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Hunger is the best spice: effect of starvation in the antennal responses of the blood-sucking bug *Rhodnius prolixus*

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Abstract

Blood-sucking insects strongly rely on olfactory cues to find their vertebrate hosts. As in other insects with different lifestyles, it has been shown that endogenous and exogenous factors modulate olfactory responses. The triatomine bug *Rhodnius prolixus* is an important vector of Chagas Disease and a classical model for studies of physiology and behavior. In this species, the behavioral response to host-derived odorants is modulated by both the time of the day and the starvation. Here I investigated the peripheral neural mechanisms underlying these modulatory effects. For this, I measured the electroantennogram (EAG) responses of insects towards different concentrations (from 0.5% to 75% vol/vol) of an attractive host-odorant, ammonia. I tested the responses of starved and fed animals during the middle of the day (when insects are inactive and aggregated in refuges) and at the beginning of the night (when insects become active and search for hosts). Regardless of the time of the day and the starvation status, EAG responses systematically increased with odorant concentration, thus accurately reflecting the response of olfactory receptor cells. Interestingly, the EAG responses of starved insects were larger than those of fed insects only during the night, with larger differences (6–7 times) observed at low-middle concentrations. This is the first, to my knowledge, reporting modulation of sensory responses at the neural level in triatomines. This modulation, considering that triatomine hosts are mostly diurnal and are also potential predators, has an important adaptive value, ensuring that insects search for hosts only when they are hungry and at appropriate times.

Keywords

olfaction; electroantennogram; *Rhodnius prolixus*; modulation; triatomine; starvation; EAG; *Triatoma*; Chagas disease; electrophysiology

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INTRODUCTION

Insects rely on a variety of sensory cues, including olfactory ones, to obtain information about biologically relevant resources such as food, mates, predators, and oviposition sites. It is known, however, that both internal and external factors such as the time of the day, the molting cycle, and the nutritional and reproductive state modulate olfactory responses (e.g. Barrozo et al., 2004, 2011; Bodin et al., 2008, 2009a, 2009b; Farhadian et al., 2012; Krishna et al., 1999; Page and Koelling, 2003; Sengupta, 2013; Siju et al., 2010; Takken et al., 2001; van der Goes van Naters et al., 1998). This behavioral modulation has a high adaptive value, as it minimizes costs and risks.

Blood-sucking insects such as mosquitoes, ticks and triatomines are vectors of a myriad of human and animal diseases (Lehane, 2005). The triatomine bug *Rhodnius prolixus*, for instance, is one of the most important vectors of Chagas disease, a parasitic disease endemic to Central and South America (World Health Organization, 2014). During the day triatomines are mostly inactive and remain aggregated inside dark refuges (Zeledón and Rabinovich, 1981). Insects became active at night and search for food using mostly thermal (Flores and Lazzari, 1996) and host-derived olfactory cues (e.g. CO₂, lactic acid, pyruvic acid, short-chain carboxylic acids, aldehydes, ammonia, etc.; Barrozo and Lazzari, 2004; Barrozo et al., 2004; Guerenstein and Guerin, 2001; Guerenstein and Lazzari, 2009, 2010; Lazzari, 2009; Taneja and Guerin, 1995, 1997). Interestingly, it has been shown that the behavioral response to heat, host-derived odorants, and aggregation pheromones is modulated by starvation (Bodin et al., 2009a; Reisenman et al., 2013), and that an unknown haemolymph factor mediates this modulation (Bodin et al., 2009a). No studies in triatomines have, to my knowledge, analyzed the underlying neural mechanisms. In other blood sucking insects such as mosquitoes, it has been shown that antennal responses towards host-derived odors are reduced after a blood meal (Davis, 1984; Takken et al., 2001), and that odorant receptor proteins are similarly down-regulated (Fox et al., 2001). Some details of the mechanisms mediating the modulatory effects of hunger in olfactory responses and food-search have are more fully understood in the fruit fly *Drosophila melanogaster* (e.g. Farhadian et al., 2012; Root et al., 2011; Sengupta, 2013).

The purpose of this work was therefore to begin elucidating the neural mechanisms controlling the modulation of olfactory responses by starvation in triatomines. I investigated this at the first level of the olfactory pathway, the antenna, using as a stimulus an attractive host-derived odorant, ammonia (Taneja and Guerin, 1997). I tested the electrophysiological antennal responses of insects at two behaviorally relevant time points, in the middle of the day, when insects are mostly inactive, and at the beginning of the night, when motivation to search for hosts is maximal (Barrozo et al., 2004; Bodin et al., 2008; Lorenzo and Lazzari, 1998). My initial prediction was that starvation would increase responses towards host-derived odorants maximally –but not solely- at nighttime.

MATERIALS AND METHODS

Experiments were conducted in the laboratory of Dr. John Hildebrand, Department of Neuroscience, University of Arizona.

Animals

Insects were fed weekly in the laboratory with bovine blood (with 2% sodium citrate added as anticoagulant) supplemented with ATP (100 mM) using an artificial feeder apparatus (Núñez and Lazzari, 1990). Although insects normally acquire their symbiotic bacteria by coprophagy (Lehane, 2005), the symbiont *Rhodococcus rhodnii* was added to the blood approximately once a month to ensure colonization and therefore adequate nutrition (Baines, 1956). Insects were reared at 24–26°C on a humidified chamber under a 12 hours light: 12 hours dark regime. About 4 days after eclosion to the fifth instar stage, insects were separated, divided in experimental groups, maintained on a light-dark cycle, and kept unfed.

Experimental groups

Experiments were conducted with independent groups of starved and non-starved insects respectively tested at 52 ± 1 and 9.4 ± 0.2 days after eclosion. Insects were assayed 4–6 hours after the beginning of their photophase or shortly (15–30 minutes) after the beginning of their scotophase. Each insect was used only once. Data was obtained from a total of 53 insects (11–16 per group).

Insect preparation and recording procedure

Nighttime insects were prepared and set-up for experiments under regular lighting conditions about 30–45 minutes before the beginning of their scotophase; laboratory lights were turned off at the beginning of the insect's scotophase except for a dimmed 25 W red incandescent lamp, which was directed away from the recording set-up (e.g., the insect remained in darkness during the experiment). Daytime insects were prepared and set-up also 30–45 minutes before the beginning of the experiment and were assayed under regular laboratory lighting conditions. I confirmed that this procedure (i.e. preparing the insects before recordings started) did not alter the amplitude of the EAG responses (not shown).

To prepare each insect for recordings, legs were removed with scissors, and each insect was glued to a glass slide by their ventral side using double-sided sticky tape. Another piece of tape was affixed to the dorsal thorax and the glass slide to minimize body movements. A small piece of melted dental wax was placed under the insect head to minimize head movements. The rostrum was extended with forceps and fixed to the glass slide with a small piece of dental wax. A small incision was opened on the head cuticle and a drop of saline was added to prevent desiccation. The insect so mounted in the glass slide was immediately transferred to the recording set-up, and the glass slide was secured with modeling clay. The reference electrode was inserted in the head incision, and the right antenna was positioned and immobilized by inserting its uncut tip inside the recording electrode. Recordings started 30–40 minutes later.

Both the reference and the recording electrodes were made from borosilicate glass capillaries with filament (reference electrode: World Precision Instruments, Sarasota, FL, 0.58 mm I.D., 1 mm O.D., catalog # 1B100F-6; recording electrode: Sutter Instruments Co., Novato, CA, 0.5 mm I.D., 1 mm O.D., catalog # BF100-50-10) on a laser puller (P-2000, Sutter Instruments Co., Novato, CA) and were filled with physiological saline solution

containing (in mM) 150 NaCl, 3 CaCl₂, 3 KCl, 10 TES buffer (pH 6.9), and 25 sucrose (Christensen and Hildebrand, 1987).

Antennal responses were amplified 100–500 fold (usually 200-fold) with an amplifier (Axoclamp-2A, Axon Instruments, Molecular Devices, Sunnyvale, CA) coupled to a DC amplifier (LPF 202A, Warner Instruments, Hamden, CT), and digitized at 20 kHz (via Datapack, Run Technologies, Mission Viejo, CA).

Odor stimulation

An L-shaped glass tube (0.8 cm I.D.; long leg: 12 cm long; short leg: 9 cm long) delivered a constant flow of humidified, charcoal-filtered air to the antenna (1.9 liters/min). The end of the L-shaped tube was flattened (4 mm I.D., 11 mm wide) and aimed at the longitudinal axis of the antenna. The tip of the glass syringe (volume: 1 ml) containing the odor stimulus was inserted into the air stream through a small hole (5 mm) in the side of the long leg of the glass tube. The odorant was injected into the air stream using a computer-activated solenoid valve (9.5 ml of odor-bearing air, duration=500 ms). A funnel connected to a negative-pressure line was positioned near and behind the preparation to remove volatiles after odorant stimulation.

The only odorant used in this study was ammonia [NH₄(OH), ammonium hydroxide, CAS # 1336-21-6, S/P Mallinckrodt #3255-500, Paris, Kentucky; 29.7% NH₃], a host-derived odorant present in vertebrate urine and human sweat which attracts triatomines (Taneja and Guerin, 1997) and other blood-sucking insects such as mosquitoes (Braks et al., 2001; Meijerink et al., 2001). Ammonia is also present in the feces of triatomine (Taneja and Guerin, 1997). The following dilutions (% vol/vol) were prepared by dissolving ammonia in distilled water: 0.5%, 1%, 2%, 2.5%, 5%, 10%, 25%, 50% and 75%. Odorant cartridges were prepared immediately before stimulation (to prevent evaporation, given the high volatility of NH₃) by inserting a 0.5 × 0.5 cm piece of filter paper loaded with 4 µl of solution (or the water control) into a 1 ml glass syringe. Each experiment started and ended with a control (water) stimulation. Dilutions were tested in ascending order to prevent adaptation effects. Although initially each insect was stimulated three consecutive times with the same odor cartridge (inter-stimulation interval: 10–12 seconds), only the first stimulation was considered for data analysis, as responses to the second and third stimulation systematically decreased in amplitude (likely due to odorant evaporation/exhaustion from the cartridge rather than antennal adaptation).

Data analysis and statistics

EAG responses were calculated by measuring the amplitude of the voltage change in response to odorant stimulation. In some animals, stimulation evoked a positive response rather than the negative voltage deflection typically observed in insect EAGs (Figure 1). This seemed to be an electronic artifact, as the amplitude of the odor-evoked EAG responses was similar regardless of the voltage change direction. Thus, the absolute EAG amplitude was considered for analysis. EAG responses (relative or absolute) were averaged across animals from the same experimental group and condition for display purposes (Figures 2–3).

For each group, the effect of concentration on the amplitude of the EAG responses was analyzed using Friedman ANOVAs followed by Dunnet's post-hoc tests (to compare the effect of each concentration vs. its control group; Zar, 1999; Figure 2). The effects of starvation on EAG responses to concentrations $\geq 2.5\%$ was analyzed separately for nighttime and daytime insects using Mann-Whitney U tests (Zar, 1999). Responses to concentrations $<2.5\%$ were not subjected to statistical analysis, as they did not evoke responses statistically different from those evoked by the water control (Figure 2). Because many Mann-Whitney U-tests were so conducted (three in Figure 2B, six in each panel of Figure 3), I used the false discovery rate method to control the proportion of false positives (Benjamini and Hochberg, 1995). Individual P -values were compared with a $(i/m) \cdot Q$ threshold, where i is the i^{th} observed p -value (ordered from smallest to largest), m is the total number of tests in each experimental series, and Q is the assigned false discovery rate (0.05). In all cases differences were considered significant if $p < 0.05$. All tests were conducted in raw data (except for Mann-Whitney U tests in Figure 2B).

RESULTS

Figure 1 shows an example of the EAG responses evoked by stimulation with different concentrations of ammonia and the water control. Typically, the amplitude of the response and the slope of the voltage deflection increased with increasing concentrations. Stimulation with the water control, either at the beginning or the end of the experiment, did not evoke measurable responses. Activation of the solenoid controlling odor stimulation produced a small square artifact, particularly noticeable at high amplifications (Figure 1, upper panel).

Figure 2 shows the normalized (data relative to the maximum response of each insect) population response of insects belonging to the four experimental groups. Regardless of the starvation status and time of the day, in all four experimental groups the amplitude of the EAG responses increased with concentration (Friedman ANOVAs, in all cases $p < 0.00001$). In the case of insects tested during their photophase, odorant concentrations 10% and 25% evoked responses statistically different from the water control in fed and starved insects, respectively (Dunnet's tests, $p < 0.05$; Figure 2A). In the case of insects tested during their scotophase, in contrast, the sensitivity threshold was lower in starved insects. Odorant concentrations 10% and 25% evoked responses statistically different from the control in starved and fed insects, respectively (Dunnet's tests, $p < 0.05$; Figure 2B).

During the day, the dose-response curves of fed and starved animals completely overlapped (Figure 2A). During the night, the shape of the dose-response curves was slightly different: while the response of starved insects increased gradually with concentration increases, the response of fed animals sharply increased at concentrations $\geq 25\%$. The relative EAG response of starved insects was larger than that of fed insects at 10% , 25% and 50% concentrations (Mann-Whitney U tests corrected for multiple comparisons, $p < 0.05$ in all three cases; Figure 2B).

Figure 3 shows the absolute response of insects belonging to the four experimental groups (same insects as in Figure 2). The amplitude of the EAG responses of starved and fed insects tested during their photophase was remarkably similar at all concentrations (Figure 3A;

Mann-Whitney U tests corrected for multiple comparisons, in all cases $p > 0.1$; Figure 3A). In contrast, in the case of insects tested during their scotophase, the response amplitude of starved insects was much larger than that of non-starved insects at all concentrations analyzed (Figure 3B; Mann-Whitney U tests corrected for multiple comparisons, in all cases $p < 0.05$; only concentrations $\geq 2.5\%$ were subjected to statistical testing, see Materials and Methods). Maximum differences in EAG amplitudes were observed at low-middle concentrations. At 5% and 2.5%, for instance, the responses of starved insects were respectively 7 and 5.7 times larger than that of fed insects, while at the highest concentration (75%) the response of starved insects was 2.3 times larger than that of fed insects.

DISCUSSION

While it has been shown that external and internal factors modulate olfactory and visually-guided behaviors in triatomines (Barrozo et al., 2004; Bodin et al., 2008, 2009a, 2009b; Reisenman et al., 1998, 2013), this is study is, to my knowledge, the first reporting modulation of neural activity by such factors in these insects. I found that the starvation status modulates antennal responses towards host-derived odorants. Interestingly, this effect was observed only during the night (Figure 3), when insects are active and search for hosts. This modulatory effect was observed at all concentrations, but was stronger in the low-middle range, with the average response of starved insects being six-seven times higher than that of fed insects (Figure 3B).

Regardless of the starvation status and the time of the day, I found that antennal responses systematically increased with odorant concentration (Figure 2), likely reflecting the response of olfactory receptor cells. The relative dose-response curves of starved and fed animals completely overlapped during the day (Figure 2A). During the night, in contrast, the response of starved insects increased more gradually with concentration than that of fed insects. This was more obvious at 10–50%, where the relative responses of fed and starved animals were statistically different from each other (Figure 2B). The absolute response of fed and starved animals was also remarkably similar during the day: again, the dose-response curves completely overlapped (Figure 3A). During the night, in contrast, the absolute responses of starved insects were larger than that of fed insects at most concentrations ($\geq 2.5\%$; Figure 3B). This difference could be attributed to changes such as variations in the sensitivity of individual olfactory receptor cells (Qiu et al., 2013; Siju et al., 2010; Van der Goes van Naters et al., 1998), although this remains to be investigated in *R. prolixus*. In sum, while in all cases antennal responses similarly increased with increasing odorant concentration (Figure 2), the antennal responses of starved insects were larger than that of fed insects at nighttime only (Figures 2B and 3B).

The EAG is an appropriate technique to study modulatory effects

Despite the time of the day and the starvation status, I found that the amplitude of the EAG response systematically increased with increasing odorant concentrations (Figure 2). The EAG response to an odorant is assumed to represent the summation of the membrane potential changes generated by the olfactory receptor cells. It is therefore reasonable to assume that the increases/decreases in EAG amplitude observed in response to stimulation

with ammonia (Figures 1–2) accurately reflect increases/decreases in the activity of olfactory receptor cells sensitive to that odorant (Mayer, 2001; Qiu et al., 2013). Indeed, triatomine insects have olfactory receptor cells within grooved-peg sensilla that respond to NH_3 in a dose-dependent manner, with sensitivity characteristics similar to those observed at the EAG level (Taneja and Guerin, 1997). Responses to ammonia have also been reported from grooved-peg sensilla in other blood-sucking insects (Meijerink et al., 2001).

Starvation modulates antennal responses at appropriate times

As expected, during the night, the antennal response of starved insects was larger than that of fed insects (Figure 3B). Although somehow an unforeseen result, no differences whatsoever were found between the antennal responses of starved and fed animals tested during the day (Figure 3A). Both of these findings are nevertheless in line with behavioral experimental evidence. First, the responses of triatomines towards host-odors follow a daily rhythm, with increased sensitivity during the night (Barrozo et al., 2004; Bodin et al., 2008). Second, even though behavioral attraction/repellence results from central nervous system processing of peripheral input, it has been shown that some of the host-derived odors that attract starved insects produce repellence (or indifference) in fed insects (Bodin et al., 2009a; Reisenman et al., 2013). Considering that triatomine hosts are mostly diurnal and also potential predators, the modulation of neural responses described here has a clear adaptive value, ensuring that insects search for hosts only when they are hungry and when their hosts are asleep.

A circadian rhythm of EAG responses to olfactory stimuli has been reported in the fruitfly *D. melanogaster* and in the cockroach *Leucophaea maderae* (Krishna et al., 1999; Page and Koelling, 2003). Contrary to the results reported here, in both flies and cockroaches, the amplitude of the EAG response was lower during the time of the day when these two insects are more active. Several explanations for these paradoxical results were proposed, including the possibility that olfactory rhythms facilitate the detection of predators when insects are inactive, and that central olfactory processing is regulated in such a way that animals are behaviorally more sensitive to odors when they are maximally active (Krishna et al., 1999; Page and Koelling, 2003). In *R. prolixus*, in contrast, the response of olfactory receptor cells and the orientation towards host-odors are directly synchronized in an adaptive manner: antennal responses are maximized at the time of the day when insects are active and search for food. In tsetse flies, as observed here, EAG responses were larger at the time of the day when insects are more active (Van de Goes van Naters et al., 1998). All these results suggest that different mechanisms operate in different species adjusting sensitivity throughout the different levels of the sensory pathway to ensure appropriate behavioral outputs.

In this work, in order to test the modulatory effects of starvation in EAG responses, the environmental conditions under which experiments were conducted were carefully controlled: animals tested during their scotophase were prepared for experiments before lights-off and were assayed under functional darkness (see Materials and Methods). As noticed by others, it is likely that this procedure limited EAG variability between animals belonging to the same experimental group (Qiu et al., 2013), allowing uncovering the modulatory effects of starvation in antennal olfactory responses.

Modulation of EAG responses towards odors

Here I tested a single host-derived odorant (ammonia) that produces behavioral attraction not only in triatomines but also in other blood-sucking insects such as mosquitoes (Braks et al., 2001; Meijerink et al., 2001; Taneja and Guerin, 1997). It would be interesting to test whether antennal responses to other behaviorally relevant host-derived odorants and odor blends are also modulated by starvation and if so, during which temporal windows. It has been shown that starved insects are attracted towards host-derived odors, but that the response sign of non-starved insects is odor dependent (i.e. some odorants cause attraction, others repellence, others indifference; Reisenman et al., 2013). Regardless, odors must evoke a response at the peripheral level to produce a behavioral effect, whether attraction or repellence, as these opposite behavioral outputs result from central nervous system processing. It also remains to be investigated whether antennal responses to odors or odor components with different behavioral meanings (e.g. sexual and aggregation pheromones) are also modulated by starvation. For instance, it has been shown that the response of starved insects towards aggregation pheromones switched from repellence to attraction as function of the hunger level (Reisenman et al., 2013).

In triatomines, pheromones are not chemically-specific as in other insects such as moths and beetles, but instead are blends of single odorants which are present in different pheromones (e.g. aggregation, alarm, sex) and in host-odor bouquets (Guerenstein and Lazzari, 2009, 2010; Lazzari, 2009). As other blood-sucking insects, triatomines use the same chemical cues in different behavioral contexts, through parsimonious use of sensory information (Lazzari, 2009). The behavioral valence of an odor signal is therefore determined by the specific blend composition (which includes chemical identity and odorant proportion), which cannot be evaluated at the periphery but rather results from central nervous system processing. Thus, it is possible that the same antennal olfactory receptor cells are involved in the detection of chemicals which are parts of blends with distinct behavioral significance.

Modulation of responses in chemosensory neurons in other insects

The modulatory effects of hunger in the activity of chemosensory neurons have been mainly investigated in the fruit fly *D. melanogaster*. For instance Root et al. (2011) showed that the sensitivity of olfactory receptor cells is increased in starved flies, and that this effect is mediated by up-regulation of the short neuropeptide F receptor. Starvation also produces numerous changes in gene expression in chemosensory organs (Farhadian et al., 2012). In satiated flies, the activity of mushroom body output neurons involved in the retrieval of olfactory memories is affected by the starvation status (Krashes et al., 2009). In the taste system, starvation modulates neuronal gustatory processing and feeding through the action of various neuropeptides and modulators (Flood et al., 2013; Inagaki et al., 2012; Marella et al., 2012; see Itskov and Ribeiro, 2013 for a review). Thus, many mechanisms at several levels of the sensory pathway underlie the modulation of chemosensory responses by the feeding status, and are likely common across nervous systems. Importantly, in the case of insects which are vectors of human diseases, this modulation can have significant implications for parasite disease transmission (e.g. Egaña et al., 2014).

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Highlights

- The EAG responses of *Rhodnius prolixus* to a host-derived odorant were studied.
- The modulatory effects of hunger and the time of the day were investigated.
- In all cases EAG responses systematically increased with odorant concentration.
- The response of starved insects was larger than that of fed insects at night only.
- This modulation was the largest (up to 7-fold) at low-middle range concentrations.

Figure 1. Examples of typical electroantennogram (EAG) responses

Shown are responses (in Volts) from a nighttime starved insect to stimulation with different concentrations of ammonia (% vol/vol) and the water control. Response amplitude increased with concentration; increasing concentrations recruited faster responses. The top and bottom plots respectively show responses to stimulation with water and several concentrations of NH_4 ranging from 0.5 to 5% (top) and from 10% to 75% (bottom); note the different calibration scales. The line below traces indicates the onset and duration (500 ms) of the

odorant stimulation. Activation of the solenoid valve produced a downward square artifact, noticeable at high amplifications (top plots).

Figure 2. EAG responses systematically increased with odorant concentration

EAG responses (mean $\pm$ SE, data relative to the maximum response of each insect) obtained from independent groups (n=11–16 in each group) of starved (triangles) and fed (circles) insects tested during the day (A) or during the night (B) in response to stimulation with increasing concentrations of ammonia (% vol/vol, log scale) and the water control. In all groups EAG responses increased with concentration (Friedman ANOVAs, $p < 0.05$) and showed similar dose-response characteristics. Asterisks indicate responses which are statistically different from the response to water stimulation (post-hoc Dunnett's tests, $p < 0.05$). The response of nighttime fed and starved insects was statistically different 10%, 25% and 50% (Mann-Whitney U tests corrected for multiple comparisons, $p < 0.05$ in all three cases).

Figure 3. EAG responses are modulated by the starvation status in nighttime insects

EAG responses (absolute values, mean $\pm$ SE) obtained from independent groups of starved (triangles) and fed (circles) insects tested during the day (A) or during the night (B) in response to stimulation with the increasing concentrations of ammonia (% vol/vol, log scale) and the water control (same insects as in Figure 2). The absolute responses of starved and fed insects tested during the day were not statistically different at any of the concentrations analyzed (Mann-Whitney U tests, $p > 0.05$). In contrast, the absolute responses of starved and fed insects tested during the night were statistically different for all concentrations ranging from 2.5% to 75% (asterisks, Mann-Whitney U tests corrected for multiple comparisons, $p < 0.05$ in all cases).