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RESEARCH ARTICLE

Circadian modulation of learning ability in a disease vector insect, *Rhodnius prolixus*

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ABSTRACT

Despite the drastic consequences it may have on the transmission of parasites, the ability of disease vectors to learn and retain information has just begun to be characterised. The kissing bug *Rhodnius prolixus*, a vector of Chagas disease, is an excellent model, particularly because conditioning the proboscis extension response (PER) constitutes a valuable paradigm to study their cognitive abilities under carefully controlled conditions. Another characteristic of these bugs is the temporal organisation of their different activities in a bimodal endogenous daily rhythm. This offers the opportunity to address the implication of the circadian system in learning and memory. Using aversive conditioning of the PER, we tested whether the ability of kissing bugs to learn and remember information varies during the day. We found that bugs perform well during the night, but not during the day: their ability to acquire information – but not their ability to retrieve it – is modulated by time. When the bugs were kept under constant conditions in order to analyse the origin of this rhythm, the rhythm continued to free run, showing its endogenous and truly circadian nature. These results are the first to evince the effect of the circadian system on the learning abilities of disease vectors and one of the few in insects in general.

KEY WORDS: Cognitive abilities, Kissing bug, Disease vector, Aversive operant conditioning, Chagas disease, Circadian clocks

INTRODUCTION

Numerous human and animal diseases are transmitted by insects whose vectorial capacity (i.e. their ability to transmit the disease) can be modulated by a variety of factors. Some of them have been well characterised (for instance, vector density or host defensive behaviour; Kelly and Thompson, 2000) and it is accepted that cognitive ability, and more precisely the ability to learn and memorise information, should have a great epidemiological impact. However, until recently, such abilities had not been clearly experimentally evinced, to the extent of making some authors wonder whether haematophagous insects could learn anything from their hosts (Alonso et al., 2003). In recent years, the number of studies devoted to analysing the learning ability of disease vector insects has increased regarding mosquitoes (McCall and Eaton, 2001; McCall et al., 2001; McCall and Kelly, 2002; Kaur et al., 2003; Alonso and Schuck-Paim, 2006; Tomberlin et al., 2006; Chilaka et al., 2012; Menda et al., 2013; Vinauger et al., 2014), tsetse flies (Bouyer et al., 2007) and triatomine bugs (Vinauger et al., 2011a,b, 2012, 2013). These studies provided direct or indirect evidence of learning ability, but as far as we know none of them dived deeper into the characterisation of how learning and memory could be modulated by endogenous factors. These factors could hopefully explain why so many studies have remained inconclusive (Alonso et al., 2003; Alonso and Schuck-Paim, 2006).

The circadian system of triatomine bugs has been characterised in both *Rhodnius prolixus* and the closely related species *Triatoma infestans*. The activity pattern is bimodal, each activity burst being controlled by an independent oscillator (Constantinou, 1984; Lazzari, 1992). Daily rhythms of ecdysis (Ampleford and Steel, 1982), egg hatching (Lazzari, 1991), refuge use (Lorenzo and Lazzari, 1998), phototactic sensitivity (Reisenman et al., 1998), adaptation to light of compound eyes (Reisenman et al., 2002) and ocelli (Lazzari et al., 2011), flight activity (McEwen and Lehane, 1993), thermopreference (Minoli and Lazzari, 2003) and sensitivity to odours (Barrozo et al., 2004; Bodin et al., 2008) have been described. All of them have been shown to have an endogenous origin (i.e. true circadian nature), with the exception of the adaptation to light of ocelli and the response to aggregation pheromones at early photophase (Bodin et al., 2008; Lazzari et al., 2011). So, it can be said that these bugs evince a strong temporal organisation of their activity and constitute good model systems for studying fundamental clock processes (e.g. Vafopoulou et al., 2010; Vafopoulou and Steel, 2012, 2014).

Daily rhythms in blood-sucking bugs have been modelled, as for other aspects of their physiology and behaviour, in accordance to the biology of their vertebrate hosts and under the strong selective pressures associated with a particularly risky way of life, i.e. haematophagy.

In insects, circadian regulation of learning ability has been demonstrated thus far in three different contexts: (i) olfactory conditioning in the honeybee *Apis mellifera* (Lehmann et al., 2011) is modulated in a circadian way; (ii) the ability of the cockroach *Rhyparobia (Leucophaea) maderae* (Decker et al., 2007) to form new olfactory memories is under the control of a circadian clock for classical but not for operant conditioning (Garren et al., 2013); and (iii) short-term memory formation in *Drosophila melanogaster* is subject to circadian modulation (Lyons and Roman, 2009).

Information about the cognitive abilities of haematophagous insects has been accumulating in recent years, but no evidence of their circadian regulation is available. Given the strong selective pressures associated with haematophagy, we can speculate that to learn and remember information about hosts is highly adaptive, but to be selective in learning only in proper temporal contexts would be even more useful. Indeed, remembering occasional events occurring at times that are not relevant would represent a waste in terms of both energy and information processing. Conversely, being able to learn and remember information about hosts only in the temporal context corresponding to host seeking would be adaptive as these abilities

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would be used in the period of time when they are salutary for the insect's survival.

To analyse whether this applies to haematophagous insects, we chose *R. prolixus* Stål 1859 as an experimental model. These hemimetabolous and obligatory blood feeders are easy to rear in the laboratory and their motivational state can be well controlled (Bodin et al., 2009a,b). Furthermore, their physiology has been well described and characterised since the seminal work of V. B. Wigglesworth in the 1930s, and the temporal organisation of these Chagas disease vectors has been extensively described (see Lazzari et al., 2013, for review). Another specificity of these insects is their stereotyped response to proximal thermal stimulation, i.e. the proboscis extension response (PER). This represents an advantage in comparison with mosquitoes or biting flies, as it makes it possible to adapt experimental tools and paradigms developed in classical models, e.g. honeybees or fruit flies, for the study of learning (Chabaud et al., 2006; Giurfa, 2007; Carcaud et al., 2009; Strube-Bloss et al., 2011), as we have recently demonstrated (Vinauger et al., 2013). In addition, as mentioned above, these bugs exhibit a high temporal organisation of their behaviour (Lazzari, 1992). By rearing bugs under a 12 h:12 h light:dark (L:D) regime (lights on at Zeitgeber time ZT0 and off at ZT12) and by training and testing them at different times, we assessed whether the ability of *R. prolixus* to learn and recall information is modulated during the day, as well as the endogenous or exogenous origin of this modulation.

RESULTS

We carried out aversive operant conditioning experiments, as described in Vinauger et al. (2013). First, the PER was elicited by exposing each insect to an appetitive thermal stimulus (a Peltier element at 35°C), upon which the extended proboscis was subjected to an aversive stimulus (i.e. the temperature of the Peltier element was raised to 50°C). The learning performance was then measured by quantifying the number of trials necessary to inhibit the PER in the presence of the 35°C appetitive stimulus (Fig. 1). Insects were exposed to two sessions and comparison of their learning performance for the first (S_{T1}, referred to as training) and the

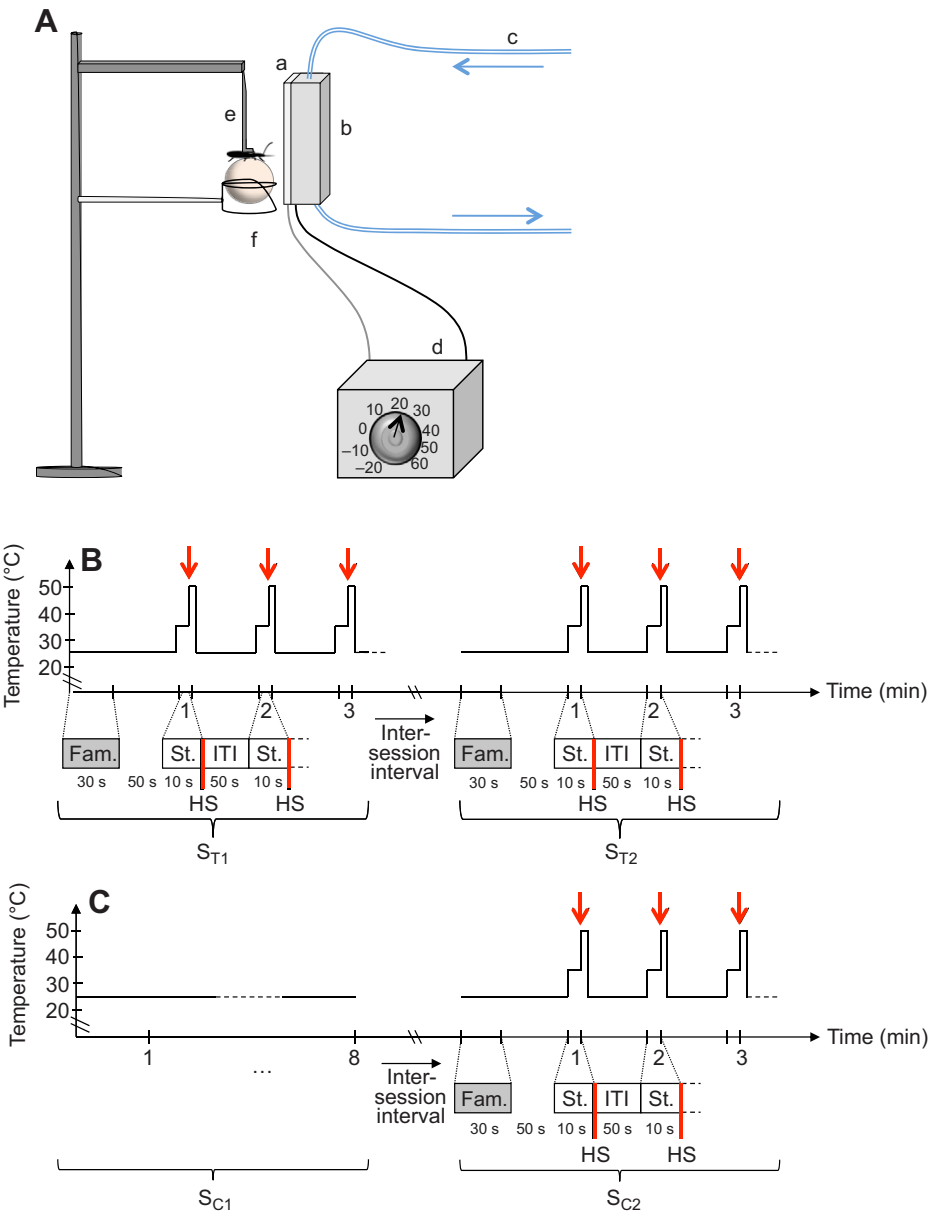

Fig. 1. Aversive operant conditioning apparatus and protocol. (A) Experimental device used for training the proboscis extension response (PER)of *Rhodnius prolixus*, allowing the delivery of thermal stimulation. a, Peltier element; b, aluminium heat-dissipating block; c, water cooling system; d, Peltier control unit; e, steel wire; f, Styrofoam® sphere (1 cm diameter). (B) Sequence of event delivery (i.e. appetitive thermal stimulation and inter-trial interval) during training sessions in the aversive conditioning of the PER. (C) Sequence of event delivery of the untrained controls. Fam., familiarisation period; St., stimulation; ITI, inter-trial interval; HS, heat shock; S_{T1}, first session of the trained group; S_{T2}, second session of the trained group; S_{C1}, first session of the control group; S_{C2}, second session of the control group.

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second session (S_{T2}, referred to as test) allowed us to assessed their memory (see Fig. 1 for details).

Effect of daytime on the ability to acquire and recall memory

To determine whether the circadian phase regulates the ability of these animals to form and recall memories, insects were trained and tested at two different moments of the day: at ZT14 (i.e. early scotophase) and ZT2 (i.e. early photophase).

At ZT14, group 1 trained insects stopped responding more rapidly during the test session than during the training session (S_{T1}: 4.2±0.5 trials; S_{T2}: 2.9±0.3 trials; Wilcoxon test, P=0.0053; Fig. 2A) and more rapidly than untrained insects exposed to the same context during their first session (S_{T2} versus S_{C2} in Fig. 2A, Mann–Whitney test, P=0.011). However, for group 2 insects at ZT2, no influence of training was observed during the test session (S_{T1}: 7.5±1.2 trials; S_{T2}: 7±1.1 trials; Wilcoxon test, P=0.24; Fig. 2B), revealing a daily modulation of learning and memory.

In addition, the difference in performance between naive insects and bugs pre-exposed to the context was not significant (S_{T1} versus S_{C2} in Fig. 2A, Mann–Whitney test, P=0.42), discarding a potential effect of exposure to the experimental context.

Effect of daytime on the ability to acquire or recall memory

The performance of insects depended on their ability to acquire and consolidate a memory during the training session and to recall the memory during the test session. In order to analyse whether the observed variation in the previous experiment was due to a modulation of only one or of both processes, i.e. the ability to acquire the information and the ability to recall the learned information, two more groups were tested (Fig. 2C,D).

Group 3 was trained during the first part of the night and tested 12 h later, during the first part of the day (ZT14–ZT2). These insects were able to recall the learned information and required fewer trials to stop responding during the second session (S_{T1}: 8.7±0.6 trials; S_{T2}: 3.2±0.6 trials; Wilcoxon test, P<0.0001). They also required fewer trials than control insects (S_{C2}: 7.7±0.6 trials; S_{T2} versus S_{C2}, Mann–Whitney test, P<0.0001). No difference was observed between naive insects (trained group, S_{T1}) and control bugs (S_{T1} versus S_{C2}, Mann–Whitney test, P=0.33).

In the same way, group 4 insects were trained during the first part of the day and tested 12 h later, during the first hours of the night (ZT2–ZT14). This time, no effect of training was observed on the performance of bugs at ZT14 (S_{T1}: 6.5±0.9 trials; S_{T2}: 7.2±1.2 trials; Wilcoxon test: P=0.68), revealing that the modulation occurs at the acquisition level and not for the recall of learned information.

Daily modulation of the behavioural response to heat stimulation

The results obtained for bugs trained at ZT14 and tested at ZT2 could, however, also be explained by a daily modulation of the bugs' motivation to respond to heat. The reduced number of trials observed at ZT2 in the previous experiment would then be due to a lack of motivation to respond and not to individual experience. To evaluate any potential temporal modulation of the response to heat,

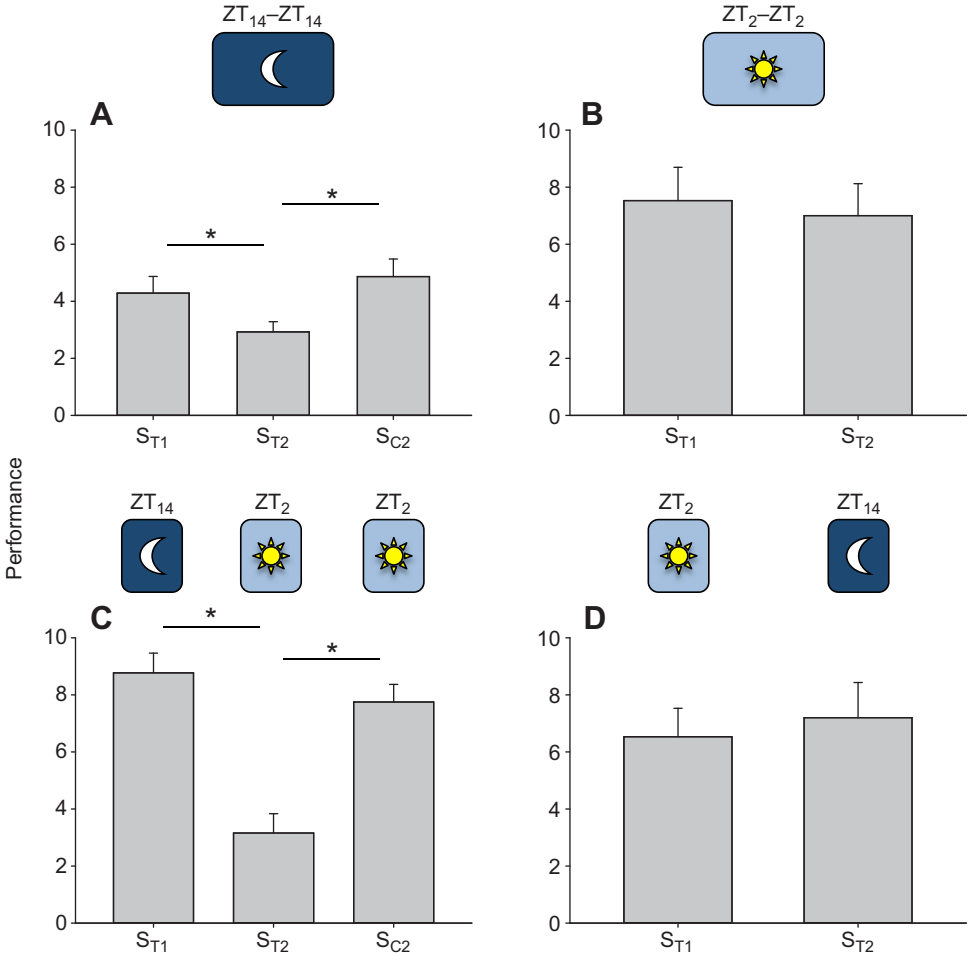

Fig. 2. Effect of daytime on the ability to acquire and/or recall memory. Performance of *R. prolixus* larvae, represented as the mean number of trials that were necessary to observe complete disappearance of the response to three consecutive stimulations. Each bar represents either a trained group during its first (S_{T1}, training) or second session (S_{T2}, test) or the associated control group (S_{C2}) when indicated. ZT, Zeitgeber time. (A) Group 1, trained and tested at ZT14, 24 h retention (trained group: N=14; control group: N=15). (B) Group 2, trained and tested at ZT2, 24 h retention (trained group: N=17). (C) Group 3, trained at ZT14 and tested at ZT2, 12 h retention (trained group: N=31; control group: N=12). (D) Group 4, trained at ZT2 and tested at ZT14, 12 h retention (trained group: N=15). *Significant difference between groups/sessions (P<0.05).

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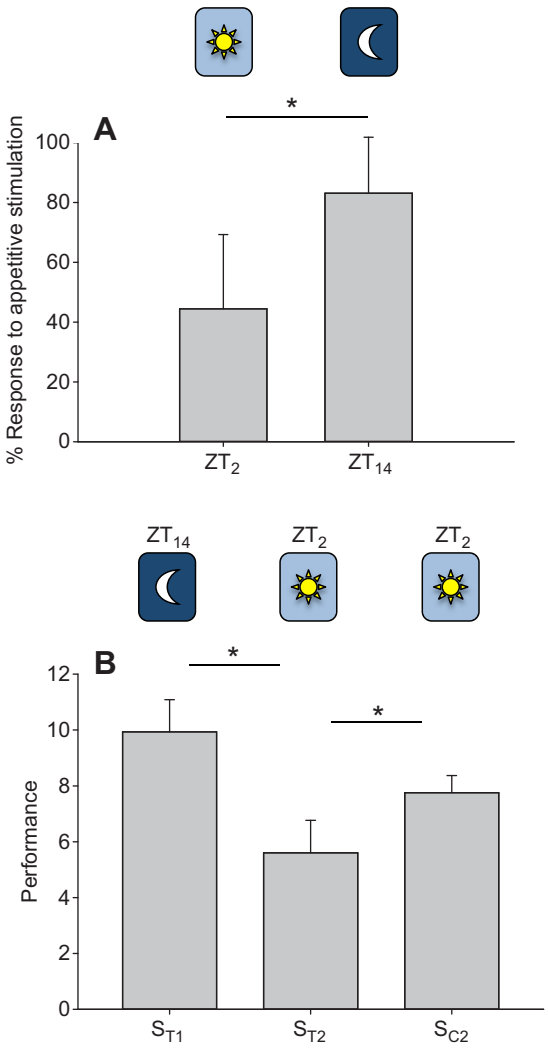

Fig. 3. Daily modulation of the response to heat stimulation. (A) Percentage of responding individuals to an appetitive stimulation (35°C), when tested at either ZT2 (*N*=54) or ZT14 (*N*=71). (B) Performance of *R. prolixus* larvae, represented as the mean number of trials that were necessary to observe a complete disappearance of the response to three consecutive stimulations. Data were corrected by excluding from analysis insects that did not respond to heat at ZT2. Each bar represents either the trained group during its first (S_{T1}; ZT14) or second session (S_{T2}; ZT2) or the associated control group (S_{C2}; ZT2) (*N*=15 for trained group and *N*=12 for control group). *Significant difference between groups/sessions (*P*<0.05).

we quantified the proportion of naive insects responding to appetitive stimulation (35°C) at ZT2 and ZT14.

We found that insects responded significantly more during the early scotophase (83.1% at ZT14, *N*=71, versus 44.4% at ZT2, *N*=54; Fisher's exact test, *P*<0.0001; Fig. 3A), confirming previous results (Fresquet and Lazzari, 2014).

Thus, in order to discriminate between (1) the effect of a modulation of the heat response and (2) an actual effect of training in bugs trained at ZT14 and tested at ZT2, the insects that did not respond at all during the second session (ZT2) were excluded from the analysis, retaining only those that were motivated to respond to heat (i.e. insects that responded by extending their proboscis during at least one of the first two stimulations). After correcting the data, the differences between S_{T1} and S_{T2} and those between S_{T1} and S_{C2} remained significant (S_{T1}: 9.9±1.1 trials; S_{T2}: 5.6±1.1 trials; S_{C2}: 7.7±0.6 trials; Wilcoxon test: S_{T1} versus S_{T2}, *P*=0.0015 and S_{T2} versus S_{C2}, *P*=0.0034; Fig. 3B), revealing a real effect of training and discarding any artefact due to the modulation of the responses to heat itself.

Assessing the nature of the rhythm

To assess the effect of the circadian system in the modulation of learning ability, two groups of insects were deprived of temporal cues by being placed under constant darkness (D:D). The first group was trained at circadian time (CT)14 and tested 23 h later (Fig. 4A). In this case, bugs required significantly fewer trials during the second session than during the first (S_{T1}: 5.7±0.6 trials; S_{T2}: 3.2±0.5 trials; Wilcoxon test, *P*=0.0002) and fewer trials than untrained insects (S_{C2}: 5.1±0.3 trials; Mann–Whitney test, *P*=0.0021), revealing a clear effect of training. No difference was observed between the performance of untrained (S_{C2}) and naive bugs (S_{T1}) (Mann–Whitney test, *P*=0.86).

The second group was trained at CT2 and tested 23 h later (Fig. 4B). This time no effect of training was evinced (S_{T1}: 4.8±0.4 trials; S_{T2}: 5.2±0.6 trials; Wilcoxon test: *P*=0.76), revealing that the modulation was maintained even in the absence of a Zeitgeber.

DISCUSSION

These results demonstrate that learning and memory in the blood-sucking bug *R. prolixus* are modulated by the circadian system, and that their effective conditioning is strongly dependent on circadian phase. Indeed, when trained and tested during the scotophase, bugs were able to use their individual experience (training) in order to stop responding more rapidly during the second session and thus avoid punishment. Conversely, when trained and tested during the photophase, no effect of training was observed.

However, the processes of learning and memorisation are composed of several distinct steps (i.e. acquisition, consolidation, retention, retrieval and performance; Margulies et al., 2005) on which the circadian system could have an impact. In the experimental paradigm that was developed here, two hypotheses were tested: the different performances observed at night and during the day were either due to a deficit in the first steps of the learning process (i.e. from acquisition to retention) or to a deficit in the last steps of memory recalling (i.e. retrieval and performance). Results obtained with bugs trained at ZT14 and tested at ZT2, and vice versa, rejected the first hypothesis as, when trained at ZT14, *R. prolixus* performed equally well in the early photophase and early scotophase. Congruently, when trained at ZT2, bugs failed to perform in both the early photophase and the early scotophase. In other words, these results revealed that (1) the ability to recall learned information and use memories to adjust behaviour was independent of circadian time, and (2) that training time was not a necessary contextual cue required for performance (time stamping).

In the present study, daily modulation might have also occurred on another level, changing the response to thermal stimulation. Indeed, the results presented in Fig. 3 show a clear fall in the percentage of insects extending their proboscis during the early photophase. When both training and testing occurred at ZT2, this modulation did not bias the data as only insects that were motivated or able to respond to heat during the photophase were selected. However, in the group of insects trained at ZT14 and tested at ZT2, the selection was made at ZT14, when non-responding bugs were discarded. Thus, when tested at ZT2, some insects might have displayed fewer responses not because of training, but simply because they were less motivated or less sensitive to respond than at ZT14. To evaluate this alternative explanation, we removed from the analysis insects that did not respond at all during the second session (i.e. at ZT2), keeping only

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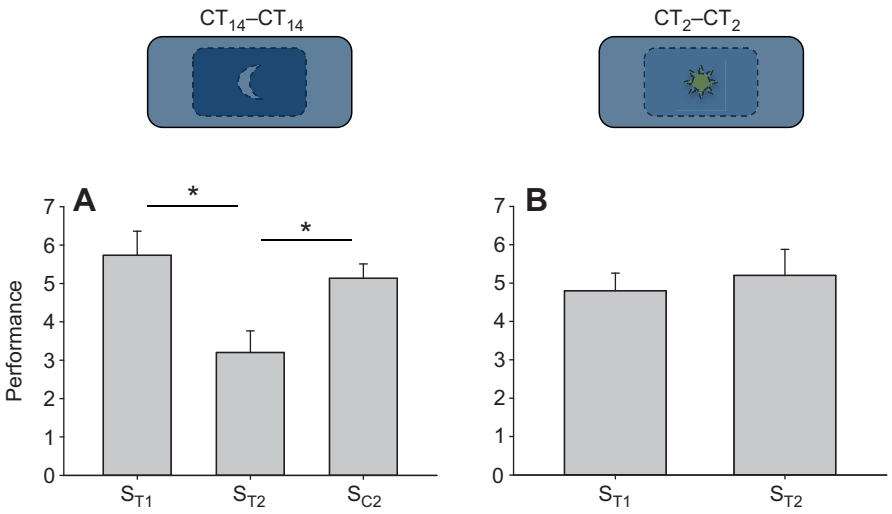

Fig. 4. Characterisation of the endogenous nature of the rhythm. Performance of *R. prolixus* larvae, represented as the mean number of trials that were necessary to observe a complete disappearance of the response to three consecutive stimulations when maintained under constant darkness. CT, circadian time. Each bar represents either a trained group during its first (S_{T1}) or second session (S_{T2}) or the associated control group (S_{C2}) when indicated. (A) Trained and tested at CT14, 23 h retention (see Materials and methods, 'Assessing the nature of the rhythm') (trained group: *N*=15; control group: *N*=15). (B) Trained and tested at CT2, 23 h retention (trained group: *N*=15). *Significant difference between groups/sessions (P<0.05).

the bugs that displayed motivation to respond to thermal stimulation at both ZT2 and ZT14 (i.e. insects that displayed a PER during at least one of the first two trials). This correction of the data revealed a true effect of training. Thus, the greater difference that was observed before correction was due to the cumulative effects of learning and of the modulation of the heat response.

To assess the involvement of an endogenous clock, we repeated the first experiment but this time the bugs were kept under constant conditions to let the circadian system run freely. Our results indicate that the rhythm in learning and memory performance is under the control of an endogenous oscillator. The persistence of the rhythm under constant darkness demonstrates that it is self-sustained and therefore endogenous, i.e. truly circadian. To our knowledge, this is the first demonstration of such circadian modulation of cognitive ability in a disease vector.

We also analysed the bugs' responses to heat under constant darkness. In these conditions, 65% of bugs extended their proboscis when stimulated at 35°C during the subjective photophase (*N*=23), whereas 86% responded during the early scotophase (*N*=35). This difference was less pronounced than under a 12 h:12 h L:D illumination regime and rendered only marginally significant results (Fisher's exact test, P=0.06).

Furthermore, it is worth highlighting that the two time points investigated in the present work were chosen because ZT14 (or CT14) and ZT2 (or CT2) correspond to the main activity peaks of *R. prolixus* (i.e. when bugs leave their shelters and start looking for a host to feed on and when they start responding to aggregation pheromones to return to the shelters, respectively), peaks separated by 12 h. This does not mean that these points correspond to the peak and trough in learning performance. Nevertheless, the persistence of higher learning performance during the subjective night relative to the subjective day, in the absence of external Zeitgeber, constitutes a basis to argue that a circadian clock is modulating PER inhibition learning. This interpretation is consistent with the biologically relevant temporal organisation of triatomine bugs – a salient characteristic of the biology of triatomines being its marked temporal organisation, largely regulated by endogenous circadian clocks (for review, see Lazzari et al., 2013). Selective pressures have acted to adjust the biting activity of blood-sucking insects to the time of the day when hosts are less active (Barrozo et al., 2004).

In the cockroach *R. maderae*, a similar temporal modulation of learning was observed when the animals were subjected to classical conditioning (Decker et al., 2007) but not when operant conditioning methods were employed (Garren et al., 2013). In the present work, the conditioning procedure that was used is close to operant conditioning as bugs learned the relationship between their own behaviour (i.e. appetitive PER) and a negative experience (thermal shock).

Regarding the adaptive value of the temporal modulation of learning ability, as formulated by Decker et al. (2007), memories are only beneficial when formed in the environmental (including temporal) context in which they will be used. A non-insect example of this is the case of the diurnal *Aplysia californica*, which performed significantly better when trained and tested during the subjective day, compared with animals trained and tested in the subjective night, in contrast to the nocturnal species, *Aplysia fasciata*, that demonstrated significant long-term memory when trained and tested during the night (Lyons et al., 2005). In both cases, circadian clocks modulate memory formation in phase with the animals' activity period. Similar observations were made in mice trained in either the day or night using different fear conditioning protocols. The mice acquired conditioning faster in the day than in the night and the recall peaked during the day for at least 3 days after training, irrespective of the time of training (Chaudhury and Colwell, 2002).

In the case of triatomine bugs, which are nocturnal insects, environment and activity are highly periodic and mostly controlled by circadian clocks (Ampleford and Steel, 1982; Constantinou, 1984; Lazzari, 1991, 1992; Lorenzo and Lazzari, 1998; Reisenman et al., 1998, 2002; Minoli and Lazzari, 2003; Barrozo et al., 2004; Bodin et al., 2008; Lazzari et al., 2011). Thus, because this haematophagous species is almost exclusively active at night and spends the daytime at rest inside shelters, it would not be profitable to form memories of the daytime environment as they would influence the bugs' activities based on information obtained at a time and in an environment irrelevant for the insect. Furthermore, as suggested by Decker et al. (2007), those memories would interfere with successful foraging during night conditions. Besides, the molecular and neurobiological processes that are involved in memory acquisition do not seem to be costless (Burger et al., 2008; Mery and Kawecki, 2003). The formation of new memories implies spending a non-negligible amount of energy and a cost in terms of neural tissues and connections devoted to the storage of information. Thus, to avoid forming new memories at times when it would not be relevant is also adaptive in terms of energy.

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In the context of the study of the cognitive ability of disease vectors and their epidemiological impact, the importance of the temporal context when dealing with learning and memory is highlighted by our results. Past failure in this field of investigation may have resulted from a lack of control regarding the time of day during which experiments were conducted. Not only do these results add another element to explain the high evolutionary success of triatomine bugs and their efficiency as disease vectors but also they help us to understand the mechanisms controlling learning and memory in haematophagous insects.

MATERIALS AND METHODS

Insects

Fifth instar *R. prolixus* larvae were used throughout the experiments. Bugs were reared in the laboratory under a 12 h:12 L:D illumination regime, at 25±2°C and 50–70% relative humidity (RH). Insects were fed weekly on heparinised sheep blood, using an artificial feeder (Núñez and Lazzari, 1990). Fourth instar larvae were blood fed and, after moulting, fifth instar larvae were isolated in individual plastic containers and starved until being tested 15 days after their moult.

Experimental apparatus

Insects were tethered by their dorsal thorax to a stiff steel wire, using double-sided adhesive tape, in an experimental room in which the temperature was kept at 25±2°C. A Styrofoam ball was placed between their legs to provide tarsal contact and reduce unnecessary stress. A Peltier element (4×4 cm, 12 V, 72 W; QuickCool, Wuppertal, Germany), representing an accurate and controllable (Peltron GmbH Peltier-Technik) heat source, was placed in front of the animals, at a distance at which they could touch the surface while extending their proboscis. This element allowed rapid temperature changes of the surface that was presented to the insects, which we used to display an appetitive heat source (35°C) or deliver negative reinforcement (50°C) (Fig. 1).

The efficiency of the Peltier element was improved by a water-cooling system placed on the back of the apparatus to cool down the dissipated heat. The temperature of the Peltier element could thus switch from 25 to 35°C and from 35 to 50°C in less than 1 s. A thermal sensor was placed in contact with the Peltier element and used to control the temperature of the device.

The assays were monitored via an infrared-sensitive camera provided with an array of infrared LEDs (emission 900 nm). This light illuminated the scene without being perceived by the bugs (Reisenman et al., 1998) and allowed us to observe proboscis movements in more detail.

PER conditioning paradigm

We carried out aversive conditioning experiments as described in Vinauger et al. (2013). Insects were first offered an appetitive stimulus (35°C stimulation), which evoked the PER, and then subjected to a 50°C thermal shock, which inhibited the PER. After a few trials, insects learned the association and stopped responding to the appetitive stimulus.

The temperatures used in these experiments correspond to the thermal preferences and tolerances of these insects (Okasha, 1968a,b; Schilman and Lazzari, 2004). The appetitive stimulus temperature was fixed at 35°C because this corresponds to the host's skin surface temperature and has been shown to elicit the PER in this species (Vinauger et al., 2013). For the negative reinforcement, 50°C represents an aversive but not lethal temperature, and is ecologically relevant as such temperatures can be encountered by the insects in their natural environment (temperatures above 60°C can be observed inside and outside houses, with objects such as cooking elements or stones exposed to sunlight, etc.).

Experimental procedures

Trained groups

At the beginning of all experiments, insects were placed individually in the experimental apparatus for a 30 s familiarisation period.

Each trained group was confronted with two training sessions, S_{T1} and S_{T2}. During each session, bugs were submitted to several trials, each of which consisted of: (a) appetitive stimulation (35°C) for 10 s; (b) in the case of a PER, i.e. if insects responded to the thermal stimulation, a heat shock was delivered to the extended proboscis at the end of the 10 s period, by increasing the temperature of the Peltier element up to 50°C, until the animal withdrew its mouthparts (less than 1 s); if no PER was displayed at the end of the 10 s stimulation, insects did not receive the negative reinforcement; and (c) an inter-trial interval of 50 s.

For each session, insects that did not respond by extending their proboscis during the first two stimulations were considered not motivated to feed and thus discarded from the analyses. A PER was recorded when the proboscis was fully extended, i.e. when it was positioned horizontally, making a 180 deg rotation from its original position. The occurrence or absence of the PER at 35°C was used to determine the percentage of insects responding to heat. Each individual was repeatedly confronted with stimulation trials until complete disappearance of the response, i.e. three successive trials without any PER.

Untrained control groups

If bugs stopped responding more rapidly in the second test session than in the first one ($S_{T1}>S_{T2}$), a control group was run in parallel to the corresponding trained group. Control individuals were handled in an identical manner, but not trained during the first session (S_{C1}), i.e. they were placed in the set-up and exposed to the Peltier element at a constant temperature of 25°C, during a time equal to the average duration of a training session (determined as the time necessary to observe a complete disappearance of the PER in the respective trained group, S_{T1}). Insects from the control groups were then subjected to a second session (S_{C2}), as for the associated trained groups (S_{T2}) (Fig. 1A,B). The aim of these untrained control groups was to discard any effect of contextual pre-exposure on their performance during S_{T2}.

Conditioning experiments

Effect of daytime on the ability to acquire and recall memory

One group of insects (group 1) was trained and tested 24 h later at ZT14 (i.e. early scotophase); a second group (group 2) was subjected to the same procedure at ZT2 (i.e. early photophase).

Effect of daytime on the ability to acquire or to recall memory

To test whether modulation of the ability to learn new information, the ability to recall learned information or both occur, we trained one group of insects during the first part of the subjective night and tested them 12 h later, during the first part of the subjective day (group 3, ZT14–ZT2). In the same way, we trained another group of insects during the first part of the day and tested them 12 h later, during the first hours of the night (group 4, ZT2–ZT14).

Daily modulation of the behavioural response to heat stimulation

To discard any interference from the temporal modulation of the response to heat, we also quantified the proportion of insects responding to appetitive stimulation (35°C) at two different Zeitgeber times: ZT2, i.e. the beginning of the day, and ZT14, i.e. the beginning of the night.

Assessing the nature of the rhythm

Insects were maintained for 3 days under a 12 h:12 h L:D regime and then placed under constant darkness (D:D) for another 3 days. Training occurred on the second day of constant darkness and testing on the third day of constant darkness. Experiments under D:D regimes were conducted with a dim far-red light (λ>640 nm, 12 µW cm^{-2}), which was on continuously, in order to provide sufficient light to see animals when the main lights were off. It has been shown in earlier studies of ecdysteroid-sensitive rhythms in *R. prolixus* that this dim far-red light constitutes functional darkness (Ampleford and Steel, 1982; Vafopoulou and Steel, 1998). The rationale behind the use of functional darkness is to avoid – as much as possible – giving any Zeitgeber to bugs (Ampleford and Steel, 1982).

One group of insects was trained and tested at the beginning of the subjective night (CT14) and another group at the beginning of the subjective day (CT2). The time of test periods was adjusted to 23 h post-training in order 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.

Data analysis

The learning performance of individual insects was quantified by determining the number of trials that were necessary to observe the complete disappearance of the PER in three successive trials (Braun and Bicker, 1992). Binary data (1=behaviour observed and 0=behaviour not observed) were collected and the proportion (P) of the bugs showing a response to an appetitive stimulus (35°C) was calculated. The standard deviation (s.d.) was calculated according to the following formula for binary data: s.d.=$[P(1-P)]^{1/2}$.

Mean performance was then calculated for each group. Wilcoxon tests for paired data were used to compare performance between training sessions (S_{T1} versus S_{T2}), and comparison between the performance of trained and control groups (S_{T1} or S_{T2} versus S_{C2}) was made using Mann–Whitney tests for independent data. The number of insects responding to an appetitive thermal stimulation at ZT2 and ZT14 was compared by means of Fisher's exact test. Significance was considered at α=0.05.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

C.V. and C.R.L. designed the experiments. C.V. conducted the experiments. C.R.L. supervised the project and both authors prepared the manuscript and contributed to revising the manuscript.

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