilla are located on the ventral side of the last tarsus of the prothoracic leg. One group of these sensilla house one neuron that is sensitive to 20E, with a detection threshold of about 10^{-7} M. The same molecule is thus sensed both in larvae and adults of *L. botrana* where it respectively inhibits feeding and oviposition.

Key words *Lobesia botrana* · 20-hydroxyecdysone · taste electrophysiology · oviposition · feeding behavior · secondary compounds · host plant selection

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Introduction

Phytoecdysteroids (PE) are plant secondary compounds that include more than 200 molecules (Dinan et al., 2001). One of the most common is 20-hydroxyecdysone (20E). Phytoecdysteroids are found in about 5–6% of the species in most plant genera (Dinan 1998). Since PEs are found in primitive plants such as ferns, it was assumed that these secondary compounds appeared very early in plant evolution. PEs are structural analogs of the molting hormone of insects and are usually considered as defensive compounds against insects. Sensitive species living on plants with low levels of PEs, such as *Bombyx mori* or *Pectinophora gossypiella* (Kubo et al., 1983), are disturbed in their growth and food intake when PEs are incorporated into their diet (Tanaka and Takeda, 1993). *Manduca sexta*, which feeds on *Solanaceae* where some species contain significant level of ecdysteroids, is tolerant to PEs (Dinan 1998). Other polyphagous species are resistant, such as several Noctuidae including *Spodoptera littoralis* (Blackford et al., 1996).

Given the large botanical distribution of PEs, we hypothesize that phytophagous insects have been confronted with these defenses for a long time and that they may have evolved different strategies to cope with them. One way to escape PE intoxication by larvae or juvenile stages is to avoid exposure. This hypothesis is supported by the observation that several larval Lepidoptera are able to avoid feeding on plants or diets treated with PEs (Tanaka et al., 1994; Marion-Poll and Descoins, 2002). This is true even for resistant species such as *S. littoralis* since neonate larvae avoid PE-treated diet (Marion-Poll and Descoins, 2002). This avoidance is mediated by deterrent taste neurons that detect PEs at a low threshold (Ma 1969; Tanaka et al., 1994; Descoins and Marion-Poll, 1999). If PEs exert a selection pressure on phytophagous insects, one would expect females to avoid laying eggs on plants producing them in order to maximize the survival chances of their progeny and, thus, limit exposure of neonate larvae to the toxicant.

How could females achieve such detection? An obvious strategy would be to use the same type of taste receptors as those expressed in larvae, in order to detect PEs on plants' surfaces or within their tissues. To begin testing whether this strategy could be used in polyphagous insects, we chose the European grapevine moth (EGVM) *Lobesia botrana* Denis & Schiffermüller (Tortricidae, Lepidoptera) as a model insect. Although this species is best known as a major pest of grapes, it has been found on more than 20 plant species including rosemary, olive trees (only flowers), privet, or *Daphne gnidium*, which is sometimes considered as its ancestral host plant (Bovey 1966; Maher 2002; Thiéry 2005; Maher and Thiéry, 2006). In this species, first instars can hardly disperse and would not be able to compensate female mistakes (Torres-Vila et al., 1997; Thiéry and Moreau, 2005;). Female decisions to oviposit on different plants or different phenological stages of grapes (Thiéry and Gabel, 2000; Maher et al., 2001) have a strong influence on the fitness of future larvae. Except for grapes, most of the host plants of *L. botrana* are toxic or known for their pharmacological use, but none has been reported to produce PEs in large amounts (Dinan, personal communication). We expect all stages of EGVMs to be sensitive to PEs and possibly to avoid them, a view that is supported by earlier observations on larvae that showed that PEs, and particularly 20E, added to the diet, caused numerous abnormalities in growth, development, and metamorphosis (Mondy et al., 1997). Behavioral studies and electrophysiological observations indicated that larvae do detect PEs from plant extracts, in their diets, by using their taste receptors (Mondy et al., 1999).

In this work, we examined the feeding behavior and oviposition responses of EGVM to 20E and evaluated the sensory responses of taste sensilla of fifth instar and adult females to 20E. In doing so, we described the distribution of the contact chemoreceptors present on the

foreleg of the females of this species, and propose to segregate these taste receptors into three functional groups, based on their responses to a limited set of stimuli.

Methods and Materials

Insects A colony of *L. botrana* issued from insects sampled in Sauternes vineyard in 1998, was reared on a semisynthetic diet (Maher, 2002) at INRA Bordeaux. They were maintained without diapause at 22±1°C under a 16:8 L/D photoperiod and at 60–65% relative humidity. Electrophysiological and behavioral observations were performed on adults from this strain in 2002–2004 and on larvae in 2005.

Chemicals 20-Hydroxyecdysone was purified from *Leuzea carthamoides* (95% min purity with minor ecdysteroids mixtures of polypodine B, ajugasterone C, and inokosterone; SciTech, Prague, Czech Republic, and R. Lafont, Univ. Paris 6). It was dissolved in ultrapure water or in ethanol according to the different bioassays. For electrophysiology, test solutions were dissolved in 10^{-4} M potassium chloride (KCl, Prolabo) to ensure proper electrical conductivity. 20E was used at concentrations ranging from 10^{-8} to 10^{-3} M for electrophysiological recordings and 10^{-7} to 10^{-2} M for behavioral tests.

Two-Choice Feeding Experiments To determine 20E effects on the feeding behavior of *L. botrana* larvae, two-choice tests were conducted. Two disks of artificial food were placed diametrically opposed on the bottom of a plastic Petri dish. The size of the food disk and of the feeding arenas (Petri dish) varied according to the stage of the larvae (L2=8 mm diam× 2 mm high food disks into a 3-cm-diam Petri dish; L3=12 mm diam×2 mm high food disks into a 5.5-cm-diam Petri dish). One disk was treated with 20E 10^{-3} M in ethanol (12 μl for L2 and 22 μl for L3) and the other with ethanol only (control disk). Given the volume of the diet offered to the larvae, this corresponds to 49 $\mu g/cm^3$. A control test was conducted each time in parallel using disks treated with solvent only. Assuming that the solvent had evaporated after 30 min, four larvae starved for 4 hr were introduced into the center of each arena. We measured the number of larvae located on the disks or within a radius of 1 cm around the disks, after 2, 24, 48, and 72 hr. These experiments were repeated 15 times (240 larvae tested). The results were analyzed with nonparametric statistics. Preferences between treated and nontreated disks were compared by using the Wilcoxon test (WX). To determine if there was an increase or a decrease in the deterrency, the number of larvae placed onto or close to the treated disk at different times was analyzed with the Mann–Whitney (MW) test.

Oviposition Tests Oviposition responses of gravid females of *L. botrana* were examined by using a two-choice bioassay (Maher and Thiéry, 2004a). Test solutions were sprayed onto rows of four glass spheres (diam: 1.6 cm, spaced by 2 cm), placed on a glass plate (75× 25 mm). Each row was treated either with 0.5 ml of diluted 20E (ranging from 10^{-7} to 10^{-2} M) or with 0.5 ml of pure water (control). These spheres were dried in an incubator at 40°C for 90 min. Given the size of the spheres, the resulting concentration of 20E ranged from 0.8 ng to 80 $\mu g/cm^2$. Two rows of spheres, spaced by 6 cm, were placed into the bottom of a plastic box. This box was covered with baize, which prevents oviposition on the experimental area except on the spheres. After introducing a single female per box, each box was closed with a veil and placed into an environmental chamber maintained at 16:8 L/D photoperiod

and 23°C. Females were removed 16 hr later, and the number of eggs deposited on the spheres was counted.

Experiments were done on 3–4 day old females. Recently emerged females and males (less than 12 hr) were grouped one night in a cage. Females were isolated the following night in glass tubes and checked for oviposition in the morning. We used only females that laid eggs during this first night. By using this procedure, more than 90% of females were ready to oviposit during the experiments. Experiments that started at 4 P.M. were run on individual females with a minimum of 20 females per dose.

Oviposition scores were compared with nonparametric statistics. The total number of eggs laid during one experiment was compared by using the MW test. The preferences between treated and nontreated items in each experiment were compared by the WX test. For control experiments, the spheres were arbitrarily assigned a label A or B before experiments. Pilot experiments showed that the females ignored such labels. For convenience, data are presented in the text as mean±SE of the mean.

Electrophysiology Recordings from fifth instars were obtained from isolated heads, mounted on a reference silver electrode. The preparation was oriented in such a way that the galea was extruded, thus giving access to the lateral and medial sensilla styloconica.

Adult females were briefly narcotized with carbon dioxide, decapitated by using fine scissors, and then fixed onto a polystyrene block. A reference electrode, filled with Ringer's solution, was inserted into the abdomen. One of the prothoracic legs was taped to the support in order to expose the sensilla from the ventral side of the fifth tarsomere. Recordings were performed on sensilla chaetica type b (Maher and Thiéry, 2004b) by capping them with a capillary tube (10 μm tip diam) containing the test solution, filled just before recording.

Electrical signals were recorded with an amplifier (TastePROBE DLP-02; Syntech, Hilversum, the Netherlands) with automatic offset compensation (Marion-Poll and Van der Pers, 1996) and further amplified and filtered (CyberAmp, 320, Axon Instruments; gain: 1000; eight poles Bessel band-pass filter: 0.1–30 to 2800 Hz). Data were recorded and stored on a computer with a 16-bit A/D conversion card (DT9803; Data Translation, USA) under the control of a custom software, dbWave (Marion-Poll 1996). Each recording lasted 2 sec and was triggered by a pulse delivered by the amplifier on the initial contact of the electrode with the sensillum. Recordings were analyzed by using dbWave, in order to detect and sort spikes according to their amplitude and shape using interactive procedures. Responses to the different stimuli were evaluated by counting the total number of spikes during the first sec of the recording.

Series of recordings were done with 10^{-4} M KCl (also used as electrolyte of nonconductive solutions) and followed by an ascending series dilutions of 20E (10^{-8} to 10^{-3} M step 10). Consecutive stimuli on a sensillum were applied with an interval of at least 2 min in order to avoid adaptation. For adults, the position of each recorded sensillum was carefully noted in order to compare our results with earlier observations (Maher 2002; Maher and Thiéry, 2004b; Maher et al., 2006).

Results

Two-Choice Feeding Experiment Larvae preferentially fed on nontreated disks rather than on disks treated with 20E (Fig. 1). The difference was highly significant for all observation times for L2 and L3 larvae. Both instars spent significantly more time on or close to the food disk (treated or not) than wandering elsewhere, except during the first 24 hr. Mann–

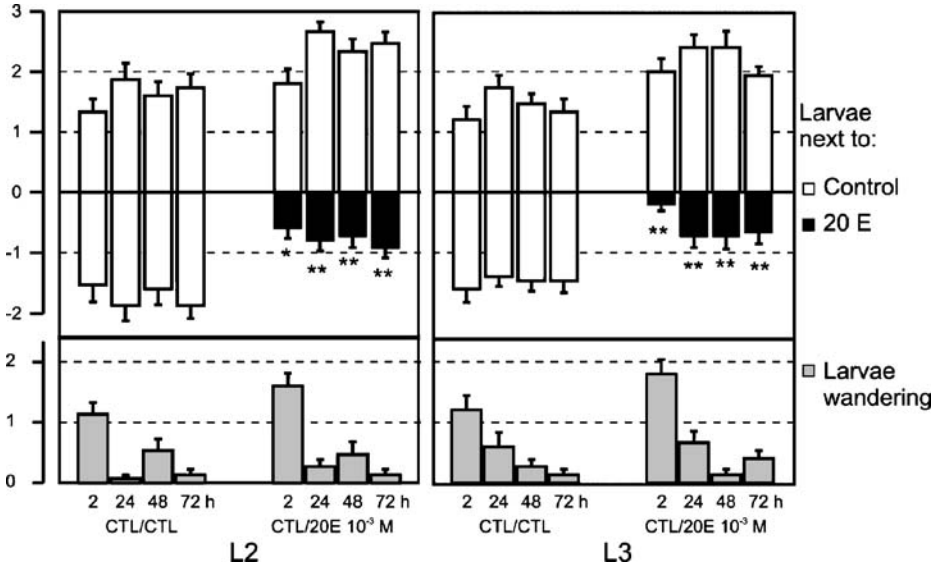

Fig. 1 Behavioral choices of second and third instars of *Lobesia botrana* between two food disks treated or not with 20E. White bars: control diet (solvent alone); black bars: treated diet (20E 10^{-3} M). Bars indicate the number of larvae (mean±SEM) found on or close to each disk at 2, 24, 48, and 72 hr after the start of the experiments. N=15 (with four larvae each). Wilcoxon test, 20E vs. control *P<0.05, **P<0.01

Whitney test showed that there was no increase or decrease in deterrence according to time. In the control tests (using disks with only solvent), L2 and L3 larvae fed equally on both disks.

Sensitivity of Larvae to 20E In fifth instars, a strong activity in response to 20E was found in the medial and lateral sensilla (Fig. 3). Responses originated from one cell that increased its activity from 10 to 80 spikes sec^{-1}, respectively, for 10^{-5} to 10^{-3} M of 20E (Figs. 2 and 3). At lower concentrations (10^{-6} and 10^{-5} M), the response was initiated after a latency of 50–100 ms as in the adults.

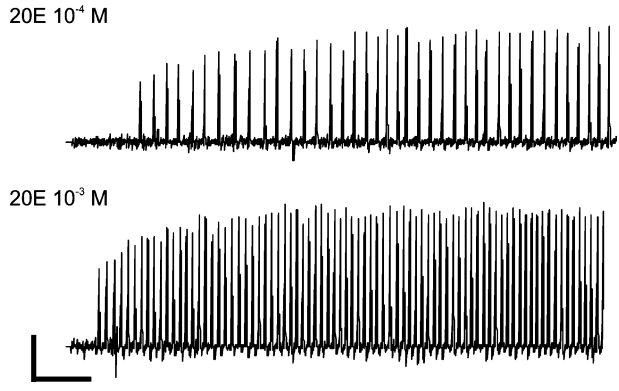

Fig. 2 Response samples of fifth instar lateral sensilla to 20E 10^{-4} and 10^{-3} M. Trace duration = 1 sec. Vertical bar = 1 mV, horizontal bar = 100 ms

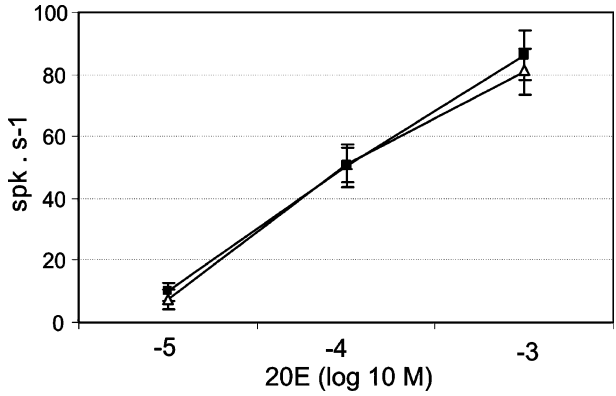

Fig. 3 Electrophysiological responses of fifth instars to ascending concentrations of 20E. Black squares: responses from medial sensilla styloconica; Open triangles: responses from the lateral sensilla styloconica. Each data point represents the average number of action potentials during the first second of the recordings (mean±SEM; N=10 stimulations)

Oviposition Tests Females laid fewer eggs on treated glass spheres (T) than on nontreated ones (NT) at intermediary doses of 20E, namely, 10^{-6} M (T: 8.1±6.9 vs. NT: 16.7±9.8; WX, P=0.003), 10^{-4} M (T: 4.7±6.9 eggs vs. NT: 19.1±14.9 eggs; WX, P<0.001) and 10^{-3} M (T: 5.2±8.6 eggs vs. NT: 16.5±15.1 eggs, WX, P<0.001). This was not the case for low 20E concentrations (10^{-7} M) nor for the highest (10^{-2} M), where the scores were not statistically different between the treated and untreated spheres (10^{-7} M: T: 14.5±10 vs. NT 20.2±8.6; 10^{-2} M T: 11.2±8.2 vs. NT: 15.1±14 (Fig. 4). The behavioral threshold to 20E is close to 10^{-6} M, a concentration that corresponds to 30 ng of 20E per sphere or 0.037 ng per mm^2. In addition, the total number of eggs deposited on both glass spheres is significantly reduced at concentrations higher than 10^{-6} M (Mann Whitney: NT vs. 10^{-6} M: P=0.002; NT vs. $10^{-4,\ 3,\ 2}$ M: P=0.008).

Tarsal Taste Sensilla On the ventral side of the fifth tarsomere, adults of both sexes bear a ring of 10 sensilla and four medial sensilla that were described as sensilla chaetica type I, or "sensilla b" (Maher, 2002; Maher and Thiéry 2004b). Although they look morphologically

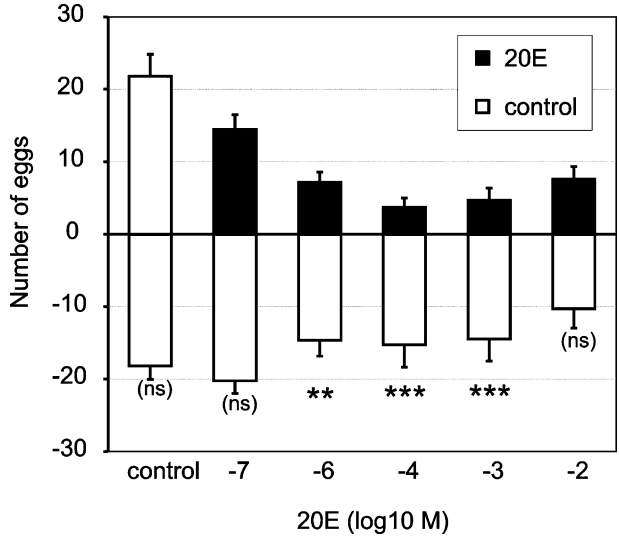

Fig. 4 Behavioral choices of females *L. botrana* given a choice between glass surrogates treated or not with 20E (from 10^{-7} to 10^{-2} M). Number of eggs laid by one female according to the substrate (N>20). Wilcoxon test: 20E vs. control **P<0.01, ***P<0.001

identical, they diverge in their responses to KCl. We propose to distinguish three groups among them (Fig. 5):

(a) Group I sensilla (Fig. 6a) that fires a few spikes of large amplitude at the beginning of the stimulation (amplitude = 1.5–2 mV; 1.7±0.2 spikes sec^{-1} (average±SEM), N=32).
(b) Group II sensilla responds tonically with a single class of spikes (0.3 mV; 57.2± 2.7 spikes sec^{-1}, N=27).
(c) Group III sensilla (Fig. 6b) that responds phasitonically with two classes of spikes: one of large amplitude (1–2 mV; 2.6±0.3 spikes sec^{-1}, N=6 recordings) and a second one of smaller amplitude (0.2 mV; 48.4±6.4 spikes sec^{-1}). On some recordings, this second cell started to fire after a variable delay.

The large amplitude spikes observed at the beginning of the recordings (Fig. 6a, b) differ from the activity of mechanoreceptors. When a deflection was imposed on the hair by moving the capping electrode laterally, these mechanoreceptors elicited spikes of much smaller amplitude (1–2 mV) superimposed on a downward deflection of the baseline during the bending (data not shown). Only sensilla of group II responded to 20E; therefore, all subsequent recordings were performed on them.

Sensitivity of Adults to 20E Although two spike amplitudes were observed in the responses of group II sensilla to 20E, we consider that only one neuron was activated (Fig. 7a, b). One neuron consistently fired spikes of medium amplitude (0.3–0.4 mV), but its activity did not vary with changes of 20E concentration (Fig. 8a), except by a small decrease of activity at 10^{-3} M (46.5±3.02 spikes sec^{-1}). This cell fired also in the presence of NaCl or of sugars (data not shown).

A second cell fired spikes of smaller amplitude (0.2–0.3 mV) in the presence of 20E, starting at concentrations of 10^{-8} or 10^{-7} M, depending on the preparation. Its activity increased with 20E concentration, ranging from 8.25±1.61 spikes sec^{-1} at 10^{-7} M to a maximum of 22.5±2.79 spikes sec^{-1} at 10^{-5} M (Fig. 8b). Its response decreased from 10^{-4}

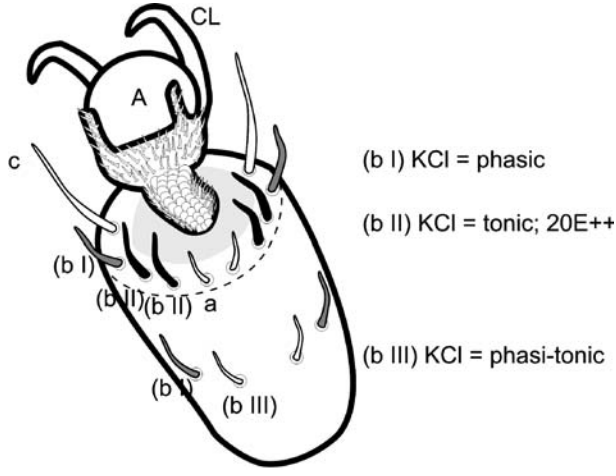

Fig. 5 Schematic diagram of the fifth tarsomere of *L. botrana* viewed from the ventral side and sensilla types according to their shape and their electrophysiological responses. CL: claw; A: arolium; a, c: sensilla chaetica type II; bI, II, III: sensilla chaetica type I

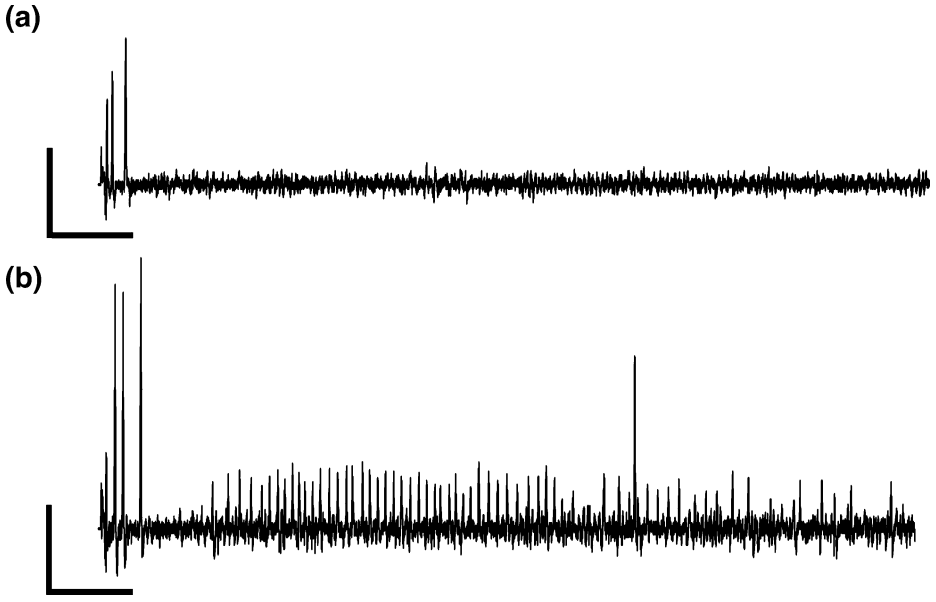

Fig. 6 Response samples of "b" sensilla to 10^{-4} M KCl: (a) sensilla from group I; (b) sensilla from group III. Trace duration=1 sec. Vertical bar=400 μV; horizontal bar=60 msec

to 10^{-3} M. The responses were tonic and regular over the observation period (2 sec). At low concentrations of 20E, it started to fire with a delay of 200–300 ms. This delay shortened at higher concentrations of 20E.

Discussion

In this work, we demonstrate that *L. botrana* adults and larvae can detect 20E. Electrophysiological recordings indicate that taste receptors responding to 20E are present

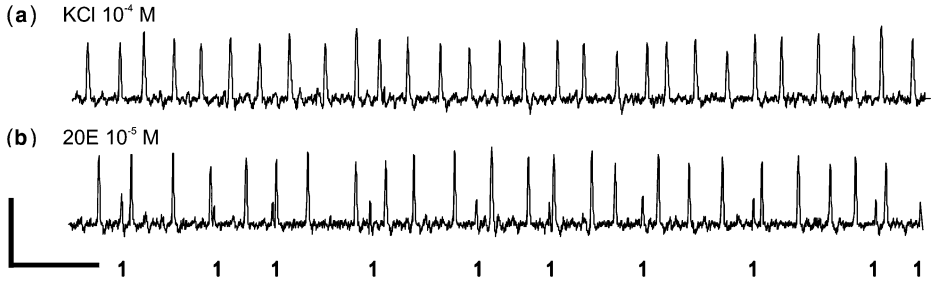

Fig. 7 Response samples of sensilla chaetica b to (a) 10^{-4} M KCl and to (b) 10^{-7} M 20E. Trace duration = 500 msec; vertical bar=400 μV; horizontal bar=50 msec. Spikes amplitudes fall into two classes: medium-amplitude spikes (class 0) are observed in both types of recordings; small-amplitude spikes (class 1) are observed only in the presence of 20E

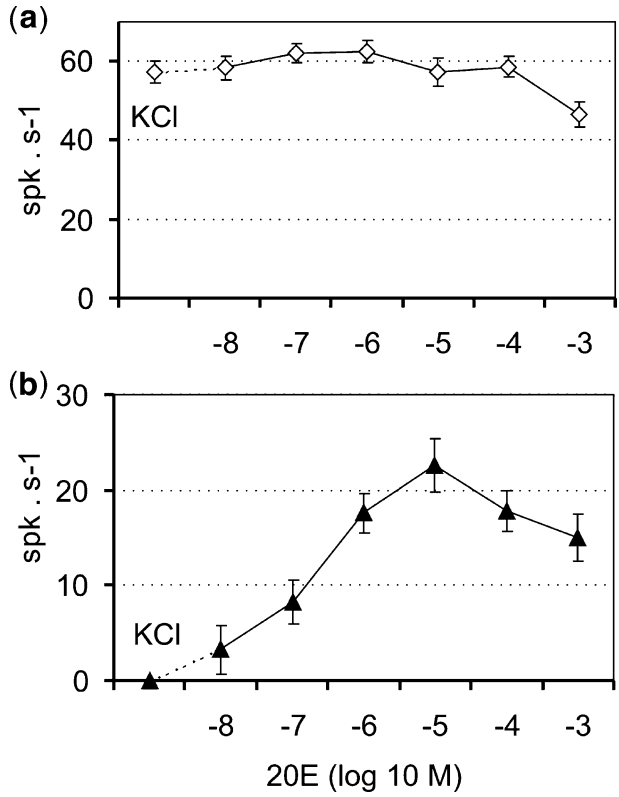

Fig. 8 Electrophysiological responses of sensilla chaetica type I (sensilla b—group II) to 10^{-4} M KCl and to ascending concentrations of 20E. (a) White diamonds: responses from class 0 spikes (of medium amplitude). (b) Black triangles: responses from class 1 spikes (of small amplitude). Each data point represents the average number of action potentials during the first second of the recording (mean± SEM; *N*=12–25 recordings)

in both stages, and our behavioral studies show that 20E has a deterrent effect on feeding and oviposition. These observations are new because the impact of phytoecdysteroids has been mostly evaluated on larval insects. Using behavioral and toxicity tests on larvae, Blackford and Dinan proposed that species can be rated as sensitive, semitolerant, or tolerant to PEs (Blackford et al., 1996, 1997; Blackford and Dinan, 1997a, b, c). Our own observations on second and third instars as well as former studies on *L. botrana* (Mondy et al., 1997) suggest that this species is sensitive to 20E. These behavioral responses are probably mediated by taste neurons located in the lateral and the medial sensilla of the maxillary palps in which we recorded vigorous and sustained monocellular responses to 20E, at a threshold of 10^{-5} M.

Because larvae are sensitive to 20E, we expected adults to avoid ovipositing on a substrate in the presence of 20E. This hypothesis was tested by monitoring eggs laid on berry surrogates treated with 20E. We observed that 20E-treated berries were avoided and that females laid fewer eggs in the presence of 20E. The most effective concentration of 20E was 10^{-4} M, which is equivalent to 6.3 μg of 20E per sphere, i.e., 0.8 μg of 20E per cm^2. At higher concentrations of 20E, discrimination between treated and nontreated spheres was less effective. The total number of eggs deposited during the observation period decreased with 20E in a dose-dependent manner, even at higher doses when females did not seem to discriminate between treated and nontreated surfaces.

As in larvae, detection of 20E in adults is certainly mediated by taste sensilla. We studied taste sensilla located on the distal tarsi. From the 16 sensilla present on the ventral

side of the last tarsus of the prothoracic legs, only four were found to house a neuron stimulated by 20E. This neuron fired spikes of small amplitude and responded with a detection threshold of 10^{-7} M. Its response was maximal at 10^{-5} M and decreased slightly at higher concentrations. This decrease is probably attributable to adaptation and to our experimental protocol, because we presented 20E as a series of increasing concentrations. It is likely that we would need to respect resting intervals longer than 2–3 min at these high doses. While 20E stimulated this cell firing small amplitude spikes, a second cell was present in the recordings, characterized by spikes of medium amplitude. Its firing activity did not change in the presence of 20E, except at high concentrations where its activity decreased. This cell could correspond to the ubiquitous water cell found in many taste sensilla of other insects (Inoshita and Tanimura, 2006).

Our anatomical and electrophysiological observations complete earlier descriptions of taste tarsal sensilla in *L. botrana* (Maher, 2002; Maher and Thiéry, 2004b; Maher et al., 2006). By using KCl and 20E as test stimuli, the responses of these taste receptors could be classified into three groups, with only group II sensilla responding to 20E. It remains to be determined if group I–III sensilla respond to other deterrent compounds or if group II sensilla are specialized in triggering an avoidance behavior. The functional arrangement of these tarsal sensilla is reminiscent of what we found with *Ostrinia nubilalis* (Marion-Poll et al., 1992; Marion-Poll and Calas, unpublished data) and with *Drosophila melanogaster* (Meunier et al., 2003), where only a subset of the tarsal sensilla are sensitive to a given deterrent molecule. This means that behavioral decisions taken in the presence of deterrent compounds is taken from integration of responses from several types of sensilla from one or more appendages.

Although similar taste cells responding to 20E have been found in *L. botrana* larvae, as well as in larvae of several other species of Lepidoptera (Ma, 1969; Tanaka et al., 1994; Descoins and Marion-Poll, 1999; Marion-Poll and Descoins, 2002), it is the first time that responses to this compound have been demonstrated in adult Lepidoptera. We postulated that if 20E presented a negative effect on growth and development of the larvae of *L. botrana* (Mondy et al., 1997), this ecdysteroid could be perceived by adults and particularly females as a deterrent compound for oviposition. That females could detect deterrents avoided by larvae is ecologically relevant, since females are much more mobile than larvae. A similar situation was observed in *Trichoplusia ni*, where oviposition of adult females was deterred by toosendanin, a limonoid from the bark of *Melia azedarach*, which inhibits feeding of larvae of this species (Akhtar and Isman, 2003).

Although we have examined the effect of 20E on adults only in regard to oviposition, and have speculated that detection of 20E is relevant in avoiding plants that would lessen the chance of larvae survival, it is still possible that 20E could impact directly on adults, a hypothesis that was not tested in this work. Adults may need to avoid ingesting phytoecdysteroids present in the nectar of flower or from guttation liquids, especially considering that the maturation of the ovocytes requires 20E. Avoiding 20E has been noted in Heteroptera, where 20E was found to deter drinking in three species among four tested (Schoonhoven and Derksen-Koeppers, 1973).

The ecological significance of 20E avoidance in the European grapevine moth may have to be considered in a broader frame than the relations between the EGVM and its host plants. Given the number of plants producing PEs in different genera and scattered observations in the literature concerning the detection of PEs, it is possible that PEs have been conserved in many phytophagous insect species, not only in larvae as discussed by Blackford and Dinan (1997a), but also in adult insects.

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