STATE DEPENDENCE OF HOST-SEEKING IN BLOOD-SUCKING INSECTS: BEHAVIOUR AND PHYSIOLOGY

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SUMMARY

Blood from vertebrates constitutes a resource necessary for growth and reproduction for haematophagous insects. Provided that hosts play the double role of food-sources and predators, feeding on their blood expose these insects to a high predation risk. So, host-seeking behaviour should be modulated in order to make the insect feed, only when it is necessary. In this work we analyze how the nutritional status affects the response to host-associated cues in the bug *Rhodnius prolixus*. We show that the responsiveness to CO_2 stimulation, to thermal stimulation and the motivation to feed depends on the time elapsed since a blood meal. In the case of CO_2, the same concentration may attract or repel, depending on the moment. As far as we know, this is the first time that a host-signal is shown to be repellent for an haematophagous insect. The response to heat is also modulated, but no repellence was observed. When blood was replaced by saline solution as food, a significant inhibition of the response to both signals was evinced, but not repellence. The injection of haemolymph of fed insects in starved bugs inhibited the response of receivers to both signals, but not the injection of saline solution or haemolymph of non-fed insects. This is the first time that the modulation of feeding behaviour is analyzed in an hemimetabolous blood-sucking insect, excluding any effect of other processes, such as reproduction.

Keywords: motivation, orientation, feeding behaviour, Chagas disease

INTRODUCTION

The transmission of diseases by blood-sucking insects depends on their ability to find a potential host and then to begin taking a blood meal from it. Indeed, host-seeking constitute a main activity in the life of haematophagous insects, which devote to it a high portion of their sensory abilities and much of their time. This activity allows them to obtain blood nutrients, necessary for growth and reproduction. The obtainment of a blood meal, however, constitutes a dangerous task, provided that hosts play the double role of food-sources and predators. To avoid unnecessary risks, the motivation to feed should be modulated in order to make the insect feed only when needed. This is the case, for example, of mosquito females, which are not sensitive to host cue during egg production and they recover their ability to seek for food, once eggs are mature or have already been laid. This alternance between feeding and reproduction expresses as a gonotrophic cycle which has been well characterised in different mosquito species (Klowden, 1981; Klowden, 1990; Klowden and Briegel, 1994; Klowden 1995; Takken *et al*., 2001). Actually, if a first meal is sufficient for the production of a batch of eggs, a supplementary one would not necessarily increase fecundity (Lea *et al*., 1978; Edman and Scott, 1987).

Concerning hemimetabolous insects, obligatory haematophagous along their whole life, to our knowledge, no information is at present available. Even when reproduction and ovarian activity may modulate the feeding activity of females, this would not be the case of males and larvae, which are submitted to the same trade-off between feeding and avoiding predation. To shed some light on this problem, we studied the response of the blood-sucking bug *Rhodnius prolixus* to host-associated cues, as well as their motivation to feed, as a function of the time elapsed since the last meal. We have previously shown that internal factors, such as circadian clocks and the moult cycle affect the responsiveness of these bugs to host cues (Bodin *et al*., 2008; S. P. Bodin *et al*., unpublished).

First, we studied the response of larvae and adult males and females to carbon dioxide and thermal stimulation, as well as their motivation to feed, at different times after feeding and, in the case of the larvae, until their ecdysis. Then, we analysed the effect of abdominal distension on the modulation of the responses and, finally, we tested the influence of haemolymph-borne factors on the behavioural response to host-associated stimuli in these bugs.

MATERIAL AND METHODS

Insects

Larvae of *Rhodnius prolixus* were reared in the laboratory under 12/12 h light/dark illumination regime, at 28°C and 60 - 70 % RH. Insects were fed weekly, with sheep heparinised blood, using an artificial feeder (Núñez and Lazzari, 1990). Fifth instar larvae, adult males and females that had just moulted were isolated in individual plastic containers and starved until the tests. It has previously been shown that these bugs hatch at the end of the night (Ampleford and Steel, 1982). For our experiments, bugs were collected in the morning following ecdysis. They are recognizable by their characteristic pale-pink colour. In order to avoid any eventual interference of reproduction, adults of both sexes were kept virgin along the experiments.

Bioassay protocols

Experiment 1: Nutritional state and host-seeking behaviour

To investigate the potential modulation of the behavioural response of *R. prolixus* to host signals after a blood meal, we tested the behavioural response to CO_2 and heat, as well as the motivation to feed, at different times after a blood meal. Bugs were fed using an artificial feeder (Lazzari and Núñez, 1989), 15 days after their ecdysis either to the fifth instar or to the adulthood, in order to assure that they were highly motivated to feed. During the 24^{th} hours after feeding, bugs' weight varies rapidly because of the excretion of urine (Maddrell, 1963; Maddrell, 1964a; Maddrell, 1964b). Thus, in order to estimate the volume of ingested blood, insects were weighted 24 hours after feeding. Insects that had not fed at repletion were discarded in order to constitute homogenous groups.

After a complete blood meal in 5^{th} instar larvae, the behavioural responses to CO_2 and heat were tested each day during 8 days, and the 10^{th}, 15^{th}, 20^{th} and 27^{th} day, to characterize their responses during the entire larval stage. The 27^{th} day corresponds to the moment 5^{th} instar larvae start to moult to adults, i.e., imaginal ecdysis (Rabinovich *et al*., 1979). In male and female adults, the behavioural responses to CO_2 and heat after a complete blood meal were tested every 2 days during 20 days. The responses of the tested fed groups were compared with control, highly motivated bugs, which were starved for 15 days after ecdysis

(S. P. Bodin *et al.*, unpublished). Each insect was tested only once and discarded from experiments (N= 951).

All the assays were conducted in a room maintained at 25 ± 2°C; 40 – 60 % RH, 500 ± 100 ppm of CO_2 and under functional darkness for the insects, i.e. under infrared illumination (Reisenman *et al*., 1998). The experiments were carried out during the first hours of the scotophase, since triatomines display a peak of activity throughout this period, corresponding to the moment bugs get out from their refuges following host emitted cues in order to have a blood meal (Lazzari, 1992). Also their attractiveness to CO_2 is limited to this temporal window (Barrozo *et al*., 2004; Bodin *et al*., 2008).

Experiment 2: Abdominal distension and host-seeking-behaviour

To assess the potential role of the abdominal distension on the host-seeking modulation, we tested the response of 5th instar larvae of *R. prolixus* to CO_2 and heat during 3 days after feeding them on Ringer solution. As previously, an artificial feeder was used to feed the bugs 15 days after their ecdysis. The solution used was Ringer Locke (for 1 l of solution: 9 g NaCl; 0.42 g KCl; 0.24 g $CaCl_2$ $2H_2O$; 0.15 g $NaHCO_3$; 1 g glucose and distilled water to complete the volume). Just before feeding, adenosine triphosphate (ATP) was added to the solution to reach a concentration of 10^{-3} M. *R. prolixus* does not normally gorge on saline solutions (Friend and Smith, 1977) if not supplemented with ATP or some other related compound (Friend, 1965; Friend and Smith, 1977; Guerenstein and Núñez, 1994; Smith and Friend, 1982). These compounds are "phagostimulants" and a cue used by these insects to identify the presence of blood on the food source.

The rationale of this experiment was to test the unique intervention of the abdominal distension and not the presence of blood nutritive elements like proteins, lipids etc. in the gut. In order to estimate the volume of ingested solution, insects were weighted 24 hours after feeding and every experimental day. Bugs' weight was compared with that of blood-fed insects to assure identical abdominal distension, insects insufficiently fed were discarded to ensure a homogenous group of bugs. The experiments were carried out during the first hours of the scotophase because the attractiveness of the bugs to CO_2 is limited to this temporal window (Barrozo *et al*., 2004; Bodin *et al*., 2008). Each insect was tested only once then discarded from experiments (N= 95). We have chosen to feed bugs on saline solution to induce abdominal distension and not injecting the solution into the cavity of the insects body for the following reasons: 1) make the insects feed by themselves represents a non-invasive,

less deleterious manner to distend the abdomen (Wintle and Reinhardt, 2008) and 2) to get full distension, the abdomen of triatomines needs to be "plasticized" (i.e., to change its mechanical properties), a process triggered by stimuli associated to biting (Ianowsky *et al*., 1998).

Experiment 3: The role of haemolymph-borne factors

To explore the existence of humoral factors affecting the post-feeding modulation of the host-seeking behaviour, we analyzed the behavioural response to CO_2 and heat, as well as the motivation to feed in non-fed 5th instar larvae to which the haemolymph of fed donors was transferred. As previously, the non-fed insects were starved for 15 days for highly motivation to feed (S. P. Bodin *et al*., unpublished). Two groups of 15 fed donors were constituted, the first one composed of insects fed 1 day (I1) and the second composed of insects fed 4 days (I4) before haemolymph extraction. The collection of haemolymph for these groups was accomplished by securing the bugs by its back on adhesive tape, removing the legs, and applying a slight pressure to the abdomen until a drop of haemolymph extruded from the leg cuts. Collections were performed with a graduated micro-capillary connected to a manual pump, under a stereomicroscope. Using the same apparatus, non-fed insects were injected with 1 µl of I1-haemolymph or I4-haemolymph, and different groups of bugs were tested for their response either at day 1 (T1), 2 (T2) and 3 (T3) after the injection. The experiments were carried out during the first hours of the scotophase because the attractiveness of the bugs to CO_2 is limited to this temporal window (Barrozo *et al*., 2004; Bodin *et al*., 2008). Each insect was tested only once then discarded (N= 143). Three control experiments were conducted: 1) non-injected non-fed bugs (A group); 2) non-fed bugs injected with 1 µl of saline solution (B group) and 3) non-fed bugs injected with 1 µl of haemolymph obtained from non-fed bugs (C group).

Response to CO_2

The behavioural response of *R. prolixus* to CO_2 was recorded in an open-loop design for translation on a locomotion compensator and the walking paths of the bugs were reconstructed and analyzed in their spatio-temporal components as previously described by Barrozo and Lazzari (2004a). Before the beginning of each test, the insect remained in still air on the locomotion compensator for 2 minutes to familiarize to the experimental situation,

after which the airstreams (control and stimulus) were presented during 3 minutes. The assays were monitored from the outside of the experimental room thanks to an infrared-sensitive camera equipped with an array of infrared LEDs (emission 900 nm). This light illuminated the scene without being perceived by the bugs (Reisenman *et al*., 1998).

Provided that in these conditions triatomines exhibit spontaneous anemotaxis to odourless airstreams (Barrozo *et al*., 2003), a simultaneous-discrimination bioassay was conducted, similar to that previously used by us (Barrozo *et al*., 2004; Barrozo and Lazzari, 2004a, Barrozo and Lazzari, 2004b; Bodin *et al*., 2008; S. P. Bodin *et al*., unpublished). Individual bugs were exposed to two opposite horizontal charcoal-filtered airstreams (180°), one bearing 1200 ppm of CO_2, over the environmental concentration of 500 ± 100 ppm, while the other was kept clean (control versus test). Thus, each bug could choose to walk towards one of the two streams or could exhibit a non-oriented behaviour, i.e. to walk randomly. Both airstreams were blown over the insects through glass tubes (0.6 cm inner diameter, 14 cm length) placed at 3 cm from the insect at constant velocity (4.2 $cm.s^{-1}$), temperature (25 ± 2°C) and relative humidity (40 ± 5%). The production of CO_2 was achieved as previously described by Barrozo and Lazzari (2004a). To avoid eventual environmental biases, the positions of the stimulus and the control air-streams were changed randomly along the experiments.

The walking pathways described by the insects on the locomotion compensator were analyzed by means of circular statistics (Zar, 1984; Fisher, 1993). The mean walking angle (α_i) displayed by each insect along the experimental time was computed and subsequently, for every experimental group a mean angle (α_m) and the length of the resultant mean vector (r) were calculated. The angle α varied between 0 and 360° and r varied between 0 and 1 (0 indicating a non-defined mean direction and 1 a straight path to a given direction). The position of the stimulus-delivered current was conventionally designated as 0° and the control current as 180°. The V-test (Zar, 1984) was conducted to test if the mean angle (α_m) was significantly different from the stimulus direction (0°). Additionally, for an easier visualization of the data, an orientation index (OI) was calculated by multiplying the cosine of the mean angle (α_m) by the length of the vector (r), as cos (α_m) x r. This index fluctuates between 1 and -1, indicating orientation directly towards or away from the stimulus position, respectively. We also tested the pathways for eventual bimodal axial directions (i.e. opposite directions vs. uniformity) by means of the Rao's Spacing test (Fisher, 1993).

Response to heat and feeding motivation

In order to study the impact of a blood meal on the behavioural response to heat and the motivation to feed, we tested the bugs' ability to respond to a thermal source which also served as feeder. We set up an artificial feeder allowing us to observe independently 10 insects at a time (S. P. Bodin *et al*., unpublished). The artificial feeder consisted of ten 1 ml Eppendorf® tubes whose rear ends were cut and replaced by Parafilm® through which insects were able to bite. The tubes contained 0.5 ml of sheep heparinised blood and were placed in a tapped aluminium block (35 x 5 x 1.3 cm) equipped with a flat electric resistance. A thermostat kept blood at constant temperature, 33 ± 1°C, which roughly corresponds to the temperature of the surface of a host body. The aluminium block was isolated with a polystyrene foam plate which was pierced to let the tubes accessible. Thus, the lower side of the polystyrene plate was at ambient temperature (25 ± 2°C). The insects were placed in plastic containers (11.7 cm height and 3 cm diameter), which top was covered with a fabric mesh, allowing the insects to bite the blood containers. These tubes contained a piece of filter paper serving as substrate for the bugs and allowing them climbing up to approach the feeder. Before a test began, each insect was allowed to habituate for 2 minutes to the experimental situation without stimulation. After this time, the artificial feeder was placed over the containers and the response of the insects recorded for 15 minutes. Three parameters were recorded: a) approaching (the bug climbed up approaching the feeder), b) PER (proboscis extension response) and c) feeding (the bug increased noticeably its abdominal volume).

Binary data (1 = behaviour observed and 0 = behaviour not observed) were collected and a proportion of response (p) was calculated for each behaviour. The standard deviation (s) was calculated by the following mathematic formula for binary data: $s = (p\,(1-p))^{½}$. When needed, a non-parametric Mann-Whitney U-test was performed for comparing groups adjusting the value of α in the case of multiple comparisons.

RESULTS

Experiment 1: Nutritional state and host-seeking behaviour

Behavioural response to CO_2 and heat in 5th instar larvae

The Fig. 1A represents the response to CO_2 in 5th instar larvae of *R. prolixus*. A variation in the orientation response of the bugs to this long-range host cue could be observed,

as a function of the time elapsed since the blood-meal. Before taking a meal, bugs showed a strong attraction response to CO_2 (V-test, $p<0.05$), displaying a preferred walking direction to the stimulus. Then, after feeding we have observed 4 phases of response. First, during the two first days following feeding (T1 and T2), bugs showed a behavioural inhibition phase. They walked randomly on the sphere showing no orientation preference, i.e. the pathways of the insects were uniformly distributed between 0° and 360° (V-test not significant in all cases). Second, during the tree following days (T3, T4 and T5), bugs showed a behavioural repulsion phase, displaying a preferred walking direction opposite to the stimulus (V-test, $p<0.05$ in all cases). Third, from T6 to T15, bugs displayed again behavioural inhibition, with no orientation preference on the locomotion compensator (V-test not significant in all cases). Finally, in both cases, at T20 and T27 bugs displayed once more a preferred walking direction opposite to the stimulus (V-test, $p<0.05$), showing another behavioural repulsion phase. The comparison of the walking duration among all the different groups, revealed in all cases, no significant differences, indicating that the activity on the locomotion compensator was similar for all the groups (ANOVA not significant). The Rao's Spacing test did not revealed axial orientation in any case, i.e., non-significant.

The Fig. 2A represents the behavioural response of 5th instar larvae of *R. prolixus* to a short-range stimulation by heat. It represents the proportion of the different behaviours realized by the bugs, a) approaching the heat source, b) PER, and c) feeding, as a function of the time elapsed since the blood-meal. For each day, the number of insects that presented one of the behaviours studied was expressed as a proportion of the total number of bugs tested. Our results showed that approaching and PER are associated, since all the insects that approached the thermal source (the artificial feeder), extended their proboscis. Before feeding, we observed a great proportion of insects approaching the heat source and extending their proboscis (90 ± 4.8 %), and an important proportion fed (77.5 ± 6.8 %). Then, after feeding and during all the experiments, there was an important decrease in the proportion of insects responding to heat and in the motivation to feed. The proportion of insects approaching the heat source and displaying the PER has never exceeded 35 %. The maximum responses were observed between T7 and T15. Concerning the proportion of insects that fed from the artificial feeder, it never exceeded 15 %, with maximum responses also between T7 and T15, corresponding to the inhibition phase of the response to CO_2 that follows the repulsion period.

Behavioural response to CO_2 and heat in females

The Fig. 1B represents the response to CO_2 in females of *R. prolixus*. As for larvae, a variation in the orientation response of the bugs to this cue could be observed as a function of the time elapsed since the blood-meal. Before taking a meal, bugs showed a strong attraction response to CO_2 (V-test, $p<0.05$), displaying a preferred walking direction to the stimulus. As for 5th instar larvae, we observed different phases of response during the days after feeding. First, during the four first days following feeding (T2 and T4), females showed a behavioural inhibition phase. The pathways of the insects were uniformly distributed between 0° and 360° (V-test not significant in all cases), i.e. they walked randomly on the sphere showing no orientation preference. Second, between T6 and T10, bugs showed a behavioural repulsion phase, displaying a preferred walking direction opposite to the stimulus (V-test, $p<0.05$ in all cases). Third, from T12 to T18, females displayed no orientation preference on the locomotion compensator (V-test not significant in all cases) suggesting a behavioural inhibition phase. Finally, in T20 they displayed a preferred walking direction to the stimulus (V-test, $p<0.05$), showing a behavioural attraction response. These results revealed that virgin females needs at least 20 days before responding to CO_2 after a blood-meal. The comparison of the walking duration among all the different groups, revealed in all cases, no significant differences, indicating that the activity on the locomotion compensator was similar for all the groups (ANOVA not significant). The Rao's Spacing test did not revealed axial orientation in any case, i.e., non-significant.

The Fig. 2B represents the behavioural response of females of *R. prolixus* to heat. As in the case of larvae, all the insects that approached the thermal source extended their proboscis. Before feeding, we observed a great proportion of insects approaching the heat source and extending their proboscis (65 ± 12.5 %), and an important proportion of insects that fed (60 ± 9.6 %). These proportions were statistically different to those observed in larvae (U= 2.84, $p<0.05$ and U=-2.17, $p<0.05$, respectively for the proportion of insects attracted (approaching and PER) and for insects that fed through the feeder). Then, after the blood-meal and during all the experiments, there was a great decrease in the proportion of insects responding to heat. The proportion of insects approaching the heat source and displaying the PER has never exceeded 30 %. The maximum responses were observed between T6 and T10. Concerning feeding motivation, no insect was observed trying to feed from the artificial feeder during the experimental time.

Behavioural response to CO_2 and heat in males

The Fig. 1C represents the response to CO_2 in males of *R. prolixus*. As for larvae and females, we observed a variation in the orientation response of the bugs as a function of the time elapsed since the blood-meal. Males showed a strong attraction response to CO_2 before taking a blood-meal (V-test, $p<0.05$), displaying a preferred walking direction to the stimulus. Contrary to what observed in larvae and females, males showed no orientation response along the experiments, i.e., neither attraction, nor repulsion. The pathways of the insects were uniformly distributed between 0° and 360° (V-test not significant in all cases), i.e. they walked randomly on the sphere showing no orientation preference. The comparison of the walking duration among all the different groups, revealed in all cases, no significant differences, indicating that the activity on the locomotion compensator was similar for all the groups (ANOVA not significant). The Rao's Spacing test did not revealed axial orientation in any case, i.e., non-significant.

The Fig. 2C represents the behavioural response of males of *R. prolixus* to heat. All the insects that approached the thermal source extended their proboscis. Our results are similar to those obtained with females. Before feeding, we observed most insects to approach the heat source and to extend their proboscis (62 ± 10.2 %), as well as an important proportion that fed (59 ± 9.3 %). These proportions were statistically different to what observed in larvae (U= 2.15, $p<0.05$ and U=-2.29, $p<0.05$, respectively for the proportion of insects attracted (approaching and PER) and for insects that fed through the feeder) but not from females (Mann-Whitney not significant for all behaviours observed). Then, after the blood-meal, there was a great decrease in the proportion of insects responding to heat. The proportion of insects approaching the heat source and displaying the PER has never exceeded 20 %. The maximum responses were observed between T8 and T16. As for females, we never observed insects trying to feed from the artificial feeder.

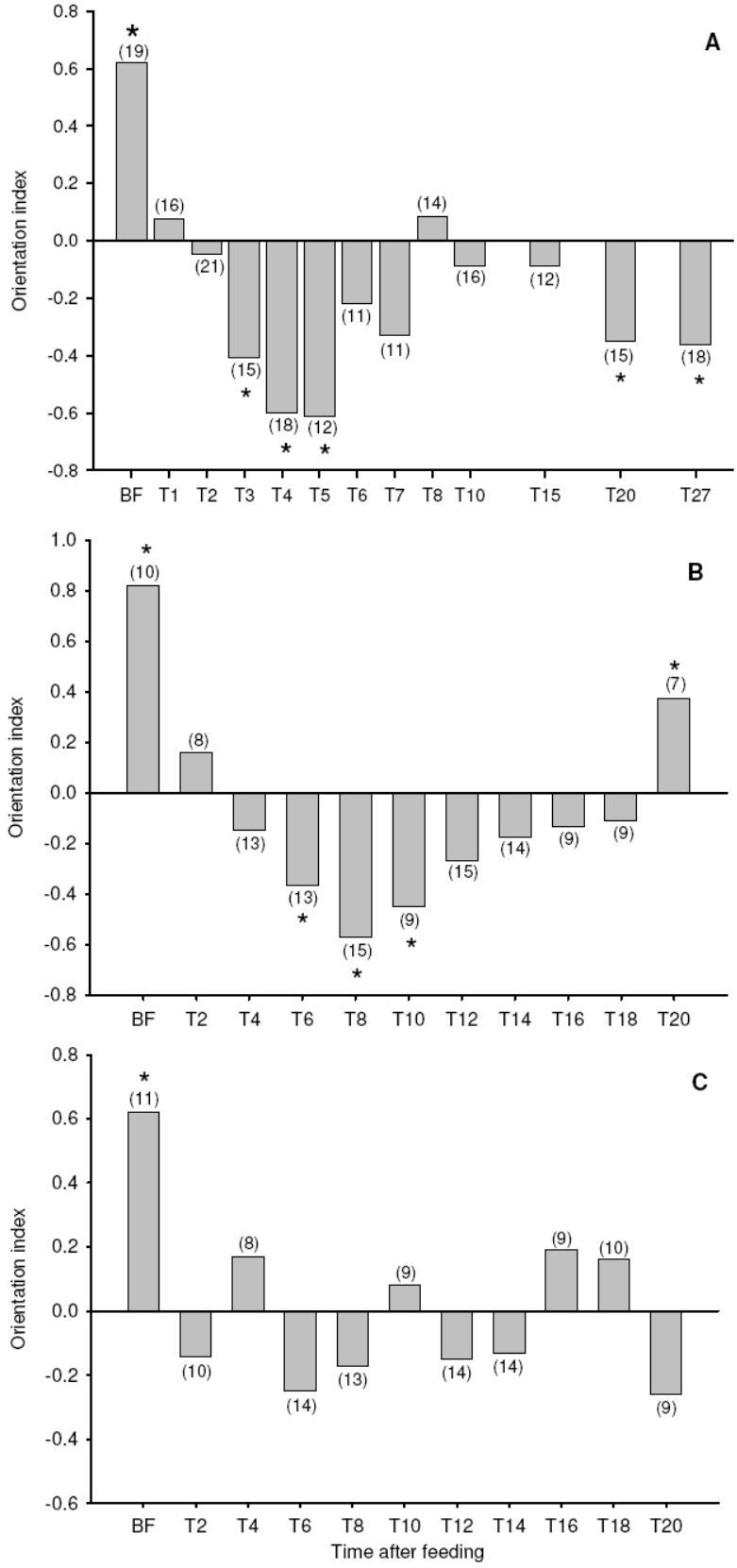

Figure 1: Orientation response of *R. prolixus* larvae (A), adult females (B) and males (C) to airstreams loaded with 1200 ppm of CO_2 above the background (500 ± 100 ppm), at different times after a complete blood-meal. Asterisks denote a statistically significant preferred walking direction towards or against the stimulus location (0°) (*V* test, $p<0.05$). Orientation index varies from -1 (orientation against the stimulus position) to 1 (orientation towards the stimulus location). The number of insects tested is shown in brackets.

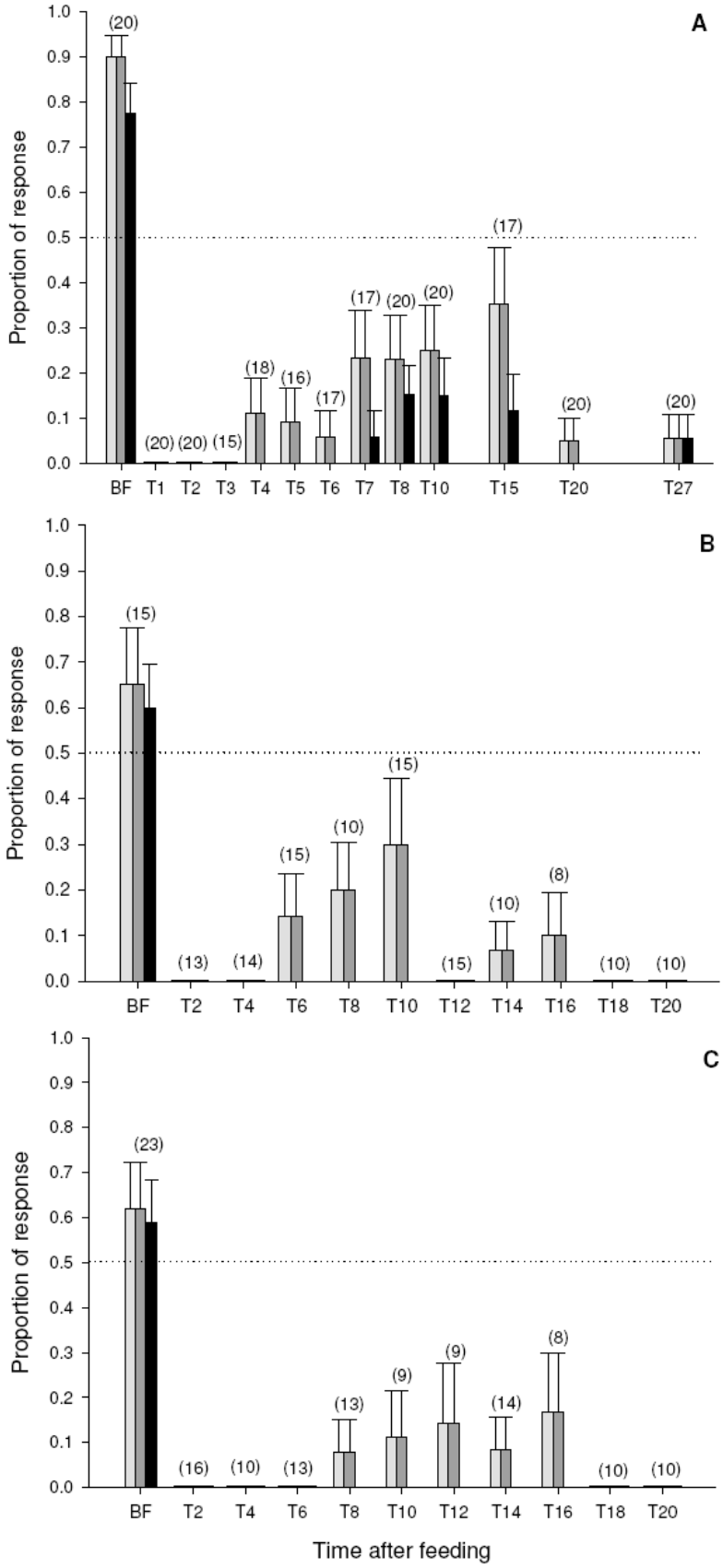

Figure 2: Behavioural response to heat and motivation to feed in *R. prolixus* larvae (A), adult females (B) and males (C) at different times after a complete blood-meal. White bars represent the proportion of insects that approached the heat source, gray bars represent the proportion of insects that performed proboscis extension response (PER) and the black ones represent the proportion of insects that fed. Each bar represents the mean response ± S.E.M. The dot line represents the level of 50 % of response. The number of insects tested is shown in brackets.

Experiment 2: Abdominal distension and host-seeking-behaviour

One day after feeding with both, blood or saline solutions, the weight of the insects had increased by a factor of approximately 6.5 (29.7 ± 1.4 mg before feeding and 195.9 ± 3.6 mg after) and stayed constant during the three days of experiment and no statistically difference between the weights of the two groups of insects were observed (Mann-Whitney not significant in all cases).

The Fig. 3A represents the response to CO_2 after feeding with saline solution in 5th instar larvae of *R. prolixus*. The results obtained before feeding correspond to those obtained in the experiment 1 (see above). During the three first days after feeding, they showed no orientation tendency towards the stimulus (V-test not significant in all cases). The comparison of the walking duration among all the different groups, revealed in all cases, no significant differences, indicating that the activity on the locomotion compensator was similar for all the groups (ANOVA not significant). The Rao's Spacing test did not revealed axial orientation in any case, i.e., non-significant.

The behavioural response to heat after an artificial feeding with saline solution is represented in Fig. 3B. The results before feeding are the same as in the experiment 1 (see above). After the artificial meal, we observed no response during the 3 days of experiments. The bugs even didn't move in their containers.

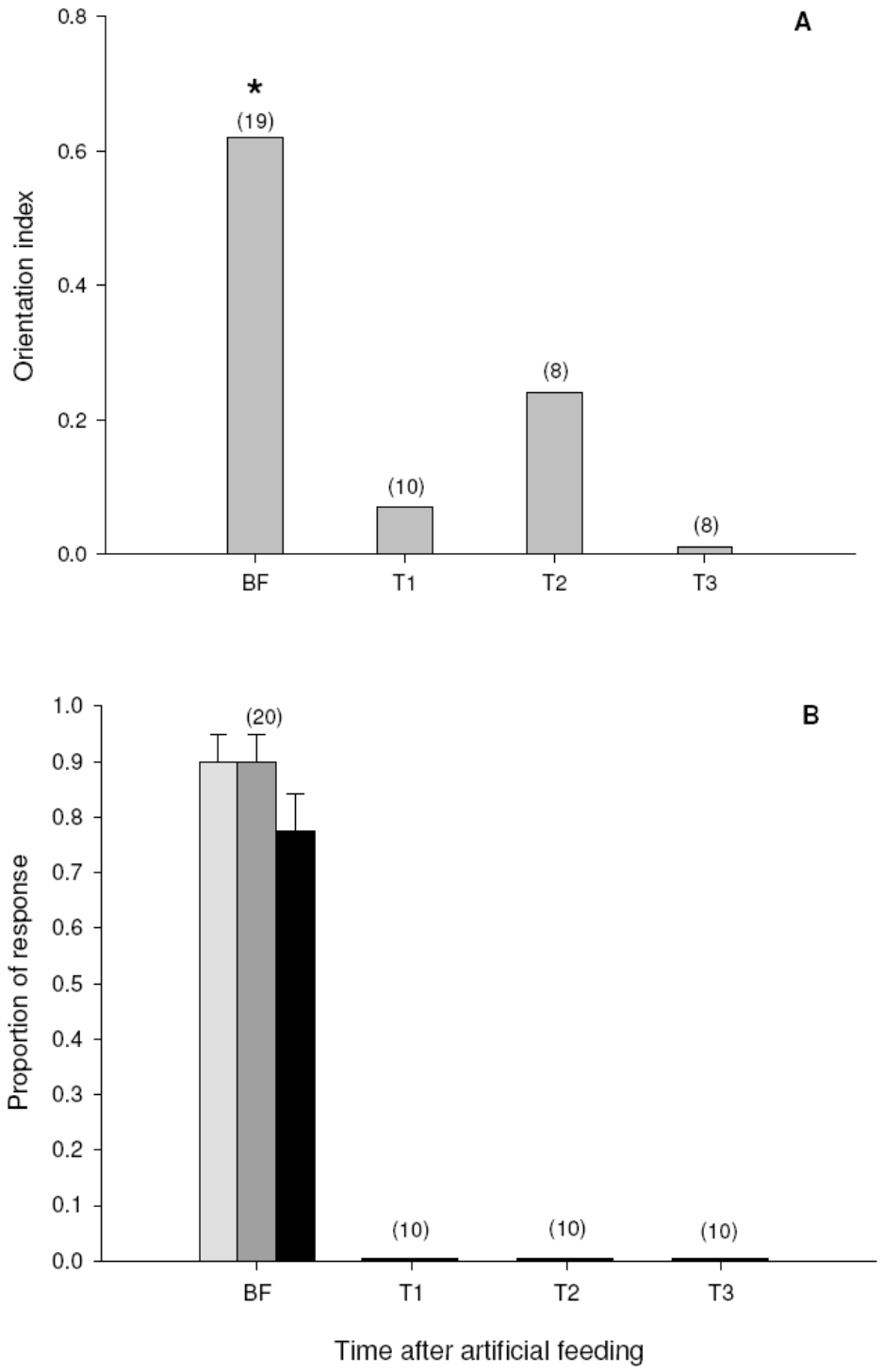

Figure 3: Behavioural response of *R. prolixus* larvae to different host-emitting cues during three days after an artificial meal with a saline solution. (A) represents the behavioural response of the bugs to 1200 ppm of CO_2 above the background (500 ± 100 ppm) before and after feeding on saline, asterisks denote a statistically significant preferred walking direction towards stimulus location (0°) (*V* test, $p<0.05$). Orientation index varies from -1 (orientation against the stimulus position) to 1 (orientation towards the stimulus location). (B) represents the behavioural response of the bugs to heat and their motivation to feed before and after feeding on saline, white bars represent the proportion of insects that approached the heat source, gray bars represent the proportion of insects that performed proboscis extension (PER) and the black ones represent the proportion of insects that fed. Each bar represents the mean response ± S.E.M. The number of insects tested is shown in brackets.

Experiment 3: The role of haemolymph-borne factors

The Fig. 4A represents the behavioural response to CO_2 of non-fed 5th instar larvae of *R. prolixus* injected with haemolymph of fed ones. Larvae were injected with haemolymph of insects fed for 1 day (I1) or with haemolymph of insects fed for 4 days (I4). The results of the control A (non-fed insects without injections) corresponds to that obtained in the experiment 1 before feeding (see above). The two other controls, B (non-fed insects injected with saline solution) and C (non-fed insects injected with haemolymph of non-fed insects), showed that bugs displayed an orientation preference to the stimulus after being injected (V-test, $p<0.05$ in all cases) excluding any possible negative effect of the injections.

It should be noted that all the bugs of group I1 died between 24 and 48 hours after injection. So, data could only be obtained for day 1 after injection (I1-T1) and showed that the bugs were not attracted to the stimulus. A certain tendency to repulsion by the stimulus was observed, but it revealed as statistically not significant (V-test not significant). Results obtained from the group I4 are quite different from I1. All injected bugs survived during experiments and beyond, indicating that the haemolymph rather than the injection itself was the deleterious factor. In I4-T1, I4-T2 and I4-T3 after injections, bugs showed no orientation preference when confronted to the stimulus (V-test not significant in all cases). The comparison of the walking duration among all the different groups (I1 and I4) and days, revealed in all cases, no significant differences, indicating that the activity on the locomotion compensator was similar for all the groups (ANOVA not significant). The Rao's Spacing test did not revealed axial orientation in any case, i.e., non-significant.

The Fig. 4B represents the behavioural response to heat of non-fed 5th instar larvae of *R. prolixus* injected with haemolymph of fed ones. The results of the control A (before injections) are the same of the experiment 1 (see above). For the control B (non-fed insects injected with saline solution) and C (non-fed insects injected with haemolymph of non-fed insects), the three behavioural responses (approaching, PER and feeding) did not differ from those of the control A (Mann-Whitney, not significant in all cases). As explained before, data from the group I1 were obtained only for 1 day after injection (T1) and showed that the bugs were not attracted to heat. Insects even didn't move in their containers. Results obtained from the group I4 are very different, a progressive attraction response to heat was observed. Observations revealed that few insects responded to the heat stimulus 1 day after injection. These proportions of response increased during the following days (I4-T2 and I4-T3) to nearly reach the proportions of response of the control A. In I4-T1 and I4-T2, the proportions of insects that responded to heat and fed from the feeder were statistically different from those

obtained for the control A (Mann-Whitney, $p<0.05$ in all cases). The proportions of response obtained in I4-T3 were not different from the control A (Mann-Whitney, not significant). Our results also show that all the insects that approached the thermal source (the feeder), extended their proboscis.

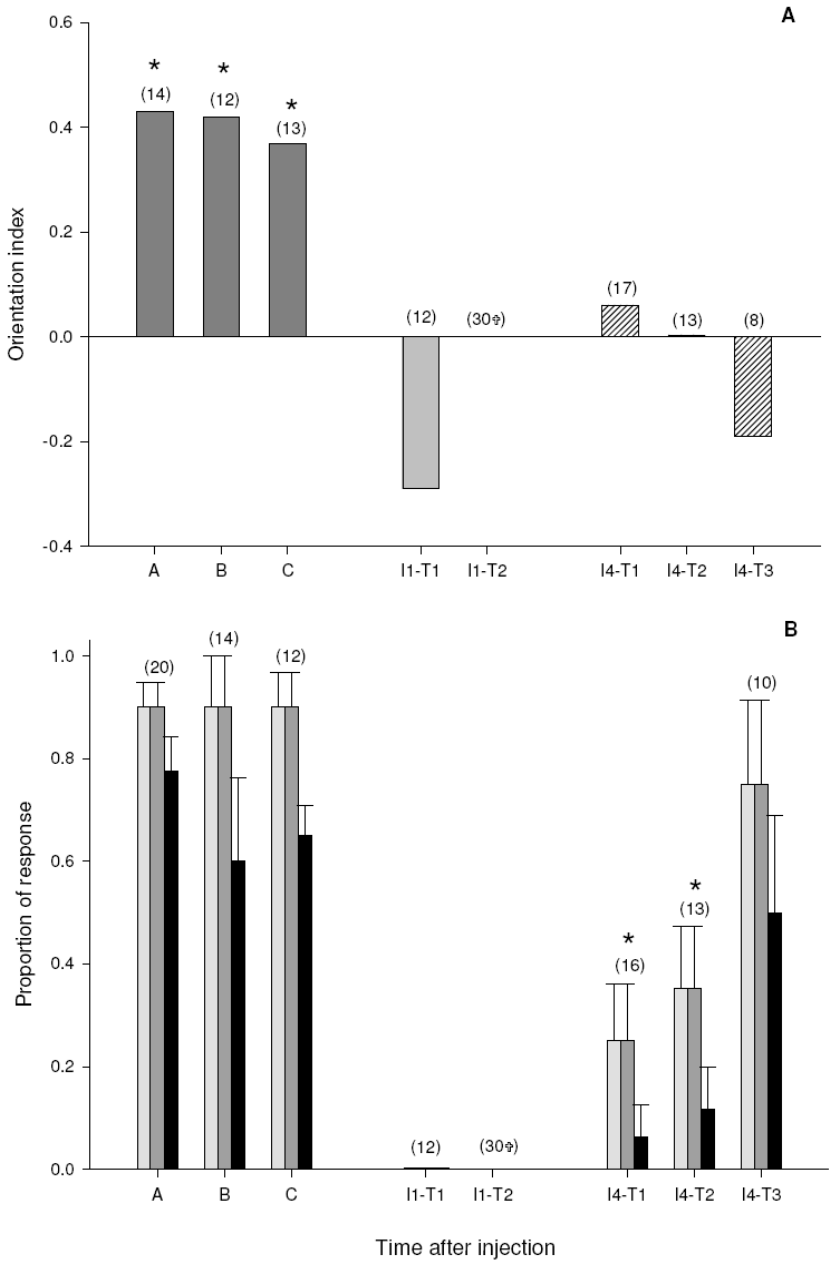

Figure 4: Behavioural response of non-fed larvae of *R. prolixus* to different host-emitting cues at different times after injection of haemolymph of insects fed for 1 day (I1) or 4 days (I4). (A) represents the behavioural response of the bugs to 1200 ppm of CO_2 above the background (500 ± 100 ppm), asterisks denote a statistically significant preferred walking direction towards the stimulus location (0°) (*V* test, $p<0.05$). Orientation index varies from -1 (orientation against the stimulus position) to 1 (orientation towards the stimulus location). (B) represents the behavioural response of the bugs to heat and their motivation to feed, white bars represent the proportion of insects that approached the heat source, gray bars represent the proportion of insects that performed proboscis extension (PER) and the black ones represent the proportion of insects that fed. Each bar represents the mean response ± S.E.M. The asterisk indicates a significant difference in the feeding response (Mann-Whitney test). The number of insects tested is shown in brackets. The cross means that all insects dead before the day of experiment.

DISCUSSION

Modulation of the responsiveness to host cues and feeding behaviour

Our experiments have shown that the responsiveness of blood-sucking insects to host cues is modulated by the nutritional state of the animal. This modulation affects the response of the insect to cues of different modalities, as well as the motivation to feed. So, the oriented response to CO_2 varied as a function of the time elapsed since the last blood-meal, as did the approaching to a thermal source and the motivation to feed.

A very interesting finding of our work was that the response to a host cue may be attraction, indifference or, quite surprisingly, a significant repellence. As far as we know, this is the first time that a repellent effect to a host-associated cue is demonstrated for an haematophagous insect. The theoretical analysis of mechanisms of spatial orientation (Jander, 1963) indicates that a same stimulus may mediate responses of opposite signs, as indicated by Núñez (1985): "The possibility that some of the sensory cues that served to locate the host would be used to escape or to move away from it, given a change of the sign of the orientation vector (see Jander, 1963). No experimental data are at present available on this aspect". This behaviour became evident in *R. prolixus* at different moments or "physiological contexts". In larvae, repulsion appeared about 72hs after feeding lasting for two days (Fig. 1A, T3 to T5), and also in the proximity of the ecdysis (Fig. 1A, T20 - T27; Lent and Valderrama, 1977; Rabinovich *et al*., 1979). In females, it appeared between T6 and T10 (Fig. 1B), when their motivation to oviposit is expected to be maximal (Davey, 1965). The fact that males did not show repellence to CO_2 seems to be consistent with the fact that in females it could only be related to oviposition and not to feeding as in larvae.

Only a reduction of the motivation to approach, but not repulsion to heat was observed in the bugs. In all cases, larvae, males and females, a strong reduction of both, the responsiveness to heat and to feeding motivation was verified (Fig. 2). In all cases, a complete inhibition of the response occurred just after a blood-meal (i.e., during the repulsion phase to CO_2), to recover gradually along the following days. It should be noted that approaching and PER were associated in 100 % of the cases (i.e. every time the bugs approached the heat source, they extended their proboscis), evincing once more the crucial role of heat for biting in triatomines (Flores and Lazzari, 1996). Nevertheless, PER did not necessarily implicate feeding. In contrast to chemical stimulation, which relies on

anemotaxis, heat orientation occurred in close proximity (Lazzari and Núñez, 1989). So, it is not surprising that bugs responded trying to bite (i.e., PER) a heat source when they are in contact or almost, even when their motivation to feed repress them from taking blood.

The physiological mechanisms

To explore the physiological basis of the state-depence to feed, we performed two further experimental series. The first one was addressed to test whether the change was induced by the mechanical distension of the abdomen or by chemical composition of food. For this, we fed a group of bugs on saline solution enriched with phagostimulants. A strong inhibition of both, the response to CO_2 and to heat was verified, as shown in Fig. 3. Nevertheless, no repulsive response was observed; only a blood meal was able to induce repellence, but not saline, suggesting that negative orientation to CO_2 is triggered by one or more chemical components of blood.

The last experiment tested whether the factors responsible for the modulation of the response to host cues are transported by the haemolymph. By injecting the haemolymph of just fed insects to starved ones, we were able to reduce their responsiveness to both, CO_2 and heat (Fig. 4). The duration of this effect on the receivers could not be measured because all of them indefectibly died 48hs after receiving the injection. Neither the change in the response, nor death occurred when the starved insects received saline solution or the haemolymph of non-fed insects (Fig. 4). The reason for the death of receivers of the haemolymph of just fed insects could be related to the important physiological changes occurring at feeding. To handle blood meals making several times their own body weight, these bugs experience dramatic physiological modifications, including the flow of a big amount of blood components and water through the crop wall towards the haemolymph. At the same time, diuretic hormones assure the rapid elimination of many of them during the hours following feeding (Maddrell, 1963). We can imagine that receivers got all this factors, including some toxic ones, without being able to eliminate them, because, even though diuretic hormones were also transferred, they do not suffice to induce diuresis in starved insects (Gomez and Balderrama, 1986).

In the last experiment we injected to starved insects, the haemolymph of donors fed 4 days before. This experiment allowed us to test two things. The first one was whether during the repulsive phase to CO_2, this response could be reproduced in receivers. The second aim was to test whether the lethal effect on receivers of the haemolymph of just fed donors

persisted. The receivers of this haemolymph were inhibited in their response to both, CO_2 and heat (Fig. 4), but no significant repulsion could be verified (Fig. 4B). These receiver bugs did not die after the injection and their response could be followed, revealing a recovering of the responsiveness to heat and the persistence of the lack of response to CO_2 for at least 3 days. These results show that the haemolymph-borne factors are responsible for the inhibition of the behavioural response of the bugs and also that these factors persist for several days to slowly lose their activity. The survival of these receivers supports the idea that what caused the death of receivers of the haemolymph of just fed insects was some factor associated to physiological changes occurring just after feeding.

The influence of the nutritional state on host-seeking by haematophagous insects has been analysed in detail in female mosquitoes and particularly concerning the interaction between the nutritive and the reproductive state. In the female mosquito, feeding triggers endocrine processes controlling the ovarian function (Klowden, 1997). After the initiation of a gonotrophic cycle by a blood meal, host-seeking is inhibited until egg maturation or oviposition (Klowden and Briegel, 1994). The inhibition is the result of the interplay of two mechanisms, one nervous and one humoral. The distension of the abdomen stimulates abdominal mechanoreceptors ("distension induced inhibition", Klowden and Lea, 1979a). If the blood meal suffices for triggering egg production, the humoral mechanism is activated ("oocyte-induced inhibition", Klowden and Lea, 1979b), where different organs are involved, such as the ovary, fat body and neurosecretory cells. In contrast to our findings, no repulsion seems to occur. Concerning the physiological mechanism, it has been shown in *Aedes aegypti* that the response of lactic-acid receptors is inhibited by humoral factors present in the haemolymph after feeding (Brown *et al.*, 1994).

In conclusion, we have shown that the response of haematophagous insects to their host is modulated by their physiological state. Feeding, moult and oviposition seem all three to be affecting the responsiveness to both, chemical and physical cues associated to vertebrate hosts. This modulation seems to be triggered by both, the distension of the abdomen and also chemical components of the blood ingested. Only a blood meal is able to induce repulsion and could be directly or indirectly responsible of the release of haemolymph-borne factors, which can be transferred from an insect to another. Further work should reveal the exact origin and targets of this factor.

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