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RESEARCH ARTICLE

Bitter stimuli modulate the feeding decision of a blood-sucking insect via two sensory inputs

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ABSTRACT

The gustatory system of animals is involved in food quality assessment and controls the feeding decision of an individual confronted with a potential alimentary source. Triatomines are haematophagous insects that feed on vertebrate blood. Once they reach a potential host, they walk over the host skin searching for an adequate site to pierce. Then, they insert their stylets and take a first sampling gorge to decide whether food is acceptable. Our work reveals that the presence of bitter compounds inhibits the feeding behavior of these bugs. Firstly, triatomines decreased their feeding behavior if substrates spread with quinine or caffeine were detected by external receptors localized exclusively in the antennae. Morphological inspections along with electrophysiological recordings revealed the existence of four gustatory sensilla located in the tip of the antenna that respond to both bitter tastants. The absence of these bitter detectors by antennal ablation reversed the observed feeding inhibition evoked by bitter compounds. Secondly, once triatomines pumped the first volume of food with bitter compounds (quinine, caffeine, berberine, salicin), a decrease in their feeding behavior was observed. Morphological inspections revealed the existence of eight gustatory sensilla located in the pharynx that might be responsible for the internal bitter detection. Finally, we found that a brief pre-exposure to bitter compounds negatively modulates the motivation of bugs to feed on an appetitive solution. Results presented here highlight the relevance of bitter taste perception in the modulation of the feeding behavior of a blood-sucking insect.

KEY WORDS: Feeding behavior, Bitter, Taste sensilla, Plasticity, Blood-sucking

INTRODUCTION

Taste provides reliable information about the quality of food and can contribute to the discrimination between nutritious and harmful feeding sources. If food quality assessment is followed by an associated decision-making, this process might acquire important physiological consequences for animals. For example, to prevent the ingestion of toxic food, the gustatory system of an individual can detect the presence of particular substances or tastes that signal toxicity. Many toxins or poisonous substances have a bitter taste for humans. Although there is no unique chemical identity for bitter compounds (they are defined anthropocentrically as substances perceived by our gustatory sense as bitter), most have been shown to elicit rejection or aversive behaviors in many mammals and insects (Yarmolinsky et al., 2009). Bitter perception might have then evolved as a key defense mechanism against the ingestion of harmful substances.

In insects, the detection of tastants starts primarily at the gustatory receptor neurons (GRNs) located within taste hairs or sensilla that occur externally in different parts of the body and appendages (e.g. legs, antennae, proboscis, margin of wings or ovipositor, among others) (reviewed in Chapman, 2003). Each GRN is tuned to a particular taste modality (e.g. salty, sweet, bitter) by the presence of specific membrane gustatory receptor proteins (Clyne et al., 2000; Dunipace et al., 2001; Scott et al., 2001; Robertson et al., 2003). Different groups of phytophagous insects belonging to different orders such as Orthoptera, Lepidoptera, Coleoptera and Diptera (Chapman et al., 1991; Schoonhoven and van Loon, 2002; Messchendorp et al., 1998; Meunier et al., 2003) have bitter-sensitive GRNs that elicit aversive responses when activated. Bitter substances are biologically relevant in animal–plant relationships, as many plants produce these substances for protection from herbivores and insect pests (Wittstock and Gershenzon, 2002), and can modulate the feeding behavior of phytophagous insects (Bernays and Chapman, 2000; Gegear et al., 2007; Glendinning et al., 2001).

The gustatory perception of blood-sucking insects has so far received little attention. During the late 1960s and up to the 1980s, several research groups focused on identifying an adequate dietary composition to artificially breed haematophagous insects. As a result, the characterization and identification of phagostimulants have been largely reported in different groups of blood-feeders (Galun, 1967; Galun and Kindler, 1968; Friend and Smith, 1971; Friend and Stoffolano, 1983; Galun et al., 1988). In most of these cases, the presence of adenosine nucleotides, such as ATP or other similar purinergic compounds, seemed to be decisive for food acceptance (Friend, 1965; Friend and Smith, 1971; Smith and Friend, 1982; Galun et al., 1985). On the contrary, less information is available about the existence of anti-feedant compounds for haematophagous insects and their influence on food preference (Ignell et al., 2010; Kessler et al., 2013). New data in mosquitoes showed the occurrence of *Drosophila* orthologous gustatory receptor genes (Kent et al., 2008; Bohbot et al., 2014; Sparks et al., 2014) that might share a similar function, such as the one required for caffeine detection (Sparks et al., 2013).

Rhodnius prolixus Stål is a triatomine bug, vector of Chagas disease in Latin America (WHO, 2012). As in many other blood-feeders, they find their hosts by following host-emitted cues such as CO_2, chemical volatiles (short-chain carboxylic acids, L-lactic acid), water vapor and heat (Flores and Lazzari, 1996; Guerenstein and Guerin, 2001; Barrozo et al., 2003; Barrozo and Lazzari, 2004a; Barrozo and Lazzari, 2004b; Barrozo and Lazzari, 2006). Triatomines feed exclusively on vertebrate blood. Once they find a

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List of abbreviations

ANT–	blocked antennal input
AS	appetitive solution
BER	berberine chloride hydrate
CAF	caffeine
GRN	gustatory receptor neuron
INT	intact animal
LEG–	blocked leg input
QUI	quinine hydrochloride
SAL	D-(-)salicin
SEM	scanning electronic microscopy
WAT	water

potential host, triatomines start a feeding process that involves several steps. First, they walk over the host skin and search for a place to puncture in search of blood, using mainly their fine thermal sense to find subcutaneous hot blood vessels (Ferreira et al., 2007). However, up to now it was still uncertain whether these insects make use of other sensory inputs (e.g. gustatory or olfactory) to determine the quality of the substrate. Then, if the insect decides to puncture the host's skin, and once the maxillae and mandible are inside the host body forming the alimentary canal, the cibarial pump musculature produces contractions, sucking firstly a small quantity of blood. Only if the ingested blood fulfills the insect's feeding requirements does the animal continue feeding; if not, the animal leaves the host and searches for another (Smith and Friend, 1970).

Despite the accumulated knowledge about how blood-feeders find a host and which are the olfactory-relevant host-emitted cues used to accomplish this task, much less information is available about how these insects assess the quality of food and ultimately how they choose a host based on their gustatory preferences. We postulate that the gustatory sense might be important at two different periods of the feeding behavior: (1) once the insect reaches the host skin and has to decide whether to pierce; and (2) once it takes a first gorge of blood and has to decide whether the diet is adequate.

In this work, we investigated the effects that different bitter tastants might exert in modulating the decision-making of triatomines during two discrete phases of the feeding process: the substrate probing phase and the sampling phase. Furthermore, we looked for the chemosensory organs involved in the detection of these aversive compounds at both levels. Finally, we evaluated whether the feeding response of these insects can be modulated by a previous chemical experience to bitter compounds.

RESULTS

In this work we analyzed the role of the gustatory sense in the feeding decision of a blood-feeding insect. We studied how *R. prolixus* assesses the food quality at two moments of the feeding process: (1) once the insect reaches the host skin and by external contact estimates the quality of a potential food source (substrate probing phase); and (2) once the bug has pierced the host skin and taken a first gorge of blood to decide whether the diet is adequate (sampling phase). In particular, we analyzed whether these haematophagous insects perceive bitter compounds and how these compounds modulate their feeding behavior.

Can an external chemical assessment of the substrate modulate the feeding decision of insects?

The decision of a haematophagous insect of whether to pierce might be mediated by taste receptors that could be present in any part of their body. To date, no reports have focused on the importance that external taste sensors might have as a primary detection system controlling food preferences in blood-sucking insects. Likewise, it is unknown which compounds might be detectable and how this gustatory input might affect the feeding decision of triatomines.

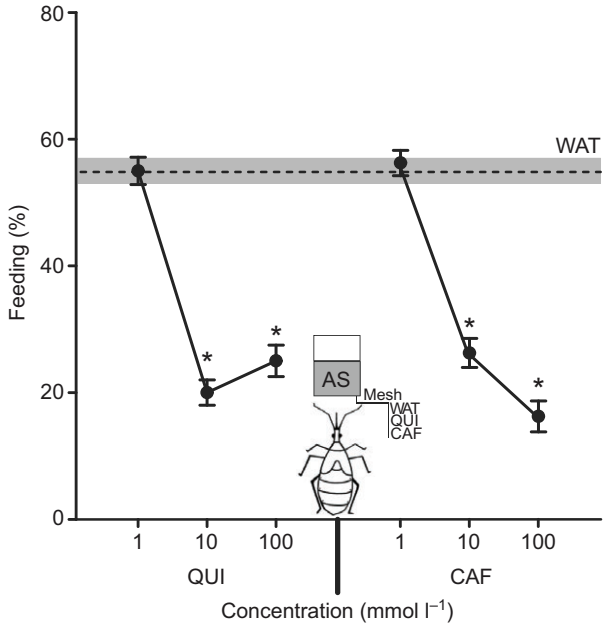

Fig. 1. Effect of external bitter detection on the feeding behavior of *Rhodnius prolixus*. Percentage of insects that ingested at least their own mass on the appetitive solution (AS) when the piercing mesh was spread with water (WAT; dashed line), quinine (QUI) or caffeine (CAF) at different concentrations. The addition of high doses of QUI and CAF over the piercing mesh elicited an inhibitory effect on feeding behavior. Asterisks denote significant differences from the WAT group (Pearson chi-square, $*P<0.05$). Twenty replicates were carried out for each concentration.

Effect of bitter compounds on the external assessment of a potential food source

This series of experiments was designed to determine whether the presence of bitter compounds spread over the piercing mesh (and not in the feeding solution) can prevent the feeding response of insects offered an appetitive solution (AS).

Approximately 55% of the insects ingested at least their own mass of AS (Fig. 1, horizontal line) when the piercing mesh was spread with water (WAT). The addition of 10 or 100 mmol l^{-1} of quinine (QUI) or caffeine (CAF) to the piercing mesh evoked a significant decrease in the feeding response of bugs as compared with WAT (QUI 10 and 100 mmol l^{-1} versus WAT: $\chi^2_1=8.94$, $P=0.002$, and $\chi^2_1=6.79$, $P=0.009$, respectively; CAF 10 and 100 mmol l^{-1} versus WAT: $\chi^2_1=4.94$, $P=0.02$, and $\chi^2_1=8.93$, $P=0.002$, respectively). In contrast, no effect of spreading the mesh with 1 mmol l^{-1} solutions of either QUI or CAF was observed. Similar response thresholds were obtained when QUI or CAF were spread over the mesh.

Location of the external bitter-compound detectors

We showed in the previous section that contact with a substrate with added QUI or CAF prevents feeding in these bugs. By selectively blocking the sensory inputs of their legs or antennae, we analyzed which chemosensory organs might be involved in the gustatory input associated with feeding. In a control group, insects were kept intact (INT). In another group, to obstruct putative gustatory inputs coming from the legs, tibiae and tarsi were painted with acrylic paint

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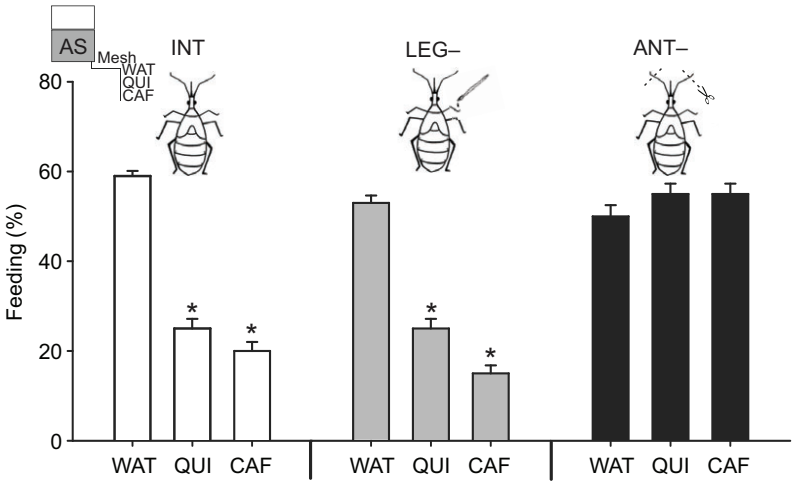

Fig. 2. Identification of the sensory structures involved in the feeding inhibition of *R. prolixus*. Percentage of insects that ingested at least their own mass on AS when the piercing mesh was spread with WAT, QUI or CAF. INT, intact animals; LEG–, animals deprived of leg inputs; ANT–, animals deprived of antennal inputs (last flagella). Feeding inhibition evoked by externally contacting a bitter substrate occurred in the INT and LEG– groups, and not in the ANT– group, suggesting that bitter sensing takes place through the taste inputs of the antennae of these insects. Asterisks denote significant differences from the corresponding WAT group (Pearson chi-square, $P<0.05$). Twenty replicates were carried out for each condition.

24 h before the assays (LEG–). In a third group, to block gustatory inputs from the antenna, the last segment was cut off 24 h prior to the feeding tests (ANT–). Different methodology to block peripheral inputs from legs and antennae was applied because preliminary experiments showed that insects could easily remove the acrylic paint from their antennae (but not from their tarsi) with their forelegs. In contrast, cutting the tarsi impeded the correct locomotion of bugs. All insects were then allowed to feed from an AS with the piercing mesh spread with WAT, QUI (100 mmol l^{-1}) or CAF (100 mmol l^{-1}) (Fig. 2).

As shown before, the presence of QUI or CAF over the piercing mesh inhibited feeding of INT animals. When the gustatory input from their legs was blocked (LEG–), a similar inhibition was evoked for QUI and CAF as compared with WAT (QUI $\chi^2_1=3.94$, $P=0.04$ and CAF $\chi^2_1=7.48$, $P=0.0062$). Conversely, insects deprived of their last antennal flagellum ingested as much AS in WAT assays as in QUI or CAF assays (ANT–, n.s.). These results suggest that the antennal gustatory input but not the information coming from legs or proboscis is involved in external bitter detection in these haematophagous insects.

Bitter detection of antennal taste sensilla

The morphology of the whole antennae of *R. prolixus* has already been described by other authors (Catalá, 1998; Insausti et al., 1999). In our work, the screening of the last flagellum of the antennae by means of scanning electron microscopy (SEM) revealed the presence of four chaetic sensilla with a terminal pore that surpass the edge of the antenna (Fig. 3A, inset). Although the morphology of these sensilla suggests a contact chemoreception or gustatory function, before the present study there had been no functional studies that confirmed this assumption.

In single-sensillum recordings we stimulated these four sensilla with KCl (a conductive electrolyte), QUI or CAF (Fig. 3). We found that both bitter compounds tend to modify the activity of sensory neurons in a dose-dependent manner (Fig. 3A), although statistical differences were only detected for 1 mmol l^{-1} CAF ($W=40$, $P=0.01$). However, the low response of neurons stimulated with 1 mmol l^{-1} QUI was surprising. With our results we cannot affirm whether there is an inhibitory effect in the firing rate of the neuron or instead whether a deleterious effect is occurring. These results show for the first time a gustatory function of these chaetic sensilla and establish their capacity of detecting bitter compounds.

Effect of previous antennal contact with bitter compounds on subsequent feeding decisions

Here, we analyzed whether a brief pre-exposure to bitter compounds can modulate the motivation of bugs to feed on AS. During pre-exposure, insects were allowed to walk for 30 s over the piercing

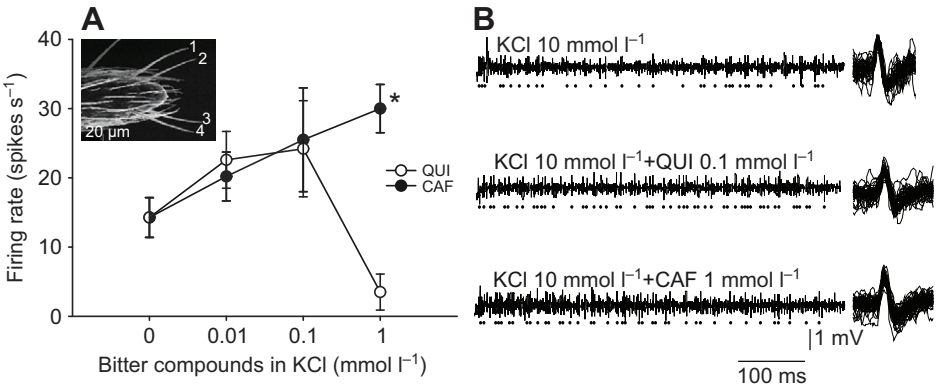

Fig. 3. Bitter detection by antennal taste sensilla of *R. prolixus*. (A) Mean spike frequency of gustatory neurons from these four sensilla stimulated with QUI or CAF at different concentrations. Inset shows the last flagellum of the antenna under SEM, showing four chaetic gustatory sensilla. Asterisk denotes a significant differences from the control (KCl) (Wilcoxon test, $P<0.05$). Each dot represents the median ± s.e. of eight sensilla. (B) Typical single-sensillum recordings showing a gustatory receptor neuron excitatory response to KCl (control), QUI and CAF; spikes are denoted by a dot beneath the trace. CAF tend to increase the firing rates of the neurons, although only the highest concentration of CAF was statistically significant.

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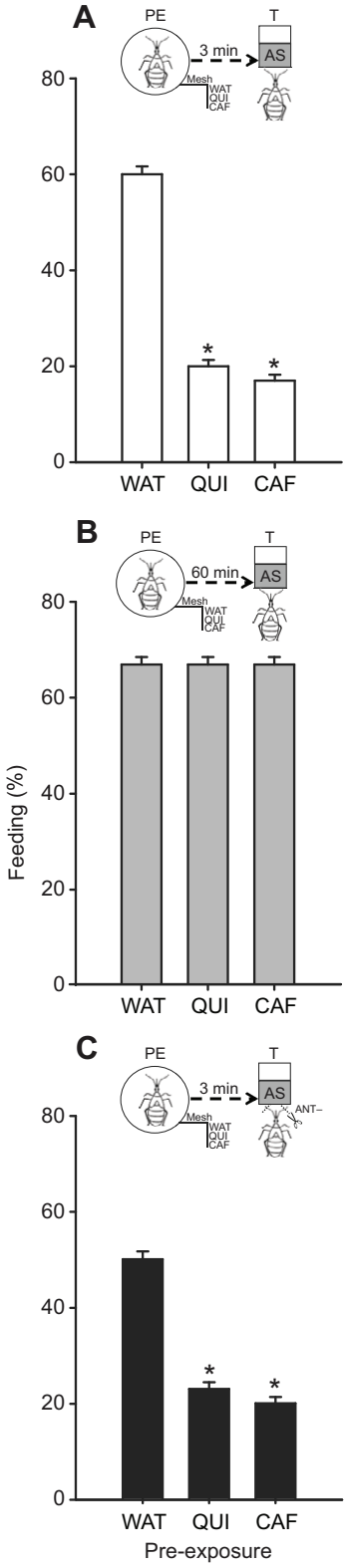

Fig. 4. Modulation of the feeding behavior of insects by a previous contact with bitter compounds. The percentage of insects that ingested at least their own mass on AS is represented. Insects were pre-exposed to a mesh spread with WAT, QUI or CAF and then tested 3 min (A) or 60 min (B) later. An external pre-exposure to bitter compounds evoked a feeding deterrence that lasted more than 3 min but less than 60 min. In C, the feeding test was carried 3 min after pre-exposure but the last flagella the antennae of bugs were cut off immediately after pre-exposure (ANT–). The ANT– group pre-exposed to QUI and CAF also showed feeding inhibition to bitter compounds, suggesting a central processing of the bitter sensory information and not overstimulation of taste sensilla. Asterisks denote significant differences from the corresponding WAT group (Pearson chi-square, **P*<0.05). Thirty replicates were carried out for each condition. PE: pre-exposure, T: feeding test.

mesh spread with WAT, QUI or CAF and were then transferred to a clean insect receptacle for either 3 or 60 min, until the feeding tests were carried out. In tests carried out 3 min after pre-exposure (Fig. 4A), significantly fewer insects fed on AS when pre-exposed to QUI or CAF as compared with those pre-exposed to WAT (χ^2_1=10, *P*=0.0016 and χ^2_1=11.92, *P*=0.0006, respectively). Note here that feeding avoidance occurred even if bitter compounds were absent during tests. In addition, this inhibitory effect vanished 60 min after pre-exposure, and no significant differences with insects pre-exposed to WAT were observed (Fig. 4B).

To verify that these inhibitory results were not due to the persistence of QUI or CAF from the pre-exposure procedure in the peripheral receptors, immediately after pre-exposure to QUI or CAF the last flagellum of both antennae were cut off. The insects' feeding behavior was then tested in the artificial feeder. Results show that bugs pre-exposed to QUI or CAF still fed less frequently over clean mesh than did bugs pre-exposed to WAT (χ^2_1=4.59, *P*=0.032 and χ^2_1=5.93, *P*=0.016, respectively; Fig. 4C), even if during tests there were no longer antennal inputs. The feeding inhibition evoked by a previous contact with bitter compounds seems to be under brain control rather than under peripheral modulation.

Can internal bitter detection during food ingestion modulate the feeding decision of insects?

In the previous section we showed that external chemoreception plays a relevant role in the assessment of a potential food source in triatomines. According to a previous report, once triatomines pierce their host's skin, they first pump a small quantity of blood, presumably to assess its properties (Bennet-Clark, 1963) and then decide whether to continue with the alimentation. The presence of internal chemosensory structures in the epipharynx of other species of triatomines has been reported previously (Barth, 1952; Bernard, 1974).

Effect of bitter compounds on the internal assessment of a food source quality

In this series of experiments, different bitter compounds were added to the AS (and not over the piercing mesh) and the feeding response of insects was analyzed. Insects were individually placed in the artificial feeder filled with either the AS alone or the AS with QUI, CAF, BER or SAL (0.000001 to 1 mmol l^{-1} in all cases).

As previously observed, a high percentage of insects (55%) fed on the AS (Fig. 5A, horizontal line). However, an inhibitory feeding effect was found when bitter compounds were individually added to the AS. QUI and SAL were the most potent inhibitory compounds presenting the lower thresholds of aversion, i.e. <0.00001 mmol l^{-1} (lower dose significantly different from AS, QUI 0.00001 mmol l^{-1}, χ^2_1=8.94, *P*=0.0028; SAL 0.00001 mmol l^{-1}, χ^2_1=4.94, *P*=0.02). The other bitter compounds also exhibited inhibitory effects, although with response thresholds below 0.01 mmol l^{-1} for CAF (lower dose significantly different from AS, χ^2_1=4.94, *P*=0.02) and below 0.0001 mmol l^{-1} for BER (χ^2_1=8.94, *P*=0.0028).

The internal detection of bitter compounds present in the food should take place somewhere in the alimentary canal (Fig. 5B). Although no functional studies have been performed as of yet, we

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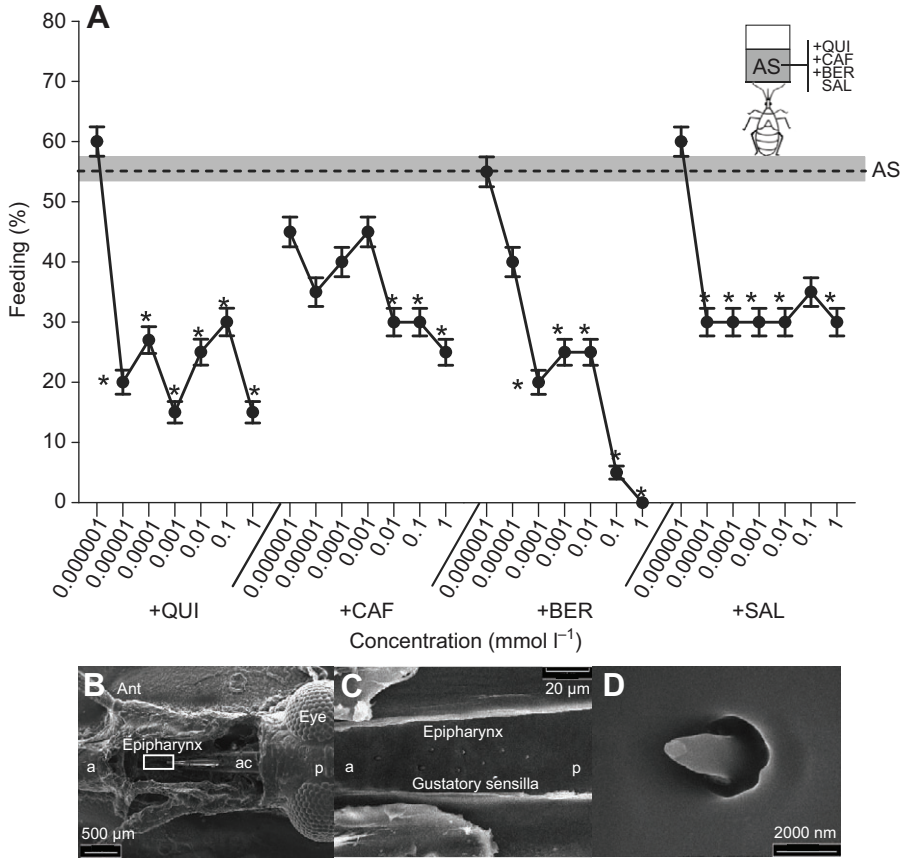

Fig. 5. Effect of internal bitter detection on the feeding behavior of *R. prolixus*. Percentage of insects that ingested at least once their own mass on AS alone (dashed line) or on AS with QUI, CAF, berberine chloride hydrate (BER) or D-(-)salicin (SAL) at different concentrations. (A) The addition of QUI, CAF, BER and SAL to the AS elicited an inhibitory effect on the feeding behavior of insects, although at different concentrations. (B) Photograph of the head showing the base of the antennae (ant), the alimentary canal (ac), the epipharynx and the eyes (a, anterior; p, posterior) under SEM. (C) Photograph of the epipharynx showing eight short-peg gustatory sensilla. (D) Detail of one gustatory sensillum bearing an apical pore. Asterisks denote significant differences with the AS group (Pearson chi-square, **P*<0.05). Twenty replicates were carried out for each concentration.

revealed by means of SEM the existence of eight short-peg sensilla (2–3 µm height and 2 µm at the base) with a unique pore at the end, localized antero-dorsally inside the alimentary canal (epipharynx) of *R. prolixus* (Fig. 5C,D).

Effect of previous ingestion of bitter compounds on subsequent food acceptance

Here, we analyzed whether a brief ingestion of QUI and CAF before the feeding tests could modulate the posterior ingestion of the AS. Insects were allowed to feed for 30 s on the AS alone (control group) or on the AS with added QUI (0.00001 mmol l^{-1}) or CAF (0.01 mmol l^{-1}). Given the fine-scale sensitivity of these insects to thermal cues, a group was pre-exposed only to the heat emanated by the artificial feeder (HT). Feeding tests on the AS were carried out after 3 or 60 min following the pre-exposure.

Approximately 65% of the insects fed on the AS after pre-exposure to HT or AS, and no differences were detected among these groups (Fig. 6A). Conversely, previous ingestion of QUI or CAF 3 min before the feeding tests led to a decrease in the percentage of insects feeding on the AS (QUI versus AS χ^2_1=13.13, P=0.0003 and CAF versus AS χ^2_1=15.15, P=0.0001; Fig. 6A). Note that this feeding avoidance was persistent even if bitter compounds were absent during tests. This inhibition disappeared after 60 min (Fig. 6B), suggesting the existence of a memory component that lasts more than 3 min but less than 60 min.

DISCUSSION

In the present study, we showed for the first time that the bitter modality in the blood-sucking bug *R. prolixus* is functional and active during feeding. Notably, the detection of bitter compounds occurs via two sensory paths working with different thresholds of responsiveness: one starting externally at the tip of the antennae and the other inside the alimentary canal, probably at the epipharynx. While antennal taste receptors interact solely with the host skin and never get in contact with the blood of the host, internal gustatory receptors are confined to the alimentary canal and are therefore exclusively bathed with the ingested blood during sampling phase and feeding.

Recognition of an adequate substrate

Like most haematophagous invertebrates, triatomines exploit olfactory and thermal cues emanated by their vertebrate hosts to localize them (Barrozo et al., 2003; Barrozo and Lazzari, 2004a; Barrozo and Lazzari, 2004b; Bodin et al., 2008). As soon as bugs reach a potential host, they search for a zone of the skin to pierce, a process that involves the thermal sense (Lazzari and Núñez, 1989; Flores and Lazzari, 1996; Ferreira et al., 2007). However, it was still unknown whether these insects were able to assess the gustatory quality of the substrate before piercing the skin. Results from the present study show that this is actually the case. We found that before feeding, *R. prolixus* undergoes a substrate probing phase in which it evaluates the taste properties of a potential food source and consequently decides whether to continue the feeding process. In our experiments, we observed a decrease in the feeding response of those insects that reached and contacted a piercing surface impregnated with bitter compounds such as QUI and CAF, even if the offered food was an appetitive solution. Both substances elicited similar aversive effects at similar concentrations, i.e. 10 mmol l^{-1} (Fig. 1).

Moreover, we found that the external sensory organs involved in bitter detection during feeding are located in the antennae and not in the legs or proboscis (Fig. 2). Based on our electrophysiological

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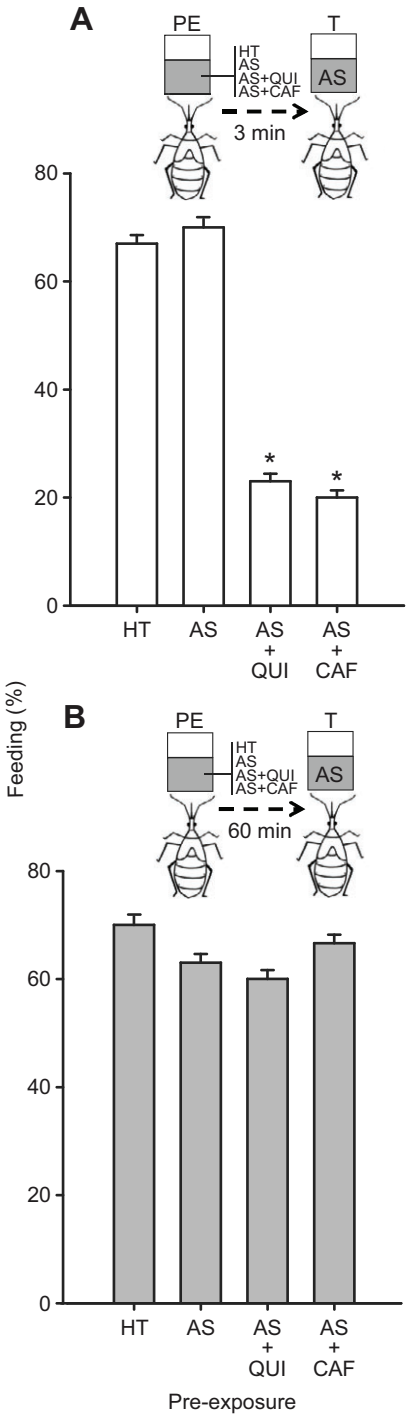

Fig. 6. Modulation of the feeding behavior of insects by a previous ingestion of bitter compounds. The percentage of insects that ingested at least their own mass on AS is represented. Bugs were pre-exposed to heat (HT), AS, AS+QUI or AS+CAF. Feeding tests on AS were carried out 3 min (A) or 60 min (B) after pre-exposure. A brief pre-ingestion of bitter compounds evoked a feeding avoidance of the AS that lasted more than 3 min but less than 60 min. Asterisks denote significant differences from the corresponding AS group (Pearson chi-square, **P*<0.05). Thirty replicates were carried out for each condition.

results, we confirmed the gustatory function of four chaetic sensilla located in the second flagellum (last segment) of the antennae of *R. prolixus*. We showed that these taste sensilla respond to QUI and CAF (Fig. 3). Further studies are needed to determine the number of GRNs inside these sensilla and to extend the spectrum of taste modalities these insects detect. For example, we observed electrophysiological dose-dependent responses also to salts such as NaCl and KCl (data not shown).

Bitter detection at the periphery normally starts in motion an aversive behavioral response in insects. The presence of bitter-specific sensitive taste cells has been described before in insects (Glendinning et al., 1999; Schoonhoven and van Loon, 2002; Meunier et al., 2003; Weiss et al., 2011). However, there are several examples in which bitter substances do not act directly via specialized bitter detectors but instead interfere in the normal perception of phagostimulant receptors (see Chapman, 2003). In the case of *R. prolixus*, both scenarios could occur: it may be that insects have bitter receptors in their antennae, or that bitter substances modulate the response of other gustatory neurons. In our behavioral experiments we showed that even in the absence of chemical compounds over the piercing substrate, these insects fed on the AS (e.g. Fig. 1, see WAT group), indicating that they do not need external contact with phagostimulants to do so. We also showed that the addition of bitter tastants inhibited this feeding behavior, suggesting that in these bugs, bitter compounds are acting independently, probably via specific bitter receptors instead of interfering in the response of other gustatory neurons.

Recognition of an adequate food

The assessment of food through antennal taste inputs constitutes the first examination made by insects giving way to the first decision: to accept or reject a potential food source before ingestion starts (Figs 1, 2). A small gorge of food will be then ingested during the sampling phase, as described by Smith and Friend (Smith and Friend, 1970). In our work we observed throughout the experiments that an insect can ingest between 100 and 280 µl of the AS presented alone, increasing up to 15 times its initial mass during a 10 min alimentation. However, when different bitter tastants (three alkaloids: quinine, caffeine and berberine; and one phenolic glycoside: salicin) were added to the AS, the insects dramatically decreased ingestion in a dose-dependent manner (Fig. 5), even up to a total inhibition. The threshold of feeding rejection found for *R. prolixus* ranged from 0.00001 mmol l^{-1} (for QUI and SAL) to 0.01 mmol l^{-1} (for CAF). Sensitivity thresholds found for compounds that stimulate bitter-sensitive cells in phytophagous insects vary from 0.1 to 10 mmol l^{-1} (see Chapman, 2003) and in humans from 0.00001 to 50 mmol l^{-1} (Meyerhof et al., 2005).

Although most insects have internal taste organs in different parts of their alimentary canal or mouthparts, their physiology is far less studied than external receptors, mainly because of difficulties in accessing them with recording electrodes. In triatomines, Barth (Barth, 1952) was the first to suggest the existence of a group of chemosensory structures present in the alimentary canal of *Triatoma infestans*, a species related to *R. prolixus*, particularly in the epipharynx. In other insects, structures with similar functions have been described, for example, the cibarial organ of simulids, tsetse flies and ticks (Rice, 1973; John, 1979; McIver and Siemicki, 1981; Foster et al., 1983; Backus and McLean, 1985; Jefferies, 1987). We propose here that the eight short-peg uniporous sensilla observed in the ephipharynx of *R. prolixus* (Fig. 5B–D) are responsible for bitter sensing. However, only an electrophysiological approach would determine unequivocally this fact.

Plasticity of the taste sense

Gustatory stimuli coming from the environment can induce memories in an animal that may allow them to learn how to

discern between good or and bad food sources (Bernays and Chapman, 2000). This experience-dependent cognitive modulation of the behavior may be guided by either an associative or a non-associative process. Associative learning is a complex process that allows an individual to convert a previously neutral stimulus into a predictor of the occurrence of a relevant event. Non-associative processes are simpler forms of learning that can help an individual to be more prone to respond to a recently perceived stimulus (sensitization) or to filter out information that is no longer informative (habituation). Here, we show that both the substrate probing phase and the sampling phase of the feeding process of *R. prolixus* are modulated by a previous sensory experience to bitter compounds. We found that a simple chemical pre-exposure to QUI and CAF during both phases inhibited the posterior feeding behavior of *R. prolixus*, even if the bitter compounds were no longer present during tests. This effect lasted for a brief period (between 3 and 60 min) (Figs 4, 6).

Although a clear modulation of the behavior of the insects was observed after a non-associative experience (i.e. a chemical pre-exposure to bitter compounds), the results presented here do not fit into a typical habituation or sensitization category. In these processes, the response to a particular stimulus A decreases or increases after pre-exposure to the same stimulus A. In our case, pre-exposure to A (e.g. any of the tested bitter compounds) decreased the feeding behavior of bugs in the absence of A. And this decrease was not caused by an impregnation of the antennal taste receptors with bitter compounds during tests, but mostly to a central integration of aversive input information. This was shown in the experiments in which we deprived the animals of their antennal tips after pre-exposure and they still did not feed (Fig. 4B). This result indicates that aversive input centrally modulates the final decision of the insect after a noxious experience, i.e. not to feed.

In nature, this short feeding deterrent memory might allow animals to stop probing around once a toxic food source is perceived. This plasticity might be important as whenever a toxic source is found, there is a certain probability to find another toxic one or even to be still over the same source than before.

Bitter compounds for haematophagous insects?

Although bitter is a relevant taste modality involved in the modulation of the decision-making process about whether to accept a potential food source for many animals, the fine-scale and highly sensitive perception system of *R. prolixus* to bitter substances was quite surprising to us. What might be the reason for the existence of a bitter detection system in an obligatory blood-sucking insect? *Rhodnius prolixus* feeds exclusively on vertebrate blood, a medium that intrinsically lacks caffeine, quinine, berberine or salicin. However, if these compounds are ingested by these host animals, they can become an active part of their blood. For example, when herbivores eat hosts plants that produce bitter compounds, or more recently in evolutionary time, when humans ingest a normal cup of coffee, a peak of caffeine can be found in their plasma. The peak of caffeine after a single cup of coffee for men is estimated to be between 0.001 to 0.01 mmol l^{-1} (Fredholm et al., 1999), which encompasses the doses detected by *R. prolixus*. However, these haematophagous insects evolved from predatory ancestors, for which the adaptive pressure of sensing bitter tastants was probably higher. As such, insects may have conserved from past ancestors the fine detection system tuned to bitter tastants. In mosquitoes, which feed on plants (males and females) but also on vertebrate blood (only females), recent reports showed behavioral and neuronal responses to quinine (Sanford et al., 2013; Kessler et al., 2013). Although the importance of the gustatory system in blood-sucking vector-borne diseases during host recognition and feeding has been neglected in the past, it has lately become an area of interest (Kessler et al., 2013; Sanford et al., 2013; Bohbot et al., 2014; Sparks et al., 2013; Sparks et al., 2014). The development of new strategies targeting the gustatory system of haematophagous insects, by using anti-feedants or bitter compounds, could help to diminish host–vector interactions and thus to prevent the vectorial transmission.

The balance between positive and negative inputs

The feeding response of insects is finally governed by the contrast between the presence of phagostimulatory and aversive inputs. Our study shows that *R. prolixus* has two sensory stages working at different avoidance thresholds: antennal input exerts a modulatory bitter signalling at higher doses (10 mmol l^{-1}) than internal sensors bathed with feeding solution, whose bitter threshold is approximately six orders of magnitude below for QUI and three for CAF. The results obtained from the present study are summarized and depicted in a flow chart (Fig. 7). The first assessment of the adequateness of a potential food source takes place at the antennal receptors, during the substrate probing phase (A). If bitter compounds are detected at this point (1), the animal will not insert its biting mouthparts in the host skin and will not feed, restarting a new cycle at the substrate probing phase. Conversely, if no aversive compounds are detected (2), the next step is to pierce the skin and insert their mouthparts in the host [i.e. piercing phase (B)]. Subsequently, during the sampling phase (C), a small quantity of food is ingested for an internal quality assessment. If no phagostimulants are detected (3), the animal will simply not feed. Conversely, if the ingested solution contains phagostimulants, such as ATP and salts (4), the insect will continue with the engorgement (D) up to repletion. However, if bitter compounds are detected (5) together with the phagostimulants, the animal will not feed, and moves backwards in the cycle up to the piercing phase or the substrate probing phase to restart the feeding process. We found that for an extended range of doses, bitter detection was more important in the central decision about whether to feed than was the phagostimulatory input. Any interactions between chemicals and neurons that occur at the periphery will alter the phagostimulatory or aversive inputs, significantly changing the balance. The insect's final decision related to host selection will depend on this balance.

Conclusions

Here we demonstrated that *R. prolixus* have taste sensilla localized in the tip of their antennae that showed electrophysiological sensitivity to bitter compounds such as caffeine and quinine. The perception of bitter stimuli via these external receptors caused an inhibition of the feeding behavior of bugs during the substrate probing phase. Similarly, inside their alimentary canal, this species bears eight sensilla that might be involved in the detection of bitter compounds during the sampling phase, which also inhibited ingestion. The feeding inhibition observed in response to bitter compounds acts via these two sensory inputs working at different thresholds of tolerance. Finally, by applying a cognitive approach, we found that the feeding behavior of triatomines can be negatively modulated by previous experience with bitter tastants. These results highlight the relevance of bitter taste perception in the modulation of the feeding behavior of a blood-sucking insect. Thus, our work is significant in the frame of the development of novel tools that can help in the surveillance and control of this insect vector.

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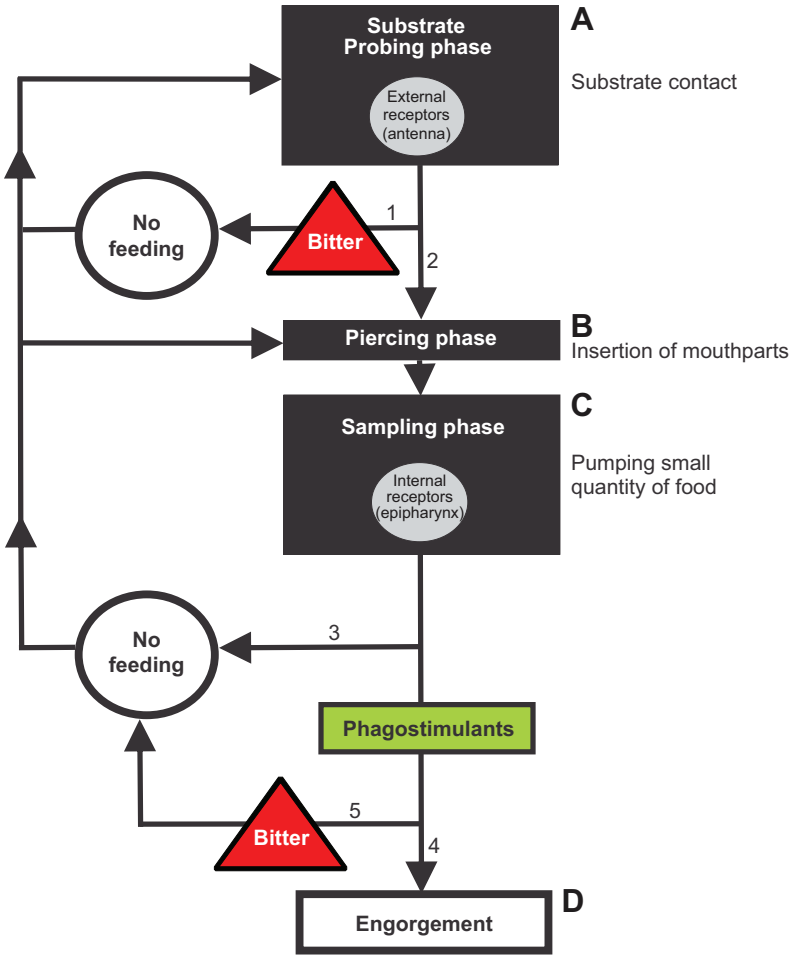

Fig. 7. Flow chart showing the feeding phases of *R. prolixus* and its modulation by bitter compounds. During the substrate probing phase (A), if external receptors of the antennae detect bitter compounds in the substrate (1), insects interrupt the normal feeding process (2) that leads them to the piercing phase (B). During the sampling phase (C), once the first gorge of blood is pumped, if no phagostimulants are detected by internal receptors in the alimentary canal (3), feeding does not continue. Conversely, if phagostimulants are detected (4) the engorgement (D) starts. However, feeding is inhibited if bitter compounds are detected in the ingested food (5). Non-fed animals can restart the feeding cycle at the substrate probing phase (A) or the piercing phase (B).

MATERIALS AND METHODS

Animals and rearing conditions

Fifth-instar larvae and adults of *R. prolixus* used throughout the experiments were obtained from a laboratory colony, reared at 28°C, ambient relative humidity and under a 12 h:12 h light:dark cycle. Following ecdysis as fifth instars or adults, insects did not have access to food. Experiments were carried out 15±2 days post-ecdysis.

Artificial feeder

We quantified the mass gained by *R. prolixus* fed with different solutions using an artificial feeder. The ad hoc feeding device consisted of two parts: the feeding receptacle, made of a plastic cylinder (1 cm diameter×2 cm height) with its lower opening closed with a latex membrane (0.125 mm thick), filled with an appetitive solution (AS) alone or with different bitter compounds; and the insect receptacle, which was a plastic vial (3 cm diameter×3.5 cm height) in which bugs were individually placed whose upper openings were covered with a tissue mesh. A piece of filter paper (1.5×3.5 cm) placed vertically inside the vial helped the insects to climb in order to reach the tissue mesh. The mesh was either unembedded or embedded with different bitter compounds.

The feeding receptacle was placed close to an aluminium plate connected to a thermostatized resistance that heated the feeding solution to 35°C to match the mean temperature of the triatomines' hosts. The latex membrane in contact with the solution also acquired the same temperature, mimicking the temperature of host skin and acting as a piercing membrane.

Feeding experiments started when the tissue mesh of the insect's receptacle was carefully put into contact with the piercing membrane of the feeding receptacle (triatomines could easily perforate both with their mouthparts). The feeding assays lasted in all cases 10 min.

Gustatory stimuli

Preliminary feeding assays carried out in our laboratory in accordance with previous reports by other authors (Friend and Smith, 1971) showed that a solution of 1 mmol l^{-1} ATP in 0.15 mol l^{-1} NaCl evokes a high feeding response in *R. prolixus*. Therefore, for this study we used this as the appetitive solution (AS) and as a standardized feeding solution.

ATP, quinine hydrochloride (QUI), berberine chloride hydrate (BER) and D-(-)salicin (SAL) were purchased from Sigma-Aldrich (St Louis, MO, USA). Sodium chloride and anhydrous caffeine (CAF) were purchased from Biopack (Buenos Aires, Argentina). All solutions were prepared weekly and stored at −18°C. In all cases, the pH of the solutions was verified and adjusted to 7 when necessary with 1 mol l^{-1} NaOH.

Experimental protocols

All experiments were carried out at the beginning of the insects' scotophase, the time of the day in which triatomines display their maximal motivation to search for a host and feed (Lorenzo and Lazzari, 1998; Barrozo et al., 2004; Bodin et al., 2008). In each assay, an unfed larva was weighed before (initial mass, M_i) and after (final mass, M_f) the feeding tests. A normalized mass gain was calculated as: $(M_f–M_i)/M_i$. We then registered the percentage of insects whose normalized mass gain was higher than 1 (i.e. insects that ingested at least their own mass).

The effect of the presence of bitter compounds was studied at two different phases of the feeding process of triatomines. First, during the substrate probing phase, bitter stimuli were added to the substrate: in the control group, 50 µl of distilled water were spread over the mesh (WAT). Bitter stimulation was achieved by spreading 50 µl of 1, 10 or 100 mmol l^{-1} of QUI or CAF (both prepared in water) over the mesh of the insect receptacle. Then, the vial was placed in the artificial feeder and the insect was allowed to feed on the AS for 10 min (see Results). Additionally, the

effect of a previous experience with bitter compounds on the feeding behavior of insects was studied. Insects were pre-exposed by allowing them to walk for 30 s over a substrate with added WAT, QUI or CAF (10 mmol l^{-1}), and 3 or 60 min after, their feeding acceptance of the AS was tested. Second, during the sampling phase, bitter stimuli were added to the AS: in the control group, no bitter compounds were added to the AS. Different doses of QUI, CAF, BER and SAL (0.000001–1 mmol l^{-1}) were added to the AS in the feeding receptacle and offered to the insects in the artificial feeder for 10 min. In these experiments the substrate was always clean (see Results).

In addition, we analyzed the effect of a brief pre-ingestion of bitter compounds on the feeding behavior of insects in response to the AS. Insects were allowed to shortly feed (30 s, accounting from the moment the insect pierced the mesh of the artificial feeder and kept the proboscis inserted) on the AS alone or on AS with QUI (0.00001 mmol l^{-1}) or CAF (0.01 mmol l^{-1}), and 3 or 60 min later their feeding response to the AS was evaluated.

Data analysis

Data from behavioral experiments were analyzed by means of contingency tables of independence (Sokal and Rohlf, 1995). The percentage of insects that exhibited a normalized mass gain higher than 1 was registered. We statistically tested whether the feeding responses of insects were independent of the different experimental conditions. A global comparison including all treatments was assessed by means of a Pearson's chi-squared test. Whenever the global test was statistically significant (α=0.05), individual *post hoc* comparisons were performed. The standard deviations of percentages were calculated as $\sqrt{p(1-p)/N}$, where p is the proportion of responses and N is the number of animals tested. Electrophysiological data were statistically analyzed using the Wilcoxon test (W). The InfoStat v2012 statistical package was used for analyses (http://www.infostat.com.ar).

Scanning electron microscopy

The external structures of the tip of the antennae and the interior of the epipharynx (anterior part of the alimentary canal) of adult *R. prolixus* were scanned by means of SEM to search for taste sensilla, putative candidates involved in substrate/food recognition. The antennae were cut at the base and mounted horizontally with double-sided tape on a standard aluminium stub. The epipharynx was exposed by making a small ventral opening in the anterior part of the head of the insects. Then the lumen of the alimentary canal was exposed by cutting a second opening that uncovered the internal sensilla. The head was then mounted on an aluminium stud. All samples were coated successively during 180 s with gold/palladium (40/60%) before examination under a Philips XL 30 scanning electron microscope.

Single-sensillum electrophysiological recordings

The morphological identification of gustatory structures present in the antennae of adult *R. prolixus* allowed us to carry out electrophysiological recordings on putative taste sensilla that showed a pore at their tip. Recordings were carried out from the four most apical hairs placed in the last segment of the antennae by measuring the activity of the sensory neurons housed inside these hairs in response to KCl, QUI or CAF.

Insects were secured with wax inside plastic conic supports, with their antennae kept outside, immobilized with double-sided tape. Following the recording method of Hodgson et al. (Hodgson et al., 1955), animals were grounded via a silver wire to the left eye (reference electrode) and an individual sensillum was inserted for 3 s in a glass electrode (recording electrode) containing the electrolyte alone (10 mmol l^{-1} KCl) or with the bitter stimuli (QUI or CAF 0.01, 0.1 and 1 mmol l^{-1} presented in ascending order) added. Each sensillum was tested first with KCl and then with CAF or QUI in a random order. The time between subsequent stimulations was fixed to 1 min.

The recording electrode (20–30 µm diameter) was connected to a preamplifier (gain ×10, TastePROBE DTP-02, Syntech) and the biological signals were further amplified, filtered and digitized by means of an IDAC4 (Syntech) (gain ×100, eighth-order Bessel pass-band filter: 1–3000 Hz, sampling rate: 10 kHz, 16 bits). The data were stored on a computer. Spike detection and analysis were performed off-line using Autospike (Syntech). The number of spikes was counted to the first second of stimulation.

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Competing interests

The authors declare no competing financial interests.

Author contributions

G.P., S.M. and R.B.B. conceived and designed the research; G.P., S.M., I.O.I., M.G.B.S. and R.B.B. collected the data; G.P., S.M., M.G.B.S. and R.B.B. analyzed the data, interpreted the results, and drafted and revised the article.

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