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RESEARCH ARTICLE

Learning and memory in *Rhodnius prolixus*: habituation and aversive operant conditioning of the proboscis extension response

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SUMMARY

It has been largely accepted that the cognitive abilities of disease vector insects may have drastic consequences on parasite transmission. However, despite the research effort that has been invested in the study of learning and memory in haematophagous insects, hitherto few conclusive results have been obtained. Adapting procedures largely validated in *Drosophila*, honeybees and butterflies, we demonstrate here that the proboscis extension response (PER) of the haematophagous insect *Rhodnius prolixus* can be modulated by non-associative (habituation) and associative (aversive conditioning) learning forms. Thermal stimuli were used as both unconditional stimulus (appetitive temperatures) and negative reinforcement (thermal shock). In the first part of this work, the PER was habituated and dishabituated to thermal stimuli, demonstrating the true central processing of information and discarding motor fatigue or sensory adaptation. Habituation was revealed to be modulated by the spatial context. In the second part, bugs that were submitted to aversive operant conditioning stopped responding with PER to thermal stimulation more quickly than by habituation. They were able to use their training experience when tested up to 72 h later. Our work constitutes the first demonstration of PER habituation and conditioning in a blood-sucking insect and provides reproducible experimental tools for the study of the mechanisms underlying learning and memory in disease vectors.

Key words: haematophagous insect, cognitive ability, memory retention, disease vector.

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INTRODUCTION

In some insect species feeding on liquid food, mouthparts have evolved towards a tubular feeding and sucking organ, known as the proboscis, latinization of the Greek *proboskis*, which comes from *pro* 'forth, forward, before' and *bosko*, 'to feed, to nourish'. The organization of the different mouthparts (i.e. mandibulae, maxillae, etc.) that form this proboscis varies across insect groups. It usually remains retracted (flies, honeybees), rolled (butterflies) or folded (bugs) and thus, to obtain their food, insects equipped with a proboscis extend it in a stereotyped behaviour, referred to as the proboscis extension response or PER. This behavioural response to food-related signals has been widely used in insect gustative physiology studies (Frings, 1941; Frings, 1944; Hayes and Liu, 1947; Grabowski and Dethier, 1954) and turned out to be a key paradigm in the study of the behavioural and cognitive plasticity of insects (Takeda, 1961; Bitterman et al., 1983; Giurfa and Sandoz, 2012).

Major advances in this field of knowledge were made in the honeybee *Apis mellifera* by means of classical appetitive conditioning procedures (Bitterman et al., 1983; Menzel and Muller, 1996; Erber et al., 1997; de Brito Sanchez et al., 2005) and also more complex conditioning forms (e.g. second-order conditioning, differential conditioning, etc.) (Deisig et al., 2002; Giurfa and Malun, 2004; Châline et al., 2005; Giurfa and Sandoz, 2012). In dipterans, the fruit fly *Drosophila melanogaster* also constitutes an excellent model to unravel the mechanisms of learning and memory by means of aversive conditioning of PER (Vaysse and Médioni, 1976; DeJianne et al., 1985) or aversive olfactory conditioning (Holliday and Hirsch, 1986; Fresquet, 1999; Chabaud et al., 2006; Busto et al., 2010).

In haematophagous insects, blood feeding consists of accessing fluid that is hidden under the host skin. To do so, blood-sucking insects have to locate blood vessels and extend their proboscis to reach them by piercing the skin (Ferreira et al., 2007). Host approaching is achieved thanks to behavioural responses to olfactory and thermal signals (Lehane, 2005), whereas biting is mediated solely by thermal cues (Ferreira et al., 2007; Lazzari, 2009). The feeding success of a blood-sucking insect depends on the ability of a given host to defend itself from biting, making this task a dangerous one. Thus learning to recognize the less defensive hosts (i.e. the easiest to feed on) would be very adaptive and one would expect to observe well-developed cognitive abilities in these insects as well (McCall and Kelly, 2002; Alonso et al., 2003). Furthermore, it has been largely accepted that learning and memory are two key factors that explain the heterogeneous distribution of vectors among host species and populations (Kelly and Thompson, 2000; Kelly, 2001; McCall and Kelly, 2002). In terms of epidemiology, such heterogeneities in the biting strategies of insects mean heterogeneities in the transmission of infections agents. Woolhouse et al. (Woolhouse et al., 1997) suggested that 20% of the host population contributes 80% of the net transmission potential. In other words, learning and memory are two factors participating in the creation of extreme transmission 'hot spots' and 'cold spots' (Kelly, 2001).

Consequently, an important research effort has been invested so far to study the cognitive abilities of blood-sucking insects in the laboratory, as well as in the field. Unfortunately, only few studies have provided clear experimental demonstrations of learning and memory in haematophagous insects. Alonso and Schuck-Paim

(Alonso and Schuck-Paim, 2006) present a critical analysis of the evidence. Most available studies were conducted under natural or partially controlled conditions (Mwandawiro et al., 2000; McCall and Eaton, 2001; Bouyer et al., 2007) that render an insight into underlying mechanisms difficult. Standardized and practical methodological tools need to be developed for the study of learning and memory in this group of insects. It is worth mentioning that methods validated in sugar-feeders cannot be directly transferred, because of specific constraints imposed by haematophagy (Vinauger et al., 2011a).

In triatomine bugs, vectors of the Chagas disease, the responses of *Rhodnius prolixus* to a single olfactory stimulus can be modified by either appetitive or aversive conditioning (Vinauger et al., 2011a; Vinauger et al., 2011b). Similarly, their host preference has been demonstrated to be under the influence of previous individual experience (Vinauger et al., 2012). These studies aimed at testing the ability of these insects to learn information about their hosts. They were thus designed to place the insects in an experimental context that was as favourable as possible for the observation of learning abilities, but not to allow a rapid and detailed analysis of learning and memory processes.

The general biology and physiology of *R. prolixus* have been relatively well described, including its appetitive PER to thermal stimuli whose temperature corresponds to that of the skin surface of potential vertebrate hosts (Fresquet and Lazzari, 2011). Furthermore, heat constitutes the only necessary and sufficient signal to trigger the PER (Flores and Lazzari, 1996). Here we explored the possibility of using PER in learning bioassays in order to facilitate controlled and standardized studies on learning and memory in *R. prolixus*. Specifically, we conducted two series of experiments aimed at characterizing two distinct forms of learning, habituation and aversive operant conditioning.

MATERIALS AND METHODS

Insects

Fifth-instar larvae of *Rhodnius prolixus* Stål 1859 were used throughout the experiments. Bugs were reared in the laboratory under a 12 h:12 h light:dark illumination regime, at 27±2°C and 60–70% relative humidity (RH). Insects were fed weekly on sheep heparinized blood, using an artificial feeder (Núñez and Lazzari, 1990). Fifth-instar larvae that had just moulted 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 whose temperature was kept at 25±2°C (Fig. 1). A Styrofoam ball was placed between their legs in order to provide tarsal contact and reduce, in this way, stress. A Peltier element (4×4 cm, 12 V, 72 W, Conrad, Lille, France) coupled to a controller (Peltron, Fürth, Germany) (Fig. 1), representing an accurate and controllable heat source, was placed in front of the animals, at a distance from which they could reach and contact the Peltier surface with the tip of their extended proboscis. The Peltier element allowed rapid temperature changes of the surface presented to the insects. In this way, we could display an appetitive heat source, apply a negative reinforcement, or maintain the Peltier at room temperature. The efficiency of the Peltier element was improved by a water cooling device that dissipated heat from the backside. Thus the temperature of the Peltier element could switch up and down very quickly (Δ25°C in less than 1 s). A thermal sensor was placed in contact with the Peltier element and used to monitor the temperature of the device. A thermographic camera allowed measurement of the dynamics of the temperature changes. The assays were monitored with the aid of a small charge coupled device (CCD) camera provided with a macro lens to observe proboscis movements in great detail.

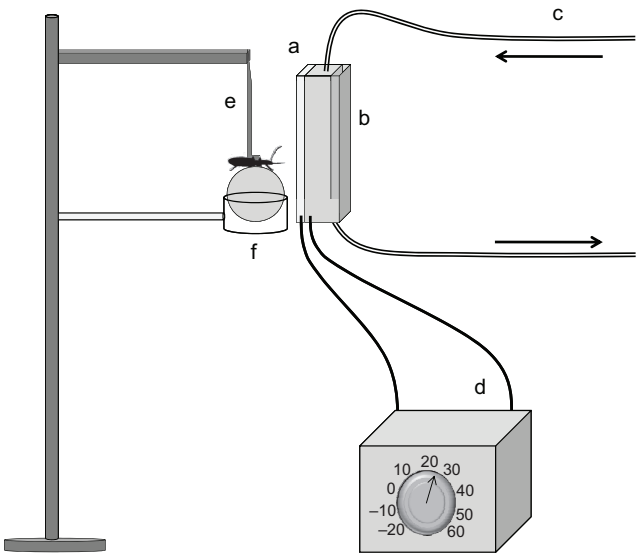

Fig. 1. Experimental device used for training the PER of *Rhodnius prolixus*. It allows the delivery of thermal stimulation. a, Peltier element; b, aluminium heat-dissipating block; c, enclosed water based cooling; d, Peltier control unit; e, steel wire; f, Styrofoam sphere (1 cm diameter).

Two distinct series of experiments were conducted in order to study two different forms of learning, i.e. habituation and aversive operant conditioning. In the first series, the habituation of the PER along successive stimulation at 35°C was studied. In the second series, we studied whether or not bugs learn to inhibit PER induced by an appetitive thermal stimulus (35°C) upon receiving an aversive heat shock (50°C) after proboscis extension. In both experiments, the temperatures were chosen according to our knowledge on the response of bugs to thermal sources (Fresquet and Lazzari, 2011). The appetitive temperature, of 30 or 35°C, depending on the experiment, roughly corresponds to the temperature at the surface of the host skin. The aversive temperature, of 50°C, is not harmful but represents objects too hot to be a natural host.

At the beginning of each experiment, bugs were placed individually in the device and familiarized for 30 s with the experimental situation. During this period, the temperature of the Peltier element was fixed at 25°C, corresponding to the room temperature. The bugs were then submitted to several successive trials, separated by 50 s inter-trial intervals (ITI). During trials, the occurrence or absence of the PER was noted, and the percentage of insects responding to appetitive heat stimulation was calculated. In both kinds of experiments, habituation and aversive conditioning, each individual was repeatedly submitted to trials until complete disappearance of the response, i.e. until no PER was visible during three consecutive trials.

Insects that did not respond to appetitive heat stimulation during the first two trials were considered as not motivated and were discarded from analyses. A PER was counted when the proboscis was fully extended, i.e. when displaying an angle of 180 deg from its initial position.

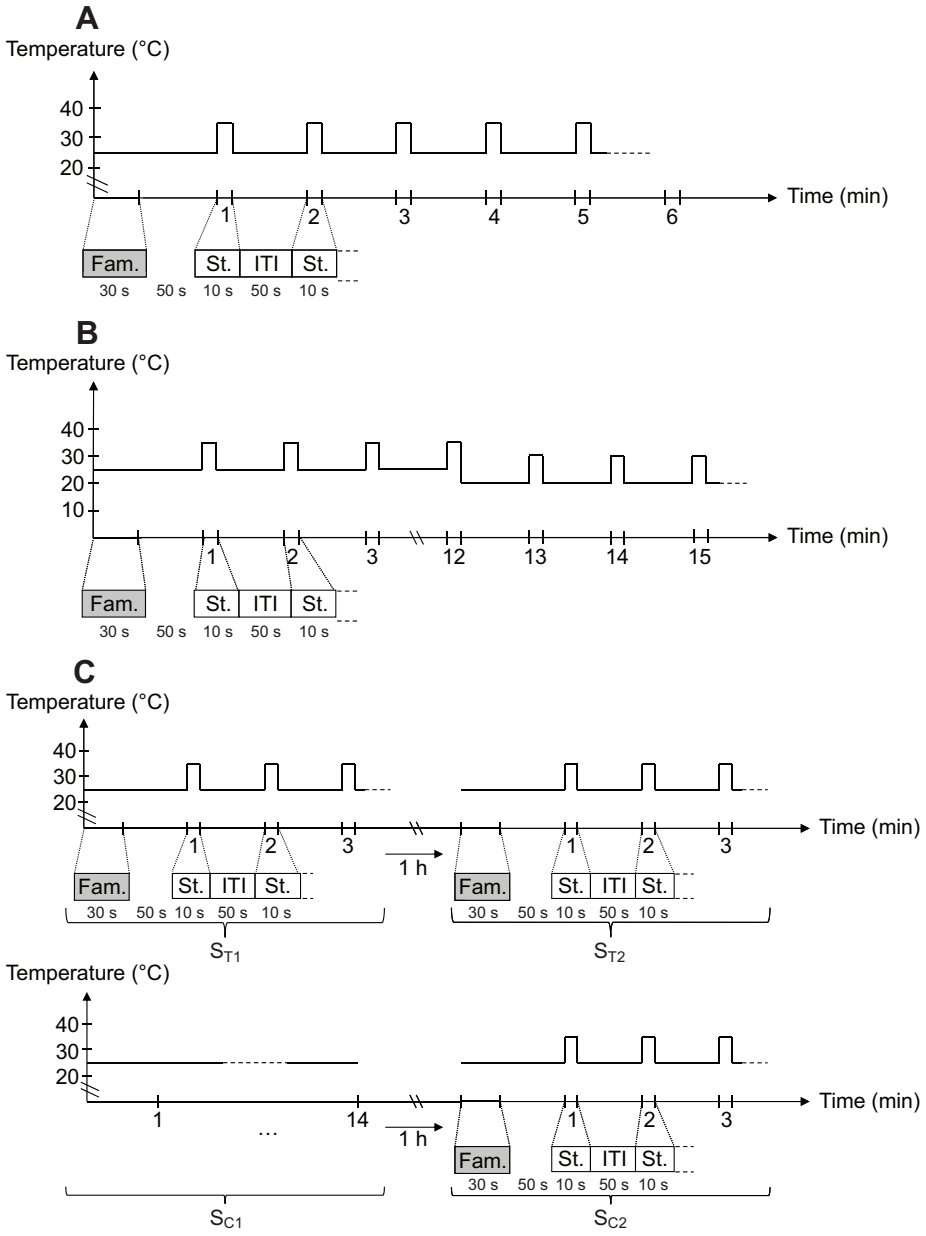

Fig. 2. Sequence of event delivery (i.e. appetitive thermal stimulation and inter-trial interval) during training sessions of the different experimental groups: (A) PER habituation; (B) PER dishabituation; (C) retention and context effect. Fam., familiarization period; St., stimulation; ITI, inter-trial interval; 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.

Habituation

Three experiments were carried out (Fig. 2).

Experiment 1a: PER habituation

Each individual was placed in the experimental device while the Peltier was at room temperature (25°C). After the familiarization period, the bug was submitted to successive trials during which the temperature of the Peltier was increased to 35°C over 10 s. Trials were separated by a 50 s ITI during which the Peltier was brought back to room temperature (Fig. 2A). Insects remained in the device until the end of the session, i.e. until complete disappearance of the response to the appetitive stimulus.

In order to test if the PER disappearance was due to peripheral (sensory adaptation or motor fatigue) or central (habituation) processes, a dishabituation experiment was conducted (Fig. 2B). The first 12 trials of this experiment were similar to the habituation procedure (i.e. room temperature at 25°C and a stimulation at 35°C over 10 s), then, from the 13th trial on, both the thermal stimulus and the temperature of the Peltier during ITIs were modified to 30 and 20°C, respectively. The other parameters were kept unchanged.

The choice of the 13th trial to begin the dishabituation period was made according to the mean number of trials that were necessary to observe the habituation of the PER during the first experiment. The habituation and dishabituation phases of the experiment were only separated by the duration of an ITI (i.e. 50 s).

Experiment 1b: retention experiment

To test whether or not habituation gives place to a mnesic process, we tested the influence of a first habituation session (trained group, session 1: S_{T1}) on the performances during a second habituation session (trained group, session 2: S_{T2}) performed 1 h later. Procedures and temperatures were the same as in Experiment 1a.

Performances were compared with control groups that were not trained during the first session (control group, session 1: S_{C1}), but equally manipulated and kept in the same context, to be tested 1 h

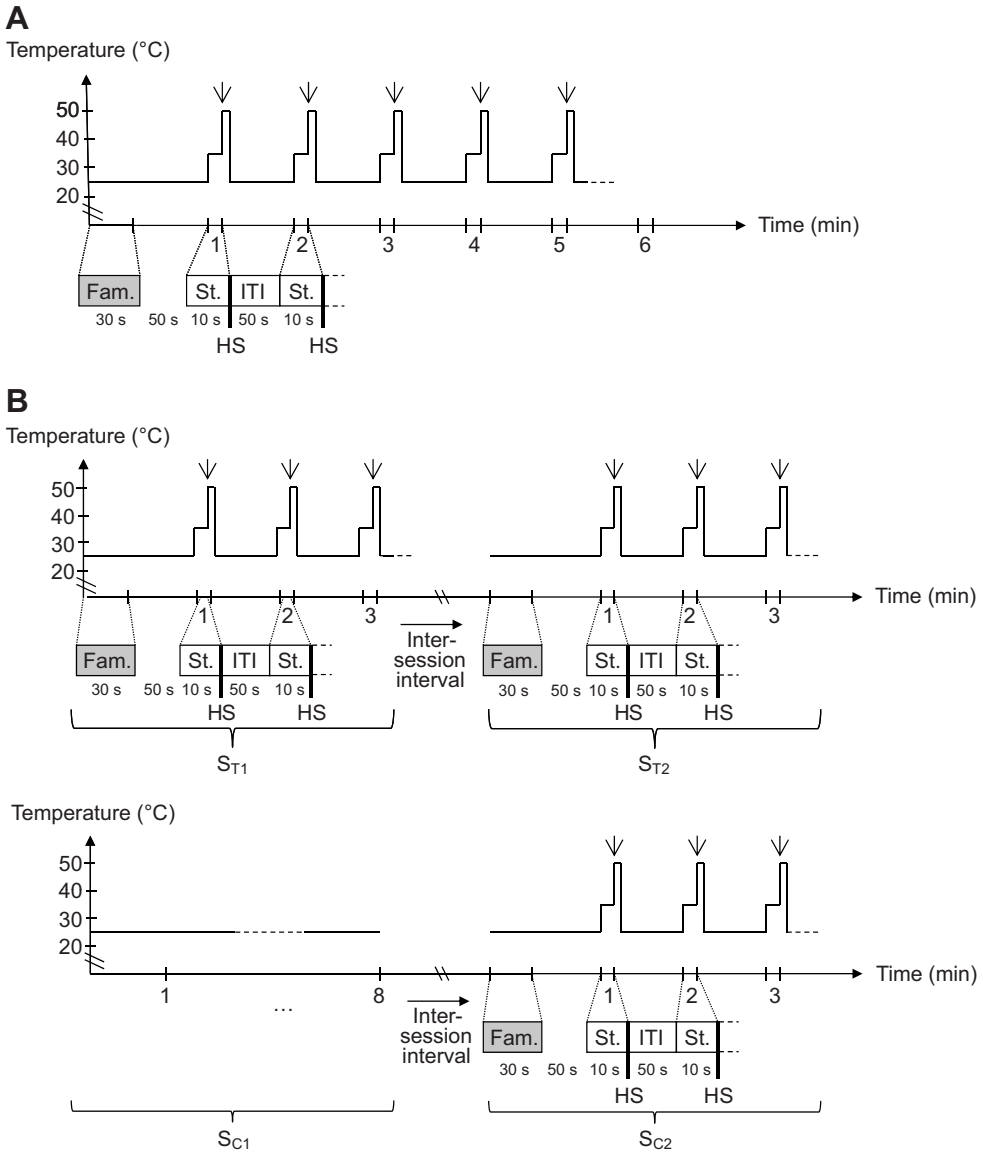

Fig. 3. Sequence of event delivery (i.e. appetitive thermal stimulation and inter-trial interval) during training sessions of the different experimental groups: (A) aversive conditioning of PER; (B) retention experiments. Fam., familiarization period; St., stimulation; ITI, inter-trial interval; HS, 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.

later (control group, session 2: S_{C2}) (Fig. 2C). During the first session (S_{C1}), the Peltier element was kept at room temperature (25°C) all the time.

Experiment 1c: context influence

The influence of the experimental context on memory retention was tested in this experiment. Two groups of insects were used as in Experiment 1b: trained and control groups. Procedures were identical to the retention experiment (Fig. 2C) except that during the first session (S_{C1}) the control group was not exposed to the same environmental context, but placed in darkness in an opaque plastic jar (5 cm height, 3 cm diameter), with a small piece of paper between their legs, instead of the polystyrene sphere. The duration of this first session was set as the mean time necessary to observe a complete habituation of PER in the first session of the trained group (S_{T1}).

As in the retention experiment, the first and second sessions were separated by 1 h for both trained and control bugs.

In all cases, between training and test sessions, insects were placed in individual plastic jars and brought back to the rearing room.

Aversive operant conditioning

We carried out aversive operant conditioning in order to test the ability of *R. prolixus* to inhibit PER triggered by an appetitive stimulus when this response was immediately followed by aversive heat reinforcement. Three experiments were carried out (Fig. 3).

Experiment 2a: aversive operant conditioning of PER

Bugs of this experimental group were submitted to repeated conditioning trials, after a 30 s familiarization period (Fig. 3A). Each trial consisted of: (1) appetitive stimulation (35°C) over 10 s; (2) in case of PER, i.e. insects responding to the appetitive 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 to 50°C. If no PER was displayed, at the end of the 10 s stimulation, insects did not receive any reinforcement. Trials were separated by an ITI of 50 s.

Insects reacted to the heat shock by retracting their proboscis and by displaying stress-associated behaviours (e.g. rapid movements of legs, head and antennae).

Once the proboscis was folded back, the temperature of the Peltier was reduced to 25°C. Results were compared with those obtained in the habituation experiment (Experiment 1a) in order to assess the

influence of a negative reinforcement on learning performances and, in particular, on acquisition speed.

Either yoked or omission procedure should have been used as controls because of the operant nature of our conditioning protocol in which the negative reinforcement was contingent to the animal's response. The yoked procedure consists of the use of a second group of animals (i.e. yoked group) that is reinforced in association with the history of reinforcement experienced by a first experimental group. In this way, the reinforcement experience of the yoked animal is not necessarily contingent with their own response. This standard procedure is not possible here since there would be no way to stimulate with a heat shock a folded proboscis of yoked animals that remain distant from the heat source. The omission procedure consists of suppressing reinforcement (heat shock in this case) any time the animal produced the PER response. Conversely, reinforcement should be delivered only when the animal fails to respond. As the operant contingency would be suppressed, no operant learning should then occur. Again, here it would not be possible to achieve the later component (i.e. to deliver reinforcement when the animal fails to respond; see above). Thus since these procedures were not applicable in this experimental protocol, the only option left is not to deliver reinforcement when the animal responds. Assuming that the animal always responds with PER to the triggering stimulus of 30°C, what remains is thus a non-canonical comparison with the habituation experiment, the timing of triggering the thermal stimulus (30°C) being exactly the same in both experiments.

Experiment 2b: retention experiment

Trained groups of bugs underwent two sessions, S_{T1} and S_{T2}, following the same procedure as in Experiment 2a. Four trained groups were constituted in order to test: (1) if training influences the performance during a subsequent test session, and (2) the maximal retention time length (Fig. 3B). Thus for each group, training and test sessions were separated by a different time interval: 1, 24, 72 or 96 h.

As in habituation experiments, control groups were run in parallel to the corresponding experimental 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 at a constant temperature of 25°C, during the mean time of a training session (determined as the time necessary to observe complete disappearance of the PER in the respective trained groups, S_{T1}). Insects of the control groups were then submitted to a second session (S_{C2}), as the associated trained groups (S_{T2}) (Fig. 3B).

Data analysis

Learning performance of individual insects was quantified by determining the number of trials required to observe the disappearance of the PER in three consecutive trials (Braun and Bicker, 1992). A mean performance was then calculated for each group. Given that not all the samples were normally distributed, non-parametric statistics were used throughout. The Wilcoxon signed-rank test for paired data was used to compare performances between first and second sessions of the same group (S_{T1} *vs* S_{T2}) and the comparison between the performances of trained and untrained control groups (S_{T1} *vs* S_{C2} and S_{T2} *vs* S_{C2}) was made using the Mann–Whitney test for independent samples.

RESULTS

Habituation

Experiment 1a: PER habituation

Habituation of PER is represented in Fig. 4A. With the repetition of thermal stimulation, the percentage of bugs extending their proboscis in response to the appetitive stimulus progressively decreased through trials, down to zero. A mean (±s.e.m.) of 25.6±4.7 trials were necessary to observe a complete absence of PER during three successive trials (n=16).

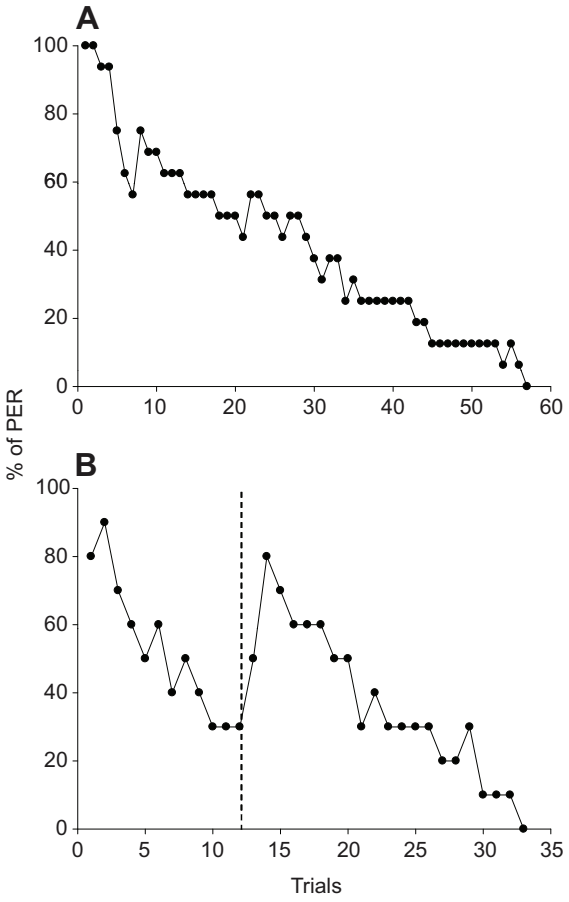

Fig. 4. Percentage of *Rhodnius prolixus* larvae responding to the appetitive stimulation (35°C) during trials. (A) Habituation of PER (N=16); (B) Dishabituation of PER (N=10).

To discard the influence of peripheral processes such as sensory adaptation or motor fatigue, we tested whether a change in the experimental parameters (i.e. stimulus and ITI temperatures) could restore the initial reactivity to appetitive thermal stimulation by dishabituation. First, insects of this experimental group were stimulated as in the previous experiment and displayed a typical habituation response during the first 12 trials (from 80% of PER at the first trial to 30% at the 12th; Fig. 4B; n=10). Then temperatures of stimulus and ITI were reduced from 35 to 30°C and from 25 to 20°C, respectively. We then observed that the percentage of responses increased to 50% at the 13th trial and 80% at the 14th trial, i.e. the same level of responsiveness as at the beginning of the habituation phase to gradually decrease afterwards.

These results demonstrate that the decrease in the response was due to true habituation rather than to peripheral processes, because the sensory receptors involved and the motor response were the same in both phases of the experiment.

Experiment 1b: retention experiments

Results are depicted in Fig. 5A. In the 1 h retention test, trained bugs required significantly fewer trials to stop responding to the stimulus

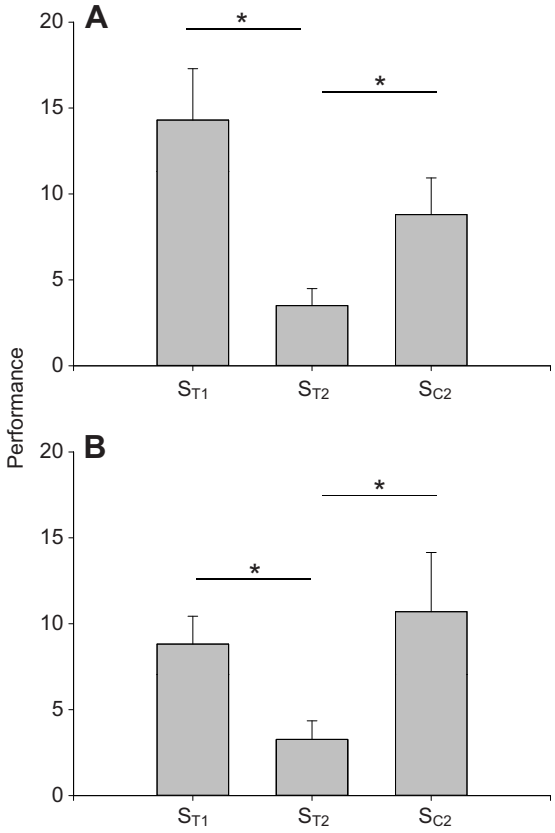

Fig. 5. Performances of *Rhodnius prolixus* larvae, represented as the mean number of trials that were necessary to observe a complete disappearance of the response. Each bar represents either a trained group during its first (S_{T1}) or second session (S_{T2}) or the associated control group (S_{C2}). (A) Results of the retention experiment (N=10 for trained and control groups); (B) Results of the context effect experiment (trained group: N=11; untrained group: N=10). *Significant differences (P<0.05).

during the second session than during the first session (S_{T1}: 14.3±3 trials; S_{T2}: 3.5±1 trials; Wilcoxon test: P=0.002; n=10). In addition, the number of trials required to observe a complete habituation was significantly different between the control group and the test session of the trained group (S_{T2} *vs* S_{C2}; n=10; Mann–Whitney test: P=0.019). The number of trials required by the control group (8.8±2.13 trials, S_{C2}) was different, although not statistically significant, from the performance of trained bugs during their first session (S_{T1} *vs* S_{C2}; Mann–Whitney test: P=0.074), suggesting that other factors, such as the spatial context, could play a role.

Experiment 1c: context influence

To test the potential context effect we performed a similar experiment but, this time, exposing control bugs during S_{C1} to a context that was different from that of the experimental group. Testing (S_{C2}) occurred in the same context as the experimental group, 1 h later. Results are depicted in Fig. 5B. Trained bugs displayed similar results as in the previous experiment and stopped responding sooner during the second session than during the first session (S_{T1}: 8.9±1.6 trials; S_{T2}: 3.5±1.1 trials; Wilcoxon test: P=0.006; n=11). However, this time no difference could be observed between performances of the control group (10.7±3.5 trials; n=10) and the first session of the trained group (S_{T1} *vs* S_{C2}; Mann–Whitney test: P=0.40). Furthermore, since insects of both groups (trained and control) were submitted to the same manipulations, and taking into account the fact that performances were significantly different between control and tested bugs (S_{T2} *vs* S_{C2}; Mann–Whitney test: P=0.006), we can discard a potential effect of manipulation on learning performances.

Aversive operant conditioning

Experiment 2a: aversive operant conditioning of PER

In comparison with the non-reinforced group, i.e. habituated group, we observed a more rapid decrease in the percentage of PER per trial in the conditioned group (Fig. 6). In both groups 100% of bugs extended their proboscis during the first trial. However, while they required a mean of 26 trials to observe a 50% reduction of the response in the habituation test (n=16), this decrease was observed at the fifth trial in the negatively reinforced group (n=16). Similarly, the mean number of trials that were necessary to observe a complete disappearance of the PER was significantly lower in the reinforced group (7.3±0.9 trials) than in the habituated group (25.6±4.7 trials; Mann–Whitney test: P=0.004). These results reveal that *R. prolixus* is able to associate its behaviour with a negative reinforcement and to stop responding in order to avoid heat shocks.

Experiment 2b: retention experiment

Memory was first tested 1 h post-training (Fig. 7A). The percentage of bugs responding with PER to thermal stimulation at the first trial was higher during the first session (S_{T1}: 100%) than during the second trial (S_{T2}: 33%). In the untrained group (S_{C2}), 80% of PER was observed during the first trial. Regarding the mean number of trials that were necessary to observe a complete disappearance of the PER, we observed that bugs stopped responding sooner in the second than in the first session (S_{T1} *vs* S_{T2} in Fig. 7A; Wilcoxon test: P=0.004; n=12). Furthermore, the significant differences between the performance of the trained insects and the untrained insect exposed to the same context during their test session (S_{T2} *vs* S_{C2} in Fig. 7A; Mann–Whitney test; P=0.009; n=10), revealed that a memory trace has been developed during the training phase as a consequence of the association between the PER and the thermal shock. In addition, the difference in performances between naïve insects and bugs just pre-exposed to the context was not significant (S_{T1} *vs* S_{C2} in Fig. 7A; Mann–Whitney test; P=0.076).

When the interval between training and testing was increased to 24 h, we still observed a significant effect of training on the performances during the second session (S_{T1} *vs* S_{T2}; Wilcoxon signed rank test: P=0.0054; n=14; Fig. 7B). Performance during the second session was also significantly better (i.e. required fewer trials) than the performance of untrained bugs (S_{T2} *vs* S_{C2} in Fig. 7B; Mann–Whitney: P=0.011; n=15). No significant difference was observed between naïve insects during their first session and bugs that were pre-exposed to the experimental context (S_{T1} *vs* S_{C2} in Fig. 7B; Mann–Whitney test: P=0.424), discarding any effect of context in this retention test.

Similar results were obtained when the retention was tested 72 h after training (Fig. 7C). Performances were improved by learning since insects required fewer trials during the second session than during the first session (S_{T1} *vs* S_{T2} in Fig. 7B; Wilcoxon test: P=0.019; n=12) and fewer trials than untrained bugs (S_{T2} *vs* S_{C2} in Fig. 7B; Mann–Whitney test: P=0.006; n=12). No significant difference was observed between performances of naïve insects (S_{T1}) and bugs pre-exposed to the experimental context (S_{T1} *vs* S_{C2} in Fig. 7C; Mann–Whitney test: P=0.53), discarding any context effect in this retention test.

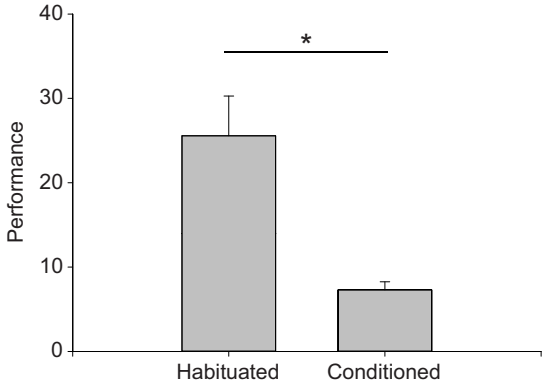

Fig. 6. Performances of *Rhodnius prolixus* larvae, defined as the mean number of trials that were necessary to observe a complete disappearance of the response, submitted to habituation (N=16) and aversive conditioning (N=16) procedures. *Significant differences (P<0.05).

Conversely, when tested 96 h after training, the difference between groups started to disappear. Indeed, no significant effect of training was observed on the performances of bugs 96 h post-learning (S_{T1} *vs* S_{T2} in Fig. 7B; Wilcoxon test: P=0.078; n=14; Fig. 7D).

DISCUSSION

The PER constitutes a main behavioural tool to study learning processes in *Drosophila*, honeybees and other insects. The exploitation of the PER in learning paradigms in blood-feeding models requires overcoming some major constraints associated with haematophagy. For instance, in nectar feeding insects, PER can be elicited via the direct contact of sugar solutions (unconditional stimulus, US) on taste receptors located in any part of their body (i.e. mouthparts, antennae, tarsi, etc.). It is then relatively easy to pair an odour or other stimulus (conditional stimulus, CS) with the US and test whether or not the insect has associated both stimuli and extends its proboscis only to the delivery of the CS. In haematophagous bugs, PER is only triggered by appetitive thermal stimulation (US). In other words, heat is the only stimulus both necessary and sufficient to evoke the PER (Flores and Lazzari, 1996), which implies that the direct contact with food (blood) is not able to evoke PER at all (Lazzari, 2009). Besides, in order to obtain their reward, mosquitoes and other blood-sucking insects need to pierce the skin of their hosts to recover their food from inside blood vessels or through the membrane of an artificial feeder. As a consequence, they never drink blood from drops, nor do they respond to blood odour or to cold objects. Despite these particularities, we succeeded, in the present work, in adapting the PER for the study of learning and memory in haematophagous insects.

In the first part of this work, the response of the bugs to an appetitive thermal stimulus progressively decreased with the repeated presentation of the stimulus in absence of reward. This decrease ended with the complete disappearance of the response. The reappearance of PER after shifting stimulation and ITI temperatures, but keeping the same difference, revealed the central basis of this phenomenon. In other words, disappearance of PER was due to real habituation and not to peripheral processes, such as motor fatigue or sensory adaptation. Furthermore, it is worth mentioning that to evince dishabituation, both the sensory receptors involved and the motor response evaluated remained identical, the intensity of the stimulus being the only difference. So, both sensory adaptation and motor fatigue could be tested in only one step. This kind of simultaneous control is not always possible in other models where two steps are required. For instance, to test motor fatigue the same response is tested by using a different stimulus (e.g. in olfactory conditioning a different odour) and to test for sensory adaptation the ability of the same stimulus to evoke a different response is measured.

In addition, our results show that at least 1 h after the habituation procedure, training has an effect on the performances observed

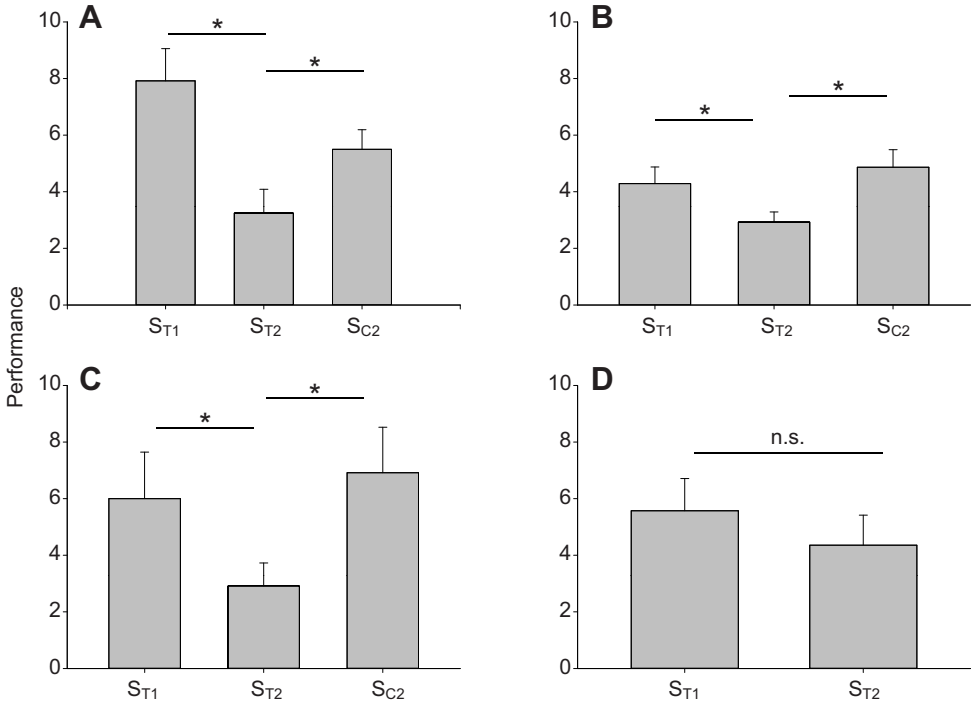

Fig. 7. Performances of *Rhodnius prolixus* larvae, represented as the mean number of trials that were necessary to observe a complete disappearance of the response. Each bar represents either a trained group during its first (S_{T1}) or second session (S_{T2}) or the associated control group (S_{C2}) when indicated. (A) 1 h retention (trained group: N=12; control group: N=10); (B) 24 h retention (trained group: N=14; control group: N=15); (C) 72 h retention (trained group: N=12; control group: N=12; (D) 96 h retention (trained group: N=14). *Significant differences (P<0.05).

during a subsequent habituation session. Thus habituation induced the formation of a mnesic trace.

In the second part of this work, we present an aversive conditioning paradigm of the PER. Bugs learned to stop responding to appetitive stimulation more rapidly than by habituation, to avoid being punished by the thermal shock. This conditioning paradigm is revealed as being simple and reproducible enough to go deep into the analysis of learning and memory. By using the aversive conditioning of the PER, we were able to obtain some insights into memory persistence. Indeed, an effect of training was verified up to 72 h later. When tested 96 h after the first session, the effect of training started to disappear. Provided that the physiological mechanisms underlying memory retention remain to be analysed (i.e. dependence or independence of protein synthesis), we prefer to avoid here any reference to short-, mid- or long-term memory. Further work manipulating protein synthesis should provide a definitive answer and allow one to use any mechanistic definition.

As in the habituation experiment, during aversive conditioning an apparent (but only marginally significant) effect of the context was observed. In both cases, this tendency was limited to retention tests performed 1 h after training. It consisted of a reduction in the number of trials necessary to stop responding for insects only exposed to the context, but not stimulated in the first session (i.e. untrained control group during S_{C1}), compared with the first session of the trained group (i.e. S_{T1}). This effect was not visible when retention tests were performed 24 or 72 h post-training, which indicates that context memory lasts for less than 24 h in *R. prolixus*.

If we now focus our analysis on the insect model itself, our results suggest that the extension of the proboscis, which is easily triggered by heat stimulation (US), is not a fully stereotyped response but a plastic one submitted to the control of superior centres instead. From an ecological point of view, such a behavioural plasticity appears as highly adaptive, if we take into account the diversity of hot non-host objects that these insects may encounter in their habitat. Indeed, *R. prolixus* is able to establish a close association with the human habitat, where warm objects other than hosts are present. Triatominae bugs exhibit a high sensitivity to heat that they use to find a potential food source (i.e. endothermic vertebrates) and that is able to trigger the PER (Flores and Lazzari, 1996; Lazzari, 2009). Thus to be capable of stopping responding to thermal stimulation that does not provide food seems as adaptive as the possibility to dishabituate the PER to start responding again to warm objects at a different temperature.

Another relevant point from an ecological perspective is the role played by the spatial context in learning. Our results suggest that the context could be important in the habituation of the PER. In their natural habitat, bugs may encounter many warm objects, some being hosts and the others not. To take into account the context in which bites are ineffective reduces the possibility of not responding later, when a true host is found in a different place. This link between the learning context and memory has already been demonstrated in other invertebrates such as the nematode *Caenorhabditis elegans* (Rankin, 2000), the crab *Chasmagnatus granulatus* (Hermitte et al., 1999) and in *Aplysia* (Colwill et al., 1988).

It is worth mentioning that *R. prolixus* is able to perform forms of learning other than those described here. Recently, we succeeded in applying Pavlovian conditioning procedures and made bugs associate the same olfactory stimulus with either a positive (i.e. appetitive conditioning) or a negative reinforcement (i.e. aversive conditioning). Bugs were also able to use the association learned in one context (contingency of an odour with food or punishment) in a different one (spatial orientation) (Vinauger et al., 2011a; Vinauger et al., 2011b). It must be said, however, that olfactory conditioning of haematophagous insects is not very practical for intensive studies. Indeed, it is time consuming and has many specific constraints (e.g. precise control of rewards to keep motivation constant and homogenous across individuals and many others) (Vinauger et al., 2011a; Vinauger et al., 2011b; Vinauger et al., 2012). In contrast, the experimental protocols based on the PER, in particular the aversive conditioning of the PER, are easier to set up and more adequate to tackle questions that require precise parameter control.

We believe that our characterization of the PER of *R. prolixus* is useful for several reasons. First, because it allows us to apply a simple, easily reproducible and largely validated learning protocol to haematophagous insects vectors of diseases. As indicated before, learning abilities are supposed to play a key role in parasite transmission, but the experimental evidence is scarce because of experimental constraints. Second, once characterized, the PER protocol furnishes the possibility to explore the neurobiological basis of learning and memory in experimental models biologically and phylogenetically distant from classical ones (i.e. *Drosophila* and bees). Third, it allows using the same sensory modality, i.e. the thermal one, as US and CS. Fourthly, given the difficulties for setting up simple and reproducible protocols for studying learning and memory in mosquitoes (by far the most important disease vector insects), the PER of *R. prolixus* offers a model system to analyse biologically relevant questions concerning haematophagy in general. In this sense, it should not be forgotten that even having evolved many times among insects, the haematophagous way of life imposes similar selection pressures on all blood-sucking arthropods (e.g. host detection and selection, avoiding the most defensive ones, etc.). How these pressures have modelled the cognitive abilities of this group is just starting to be unravelled. Taking into account the important epidemiological relevance of such abilities, we believe that it is worth investing in the study of haematophagous cognition, an effort equivalent to the one made in the study of sugar-feeders. It should be emphasized, however, that the only way to obtain reliable knowledge is to respect the strict conditions imposed by experimental psychology. Otherwise, we will risk repeating mistakes and compromise reproducibility, as pertinently highlighted by Alonso and Schuck-Paim (Alonso and Schuck-Paim, 2006).

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