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Kissing bugs can generalize and discriminate between different bitter compounds

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ABSTRACT

Animals make use of contact chemoreception structures to examine the quality of potential food sources. During this evaluation they can detect nutritious compounds that promote feeding and recognize toxins that trigger evasive behaviors. Although animals can easily distinguish between stimuli of different gustatory qualities (bitter, salty, sweet, etc.), their ability to discriminate between compounds of the same quality may be limited. Numerous plants produce alkaloids, compounds that elicit aversive behaviors in phytophagous insects and almost uniformly evoke a bitter taste for man. In hematophagous insects, however, the effect of feeding deterrent molecules has been barely studied. Recent studies showed that feeding in *Rhodnius prolixus* can be negatively modulated by the presence of alkaloids such as quinine (QUI) and caffeine (CAF), compounds that elicit similar aversive responses. Here, we applied associative and non-associative learning paradigms to examine under two behavioral contexts the ability of *R. prolixus* to distinguish, discriminate and/or generalize between these two bitter compounds, QUI and CAF.

Our results show that bugs innately repelled by bitter compounds can change their behavior from avoidance to indifference or even to preference according to their previous experiences. After an aversive operant conditioning with QUI or CAF, *R. prolixus* modified its behavior in a direct but also in a cross-compound manner, suggesting the occurrence of a generalization process between these two alkaloids. Conversely, after a long pre-exposure to each alkaloid, bugs decreased their avoidance to the compound used during pre-exposure but still expressed an avoidance of the novel compound, proving that QUI and CAF are detected separately. Our results suggest that *R. prolixus* is able to discriminate between QUI and CAF, although after an associative conditioning they express a symmetrical cross-generalization. This kind of studies adds insight into the gustatory sense of a blood-sucking model but also into the learning abilities of hematophagous insects.

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1. Introduction

Animals rely on the gustatory system to recognize toxins and poisons, alerting the central nervous system, which can in turn elicit appropriate aversive behaviors. Many compounds are produced by plants as defense mechanisms against herbivory, and many of them usually taste bitter to humans. Besides this anthropocentric view of the gustatory sense, bitter perception comprises many different molecules that have deterrent effects in animals. It is generally assumed that bitter detection is a quite conserved feature

among animals that dissuades the ingestion of potentially toxic food (Yarmolinsky et al., 2009; Freeman and Dahanukar, 2015). Feeding interference can be induced by chemicals of numerous identities, but especially alkaloids have been described as extremely potent deterrent agents in a variety of insects (Chapman, 2003; Schoonhoven, 1982). Feeding can be inhibited if an individual perceives a bad taste (i.e. not noxious) in food, or if after eating it undergoes a particular malaise or physiological constraint (i.e. not distasteful) (Ayestaran et al., 2010; Hurst et al., 2014). Generally non-palatability and toxicity come together in nature.

Gustatory discrimination between taste stimuli of different qualities (i.e. salty, bitter, sweet, sour and umami) can be achieved by the selective activation of segregated neuronal populations encoding different taste qualities (Chen et al., 2011; Marella et al., 2006; Thorne et al., 2004; Zhang et al., 2003). Accordingly, bitter-sensitive neurons project their axons to areas of the brain

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different from those where sugar-sensitive neurons project (Harris et al., 2015; Marella et al., 2006; Wang et al., 2004). However, the ability to distinguish among compounds of the same qualities is still controversial (Masek and Scott, 2010). Nevertheless, gustatory receptor neurons (GRNs) can encode incoming information from the peripheral detection of bitter chemicals with different temporal or spatio-temporal codes, making possible the discrimination between tastants of the same quality (Dethier and Crnjar, 1982; Glendinning et al., 2002, 2006; Reiter et al., 2015; Schoonhoven et al., 1992; Weiss et al., 2011).

Bitter detection starts at the GRNs housed inside sensory organs or sensilla distributed along the insects' body (Chapman, 1982). In the fruit fly *Drosophila melanogaster*, among the gustatory receptors (GRs) expressed in the membrane of the GRNs, some are known to detect bitter compounds (Lee et al., 2009, 2012, 2015; Ling et al., 2014; Moon et al., 2006; Shim et al., 2015; Weiss et al., 2011; for a detailed review of *Drosophilas's* bitter taste see French et al., 2015). Once activated, the bitter-sensitive neurons trigger aversive behaviors in insects (Meunier et al., 2003; Thorne et al., 2004; Weiss et al., 2011). Moreover, bitter stimuli can work as reinforcers, producing gustatory memories that affect insect's decisions (El-Keredy et al., 2012). In hematophagous animals the role of bitterness in modulating insects' behavior remains almost unexplored. It was only recently that it was shown that bitter compounds may have an aversive effect on the feeding behavior of mosquitoes (Ignell et al., 2010; Kessler et al., 2013, 2014). Moreover, based on sequence-homology comparisons with *D. melanogaster* (Kent et al., 2008) and on electrophysiological recordings (Sanford et al., 2013), the most abundant GR expressed in the mouthparts of the mosquito *Aedes aegypti* seems to be a bitter receptor (Sparks et al., 2013). This bitter-respondering neuron is sensitive to quinine, among other deterrents (Sanford et al., 2013; Sparks and Dickens, 2016a,b). In the kissing bug *R. prolixus*, vector of Chagas Disease in Latin America, it was shown that bitter compounds can affect feeding at two independent moments of the feeding behavior: when the insects contact the host skin, and when they ingest the blood (Pontes et al., 2014). Contact with surfaces embedded with quinine or caffeine inhibited feeding in *R. prolixus*. This inhibition, however, disappeared when the distal flagellomere of the antennae was ablated, indicating that quinine and caffeine are detected by tuned gustatory sensilla within this antennal segment (Pontes et al., 2014). Moreover, electrophysiological recordings of GRNs of the antenna of *R. prolixus* revealed a dose-dependent response to quinine and caffeine (Pontes et al., 2014). The recent annotation of the *R. prolixus* genome revealed 28 GRs, where GR5-11 might putatively encode bitter taste receptors (Mesquita et al., 2016). Although quinine and caffeine evoked aversive behaviors in *R. prolixus* (Pontes et al., 2014), we still do not know whether these insects are able to distinguish, discriminate or generalize among these two alkaloids. Along our work, we will consider *to distinguish* as the ability to detect two stimuli as different; *to discriminate* as the ability to express two different behavioral outputs when confronted to different stimuli; and *to generalize* as the ability to consider two different stimuli as belonging to the same category.

In this work we evaluated if *R. prolixus* is able to distinguish between quinine and caffeine, and to discriminate and/or generalize between them. Since the behavioral outputs generated by both bitter compounds in these hematophagous bugs are quite similar, associative (i.e. operant aversive conditioning) and non-associative (i.e. chemical pre-exposure) protocols were applied to uncover if they are able to detect these alkaloids as different compounds or not. The experiments were carried out by analyzing the effect of bitterness in the behavior of *R. prolixus* in two different contexts: (A) feeding, by using an artificial feeder, or (B) exploratory walking activity, by using a two-choice arena.

2. Materials and methods

2.1. Insects

Fifth instar larvae of *R. prolixus* (Heteroptera: Reduviidae: Triatominae) reared in our laboratory (constant 28 °C, 50–90% relative humidity, 12 h:12 h L/D cycle) were used along this work. Insects were kept unfed for 14 days after 4th to 5th instar ecdysis and then used in the different experiments.

2.2. Experiment A: bitterness and feeding behavior

2.2.1. Artificial feeder

The effect that bitter compounds might have in the feeding behavior of larvae of *R. prolixus* was analyzed using an artificial feeder. It consisted of a plastic feeding recipient (1 cm diameter, 2 cm height) with its base replaced by a latex membrane (0.125 mm thick). This recipient was filled up with a feeding solution and placed close to a thermostated aluminum plate that heated the solution to 35 °C. The latex in contact with the solution also acquired the same temperature. Separately, an insect was placed inside a second plastic recipient (3 cm diameter, 3.5 cm height) with its upper opening covered with a tissue mesh. A piece of filter paper (1.5 × 3.5 cm) placed vertically inside this recipient helped the animals to climb in order to reach the tissue mesh. Feeding experiments started when the tissue mesh of the insect's recipient was carefully put into contact with the latex membrane of the feeding recipient. *R. prolixus* could easily perforate both layers (i.e. the tissue mesh and the latex membrane) with their mouthparts and ingest the feeding solution.

An appetitive solution (AS) of 1 mM ATP and 0.15 M NaCl was offered as food source along the experiments, since previous works reported that such solution evokes a high feeding response in *R. prolixus* (Pontes et al., 2014). To analyze the effect of the detection of bitter compounds in the feeding behavior of triatomines, the tissue mesh of the insect's recipient was embedded with 50 µl of distilled water (WAT), 0.01 M quinine (QUI) or 0.01 M caffeine (CAF). Note that QUI or CAF were added to the piercing membrane but not to the appetitive feeding solution.

All experiments were carried out in a dark room, during the insects' scotophase. Each individual was weighed before (initial mass, Mi) and after (final mass, Mf) the feeding test, which lasted for 10 min. A normalized mass-gain or feeding index (FI) was calculated as (Mf-Mi)/Mi. The mean feeding index (FI) of each experimental group was compared to the expected feeding index of naïve animals fed on AS with the mesh embedded with water (Fig. 1, dashed line) by means of one sample *t*-tests ($\alpha = 0.05$).

2.2.2. Pre-exposure to QUI or CAF

To analyze the effect of a previous experience with bitter compounds on the feeding behavior of *R. prolixus*, they were individually pre-exposed to QUI or CAF by placing them inside a recipient (similar to the insect's recipient of the artificial feeder) whose tissue mesh was embedded with 50 µl of WAT, 0.01 M QUI or 0.01 M CAF (using a micropipette). Immediately after, insects were placed and left inside this recipient in contact with the bitter compounds (or the water) during 30 s, then transferred to a new clean recipient, and 3 min after placed in the artificial feeder to test their feeding behavior in presence of QUI or CAF as explained in Section 2.2.1.

2.3. Experiment B: bitterness and exploratory walking behavior

2.3.1. Two-choice walking arena

The effect that the detection of bitter compounds spread over the walking substrate of *R. prolixus* might exert on its exploratory

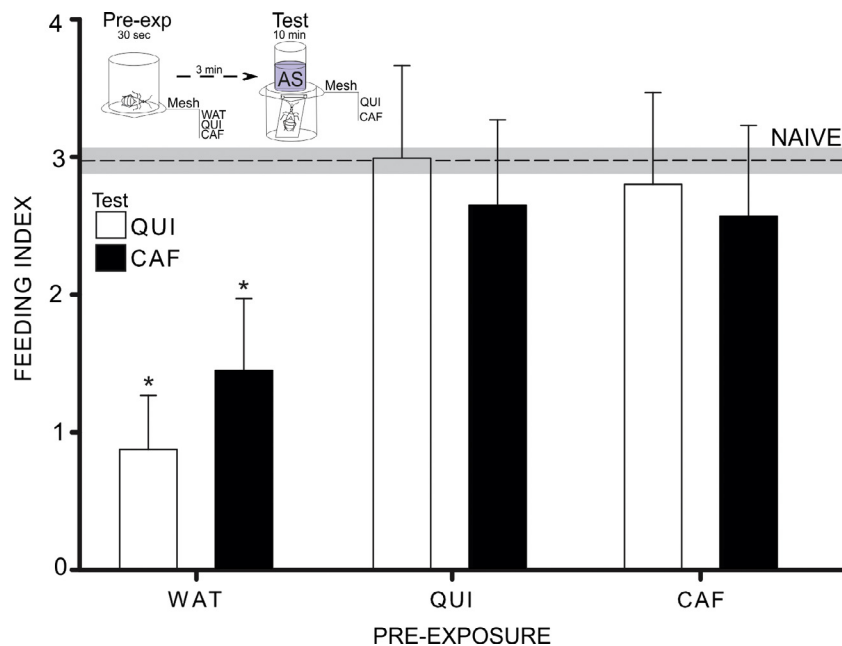


Fig. 1. Non-associative modulation of the feeding deterrence by bitter compounds. QUI and CAF spread over the piercing membrane interfered with the feeding of WAT pre-exposed *R. prolixus*. A pre-exposure to QUI or CAF evanished such deterrence in a non-specific compound manner. The Feeding Index expresses how many times their own weight each insect ingested. Each column represents the mean and standard error of 30 replicates. Horizontal dashed line shows the mean Feeding Index (SE in grey) of naive insects feeding over a clean mesh. AS = appetitive solution. WAT = distilled water. QUI = quinine. CAF = caffeine.

behavior was studied by individually releasing insects in a two-choice experimental arena. The arena consisted of a rectangular acrylic box (8×4 cm) divided in two equal zones. Each zone was covered with a filter paper that could be loaded with 100 μ l of WAT, 0.1 M CAF or 0.1 M QUI. All solutions were loaded on the paper with a micropipette immediately before releasing the insects. As a result, larvae were confronted to the following chemical two-choices: WAT/QUI, WAT/CAF or QUI/CAF.

All experiments were carried out in a dark room, during the insects' scotophase. One insect was gently released at the center of the arena and its preference in relation to the position of the added stimuli (i.e. WAT, QUI or CAF) was recorded during 4 min using an infrared-sensitive video-camera connected to a digital recorder. The time spent at each side of the arena was registered separately during training and test. A preference index (PI) was then calculated as the difference between the time spent at each side of the arena divided by the total experimental time. PIs near 0 indicate lack of preference. PIs close to -1 or 1 show preference for one side of the arena. PIs were compared against 0 (i.e. no preference) by using one sample *t*-tests ($\alpha = 0.05$).

2.3.2. Operant aversive conditioning

The effect of applying an operant aversive protocol, in which WAT, QUI or CAF were negatively reinforced with a mechanical disturbance, was analyzed in the two-choice arena. The negative reinforcement was delivered by attaching the experimental arena to a vortex mixer, which generated a mechanical vibration that disturbed the insects. One larva was gently released at the center of the arena and the mechanical disturbance was delivered every time the insect entered a previously defined punished-side of the arena, and turned off when they occupied the opposite side. The negative reinforcement was delivered associated with WAT, with QUI or with CAF in different experimental series. Four experimental training series were applied (the symbol "(–)" minus indicates the punished side of the arena during training): (1) QUI(–)/CAF: half of the arena contained QUI, the other half contained CAF, the mechanical disturbance was only applied when the insect entered the QUI side, (2) QUI/CAF(–): idem 1, but the CAF side was pun-

ished; (3) WAT(–)/QUI: half of the arena contained WAT, the other half contained QUI, the mechanical disturbance was only applied when the insect entered the WAT side, (4) WAT(–)/CAF: idem 3, but the non-punished side contained CAF. These associative trainings were applied during 4 min. Pseudoconditioning series, in which the mechanical disturbance was applied independently from the position of the insect, were run in parallel. Following trainings, the insects were removed from the arena and placed in a clean flask for 1 min. After this time, their preference over the two-choice arena was tested as explained in Section 2.3.1. Note that during tests no mechanical disturbance was applied.

2.3.3. Pre-exposure to QUI or CAF

The effect of a long pre-exposure to bitter compounds in the exploratory walking activity of *R. prolixus* was analyzed in the two-choice arena. Insects were individually placed inside a plastic recipient (2 cm diameter, 3 cm height) whose floor was covered with a filter paper embedded with 100 μ l of WAT, 0.1 M CAF or 0.1 M QUI. After 60 min of such chemical pre-exposure, the animals were transferred to the two-choice arena where their preferences in relation to the position of QUI or CAF were registered as pointed in Section 2.3.1.

2.4. Stimuli

ATP (adenosine triphosphate) and QUI (quinine hydrochloride dihydrate) were purchased from Sigma-Aldrich (St Louis, MO, USA). NaCl (sodium chloride) and CAF (caffeine anhydrous) were purchased from Biopack (Buenos Aires, Argentina). The pH of the appetitive feeding solution was verified and adjusted to 7 when necessary with NaOH 1 M. All solutions were prepared weekly and stored at -18°C .

3. Results

In this work we analyzed the chemical specificity of the aversive effect evoked by two different alkaloid bitter compounds, QUI and

CAF, in *R. prolixus*. Applying non-associative and associative protocols in different behavioral contexts (i.e. feeding and exploratory walking activity) we show that *R. prolixus* can detect QUI and CAF as different compounds and that it can generalize or discriminate among them according to the behavioral context.

3.1. Bitterness-mediated modulation of the feeding behavior

3.1.1. Non-associative pre-exposure to QUI or CAF

In control series where insects were pre-exposed to water and the piercing membrane was embedded with water (i.e. naïve), insects fed about 3 times their own weight (Fig. 1, horizontal dashed line). But, if QUI or CAF were added to the piercing membrane, the feeding intake of insects pre-exposed to WAT comparatively decreased ($t_{\text{QUI}} = -5.34$, $p < 0.001$; $t_{\text{CAF}} = -2.90$, $p = 0.006$). However, this bitterness-mediated feeding inhibition disappeared after a pre-exposure to QUI ($t_{\text{QUI}} = 0.03$, $p = 0.974$; $t_{\text{CAF}} = -0.51$, $p = 0.610$) or CAF ($t_{\text{QUI}} = -0.25$, $p = 0.802$; $t_{\text{CAF}} = -0.60$, $p = 0.548$).

It is worth noting that the aversive effects of spreading QUI or CAF on the piercing mesh were quite similar: a marked feeding deterrence was observed in both cases. Similarly, the pre-exposure to QUI or CAF also rendered similar results: in both cases a decrease in the feeding deterrence was observed. Moreover, this modulation effect occurred in an intra-compound manner (i.e. QUI-QUI and CAF-CAF) but also in an inter-compound way (i.e. QUI-CAF and CAF-QUI). These results do not allow us to ascertain if animals cannot distinguish between QUI and CAF or if conversely, they can detect them as different compounds but they generalize among them.

3.2. Bitterness-mediated modulation of the exploratory walking behavior

3.2.1. Innate avoidance of bitter compounds

In the two-choice experiments carried out in the walking arena *R. prolixus* avoided both, QUI and CAF when confronted to water (i.e. WAT/QUI or WAT/CAF, Fig. 2, $t_{\text{WAT/QUI}} = -3.36$, $p = 0.002$, $t_{\text{WAT/CAF}} = -5.57$, $p < 0.001$). These results reveal that bugs can perceive QUI and CAF in the substrate and behave in consequence avoiding them. However, when QUI and CAF were presented simultaneously at each side of the arena, no preference for any of the bitter compounds was exhibited (Fig. 2, $t_{\text{QUI/CAF}} = -0.05$, $p = 0.960$). This lack of preference could be a consequence of two opposite situations. On one hand, these insects could be simply unable to distinguish between QUI and CAF. But on the other, they could be detecting them as different compounds but responding in

a similar manner (i.e. similar intensity of avoidance) to both compounds.

3.2.2. Associative modulation of the bitterness avoidance behavior: operant aversive conditioning

In these experiments, different operant aversive conditioning protocols were applied before testing the responses of *R. prolixus* confronted to WAT, QUI or CAF (Fig. 3). During trainings QUI(-)/CAF and QUI/CAF(-), insects avoided the punished side of the arena, verifying that the mechanical disturbance was indeed a negative reinforcement (Fig. 3A, triangles, $t_{\text{QUI(-)/CAF}} = 2.35$, $p = 0.025$, $t_{\text{QUI/CAF(-)}} = -3.58$, $p = 0.001$). However, insects did not modify their lack of preference in subsequent tests in which the choice QUI/CAF was presented (Fig. 3A, circles, $t_{\text{QUI/CAF}} = -0.68$, $p = 0.499$, $t_{\text{QUI/CAF}} = 0.15$, $p = 0.874$).

In order to discard that the applied conditioning protocols were not adequate for establishing an association between the bitter compound and the negative reinforcement, we applied two different conditionings protocols: WAT(-)/QUI and WAT(-)/CAF (Fig. 3B). During training, the insects avoided the punished side of the arena (WAT side), even if the alternative side contained aversive compounds such as QUI or CAF (Fig. 3B, triangles, $t_{\text{WAT(-)/QUI}} = 2.14$, $p = 0.040$, $t_{\text{WAT(-)/CAF}} = 2.40$, $p = 0.022$). Then, during tests (in which mechanical vibrations were no longer delivered) insects continued to avoid the WAT side, preferring to remain at the QUI or CAF side (Fig. 3B, circles, $t_{\text{WAT/QUI}} = 2.09$, $p = 0.045$, $t_{\text{WAT/CAF}} = 3.00$, $p = 0.005$). These results show that these insects are able to learn to avoid previously punished zones of the arena.

Then, insects were again trained in a WAT(-)/QUI protocol, but then tested in a WAT/CAF test, i.e. a context in which a novel bitter compound was presented (Fig. 3C). Insects preferred the QUI side during training (Fig. 3C, triangles, $t_{\text{WAT(-)/QUI}} = 3.24$, $p = 0.002$) and then preferred the novel compound side (CAF) during test (circles, $t_{\text{WAT/CAF}} = 3.16$, $p = 0.003$). Similarly when animals were trained to WAT(-)/CAF, they preferred the CAF side during training (triangles, $t_{\text{WAT(-)/CAF}} = 2.28$, $p = 0.030$) and then the QUI side during the test (circles, $t_{\text{WAT/QUI}} = 2.41$, $p = 0.022$).

Finally, insects trained to WAT(-)/QUI or WAT(-)/CAF avoided the punished side during trainings (Fig. 3D, triangles, $t_{\text{WAT(-)/QUI}} = 4.32$, $p < 0.001$; $t_{\text{WAT(-)/CAF}} = 4.85$, $p < 0.001$) but exhibited no significant preferences for any side of the arena when tested to QUI/CAF (circles, $t_{\text{QUI/CAF}} = -0.41$, $p = 0.678$; $t_{\text{QUI/CAF}} = 0.94$, $p = 0.351$).

All pseudoconditioning series rendered random distribution of insects over the arena during trainings (Fig. S1) suggesting that the random delivery of vibration inhibits the expression of the

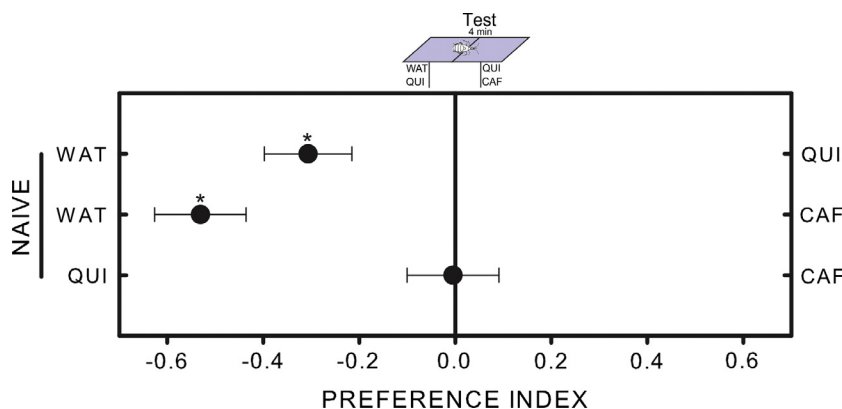


Fig. 2. Innate avoidance behavior of bitter compounds. QUI and CAF spread over the walking substrate repelled *R. prolixus* when confronted with WAT in a two-choice arena. However, when presented simultaneously insects exhibited no preference. The Preference Index expresses the time spent at each side of the arena: 0 = half time at each side, -1 and 1 = preference for one side of the arena. Each point represents the mean and standard error of 30 replicates. WAT = distilled water. QUI = quinine. CAF = caffeine.

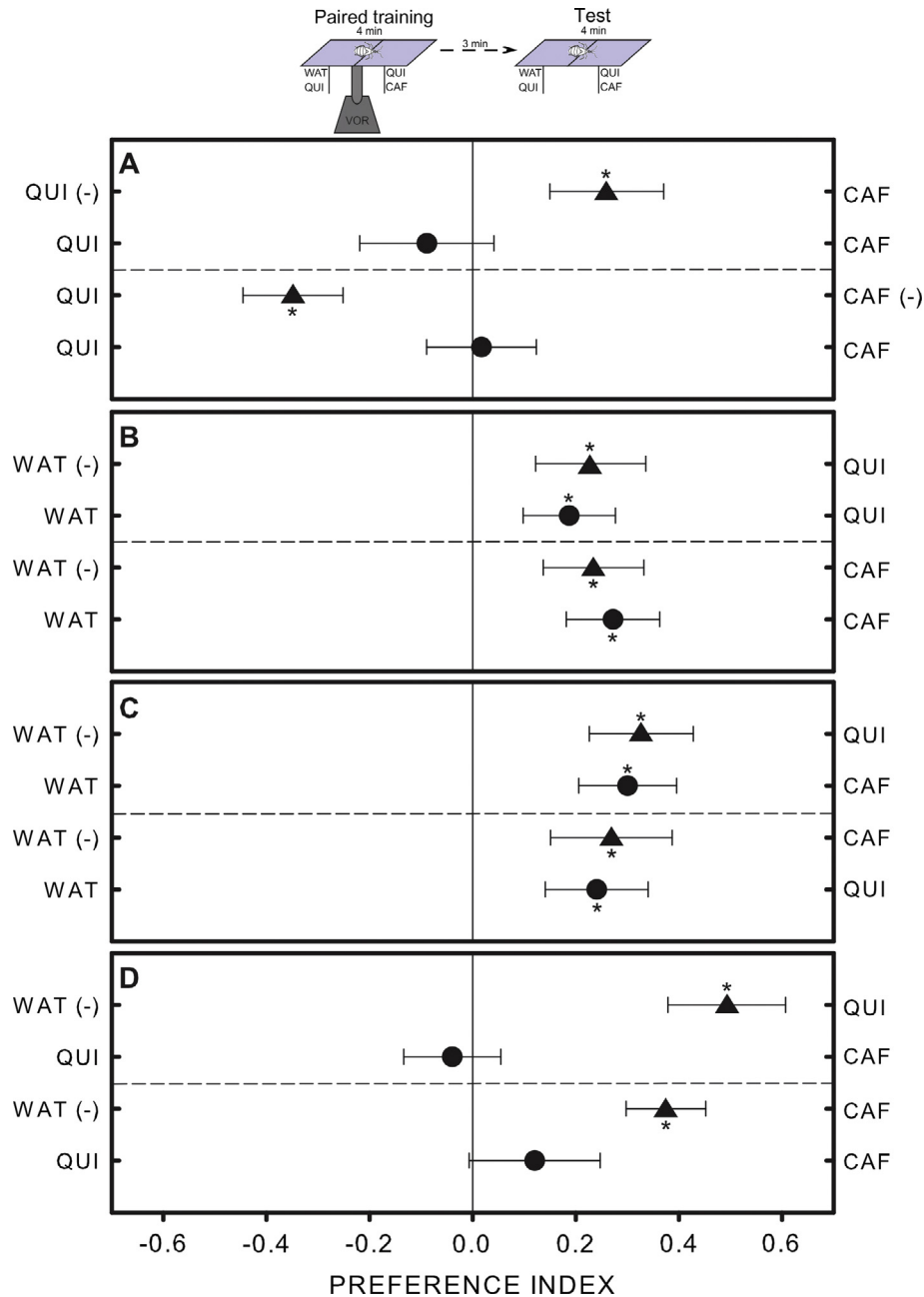


Fig. 3. Associative modulation of the bitterness avoidance behavior. (A) The lack of preference when QUI and CAF were presented simultaneously was not modified by an aversive conditioning QUI(-) or CAF(-). (B) The aversion to QUI and CAF confronted to WAT was reverted by an aversive conditioning with the same bitter compound or (C) with the novel bitter compound. (D) The lack of preference when QUI and CAF were presented simultaneously was not modified by an aversive conditioning WAT(-). The Preference Index expresses the time spent at each side of the arena: 0 = half time at each side, -1 and 1 = preference for one side of the arena. Each point represents the mean and standard error of 30 replicates. Triangles = training. Circles = test. The (-) shows the punished compound during training. VOR = vortex mixer. WAT = distilled water. QUI = quinine. CAF = caffeine.

naïve bitterness-avoidance behavior. However, during tests, all of them behaved as naïve animals, avoiding the bitter compounds when confronted to WAT and not presenting a preference when QUI and CAF were presented simultaneously at each side of the arena. These results confirm that in the conditioning series presented above, an associative learning was responsible for the modulation of the bitterness avoidance.

Results of these series show that an associative process can be established in *R. prolixus* after an operant aversive conditioning. However, this modulation was not compound specific. So, to analyze if these insects are not able to distinguish between QUI and CAF or if instead they are indeed capable of doing it but they generalize during the associative process, next series were designed.

3.2.3. Non-associative modulation of the bitterness avoidance behavior: pre-exposure to QUI or CAF

Insects were exposed during 60 min to WAT, QUI or CAF before their performances in the two-choice arena were tested. Insects pre-exposed to WAT behaved as naïve ones, avoiding QUI in WAT/QUI tests and CAF in WAT/CAF tests, and exhibiting no preference when both bitter compounds were presented together, i.e. QUI/CAF (Fig. S2). These results show that a pre-exposure to water does not change the repellence of the bitter compounds substantially. However, a pre-exposure to QUI or CAF evoked a decrease in the repellence produced by the same but not by the novel bitter compound (Fig. 4A): i.e. a pre-exposure to QUI decreased the repellence of QUI in WAT/QUI tests ($t_{\text{QUI}} = 1.19$, $p = 0.241$) but not the

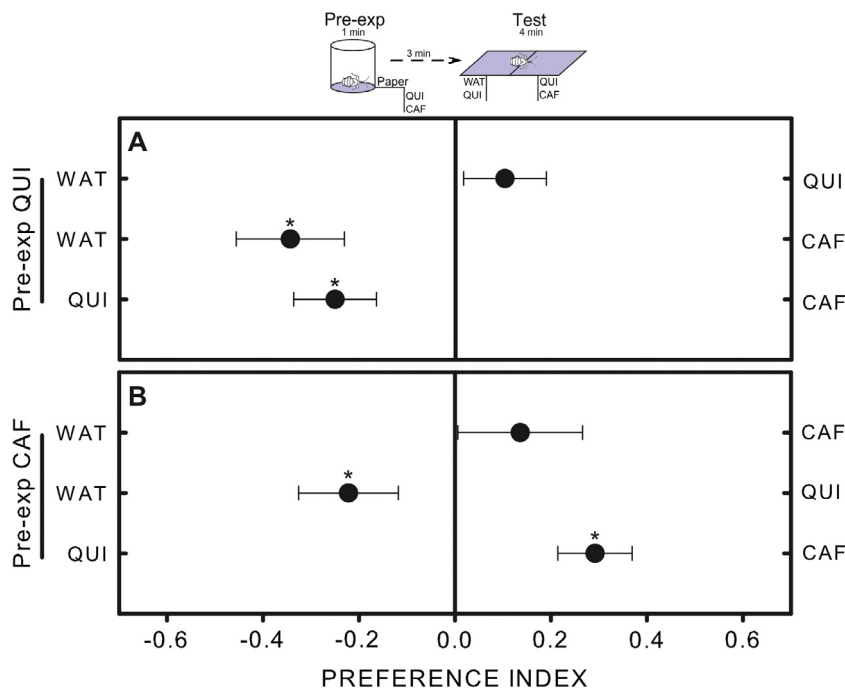


Fig. 4. Non-associative modulation of the bitterness avoidance behavior. A pre-exposure to QUI (A) or CAF (B) reduced the aversion of *R. prolixus* to the same but not to the novel bitter compound. The Preference Index expresses the time spent at each side of the arena: 0 = half time at each side, -1 and 1 = preference for one side of the arena. Each point represents the mean and standard error of 30 replicates. WAT = distilled water. QUI = quinine. CAF = caffeine.

repellence of CAF in WAT/CAF ($t_{CAF} = -3.05$, $p = 0.004$). Similarly, a pre-exposure to CAF decreased the repellence of CAF in WAT/CAF (Fig. 4B, $t_{CAF} = -2.13$, $p = 0.304$) but not that of QUI in WAT/QUI ($t_{QUI} = 1.04$, $p = 0.041$). Moreover, insects pre-exposed to QUI and tested with both bitter compounds presented simultaneously (i.e. QUI/CAF) avoided the CAF side of the arena (Fig. 4A, $t_{QUI} = -2.91$, $p = 0.006$), revealing a compound specific modulation of the repellence. Similarly, when pre-exposed to CAF, bugs avoided the QUI side during tests QUI/CAF (Fig. 4B, $t_{QUI} = 3.77$, $p < 0.001$).

These results, in which the decrease in the bitterness-avoidance behavior of *R. prolixus* produced by a pre-exposure is compound-specific, prove that *R. prolixus* can detect QUI and CAF as different compounds. We suggest then that, at least after a chemical pre-exposure to QUI or CAF, these bugs can discriminate between these two alkaloids.

4. Discussion

In this work we addressed the capacity of *R. prolixus* to distinguish between two bitter alkaloids, QUI and CAF. First, we evaluated the innate response of insects to both compounds in a feeding context and while animals explored a two-choice arena. Then we applied associative and non-associative protocols to analyze if the modulation of the aversion observed was compound-specific or not.

Our results confirm that feeding in *R. prolixus* is inhibited by the presence of quinine and caffeine over the piercing membrane (Pontes et al., 2014). Besides, both alkaloids produced a repellent effect in these insects over the two-choice walking arena. Although bitter perception in *R. prolixus* was demonstrated, the existence of bitter receptors in a blood-sucking insect that feeds mainly on blood of vertebrates (i.e. a medium that intrinsically lacks of caffeine and quinine) is quite intriguing. Hematophagous hemipterans evolved from common phytophagous ancestors, for which the adaptive pressure of sensing bitter compounds is well

described, as plants can produce toxic alkaloids as a way of minimizing herbivory. It is likely then that triatomines could have retained this gustatory capability from their ancestors. Mosquitoes, which feed on plants (males and females) but also on vertebrates' blood (only females), also present neuronal and behavioral responses to quinine (Ignell et al., 2010; Kessler et al., 2014, 2013; Sanford et al., 2013).

Bitter taste discrimination and generalization processes have been widely studied in vertebrates and insects (e.g. Brassler et al., 2005; Glendinning et al., 2002; Masek and Scott, 2010; Spector and Kopka, 2002). In the major part of these studies, learning has been a useful experimental tool to analyze if animals can distinguish between two stimuli. It is assumed that if after an associative conditioning or a habituation process, animals modify their responses to the stimulus used along training, but not to a novel stimulus, both compounds are discriminated. However, if the modulation of the response is not stimulus-specific, animals could be incapable of distinguishing them, or alternatively they could detect them as different stimuli but generalize them at a further processing stage. Regarding the perception of bitter compounds, different studies demonstrated perceptual distinctions among species. In hamsters, conditioned taste aversions to quinine and denatonium cross-generalize with each other (Spector and Kopka, 2002). Likewise, mice and rats can discriminate quinine from nicotine (Oliveira-Maia et al., 2009). Contrarily, fruit flies cannot discriminate among bitter compounds (Masek and Scott, 2010). To our knowledge, this is the first report describing the bitter-taste perceptual capacities of triatomines.

In our feeding experiments, after a short pre-exposure to QUI or CAF, insects fed over bitter substrates that they avoided before. This modulation resulted to be non-specific, as insects also fed when the membrane was embedded with the novel bitter compound. These results still did not allow us to discern if the insects were not distinguishing between QUI and CAF, or if instead they were generalizing across them. Note that a generalization implies that animals are indeed detecting both stimuli as different, but

the learned response is transferred from one stimulus to another. In any case, the detection of bitter tastes on the piercing membrane is achieved by specific receptors housed in the antennae of these insects. Previous work shows that cutting the distal flagellomere of antennae vanishes the deterrent effect of bitter compounds (Pontes et al., 2014). Electrophysiological recordings from the 4 chaetic hairs in the distal flagellomere of the antennae of *R. prolixus* confirmed the response to quinine and caffeine in a dose-dependent manner (Pontes et al., 2014). In several insects, an exposure-induced desensitization of bitter-sensitive taste cells explained the loss in responsiveness (Glendinning et al., 2001; Zhou et al., 2010, 2009). In Section 3.2.3., we showed that *R. prolixus* is capable of detecting QUI and CAF as different compounds. Knowing this, the unspecific loss in responsiveness to bitter compounds after a QUI or CAF pre-exposure strongly suggests that a cross-generalization (from QUI to CAF and vice versa) of an habituation process is involved in the results of the feeding experiments (Section 3.1.1.).

Then, by applying an operant aversive conditioning we continued to analyze if *R. prolixus* is able to discriminate or generalize between QUI and CAF. These insects exhibited no preference for QUI or CAF when both alkaloids were presented simultaneously in the two-choice arena. Moreover, animals conditioned to avoid a bitter side of the arena, QUI(–) or CAF(–), still did not show a preference for one or the other alkaloid presented simultaneously. In all associative experiments performed, insects behaved similarly in response to QUI and CAF independently if the conditioning and test were performed with the same or with the other bitter compound. Then again, knowing that *R. prolixus* can distinguish between QUI and CAF peripherally (see Section 3.2.3.), we strongly suggest that they generalize across them. However, in series where the WAT side was punished (see Fig. 3B–D) we cannot completely discard that insects simply learned to avoid the clean side independently from the chemical stimulus added on the opposite side.

The experiments in which we exposed insects during 1 h to QUI or CAF before testing their performances over the two-choice arena containing bitter compounds helped us to unambiguously establish that these insects can distinguish between QUI and CAF. We found that the long (60 min) and strong (0.1 M QUI or CAF) pre-exposure to each bitter compound produced a decrease in the avoidance response to the same but not to the novel compound (i.e. pre-exposure to QUI inhibits detecting QUI but not CAF and vice versa). This can only occur if individuals sense these two alkaloids as different stimuli. However, this decrease could be the consequence of a peripheral impairment of the detection (i.e. sensory adaptation) or of a central modulation of the behavioral output (i.e. habituation). In any case, discrimination among different bitter compounds must start at the periphery. However, whether bitter detectors present in the antennae and/or legs are responsible for the avoidance observed in our device needs still to be unveiled. As in *R. prolixus* QUI and CAF receptors responsible for feeding deterrence were already described to be present in the antennae (Pontes et al., 2014), we suggest that same receptors might be involved in the avoidance behavior described in our work. Moreover, notwithstanding the peripheral organ stimulated, different bitter molecules can either activate different bitter-sensory cells or activate a common bitter-taste cell that contains at least two excitatory transduction pathways (Glendinning et al., 2002). This duality remains undisclosed for us. Differences in the temporal pattern of discharge of the bitter neuron could also contribute to taste discrimination of bitter stimuli (Glendinning et al., 2006; Reiter et al., 2015; Weiss et al., 2011). Beyond the peripheral bitter-specific detection capabilities of *R. prolixus*, it is important to remark that when both aversive bitter stimuli were presented simultaneously insects did not show a preference or avoidance for one or the other. Therefore, a central processing must be con-

trolling this decision making in *R. prolixus* with respect to bitter compounds. These results gave us the key to unambiguously conclude that *R. prolixus* can detect QUI and CAF as different compounds and can differentially react to them after a compound-specific chemical exposure. Then, knowing that *R. prolixus* is able to peripherally distinguish between QUI and CAF, we conclude that in the associative experiments in which the learned behavior was unspecific for each compound, a generalization process occurred. Similarly, in feeding experiments, a habituation process followed by a symmetric cross-generalization between QUI and CAF was responsible for the unspecific decrease of the deterrence generated by bitterness.

Interestingly, our results show that *R. prolixus* can learn. Learning and memory in triatomines has been studied only recently. Vinauger et al. (2011) showed that *R. prolixus* can learn to walk towards or against an originally neutral odor after an appetitive or aversive conditioning, respectively. Moreover, the same authors found that even if *R. prolixus* did not show a preference when odors from a live rat or quail are presented simultaneously, an aversive conditioning generated an aversion to odors from the host previously punished (Vinauger et al., 2012). Taking advantage of the proboscis extension response elicited by triatomine insects confronted to a warm surface, Vinauger et al. (2013) showed that *R. prolixus* is capable of associating previously neutral odors to novel contexts if they are positively rewarded. In a completely different context, Minoli et al. (2013) found that the escape response of *R. prolixus* to the alarm pheromone can be widely modulated by associative and non-associative conditioning protocols. All these results clearly show that hematophagous bugs are capable of learning.

Up to date, specific repellency in triatomines was only reported using DEET as an odor source (Zermoglio et al., 2015; Sfara et al., 2011). In this work we describe the contact repellency of two bitter compounds (QUI and CAF) in *R. prolixus*. Our results provide relevant and original information that might help in developing new strategies that could help diminishing vector-hosts interactions and thus disease transmission. Moreover, we add knowledge about the learning abilities of these hematophagous insects showing that under different protocols *R. prolixus* can change its behavior after a previous experience, which could be associative or non-associative.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jphysparis.2016.11.006>.

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