TEMPORAL MODULATION AND ADAPTIVE CONTROL OF THE BEHAVIOURAL RESPONSE TO ODOURS IN *RHODNIUS PROLIXUS*

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ABSTRACT

It has been demonstrated in several insect species that a circadian clock makes the whole of antennal chemoreceptors more sensitive during a particular temporal window every day. This assessment raises the question about how insects exhibiting bimodal activity handle their sensitivity to odours which are relevant at different moments of the day. To shed some light on this problem, we studied in *Rhodnius prolixu*s the daily dynamics of their responsiveness to CO_2 (host-associated cue) and aggregation cues (refuge-associated), which are relevant at dusk and dawn, respectively. We analysed: 1) whether a temporal modulation of the responsiveness to odours does exist in *R. prolixus*, 2) if this modulation is a general one or it is specific for each type of volatile, and 3) if it is controlled by exogenous or endogenous mechanisms. We found that the responsiveness to CO_2 only occurs at dusk and that to assembling odours is restricted to dawn. Experiments under free-running conditions revealed that only the responsiveness to CO_2 is controlled by a circadian clock, but not that to assembling signals. Thus, by combining endogenous and exogenous mechanisms, sensitivities to different odours are adjusted according to their associated behavioural context and moment of the day.

Key words: olfaction, CO_2, aggregation pheromone, chronobiology.

INTRODUCTION

In nature, animals are continuously exposed to a variety of physical and chemical cues perceived by different sensory systems (olfactory, visual, etc.). Many of these cues play a relevant role in animals' survival, helping them to find food, mates, refuges and other resources, to avoid predators, and also mediating communication with con-specifics. In particular, the detection of odours represents a crucial task for animals, provided the diversity, dispersion ability and informational value of volatile molecules. In many cases the presence of certain olfactory cues is limited to a certain time of the day. For instance, female moths release sexual pheromones at particular temporal windows every day, during which males become active (Baker and Cardé, 1979). In other cases, odours can be more or less continuously present, but the response occurs during a given daytime, as in the case of mosquitoes, which search for food when their hosts are resting (Brady and Crump, 1978).

Daily rhythms of olfactory responses have been repeatedly reported at both, the sensory and the behavioural levels (see Saunders *et al.*, 2002). In moths, for instance, numerous examples from studies on female release and male behavioural response to the pheromone indicate that the timing of this behaviour is governed by a circadian mechanism (Baker and Cardé, 1979; Cardé and Webster, 1981; Castrovillo and Cardé, 1979; Haynes and Birch, 1984; Linn *et al.*, 1996; Rosén *et al.*, 2003). In the cockroach, *Periplaneta americana*, males maintained in a light/dark cycle show a daily rhythm in responsiveness to the female sex pheromone with a peak of sensitivity during the early night hours (Hawkins and Rust, 1977; Zhukovskaya, 1995). Daily rhythms of behavioural response to different sensory stimuli have also been reported in tsetse flies. When stimulated with host-associated odours, flies show a daily bimodal pattern of responsiveness, with peaks at dawn and dusk (Brady, 1975). Furthermore, the chemoreceptors located on the antennae of *Glossina morsitans* and *G. fuscipes* seem to respond to host-related stimuli with a daily rhythm in synchrony with much of its behavioural repertoire (Van der Goes van Naters *et al.*, 1998). In another blood-sucking insect, the bug *Triatoma infestans*, it has been shown that the rhythm of behavioural responsiveness to host odours is under the control of an endogenous circadian clock (Barrozo *et al.*, 2004). Rhythms of sensory sensitivity have been also well established in *Drosophila melanogaster* (Krishnan *et al.*, 1999) and in *Leucophaea maderae* (Page and Koelling, 2003). In both species, a general modulation of chemosensitivity has been postulated, i.e., the animal's sensitivity does not depend on odour identity. Paradoxically, in both cases, maximal

sensitivity to odours does not occur at the time during which insects are behaviourally active (i.e., when a given odour becomes biologically relevant), but during their resting periods.

These findings raise some relevant chronobiological questions about how insects, many of which exhibit different discrete activity periods (i.e., bimodal activity), modulate their responsiveness to different odours which are relevant at different moments of the day. The first question is whether or not the behavioural responsiveness to odours is modulated. Second, if so, is it maximal during just one or both activity periods? Third, if different odours are associated to different activity periods, are specific sensitivities modulated together or selectively? Finally, if modulation occurs, is it controlled by an endogenous mechanism or is it under the direct influence of exogenous mechanisms?

Trying to shed some light on these questions, we analysed the chronobiological basis of the responsiveness to odours in a blood-sucking bug *Rhodnius prolixus* (Heteroptera: Reduviidae). Our choice lies on several biological characteristics of these insects, which make them particularly adequate for this kind of study. Triatomine insects exhibit a marked circadian organisation of many activities, *e.g.* locomotor activity, egg hatching, ecdysis, oviposition, thermopreference, visual sensitivity (Ampleford and Steel, 1982; Constantinou, 1984; Lazzari, 1991, 1992; Minoli and Lazzari, 2003; Reisenman *et al.*, 1998, 2002). These different physiological and behavioural processes take place at either one or two temporal windows, one at the beginning and the other at the end of the scotophase. They exhibit a maximal motivation to feed at dusk (Lorenzo and Lazzari, 1998), and during this time they left their shelters and seek for a blood meal guided by carbon dioxide, a main olfactory cue in host searching (Barrozo *et al.*, 2004). At dawn, the bugs return to their shelters using the aggregation pheromone present in their excrements as chemical landmarks to guide them back to refuges (Lorenzo Figueiras *et al.*, 1994). For this, they deposit faecal drops at the entrance of their shelters (Lorenzo and Lazzari, 1996).

Given the bimodal activity pattern and the use of different olfactory signals guiding specific behaviours at different moments of the day (i.e. host finding at dusk and refuge search at dawn), triatomine insects constitute an appropriate model to analyse how olfactory sensitivities are organised in time and how are they controlled. To investigate these questions, we analysed the orientation response of *R. prolixus* to carbon dioxide and to the faeces, the source of the aggregation pheromone during different moments of the day under both, temporally synchronized and free-running conditions.

MATERIAL AND METHODS

Insects

Fifth-instar larvae of *R. prolixus* were used throughout the experiments. They were reared in our laboratory at 27°C, 50-70% RH, and maintained under an artificial 12/12h light/dark illumination regime. Insects were fed weekly with sheep heparinised blood, using an artificial feeder (Núñez and Lazzari, 1990). After their ecdysis to the fifth-instar, insects were starved for 20-30 days and then used for the experiments.

Illumination regimes and recording times

Bugs were entrained for 3 days in a 12:12 L/D regime and then kept another 3 days either under the same L/D regime or in constant darkness (D/D). The seventh day, the orientation response of *R. prolixus* to CO_2 (host-related odour) or to the aggregation pheromone was tested at different times (see below).

The behaviour of bugs was studied in different temporal windows along the day. The light onset was considered as Zt (*Zeitgeber* time)= 00:00h. In L/D conditions, four experimental time intervals were defined, i.e. *early* (Zt= 00:00 to 02:00h) and *middle* (Zt= 04:00 to 06:00h) photophase, and *early* (Zt= 14:00 to 16:00h) and *late* (Zt= 22:00 to 00:00h) scotophase. In D/D conditions, the 2 intervals corresponding to the *early* and *middle* (subjective) photophase were the same as for animals under L/D. However, when the response to CO_2 was tested, two intervals were considered for the *early* (subjective) scotophase, i.e. Zt= 12:00 to 14:00h and Zt= 14:00 to 16:00h. The *late* scotophase remained the same as for the L/D condition. When the response to the aggregation pheromone was studied, the interval *early* (subjective) photophase remained equal to the L/D condition, however the *late* (subjective) scotophase was divided in two recording intervals i.e. Zt= 20:00 to 22:00h and 22:00 to 00:00h. The time of both, the *early* and *late* (subjective) scotophase test periods, were adjusted to compensate for the expected shortening of the free running period under D/D conditions (Lazzari, 1992) and, to confirm that the absence of a response under D/D was not due to a more important shift of the rhythm under free-running conditions

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Recording of walking pathways

A locomotion compensator was used to analyze the orientation behaviour of *R. prolixus* (Barrozo and Lazzari, 2004). Briefly, it consists of a hollow Styrofoam sphere (9.7 cm diameter, 2.5 g weight) suspended by a vertical air-stream generated by an air-pump. The bugs were maintained at the apex of the sphere attached by their dorsal abdomen to a freely rotating stiff steel wire, using double-sided sticky tape. The animals started to normally walk when contacting the surface of the sphere, thus displacing it with their legs. Bugs on the locomotion compensator could walk and rotate freely, changing its direction without modifying its relative distance to the stimulus location, i.e. in an open-loop condition for translation, but closed-loop for rotation. The sphere movement was detected by an optic sensor and the signal fed to a computer every 200 ms as *x*-, *y*-co-ordinates. The walking paths of the bugs were reconstructed and analyzed in their spatio-temporal components as previously described (Barrozo and Lazzari, 2004).

All the experiments were performed in an experimental room (25±2°C) under conditions of functional darkness for the insects (see below). Before starting an experiment the insects remained in still air on the locomotion compensator for 120s to habituate them to the experimental situation, after which the airstreams (control and stimulus) were presented during 180s. The assays were monitored from the outside of the experimental room using an infrared-sensitive camera with its own illumination system composed by IR-emitting LEDs (900 nm). It has been previously shown that this light is not perceived by the bugs (Reisenman *et al.*, 1998). Each individual insect was tested only once and discarded afterwards.

Stimulus delivery

Insects were confronted to two simultaneous opposite (180°) horizontal charcoal-filtered airstreams, one bearing the test stimulus while the other kept clean (test versus control) (Barrozo and Lazzari, 2004). Insects either walked towards one of the two streams or did not exhibit an odour-orientated behaviour, i.e. walked randomly when both currents had identical composition. The two airstreams, being at constant temperature (25±2°C) and relative humidity (40±5%), reached the insects through glass tubes (0.6 cm inner diameter, 14

cm length) whose were 3 cm from the insect. The velocity of the air measured at the exit of the glass tube was 4.2 $cm.s^{-1}$.

CO_2 was chemically generated ($Na_2CO_3 + H_2SO_4 \rightarrow NA_2SO_4 + H_2O + CO_2$). For this, a synchronous-motor driven syringe injected a 0.3 M solution of Na_2CO_3 at a rate of 0.08 $ml.min^{-1}$ into a glass bottle containing 100 ml of 1 M H_2SO_4. The mixture was continuously stirred to ensure a stable CO_2 production. An airstream (97.5 $ml.min^{-1}$) passed over the surface of the reaction mixture and thus loaded with CO_2, resulting in an increase of 1200 ppm of CO_2 over the ambient level (500±30 ppm). To measure the CO_2 concentration in the air, a non-dispersive infrared sensor was used (PP Systems, model EGM-3, range 0-5000 ppm, and accuracy 0.5 %). The control airstream, carrying ambient levels of CO_2 (500±30 ppm) was produced by an identical arrangement, but lacking from the injection of Na_2CO_3 (for further details see Barrozo and Lazzari, 2004). This arrangement made possible to keep in both currents an identical air speed, relative humidity and temperature.

Faeces from fed insects, the source of the aggregation pheromone (Lorenzo Figueiras *et al*., 1994), were collected on filter paper (Whatman paper N°1, 1 x 3 cm) by gently pressing their abdomen with forceps. Insects did not contact the filter paper to avoid contamination with cuticular cues. Collected faeces were stored in a chamber at 28°C and 50-70% humidity and used after 4 days. It has been previously shown that the aggregation signal remains attractive from 3h to 10 days (Lorenzo Figueiras and Lazzari, 2000). A filter paper impregnated with faeces was placed in one of the stimulation tubes, while in the opposite an identical but clean filter paper served as control. Every 5 insects tested, the paper impregnated with faeces was substituted by a new one, to avoid differences in the concentration of the aggregation pheromone between the insects along the assays. In all cases, to avoid eventual environment biases, the sides of the test and the control currents were changed between assays.

Data analysis

The pathways followed by the insects were analysed by means of circular statistics (Batschelet, 1965; Zar, 1984; Fisher, 1993), since standard "lineal" statistical analysis is not applicable. 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 relative position of the stimulus delivery current was conventionally designated as 0° and the control current as 180°. Whereas

α extends from 0 to 360°, r varies between 0 and 1 (0 indicating a non-defined mean direction and 1 a straight path to a given direction). The V-test (Zar, 1984) was carried out to assess whether the mean angle calculated from the sample was statistically distant from the stimulus direction (0°). Additionally, for an easier visualization of the data, an orientation index was calculated, multiplying the cosine of the mean angle (α_m) by the length of the mean resultant vector (r), as cos (α_m) × r. The orientation index varies between -1 and 1 (-1 indicates orientation away from the stimulus and 1 orientation towards the stimulus location). 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).

RESULTS

Daily modulation of the oriented response to odours

R. prolixus larvae showed a daily variation in their orientation response to both, CO_2 and aggregation pheromones (Fig. 1). In both cases a preferred walking direction was observed at only one time of the day. Insects oriented towards the CO_2 only during the *early* scotophase, i.e. between the second and the third hour after lights-off (Fig. 1A, OI= 0.35, *V*-test, $u_{(21)}$= 2.3, p= 0.012). In the other intervals tested (i.e. *early* and *middle* photophase and *late* scotophase), the pathways of the insects were uniformly distributed between 0° and 360°, i.e. they showed no orientation tendency, walking randomly on the sphere (*V*-test not significant in all cases). The response to the aggregation pheromone was observed in a different temporal window as compared with the response to CO_2 (Fig. 1B). The bugs were attracted by the pheromone during the first two hours of daylight, i.e. the *early* photophase (OI= 0.38, *V*-test, $u_{(21)}$= 2.5, p= 0.006) but any oriented response was observed neither during the *middle* photophase, nor throughout the scotophase (*V*-test, p> 0.05 in all cases).

When the data of each experimental group were submitted to the Rao's spacing test some additional differences between groups appeared. The group of bugs tested at the end of the scotophase for their response to carbon dioxide evinced an axial distribution of their preferred orientation direction (p< 0.01); it means, bugs walked either towards or against the air-stream loaded with the stimulus. The same result was obtained for the group tested for their response to the odour of faeces at the beginning of the night (p< 0.01). This kind of

response was not observed for the rest of the temporal windows tested using the two stimuli ($p > 0.05$).

Endogenous vs. exogenous control of the response

Figure 2 depicts the orientation response of *R. prolixus* larvae maintained under constant darkness, to both CO_2 and the source of aggregation pheromones. Insects displayed a preferred walking direction to CO_2 during the first two hours of the *early* subjective scotophase (Fig. 2A) (OI= 0.37, V-test, $u_{(21)}= 2.4$, $p= 0.008$). The orientation towards CO_2 occurred about one hour earlier than in the group kept under the L/D regime, confirming the expected shortening in the period of the rhythm under free-running conditions. Like in L/D conditions, insects walked randomly on the locomotion compensator in the other intervals tested (*V*-test, all cases not significant). In contrast, no changes in the responsiveness towards the aggregation pheromone were observed for insects kept under D/D conditions (Fig. 2B), and the observed response during the *early* photophase in L/D disappeared.

When the data of each experimental group were submitted to the Rao's spacing test no difference among groups was observed, neither as a function of the stimulus (CO_2 or faeces), nor comparing temporal windows.

Walking activity

The comparison of the duration of walking among the different temporal windows, corresponding to each stimulus, revealed, in both cases, no significant differences, indicating that the activity on the locomotion compensator was similar for all the groups (ANOVA, n.s.).

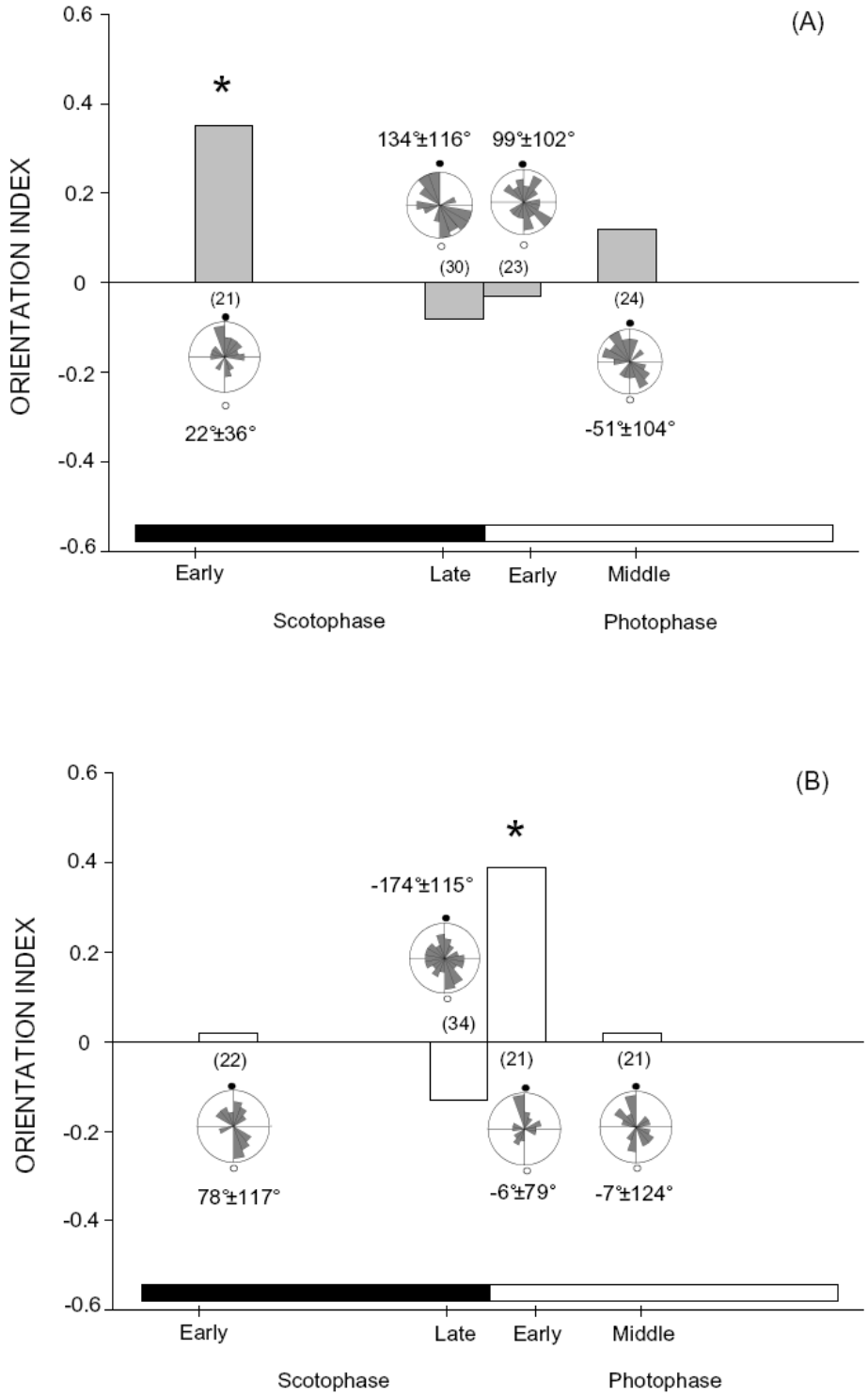

Figure 1. Orientation response of *R. prolixus* larvae to airstreams loaded with 1200 ppm of carbon dioxide above the background (500±30 ppm) (A) or aggregation pheromones (B), at different moments of the day, i.e. early and late scotophase and early and middle photophase. Asterisks denote a statistically significant preferred direction around 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 circular histograms (rose diagrams) represent the frequency of the angles displayed by the animals, which is proportional to the area of the wedge (bar width of 10°). Open dots indicate control airstreams position and filled dots denote test airstream location. Number of insects tested is shown in brackets. The mean vector angle ± the circular mean deviation are indicated.

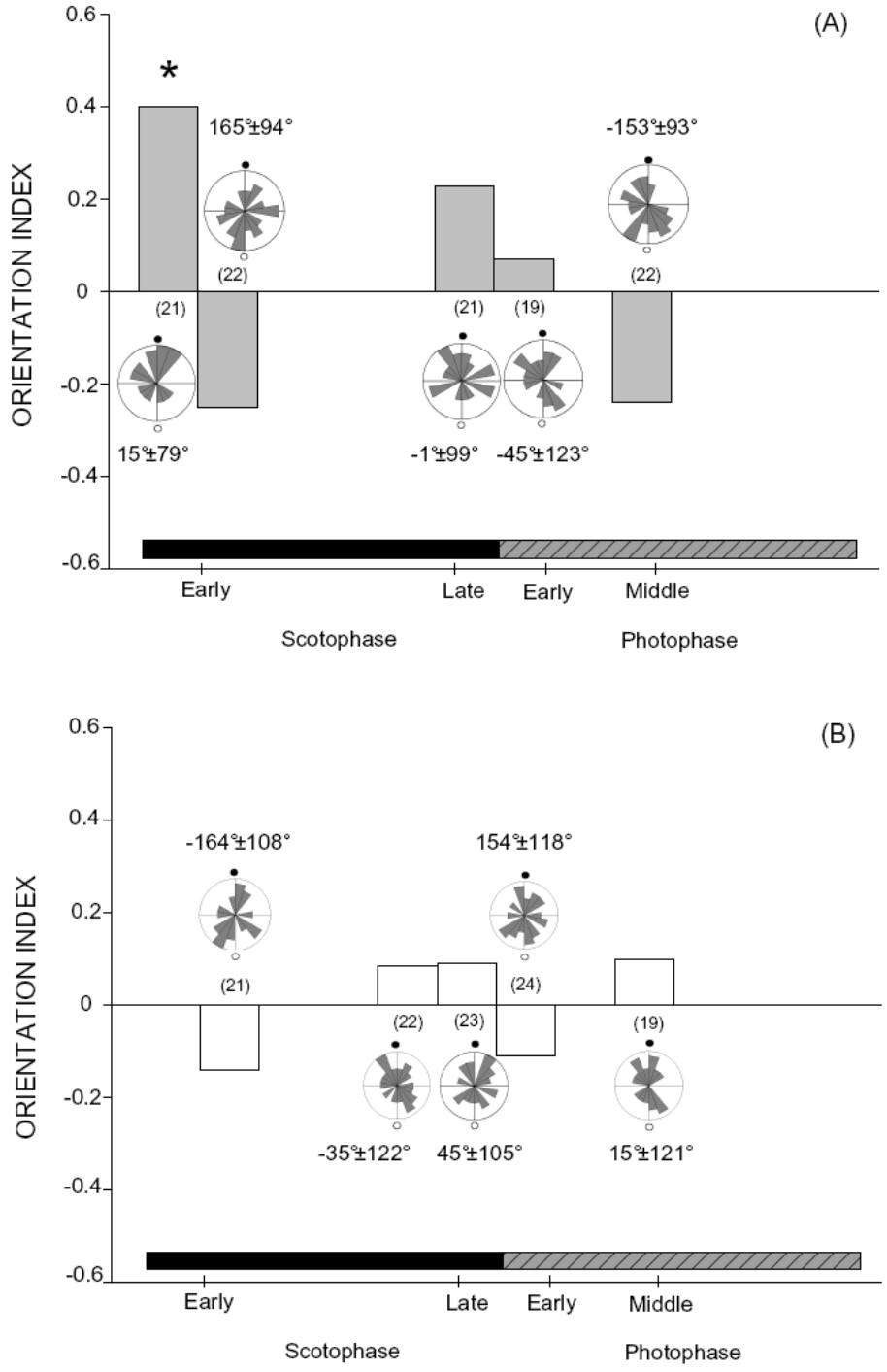

Figure 2. Orientation response to CO_2 (A) and aggregation pheromones (B) of *R. prolixus* larvae maintained under constant darkness (i.e., free-running). Bugs were entrained for 3 days in L/D cycles (12h:12h) and then transferred to D/D conditions for three more days. Asterisks denote a significant mean walking direction around 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 circular histograms (rose diagrams) represent the frequency of the angles displayed by the animals, which is proportional to the area of the wedge (bar width of 10°). Open dots indicate control airstreams position and filled dots denote test airstream location. Number of insects tested is shown in brackets. The mean vector angle ± the circular mean deviation are indicated.

DISCUSSION

The expression of odour sensitivity rhythms in insects

Several studies at the peripheral sensory level revealed a daily rhythm of sensory sensitivity in insects. This modulation has been verified to occur in the olfactory and in the visual system (Reisenman *et al.*, 1998; Krishnan *et al.*, 1999; Reisenman *et al.*, 2002; Page and Koelling, 2003). Nevertheless, in the case of olfactory reception, paradoxically, in the few species studied up to now, insect chemoreceptors are consistently more sensitive during resting periods than when they are active (Krishnan *et al.*, 1999; Page and Koelling, 2003; Merlin *et al.*, 2007). In *Drosophila*, it has been proposed that this could also be the case for the behavioural responsiveness (Zhuo *et al.*, 2005), but again, this appears as quite paradoxical. Different hypotheses have been raised to explain this paradox, being the most accepted that insects need to be more sensitive to signals appearing during moments where their reactivity threshold is the lowest. It is argued that a higher olfactory sensitivity would be more adaptive for predator avoidance or opportunistic feeding at a time when the animals are normally asleep and another is that higher sensory sensitivity at resting is more likely to arouse the "sleeping brain" (Merritt, 2007). This hypothesis seems quite sound concerning food odours. Nevertheless, the same inconsistency between sensory sensitivity and behaviour has been recently observed in moths, concerning pheromone reception (Merlin *et al.*, 2007). This case is less easy to understand in adaptive terms, since moths use sexual communication in a highly synchronous fashion. Both, the release of sexual pheromones by the emitter and the search for the source by the receptor sex are finely tuned to be accomplished during the same temporal window (Saunders *et al.*, 2002). In the case of sympatric species using similar pheromonal blends, the temporal allocation of the sexual call even constitutes a reproductive barrier (Cardé *et al.*, 1977). Thus, to react to the right pheromonal odour, when it is present in the environment at a moment that is not the right one for the species represents a waste of effort and a risk of being predated, without a reproductive success assured. In other cases, however, when olfactory behaviour is analysed, this paradox is not present, being insects more responsive to odours at the right time to look for food, for mate or even in relation with olfactory learning (Decker *et al*., 2007).

Thinking in terms of natural selection, it seems more adaptive that maximal responsiveness occurs during active periods and moreover, when a given odour becomes biologically relevant. This is what we have observed in *R. prolixus*, where maximal olfactory

responsiveness to different odours coincides with the precise temporal windows at which the behaviour is known to occur. In their natural habitat, triatomine bugs are permanently exposed to both host odours and long-persistence assembling pheromones. Nevertheless, we found that they respond to one or the other at a particular time of the day. Bugs spend daylight hours hidden in shelters. At dusk, they are attracted by host odours like CO_2 (Fig. 1A), at the time the insects leave their refuges to search for a blood meal. In contrast, insects are only attracted to the aggregation pheromone at dawn; even when host odours are also present (Fig. 1B). This temporal window corresponds to the moment at which bugs return to their refuges in order to remain hidden to predators. Therefore, the time at which the maximal behavioural responses to a given odour occurs, matches the behavioural context to which that odour is associated with.

General vs. selective modulation of olfactory sensitivity

In their natural environment, animals are confronted to different stimuli, whose presence or behavioural relevance may change along the day. For example, two behaviourally-relevant odours, A and B, could be each associated to two behavioural contexts allocated at different temporal windows. On the other hand, as indicate above enough evidence has been gathered showing that odour sensitivity does not remain constant along the day. Theoretically, two types of variation in chemosensitivity can be postulated. In the case of a *general* modulation of the responsiveness to chemical cues, the animals would exhibit the highest sensitivity to all odorants at a given daytime, thus remaining the relative sensitivity to A and B the same at any moment of the day. This is the kind of variation that has been revealed, for example, in the sensitivity of the antennae in *Drosophila* flies and *Leucophaea* cockroaches (Krishnan *et al.*, 1999; Page and Koelling, 2003). Conversely, if the response to odours is modulated in a *selective* fashion, the response to odours would be maximal to A only at the time when A is behaviourally relevant, but not to B, even if it is also present in the environment, and vice-versa. In other words, the animals would express a maximal sensitivity for each odorant at the time that the odour is present and behaviourally relevant. This kind of modulation has not been previously described or postulated in any insect species, even though it would be much more adaptive to modulate differentially the responsiveness to each odour according to a behavioural context, rather than to become more or less generally sensitive, i.e., increase their responsiveness to any given odour. Thus, insects could keep a maximal sensitivity to a given odour, without responding to other odours that albeit present in the environment, are relevant in a different behavioural and/or temporal context.

Rhodnius prolixus revealed as a good model to test general vs. specific modulation of responsiveness to odours, because its bimodal daily activity and since as indicated this species uses different chemical cues at different moments of the day. Our experiments evinced that the responsiveness to odours is modulated in this species in a specific fashion, being the insect behaviourally more sensitive to the odour corresponding to a given behavioural context only during the temporal window when such behaviour takes place, i.e. food-search at dusk and return to the refuge at dawn.

An interesting but puzzling result of our study is that insects responded to both odours at dusk and dawn either unimodaly (i.e., one preferred direction) or in an apparent bimodal way (i.e. towards and against). So, whereas the orientation towards a CO_2 source is maximal only at dusk, they did remain indifferent most, but not all, the rest of the time. The Rao's spacing test revealed that at dawn they seem to have responded in opposite directions; i.e., approaching and avoiding the odour source. Exactly the same result was obtained when bugs were tested against faeces odours, but inversing the temporal windows. This suggests, either that the sensitivity to one odour is not "switched-off" outside the specific window when this odour is behaviourally relevant, or that the anemotactic behaviour is not uniform along the day. To speculate about the exact mechanism, more data seem to be necessary.

Endogenous and exogenous control of the response to odours

Increasing amount of evidence supports the fact that the odour sensitivity in insects changes rhythmically, and may be endogenously controlled by circadian clocks (Krishnan *et al.,* 1999; Page and Koelling, 2003; Barrozo *et al.,* 2004). This temporal modulation has been demonstrated at the sensory level, expressed as a temporal change in the sensitivity of chemosensitive cells (Van der Goes van Naters *et al.,* 1998; Krishnan *et al.,* 1999; Tanoue *et al.,* 2004) and also at the behavioural level as the responsiveness of the insects to odours (Brady, 1975; Barrozo *et al.,* 2004).

Our results indicate that the rhythm of responsiveness of *R. prolixus* to CO_2 is under the control of an endogenous oscillator (Fig. 2A). The persistence of the rhythm of responsiveness to odours under constant darkness demonstrates that it is self-sustained and therefore truly circadian. As can be expected for a nocturnal animal (Aschoff, 1989) and as observed for other circadian rhythms already described in this species, the period of the rhythm under D/D is shorter than the period of the rhythm under L/D conditions. Our results are also in agreement with previous data on a related species of haematophagous bug

Triatoma infestans, which responsiveness to CO_2 is endogenously controlled and limited to a narrow temporal window at the beginning of the night (Barrozo *et al*., 2004). Contrarily to the responsiveness to CO_2, the olfactory response to the aggregation pheromone is not under the control of an endogenous circadian clock (since it disappears under constant darkness) but is under the direct influence of exogenous variables (e.g. the environmental light cycle).

In summary, the relationship between sensory sensitivity and behavioural response to stimuli is more complex than believed and deserves further work to be fully understood. Actually, before circadian rhythms of olfactory sensitivity were described by the first time in insects, Blaney *et al*. (1986) already pointed out concerning sensitivity variations in general that "…correlations between receptor changes and behavioural changes do not necessarily indicate direct relationships".

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CHAPITRE 2

STATE DEPENDENCE OF HOST ORIENTATION IN *RHODNIUS PROLIXUS*: THE POST-ECDYSIS TIME

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Soumis

SUMMARY

The source of blood of most haematophagous insects plays at the same time the double role of host and predator. Thus, the insect motivation to feed should be modulated in trigger feeding behaviour only when necessary and occurring as fast as possible. From an epidemiological point of view, this modulation has an impact on the feeding frequency of disease vectors and, as a consequence, on the transmission of parasites. At present, not much data are available on the influence of the physiological state on the motivation to feed, and mostly limited to a few mosquito species. We analyzed the motivation to feed in *Rhodnius prolixus* after ecdysis, by testing the response of larvae to a blood source, and long- (CO_2) and short-range (heat) orientation cues associated to their vertebrate hosts. Our experiments demonstrated that during the first days following the ecdysis insects do not respond to any stimuli. The ability to follow chemical and physical cues increases either gradually (heat) or step-wise (CO_2) with post-ecdysis time. Insects feed as soon as seven days after ecdysis but showed the stronger feeding motivation at day 10^{th} after the ecdysis. The reasons for the "maturation period" in feeding behaviour of *R. prolixus* are discussed.

Key words: modulation, orientation, heat, odours, Chagas disease.

INTRODUCTION

In nature, haematophagous insects use different types of signals from their environment in order to communicate or to search for food or protected resting places. Because the blood within a host is not detectable from a distance, these insects have evolved the ability to detect host-emitted cues. For instance, they associate metabolic by-products of vertebrates with the presence of blood and use them to find a potential host. Many of these kairomones in several blood-sucking species have been identified, as well as their roles in long- and short-range attraction. CO_2 is the most universal cue mediating long-range attraction in blood-sucking insects, with changes in its concentration being in many cases mediating oriented searching behaviour (e.g. Barrozo and Lazzari, 2004a). In addition to chemical cues, haematophagous insects also use physical signals for the location of a host like heat and humidity (Lazzari and Núñez, 1989; Barrozo *et al.*, 2003; Lehane, 2005). Host-seeking behaviour is further controlled by endogenous mechanisms such as circadian clocks, with insects actively searching for blood when their hosts are asleep (e.g., Barrozo *et al.*, 2004; Bodin *et al.*, 2008). Moreover, host-seeking behaviour should be regulated in such a way that insects search a host only when they are physiologically capable to obtain and handle a blood meal.

Factors affecting the motivation to feed have been analyzed only in a few species. Up to now, all the studies focusing on the impact of the physiological state on the host-seeking behaviour of blood-sucking insects were conducted in holometabolous insects that are haematophagous only in the adult stage, like mosquitoes (Davis, 1984a). Hemimetabolous blood-sucking insects, in contrast, are obligatory haematophagous during their whole life, i.e., through all the developmental stages. Thus, hemimetabolous blood-sucking insects can acquire pathogen parasites early in life and hence transmit them each time they subsequently feed, i.e. for many months. It is largely accepted that the frequency of feeding is an important factor determining the competence of disease vector insects for transmitting pathogens. This frequency is determined by the insect motivation to respond to host cues, which depends, in turn, on the physiological state. In hemimetabolous insects each moult is triggered by the nutritional stage of the individual and, conversely, moulting should itself affect the insect's feeding ability.

Triatomines are hemimetabolous insects and obligatory haematophagous during their whole life and thus constitute an appropriate model to study how the motivation to feed

changes throughout development. Many triatomine species are vectors of the parasite *Trypanosoma cruzi*, the causative agent of Chagas Disease, one of the most important sanitary problems in Latin America (WHO, 2002). Chagas Disease vectors feed mainly on humans and on domestic animals. During the day these insects display little activity and are usually found in a quiescent state or "akinesis" inside refuges. During the night they display most of their activity, e.g. they search for food, mate, oviposition sites, etc. (Lazzari, 1992; Lorenzo and Lazzari, 1996). Their locomotion activity is divided in two endogenously controlled temporal windows: one at dusk and another at dawn (Lazzari, 1992). These two peaks of activity are respectively associated with host seeking (dusk) and refuge search (dawn), (Lazzari, 1992; Lorenzo and Lazzari, 1998). Heat and carbon dioxide emitted by warm-blooded animals are among the most important cues guiding triatomines to their food source (Wigglesworth and Gillett, 1934; Lazzari and Núñez, 1989; Flores and Lazzari, 1996; Barrozo *et al.*, 2004). Up to date, several studies have focused on the identification of the chemical cues implicated in host location (e.g. Guerenstein and Guerin, 2001), but no data are available about the physiological modulation of the host-seeking behaviour, with the exception of the influence of circadian clocks on the response to host odours (Barrozo *et al.*, 2004; Bodin *et al.*, 2008).

We investigated the effect of endogenous factors on the feeding behaviour of the blood-sucking bug *Rhodnius prolixus* (Heteroptera: Reduviidae), namely the impact of the physiological state on the responsiveness to different host cues. Here we present a quantitative analysis of the behavioural response of *R. prolixus* larvae to two main host cues, carbon dioxide and heat, at different times after the ecdysis, as well as their motivation to feed.

MATERIAL AND METHODS

Insects

Fifth-instar larvae of *R. prolixus* were used throughout the experiments. Larvae were reared in our laboratory under a 12/12 h light/dark illumination regime, at 28°C and 60-70% of relative humidity (RH). Insects were fed weekly on sheep heparinised blood, using an artificial feeder (Núñez and Lazzari, 1990) until their ecdysis to the fifth-instar. Since insects hatch at the end of the night (Ampleford and Steel, 1982), we collected fifth-instar larvae (L5)

every morning (i.e., some hours after their ecdysis). Newly emerged bugs were recognizable by their characteristic pale-pink colour. Bugs were individualised and starved until the assays.

Bioassay protocol

We tested the insect's attractiveness to CO_2 and heat, as well as their motivation to feed at different times post-ecdysis. All the assays were conducted in a room maintained at 25 ± 2°C; 40-60% RH, 500 ± 100 ppm of CO_2 and under infrared illumination, i.e. under functional darkness for the insects (Reisenman *et al.*, 1998). The experiments were carried out only during the first hours of the scotophase, which corresponds to the time of the day during which insects leave their shelters to search for food (Lazzari, 1992; Lorenzo and Lazzari, 1998). The attractiveness to CO_2 in triatomines is also limited to this temporal window (Barrozo *et al.*, 2004; Bodin *et al.*, 2008). Each individual was tested only once between the first (T0, the first night after ecdysis) and the 15^{th} day (T15) after ecdysis and discarded afterwards. A positive control of highly motivated bugs was tested using insects starved during 60 days after ecdysis (T60).

Response to CO_2

A locomotion compensator was used to analyze the walking behaviour of *R. prolixus* in response to CO_2 (Barrozo *et al.*, 2004; Barrozo and Lazzari, 2004a, Barrozo and Lazzari, 2004b; Bodin *et al.*, 2008). It consists of a hollow polystyrene sphere (9.7 cm diameter, 2.5 g weight) suspended by a vertical air-stream generated by an air-pump. Insects were tethered by their dorsal thorax using double-sided adhesive tape, to a freely rotating stiff steel wire and placed at the apex of the sphere. Insects started to walk as soon as their tarsi contacted the surface of the sphere, thus displacing it with their legs. Insects on the locomotion compensator could walk and rotate freely, changing its direction without modifying its relative distance from the stimulus location, i.e. in an "open-loop" condition for translation, but "closed-loop" for free rotation. The locomotion compensator included an optic sensor which detected the movements of the sphere and this signal was transferred to a computer every 200 ms as *x-y* co-ordinates. The walking paths of the bugs were reconstructed and analyzed in their spatio-temporal components as previously described by Barrozo and Lazzari (2004a). Before starting a test each insect remained in still air on the locomotion compensator

for 2 minutes to familiarize it 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 using an infrared-sensitive camera and an array of infrared LEDs (emission 900 nm). This light illuminated the scene without modifying the normal activity of insects, i.e., functional darkness (Reisenman *et al.*, 1998).

The insects were tested on a simultaneous-discrimination bioassay similar to that previously used (Barrozo *et al.*, 2004; Barrozo and Lazzari, 2004a, Barrozo and Lazzari, 2004b; Bodin *et al.*, 2008). Insects 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). We used two airstreams (control vs. test) because insects exhibit spontaneous anemotaxis to odourless airstreams. In this way, insects exposed to two opposite identical airstreams will orient randomly, but if one of the streams is loaded with an attractive stimulus they will orientate their trajectory towards one of them (Barrozo *et al.*, 2003; Barrozo and Lazzari, 2004a, Barrozo and Lazzari, 2004b, Bodin *et al.*, 2008). In this situation, each bug could choose to walk towards one of the two streams or could exhibit a non-oriented behaviour, i.e. random walk. Two identical constant flow airstreams (4.2 $cm.s^{-1}$; 25 ± 2°C; 40 ± 5% relative humidity), were blown over the insects through glass tubes (0.6 cm inner diameter, 14 cm length) placed 3 cm in front of the insect. CO_2 was generated as previously described by Barrozo and Lazzari (2004a). In order to avoid any environmental asymmetry, the position of the stimulus and the control air-stream were changed randomly between tests. To test for any bias in our dispositive, a control experiment was performed with insects exposed to two opposite and identical odourless airstreams (control, 0 ppm of CO_2 over the ambient).

The insects walking trajectories were analyzed by means of circular statistics (Zar, 1984; Fisher, 1996). 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-bearing 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, respectively indicating orientation

directly towards or away from the stimulus position. We also tested the pathways for eventual bimodal axial directions (i.e. opposite directions vs. uniformity) using the Rao's Spacing test (Fisher, 1993).

Response to heat

In order to study the behavioural response to a thermal stimulus, we tested the bugs' ability to respond to heat and their motivation to feed after their moult to 5^{th} instar. We set up an artificial feeder which allowed us to individually test the response of 10 insects at a time. The artificial feeder (Fig. 1) consisted of ten 1 ml Eppendorf® tubes whose rear ends were cut and replaced with Parafilm® in order to allow insects to bit and feed on the blood contained in the tubes. The tubes contained 0.5 ml of fresh sheep heparinised blood and were placed in a tapped aluminium block (35 x 5 x 1.3 cm) equipped with a flat electric heater. A thermostat kept blood at a constant temperature, 33 ± 1°C, which corresponds roughly to the surface temperature of a host body. The underneath of the aluminium block was isolated with a polystyrene foam plate which held the tubes. Thus, the bottom surface of the feeder remained at ambient temperature (25 ± 2°C), except the Parafilm® surface. The insects were individually placed in plastic containers (11.7 cm height and 3 cm diameter). These tubes contained a filter paper which allowed insects to reach the blood; the top of the feeding tubes was covered with a fabric mesh that allowed them to bite through the Parafilm® of the blood containers. Before a test began, each insect was allowed to familiarize during 2 minutes with the experimental situation (i.e. without stimulation), during which the plastic containers with insects were placed in still air in the experimental room at 25 ± 2°C and 40 ± 5% relative humidity. The artificial feeder was then put in contact with the containers and insects were tested during 15 minutes. Three parameters were recorded: a) approaching to the feeding tubes; b) proboscis extension response (PER); and c) feeding.

Binary data (1 = behaviour observed and 0 = behaviour not observed) were collected and the proportion (p) of animals responding was calculated for the three behavioural parameters described above. For binary data, the standard deviation (s) was calculated as: $s = (p\,(1-p))^{½}$ (Le, 2003). The proportions of insects responding to heat were compared by means of the Mann-Whitney *U*-test.

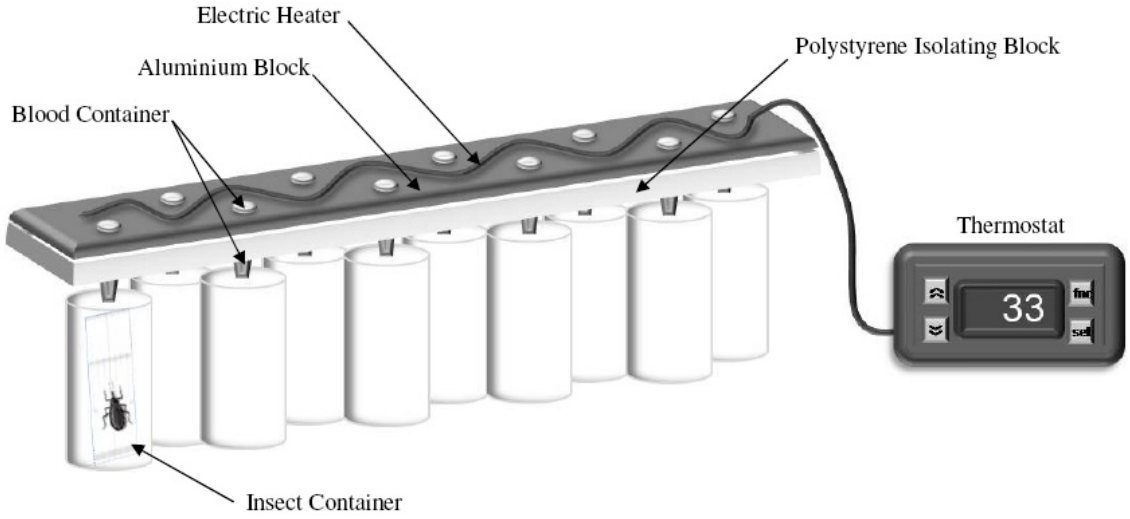

Figure 1. Artificial feeder used to test the behavioural response to heat and the motivation to feed of 5th instar larvae of *Rhodnius prolixus*.

RESULTS

Response to CO_2

The Fig. 2 shows that the orientation response of insects to CO_2, a long-distance cue, depends on the time elapsed since their ecdysis to the 5th stage. No data could be obtained from larvae at T0 (right after ecdysis) since they didn't move at all on the sphere. From T1 to T6, the pathways of the insects were uniformly distributed between 0° and 360°, i.e. they showed no orientation tendency, walking randomly on the sphere (V tests, $p>0.05$ in all cases). A statistically significant attraction response was observed at T7 and this response stayed constant until T60 (V tests, $p<0.05$ in all cases). When insects were stimulated with two opposite and identical odourless airstreams (control, 0 ppm of CO_2 over the ambient) no oriented behaviour was observed (V-test, $p>0.05$, Fig. 2; Table 1). When non-statistically significant data were re-analyzed using the Rao's Spacing test, we found that the insects tested at T5 and T6 showed an axial distribution of their preferred orientation direction (Rao's Spacing test, $p< 0.05$ for T5 and $p<0.01$ for T6), indicating that insects walked either towards or against the air-stream loaded with the stimulus, rather than randomly (Table 1). Comparison of the walking duration did not revealed significant differences, indicating that the activity on the locomotion compensator was similar for all groups (ANOVA, n.s.).

Table 1. Statistical analysis of the behavioural response to 1200 ppm of CO_2 of 5th-instar larvae of *R. prolixus* as a function of the post-emergence time. No insect walked at T0. RS, Rao's Spacing test; MA, mean walking angle; SMD, standard mean deviation.

	V-test			RS test	Walking angle
Time after ecdysis	n	u	p	p	MA ± SMD
control	70	-0.19	*n.s.*	*n.s.*	95 ± 107
T0	15	-	-	-	-
T1	12	-0.45	*n.s.*	*n.s.*	-125 ± 105
T2	13	-0.9	*n.s.*	*n.s.*	-139 ± 97
T3	12	-0.18	*n.s.*	*n.s.*	-99 ± 96
T4	13	0.46	*n.s.*	*n.s.*	-53 ± 111
T5	14	-0.29	*n.s.*	*p< 0.05*	111 ± 112
T6	23	0.35	*n.s.*	*p< 0.01*	9 ± 138
T7	15	2.49	*p< 0.01*	-	-33 ± 18
T8	16	1.86	*p< 0.05*	-	24 ± 81
T10	18	2.45	*p< 0.01*	-	-41 ± 63
T15	19	3.83	*p< 0.001*	-	-23 ± 51
T60	21	2.74	*p< 0.001*	-	-25 ± 56

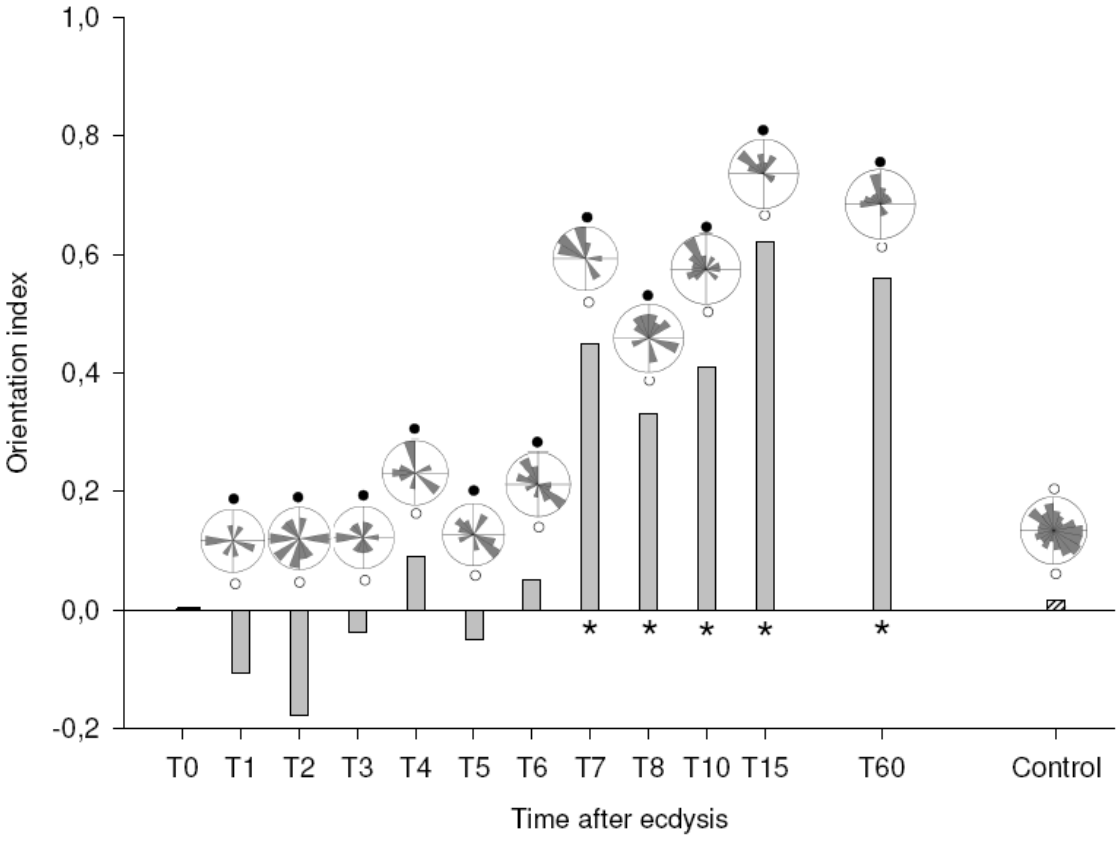

Figure 2. Orientation response of *R. prolixus* larvae to airstreams loaded with 1200 ppm of carbon dioxide above the background (500 ± 100 ppm), at different times after their 5th instar ecdysis. Asterisks denote a statistically significant preferred walking direction towards stimulus location (0°) (V test, p< 0.05). Orientation index varies from -1 (orientation against the stimulus position) to 1 (orientation towards the stimulus location). The circular histograms (Rose diagrams) represent the frequency of the angles displayed by the animals, which is proportional to the area of the wedge (bar width of 10°). Open dots indicate control airstreams position and filled dots denote test airstream location.

Response to heat

The Fig. 3 shows the orientation response (approaching, PER and feeding) of insects to heat, a short-range host cue as a function of the time elapsed since the ecdysis. We found that all the insects that approached the thermal source (the feeder) extended their proboscis, indicating that these two behaviours are associated. The behaviour of insects after ecdysis could be divided in different phases: (1) no attraction was observed before T2 (insects did not move); (2) insects become progressively attracted between T2 and T4, with about 20% and 50% of the insects approaching the feeding in T2 and T4 respectively; (3) the attraction response remained constant at 50% between T4 and T7; (4) the attraction response reached a 90% level between T10 and T15 and stayed constant until T60.

Regarding the motivation of insects to feed, there was a progressive variation as a function of the time post-ecdysis (Fig. 3), and also different phases could be observed. First, no insect fed during the two first nights after ecdysis (0% in T0 and 0% in T1). Second, a progressive response was observed from T2 to T6 with a small increase in the proportion of insects that took a blood-meal (from 10% in T2 to 25% in T6). Finally, we observed a significant increase in the proportion of insects that fed after T7. The number of insects that took a blood-meal increased twice between T6 and T7 (25% to 50% respectively; U= 1.96, p= 0.016), and reached a maximum level between T10 to T60 (about 80% of insects took a meal).

The Fig. 4 depicts the proportion of insects that fed among those that were attracted to heat. Not every insect that was attracted to heat took a blood-meal. We observed two phases in the dynamics of the response, the first one, from T0 to T6, when about 45% of the insects attracted to heat took a blood meal and the second one from T7 to T60, where almost every insect attracted fed (90%). The number of insects that took a blood-meal after their moult increased twice between T6 and T7 (from approximately 45% to 90% respectively, U= 1.96, p= 0.004).

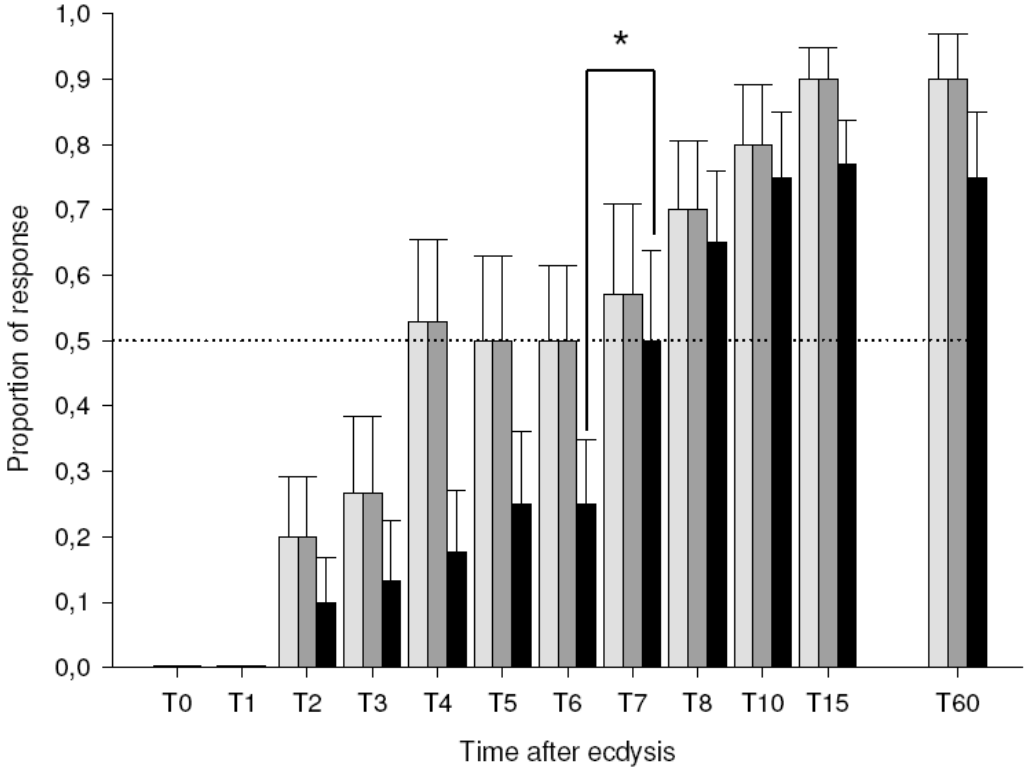

Figure 3. Behavioural response to heat of *R. prolixus* larvae at different times after their ecdysis to the 5th instar. White bars represent the proportion of insects that approached the heat source, gray bars represent the proportion of insects that performed proboscis extension (PER) and the black ones represent the proportion of insects that fed. Each bar represents the mean response ± S.E.M. The dot line represents the level of 50 % of response. The asterisk indicates a significant difference in the feeding response (Mann-Whitney test).

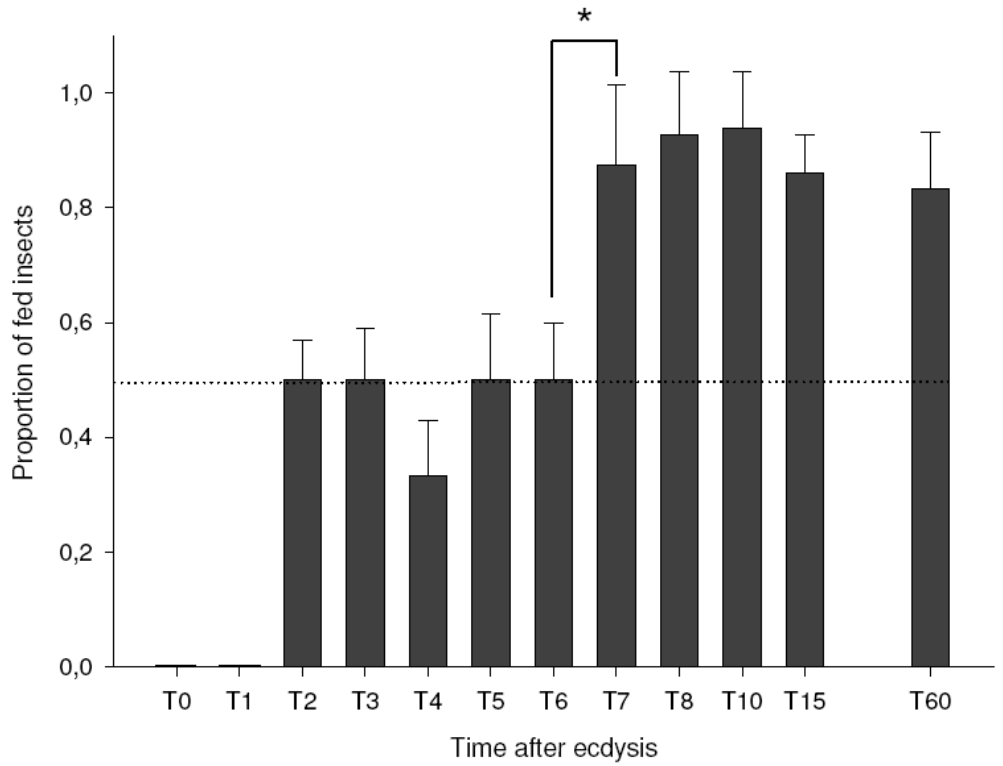

Figure 4. Feeding response of 5th instar larvae of *R. prolixus*. The feeding response is represented as the proportion of insects fed among the insects that were attracted by the heat source, as a function of the time elapsed from their 5th instar ecdysis. Each value corresponds to the mean response ± S.E.M. The dot line represents the threshold of 50 % of response. The asterisk indicates a significant difference in the feeding response (Mann-Whitney test).

DISCUSSION

In the present work, we studied the development of host-seeking behaviour in newly emerged 5th instar larvae of *R. prolixus*. We analyzed the behavioural response of insects to two different cues associated with host localization at long and short distance, as well as their motivation to feed on blood. Insects presented a simultaneous development of their behavioural response to both kinds of cues, i.e. long- (CO_2) and short-range (heat) host cues. Our results showed that the development of the insects' response to these cues is clearly age-dependent. We found that the evolution of the response to CO_2 is not gradual, but all-or-none instead. In other words, insects remained indifferent to this cue during the first week post-ecdysis, and become highly attracted afterwards. In contrast, the response to heat progressed gradually, with the attractiveness to heat increasing steadily for about 10 days until reaching a maximum level.

The development of the behavioural response to heat is thus different from the one to CO_2. In natural conditions, heat constitute a short-range host cue, acting during the final approach of the insect to its host, i.e. after having been guided to their proximity by host-derived chemical cues and anemotaxis. In our experiments, when the insects were exposed to heat, the chemical host-seeking step in the chain of responses preceding blood-feeding was bypassed. Thus, the stimulation with heat indicated to the insect that a host can be easily contacted, even in the case of insects that are not yet motivated to actively seek for a host. Thus, host-seeking *per se* is not necessary for feeding, if the food appears as easily accessible. This is in accordance with the fact that *R. prolixus* started to respond earlier to heat than to CO_2., with 50% of the insects responding by the 4th day (Fig. 3), but with a great increase in the response by the 7th day, when bugs start to be attracted to CO_2 (Fig. 3). When our results are compared to those obtained in mosquitoes, some similarities become evident, despite phylogenetic distances and the fact that *R. prolixus* is an hemimetabolous insect and obligatory haematophagous during all stages of development, whereas mosquitoes are holometabolous and only adult females feed on blood (but not exclusively). In *Culex* mosquitoes, Mitchell (1981, 1983) has shown the effect of the access to the host on feeding behaviour. He found that diapausing female *Culex* spp fed on a host animal when they were placed in a small cage but did not in a large cage. The author concluded that in a large cage most mosquitoes must engage in host-seeking behaviour in order to contact the host, whereas a small cage assures host contact even by mosquitoes that are not actively looking for a host. Roth (1957) observed that female *Aedes aegypti* and *Anopheles quadrimaculatus* would not

orient to a human arm after bilateral antennectomy, but if they encountered the arm by chance, they would settle and feed.

An interesting observation concerning the development of the behavioural response to CO_2 was that during the two days previous to the establishment of a clear-cut attraction response (i.e., T5 and T6), insects displayed a bimodal response. The Rao's Spacing test revealed that insects oriented in opposite directions; i.e., both approaching and avoiding the odour source, rather than walking randomly (Table 1). This phenomenon has been recently reported to occur under similar stimulation conditions, when insects were exposed to CO_2 at a time of the day when they are not supposed to search for food but for refuge (Bodin *et al.*, 2008). So, three different phases could be observed in the insects' response to CO_2 after ecdysis. From T0 to T4, the insects displayed a non-oriented response being indifferent to this signal. At T5 and T6, insects displayed a bimodal axial orientation with half of the bugs oriented equally towards the stimulus and against it. Then, after T7 and until two months after ecdysis (T60), insects were highly motivated to find a host and strongly attracted to CO_2 (Fig. 2). The 7^{th} day appeared to be a transition day where the attraction to CO_2 clearly started.

In *R. prolixus*, the modulation of the response to different kinds of host signals (long and short-range cues) is consistent with the context in which they intervene. At a distance from a host, insects seek for host-emitted cues by means of a non-oriented behaviour which allow them to encounter these attractive signals in their environment. These cues trigger the searching behaviour, followed by a long-range orientation to the host. All these activities expose insects to the risk to be predated. We have recently shown that *R. prolixus* responds to CO_2 only during a narrow temporal window at the beginning of the night, when host and predators are resting (Bodin *et al.*, 2008). Besides, the host-seeking behaviour is costly in terms of energy. As a consequence, in cases in which taking a blood meal would not provide additional nutritional resources or if the insect is not physiologically ready to obtain and handle a blood meal, it would be adaptive not to respond to long-range host cues,. In the case of the experiments we present here, food is necessary to continue the development of the insect. Thus, the lack of response seems to be most probably related to the acquisition of morphological (e.g. the sclerotisation of mouth-pieces) and physiological (e.g. enzymes) abilities to obtain and handle a blood-meal.

Previous studies on holometabolous blood-sucking insects have shown that other activities that occur after adult emergence, such as reproduction, affect feeding behaviour. In this case, blood meals taken before a certain degree of ovarian maturation do not add to the

reproductive success of the insect, but visiting the host will increase the insect's chances of being damaged or killed. In mosquitoes, there is a one-two day period after adult emergence during which insects do not show seek for hosts. Blood-feeding has been reported to be initiated between 24 and 72h after a female mosquito emerges (Seaton and Lumsden, 1941; Bishop and Gilchrist, 1946; Laarman, 1955). A similar period of maturation appears to be required before the peripheral sensory organs are fully responsive (Davis, 1984a). It has been shown that the development of activity in the chemosensory afferent neurons sensitive to lactic acid in newly emerged virgin female *Aedes aegypti* mosquitoes is age-dependent, and in correlation with the development of host-seeking activity (Davis, 1984a). In *Ae. atropalpus* the receptors for lactic acid develop faster (12h), and the absence of attractive behavioural response to host components from emergence to the end of the first gonotrophic cycle in this species cannot be attributed to the delay of development of the peripheral sensory system (Bowen *et al.*, 1994a; Bowen *et al.*, 1994b). In this species, authors hypothesized that distension due to oocytes maturation in the ovaries inhibits host-seeking during the first gonotrophic cycle (Bowen *et al.*, 1994b).

Our experimental model allowed us to study, for the first time in a blood-sucking insect, the effect of the moult on feeding excluding the influence of reproduction, dispersion and other adult activities. So, the modulation of their response to host-associated cues is only due to morphological and physiological reasons associated to the insect's development. The available information does not allow us to speculate much about the mechanisms behind the inhibition of feeding behaviour following the ecdysis. As indicated above, the sclerotisation of mouthparts could need to be completed, the enzymatic machinery associated to digestion to be activated or even the sensory organs complete their development. It should be noted that the last has been shown to be the case of the simple eyes (ocelli), which complete their development only two weeks after the imaginal ecdysis, during the adult life (Insausti and Lazzari, 2000).

Another interesting result of our experiments is that the extension of the proboscis displayed by the insects is not dissociated from the approaching of the heat source. Every insect attracted by the heat source extended it proboscis. Nevertheless, proboscis extension was not necessarily associated with feeding. About 50% of the insects attracted by the heat source fed before the 7th day. It's only after this day that we observed that nearly all the bugs attracted to heat took a blood meal (Fig. 4). Thus, the extension of the proboscis does not assure that the insects would take a meal.

From an epidemiological point of view, our results provide quantitative data for computational models of the feeding rate and parasite transmission, and also allow validating previous data. For example, Rabinovich *et al.* (1979) analysed the biting frequency and blood ingestion in domiciliary *R. prolixus* in Venezuela. These authors obtained from their model what they call the "time from moult to feed", a value of 6.8 days, which corresponds quite exactly to the 7 days necessary for bugs to respond to CO_2 and to reach their maximal motivation to feed.

Summarizing, our study allow us to drawn some interesting conclusions: 1) after their ecdysis, triatomines larvae need some days before starting to look for food; 2) feeding behaviour is modulated during this period by the responsiveness of insects to chemical cues and heat; 3) the response to thermal stimuli (host approaching) is strongly associated to the PER, reinforcing the idea about the crucial role of heat for biting (Flores and Lazzari, 1996); 4) PER is not fully associated with feeding, depending the later on other signals coming from blood itself. Further work already running in our laboratory should shed more light on the influence of the physiological state of the insect (e.g., the nutritional state and reproduction) on host-seeking and feeding in blood-sucking insects.

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