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RESEARCH ARTICLE

Learning the way to blood: first evidence of dual olfactory conditioning in a blood-sucking insect, *Rhodnius prolixus*. I. Appetitive learning

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SUMMARY

It has been largely assumed that the individual experience of insects that are disease vectors might not only contribute to animal fitness, but also have an important influence on parasite transmission. Nevertheless, despite the invested efforts in testing the capacity to learn and remember information in blood-sucking insects, only little conclusive information has been obtained to date. Adapting a classical conditioning approach to our haematophagous model, we trained larvae of *Rhodnius prolixus* to associate L-lactic-acid, an odour perceived by these bugs but behaviourally neutral when presented alone, with food (i.e. positive reinforcement). Naive bugs – those exposed either to a conditioned stimulus (CS, L-lactic acid), unconditioned stimulus (US, heat) and reward (blood) alone or CS, US and reward in the absence of contingency – remained indifferent to the presence of an air stream loaded with L-lactic acid when tested in an olfactometer (random orientation), whereas the groups previously exposed to the contingency CS–US–reward (blood) were significantly attracted by L-lactic-acid. In a companion paper, the opposite, i.e. repellence, was induced in bugs exposed to the contingency of the same odour with a negative reinforcement. This constitutes the first evidence of olfactory conditioning in triatomine bugs, vectors of Chagas disease, and one of the few substantiations available to date of olfactory conditioning in haematophagous insects.

Key words: associative learning, Chagas disease, host seeking, Triatominae, haematophagous.

INTRODUCTION

To locate and assess the quality of resources, insects rely on innate behaviours that are fine-tuned by individual experience (Dukas, 2008; Raine and Chittka, 2008). Learning and memory, understood as the insects' behavioural adjustments based on previous experience, are two mechanisms that could help them to integrate and adapt to local variations in their environment (Dukas and Bernays, 2000; Menzel, 2001). These mechanisms and, more precisely, associative learning, have been particularly well studied in bees and fruit flies, which can be considered as classical models (Siwicki and Ladewski, 2003; Srinivasan, 2010), and have also been described in other insects such as cockroaches, caterpillars and hymenopteran parasitoids (Alloway, 1972; Papaj and Lewis, 1993; Horridge, 1997; Wackers and Lewis, 1999; Lucchetta et al., 2008; Costa et al., 2010). These studies have provided large amounts of information on the genetic and neurobiological bases of learning as well as on the complexity of insects' cognitive abilities (Bitterman et al., 1983; Bitterman, 1996; Dubnau and Tully, 1998; Xia et al., 1998; Menzel, 1999; Menzel et al., 2007; Menzel and Giurfa, 2001; Giurfa, 2003; Carcaud et al., 2009).

There is, however, a remarkable lack of information on the cognitive abilities of blood-sucking insects and how learning and memory could affect the transmission of parasites. In particular, we do not know to what extent host seeking by blood-feeding insects relies on innate behaviour, individual experience or a combination of both. Yet, a number of authors agree that investigations about the cognitive abilities of disease vectors could improve our understanding of the mechanisms underlying host preference (e.g. why some people are bitten more than others), as well as the heterogeneous distribution of vectors amongst host populations (Hasibeder and Dye, 1988; Kelly and Thompson, 2000; McCall and Kelly, 2002). Both the spatial distribution of a population of blood-sucking insects amongst their vertebrate hosts as well as the heterogeneity in their biting behaviour have important epidemiological consequences for the transmission of important diseases, such as malaria, leishmaniasis or Chagas disease (Dye and Hasibeder, 1986; Hasibeder and Dye, 1988).

To date, most studies examining associative learning in medically important arthropods have focused on mosquitoes. The available evidence is related to site fidelity, oviposition preference, host preference, insecticide avoidance and sugar feeding (McCall et al., 2001; McCall and Eaton, 2001; Alonso et al., 2003; Kaur et al., 2003; Jhumur et al., 2006). Nevertheless, since most studies have been conducted in natural or not completely controlled conditions, few provide unquestionable evidence of learning in this group of insects (Alonso et al., 2003; Alonso and Schuck-Paim, 2006). In haematophagous bugs, vectors of Chagas disease, some experimental studies have been conducted in order to investigate olfactory learning in these insects, but no evidence could be obtained (Abramson et al., 2005; Aldana et al., 2008).

From an evolutionary point of view, learning to recognize and remember the best hosts (e.g. less defensive and easiest to feed on) would represent an advantage. The information acquired during former foraging episodes could indicate what to avoid, as well as what to seek, during the subsequent episodes (McCall and Kelly, 2002). Olfaction plays a major role in host-seeking behaviour in haematophagous insects (Lazzari, 2009; Guerenstein and Lazzari, 2009). It allows insects to locate and recognize their host from a

secure distance and to set up adaptive responses of approach or avoidance. In this context, host odours might represent a powerful and reliable source of information, perceived at a certain distance from the host, when the latter is not yet a potential predator for the insects. It has been recently shown that the same odour may either attract or repel haematophagous insects according to their physiological state (Bodin et al., 2009a), but it remains unknown whether olfactory experience may also switch the behavioural response to a given odour.

Even though several olfactory conditioning procedures have been well standardized in some insect species, their application to haematophagous bugs is relatively difficult, because of the behavioural and physiological constraints associated with haematophagy (i.e. piercing mouthparts, heat-induced proboscis extension response and biting). In contrast to other fluid-feeding insects, such as bees, fruit flies or ants, blood-sucking insects need to pierce the skin (or a succedaneum) to obtain their food. So, to reward them in a controlled way, haematophagous insects first need to be induced to bite (i.e. indispensable thermal stimulation) and then allowed to feed on a controlled volume of blood in order to keep their motivation high to feed throughout trials.

Here we attempt to shed some light on the learning abilities of blood-sucking insects, in particular on the way individual olfactory experience can influence further host-seeking behaviour. For this, we used the triatomine bug *Rhodnius prolixus*, a vector of the human-transmitted Chagas disease, as an experimental model and tested whether larvae are able to associate a neutral odour with the perspective of obtaining a blood meal. This species was chosen because: (1) its host-seeking behaviour has been intensively studied and the roles of odours and other cues in the context of host seeking have been analysed in detail, and (2) it constitutes a classical model in insect physiology since the seminal work of V. B. Wigglesworth and followers in the 1930s. We thus used the accumulated knowledge on *R. prolixus* biology in order to adapt classical conditioning procedures, developed in bees and other non-haematophagous models, to this blood-feeding model. For this, we developed an experimental paradigm allowing us to pair a behaviourally neutral (but perceived) olfactory stimulus with a blood reward. In a second paper, we used the same neutral stimulus, but in an aversive conditioning approach (Vinauger et al., 2011).

MATERIALS AND METHODS

Insects

As experimental subjects, we used fifth-instar larvae of *Rhodnius prolixus* Stål 1859 (family Reduviidae, subfamily Triatominae) in order to exclude any interference related to reproductive behaviour. Bugs were reared in the laboratory under a 12 h:12 h light:dark illumination regime, at 26°C and 60–70% relative humidity. Bugs were fed weekly with sheep heparinised 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 ecdysis, a necessary starvation time to ensure a high motivation to feed (Bodin et al., 2009b). It has previously been shown that these bugs hatch at the end of the night (Ampleford and Steel, 1982). Thus, for our experiments, bugs were collected in the morning following ecdysis. They were recognisable by their characteristic pale-pink colour.

All of the assays were conducted in a room maintained at 25±2°C, 40–60% relative humidity. The experiments were carried out during the first hours of the scotophase (i.e. the dark period, or night-time, of the light cycle) because triatomines display a peak of activity throughout this period, which corresponds to the moment bugs leave their refuges to seek for host-emitted cues and exhibit the highest sensitivity to host odours (Lazzari, 1992; Barrozo et al., 2004; Bodin et al., 2008).

Conditioning procedure

Experiments were led using L-(+)-lactic acid (LA) as conditioned stimulus (CS). This volatile is emitted by vertebrates and has been detected on human skin (Acree et al., 1968; Bernier et al., 2000). LA was demonstrated to be the major component of perspiration (Braks et al., 1999), excreted at concentrations between 0.5 and 5 mg ml^{-1} (Eiras and Jepson, 1991; Cork and Park, 1996; Geier et al., 1996). In triatomine bugs, it is behaviourally neutral when presented alone, but lowers the response threshold of the bugs to CO_2 and fatty acids when combined with them (Barrozo and Lazzari, 2004a; Barrozo and Lazzari, 2004b). Thus, it is perceived by bugs, but no oriented response is induced, representing a good controlled stimulus for testing olfactory learning.

Training procedure: the artificial feeder

To investigate the ability of triatomines to associate an odour with a positive reinforcement, we set up a device allowing us to pair the presentation of an odorant (LA, CS) with a blood reward (appetitive conditioning), using heat as unconditioned stimulus (US) to evoke attraction and biting (unconditioned response) for recovering blood (positive reinforcement).

The device consisted of an artificial feeder (Fig. 1) allowing us to offer controlled quantities of blood and to expose 10 bugs at a time to the CS and/or the US, along with the positive reinforcement. It was composed of ten 250 µl micropipette cones whose tips were cut and sealed with Parafilm® through which bugs were able to bite (2 mm tip diameter, 3 cm height). Each tube was filled, for each trial, with 15 µl of sheep heparinised blood and placed in a taped aluminium block (48×13×0.4 cm) equipped with a flat electric resistance. A thermostat kept the temperature of the blood at 33±1°C, which roughly corresponds to a host body surface temperature. The aluminium block was isolated with a polystyrene foam plate

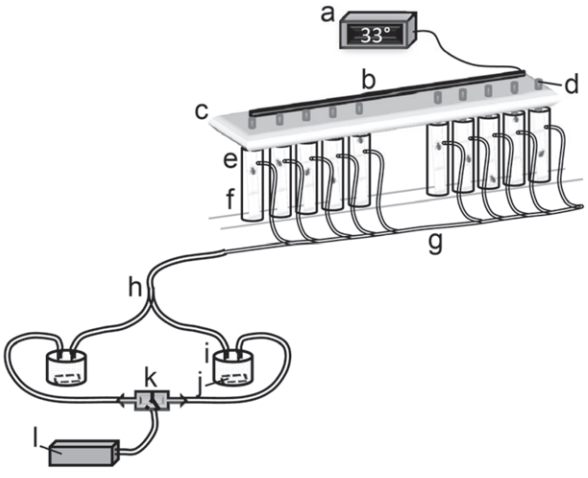

Fig. 1. Artificial feeder used in the appetitive conditioning procedure. It allows the pairing of the presentation of an odorant [L-lactic acid, conditioned stimulus (CS)] with a heat [unconditioned stimulus (US)]-induced blood reward (associative conditioning). a, Thermostat; b, electric resistance; c, isolated aluminium plate; d, blood container; e, insect container; f, filter paper; g, air supply system; h, silicone tubing; i, glass bottle; j, filter paper; k, solenoid valve; l, air pump.

(48×13×0.5 cm) that was pierced to make the tubes accessible, in order to present the thermal stimulus only through the tube membrane. The bugs were individually placed in plastic containers (11.7 cm height and 1.5 cm diameter), the tops of which were covered with a fabric mesh, allowing the insects to access the feeding tubes. A piece of filter paper inside each container allowed the bugs to climb up and reach the feeder. An individual air-delivery system was connected to each container, which could carry the CS (or not) (Fig. 2).

Air currents were generated by an air pump, and flow rate was regulated by a flow meter equipped with a needle valve. The airflow was split in two circuits. Each circuit was made of silicone tubing conducting the air current through a glass bottle containing a piece of filter paper (2.5 cm^2). One of them was soaked with 50 µl of distilled water and the other with 50 µl of LA solution (100 µg LA 50 μl^{-1} distilled water). The choice of the circuit was controlled by a solenoid valve; this enabled us to subject the bugs to streams of either clean ambient air or air loaded with LA at the same temperature, flow rate and relative humidity by activating the solenoid valve. Furthermore, it allowed a precise control of the stimulus duration.

Before the training session began, and before each trial, bugs were allowed to familiarize themselves for 2 min inside the plastic container in the absence of stimulation, except for the delivery of a clean air current. After this time, the air current loaded with LA was delivered for 1 min. The artificial feeder was then placed over the containers and the LA stimulation was maintained for one further minute. From this moment, bugs were allowed to feed until emptying the feeding blood tube and were then removed from the artificial feeder. Only bugs that took the whole amount of blood were kept and maintained in the same experimental room until the next trial; unfed or partially fed bugs were discarded. Trials were separated by 15 min. This inter-trial interval (ITI) was necessary for the bugs to reach the blood container, complete the 15 µl partial blood meal (i.e. approximately 2 to 3 min) and be motivated to feed again. In other words, ITI length was set in order to maximise the feeding success of each trial.

Conditioned bugs were submitted to three trials and thus allowed to feed on a total of 45 µl of blood during the whole training session. This amount represents approximately 26% of a full meal (mean=166 mg) and was considered to adequately reward the bugs, but kept their motivation to feed throughout the experiment high. The experimental procedure is summarised in Fig. 2.

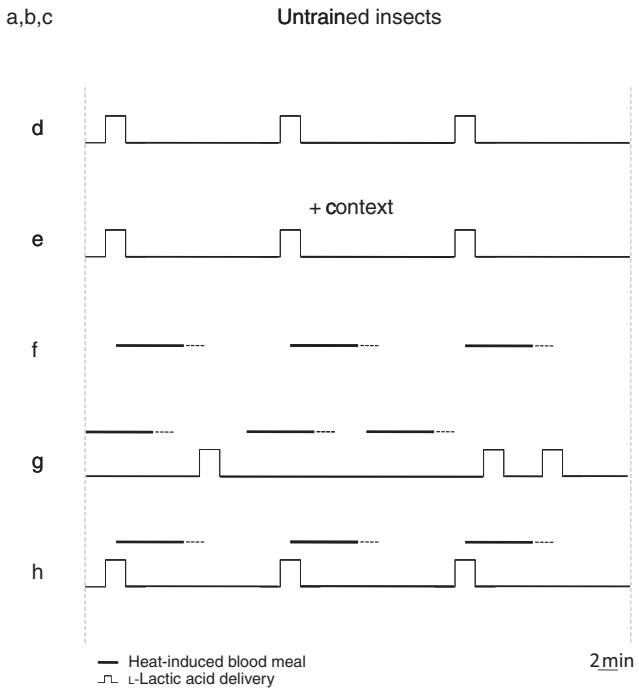

Fig. 2. Sequence of event delivery (i.e. US, CS and inter-trial interval) during training sessions of the different experimental groups: (a) neutral control (clean air); (b) positive control (yeast); (c) L-lactic acid control; (d) CS only; (e) CS context; (f) US only; (g) unpaired US–CS; and (h) appetitive conditioning (paired US–CS). All training sessions had the same duration, i.e. 45 min. Dotted lines refer to the variability in feeding duration within a group.

Testing procedure: the olfactometer

To compare the response to LA of control, untrained and trained bugs, an olfactometer was used. It consisted of an enclosed Y-maze made of Plexiglas® (Fig. 3). The angles between the arms were 120 deg. Two of the arms were connected to air inlets. The air streams were generated by two independent air pumps with independently controlled flow rates (flow rate=1.18 $cm^3 s^{-1}$, air speed=4.2 $cm s^{-1}$). Each air current flowed through a glass bottle (250 ml) containing a piece of filter paper (2.5 cm^2) on which either distilled water or LA diluted in distilled water (100 µg LA 50 μl^{-1} water) was applied. In order to avoid any pressure increase inside the maze, the third arm (starting arm) was connected to an air exhausting pump with a controlled flow rate of 3.8 $cm^3 s^{-1}$. All connections were made using silicone tubing (0.6 cm diameter).

The scene was illuminated by a red lamp (12 µW cm^{-2}), as bugs are less sensitive to red light than to other wavelengths (Reisenman and Lazzari, 2006). Temperature (25±2°C) and relative humidity (40–60%) remained constant throughout all experiments. In order to avoid environmental biases, the position of the stimulus and control currents were randomly exchanged.

At the beginning of an experiment, one bug was placed in a starting chamber located at the extremity of the starting arm, and closed by a nylon door (Fig. 3). After 1 min of familiarisation, the door was opened. Led by its positive anemotaxis (Barrozo et al., 2003), the insect walked along the starting arm and, at the bifurcation, could choose to follow one of the two air streams, one bearing the stimulus and the other only clean air, by entering into one of the two choice arms. We considered the first choice made by bugs, when they crossed an arbitrary decision line at the entry of each arm. Bugs that did not choose or did not leave the starting chamber were considered as not responding.

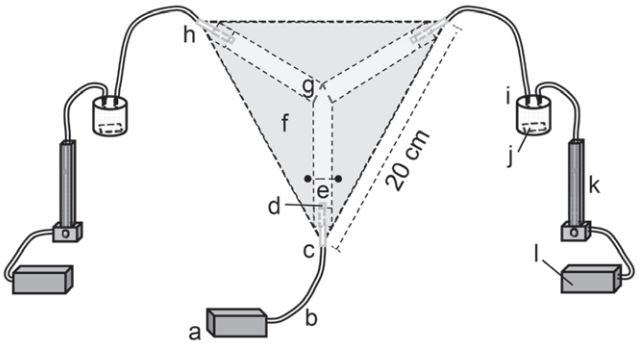

Fig. 3. Olfactometer designed for the analysis of the olfactory orientation of triatomines. a, Air-extracting pump; b, silicone tubing; c, glass tube; d, familiarisation room; e, nylon door; f, Plexiglas® olfactometer; g, decision line; h, air inlet; i, glass bottle; j, filter paper; k, flow meter; l, air pump.

The following groups of insects were tested:
(1) Neutral control. Naive bugs were tested in the olfactometer while delivering two clean air currents, in order to test for experimental biases (*N*=39).
(2) Positive control. Naive bugs were exposed to a clean air current *versus* baker's yeast fermentation odour (*Saccharomyces cerevisiae*) (*N*=22). Yeast was introduced in a glass bottle containing water (5 ml) and sugar (2.5 g), inducing the release of CO_2 and other components (Williams et al., 1981) that are attractive to triatomines (Guerenstein et al., 1995; Lorenzo et al., 1998). The aim of this test was to determine whether the apparatus was able to reveal an oriented response of bugs.
(3) L-Lactic acid control. Naive bugs were confronted to a clean air current *versus* a LA-loaded stream (*N*=22), to which, according to previous work (Barrozo and Lazzari, 2004b), they are supposed to be indifferent.
(4) CS only. Non-rewarded bugs (*N*=17) were pre-exposed to LA in a distinct plastic container, following the same procedure as the conditioned bugs but without being pre-exposed to the artificial feeder, heat or food. Bugs were tested in the olfactometer 15 min later for their response to a clean air current *versus* a LA-loaded stream.
(5) CS context. Non-rewarded bugs (*N*=18) were put inside the training containers in the artificial feeder and pre-exposed to LA only, following the same procedure as conditioned bugs except for the absence of heat and food. Bugs were tested in the olfactometer 15 min later for their response to a clean air current *versus* a LA-loaded stream.
(6) US only. Bugs (*N*=12) were submitted to the same procedure as conditioned bugs without being exposed to LA during the training phase. Bugs were tested in the olfactometer 15 min after their last partial blood meal for their response to a clean air current *versus* a LA-loaded stream.
(7) Unpaired US–CS. During the training session, bugs (*N*=17) were stimulated by heat (US) and allowed to feed on partial blood meals. They also were exposed to LA (CS), without contingency with the US. For each trial, the sequence of events (i.e. US, CS and two half-ITI) was randomly generated for each individual. An example of training sequence is given in Fig. 2. As a consequence, the duration of the training session was identical to the appetitive conditioning group (see below) and insects were submitted to the same amount of stimulation. The only differences with the appetitive conditioning group are the absence of contingency and the random presentation of the US and CS. Bugs were tested in the olfactometer 15 min after the training session for their response to a clean air current *versus* a LA-loaded stream.
(8) Appetitive conditioning (paired CS–US). Bugs (*N*=18) were trained to the contingency between LA (CS), heat (US) and blood reward. Bugs were tested in the olfactometer 15 min later for their response to a clean air current *versus* a LA-loaded stream.

Binary data collected in the olfactometer were analysed and all statistical tests were computed using R software (R Development Core Team, 2010). Comparisons were performed by means of the exact binomial test (α=0.05). For each group, the choice of the bugs in the olfactometer was compared to a random distribution of 50% on each arm of the maze. We also compared the percentage of bugs that remained in the starting chamber of the maze by means of a chi-square test.

RESULTS

From their initial position in the starting chamber of the Y-maze olfactometer, bugs displayed different behavioural responses according to their respective training experience. Results are depicted in Fig. 4. Naive untrained bugs of the neutral control group (clean air *versus* clean air) revealed no bias in the olfactometer or in the experimental room. Indeed, 51.28% of the bugs chose one arm and 48.72% chose the other. These results were not significantly different from a random distribution of 50% in each arm (binomial exact test, *P*=0.97). When they were confronted with a clean air current *versus* a current loaded with yeast odour, the naive bugs of the positive control group chose preferentially the arm delivering the odour of baker's yeast fermentation (81.82%). Thus, the device proved to be adequate for revealing an oriented response of *R. prolixus*. The difference between the distribution of bugs in this case and a random distribution was significantly different (binomial exact test, *P*=0.002). A third group of naive bugs was confronted to an air current loaded with LA *versus* a clean air current. Bugs of this group did not show any oriented response regarding LA, i.e. attraction or repulsion (LA, 54.55%; clean air, 45.45%) and their distribution in the maze was not significantly different from a random distribution (binomial exact test, *P*=0.42). This is in line with the results of Barrozo and Lazzari (Barrozo and Lazzari, 2004b).

Two groups of bugs were pre-exposed to LA. The CS-only group, pre-exposed to LA but not to the experimental context (i.e. setup, containers, etc.), did not display any oriented distribution (LA, 44%; clean air, 56%; binomial exact test, *P*=0.61). Bugs of the CS-context group exhibited a small but non-significant preference for clean air current (clean air, 66.67%; towards LA, 33.33%; binomial exact test, *P*=0.23, n.s.). In other words, pre-exposition to LA did not induce any significant change in the behavioural response to LA during a subsequent exposition. This was true when bugs were pre-exposed to LA either with or without being submitted to the same manipulations as the trained group.

Bugs of the US-only group were pre-exposed to heat and fed on three blood meals (15 µl each), an were manipulated in the same way as the conditioned bugs but without being exposed to LA. When tested in the olfactometer, they displayed a non-oriented (i.e. random) distribution (50% in each direction, binomial exact test,

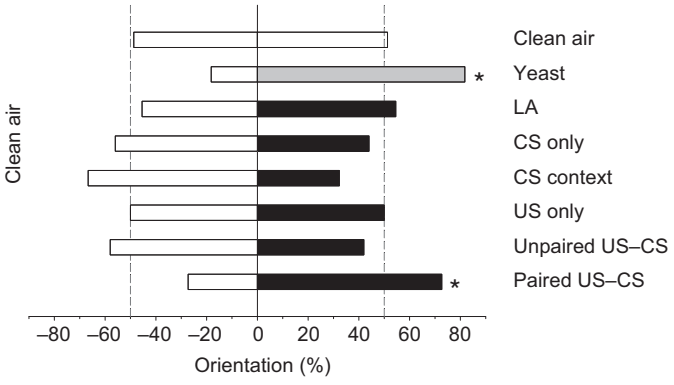

Fig. 4. Orientation response of *Rhodnius prolixus* larvae tested in the olfactometer and confronted with two air currents: clean air (white bars) and air loaded with either volatiles produced by baker's yeast (grey bar) or L-lactic acid (black bars). Orientation is represented by the percentage of bugs choosing each of the two test arms. Each bar represents an experimental group: clean air, neutral control group (*N*=39); yeast, positive control group (*N*=22); LA, L-lactic acid control group (*N*=22); CS only, CS-only group (*N*=17); CS context, CS-context group (*N*=18); US only, US-only group (*N*=12); unpaired US–CS, unpaired US–CS group (*N*=17); paired US–CS, appetitive conditioning group (*N*=18). Asterisks indicate distributions that are significantly different from random (*P*<0.05).

P=0.98), revealing no effect of the ingestion of blood on the behavioural response to LA.

For the unpaired US–CS group, the US and the CS were delivered in a random order during the training session. Bugs that were submitted to this training procedure displayed a random orientation during the test (binomial exact test, P=0.63). The arm equipped with the LA-loaded air stream was picked out by 41.2% of the bugs, whereas 58.8% of them chose to walk toward the clean air current. These results discard any cumulative effect of US and CS presentation in absence of contingency.

The last group of bugs was exposed to the contingency of a LA-loaded air current and heat-induced, controlled blood meal (Fig. 2). The majority of bugs belonging to this appetitive conditioning group chose the arm delivering the LA-loaded current (72.73%; binomial exact test, P=0.04), revealing a clearly oriented behavioural response towards LA following the training procedure, i.e. an attraction for the LA-loaded air stream.

It should be mentioned that in all tests, most bugs left the starting chamber of the olfactometer and entered into one of the decision arms. A small proportion of bugs did not respond and remained in the starting chamber (approximately 18.4±2%). This proportion was similar across the groups (chi-square test, P=0.81), and was excluded from the statistical analysis.

DISCUSSION

Until now, no evidence had been obtained regarding the ability of blood-sucking bugs to learn and, more generally, only few experimental data are available on the cognitive abilities of haematophagous insects, not all of them being conclusive (Alonso et al., 2003; Alonso and Schuck-Paim, 2006). Concerning *R. prolixus*, only negative results have been reported (Abramson et al., 2005; Aldana et al., 2008). In the present study, the accumulated knowledge on *R. prolixus* biology was exploited in order to adapt a conditioning procedure to its haematophagous way of life and to assess whether their behavioural response to a neutral odour could be modified by individual experience. The results of our experiments reveal that *R. prolixus* is able to associate a neutral stimulus with a positive (food) reinforcement, turning the odour attractive. In a companion paper we show that the same neutral odour can also be associated with a negative reinforcement (i.e. punishment represented by mechanical perturbation), making it repellent (Vinauger et al., 2011).

The association of LA (CS) with heat (US) as a predictor of food resulted in a modification of the bugs' originally neutral response to LA to a positive response. This behavioural change can be interpreted as appetitive conditioning, and indicates that *R. prolixus* are able to adapt their innate response to natural (host-associated) stimuli.

In our experiments, the groups pre-exposed to LA (i.e. when the odour was not associated to food) showed a light tendency to avoid the LA-loaded air stream. Because it would be counter-adaptive for bugs pre-exposed to a host odour without further consequences to become repulsed by this compound, we can assume that this aversion was due to handling. As a consequence, the aversive effect of handling may also be present during the appetitive conditioning procedure because bugs were manipulated in an identical way. Despite this bias, significant attraction to LA was revealed in conditioned bugs' choice (Fig. 4), and the consistent random choice across the different control groups allows us to discard any possible effect of one of the two stimuli (i.e. CS or US) acting alone, the cumulative effect of US and CS, as well as a potential effect of pre-exposure to LA.

It should be noted that most work on insect olfactory conditioning has been performed on employing sugar-feeding species, such as honeybees, flies and butterflies. In such cases, a stimulation of taste receptors on the antennae, legs or other body parts is sufficient to trigger proboscis extension and feeding on a drop of sugar solution (e.g. Bitterman et al., 1983). This is not case for blood-sucking insects, which need to pierce the host skin before gaining access to the blood, e.g. from inside blood vessels or from a wound (Lazzari, 2009). In the case of *R. prolixus*, proboscis extension is not induced by blood components, but in response to heat stimulation (Lazzari and Núñez, 1989; Flores and Lazzari, 1996). So, the US (heat) can be detached from the reward (blood), which is not case in nectar-feeding insects, for which sugar can represent both the US and the reward.

Thus, rewarding haematophagous insects requires providing blood at a temperature corresponding to that of a potential host. Another important point to be taken into account is that the consumption of blood is known to induce a change in the physiological and motivational status of the insect (Bodin et al., 2009a). We avoided this in our experiments by providing small controlled amounts of blood as positive reinforcement. Thus, our experimental design allowed us to apply Pavlovian conditioning procedures to triatomine bugs, overcoming the major difficulties associated with haematophagy, i.e. providing insects with access to the reward and maintaining a high motivational status during the conditioning procedure. A further pre-requisite was to ensure a contingency between the US and the CS while limiting handling and perturbations of insects. The device that we set up allowed us to deliver a continuous air current, to precisely control the duration of LA stimulation and to provide a partial blood meal within the same apparatus. Despite these precautions, it turned out that handling of the bugs' containers resulted in a trend (also present in the second test in the companion paper) that may indicate an effect on bugs.

As indicated above, this was not the first attempt to assess whether Pavlovian conditioning could modify the behaviour of *R. prolixus*. Nevertheless, previous work did not succeed in evincing any kind of olfactory conditioning in these bugs. Comparing our experimental approach to previous ones may shed some light on the reasons behind these contradictory results, and provide some insights for future work. In their foray, Abramson et al. (Abramson et al., 2005) and Aldana et al. (Aldana et al., 2008) also paired an olfactory CS with a thermal US using an artificial feeder. The major differences with our work lie in the way they developed their experimental design. First, insects were not allowed to feed on blood, but on saline solution. As no indication about the addition of phagostimulants is provided, saline solution may not constitute a true reward for bugs, given that these compounds are known to be indispensable for food recognition by *R. prolixus* (Friend and Smith, 1977). Secondly, the odours used as potential CS were almizcle, cinnamon, citral, ruda, raccoon and buck-lure [the rationale of the choice being that "Almizcle and ruda are common colognes used by Venezuelan men; cinnamon has been used in studies of learning by honey bees (...). The animal odours were selected because raccoon and buck are not found in Venezuela" (Abramson et al., 2005)]. It is worth mentioning that these are complex blends of which perception by the bugs' sensory system has never been tested. As a consequence, one cannot be sure that the CS or the reward was perceived as such by the bugs. In our experiments, we have chosen to employ a volatile whose purity and concentration are known, as well as the ability of the bugs to

perceive it, but to which they do not exhibit any oriented response when presented alone (Barrozo and Lazzari, 2004a; Barrozo and Lazzari, 2004b) (Fig. 4). This feature allowed us to use it in both appetitive and aversive contexts, as can be seen in Vinauger et al. (Vinauger et al., 2011).

The ability of *R. prolixus* to learn can be seen as a response to the strong selection pressures acting on haematophagous insects. Indeed, learning and memory could improve the capacity of blood-sucking insects, by narrowing their search, aiding in the selection of targets, conserving energy as well as enhancing their resource-locating efficiency (McCall and Kelly, 2002). It should be remembered that for these bugs, a host plays the double role of prey and predator (Lazzari, 2009). Although it is adaptive to recognize and remember any cues from high-quality resources, it seems equally necessary to be able to avoid the most defensive hosts (McCall and Kelly, 2002). It would be as adaptive for bugs to be able to associate particular host signals with active defensive behaviour. This will be the step developed in part II (Vinauger et al., 2011).

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