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

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Running title: Bitter perception in a blood feeder
Abstract
The gustatory system of animals is involved in the food quality assessment and controls the feeding decision of an individual confronted to a potential alimentary source. Triatomines are haematophagous insects that feed on vertebrate’s blood. Once they reach a potential host, they walk over their skin searching for an adequate site to pierce. Then, they insert their stylets and take a first sampling gorge to decide if food is acceptable or not. 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 8 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
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Introduction

Taste provides reliable information about the quality of food and can contribute to discriminate between nutrient 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, in order to prevent the ingestion of toxic food, the gustatory system of an individual can detect the presence of particular substances or tastes that signalize toxicity. Many toxins or poisonous substances have bitter taste for humans. Although there is no chemical identity uniqueness for bitter compounds (they are defined anthropocentrically as substances perceived by our gustatory sense as bitter), most of them 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 GNR is tuned to a particular taste modality (e.g. salt, sweet, bitter) by the presence of specific membrane gustatory receptor proteins (GRs) (Clyne et al., Dunipace et al., 2001, Scott et al., 2001, Robertson et al., 2003). Different groups of phytophagous insects belonging to different orders such as orthopterans, lepidopterans, coleopterans and dipterans (Chapman et al., 1991; Schoonhoven and van Loon, 2002, Messechendorp 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, 2001).

The gustatory perception of blood-sucking insects has received so far little attention. During the late 60’s and up to the 80’s, several groups were focused in 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, like ATP (adenosine triphosphate) 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 in their food preferences (Ignell et al., 2010; Kessler et al., 2013). New upcoming data in mosquitoes showed the occurrence of Drosophila orthologous GRs genes (Kent et al 2008, Bohbot et al 2014, Sparks et al 2014) that might share similar function, like the one required for caffeine detection (Sparks et al 2013).

*Rhodnius prolixus* (Stål) is a triatomine bug, vector of the Chagas disease in Latin America (WHO, 2012). As many other blood-feeders, they find their hosts by following host-emitted cues like CO₂, 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, 2004 a, b; 2006). Triatomines feed exclusively on vertebrates’ blood. Once they find a potential host, triatomines start a feeding process that involves several steps. First, they walk over the host skin and seek 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 if 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 fulfils the insect’s feeding requirements the animal continues feeding, if not the animal leaves the host and search for another one (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 do they assess the quality of the food and ultimately how do they choose a host based on their gustatory preferences. We postulate that the gustatory sense might be important at two different instances of the feeding behavior: 1- once the insect reaches the host skin and has to decide whether to pierce or not; 2- once it takes a first gorge of blood and has to decide if the diet is adequate or not.
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* (1) and the *sampling phase* (2). 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 (we named this phase as the *substrate probing phase*, results are presented in Part I); 2- once the bug has pierced the host skin and taken a first gorge of blood to decide if the diet is adequate or not (the *sampling phase*, results presented in Part II). In particular, we analyzed whether these haematophagous insects perceive bitter compounds and how do these compounds modulate their feeding behavior.

**Part I: Can an external chemical assessment of the substrate modulate the feeding decision of insects?**

The decision of a haematophagous insect about to pierce or not might be mediated by taste receptors that could be present in any part of their body. Up 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.

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

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

About 55% of the insects ingested at least one time their own weight of AS (Fig. 1, horizontal line) when the piercing mesh was spread with water (WAT). The addition of 10 mM or 100 mM of quinine (QUI) or caffeine (CAF) to the piercing mesh evoked a significant decrease in the feeding response of bugs as compared to WAT (QUI 10 mM and 100 mM vs. WAT: $X^2(1)= 8.94$, $p= 0.002$, $X^2(1)= 6.79$, $p= 0.009$, respectively; CAF 10 mM and 100 mM vs. WAT: $X^2(1)= 4.94$, $p= 0.02$; $X^2(1)= 8.93$, $p= 0.002$, respectively). Contrarily, no effect of spreading the mesh with 1 mM solutions of any of both compounds was observed (n.s. in both cases). Similar response thresholds were obtained when QUI or CAF were spread over the mesh.
We showed in the previous section that the contact with a substrate added with QUI or CAF prevents feeding in these bugs. In this section, 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, in order to obstruct putative gustatory inputs coming from the legs, tibiae and tarsi were painted with acrylic paint 24 h before the assays (LEG-). On 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 withdraw the acrylic paint from their antennae with their forelegs (and not from their tarsi). On the other hand, 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 water (WAT), QUI (100 mM) or CAF (100 mM) (Fig. 2).

As shown before, the presence of QUI or CAF over the piercing mesh inhibited feeding of intact animals (INT). When the gustatory input from their legs was blocked (LEG-), a similar inhibition was evoked for QUI and CAF as compared to WAT series ($X^2_{(1)} = 3.94$, $p= 0.04$ and $X^2_{(1)} = 7.48$, $p= 0.0062$, respectively). Conversely, insects deprived from 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 are involved in external bitter detection in these haematophagous insects.

The morphology of the whole antennae of R. prolixus has already been described by other authors (Catalá, 1994; Insauti et al., 1999). In our work, the screening of the last flagellum of the antennae by means of SEM revealed the presence of 4 chaetic sensilla with a terminal pore that surpass the edge of the antenna (Fig. 3A). Although the morphology of these sensilla suggests a contact chemoreception or gustatory function, before this work there were no functional studies that confirmed this assumption.

In single-sensillum recordings we stimulated these 4 sensilla with KCl (conductive electrolyte), QUI or CAF (Fig. 3B,C). We found that both bitter compounds tend to
modify the activity of sensory neurons in a dose-dependent manner (Fig. 3B), although statistical differences were only detected for CAF 1 mM (W= 40, p= 0.01). However, the low response of neurons stimulated with 1 mM of QUI was somehow surprising. With our results we cannot affirm whether there is an inhibitory effect in the firing rate of the neuron or instead if a deleterious effect is occurring. These results show for the first time a gustatory function of these chaetic sensilla and established their capacity of detecting bitter compounds.

**Effect of a previous antennal contact with bitter compounds in subsequent feeding decisions**

Here, we analyzed if 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 30s over the piercing mesh spread with WAT, QUI or CAF and then transferred to a clean insect’s recipient for either 3 min or 60 min, until the feeding tests were carried out. In tests carried out 3 min after pre-exposure (Fig. 4A), significantly less insects fed on AS when pre-exposed to QUI or CAF as compared to those pre-exposed to WAT ($X^2(1)= 10$, p= 0.0016 and $X^2(1)= 11.92$, p= 0.0006, respectively). Notice here that feeding avoidance occurred even if bitter compounds were absent during tests. In addition, this inhibitory effect vanished 60 min after pre-exposure as no significant differences with WAT-pre-exposed insects were observed (Fig. 4B, n.s.).

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 flagella of both antennae were cut off. Then, the insects’ feeding behavior was tested in the artificial feeder. Results show that bugs pre-exposed to QUI or CAF still fed less frequently over a clean mesh than WAT-pre-exposed bugs (Fig. 4C, $X^2(1)= 4.59$, p= 0.032 and $X^2(1)= 5.93$, p= 0.016, respectively), 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.

**Part II: 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 with previous
reports, once triatomines pierce their host’s skin, they first pump a small quantity of
blood, presumably to assess its properties (Bennet-Clark, 1963) and decide whether to
continue with the alimentation or not. The presence of internal chemosensory structures
in the epipharynx of other species of triatomines has been reported (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 the AS alone or added with QUI,
CAF, BER or SAL (0.000001 to 1 mM 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 mM
(lower dose significantly different from AS, QUI 0.00001 mM, $X^2_{(1)}= 8.94$, p= 0.0028;
SAL 0.00001 mM, $X^2_{(1)}= 4.94$, p= 0.02). The other bitter compounds also exhibited
inhibitory effects, although with response thresholds below 0.01 mM for CAF (lower
dose significantly different from AS, CAF 0.01 mM, $X^2_{(1)}= 4.94$, p= 0.02) and 0.0001
mM for BER (BER 0.0001 mM, $X^2_{(1)}= 8.94$, 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 were done
so far, we revealed by means of SEM the existence of 8 based-articulated 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 a previous ingestion of bitter compounds in subsequent food acceptance**

Here, we analyzed if a brief ingestion of QUI and CAF before the feeding tests could
modulate the posterior ingestion of the AS. Insects were allowed to feed during 30 s on
the AS alone (control group) or on the AS added with QUI (0.00001 mM) or CAF (0.01
mM). Provided the fine sensitivity of these insects to the 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 min or 60 min following the pre-exposure.
About 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, n.s.). Conversely, the 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 (Fig. 6A, QUI vs. AS $X^2(1)= 13.13, p= 0.0003$ and CAF vs. AS $X^2(1)= 15.15, p= 0.0001$). Note that this feeding avoidance was persistent even if bitter compounds were absent during tests. This inhibition disappeared after 60 min (Fig. 6B, n.s.), suggesting the existence of a memory component that lasts more than 3 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,b; Bodin et al., 2008). As soon as bugs reach a potential host, they search for a zone of the skin to pierce, a process which involves the thermal sense (Lazzari and Nuñez, 1989; Flores and Lazzari, 1996; Ferreira et al., 2007). However, it was still unknow whether these insects could assess the gustatory quality of the substrate or not before piercing the skin. Results presented along our work show that they actually do this. 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 or not. 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 like QUI and CAF, even if the offered food was an appetitive solution. Both substances elicited similar aversive effects at similar concentrations, i.e. 10 mM (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 in our electrophysiological results, we confirmed the gustatory function of 4 chaotic 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 like 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 have 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 might happen 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 group tested to WAT condition), showing that they do not need external contact with phagostimulants to do it. 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 through antennal taste inputs constitutes the first examination done by insects giving place to the first decision making: to accept or reject a potential food source before ingestion starts (Figs. 1, 2). Then, a small gorge of food will be ingested during the sampling phase as described by Smith and Friend (1970). In our work we observed all along the experiments that an insect can ingest between 100 and 280 µl of the AS presented alone, increasing up to 15 times its initial weight during a 10 min alimentation. However, when different bitter tastants (three alkaloids: quinine, caffeine, berberine and one phenolic glycoside: salicin) were added to the AS the insects decreased dramatically the 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 mM (for QUI and SAL) to 0.01 mM (for CAF). Sensitivity thresholds found for compounds that stimulate bitter-sensitive cells in phytophagous insects varied from 0.1 to 10 mM (see Chapman 2003) and in humans from 0.00001 mM to 50 mM (Meyerhof et al., 2005).

Although most insects have internal taste organs in different parts of their alimentary canal or mouthparts, their physiology is by far less studied than external receptors, mainly due to difficulties found to access them with the recording electrodes. In
triatomines, Barth (1952) was the first to suggest the existence of a group of chemosensory structures present in the alimentary canal of *Triatoma infestans*, a related species to *R. prolixus*, particularly in the epipharynx. In other insects, structures with similar functions have been described, as 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 8 short-peg uniporous sensilla observed in the epipharynx of *R. prolixus* (Fig. 5B,C,D) would be responsible for bitter sensing. However, only an electrophysiological approach would serve to 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 in 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 which is not 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 not longer present during tests. This effect lasted for a brief period (between 3 min 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), results presented here do not fit in 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, a 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 from
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 was 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 to accept or not a potential food source for many animals, the fine and highly sensitive perception system of *R. prolixus* to bitter substances was quite surprising for us. What might be the reason for the existence of a bitter detection system in an obligatory blood-sucking insect? *R. prolixus* feeds exclusively on vertebrate’s blood, a feeding media 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 in their plasma can be found. The peak of caffeine after a single cup of coffee for men is estimated between 0.001 to 0.01 mM (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 was probably higher. It might occur then that insects conserved from past ancestors the fine detection system tuned to bitter tastants. In mosquitoes, which feed on plants (males and females) but also on vertebrates 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, 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

Insect’s feeding response is finally governed by the fine contrast between the presence of phagostimulatory and aversive inputs. Our study shows that R. prolixus has two sensory stages working with different avoidance thresholds: antennal input exerts a modulatory bitter signalling at higher doses (10 mM) than internal sensors bathed with feeding solution, whose bitter threshold is about 6 orders of magnitude below for QUI and 3 for CAF. Results obtained along this work were summarized and depicted in a flow chart (Fig. 7). The first assessment of adequateness of a potential food source takes place at the antennal receptors, during the here named 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, 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 move 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 attained a more relevant weight in the central decision about to feed or not, than the phagostimulatory input. Any interactions between chemicals and neurons that occur at the periphery will alter the phagostimulatory or aversive inputs changing significantly the balance. 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 like 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, this species bears 8 sensilla inside their alimentary canal which might be involved in the detection of bitter compounds during the sampling phase, which also inhibited the ingestion. The feeding inhibition observed to bitter compounds acts via these two
sensory inputs working with different thresholds of tolerance. Finally, by applying a cognitive approach, we found that the feeding behavior of triatomines can be negatively modulated by a 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 acquires a significant importance in the frame of the development of novel tools that can help in the surveillance and control of this vector insect.

**Materials and Methods**

**Animals and rearing conditions**

Fifth instars larvae and adults of *R. prolixus* used throughout the experiments were obtained from the laboratory colony, reared at 28°C, ambient relative humidity (RH), 12h:12h L/D cycle. Following ecdysis as 5th instars or adults, insects did not have access to food. Experiments were carried out 15±2 days post-ecdysis.

**Artificial feeder**

Along this work we quantified the weight gained by *R. prolixus* fed with different solutions using an artificial feeder. The *ad hoc* feeding device consisted of two parts: the *feeding recipient*, made of a plastic cylinder (1 cm diameter x 2 cm height) with its lower opening closed with a latex membrane (0.125 mm thick) filled-up with an appetitive solution added or not with different bitter compounds, and the *insect’s recipient*, which was a plastic vial (3 cm diameter x 3.5 cm height) where bugs were individually placed, whose upper openings were covered with a tissue mesh. A piece of filter paper (1.5 cm x 3.5 cm) placed vertically inside the vial helped the animals to climb in order to reach the tissue mesh. The mesh could be embedded or not with different bitter compounds.

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

Then, feeding experiments started when the tissue mesh of the insect’s recipient was carefully put in contact with the piercing membrane of the feeding recipient (triatomines
could easily perforate both with their mouthparts). The feeding assays lasted in all cases 10 minutes.

**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 mM ATP in 0.15 M NaCl evokes a high feeding response in *R. prolixus*. Therefore, for this work we named it arbitrarily as *appetitive solution* (AS) and we used it along as a standardized feeding solution.

Adenosine 5´-triphosphate disodium salt hydrate (ATP), quinine hydrochloride (QUI), berberine chloride hydrate (BER) and D-(-) salicin (SAL) were purchased from Sigma-Aldrich (StLouis, MO, USA). Sodium chloride and caffeine anhydrous were purchased from Biopack (Buenos Aires, AR). All the solutions were prepared weekly and stored at -18 ºC. In all cases, the pH of the solutions was verified and adjusted when necessary to 7 with NaOH 1 M.

**Experimental protocols**

All the experiments were carried out at the beginning of the insects’ scotophase, 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 weight, Wi) and after (final weight, Wf) the feeding tests. A normalized weight gain was calculated as: (Wf-Wi)/Wi. We registered then the percentage of insects whose normalized weight gain was higher than 1 (i.e. bugs that ingested at least one time their own weight).

The effect of the presence of bitter compounds was studied at two different phases of the feeding process of triatomines:

1- 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 mM of QUI or CAF (both prepared in water) over the mesh of the insect’s recipient. Then, the vial was placed in the artificial feeder and the insect was allowed to feed on the AS for 10 min (Part I in 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 added with WAT, QUI or CAF (10 mM), and 3 min or 60 min after, their feeding acceptance of AS was tested.

2-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, SAL (0.000001 mM to 1 mM) were added to the AS in the feeding recipient and offered to the insects in the artificial feeder for 10 min. In these experiments the substrate was always clean (Part II in Results).

Besides, we analyzed the effect of a brief pre-ingestion of bitter compounds on the feeding behavior of insects 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) with the AS alone or added with QUI (0.00001 mM) or CAF (0.01 mM), and 3 min 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 weight gain higher than 1 was registered. We statistically tested if the feeding responses of insects were independent from the different experimental conditions. A global comparison including all treatments was assessed by means of a Pearson's Chi-squared test ($X^2$). Then, whenever the global test was statistically significant ($\alpha = 0.05$), individual post hoc comparisons were done. The standard deviations of percentages (s.d.) were calculated as $\sqrt{p(1-p)/N}$; p: proportion of response; N: number of animals tested. Electrophysiological data were statistically analyzed by using the Wilcoxon test (W). The InfoStat v2012 statistical package was used for the analyses (Di Rienzo JA, Casanoves F, Balzarini MG, Gonzalez L, Tablada M, Robledo CW. Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina (URL [http://www.infostat.com.ar](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 adults of *R. prolixus* were scanned by means of a scanning electronic microscopy (SEM) in order to search for taste sensilla, putative
candidates involved in the substrate/food recognition. The antennae were cut at the base and mounted horizontally with double-sided tape on a standard aluminum stub. The epipharynx was exposed by performing a small ventral opening in the anterior part of the head of the insects. Then the lumen of the alimentary canal was exposed cutting a second opening that uncovered the internal sensilla. The head was then mounted on an aluminum stud. All samples were coated successively during 180 s with gold/palladium (40/60%) before examination in a scanning electron microscope Philips XL 30.

**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 4 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 Hodgson et al. (1955) recording method, animals were grounded via a silver wire to the left eye (reference electrode) and an individual sensillum was inserted for 3 seconds in a glass electrode (recording electrode) containing the electrolyte alone (10 mM KCl) or added with the bitter stimuli (QUI or CAF 0.01, 0.1 and 1 mM presented in ascending order). Each sensillum was tested first with KCl and then with CAF or QUI in an random order. Time between subsequent stimulations was fixed to 1 minute.

The recording electrode (20-30 µm diameter) was connected to a preamplifier (gain x10, TastePROBE DTP-02, Syntech) and the biological signals were further amplified, filtered and digitalized by means of an IDAC4 (Syntech) (gain x100, eight-order Bessel pass-band filter: 1–3000 Hz, sampling rate: 10 kHz, 16 bits). The data were stored on computer. Spike detection and analysis were done off-line by using Autospike (Syntech). The number of spikes was counted to the first second of stimulation.
Acknowledgments
This work was funded by the ANPCyT, FONCyT, University of Buenos Aires (grant number PICT PRH 2009-081 to RBB and PICT PRH 2009-029 to SM), CONICET (PIP 1053 to RBB and SM, and a postdoctoral grant to GP) and Cesar Milstein (to MGdBS).

Author Contributions

Competing Interests
The authors have no competing interests.
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**Figure legends**

**Figure 1- Effect of external bitter detection on the feeding behavior of *R. prolixus.***

The percentage of insects that fed at least one time their own weight on AS is represented when the piercing mesh was spread with WAT (dashed line), QUI or CAF at different concentrations. The addition of high doses of QUI and CAF over the piercing mesh elicited an inhibitory effect on their feeding behavior. Asterisks denote statistical differences with the WAT group (Pearson Chi-Square, p<0.05). 20 replicates were carried out for each concentration. AS: appetitive solution, WAT: water, QUI: quinine, CAF: caffeine.

**Figure 2- Identification of the sensory structures involved in the feeding inhibition of *R. prolixus.*** The percentage of insects that fed at least one time their own weight on AS is represented when the piercing mesh was spread with WAT, QUI or CAF. INT: intact animals, LEG-: animals deprived from legs inputs, ANT-: animals deprived from antennal inputs (last flagella). Feeding inhibition evoked by externally contacting a
bitter substrate occurred in INT and in LEG- groups, and not in ANT-, suggesting that bitter sensing takes place through taste inputs of the antennae of these insects. Asterisks denote statistical differences with the corresponding WAT group (Pearson Chi-Square, p<0.05). 20 replicates were carried out for each condition. AS: appetitive solution, WAT: water, QUI: quinine, CAF: caffeine.

Figure 3- Bitter detection by antennal taste sensilla of *R. prolixus*. A: photograph of the last flagellum of the antenna under SEM showing 4 chaetic gustatory sensilla. B: mean spike frequency of gustatory neurons from these 4 sensilla stimulated with QUI or CAF at different concentrations. C: typical single-sensillum recordings showing a gustatory receptor neuron excitatory response to KCl (control), QUI and CAF; spikes are denoted with 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. Asterisk denotes statistical differences with the control (KCl) (Wilcoxon test, p<0.05). Each dot represents the media and ±s.e. of 8 sensilla. QUI: quinine, CAF: caffeine.

Figure 4- Modulation of the feeding behavior of insects by a previous contact with bitter compounds. The percentage of insects that fed at least one time their own weight 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 3min after pre-exposure but the antennal last flagella of bugs were cut-off immediately after pre-exposure (ANT-). 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 statistical differences with the corresponding WAT group (Pearson Chi-Square, p<0.05). 30 replicates were carried out for each condition. AS: appetitive solution, WAT: water, QUI: quinine, CAF: caffeine, PE: pre-exposure, T: feeding test.

Figure 5- Effect of internal bitter detection on the feeding behavior of *R. prolixus*. The percentage of insects that fed at least one time their own weight on AS alone (dashed line) or added with QUI, CAF, BER or SAL at different concentrations is represented. 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 8 short-peg gustatory sensilla. D: detail of one gustatory sensillum bearing an apical pore. Asterisks denote statistical differences with the AS group (Pearson Chi-Square, $p<0.05$). 20 replicates were carried out for each concentration. AS: appetitive solution, QUI: quinine, CAF: caffeine, BER: berberine, SAL: salicine.

**Figure 6- Modulation of the feeding behavior of insects by a previous ingestion of bitter compounds.** The percentage of insects that fed at least one time their own weight on AS is represented. Bugs were pre-exposed to HT, AS, AS+QUI or AS+CAF. Feeding tests to AS were carried out 3min (A) or 60min (B) after pre-exposure. A brief pre-ingestion of bitter compounds evoked a feeding avoidance to the AS that lasted more than 3min but less than 60min. Asterisks denote statistical differences with the corresponding AS group (Pearson Chi-Square, $p<0.05$). 30 replicates were carried out for each condition. HT: heat, AS: appetitive solution, QUI: quinine, CAF: caffeine, PE: pre-exposure, T: feeding test.

**Figure 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).
FIGURE 2

The diagram shows a bar chart illustrating feeding percentages under different conditions. The x-axis represents different treatments labeled as WAT, QUI, and CAF for each category: INT, LEG-, and ANT-. The y-axis represents feeding percentages ranging from 0 to 80. The bars are accompanied by error bars indicating the variability. Asterisks (*) indicate significant differences. The mesh in the diagram suggests an experimental setup with labeled controls (WAT, QUI, CAF) and treatments.
FIGURE 3

Bitter compounds in KCl (mM)

Firing rate (spikes/s)

A

B

C

KCl 10mM

KCl 10mM+QUI 0.1mM

KCl 10mM+CAF 1mM

100ms

1mv
FIGURE 4

Feeding (%) vs. Pre-exposure

A

WAT  QUI  CAF

B

WAT  QUI  CAF

C

WAT  QUI  CAF

* *
FIGURE 5

A

Feeding (%) vs. concentration of different substances.

B

Microscopic images showing epipharynx and gustatory sensilla.

C

Epipharynx and gustatory sensilla at higher magnification.

D

Image of a structure at a lower magnification.
FIGURE 6
FIGURE 7

A. Substrate contact
B. Insertion of mouthparts
C. Pumping small quantity of food
D. Engagement

1. External receptors (antenna)
2. Substrate contact
3. NO FEEDING
4. Internal receptors (epiph.)
5. PHAGOSTIMULANTS

NO FEEDING

BITTER

External receptors (antenna)

PHAGOSTIMULANTS

ENGORGEMENT