



Salt controls feeding decisions in a blood-sucking insect



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ABSTRACT

Salts are necessary for maintaining homeostatic conditions within the body of all living organisms. Like with all essential nutrients, deficient or excessive ingestion of salts can result in adverse health effects. The taste system is a primary sensory modality that helps animals to make adequate feeding decisions in terms of salt consumption. In this work we show that sodium and potassium chloride salts modulate the feeding behavior of *Rhodnius prolixus* in a concentration-dependent manner. Feeding is only triggered by an optimal concentration of any of these salts (0.1–0.15 M) and in presence of the phagostimulant ATP. Conversely, feeding solutions that do not contain salts or have a high-salt concentration (>0.3 M) are not ingested by insects. Notably, we show that feeding decisions of insects cannot be explained as an osmotic effect, because they still feed over hyperosmotic solutions bearing the optimal salt concentration. Insects perceive optimal-salt, no-salt and high-salt solutions as different gustatory information, as revealed the electromyogram recordings of the cibarial pump. Moreover, because insects do a continuous gustatory monitoring of the incoming food during feeding, sudden changes beyond the optimal sodium concentration decrease and even inhibit feeding. The administration of amiloride, a sodium channel blocker, noticeably reduces the ingestion of the optimal sodium solution but not of the optimal potassium solution. Salt detection seems to occur at least through two salt receptors, one amiloride-sensitive and another amiloride-insensitive. Our results confirm the importance of the gustatory system in *R. prolixus*, showing the relevant role that salts play on their feeding decisions.

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1. Introduction

Ions are vital components in a variety of physiological functions like electrolyte homeostasis and neural processes. In animals, salt balance is achieved by controlling salt ingestion and excretion (Chapman, 1982). Therefore, quantitative detection of salts in food becomes crucial for animal's survival, task in which the taste system is a relevant actor (Contreras and Lundy, 2000). Low levels of salts in the food are, in general, beneficial for most animals, however high salt levels can be harmful. Therefore, for most animals salt consumption will be the result of an appropriate balance between appetitive and aversive behaviors. The cellular, molecular and neuronal mechanisms by which animals can discern between low and high salt concentration are not completely understood. Nevertheless, different gustatory pathways seem to contribute for low and high salt detection in animals across phyla (e.g. rodents and Diptera) (Roper, 2015; Liman et al., 2014 and Refs. therein). In mice and in fruit fly larvae, low salt detection requires

amiloride-sensitive epithelial sodium channel family members that are present in taste cells and whose activation mediates behavioral attraction to salts (Chandrashekar et al., 2010; Liu et al., 2003). In contrast, aversive gustatory pathways such as the bitter and sour-sensing taste cells seem to be involved in high salt detection in rodents (Oka et al., 2013). Similarly in adult flies feeding results from the competition of gustatory neurons tuned to high salt and low salt concentration (Zhang et al., 2013).

The kissing bug *Rhodnius prolixus* is a hematophagous insect. They assess for the quality of a potential host by probing its blood (Smith and Friend, 1970; Friend and Smith 1971a). Knowing that salts are, among others, major constituents of the vertebrates' blood, several authors considered the importance of salts to elicit engorgement (Friend, 1965; Friend and Smith, 1971b; Guerenstein and Nuñez, 1994). Salts, nucleotides, and most likely other molecules present in the blood are possibly detected by the taste system of *R. prolixus*. However, still the gustatory capacities of kissing bugs and of other blood feeders have been neglected in comparison with other groups of insects (e.g. phytophagous). A recent study demonstrated that *R. prolixus* use antennal gustatory sensilla to assess the quality of a biting substrate (Pontes et al.,

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2014). Consequently, following this taste assessment, insects make a decision: to bite or not. If they bite, insects search for a blood vessel and then blood pumping starts (Friend and Smith, 1971a). The cibarial pump, a large structure associated to a complex of muscles located in the head of the insect, makes possible blood sucking, although a second small pharyngeal pump is present. Then, while the blood enters the pharynx, epipharyngeal internal receptors probably detect the different components of the incoming food (Friend and Smith, 1971b; Pontes et al., 2014). Thus, animals make another decision: to continue feeding or to abandon the site (Friend and Smith, 1971a; Pontes et al., 2014).

Here, we studied the role that salts might have on the feeding behavior of *R. prolixus* and we investigated mechanisms involved in salt perception. In particular we examined how the salt content of a feeding solution (chemical identity, concentration and osmolarity) can drive insects to accept or to reject a potential feeding source. Additionally, we studied whether salt perception in *R. prolixus* could be sensitive to amiloride blockade likewise to mammals and in fruit flies. Finally, we asked whether ingested food is continuously assessed or not, and if feeding decisions can be modified whenever the gustatory information changes.

2. Material and methods

2.1. Animals

Fifth-instar larvae of *R. prolixus* were used throughout the experiments. Insects, from an established colony of ca. 20 years, were reared at 28 °C, ambient relative humidity (30–60%) and a 12 h: 12 h L:D illumination cycle. Insects were fed on hen's blood and following ecdysis as fifth instars insects did not have access to food and experiments were carried out 15 ± 2 days post-ecdysis. Animals were handled according to the biosafety guidelines of the Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina.

All the experiments were carried out at the beginning of the insect's scotophase (0–6 h after lights switched off), time of the day in which triatomines display their maximal motivation to search for a host and feed (Barrozo et al., 2004; Bodin et al., 2008).

2.2. Artificial feeder: measurement of ingested volume

An artificial feeder was used to examine the feeding preferences of insects for different solutions. The device was described in Pontes et al. (2014). Briefly, it consists of a feeding recipient (1 cm diameter × 2 cm height) with its base closed with a latex membrane (0.125 mm thick), and it is filled with the feeding solution to be tested. The feeding solution is heated to 35 °C to match the average temperature of insects' host body. The latex membrane in contact with the solution also acquires the same temperature, mimicking the host skin and acting as a biting membrane. Insects could easily perforate the membrane with their mouthparts. Separately, a single insect is placed inside an acrylic flask (3 cm diameter × 3.5 cm height) whose upper opening is covered with a tissue mesh. A filter paper (1.5 × 3.5 cm) placed vertically inside this recipient helps the animal to climb and to reach the tissue mesh. The feeding experiments start once the tissue mesh of the insect recipient is put in contact with the latex membrane of the feeding recipient.

Unfed insects were weighed before (initial mass, M_i) and after (final mass, M_f) the feeding trials that lasted 10 min. 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 initial mass).

2.3. Electromyogram (EMG) of the cibarial pump: measurement of pumping activity

The feeding behavior of insects was additionally characterized by registering the activity of the cibarial pump muscles while the animal is feeding. The methodology used is similar to the described in Araujo et al. (2011). Briefly, one silver electrode is fixed externally to the insects' thorax by using a sticky tape. A second silver electrode is placed inside the feeding recipient of the artificial feeder in contact with the solution. Both electrodes are connected to a differential amplifier (HotBit HB-3600). When the insect inserts its mouthparts in the feeding recipient, it closes the circuit generating a base conductance. When the animal starts pumping, the base line signal changes as a result of the contractions produced by the cibarial muscles. Because insects remain completely immobile while feeding, exclusively the contractions of the cibarial pump musculature are registered. Recorded signals were filtered by means of low pass filter (17 Hz), amplified (×210) and digitalized with a data acquisition plate (ADC100®-Pico Technology Limited, UK) connected to a PC. Recorded data were analyzed with the aid of a software designed *ad hoc* (Diego Anfossi, unpubl.).

Different feeding parameters- number of bites, biting time and the pumping frequency - were quantified and analyzed during the 10 min assay. The number of bites was defined as the times an insect inserted their mouthparts in the feeder. The biting time was defined as the total time the insect's mouthparts remained inside the feeder. The pumping frequency was calculated as the number of peaks (each peak represents a muscle contraction) during the biting time. The instantaneous pumping frequency was calculated every 1 min as the mean frequency of 30 s.

2.4. Feeding solutions

Two inorganic salts, NaCl and KCl, were used as feeding solutions. Distilled water was also used as a feeding solution. It is important to note that all the solutions contained 1 mM adenosine triphosphate (ATP). The ATP is a potent phagostimulant of *R. prolixus*, therefore, its utilization assured a high feeding motivation of insects (Friend and Smith, 1971a). The solutions were prepared immediately before being used in experiments. The pH of all solutions was adjusted to 7 with 10 µl of 1 M NaOH when necessary. NaCl and KCl solutions were prepared at the following concentrations: 0.1, 0.15, 0.3, 0.6 M.

The osmolarity of a solution can be calculated as the number of free charges in solution multiplied by its molar concentration (i.e. osmolarity = molarity × i ; i is the number of particles) (Stoker, 2016). Thus, a 0.15 M NaCl solution produces an osmolarity of 300 mOsm (e.g. $\text{NaCl} \rightarrow \text{Na}^+ + \text{Cl}^-$; osmolarity (mOsm) = $0.15 \text{ M} \times 2 \times 1000 = 300 \text{ mOsm}$). Tricholine citrate (TCi) solutions at different concentrations (0.05, 0.075 and 0.15 M) were additionally used to produce feeding solutions bearing different osmolarities. Because the TCi dissociates in 4 particles in solution, the osmolarities generated by those concentrations were 200, 300 and 600 mOsm, respectively. Then, when these TCi solutions were combined with 0.15 M NaCl, the collective effects of both solutes were considered as the sum of the respective osmolarities, therefore the final osmolarities obtained were: 500, 600 or 900 mOsm.

The amiloride was added to the feeding solutions, at a concentration of 1 mM.

NaCl and KCl were purchased at Biopack (Buenos Aires, AR). Adenosine 5'-triphosphate disodium salt hydrate (A26209), tricholine citrate (T0252) and amiloride hydrochloride hydrate (A7410) were purchased at Sigma-Aldrich (St Louis, MO, USA).

2.5. Data analysis

The percentage of insects fed on different solutions was compared by means of contingency tables of independence. The statistical used was Pearson's Chi-squared test (X^2). The standard deviations of percentages were calculated as $\sqrt{p(1-p)/N}$, where p is the proportion of response and N is the number of animals tested.

The feeding parameters such as number of bites, biting time and mean pumping frequency and instantaneous pumping frequency, obtained from the EMG recordings were statistically analyzed by using Wilcoxon and Kruskal-Wallis tests.

Except otherwise stated, statistical differences were established for p values lower than $\alpha = 0.05$. However, when multiple comparisons with the same data set were necessary, the Bonferroni's correction was applied to avoid for the experimental wise error (α') (Sokal and Rohlf, 1995). Then, the α' value was calculated as $\alpha' = \alpha/k$, where k was the number of comparisons done.

The InfoStat v2012 statistical package was used for all the analyses (<http://www.infostat.com.ar>).

3. Results

3.1. Different salts, different concentrations, different osmolarities

We evaluated whether the salt identity, the concentration and/or the osmolarity of the feeding solution affects the feeding behavior of *R. prolixus*. Firstly, we examined in the artificial feeder the response of insects to NaCl and to KCl, at different concentrations. All feeding solutions contained 1 mM ATP in order to ensure insects' motivation to feed.

The feeding response of insects to NaCl and to KCl solutions was similar along the entire range of concentrations tested (Fig. 1A). Because no statistically differences were found among salts (n.s.), we analyzed the concentration effect as the main factor. The highest percentages of feeding acceptance were observed at 0.1 M and 0.15 M. On the other hand, significantly low percentages of insects

fed on solutions with no salts added (i.e. distilled water) or with high-salt concentrations as 0.3 M and 0.6 M (0.1 and 0.15 M vs. 0 M $X^2 = 7.68$, $p = 0.0056$; 0.1 and 0.15 M vs. 0.3 M $X^2 = 10.84$, $p = 0.001$; 0.1 and 0.15 M vs. 0.6 M $X^2 = 13.9$, $p = 0.0002$; $\alpha' = 0.01$).

These results show the narrow range of salt tolerance of *R. prolixus*. The optimal-salt concentration that triggers feeding is around 0.1–0.15 M NaCl or KCl.

Salts dissociate into their inorganic ions in aqueous solution affecting the osmotic pressure of the solution. Then, both the osmolarity and the concentration of dissolved inorganic salts in solution are inevitably linked. Therefore, in Fig. 1A, insects could be sensing the osmolarity of the solution instead of its salt concentration or both. To uncover this question, we designed the next experiment. We used TCi to change the osmolarity of the feeding solution, without adding inorganic ions. Three solutions of TCi were prepared to achieve 200, 300 or 600 mOsm. Besides, these solutions could be combined with 300 mOsm (0.15 M) NaCl raising the osmolarity of the feeding solution to 500, 600 or 900 mOsm (Fig. 1B).

Independently of the osmolarity generated, only weak feeding responses were observed for insects offered with TCi solutions (200, 300, 600 mOsm TCi vs. 200 and 300 mOsm NaCl: $X^2 = 28.83$, $p = 0.0001$, $\alpha' = 0.01$) (Fig. 1B, white bars). In contrast, high acceptance levels were found when TCi solutions were combined with 300 mOsm NaCl, even if the osmolarity of these solutions increased markedly (e.g. 500–900 mOsm) (Fig. 1B, grey bars). These high levels of acceptance were similar to those observed for 200 and 300 mOsm NaCl solutions (TCi solutions + 300 mOsm NaCl vs. 200 and 300 mOsm NaCl, n.s.) (Fig. 1B, black bars).

These results reveal that salt concentration in food represents for *R. prolixus* a more relevant cue than its osmolarity.

3.2. Cibarial pump activity modulation by salt concentration

Results presented in the previous section show that *R. prolixus* rejects feeding on solutions with no salts added or on high-salt

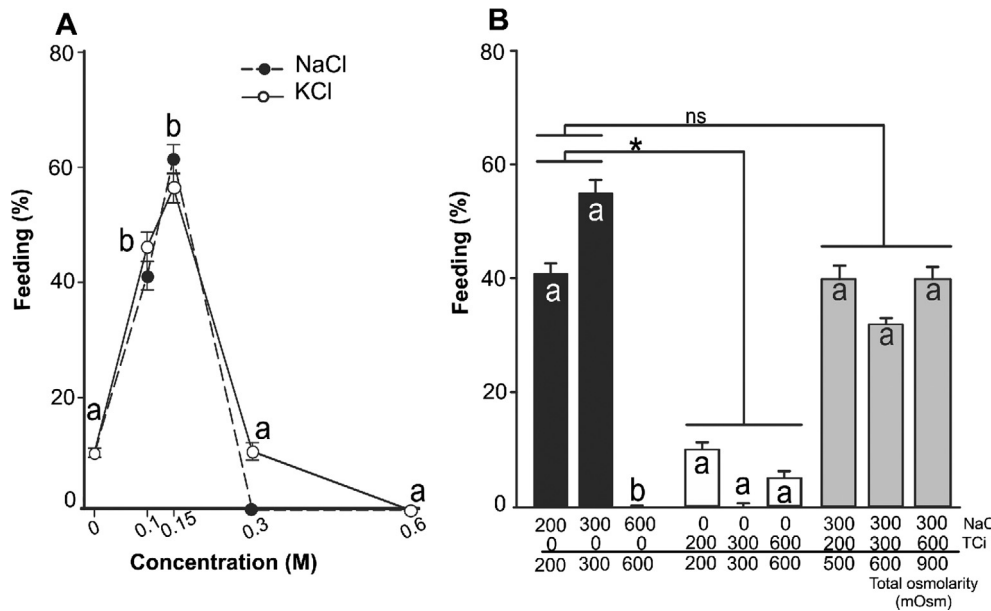


Fig. 1. Feeding behavior of *R. prolixus* to salts. A- Feeding response to NaCl and KCl at different concentrations (0; 0.1; 0.15; 0.3; 0.6 M). Insects show similar feeding behaviors to both salts, and the preferred concentrations range between 0.1 M and 0.15 M. B- Feeding response to solutions of different osmolarities (200, 300, 500, 600 and 900 mOsm) in presence or not of NaCl. Insects only feed on an optimal NaCl concentration with independence of the total osmolarity reached in the solution. All solutions contained ATP. Data are expressed in bars as the percentage of insects fed. Letters show statistical differences in each group, whereas asterisks indicate differences among groups (Pearson Chi-Square, $p < 0.01$). Vertical lines over the bars indicate the standard error. Twenty replicates were carried out at each feeding condition. TCi: tricholine citrate.

solutions. In this section, we asked whether both salt aversive conditions represent similar information for insects. Therefore we examined the activity of the cibarial pump by means of EMGs while insects fed on: no-salt (distilled water), optimal-Na and optimal-K (0.15 M) and high-Na and high-K (0.6 M). All solutions contained ATP.

Three typical examples of EMG recordings are shown in Fig. 2A–C. The visual inspection of the EMGs reveals clear differences in the pumping activity of insects to the different feeding solutions. As described earlier by Smith and Friend (1970), two phases can be easily observed to an optimal-Na solution (also observed for optimal-K, data not shown): a probing phase and an engorgement phase (Fig. 2B). The probing phase starts once the insect pierces the membrane of the feeder and begins pumping in a weak and irregular fashion (Fig. 2B, zoom 1). During this phase, the insect is probably sampling and/or assessing the quality of the food. The probing phase lasts about 52 ± 9 s for the optimal-Na solution. Immediately after, and without pulling out the mouthparts, strong and regular peaks denote the beginning of the engorgement phase (Fig. 2B, zoom 2), which ends when the insect is satiated. Conversely, when no-salt or high-Na solutions were offered as food, insects never initiated the engorgement phase (Fig. 2A, C).

The mass gained by insects at the different feeding conditions and the feeding parameters obtained from the analysis of the EMGs (mean pumping frequency, number of bites and biting time) are compared in Fig. 2D–G. As expected from the previous section, insects gained significantly more weight while feeding on optimal-Na/K solutions as compared to no-salt or high-Na/K solutions ($H = 38.98$, $p = 0.0001$; Fig. 2D).

Similarly, significantly higher mean pumping frequencies were detected when insects fed on optimal-Na/K solutions than over no-salt or high-Na/K solutions ($H = 38.93$, $p = 0.0001$; Fig. 2E). For no-salt or high-Na/K conditions the mean pumping frequency was calculated over those short periods of time in which the insects pumped.

Significantly more bites were elicited by insects at high-Na/K solutions than at optimal-Na/K and no-salt solutions ($H = 36.39$, $p = 0.0001$; Fig. 2F).

Finally, the time that insects kept their mouthparts inside the feeder also varied with salt concentration. Insects exposed to high-Na/K solutions showed significantly lower biting times than those feeding over optimal-Na/K or no-salt ($H = 39.4$, $p = 0.0001$; Fig. 2G). Under the latter two feeding conditions, the animals kept their mouthparts inside the feeder for similar and longer periods of time than to high-Na/K (Fig. 2G).

Results presented here reveal that even if two extremely opposite salt conditions: no-salt and high-salt render the same output, i.e. insects do not gain body mass, the performance of the cibarial pump is markedly different. Under a no-salt condition animals bite a few times (i.e. 1–2), and despite they keep their mouthparts inside the feeder along the experiment, do only low frequency pumpings. At a high-salt feeding solution, insects, in contrast, do numerous bites with frequent insertions and withdrawals of the mouthparts from the feeder.

3.3. Amiloride and salt perception

We asked whether in salt perception could be involved amiloride-sensitive receptors, similarly to what happens in mammals and fruit flies. Thus, by means of EMG recordings, we examined the feeding behavior of animals to optimal-Na/K solutions in the presence (or not) of 1 mM amiloride (a potent inhibitor of sodium channels). Because it was uncertain the time necessary for the amiloride to cause an effect (if there was any), we analyzed the instantaneous pumping frequency along the 10 min the experiment lasted.

The pumping activity of insects feeding on the optimal-Na solution was markedly affected by the presence of amiloride (Fig. 3A). As a result a significant reduction in the instantaneous pumping frequency was observed all along the recording time as compared to the optimal-Na condition without amiloride ($p < 0.001$ for all comparisons at each time; $\alpha' = 0.0055$). This was also evident when comparing the mean pumping frequency ($Z = 55$, $p = 0.0002$). However, neither the instantaneous nor the mean pumping frequency of insects fed on the optimal-K solution was altered by the presence of amiloride (n.s.) (Fig. 3B).

These results show that amiloride blocks the perception of sodium solutions but not of potassium solutions in *R. prolixus*.

3.4. Ingested food and its continuous evaluation

The feeding response of *R. prolixus* is generally accepted to be all-or-nothing (Friend and Smith, 1977; Guerenstein and Nuñez, 1994). This means that once the insect decides to ingest a blood meal (or an artificial diet), it does until repletion. In this section, we examined whether the food is continuously gustatory evaluated or not. Thus, we asked if a feeding decision can be changed as a result of this assessment. In these experiments, insects were allowed to feed on an optimal-Na solution, and once they reached the engorgement phase the feeding solution was altered by adding amiloride (1 mM) or by increasing the concentration of NaCl (high-Na condition) in the feeder (Fig. 4A, B). Besides, a control series was carried out by adding optimal-Na solution instead of the treatment solutions (Fig. 4C). The instantaneous pumping frequency was calculated previously to the treatment addition (i.e. time before) and during (i.e. 0 min) and after the application of the test solutions (i.e. 2 and 5 min).

The pumping activity of insects was considerably modified by changing the composition of the optimal-Na solution (Fig. 4A, B). Following the addition of amiloride (0–2 min), the instantaneous frequency showed a reduction, although it was not statistically significant (n.s.) (Fig. 4A). However, 5 min later the frequency decreased significantly in comparison to the time before the amiloride application (Before vs. 5 min: $Z = 2.31$ $p = 0.0016$, $\alpha' = 0.016$) (Fig. 4A). High-Na addition (i.e. 0.6 M NaCl) to the feeding recipient, in contrast, reduced immediately the cibarial pumping activity (Before vs. 0 min: $Z = 2.2$ $p = 0.0098$; Before vs. 2 min: $Z = 2.2$ $p = 0.0018$; Before vs. 5 min: $Z = 2.2$ $p = 0.0018$; in all cases $\alpha' = 0.0016$) (Fig. 4B). Any possible artifact effects produced by the addition of the treatment solutions (Fig. 4A, B) were discarded by adding the optimal-Na solution inside the feeder (Fig. 4C). No change in the instantaneous pumping frequency was observed under this control condition (Fig. 4C).

These results demonstrate that even if insects are engaged in feeding, they still do assess for the gustatory quality of the incoming food.

4. Discussion

In this work, we show that feeding decisions in *R. prolixus* are heavily affected by salt concentration. Opposite feeding behaviors are observed depending on the salt concentration, while an optimal-Na/K concentration (0.1–0.15 M) produces appetitive responses, high-Na/K levels (0.3–0.6 M) elicits feeding rejection in insects (Fig. 1A). However, not only high-Na/K concentrations caused feeding aversion in *R. prolixus*. The lack of ions in the feeding solution has a negative valence for insects, even if the feeding solution contains the main phagostimulant ATP (Fig. 1). NaCl and KCl solutions, at the range of concentrations used in this work, evoke similar feeding responses in *R. prolixus*. It was previously shown that *R. prolixus* also accept feeding on solutions of NaBr,

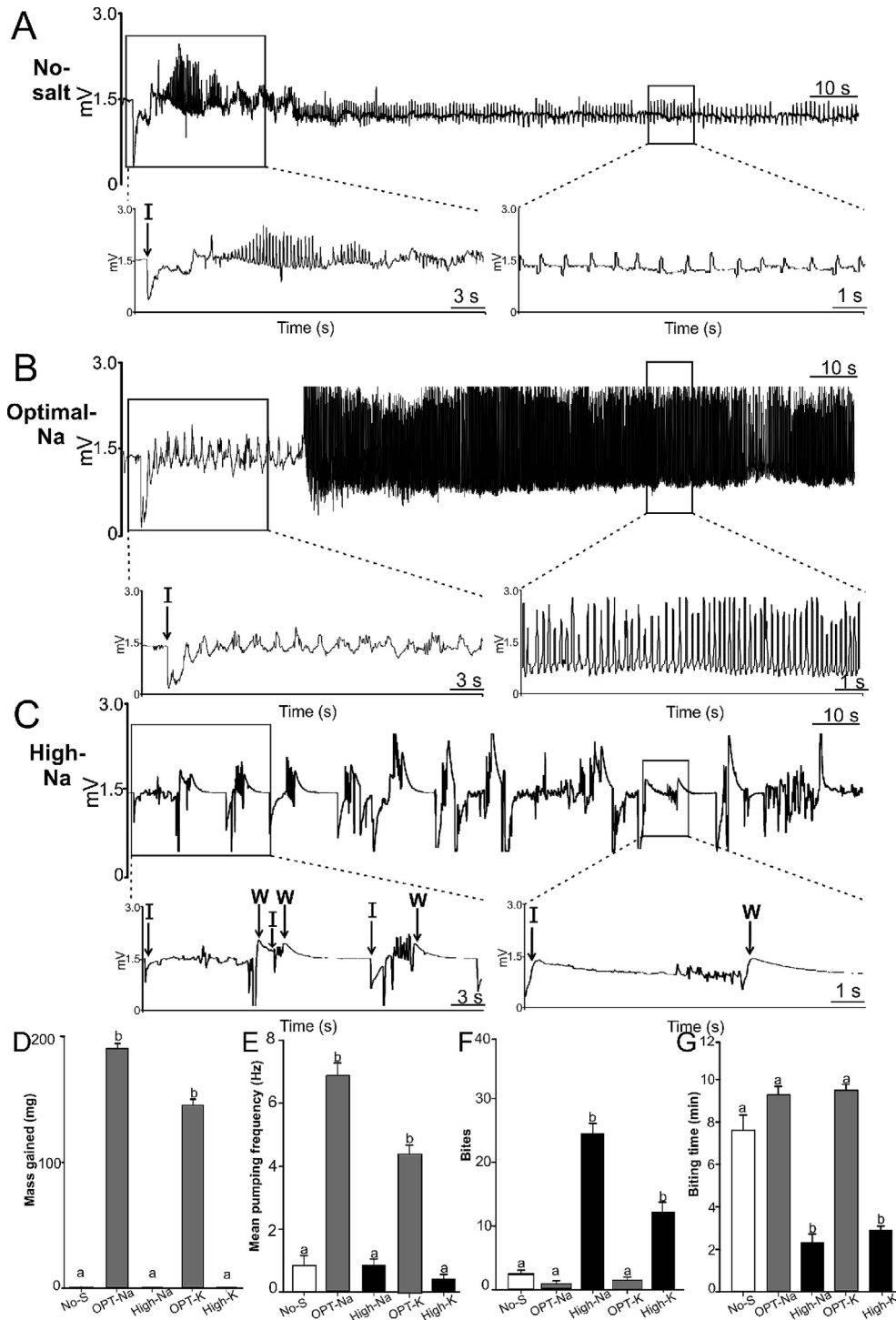


Fig. 2. Cibarial pump activity of *R. prolixus* under different salt feeding conditions. A–C Examples of electromyograms of 3 insects feeding on A– no-salt, B– optimal-Na and C– high-Na solutions. D–G Quantitative analysis of the feeding parameters, such as D– mass gained, E– mean pumping frequency, F– number of bites and G– biting time, of insects offered with sodium and potassium salts at the 3 different concentrations. The pump performance is similar salts, although it varies significantly with the concentration. All solutions contained ATP. Different letters denote statistical differences (Kruskall-Wallis followed by *a posteriori* comparisons, $p < 0.05$). Bars show the mean and the standard error. Ten replicates were carried out at each feeding condition. I = insertion of mouthparts, W = withdrawal of mouthparts. No-salt = distilled water, OPT-Na/K = 0.15 M NaCl or KCl; high-Na/K = 0.6 M NaCl or KCl.

KBr, NaI, and KI, although the acceptance level is considerably lower with respect to NaCl solutions (Friend and Smith, 1977). Moreover, if *R. prolixus* is considerably fasted (more than 8 weeks), can even feed on saline solutions in absence of ATP (Guerestein and Nuñez, 1994). In many blood feeders, artificial diets composed by NaCl are most favorable to elicit feeding rather than other elec-

trolites (see Friend and Smith, 1977). In this work, the highest feeding response of insects was observed between 0.1 and 0.15 M, interestingly, it is close to the sodium concentration found in the human blood (i.e. 0.145 M) (Haynes, 2012).

Changing the concentration of solutes in a solution is concomitantly associated to a change in the osmolarity of the solution. Our

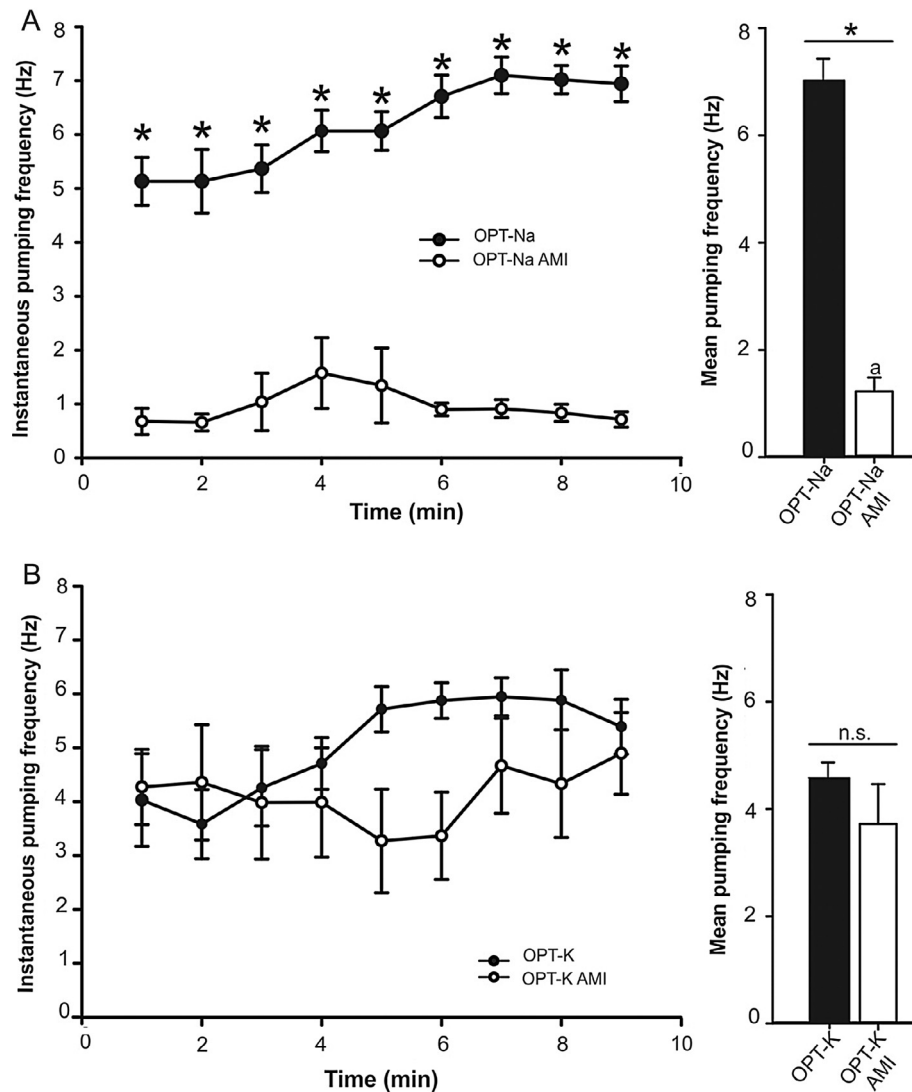


Fig. 3. Amiloride effect on the feeding response of *R. prolixus* to salts. Cibarial pumping activity to optimal feeding solutions of: A- 0.15 M NaCl (OPT-Na) and B- 0.15 M KCl (OPT-K) in presence (white circles) or in absence (black circles) of amiloride (AMI). Amiloride affects the response (instantaneous and mean pumping frequency) to the OPT-Na solution but not to the OPT-K solution. All solutions contained ATP. Bars show the mean and the standard error. Asterisks denote statistical differences (Wilcoxon test, $p < 0.0055$). Ten replicates were carried out at each feeding condition.

results show that *R. prolixus* can feed on solutions bearing high osmolarities (500–900 mOsm), but only if these solutions contain the optimal-Na concentration (Fig. 1B). Salt concentration, but not osmolarity, appears to be a key factor to trigger feeding in *R. prolixus*. The presence of osmotic detectors was demonstrated in *Drosophila melanogaster*, as these flies respond to water (or hypo-osmotic solutions) through gustatory neurons housed on sensilla of the proboscis (Cameron et al., 2010; Chen et al., 2010; Inoshita and Tanimura, 2006). Many blood feeders like mosquitoes, simuliids, tabanids consume water besides nectar and blood (Friend and Smith, 1977 and Refs. therein). Clearly, water consumption, like any other essential nutrient, is required for the correct maintenance of the osmotic balance in the body. However, in this work, we show that water (although added with ATP) is not an appetitive or a positive gustatory stimulus for *R. prolixus*. The hematophagous habits of these insects force them to ingest large quantities of water while taking a blood meal. Therefore, water might not represent a limiting resource for them. Moreover, *R. prolixus* can produce its own weight of urine and eliminates 75 per cent of the ingested

water within a blood meal (Wigglesworth, 1931). Thus, the water requirement of *R. prolixus* could be completely fulfilled by a blood meal intake. This could explain the feeding behavior of *R. prolixus* to water, even in the presence of ATP (Figs. 1A, 2A).

R. prolixus display a typical feeding pattern, composed by a probing and the engorgement phase (Smith and Friend, 1970; Friend and Smith, 1971a; Araujo et al., 2011). By measuring the electrical resistance changes -between the insect and the feeder- during feeding is possible to characterize the entire process. Thus, it was possible to distinguish once: 1- the insect pierces the membrane of the feeder, 2- it probes the solution, 3- it engages in true ingestion. We show that the probing phase is greatly affected by the composition of the feeding solution, and consequently the engorgement phase is affected. Only if optimal-Na/K solutions are offered, *R. prolixus* exhibit the two feeding phases (Fig. 2B). Contrarily, at no-salt or high-Na/K conditions the probing phase is prolonged, moreover, insects never start with the engorgement phase (Fig. 2A, C). At the no-salt condition, insects pierce the feeder once or twice maintaining their mouthparts inside the feeder for

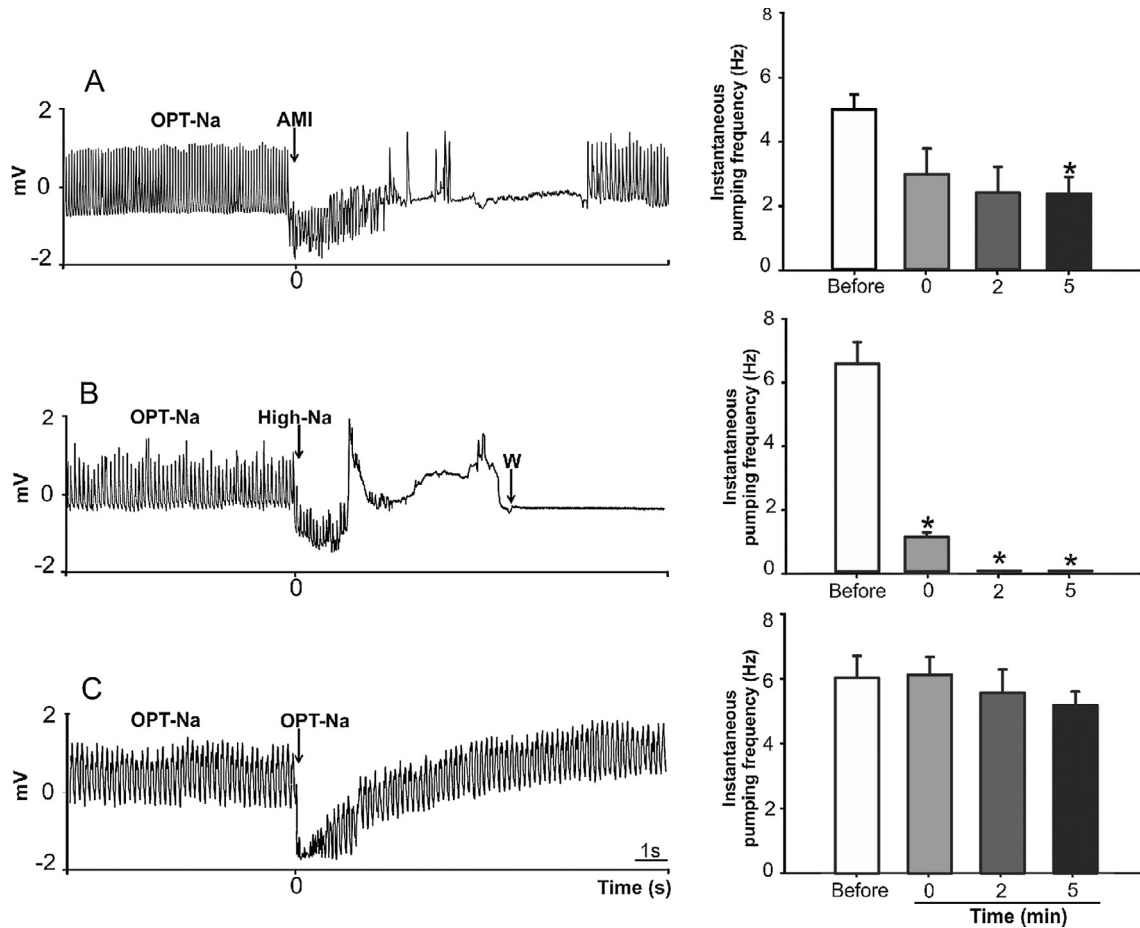


Fig. 4. Gustatory monitoring of salt concentration in the feeding solution. Electromyograms of insects engaged in feeding over an OPT-Na solution (0.15 M NaCl) and the subsequent addition of A- amiloride (AMI), B- high-Na (0.6 M NaCl) and C- OPT-Na (0.15 M NaCl). The mean pumping frequency decreases in a time dependent manner with the addition of both amiloride and high-Na solution. All solutions contained ATP. Bars show the mean and the standard error. Asterisks denote statistical differences (Wilcoxon test, $p < 0.016$). Ten replicates were carried out at each feeding condition.

the whole experimental time without feeding (Fig. 2D, E). In this case, insects are sampling continuously the feeding solution at a low pumping frequency, probably assessing the presence of ions. However, when a high-Na/K solution is offered, insects insert, withdraw and reinsert their mouthparts into the feeder numerous times (Fig. 2C, F). Thus, at high-salt, animals are also continuously sampling the feeding solution as observed for no-salt feeding condition. However, the detection of high levels of Na^+ and K^+ elicits the mouthparts withdrawal of the feeder. Notably albeit the aversive behavior evoked by high-salt levels, insects continue piercing the membrane of feeder frequent times (i.e. more than 10 and 20 bites for high-K and high-Na, respectively). However the low-effective time the insects kept their mouthparts inside the feeder, denotes the foremost degree of aversiveness evoked by the high-Na/K solutions (Fig. 2G). The fact that *R. prolixus* did numerous biting over a high-salt solution was not completely surprising. Previous studies have shown that *R. prolixus* withdraw the stylets from an artificial diet only to reinsert repeatedly in a different location whenever the diet does not fulfill with insects' requirements (Smith and Friend, 1970; Friend and Smith, 1971a).

In insects, besides the external gustatory detectors located in taste sensilla along their body, there are those that respond to chemicals with gorging activity, located internally to the food canal. Pharyngeal sensilla are placed in an appropriate position to monitor the composition of the incoming food. Therefore these internal sensilla could play a major role in determining the acceptability and destination of a meal. Different electrophysiological

evidences in blood-sucking insects support this assumption (Rice et al., 1973; Bernard, 1974; Lee, 1974; McIver and Siemicki, 1981; Jefferies, 1987). In *R. prolixus* a group of 8 uniporous sensilla was previously described in the anterior region of the pharynx, the epipharynx (Kraus, 1957; Bennet-Clark, 1963; Pontes et al., 2014). Thus, when the insect takes a sip of the feeding solution it brings this solution in contact with the epipharyngeal sensilla. If the incoming food has the adequate salt and ATP composition, the gustatory neurons may inform the brain. Consequently the cibarial musculature will start pumping to fill the gut. Therefore, gustatory information collected by epipharyngeal sensilla could supply the brain with the necessary information for controlling the sucking activity of insects.

In both mammals and insects, salts in the diet can elicit appetitive or aversive behaviors (Liu et al., 2003; Chandrashekar et al., 2010; Zhang et al., 2013; Liman et al., 2014; Alves et al., 2014; etc.). Low-salt content in the diet leads to appetitive behaviors in most animals, contrarily to high-salt content that is mostly rejected. This duality assures animals to maintain the body homeostasis. Salts are not stored therefore animals consume salts when necessary, although excessive salt consumption can be deleterious. In mammals, it is actually accepted that low-salt sensing occurs through the epithelial sodium channel receptors (ENaC), which are highly sensitive to amiloride blockade (Chandrashekar et al., 2010; Kellenberger and Schild, 2002; Hettinger and Frank, 1990). In *D. melanogaster* larvae, low-salt detection seems to be possible through two pickpocket genes: ppk11 and the ppk19 (Liu et al.,

2003), both coding for homologs of ENaC of mammals. In *R. prolixus*, the feeding behavior on optimal sodium solutions is negatively affected by amiloride (Fig. 3A). These results suggest that sodium detection in *R. prolixus* can be possible through amiloride-sensitive receptors. The recent annotation of the genome of *R. prolixus* revealed the presence of a set of amiloride-sensitive sodium channels (Mesquita et al., 2016). Whether epipharyngeal sensilla of *R. prolixus* express these sodium receptors remains to be confirmed. On the other hand, amiloride does not interfere with the feeding response of *R. prolixus* over an optimal potassium solution (Fig. 3B). Similar results were previously found in mammals, where taste responses to KCl cannot be blocked by amiloride (Kellenberger and Schild, 2002; Brand et al., 1985). Altogether our results suggest the existence of at least two different salt receptors participating in salt signaling. On one hand, sodium detection might be possible through amiloride-sensitive salt receptors, and on the other, potassium detection might undergo an amiloride-insensitive sensing pathway.

5. Conclusions

Here we demonstrate that salt concentration, and not osmolarity, plays a crucial role helping *R. prolixus* to make adequate feeding decisions. Salt detection has a relevant impact on the biting and pumping activity of insects. High- and no-salt solutions are rejected by insects; however, both saltiness conditions evoke different aversive behaviors. Because the food quality is continuously assessed feeding decisions can be modified as the gustatory information changes. Amiloride-sensitive and insensitive salt receptors seem to be involved in salt recognition in *R. prolixus*.

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