Circadian rhythm of behavioural responsiveness to carbon dioxide in the blood-sucking bug *Triatoma infestans* (Heteroptera: Reduviidae)

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**Abstract**

The temporal modulation of the behavioural response to carbon dioxide and its chronobiological basis were investigated in larvae of *Triatoma infestans*. We analysed the orientation towards CO\(_2\) of insects kept under three different illumination regimes: (1) 12 h light/12 h darkness cycles (L/D), (2) constant darkness (D/D) and (3) constant light (L/L). When maintained under L/D conditions, insects exhibited an oriented response towards airstreams added with 1500 ppm of CO\(_2\) during the first hours of the scotophase only. Bugs maintained under D/D also showed a positive orientation response towards CO\(_2\) during the first hours of the subjective night, while bugs kept under L/L did not show a rhythmic oriented behaviour. Thus, *T. infestans* displayed a daily rhythm of orientation towards CO\(_2\) (i.e. a potential food source) only at the beginning of the scotophase. The persistence of the rhythm under constant darkness reveals the existence of an endogenous circadian control of this behaviour.

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1. **Introduction**

Triatominine bugs are vectors of the Chagas disease, one of the most important sanitary problems in Latin America. Currently, more than 18 million people are infected by this disease and about 90 million are in the risk of becoming infected (WHO, 1997). *Triatoma infestans* is the main vector of this disease in southern South America. This species is almost restricted exclusively to human dwellings, with only one known wild population in Cochabamba, Bolivia (Dujardin et al., 1987; Bermúdez et al., 1993). Inside human houses, these haematophagous bugs inhabit crevices in walls and roofs, and feed mainly on humans, dogs and hens (Gürtler et al., 1997). During the day they are mostly inactive and are usually found in a quiescent state or *akinesis*, aggre-
Carbon dioxide (CO₂), has been largely described as an attractant or activator for many haematophagous insects (see Lehnane, 1991), including T. infestans (Wiesinger, 1956; Taneja and Guerin, 1995). Recently (Barrozo, 2003; Barrozo and Lazzari, submitted), we studied in our laboratory the response of this species to CO₂, determining the behavioural threshold of responsiveness to this gas and analysing the effects of CO₂ when associated with other chemicals. Additionally, evidence was obtained indicating that the attractiveness to CO₂ by this species is limited to the early scotophase. These changes in olfactory sensitivity over the day are not fully surprising since they have also been reported for other insects. For instance, daily rhythms of sensitivity and orientation towards host-related cues have been reported in the haematophagous tsetse flies (Brady, 1975; Van der Goes van Naters et al., 1998). Furthermore, phylogenetically distant insects, like moths and cockroaches, exhibit daily rhythms of pheromone orientation which were demonstrated to be under endogenous control (Baker and Cardé, 1979; Liang and Schal, 1990; Linn et al., 1996). Such temporal modulation could be either a direct response to environmental changes, i.e. consequence of illumination and/or temperature cycles, or under an endogenous control, i.e. commanded by internal oscillator/s. The goal of this work was to test the existence of a daily rhythm of responsiveness to CO₂ in T. infestans and, furthermore, whether it would be under exogenous or endogenous control.

2. Materials and methods

2.1. Insects

Fourth-instar larvae of T. infestans were used throughout the experiments. Insects were obtained as third-instar from the Servicio Nacional de Chagas (Córdoba, Argentina), kept under a 12:12 h L/D regime, 26 °C, 30–50% RH and fed on hens weekly until molting. After ecdysis, bugs were starved for 10–20 days until the experiments.

2.2. Illumination regimes

Insects were entrained in a 12:12 L/D illumination regime for 3 days and then kept under one of the following: (1) another 3 days under the same L/D regime, (2) 3 days in constant darkness (D/D), or (3) 3 days in constant light (L/L). Temperature was kept at 26 °C. The fourth day, different larvae were assayed at different times to cover a period of 24 h. The data were pooled every two-hour intervals.

2.3. Recording of walking pathways

Behavioural responses were recorded in an open-loop design for translation on a locomotion compensator (modified from Dahmen, 1980). The bugs were tethered by their dorsal thorax and abdomen to a freely rotating stiff steel wire centered at the apex of a Styrofoam sphere (97 mm diameter, 2.5 g weight), which was suspended by a vertical airstream. The animals started to walk when contacting the surface of the sphere thus displacing it with their legs. The bugs could freely walk and rotate, changing their direction of locomotion without modifying its distance to the stimulus. The movements of the sphere were detected by an optic sensor and the signals sent to a computer every 0.2 s as x-, y- co-ordinates with the aid of a software designed ad hoc (Diego Anfossi, unpublished). The walking paths of the bugs were reconstructed and analysed in their spatio-temporal components.

2.4. Stimulus delivery

Given that under our experimental conditions T. infestans exhibits a spontaneous positive anemotaxis to odourless air-currents (Barrozo et al., 2003), we developed a simultaneous-discrimination bioassay. Thus, the animals were confronted simultaneously to two opposite horizontal charcoal-filtered airstreams. Both currents were identical in velocity, temperature and humidity content, but one carried the test stimulus (1500 ppm of CO₂ above the ambient levels), while the other was kept at ambient CO₂ levels. In this way, the test insect could choose to walk preferentially towards one of the two-streams presented.

The air (26 ± 1 °C, 85 ± 1% RH and 6 cm s⁻¹ at the exit of the glass tube) was blown over the insect through glass tubes (0.67-cm inner diameter, 5-cm length), the tips of which were at 3 cm from the insect. The glass tubes were connected to silicone tubes each passing through a different glass bottle (250 ml) in which either CO₂ (test stream) or nothing (control stream) was added to the air. The test and control streams were interchanged between sides randomly to prevent eventual environmental bias.

The CO₂ was chemically generated according to the following reaction: \( \text{Na}_2\text{CO}_3 + \text{H}_2\text{SO}_4 \rightarrow \text{Na}_2\text{SO}_4 + \text{H}_2\text{O} + \text{CO}_2 \). A synchronic-motor driven syringe injected a 0.2 M solution of Na₂CO₃ (0.08 ml min⁻¹) in a 250 ml glass bottle containing 100 ml of 1 M H₂SO₄. The mixture was continuously stirred to ensure a stable CO₂ production. The charcoal-filtered airstream (90 ml min⁻¹)
passed over the surface of the reaction mixture, where it was loaded with CO$_2$, resulting in an increase of 1500 ppm of CO$_2$ over the ambient level (400 ± 20 ppm). A non-dispersive infrared sensor (PP Systems, model EGM-3, range 0–5000 ppm, accuracy 0.5%) was used to measure the CO$_2$ concentration in the air. The control stream passed through an identical glass bottle filled with 100 ml of 1 M H$_2$SO$_4$. This disposition allowed maintaining identical conditions (relative humidity and temperature) in both currents.

2.5. Bioassay protocol

Before starting an experiment the insects remained in still air on the locomotion compensator for 120 s to habituate to the experimental situation, after which the airstreams (control and CO$_2$ streams) were presented during 180 s.

All assays were conducted in a room maintained at 26 ± 1 °C and illuminated only by an array of infrared LEDs (900 nm), whose emission is not perceived by the bugs (Reisenman et al., 1998). The animals were monitored from the outside of the experimental room with the aid of an infrared-sensitive camera.

Each individual insect was tested only once and discarded afterwards.

2.6. Data analysis

The walking pathway described by insects on the locomotion compensator was analysed by means of circular statistics (Batschelet, 1965; Zar, 1984). We measured the mean walking angle displayed by each individual ($\theta_i$). For each two-hour interval and regime, the $\theta_i$ of insects subjected to the same treatment was averaged resulting in a mean vector, constituted by a mean walking angle ($\bar{\theta}_m$) and a vector length ($r$). The angle $\theta$ varied between 0 and 360° and $r$ varied between 0 and 1 (0 indicating a non-defined mean direction and 1 a straight path). The position of the CO$_2$ current was conventionally designated as 0° and the control current as 180°. The Rayleigh test was performed to evaluate the uniformity of the sampled population around a circle. Subsequently, the V test was conducted with samples that differed significantly from uniform distribution to test if the mean angle ($\bar{\theta}_m$) calculated from every two-hour interval was significantly distant from the stimulus direction (0°). Additionally, an orientation index (OI) was calculated for an easier visualisation of the data, by multiplying the cosine of the mean angle ($\bar{\theta}_m$) by the length of the vector ($r$). This index fluctuates between 1 and −1, indicating orientation directly towards or away from the stimulus position, respectively.

Finally, as a measure of activity, the total walked distance (cm) during the 180 s in the presence of stimulus was calculated for every insect from the individual pathways. This parameter was analysed throughout the different two-hour intervals and illumination regimes by means of linear statistics (one-way ANOVA) (Zar, 1984).

3. Results

Fig. 1 depicts the orientation index of *T. infestans* larvae subjected to different illumination regimes after entrained in L/D, and tested with airstreams loaded with 1500 ppm of CO$_2$ above the ambient levels. Bugs maintained under L/D exhibited a daily temporal modulation in their response to CO$_2$ (Fig. 1a). The variation was also evident in bugs subjected to D/D conditions, as depicted in Fig. 1b. Conversely, no daily change in responsiveness was observed for insects maintained under constant illumination (L/L, Fig. 1c).

In all cases, during either the actual (L/D) or subjective day (D/D and L/L), the pathways of the insects were uniformly distributed between 0° and 360°, i.e. they showed no orientation tendency (Rayleigh test, all cases n.s.). However, during either the night (insects subjected to L/D) or the subjective night (insects kept under D/D), a preferred walking direction towards the CO$_2$-enriched current was observed, but taking place only at certain times. Bugs subjected to L/D displayed a main walking direction towards the CO$_2$-loaded stream during the second interval of measurement after “lights off” (V test, $u_{(20)} = 4.3$, $p<0.0005$), i.e. between the second and the fourth hour after the beginning of the scotophase. Insects walked randomly on the sphere during the rest of the night (Fig. 1a). Under constant dark conditions (D/D), bugs displayed a strong orientation towards CO$_2$ during the first two-hour interval of the subjective night ($V$ test, $u_{(25)} = 3.6$, $p<0.0005$) (Fig. 1b). Thus, in insects maintained under D/D conditions, the orientation towards CO$_2$ occurred about two hours earlier than in the group kept under L/D regime, evincing the shortening in the period of the rhythm. In both cases, insects oriented to CO$_2$ in a specific temporal window during the night. On the contrary, insects kept under constant conditions of illumination (L/L) showed a non-rhythmic oriented response (Rayleigh test, all cases n.s.) (Fig. 1c).

The total walked distance did not vary with the time of the day under any of the three illumination regimes (line plots in Fig. 1a–c; ANOVA, in all cases n.s., range under L/D: 100–210 cm; D/D: 123–205 cm and L/L: 120–198 cm).

4. Discussion

Our results demonstrate that the responsiveness of *T. infestans* to CO$_2$, one of the main host-associated
stimuli, is endogenously controlled and limited to a narrow temporal window at the beginning of the night. The spontaneous locomotor activity of *T. infestans* can be observed during the whole night, with two main bursts, similar in intensity, at dusk and dawn, respectively (Lazzari, 1992). Lorenzo and Lazzari (1998) showed that more bugs fed if food was offered at the beginning of the scotophase than if it was offered at the end. It should be mentioned that these authors offered food in an artificial feeder that exploited the response of these bugs to heat, but in the absence of chemical cues, such as CO₂ or others. We went a step further by demonstrating the motivation of bugs to orient towards CO₂—a potential food source—independedently of their activity pattern. Moreover, our results show that the rhythm of responsiveness to CO₂ is not just a direct response to environmental factors (i.e. the light–dark cycle), but instead, it is under the control of an endogenous oscillator. The persistence of the rhythm under constant darkness demonstrates that it is self-sustained and hence 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. As a result, in our assays, which were performed after 3 days in D/D, we observed a maximal response of insects during the previous two-hour intervals than the entrained bugs. As with several other circadian rhythms in these and other insects, the rhythm of responsiveness to carbon dioxide vanished under L/L. However, this fact does not necessarily mean that the responsiveness of *T. infestans* to CO₂ remained at low levels during the whole day, as might be wrongly deduced from a cursory inspection of Fig. 1c. This figure represents the average response of groups of insects, rather than individual performances. Then, in the absence of a synchronised rhythm by external or internal signals, each individual might be expressing an oriented response to CO₂ at different times of the day. Conversely, in L/D or D/D, the insects coincide temporally in expressing a peak of responsiveness at certain hours of either the real or the subjective night. A general decrease in activity under L/L can also be excluded. As shown in Fig. 1a–c, the walking activity of bugs remained the same both, along the day and under the three illumination regimes, i.e. L/D, D/D and L/L. It is worth mentioning that the bioassay described here assured a similar level of locomotor activity of insects, since all of them depart from the state of akinesis in the course of the experimental manipulation. Therefore, the addition of an orienting stimulus would just modify the direction, but not the speed of displacement.

The existence of circadian rhythms has been demonstrated mainly for locomotor activity, in other haematophagous insects, like mosquitoes and tsetse flies (Taylor and Jones, 1969; Brady, 1972; Jones et al., 1972; Jones, 1976; Jones, 1982; Rowland, 1989; Pandian, 1994). However, to our knowledge, this is the

![Fig. 1. Orientation towards CO₂ in *T. infestans* larvae. Bugs were entrained for 3 days in L/D (denoted with the white and black portions of the upper horizontal bar) and then kept under L/D (1a), D/D (1b) or L/L (1c) (indicated with the lower horizontal bar) for three more days. Afterwards, their orientation towards CO₂ was tested under functional darkness. Bars represent the orientation index of about 15–28 (L/D), 15–27 (D/D) and 15–25 (L/L) insects. Asterisks indicate simultaneous occurrence of statistically significant differences from a uniform distribution (Rayleigh test, *p* < 0.05) and a significant mean direction around the stimulus position (Θ’ (V test, *p* < 0.05). Orientation index varies from −1 (orientation against the stimulus position) to 1 (orientation towards the stimulus location). The line plots indicate the mean total distance walked (cm) at every two-hour interval for each illumination regime.](image)
first report demonstrating the existence of a truly circadian (i.e. endogenous) rhythm of responsiveness to a chemical cue, either pheromonal, ovipositional or host-related, in a haematophagous insect. Circadian control of behaviour provides animals temporal orientation independently from environmental cues for the occupation of temporal niches or in the co-ordination of activities between individuals and species (see Aschoff, 1989). It has been shown that circadian clocks may not only play a role in regulating olfactory behaviours in insects, but also may control the sensory reception (Krishnan et al., 1999; Page and Koelling, 2003).

Concerning triatomines, the question arises about the biological significance of limiting responsiveness to a particular temporal window. These insects have shown a high temporal organisation of several activities, e.g. dispersion, feeding, outgoing from shelters, flight initiation and oviposition which take place during the early night, whereas egg hatching, ecdysis and return to refuges take place at the end of the scotophase (Ampleford and Steel, 1982; Constantinou, 1984; Lazzari, 1991; Mc Ewen and Lehane, 1993; Lorenzo Figueiras et al., 1994; Lorenzo and Lazzari, 1998).

Only the spontaneous locomotor activity is expressed in a bimodal fashion. Therefore, a given process can be observed to occur in just one temporal window, either at dawn or at dusk.

During the day, the insects remain immobile and assemble inside wall crevices in human housings, becoming active during the night. This fact can be explained by the diurnal habits of their hosts, which may also play the role as predators when awake. Thus, in addition to a positive selective pressure to obtain food when hosts are resting, a negative pressure to avoid exposure during daylight hours might be postulated as a defence mechanism against predation. However, this argument does not explain why the response to CO2 is restricted to the first hours of the night and does not occur along the entire scotophase. After feeding, the bugs remain outside of the refuges, where they experience an abundant diuresis before returning to their shelters where they will remain inactive during the daylight hours. If the motivation to respond to host-related stimuli and to feed remain the same (high) during both activity bouts, many bugs would feed during the end of the night. As a consequence, they could be surprised by the day and awakening of the host/predator. Moreover, they would eliminate faeces and urine inside refuges. The importance of the elimination of excretion products outside the shelters has been discussed previously (Lorenzo and Lazzari, 1996). Faeces act as chemical marks of the entrance to refuges, helping bugs to find them. In addition, defecating outside refuges would diminish the presence of toxic waste products inside them. Besides, it is consistent with a temporary co-ordination of activities like feeding and returning to the refuge. Thus, the biological sense of limiting the responsiveness to host stimuli to the early night can be hypothesised, but only in the frame of the whole temporal organisation of triatomines’ life.

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