Behavioural and physiological state dependency of host seeking in the blood-sucking insect *Rhodnius prolixus*

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SUMMARY

Vertebrate blood is essential for the growth and the reproduction of haematophagous insects. Provided that hosts play the double role of food sources and predators, feeding on their blood exposes these insects to a high predation risk. Therefore, it is expected that host seeking occurs only when insects need to feed. In the present study, we analyse how the feeding status affects the response to host-associated cues in the blood-sucking insect *Rhodnius prolixus*. We show that the responsiveness to host-associated cues, such as CO_2 and heat, and that the motivation to feed depend on the time elapsed since a blood meal. Depending on the time elapsed after feeding, the same concentration of CO_2 may attract or repel the insects. As far as we know, this is the first time that a host signal has been shown to be repellent for a 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 reduction of the response to both signals was evinced but repellence was not observed. The injection of haemolymph from fed insects into starved insects inhibited the response of the receiver insects to both signals but this was not observed after the injection of saline solution or of haemolymph from non-fed insects. This is the first time that the modulation of feeding behaviour by the feeding status has been analysed in a hemimetabolous blood-sucking insect, fully excluding any effect of other processes, such as reproduction.

Key words: motivation, orientation, feeding behaviour, Chagas disease.

INTRODUCTION

The transmission of diseases by blood-sucking insects depends on their ability to find and feed from a potential host and, then, to begin taking a blood meal from it. Indeed, host seeking constitutes a main activity in the life of haematophagous insects, as indicated by the high portion of their sensory abilities and time devoted to it. Through this activity, they obtain the blood nutrients that are necessary for their growth and reproduction. Obtaining 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, such as insects feed only when needed. This is the case, for example, of mosquito females, which are not sensitive to host cues during egg production and which recover their ability to search for food once eggs are mature or they have already been laid. This alternation between feeding and reproduction expresses itself 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 to produce a batch of eggs, a supplementary meal would not necessarily increase fecundity (Lea et al., 1978; Edman and Scott, 1987).

Concerning hemimetabolous insects, which are obligatory haematophagous throughout all of their life stages, to our knowledge, no information is presently available. Even when reproduction and ovarian activity may modulate the feeding activity of females, this would not be the case in males and in larvae, which are subjected to the same trade-off between feeding and avoiding predation. To shed some light on this problem, we studied the response of blood-sucking insects to host-associated cues and 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 insects to host cues (Bodin et al., 2008; Bodin et al., 2009). By analysing the effect of feeding, we expand the characterisation of the effects of the physiological state on host-seeking and host-feeding behaviour. This knowledge is relevant to achieve a better comprehension on how feeding frequency is modulated in blood-sucking insects; a main factor affecting the transmission of parasites.

As an experimental model, we chose *Rhodnius prolixus*, because in addition to fulfilling the conditions for the study (i.e. hemimetabolous development, and larvae, males and females are obligatory haematophagous), it is a classical model in insect physiology and one of the main vectors of Chagas disease in Central and South America. First, we studied the response of larvae and adult males and females to CO_2 and heat, and their motivation to feed at different times after feeding and, in the case of the larvae, until their ecdysis. Second, we also analysed the effect of abdominal distension and the influence of haemolymph-borne factors on the behavioural responses to host-associated stimuli in these insects.

MATERIALS AND METHODS
Insects

Rhodnius prolixus Stahl were reared in the laboratory under 12h:12h light:dark illumination regime, at 28°C and 60–70% RH (relative humidity). 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 insects hatch at the end of the night (Ampleford and Steel, 1982). For our experiments, insects

were collected in the morning following ecdysis. They are recognisable by their characteristic pale-pink colour. In order to avoid any eventual interference of reproduction, adults of both sexes were kept virgin.

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. Insects were fed using an artificial feeder (Lazzari and Núñez, 1989) 15 days after their ecdysis, either to the fifth instar or to adulthood, in order to assure that they were highly motivated to feed (Bodin et al., 2009). During the 24 h after feeding, the mass of the insects varied rapidly because of the excretion of urine (Maddrell, 1963; Maddrell, 1964a; Maddrell, 1964b). Insects were weighted 24 h after feeding because insects are too sensitive to manipulation to be weighed immediately after feeding. Insects that had not been fed at repletion were discarded in order to constitute homogenous groups.

In the case of fifth-instar larvae, after a complete blood meal the behavioural responses to CO_2 and heat were tested each day during eight consecutive days and on the 10th, 15th, 20th and 27th days. Fifth-instar larvae moult to adults (imaginal ecdysis) around the 27th day (Rabinovich et al., 1979). In males and females, the behavioural responses to CO_2 and heat after a complete blood meal were tested every two days during 20 days. The responses of the tested fed groups were compared with the control group; highly motivated insects, which were starved for 15 days after ecdysis (Bodin et al., 2009). Each insect was tested only once (N=951).

All of the assays were conducted in a room maintained at $25\pm2°C$, 40–60% RH, 500 ± 100 p.p.m. 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, because triatomines display a peak of activity throughout this period corresponding to the moment insects emerge from their refuges following host-emitted cues in order to have a blood meal (Lazzari, 1992). Also, their attraction 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 fifth-instar larvae of *R. prolixus* to CO_2 and heat during three days after feeding them with Ringer solution. As in the previous experiment, the insects were fed using an artificial feeder 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$_2$O; 0.15 g NaHCO$_3$; 1 g glucose and distilled water to complete the volume). Just before feeding, adenosine triphosphate (ATP) 10^{-3} mol l^{-1} was added to the solution. *R. prolixus* does not normally gorge on saline solutions unless they are 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 are a cue used by these insects to identify the presence of blood in the food source.

The rationale underlying this experiment was to test the effect of the abdominal distension as opposed to that of blood nutritive elements (proteins, lipids, etc.) in host-seeking behaviour and motivation to feed. In order to estimate the volume of ingested solution, insects were weighted 24 h after feeding and every experimental day afterwards. The mass of the insects was compared with that of blood-fed insects to assure similar abdominal distension. Insects insufficiently fed were discarded to ensure a homogenous group of insects. The experiments were carried out during the first hours of the insects' scotophase because their attraction to CO_2 is limited to this temporal window (Barrozo et al., 2004; Bodin et al., 2008). Each insect was tested only once and then discarded from the experiments (N=95). We have chosen to feed the insects on saline solution to induce abdominal distension rather than injecting the solution into the cavity of the insects' body for the following reasons: (1) making 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 'plasticised' (i.e. to change its mechanical properties), a process triggered by stimuli associated to biting (Ianowski 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 analysed the behavioural response to CO_2 and heat, as well as the motivation to feed in non-fed fifth-instar larvae to which the haemolymph of fed donors was transferred. As in the previous experiment, the non-fed insects were starved for 15 days so that they would have a high motivation to feed (Bodin et al., 2009). Two groups of 15 fed donors were constituted, the first one composed of insects fed one day (I1) and the second one composed of insects fed four days (I4) before haemolymph extraction. The collection of haemolymph for these groups was accomplished by securing the insects by their back over 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 (Leica MZ16, Wetzlar, Germany). Using the same apparatus, non-fed insects were injected with 1 µl of I1–haemolymph or I4–haemolymph, and different groups of insects were tested for their response either at day 1 (t1), day 2 (t2) or day 3 (t3) after the injection. As previously stated, the experiments were carried out during the first hours of the insects' scotophase because their attraction to CO_2 is limited to this temporal window (Barrozo et al., 2004; Bodin et al., 2008). Each insect was tested only once and then discarded (N=143). Three control experiments were conducted: (1) non-injected, non-fed insects (A group); (2) non-fed insects injected with 1 µl of saline solution (B group), and (3) non-fed insects injected with 1 µl of haemolymph obtained from non-fed insects (C group).

The 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 insects were reconstructed and analysed in their spatio–temporal components, as previously described by Barrozo and Lazzari (Barrozo and Lazzari, 2004a). Before the beginning of each test, the insect remained in still air on the locomotion compensator for 2 min to familiarise themselves with the experimental situation, after which the air-streams (control and stimulus) were presented during 3 min. The assays were monitored from the outside of the experimental room by means of an infrared-sensitive camera (Conrad, Lille, France) equipped with an array of infrared LEDs (emission 900 nm). This light illuminated the scene without being perceived by the insects (Reisenman et al., 1998).

Because triatomines exhibit spontaneous anemotaxis to odourless air-streams under these conditions (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; Bodin et al., 2009). Individual insects were exposed to two opposite horizontal charcoal-filtered air-streams (180 deg.): one bearing 1200 p.p.m. of CO_2, over the environmental concentration of 500 ± 100 p.p.m., while the other was just clean filtered air (control *versus* test). Thus, each insect could choose either walking towards one of the two streams or exhibit a non-oriented behaviour, i.e. to walk randomly. Both air-streams were blown over the insects through glass tubes (0.6 cm inner diameter, 14 cm length) placed at 3 cm from the insects at constant velocity (4.2 cm s^{-1}), temperature (25 ± 2°C) and RH (40 ± 5%). The production of CO_2 was achieved as previously described by Barrozo and Lazzari (Barrozo and Lazzari, 2004a). To avoid eventual environmental biases, the position 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 analysed 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 deg. and 360 deg., and r varied between 0 and 1 (0 indicating a non-defined mean direction and 1 indicating a straight path to a given direction). The position of the stimulus-delivered current was conventionally designated as 0 deg., and the control current as 180 deg. The *V*-test (Zar, 1984) was conducted to test if α_m was significantly different from the stimulus direction (0 deg.). Additionally, for an easier visualisation of the data, an orientation index (OI) was calculated by multiplying the cosine of the α_m by r, as $\cos(\alpha_m) \times r$. 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 *versus* 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 ability of the insects to respond to a thermal source, which also served as a feeder. We set up an artificial feeder allowing us to independently observe 10 insects at a time (Bodin et al., 2009). The artificial feeder consisted of 10 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 taped aluminium block ($35 \times 5 \times 1.3$ cm) equipped with a flat electric resistance. A thermostat kept the temperature of the blood at 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 make 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), the tops of which were covered with a fabric mesh, allowing the insects to pierce the Parafilm® and feed from the blood. Each tube contained a piece of filter paper that reached the top of the container, allowing the insects to reach the feeder. Before the test began, each insect was allowed to habituate for 2 min to the experimental situation without stimulation. After this time, the artificial feeder was placed over the containers and the response of the insects was recorded during 15 min. 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.d.) was calculated by the following formula for binary data: s.d.$=[p(1-p)]^{\frac{1}{2}}$. 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 fifth-instar larvae

Fig. 1A represents the response to CO_2 in fifth-instar larvae of *R. prolixus*. A clear variation in the orientation response of the insects to this long-range host cue was observed as a function of the time elapsed since the blood meal. Before taking a meal, insects showed a strong attraction to CO_2 (BF, *V*-test, $P<0.05$), displaying a preferred walking direction towards the stimulus. Then, after feeding, we observed four phases of response. First, during the first two days following feeding (t1 and t2), the insects walked randomly on the sphere showing no orientation preference, i.e. the pathways of the insects were uniformly distributed between 0 deg. and 360 deg. (*V*-test, n.s. in all cases). Second, during the three following days (t3, t4 and t5), the insects showed a behavioural repulsion phase, displaying a preferred walking direction opposite to the CO_2 stimulus (*V*-test, $P<0.05$ in all cases). Third, from t6 to t15, the insects again displayed no orientation preference on the locomotion compensator (*V*-test, n.s. in all cases). Finally, in both cases at t20 and t27, the insects displayed once again a preferred walking direction opposite the CO_2 stimulus (*V*-test, $P<0.05$), showing another behavioural repulsion phase. The comparison of the walking duration among all the animals tested at different times after the blood meal revealed, in all cases, no significant differences, indicating that the activity on the locomotion compensator was similar for all the groups (ANOVA, $P>0.05$, n.s.). The Rao's Spacing test did not reveal axial orientation in any situation, i.e. $P>0.05$, n.s.

Fig. 2A represents the behavioural response of fifth-instar larvae of *R. prolixus* to a short-range stimulation by heat. It represents the proportion of the different behaviours performed by the insects: (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 is expressed as a proportion of the total number of insects tested. Our results showed that approaching and PER are associated, because all of 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 a notable proportion fed (77.5 ± 6.8%). Then, after feeding and during all the experiments, there was a notable decrease in the proportion of insects responding to heat and in their motivation to feed, with the proportion of insects approaching the heat source and displaying PER never exceeding 35%. The maximal 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 random walking phase of the response to CO_2 that follows the repulsion period.

Behavioural response to CO_2 and heat in females

Fig. 1B represents the response of female *R. prolixus* to CO_2. As for larvae, a variation in the orientation response of the insects to
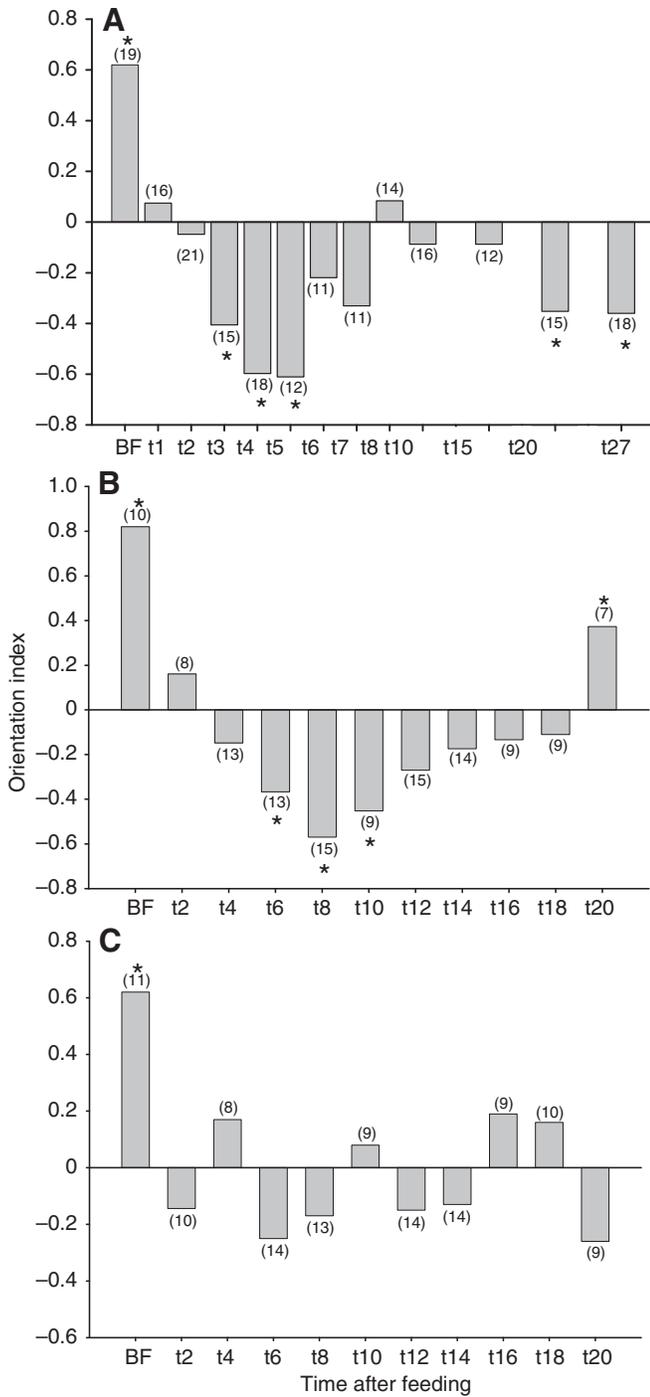
Fig. 1. Orientation response of *R. prolixus* larvae (A), adult females (B) and males (C) to CO_2 at different times after a complete blood meal. Asterisks denote a statistically significant preferred walking direction towards or against the stimulus location (0 deg.) (*V*-test, *P*<0.05). Orientation index varies from –1 (orientation against the stimulus location) to 1 (orientation towards the stimulus location). The number of insects tested is shown between parentheses. BF, before feeding; t*x*, *x* number of days after feeding.
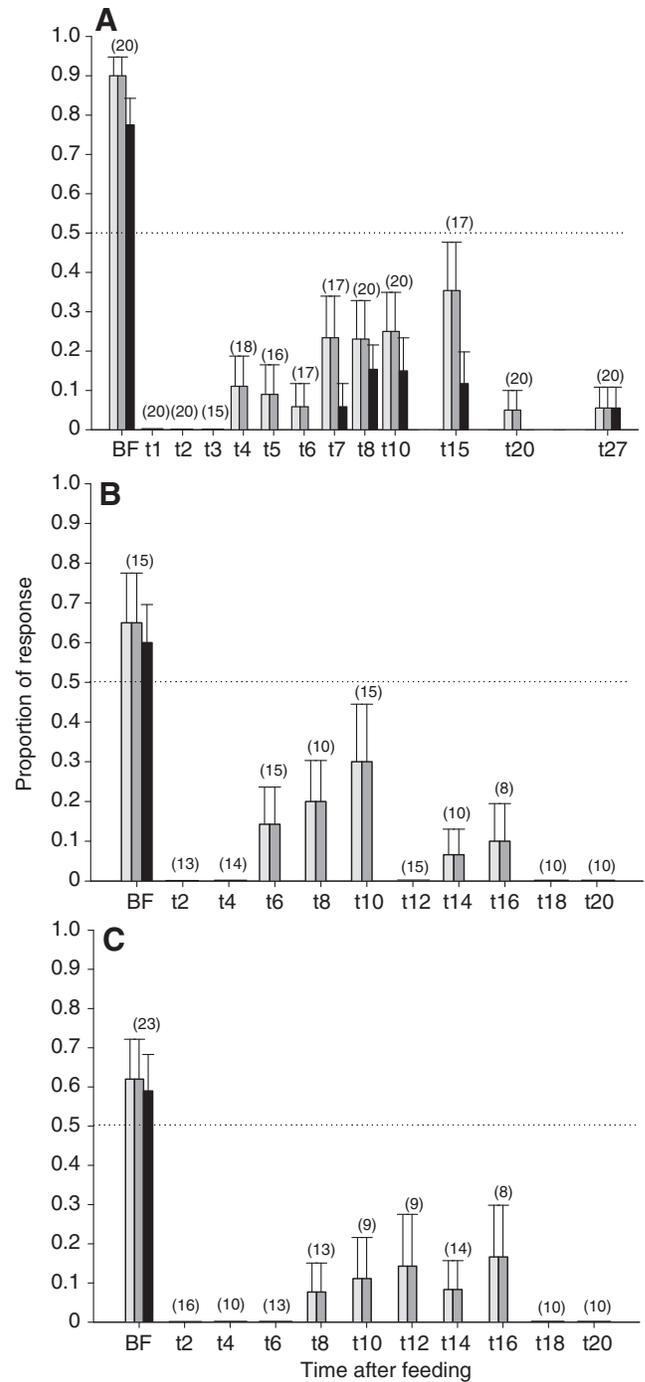
Fig. 2. Behavioural response to heat and the motivation to feed of *R. prolixus* larvae (A), females (B) and males (C) at different times after a complete blood meal. Pale grey bars represent the proportion of insects that approached the heat source, grey bars represent the proportion of insects that performed proboscis extension response (PER) and the black bars represent the proportion of insects that fed. Each bar represents the mean response ± s.d. The dotted line represents the level of 50% of response. The number of insects tested is shown between parentheses. BF, before feeding; t*x*, *x* number of days after feeding.

this cue was observed as a function of the time elapsed since the blood meal. Before taking a meal, insects showed a strong attraction response to CO_2 (*V*-test, *P*<0.05), displaying a preferred walking direction to the stimulus. As for fifth-instar larvae, we observed

different phases of response during the days after feeding. First, during the first four days following feeding (t2 and t4), females showed a behavioural non-responsive phase. The pathways of the insects were uniformly distributed between 0 deg. and 360 deg. (*V*-

test, n.s. in all cases), i.e. they walked randomly on the sphere showing no orientation preference. Second, between t6 and t10, insects showed a behavioural repulsion phase, displaying a preferred walking direction opposite 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, n.s. in all cases), suggesting a behavioural non-responsive 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 need at least 20 days before responding to CO_2 after a blood meal. The comparison of the walking duration among all tested groups revealed no significant differences, indicating that the activity on the locomotion compensator was similar in all groups (ANOVA, n.s.). Rao's Spacing test did not reveal axial orientation in any situation, i.e. *P*>0.05, n.s.

Fig. 2B represents the behavioural response of female *R. prolixus* to heat. As in the case of larvae, all of the insects that approached the thermal source extended their proboscis. Before feeding, we observed a high proportion of insects approaching the heat source and extending their proboscis (65±12.5%), and a notable proportion of insects that fed (60±9.6%). These proportions were statistically different from 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 throughout the experimental period, there was a decrease in the proportion of insects that responded to heat. The proportion of insects approaching the heat source and displaying the PER never exceeded 30%. The maximal 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

Fig. 1C represents the response of male *R. prolixus* to CO_2. As for larvae and females, we observed a variation in the orientation response of the insects 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 we had observed in larvae and females, males showed no orientated response towards CO_2, i.e. neither attraction nor repulsion. The pathways of the insects were uniformly distributed between 0 deg. and 360 deg. (*V*-test, n.s. 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 in all groups (ANOVA, n.s.). Rao's Spacing test did not reveal axial orientation in any case, i.e. *P*>0.05, n.s.

Fig. 2C represents the behavioural response of male *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 that most insects approached the heat source and extended their proboscis (62±10.2%), as well as an important proportion of insects that fed (59±9.3%). These proportions were statistically different from those 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, *P*>0.05 for all behaviours). After the blood meal, there was a decrease in the proportion of insects responding to heat. The proportion of insects approaching the heat source and displaying
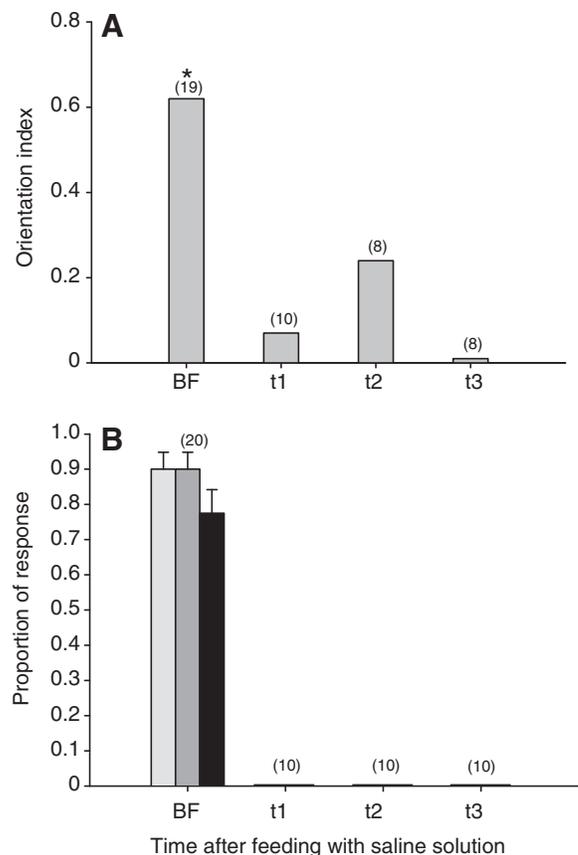

Fig. 3. Behavioural response of *R. prolixus* larvae to different host-emitted cues during three days after feeding with saline solution. (A) Represents the behavioural response to CO_2 before and after feeding on saline, asterisks denote a statistically significant preferred walking direction towards stimulus location (0 deg.) (*V*-test, *P*<0.05). Orientation index varies from –1 (orientation against the stimulus location) to 1 (orientation towards the stimulus location). (B) Represents the behavioural response of the insects to heat and their motivation to feed before and after feeding on saline. Pale grey bars represent the proportion of insects that approached the heat source, grey bars represent the proportion of insects that performed proboscis extension (PER) and black bars represent the proportion of insects that fed. Each bar represents the mean response ± s.d. The number of tested insects is shown between parentheses. BF, before feeding; t*x*, *x* number of days after feeding.

the PER never exceeded 20%. The maximal responses were observed between t8 and t16. As for females, we never observed insects trying to feed from the artificial feeder.

Experiment 2: abdominal distension and host-seeking behaviour

One day after feeding with either blood or saline solution, the mass of the insects increased by a factor of approximately 6.5 (29.7±1.4 mg before feeding and 195.9±3.6 mg after feeding) and stayed constant during the three days that the experiment lasted. No statistical difference between the masses of the two groups of insects was observed (Mann–Whitney, *P*>0.05 in all cases).

Fig. 3A represents the response of fifth-instar larvae of *R. prolixus* to CO_2 before and after feeding with saline solution. The results obtained before feeding correspond to those obtained in experiment 1 (see above). During the first three days after feeding, insects showed no orientation tendency towards the stimulus (*V*-test,

P>0.05 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 in all groups (ANOVA, n.s.). Rao's Spacing test did not reveal axial orientation in any case.

The behavioural response to heat after feeding with saline solution is represented in Fig. 3B. The results before feeding correspond to those obtained in the first experiment (see above). As with CO_2, no response to heat was observed during the first three days after feeding with saline solution. Insects remained immobile in their containers.

Experiment 3: the role of haemolymph-borne factors

Fig. 4A represents the behavioural response to CO_2 of non-fed *R. prolixus* fifth-instar larvae injected with haemolymph from fed fifth-instar insects. Larvae were injected with haemolymph of insects fed the previous day (I1) or four days before (I4) behavioural tests. The results of the control group A (non-fed insects without injections) correspond to those obtained in experiment 1. The two other control groups, B (non-fed insects injected with saline solution) and C (non-fed insects injected with haemolymph from non-fed insects), showed that insects 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 of the insects from group I1 died between 24 h and 48 h after injection. Thus, data could only be obtained for day 1 after injection (I1–t1) and showed that the insects were not attracted to the stimulus. A certain tendency to repulsion from the CO_2 stimulus was observed but it was not statistically significant (*V*-test, n.s.). Results obtained from the group I4 are quite different from I1. All injected insects survived the experimental period and beyond, indicating that an unknown factor in the haemolymph was deleterious rather than the injection itself. In I4–t1, I4–t2 and I4–t3 after injections, insects showed no orientation preference when confronted to the stimulus (*V*-test, n.s. in all cases). The comparison of the walking duration among all of the different groups (I1 and I4) and days revealed no significant differences in all cases, indicating that the activity on the locomotion compensator was similar in all groups (ANOVA, n.s.). The Rao's Spacing test did not reveal axial orientation in any case, i.e. non-significant.

Fig. 4B represents the behavioural response to heat of non-fed *R. prolixus* fifth-instar larvae injected with haemolymph from fed larvae. The results of the control A (before injections) are the same as those obtained in experiment 1 (see above). For the control B (non-fed insects injected with saline solution) and C (non-fed insects injected with haemolymph from non-fed insects), the three behavioural responses (approaching, PER and feeding) did not differ from animals in the control A (Mann–Whitney, n.s. in all cases). As explained before, data from the group I1 were obtained only for 1 day after injection (t1) and showed that the insects were not attracted to heat. Insects did not even move in their containers. Results obtained from the group I4 are very different, showing 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 from 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
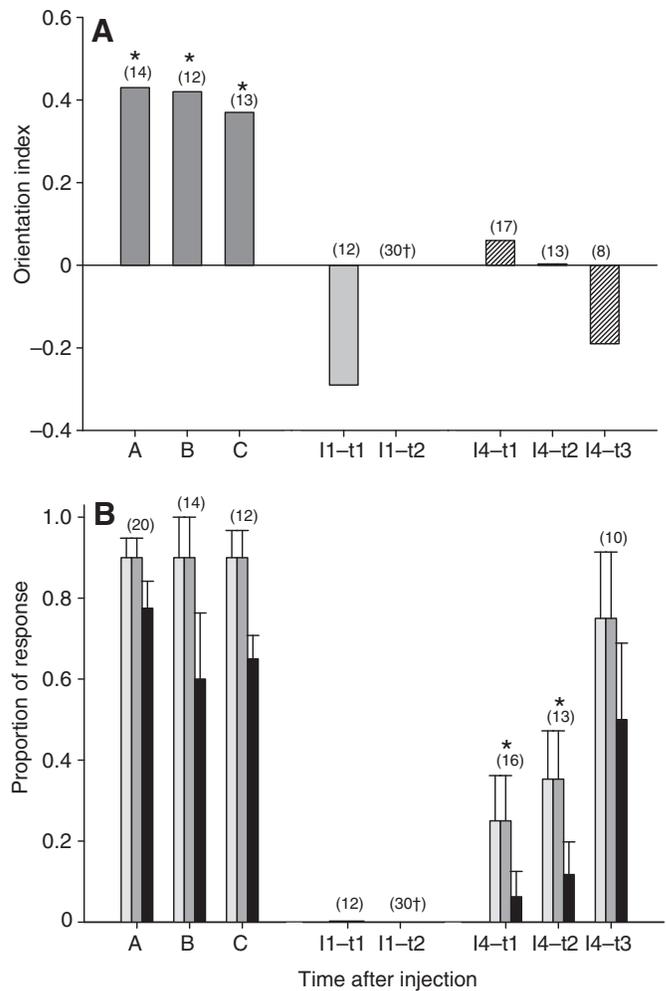

Fig. 4. Behavioural response of non-fed larvae of *R. prolixus* to different host-emitted cues at different times after injection of haemolymph from insects fed one day (I1) or four days before (I4). (A) Represents the behavioural response of the insects to CO_2, asterisks denote a statistically significant preferred walking direction towards the stimulus location (0 deg. (*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 insects to heat and their motivation to feed, pale grey bars represent the proportion of insects that approached the heat source, grey bars represent the proportion of insects that performed proboscis extension (PER) and the black bars represent the proportion of insects that fed. Each bar represents the mean response ± s.d. The asterisk indicates a significant difference in the feeding response (Mann–Whitney *U*-test). The number of tested insects is shown between parentheses. The cross means that all insects died before the day of the experiment. Control groups: A, non-fed insects without injections; B, non-fed insects injected with saline solution; C, non-fed insects injected with haemolymph from non-fed insects. BF, before feeding; t*x*, *x* number of days after injection.

(Mann–Whitney, n.s.). Our results also show that all the insects that approached the thermal source (the feeder) extended their proboscis.

DISCUSSION
Modulation of the responsiveness to host cues and feeding behaviour
Our experiments have conclusively shown that the response of blood-sucking insects to host cues is modulated by their feeding status. This modulation affects the response of *R. prolixus* to cues

of different modalities, as well as the motivation to feed. The oriented response to CO_2 and heat and the motivation to feed varied in all of groups tested (larvae, males and females) as a function of the time elapsed since the last meal.

Starved insects responded to CO_2 and heat and fed, whereas fed insects either remained indifferent or walked away from such stimuli and did not feed. Interestingly, we found 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 has been demonstrated in a haematophagous insect. The theory of spatial orientation mechanisms indicates that a same stimulus might mediate orienting responses of opposite signs (Jander, 1963). Our experiments confirm the use of the same host-associated cue for both approaching and avoiding a host.

Negative responses to CO_2 became evident in *R. prolixus* at different moments or 'physiological contexts'. In larvae, repulsion appeared about 72 h after feeding, lasting for about two days (Fig. 1A, t3–t5) and also close to the ecdysis (Fig. 1A), i.e. at t20 and t27 postfeeding when most larvae of *R. prolixus* are known to moult (Lent and Valderrama, 1977; Rabinovich et al., 1979). In females, repulsion to CO_2 appeared a little later, between t6 and t10 (Fig. 1B), when their motivation to oviposit is 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.

Concerning heat, only a reduction in the motivation of the insects to approach a thermal source was observed. Even though our assay did not allow us to verify repulsion (approach or not approach), we never observed any insect on the upper part of the tube moving down. Larvae, males and females strongly reduced both their responsiveness to heat and their motivation to feed (Fig. 2). In all cases, the response to heat completely disappeared right after the blood meal (i.e. during the repulsion phase to CO_2) and gradually recovered during the following days. It should be noted that approaching and PER were associated in 100% of the cases (i.e. every time the insects approached the heat source, they extended their proboscis), evincing once more the crucial role of heat for triggering biting in triatomines (Flores and Lazzari, 1996). Nevertheless, PER did not necessarily lead to feeding. In contrast to chemical stimulation, which relies on anemotaxis, heat orientation take place in the short range (Lazzari and Núñez, 1989), and the PER is triggered by the close proximity of a heat source (Wigglesworth and Gillett, 1934; Flores and Lazzari, 1996). Therefore, it is not surprising that insects responded trying to bite (i.e. PER) a heat source when they were in contact or almost in contact, even when their motivation to feed repressed them from taking blood.

The physiological mechanisms
In order to explore the physiological basis of the state-dependency of seeking a host and feeding in *R. prolixus*, we performed two further experimental series. The first one was aimed at testing whether the post-feeding behavioural changes were induced by the mechanical distension of the abdomen or by the composition of the food. We therefore fed a group of insects with saline solution enriched with phagostimulants. We observed a strong reduction of both the response to CO_2 and to heat (Fig. 3). However, no repulsive response to CO_2 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 the blood.

We also tested whether the factors responsible for the modulation of the response to host cues are transported by the haemolymph. Starved insects injected with haemolymph from recently fed insects showed a reduced responsiveness to both CO_2 and heat (Fig. 4), reproducing to some extent the effect of a blood meal. The duration of this effect on the receivers could not be measured because insects died within the following 48 h after being injected. Neither change in the response nor death occurred when the starved insects were injected with saline solution or haemolymph from non-fed insects (Fig. 4). The death of insects injected with haemolymph of recently fed insects can be due to changes in the haemolymph associated with the process of feeding. In order to handle blood meals that are several times heavier than their own mass, these insects experience dramatic physiological modifications, including the flow of a big amount of blood components and water through the crop wall to the haemolymph. At the same time, diuretic hormones assure the rapid elimination of a large amount of blood components during the first hours following feeding (Maddrell, 1963). It is likely that injected animals received all of these factors, including some toxic ones that could not be eliminated, because even though diuretic hormones were also transferred, they do not suffice to induce diuresis in starved insects (Gomez and Balderrama, 1986).

In our final experiment we injected starved insects with haemolymph from donors fed four days before. This experiment allowed us to test two things. The first one was to test whether, during the period of repulsion to CO_2, unknown factors in the haemolymph could induce this response in injected insects. The second was to test whether the lethal effect of haemolymph from recently fed insects persisted for several days. When tested for their response, the receivers of this haemolymph remained indifferent concerning both CO_2 and heat (Fig. 4), and no significant repulsion was observed (Fig. 4B). Injected insects did not die after the injection and their response was followed for a long time, revealing a recovery of the response to heat and the persistence of the lack of response to CO_2 for at least three days. These results show that the haemolymph-borne factors are responsible for the reduction of the insect's behavioural response and also that these factors persisted for several days and slowly lose their efficacy. The survival of the receiver insects supports the idea that what caused the death of receivers of haemolymph of recently fed insects was an unknown factor associated with the physiological changes occurring during or immediately after feeding.

The influence of the nutritional state on host-seeking behaviour in haematophagous insects has been analysed in detail in mosquitoes and has been particularly concerned with the interaction between the nutritive and the reproductive states. In female mosquitoes, 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 between a nervous and a humoral mechanism. The distension of the abdomen stimulates abdominal mechanoreceptors ['distension-induced inhibition' (Klowden and Lea, 1979a)]. If the blood meal suffices to trigger 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. Concerning the physiological mechanism, it has been shown in *Aedes aegypti* that the response of lactic acid olfactory receptors is inhibited by humoral factors present in the haemolymph after feeding (Brown et al., 1994). It is worth comparing our present results revealed in *R. prolixus* with those obtained in mosquitoes. In both cases, feeding reduces the insect motivation to

respond to host-associated cues, and humoral factors released during or after feeding seem to be involved. Concerning the modulation of the response, in contrast to our findings in triatomine bugs, no repulsion seems to occur in mosquitoes. In mosquitoes, given that only females feed on blood and that feeding influences the ovarian activity, it is difficult to discriminate between the direct effect of feeding and that of ovarian activation. This does not mean, however, that female reproduction would not influence the response of insects, because as indicated above, females exhibit a delayed repulsion to CO_2 at the time when they are probably ready to lay eggs. Nevertheless, in the case of larvae and males, we have been able to evince the effect of feeding in the absence of other influences, with the exception of larval ecdysis.

In conclusion, we have shown that the response of haematophagous insects to their host is modulated by their physiological state. Feeding, moult and oviposition (all three) seem to affect the response to both chemical and physical cues associated with vertebrate hosts. This modulation seems to be triggered by both the distension of the abdomen and chemical components of the blood meal. Only a blood meal is able to induce repulsion and could be directly or indirectly responsible for 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 these factors.

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