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RESEARCH ARTICLE

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

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SUMMARY

After having demonstrated that blood-sucking bugs are able to associate a behaviourally neutral odour (L-lactic acid) with positive reinforcement (i.e. appetitive conditioning) in the first part of this study, we tested whether these insects were also able to associate the same odour with a negative reinforcement (i.e. aversive conditioning). Learned aversion to host odours has been repeatedly suggested as a determinant for the distribution of disease vectors among host populations. Nevertheless, no experimental evidence has been obtained so far. Adapting a classical conditioning approach to our haematophagous model, we trained larvae of *Rhodnius prolixus* to associate L-lactic acid, an odour perceived by bugs but behaviourally neutral when presented alone, with a mechanical perturbation (i.e. negative reinforcement). Naive bugs and bugs exposed to CS, punishment, or CS and punishment without contingency remained indifferent to the presence of an air stream loaded with L-lactic acid (random orientation on a locomotion compensator), whereas the groups previously exposed to the contingency CS–punishment were significantly repelled by L-lactic acid. In a companion paper, the opposite, i.e. attraction, was induced in bugs exposed to the contingency of the same odour with a positive reinforcement. These constitute the first pieces of evidence of olfactory conditioning in triatomine bugs and the first demonstration that the same host odour can be used by insects that are disease vectors to learn to recognize either a host to feed on or a potentially defensive one. The orientation mechanism during repulsion is also discussed in light of our results.

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

INTRODUCTION

The ability of insects as disease vectors to learn and remember could have important consequences in the epidemiology of the diseases that they transmit. These abilities could improve the capacity of blood-sucking insects to select hosts more efficiently (McCall and Kelly, 2002). Targeting the most vulnerable hosts and avoiding the most defensive ones results in an heterogeneous distribution of biting (e.g. some people being more bitten than others) in host populations, affecting the circulation of parasites among the population members (Kelly et al., 1996; Kelly, 2001).

As previously highlighted by Alonso and Schuck-Paim (Alonso and Schuck-Paim, 2006), developing a deeper comprehension of the learning abilities of disease vectors could help us to better understand how individual experience influences vectors' hosts choices and how it might affect their potential for disease transmission. In recent literature, examples of learning-improved host selection can be found, particularly regarding mosquitoes. Tomberlin et al. (Tomberlin et al., 2006) brought experimental evidence of associative olfactory learning in *Culex quinquefasciatus* adults obtained under controlled laboratory conditions. In less controlled but more ecologically relevant ways, Charlwood et al. (Charlwood et al., 1988) have shown that *Anopheles farauti* remembered home range, and Mwandawiro et al. (Mwandawiro et al., 2000) reported that *Culex vishnui* displayed higher feeding rates when they were exposed to host species on which past blood-feeding was successful. The ability of *Rhodnius prolixus* to learn to associate a neutral host odour with a heat-associated blood meal has been demonstrated in part I of the present study (Vinauger et al., 2011).

It is worth noting, however, that for blood-sucking insects a host plays the double role of prey and predator (Lazzari, 2009). They take food from their host but, in doing so, expose themselves to the possibility of being hurt or killed by host grooming or defensive behaviours. Thus, although it is adaptive to learn and remember any cues paired with high-quality hosts, it also seems necessary to be able to learn to avoid the most defensive ones (McCall and Kelly, 2002). This could be accomplished by associating particular host signals with a high level of defensive behaviour. Because olfaction allows the insect to locate and recognize their host from a distance where the hosts are not yet a threat (Lazzari, 2009), odours represent, as developed in Vinauger et al. (Vinauger et al., 2011), a good target for learning. In previously published work, we have shown that the same host odour may either attract or repel haematophagous insects according to their physiological state (Bodin et al., 2009a), but nothing is known about the influence of experience on the significance that a given odour may have on a particular individual. Thus, the aims of the present study were to: (1) investigate whether a haematophagous insect is able to associate a host odour with its defensive behaviours and (2) assess whether the same host odour can be used in both appetitive and aversive conditioning, i.e. to test whether the same neutral odour is able to become attractive or repellent for haematophagous insects, depending on their individual experience.

MATERIALS AND METHODS

As in the first part of this study (Vinauger et al., 2011), we used triatomine bugs as experimental models and tested whether *Rhodnius prolixus* Stål 1859 larvae are able to associate the same behaviourally neutral (but perceived) odour that was used in Vinauger et al. (Vinauger et al., 2011) with the prospect of being hurt or killed by the host defensive behaviour. Here, the choice of L-lactic acid (LA) as the conditioned stimulus (CS) comes fully into play because this compound is both a biologically relevant host odour and a behaviourally neutral one that allows us to investigate the consequence of its pairing with both positive and negative reinforcement. Furthermore, because this study deals with aversive conditioning, the use of a Y-maze olfactometer [as used in Vinauger et al. (Vinauger et al., 2011)] would be incongruous. Indeed, from their initial position in the starting chamber, insects would perceive the potentially aversive odour without having the possibility to walk away from it. This means that we would expect an increase in the number of insects remaining in the starting chamber, leading to the impossibility of clearly quantifying any oriented behaviour. Thus, we used an experimental paradigm allowing us to pair LA (CS) with a mechanical perturbation [unconditioned stimulus (US)] before testing the behavioural responses of insects in an open-loop locomotion compensator.

Insects

As our experimental subject we chose *R. prolixus*, a classical model in insect physiology and also a main vector of Chagas disease in Central and South America (Dias et al., 2002). Bugs were reared in the laboratory under a 12 h:12 h light:dark illumination regime, at 28°C and 60–70% relative humidity. Insects 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 moult. 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 insects leave their refuges to search for host-emitted cues (Lazzari, 1992).

Conditioning procedure

For these experiments we used LA as the CS. This volatile is emitted by vertebrates and has been detected on human skin (Acree et al., 1968; Bernier et al., 2000), and 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). It is perceived by bugs but does not elicit any oriented response (i.e. attraction or repulsion) when presented alone. However, when combined with CO_2, LA lowers the response threshold for this gas and increases the response to fatty acids (Barrozo and Lazzari, 2004a; Barrozo and Lazzari, 2004b). Furthermore, as demonstrated previously (Vinauger et al., 2011), *R. prolixus* larvae are able to associate this compound with the possibility of obtaining a blood meal.

Training procedure: pairing CS and mechanical perturbation

In order to investigate whether *R. prolixus* is able to associate this odour with a negative reinforcement, we set up a device allowing us to pair the presentation of LA (CS) with a mechanical shock (US). We chose mechanical shock as the punishment to mimic the defensive behaviour of a host and because its contingence with odours has been shown to induce olfactory conditioning in other insects (Mery et al., 2007).

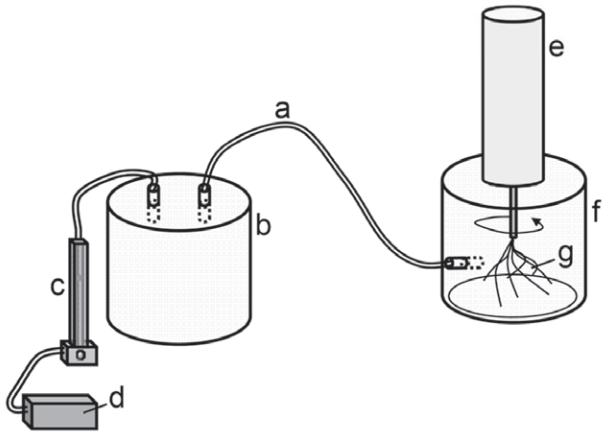

Fig. 1. Experimental device allowing either unpaired or paired presentation of L-lactic acid [LA; conditioned stimulus (CS)] and a mechanical perturbation [unconditioned stimulus (US)]. a, Silicone tubing; b, glass bottle containing either distilled water or LA (100 µg 50 µl^{-1} distilled water) on a piece of filter paper (2.5 cm^2); c, flow meter; d, air pump; e, electric motor; f, insect container; g, paper strips (0.2–0.3 cm wide, 4 cm long).

Rhodnius prolixus were placed in a plastic jar supplied with an air delivery system similar to that previously described (Vinauger et al., 2011), delivering LA-loaded air or clean air. The mechanical shock was delivered to groups of five to seven bugs by a low-speed electric motor (approximately 240 rotations min^{-1}; i.e. 4 Hz), on the axis of which 15 paper strips (2–3 mm wide; 4 cm length) were fixed (Fig. 1). This way, we were able to submit the insects to a mechanical perturbation without hurting them. Indeed, no effects on subsequent moults or any increased mortality was observed.

Before the training session began, insects were allowed to familiarise themselves with the plastic jar for 2 min, without stimulation. After this time, an air current loaded with LA was delivered for 1 min and then the LA current was paired with the mechanical shock for an additional 1 min. Bugs were submitted to five trials, separated by 5 min inter-trial intervals (ITIs). The experimental procedure is summarised in Fig. 2.

Testing procedure: use of a locomotion compensator

To test for aversive conditioning, we used a locomotion compensator to obtain the walking paths of control and trained bugs when stimulated by an air current loaded with LA *versus* a clean air current (Fig. 3). The trajectories of the bugs were recorded in an open-loop design for translation, and their spatio-temporal components were analysed, as described below (see Data analysis).

Before the beginning of each test, each bug remained in still air on the locomotion compensator for 2 min so it could familiarise itself with the experimental situation, after which two air streams coming from opposite directions were presented for 3 min. The assays were monitored from the outside of the experimental room by means of an infrared-sensitive camera (Conrad, Lille, France) equipped with an array of infrared LEDs (emission 900 nm). This light illuminated the scene without being perceived by the bugs (Reisenman et al., 1998; Reisenman and Lazzari, 2006). Because triatomines exhibit spontaneous anemotaxis to odourless air streams under these conditions (Barrozo et al., 2003), a simultaneous discrimination bioassay was conducted, similar to that used previously (Barrozo et al., 2004; Barrozo and Lazzari, 2004a; Barrozo and Lazzari, 2004b; Bodin et al., 2008; Bodin et al., 2009a; Bodin et al., 2009b).

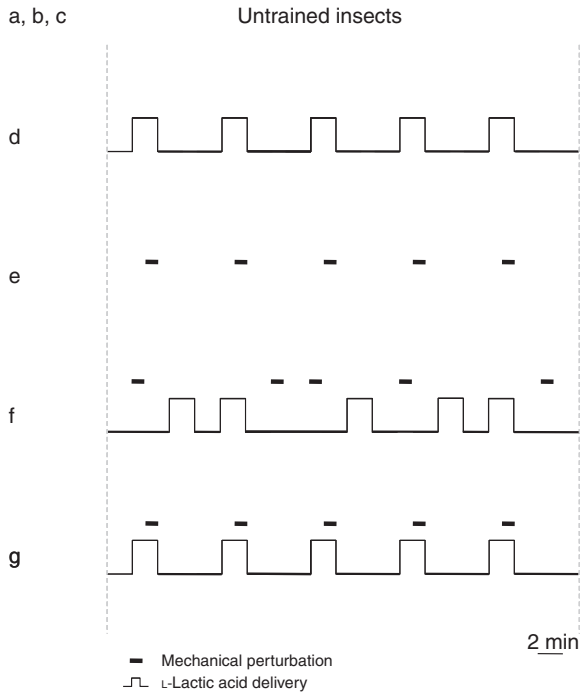

Fig. 2. Sequence of events (i.e. US, CS and inter-trial interval) during training sessions of the different experimental groups. (a) Neutral control group; (b) positive control group; (c) L-lactic acid control group; (d) CS-only group; (e) US-only group; (f) unpaired US–CS group; (g) aversive conditioning group. All training sessions had the same duration, i.e. 35 min.

Individual bugs were exposed to two different horizontal air streams (180 deg): one was loaded with LA whereas the other delivered clean air only (test *versus* control). Thus, each bug could choose either to walk towards one of the two streams or to exhibit a non-oriented behaviour, i.e. to walk randomly. Both air streams were blown over the bugs through glass tubes (0.6 cm inner diameter, 14 cm length), placed 3 cm from the bugs, at constant velocity (4.2 cm s^{-1}), temperature (25±2°C) and relative humidity (40±5%). The release of LA was achieved as previously described (see Vinauger et al., 2011). To avoid eventual environmental biases, the positions of the stimulus and the control air streams were changed randomly throughout the experiments.

The experimental groups were as follows:
(1) Neutral control group. Naive bugs (*N*=20) were exposed to two opposite clean air currents in order to test for any experimental bias.
(2) Positive control group. Naive bugs (*N*=16) were exposed to a clean air current *versus* baker's yeast fermentation odour (*Saccharomyces cerevisiae*). 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).
(3) L-Lactic acid control group. Naive bugs (*N*=18) were exposed to a clean air current *versus* a LA-loaded current.
(4) CS-only group. Non-rewarded bugs (*N*=19) were placed inside the training container and pre-exposed to LA only, after 2 min of familiarisation. Bugs were tested 5 min later on the locomotion compensator for their response to a clean air current *versus* a LA-loaded current.
(5) US-only group. Bugs (*N*=18) were placed inside the training container and the mechanical shock was delivered, after 2 min of familiarisation, in the absence of LA. Bugs were tested 5 min later on the locomotion compensator for their response to a clean air current *versus* a LA-loaded current.
(6) Unpaired US–CS group. During the training session, bugs (*N*=19) were submitted to the mechanical shock (US) and exposed to LA (CS), without contingency between US and CS. For each trial, the sequence of events (i.e. US, CS and two half-ITI) was randomly generated for each individual (an example of the sequence is given in Fig. 2). As a consequence, bugs of this group were submitted to the same amount of stimulation as the conditioned group and their training session also had the same duration. The only differences with the conditioned group were the absence of contingency and the random delivery order of US and CS. Bugs were tested on the locomotion compensator, 5 min after the training session, for their response to a clean air current *versus* a LA-loaded stream.
(7) Aversive conditioning group. Bugs (*N*=18) were trained to the contingency between LA (CS) and mechanical perturbation (US). Bugs were tested 5 min later on the locomotion compensator for their response to a clean air current *versus* a LA-loaded current.

Data analysis

The walking paths of bugs on the locomotion compensator were analysed in the following way. The mean walking angle displayed by each bug along the experimental time was computed. The position of the stimulus-delivered current was conventionally designated as

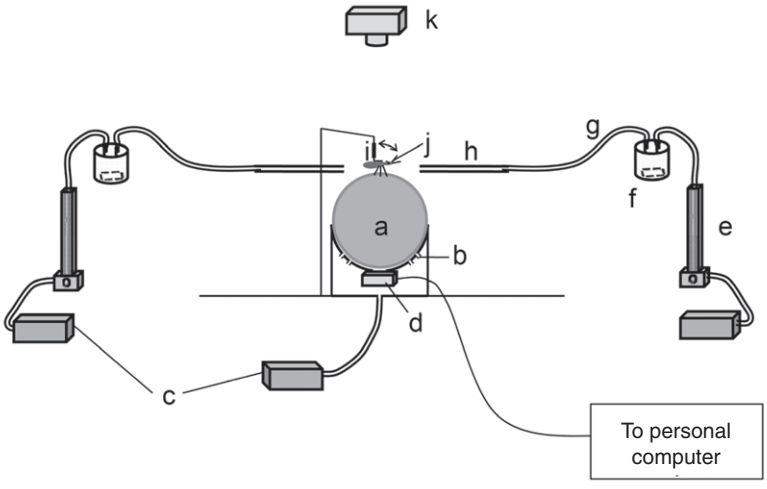

Fig. 3. Locomotion compensator designed for the analysis of the olfactory orientation of triatomines. a, Hollow Styrofoam sphere; b, sphere support with air inlets; c, air pumps; d, optical sensor; e, flow meter; f, glass bottle containing a piece of paper on which the odour can be deposited; g, silicone tubing; h, glass tube; i, 360 deg rotating bug support; j, experimental bug, tethered by its thorax with adhesive tape; k, infrared-sensitive camera equipped with infrared LEDs (emission 900 nm).

0 deg, and the control current as 180 deg. Bugs orienting in a mean direction within the hemisphere centred on 0 deg were considered to walk in the direction of the LA-loaded current whereas bugs walking in a mean direction within the hemisphere centred on 180 deg were considered to walk in the opposite direction, i.e. in the direction of the clean air control current.

Once binarised, data 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 on the locomotion compensator was compared with a random distribution of 50% in each hemisphere.

To compare attractive and aversive orientation behaviours, we performed further analysis of walking trajectories using circular statistics. Three different tests were applied: the Rayleigh test, the circular range test and the *V*-test. The rationale behind using three different tests is that each test provides different information. The Rayleigh test determines whether a circular distribution is uniform, focusing on the angular distance between individual pathways; this test was used to verify whether bugs walked randomly. The circular range test calculates the shortest arc of the circle containing the entire set of data and the *P*-value is computed using the exact distribution of the circular range under the hypothesis of uniformity, i.e. this test was used to determine whether bugs walked in a certain direction, whatever it was. The *V*-test analyses whether the bearings are randomly distributed with respect to the predicted direction, i.e. whether bugs oriented towards an expected direction, in this case, in the direction of or opposite to the stimulus.

RESULTS

Naive and untrained fifth-instar larvae confronted with two clean air currents from opposite directions walked randomly in the locomotion compensator and no oriented behaviour was revealed (binomial exact test, *P*=0.51; Fig. 4). Almost 60% of bugs oriented in one hemisphere and 40% in the other one; this orientation was not different from a random distribution. This first result revealed that neither the experimental room nor the experimental device presented any bias. In order to be sure that our experimental setup was adapted to reveal an oriented response, we exposed naive bugs of the positive control group to a clean air current *versus* an air current loaded with baker's yeast volatiles. This time, 75% of the bugs chose the hemisphere corresponding to the yeast odour current. This orientation was significantly different from a random distribution (binomial exact test, *P*=0.03; Fig. 4).

We also determined that the CS alone was not responsible for any oriented behaviour by testing the responses of naive untrained bugs confronted with a clean air current *versus* a LA-loaded current. LA did not evoke any attractive or repulsive response (binomial exact test, *P*=0.24; Fig. 4), confirming one more time the results obtained by Barrozo and Lazzari (Barrozo and Lazzari, 2004b).

In order to discard any effect of one of the two stimuli acting alone, we then pre-exposed two groups of bugs to either LA (CS) or mechanical perturbation (US). Bugs in the CS-only group did not display any oriented distribution (binomial exact test, *P*=0.18). The response of the group pre-exposed to the US was similar (binomial exact test, *P*=0.12).

The results of the experiments with the unpaired CS–US group, for which CS, US and ITIs were delivered in a pseudo-random order, indicate that there is not a cumulative effect of CS and US presentation. Bugs of this group walked towards the two hemispheres in similar proportions (53% walked towards the clean air current and 47% walked towards the LA-loaded current; binomial exact test, *P*=0.98; Fig. 4).

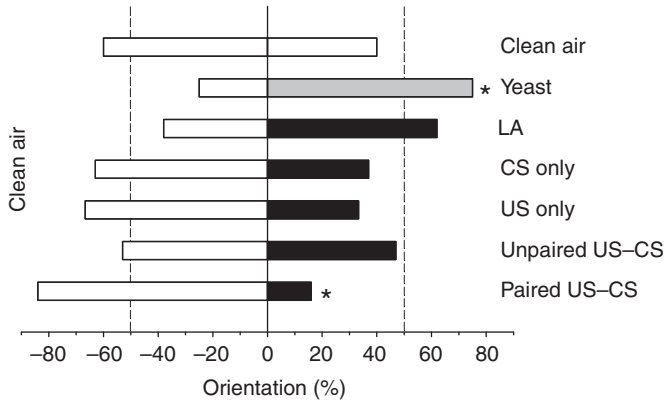

Fig. 4. Orientation response of *Rhodnius prolixus* larvae tested on the locomotion compensator and confronted with two air currents: clean air (white bars) and either air loaded with volatiles produced by baker's yeast (grey bar) or LA (black bars). Orientation is represented by the percentage of insects choosing each of the two hemispheres. Each bar represents an experimental group: clean air, neutral control group (*N*=20); yeast, positive control group (*N*=16); LA, L-lactic acid control group (*N*=18); CS only, CS-only group (*N*=19); US only, US-only group (*N*=18); unpaired US–CS, unpaired US–CS group (*N*=19); paired US–CS, aversive conditioning group (*N*=18). Asterisks indicate distributions that are significantly different from random (*P*<0.05).

The last group of bugs was trained by confronting them with the contingency between LA delivery and mechanical perturbation. After five training trials, when exposed to an LA-loaded current against a clean air current, bugs of the aversive conditioning group did not walk randomly on the locomotion compensator (binomial exact test, *P*=0.007; Fig. 4), they clearly avoided the LA-loaded current (84% of them chose the clean air current).

The analysis of walking directions (circular histograms in Fig. 5) was performed for the neutral and positive control groups and for the two groups exposed to US and CS (both unpaired and paired). The neutral control group revealed non-oriented walking (Rayleigh, *P*=0.41; circular range, *P*=0.29; *V*-test, *P*=0.59). The positive control group (i.e. yeast volatiles) revealed a non-uniform distribution of walking angles (Rayleigh, *P*=0.04); data are grouped (circular range, *P*=0.015) and showed a significant orientation towards the stimulus source (*V*-test, *P*=0.03). The unpaired US–CS group walked in a random manner (Rayleigh, *P*=0.44; circular range, *P*=0.98; *V*-test, *P*=0.37). For the aversive conditioning group, the Rayleigh test did not reveal a significant difference between the observed distribution and a uniform one (*P*=0.09), but the circular range test revealed a significantly grouped distribution of individual orientation angles (*P*=0.0017). The bugs showed a significant orientation towards 180 deg (i.e. the clean air current and the exact opposite direction of the LA source; *V*-test, *P*=0.015). A detailed analysis of the avoiding trajectory revealed, however, that bugs did not walk exactly towards 180 deg. We computed the mean angular disparity between the stimulus direction (0 deg) and the absolute value of the walking angle as 113.5±26.2 deg.

It should also be mentioned that during these experiments, only a small proportion of bugs remained immobile on the locomotion compensator (approximately 5±1.8%). This proportion was similar for all groups (chi-square test, *P*=0.89) and, as a consequence, those individuals were excluded from statistical analysis.

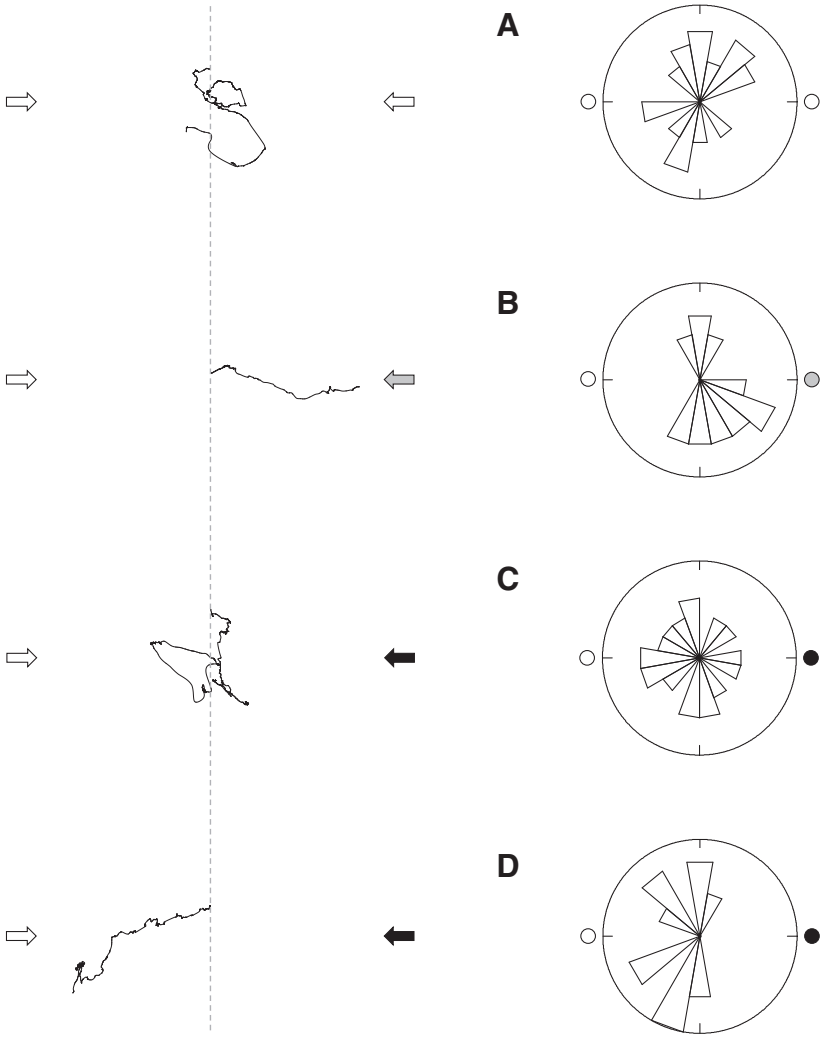

Fig. 5. (Left) Sample records of pathways described by four *Rhodnius prolixus* larvae when confronted with: (A) still air conditions, (B) control *versus* yeast-loaded airstream and (C,D) control *versus* LA-loaded airstream. Dashed line denotes the starting point of the walking bugs. Open arrows indicate the direction of the control air currents and filled arrows denote the direction of either the yeast-enriched (grey) or LA-loaded airstream (black). (Right) Orientation response of *R. prolixus* larvae tested on the locomotion compensator and confronted with two air currents: clean air (open circles) and either air loaded with volatiles produced by baker's yeast (grey circle) or LA (black circles). The circular histograms (rose diagrams) represent the frequency of the angles displayed by the bugs, which is proportional to the area of the wedge (bar width of 20 deg). Each rose diagram represents an experimental group: (A) neutral control group (N=20); (B) positive control group (N=16); (C) unpaired US–CS group (N=19); (D) aversive conditioning group (N=18).

DISCUSSION

To deal with the variability of their environment, we expect animals to gather and use information to reduce uncertainty. Learning and memory, defined as an animal's behavioural adjustment based on previous experience and the storage of this experiential information (Lorenz, 1981), are traditionally assumed to be some major contributors to animal fitness. In insects, these abilities are being evinced in a growing number of species.

Until now, no evidence had been obtained demonstrating learning abilities in blood-sucking bugs, and only few conclusive experimental data are available on the cognitive abilities of haematophagous insects in general (Alonso et al., 2003; Alonso and Schuck-Paim, 2006). In the present study, we provide the first experimental evidence that the behavioural response of *R. prolixus* to a neutral host-associated odour can be modified by the bugs' individual experiences to make the odour repellent. Taken together, both the present study and Vinauger et al. (Vinauger et al., 2011) show that *R. prolixus* is able to associate a neutral stimulus either with a positive (food) or a negative reinforcement (mechanical shock), revealing opposite consequences of olfactory experience. The association of LA (CS) with heat (US) as predictor of food and blood (reward) resulted in a modification of the originally neutral response to LA of bugs, making LA an attractive stimulus. This behavioural change can be interpreted as appetitive conditioning. Moreover, the response to the same stimulus can be conditioned in an aversive way, by associating it with punishment. Again, *R. prolixus* was able to modify its behaviour when confronted with LA, which became this time repellent, i.e. aversive conditioning.

Insects of the control group and the L-lactic acid control group walked randomly on the locomotion compensator, confirming once more that LA is behaviourally neutral when presented alone (Barrozo and Lazzari, 2004b) as well as the absence of bias in the experimental room. When we tested bugs that were submitted to the aversive conditioning procedure, we observed a non-random walking direction, i.e. bugs avoided the LA-loaded air current. The aversive olfactory conditioning was confirmed by the results of the CS-only, US-only and unpaired US–CS groups, which presented distributions that were not significantly different from random, thus discarding the potential effects of one stimulus acting alone or the cumulative effect of unpaired US and CS.

In the first part of our study (Vinauger et al., 2011), the groups that were pre-exposed to LA (i.e. when the odour was not associated to food) showed a slight tendency to avoid the air stream loaded with LA in the olfactometer. We observed a similar effect of pre-exposition in the present study (i.e. significant circular range test, but binomial and Rayleigh tests indicating random walking). One hypothesis that could explain these observations is that pre-exposed bugs have associated LA exposure due to handling of their

containers, because LA is excreted by human skin (Acree et al., 1968; Bernier et al., 2000). So, the experimenter himself would represent a source of LA that could have been associated with handling and manipulation. This could also explain the slight tendency of US-only group to avoid LA, when tested on the locomotion compensator. However, the appetitive conditioning group displayed a significant attraction despite handling (Vinauger et al., 2011), revealing that the positive reinforcement was stronger than the negative component due to handling. In other words, the perspective of completing a blood meal was stronger than the mechanical perturbation due to manipulation. Indeed, in a natural context, both types of reinforcement are present and interact.

It has often been assumed that the size of the blood meals that haematophagous insects are able to ingest is limited by the defensive behaviour of their host (Kale et al., 1972; Klowden and Lea, 1979; Day and Edman, 1984; Edman and Scott, 1987), and that both factors, meal size and host defensive behaviour, affect the distribution of vectors among host populations (Kelly et al., 1996; Kelly, 2001). In other words, when confronted with a host, haematophagous insects are submitted to the trade-off between the host defensive behaviour (i.e. negative reinforcement) and the value of the obtained blood meal (i.e. positive reinforcement). Given the high value of vertebrate blood and the important risks associated with its obtainment, strong selective pressures should have modelled haematophagous decision-making processes biasing their choice, favouring the exploitation of less defensive hosts. Hosts are sources of a plethora of physical and chemical stimuli that blood-sucking insects are able to perceive. Host-associated odours are mainly used by these insects to locate a potential food source but, as shown here, the response to these cues can be adjusted as a function of previous experience. This parsimonious (and plastic) use of host-derived information has an important value in terms of individual fitness by targeting blood sucking towards the less dangerous hosts. Thus, the question remains as to how positive (blood) and negative (host antiparasitic behaviour) factors influence future host selection when an insect is exposed to both types of reinforcement during the same feeding event.

An interesting finding that stood out from the analysis of walking pathways was that bugs that were conditioned to avoid the LA-loaded current did not walk exactly in the opposite direction (i.e. 180 deg). Indeed, despite a significant *V*-test, we analysed in detail the actual trajectory and it appeared that the direction chosen by bugs was approximately 113 deg. This angle is very close to that taken by triatomines walking away from moving objects described by Lazzari and Varjú (Lazzari and Varjú, 1990). These authors interpreted this particular fixation angle as a way for the triatomines to escape from a potential danger while keeping it within their visual field. This observation suggests that, independently of the sensory modality involved in the perception of a potential menace, they flee trying 'to keep an eye on it'. Because our experiments were conducted in darkness, we can wonder whether the bugs in the present study tried to adjust their trajectory to obtain visual contact or otherwise to optimize olfactory perception. Indeed, an airstream-borne odour coming from behind could be perturbed by the insect body before reaching the antennae.

Deviating from the exact opposite direction of the odour may thus improve olfactory detection of such odorants. Indeed, as seen in Fig. 5B, the orientation angle during stimulation with yeast-emanating volatiles is also not exactly towards 0 deg. Given that the relative position of the antennae with respect to the rest of the body in both situations, i.e. attraction and repulsion, our data are not sufficient for speculating on a relationship between walking direction and air-current perception. It should be noted, however, that no previous study has described a systematic disparity during attraction. This is a subject that deserves to be investigated in detail. The observation of deviation from the exact opposite direction raises the more general question of the application of circular statistics for the analysis of repulsion trajectories. Indeed, such tests are well adapted when dealing with oriented responses towards a stimulus, but to avoid a particular direction does not mean walking in the exact opposite direction, as revealed by our data.

Taken together, the results of the present study and those of Vinauger et al. (Vinauger et al., 2011) show that the same odours can acquire either a positive value when associated with blood or a negative value when associated with a mechanical shock, as bugs approach or avoid, respectively, the CS in orientation tests. So, we show that haematophagous bugs submitted to olfactory conditioning in a given context can use the learned information in a different situation. Bugs that learned an LA–blood association in the artificial feeder were afterwards attracted by this odour in the olfactometer. Conversely, bugs that learned an LA–mechanical shock association in a training jar clearly avoided this odour in the locomotion compensator. This ability to transfer olfactory information gained in a given experimental situation to novel situations has recently been well characterized in honeybees (Carcaud et al., 2009). Both dual olfactory conditioning and learning transfer between two contexts are demonstrated here for the first time in a blood-sucking insect.

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