J Exp Biol Advance Online Articles. First posted online on 15 April 2014 as doi:10.1242/jeb.101279
Access the most recent version at http://jeb.biologists.org/lookup/doi/10.1242/jeb.101279

Olfactory learning and memory in the disease vector mosquito, *Aedes aegypti*

Clément Vinauger[†], Eleanor K. Lutz[†] and Jeffrey A. Riffell[†,*]

[†]Department of Biology, University of Washington, Seattle, WA 98195, USA.

Keywords: Olfactory Learning, Long Term Memory, Appetitive Conditioning, Disease Vector, *Aedes aegypti*

Running head: Olfactory learning in mosquitoes

---

*To whom all correspondence should be addressed:

Jeffrey A. Riffell
Department of Biology,
University of Washington,
Seattle, WA 98195, USA
e-mail: jriffell@u.washington.edu

SUMMARY

Olfactory learning in blood-feeding insects, such as mosquitoes, could play an important role in host preference and disease transmission. However, standardized protocols allowing testing of their learning abilities are currently lacking, and how different olfactory stimuli are learned by these insects remains unknown. Using a Pavlovian conditioning paradigm, we trained individuals and groups of *Aedes aegypti* mosquitoes to associate an odorant conditioned stimulus (CS), with a blood reinforced thermal stimulus (unconditioned stimulus; US). Results showed, first, that mosquitoes could learn the association between L-lactic acid and the US, and retained the association for at least 24 h. Second, the success of olfactory conditioning was dependent upon the CS – some odorants that elicited indifferent responses in naïve mosquitoes, such as L-lactic acid and 1-octen-3-ol, were readily learned, whereas others went from aversive to attractive after training (*Z*-3-hexen-1-ol) or were untrainable (β-myrcene and benzyl alcohol). Third, we examined whether mosquitoes' ability to learn could interfere with the action of the insect repellent DEET. Results demonstrated that pre-exposure and the presence of DEET in the CS reduced the aversive effects of DEET. Last, the nature of the formed memories was explored. Experiments using cold-shock treatments within the first 6 h post-training (for testing anaesthesia-resistant memory) and a protein synthesis inhibitor (Cycloheximide; to disrupt the formation of long-term memory) both affected mosquitoes' performances. Together, these results show that learning is a critical component in odour responses in *Ae. aegypti*, and provide the first evidence for the functional role of different memory traces in these responses.

INTRODUCTION

A variety of processes, both ecological and physiological, mediate the ability of mosquitoes to transmit vector-borne diseases to their vertebrate hosts. A key factor controlling the mosquito-host interaction is the ability for mosquitoes to accurately identify their hosts by using several sensory modalities, one of which, olfaction, is a key mediator of host attraction in blood-feeding insects in general (Lehane, 2005; Lazzari, 2009; Guerenstein and Lazzari, 2009). The olfactory system can mediate innate responses to hosts (Bock and Cardew, 1996; Clements, 1999), but these responses can also be modulated by ecological factors such as vector density or host defensive behaviour (Kelly and Thompson, 2000), and physiological factors including internal- and circadian-state dependent effects (e.g. Trpis *et al.*, 1973; Klowden, 1990). In addition, it has been largely acknowledged that the cognitive abilities, and more precisely the ability to learn and retain information, have an epidemiological impact by modulating the way disease vectors will respond to host associated cues (McCall and Kelly, 2002; Alonso and Schuck-Paim, 2006).

In terms of disease transmission, one important step that learning and memory could affect is the process of host selection. Mosquitoes do not equally bite all hosts, even in the same population (Kelly, 2001). Differences in odour profiles between individuals could explain in part this heterogeneous biting behaviour, but would it be sufficient to explain that, as suggested by Woolhouse *et al.* (1997), only 20% of a host population is responsible for 80% of the transmission potential? The ability to learn to select a subset of preferred hosts could also contribute to explain the heterogeneous distribution of vectors amongst host populations (Kelly and Thompson, 2000). One can expect that it would indeed confer an adaptive advantage to mosquitoes to be able to learn and remember the cues associated with the best hosts (e.g. the easiest to feed on) since hosts play the double role of prey and predator

(Lazzari, 2009). Mosquitoes could thus benefit from their past foraging experience to determine which signals to avoid, as well as which ones to follow, during subsequent host-seeking episodes (McCall and Kelly, 2002).

During the last decade, increasing attention has been devoted towards unravelling the learning abilities of haematophagous insects, including mosquitoes (Mwandawiro *et al.*, 2000; McCall and Eaton, 2001; McCall *et al.*, 2001; McCall and Kelly, 2002; Kaur *et al.*, 2003; Alonso and Schuck-Paim, 2006; Tomberlin *et al.*, 2006; Bouyer *et al.*, 2007; Vinauger *et al.*, 2011a,b, 2012, 2013; Chilaka *et al.*, 2012; Menda *et al.*, 2013). Regarding mosquitoes, a handful of studies have provided direct or indirect evidence suggesting that learning abilities could be involved in host preference, choice of oviposition sites, and home range (see references above and McCall and Kelly, 2002; Menda *et al.*, 2013 for detailed review). While indicative of the potential for learning, these studies lacked precise temporal and quantitative control of the conditioned and unconditioned stimuli when training groups of individuals, and therefore do not allow further investigation of the mechanisms underlying these cognitive abilities (Rescorla, 1988).

Here in this study, we examine the odour-learning abilities of the yellow fever and dengue vector mosquito, *Aedes aegypti*, under controlled laboratory conditions to provide insights into how their cognitive abilities could mediate host selection. Adapting an olfactory conditioning procedure developed in the blood-sucking bug *Rhodnius prolixus* (Vinauger *et al.*, 2011a) and determining the timeframe in which *Ae. aegypti* mosquitoes were in the proper physiological state and activity level for olfactory conditioning, we were able to ask: Are individual mosquitoes able to associate a human related odour (L-lactic acid) with a blood reward, and how do those responses compare to innately attractive stimuli (e.g. carbon dioxide)? Do all odorants (host- and non-host related) have different valences in the

mosquitoes' ability to learn their association with a blood meal? And can we manipulate the formation of this memory in order to characterise it?

RESULTS

Individual training

To train mosquitoes, the presentation of an odorant (conditioned stimulus, CS) was paired with a blood reward (appetitive conditioning), using heat as unconditioned stimulus (US) to evoke attraction and blood-feeding responses (Fig. 1A). Twenty-four hours after training, the mosquitoes were tested in a Y-maze olfactometer in which they were given the choice between two stimuli (Fig. 1B) (see Materials and Methods for details).

After being released in the starting chamber of the olfactometer (Fig. 1B), individual mosquitoes displayed different behavioural responses according to their respective training experience (Fig. 2 and 3). In the absence of odours, naïve untrained females chose randomly between the two choice arms of the olfactometer (Clean air group: 52.95% in one arm and 47.05% in the other; PI=0.06; binomial exact test, p=0.86), revealing no bias in the maze or in the experimental room. But when confronted with a clean air current versus a current loaded with CO_2, naïve mosquitoes preferred the arm delivering the CO_2 (CO_2 group: 70.27%; PI=0.41; binomial exact test, p=0.02). However, naïve mosquitoes did not display any preference when confronted with a clean air current versus an air current loaded with L-(+)-lactic acid, LA (LA group: 54.29% towards LA and 45.71% towards clean air; PI=0.08; binomial exact test, p=0.74) (Fig. 3). This contrasts with the previously shown slight attraction of mosquitoes to LA (Geier and Boeckh, 1999) but not with the observations made in *Anopheles gambiae* (Dekker *et al.*, 2002) and more recently in *Aedes aegypti* (McMeniman

131 *et al.*, 2014) where LA had a synergisitic effect on the attractiveness of CO_2, skin odours and
132 skin-rubbing extracts from humans and other vertebrates.

133 Two control groups were pre-exposed to the CS or the US during the first session. The
134 CS-only group was pre-exposed to LA and to the same manipulations and experimental
135 context (i.e. setup, containers, etc.) as trained insects except that they were not fed and not
136 exposed to heat (US). When tested in the olfactometer, females of the CS-only group did not
137 display a preference for either arm of the olfactometer (LA, 42.86%; clean air, 57.14%; PI=-
138 0.14; binomial exact test, p=0.49). In other words, pre-exposure to LA was not responsible for
139 any significant change in subsequent behavioural response to LA. The mosquitoes of the US-
140 only group were pre-exposed to heat, fed on two blood meals and were manipulated in the
141 same way as trained insects, except that they were not exposed to LA during the first session.
142 When tested in the olfactometer, they displayed a random distribution as well (LA, 48.65%;
143 clean air, 51.35%; PI=-0.03; binomial exact test, p=0.62), revealing no effect of blood
144 ingestion on the behavioural response to LA.

145 In an additional control group, the unpaired US–CS group, the US and the CS were
146 delivered in a random order and without contingency during the training session (Rescorla,
147 1988). Mosquitoes submitted to this training procedure also displayed a random distribution
148 during the test (LA, 46.15%; clean air, 53.85%; PI=-0.08; binomial exact test, p=0.84). Thus
149 no cumulative effect of US and CS presentations was observed in absence of contingency.

150 For the last group of mosquitoes, females were individually exposed to the
151 contingency of LA and heat-induced blood meal. The majority of mosquitoes belonging to
152 this group chose the arm delivering the LA-loaded current (67.74%; PI=0.35; binomial exact
153 test, p=0.03), revealing a clear learned preference for LA, 24h after the training procedure.
154
155 *Group training*

If individual training provided control on the experimental conditions, group training experiments could offer a more rapid way to train mosquitoes to different odorant, allowing the assessment of their valences as CS. Thus, in addition to the individual-trained experiments, we adapted the conditioning procedure to groups of individuals, which allowed rapid training and efficient testing of different olfactory stimuli as CS. When trained in groups and tested individually to the same concentration of LA, mosquitoes displayed similar learning performances as individually trained insects, and significantly preferred LA (LA: 67.8%; PI=0.35; binomial exact test: p=0.04; Fig. 4). In addition, naïve insects that experienced a group situation displayed indifference to LA (LA: 44%; PI=-0.11; binomial exact test: p= 0.70), similarly to individually maintained animals. However, when trained to other odorants, their performances were dependent on the nature of the CS. For example, mosquitoes trained to 1-octen-3-ol (OCT) – an odorant emitted from human skin and plants and flowers (Cork and Park, 1996) – were able to develop a positive attraction to the odorant, as demonstrated by their preference for the odorant side of the olfactometer during tests (OCT: 65%; PI=0.30; binomial exact test: p= 0.04). The corresponding naïve control group showed no preference for the odour (OCT: 56%; PI=0.13; binomial exact test: p= 0.67; Fig. 4), suggesting that mosquitoes showed no innate preference for this odorant.

However, other odorants elicited different behavioural responses. For example, groups of mosquitoes were unable to learn to associate β-myrcene (MYR) with the perspective of obtaining a blood meal (trained MYR: 41.3%; PI=-0.17; binomial exact test: p= 0.22 / Naïve MYR: 34.4%; PI=-0.31;binomial exact test: p= 0.07). This volatile can be found in several fruits (Andrade *et al.*, 2000), including *Zanthoxylum piperitum* fruit oil, which tends to be a repellent for naïve *Ae. aegypti* (Naïve group: 34.5% MYR; PI=-0.31; Binomial exact test: p= 0.06; see also Choochote *et al.*, 2007). In other group-trained / group-tested assays, similar results were found when testing other plant-emitted odorants (benzyl alcohol and nonanol;

Fig. S2). For these odorants, the group-trained mosquitoes did not show a preference, or the odorants tended to elicit a seeming aversive response (Fig. S2).

For the last group-trained / individually tested experiment, mosquitoes were trained with *Z*-3hexen-1-ol (Z3H), an odorant emitted from leaves and flowers of some plants (Reddy *et al.*, 2002). We observed that mosquitoes were able to associate Z3H with the blood reward (Z3H: 75%; PI=0.50; binomial exact test: p=0.01), despite the fact that this odorant was also repellent for mosquitoes of the corresponding naïve control group (Z3H: 32%; PI=-0.35; binomial exact test: p=0.04).

Effect of learning on DEET repellency

If group-trained mosquitoes can associate aversive odours with a blood meal and thus be attracted to this odour during successive encounters, one can wonder whether mosquitoes can learn to respond positively to commonly used insect repellents. DEET (N,N-diethyl-meta-toluamide) is a strong aversive odorant for mosquitoes, but the presence of DEET in combination to a learned odorant may alter the percept of the CS, or mask the odorant (Syed and Leal, 2008). Moreover, recent work has shown that repeated DEET exposure lessens the aversion (Stanczyk *et al.*, 2013). We thus performed a series of experiments testing the effects of DEET using pre-exposed, DEET-trained and DEET- naïve animals (Fig. 2).

Given the choice between a clean air stream and an air stream loaded with LA+DEET, naïve mosquitoes significantly preferred the arm delivering clean air over the LA+DEET arm (Fig. 5; LA+DEET: 33%; PI=-0.33; binomial exact test: p=0.049). When group-trained with LA only, mosquitoes did not display a preference for LA in the presence of DEET (LA+DEET: 41.6%; PI=-0.16; binomial exact test: p=0.84). Similarly, training mosquitoes to the mixture of LA+DEET did not result in a distribution significantly different from a random one (LA+DEET: 54.2%; PI=0.08; binomial exact test: p=0.36).

When pre-exposed to DEET 1 h before being trained to LA only, insects still displayed a random distribution in the olfactometer during the test (Fig. 5; LA+DEET: 58.6%; PI=0.14; binomial exact test: p=0.22). Similarly, the distribution of mosquitoes pre-exposed to DEET 1 h before being trained to LA+DEET was not significantly different from random (LA+DEET: 60.9%; PI=0.21; binomial exact test: p=0.11).

Pre-exposure and training to DEET did not elicit distributions significantly different from random; nonetheless, these treatment groups exhibited responses that were markedly different from the naïve mosquitoes. We thus compared the distribution of the different treatment groups with the distribution of naïve females tested for their preference between LA+DEET versus clean air. The analysis revealed an effect of training on mosquitoes' responses when they were trained to LA+DEET (binomial exact test: p=0.01), pre-exposed to DEET and trained to LA (binomial exact test: p=0.005) or pre-exposed to DEET and trained to LA+DEET (binomial exact test: p=0.0003). Only the group trained to LA alone displayed a distribution that was not significantly different from the naïve group (binomial exact test: p=0.24). Thus, pre-exposure and the presence of DEET in the CS significantly reduced the aversive effects of DEET even if no significant attraction was induced by the training procedure.

Memory characterisation

The olfactory memory exhibited by mosquitoes 24 h after training may be due to two types of longer term memory, including anaesthesia resistant memory (ARM) and long-term memory (LTM). ARM is formed after multiple consecutive training trials, or massed training (repeated training sessions without a rest interval), and is not disrupted by cold induced anaesthesia but insensitive to the protein synthesis inhibitor cycloheximide (CXM). Conversely, LTM is formed after spaced training and is sensitive to CXM (see Tully *et al.*,

1994 for detailed review). In order to assess the nature of the formed memory, individual mosquitoes were subjected to either a cold-shock treatment (for testing ARM) or fed on cycloheximide, CXM, a protein synthesis inhibitor (for testing LTM) (Fig. 2).

Individual mosquitoes were submitted to cold shock anaesthesia either 1 h before training (a control for the cold-shock treatment), or 20 min, 2 h or 6 h post-training and tested 24 h post-training in all cases (Fig. 6). When submitted to the cold shock 1 h before training, mosquitoes preferred LA (72.2%; PI=0.45; binomial exact test, p=0.02), indicating that prior hypothermic exposure did not affect their ability to learn the association between the CS and US. However, when submitted to the hypothermic anaesthesia either 20 min or 2 h post-training, their distribution was no longer significantly different from random (LA arm: 56.5%, PI=0.13 and 60.7%, PI=0.21 respectively; binomial exact test, p=0.33 and p=0.17 respectively). By contrast, when cold anaesthetised 6 h post-training, trained females clearly chose LA during the test (LA: 75%; PI=0.5; binomial exact test: p=0.003), suggesting the late formation of an anaesthesia resistant form of memory.

Prior to determining the effects of CXM on the memory of mosquitoes, we performed a series of experiments to determine whether CXM had non-memory related effects on mosquitoes, such as altering their innate attraction to carbon dioxide or their flight behaviour. Mosquitoes that were fed the CXM and subsequently tested in the olfactometer for their preference for carbon dioxide or clean air showed a clear preference for the CO_2 (CO_2: 70.8%; PI=0.42; Binomial exact test: p=0.03), thus revealing that CXM-treated mosquitoes were still able to perceive odours and display an oriented response when stimulated with an innately attractive odorant. To determine whether CXM also affected mosquitoes' normal flight behaviours and odour-evoked responses, additional groups were tested in the olfactometer while their flight behaviour was recorded (Fig. S3). The analysis revealed that sucrose-fed and CXM-fed trained mosquitoes had similar flight speeds (24.84±1.22 and

21.14±1.45 cm-sec^{-1} for sucrose-fed and CXM-fed mosquitoes, respectively [values are the mean±SEM]; Student *t*-test: p=0.064; Fig. S4). Moreover, no significant differences existed in the flight speeds of trained versus naïve untreated mosquitoes (24.84±1.22 and 25.35±2.58 cm-sec^{-1}, respectively; Student *t*-test: p=0.85). Finally, the activity level and motivation to leave the starting chamber and choose between the two choice arms of CXM treated females was similar to untreated mosquitoes (on average 52.3% for CXM treated and 51.8% for untreated females).

To assess the long-term memory formation of trained mosquitoes, females were fed on 10% sucrose supplemented with CXM at 35 mM and subsequently trained to LA (Fig. 6). Results from these experiments showed that performance was impaired when tested 24 h post-training (LA: 55.2%; PI=0.10; binomial exact test: p=0.35). When the concentration of CXM was reduced by half (17 mM) to reduce its potential non-memory specific effects, performance of trained mosquitoes was also impaired when tested 24 h post-training (LA: 62.5%; PI=0.25; binomial exact test: p=0.15).

DISCUSSION

In this study, we investigated the ability of *Ae. aegypti* mosquitoes to learn to associate different olfactory stimuli with the perspective of obtaining a blood meal. Three distinct questions were addressed in the present work: At the individual scale, are mosquitoes able to perform true Pavlovian conditioning? Are different odorants equivalent in terms of CS and, in that context, are mosquitoes able to associate repellent odorants with appetitive cues? And finally, what forms of memory contribute to the performance of trained mosquitoes?

In the first part of the present work, we showed that classical, or Pavlovian, conditioning procedures previously adapted from classical models to haematophagous insects (Vinauger *et al.*, 2011a), could be used to train individual mosquitoes to respond positively to a previously neutral odour (LA). Different control treatments allowed us to discard alternative hypotheses (e.g. sensory adaptation, motor fatigue, and sensitization), by controlling for any potential effect of the CS or US acting alone or together but in the absence of contingency and thus revealing true associative learning. In addition, individual training experiments revealed that only two feeding experiences are sufficient for *Ae. aegypti* to form new memories that persist during at least 24h. In their natural environment, some individuals will fully engorge in a single blood meal and only return to feeding after developing and laying their eggs, while others will only partially feed as a result from host defensive behaviour (Klowden and Lea, 1978). In this context, forming memory of what have been successful or unsuccessful feeding events appears as highly adaptive for mosquitoes.

In the second part of this work, we adapted the conditioning procedure to groups of individuals. While individual training offers better control on the experimental conditions, results of group training experiments to LA showed that individually-tested but group-trained mosquitoes performed similarly to individually trained subjects. In contrast to previously published studies (e.g. Chilaka *et al.*, 2012; Menda *et al.*, 2013) where all the individuals of trained groups were tested simultaneously and without distinction of trained versus untrained animals in the same cohort, our experimental procedures allowed us to discard unfed females, thus only keeping individuals that were really submitted to the temporal contingency between US and CS during training.

Results of these experiments also revealed that different odorants did not have the same value to be used as potential CSs. Some odours that were not innately attractant (LA, OCT) could be associated with the possibility of obtaining a blood meal, while an innately

aversive odour (MYR) could not. Interestingly, mosquitoes learnt the innately aversive odorant Z3H as a food predictor in spite of its repulsive nature for naïve individuals. This result raised the following question: since aversive volatiles, such as DEET, are commonly used as insects repellent, to what extend would their efficacy be affected by the learning abilities of mosquitoes? The efficiency of DEET, the most commonly employed insect repellent (Fradin, 1998), relies on its ability to either "jam" an insect's olfactory perception (Bohbot and Dickens, 2010; Pellegrino *et al.*, 2011) or to trigger aversive behavioural responses (Syed and Leal, 2008; Liu *et al.*, 2010). Its efficiency in repelling mosquitoes was recently shown to be reduced in *Ae. aegypti* and *Rhodnius prolixus*, shortly after previous exposure (Sfara *et al.*, 2011; Stanczyk *et al.*, 2013). If non-associative phenomenon can impair DEET efficacy, it seems legitimate to wonder whether mosquitoes' abilities to perform associative learning could also interfere with its efficiency.

In all of the performed experiments, mosquitoes' avoidance for DEET could not be switched to attraction. However, when compared to the innate responses of naïve insects, the analysis revealed a significant effect of the treatment for insects that were trained, pre-exposed or both trained and pre-exposed to DEET. For these groups the innate aversion switched to random behavioural responses 24h post training, i.e. indifference. These results suggest that pre-exposure to DEET indeed induces a decreased repellence of *Ae. aegypti*, thus confirming results obtained by Stanczyk *et al.* (2013), and seem to indicate that learning can also play a role in decreasing DEET repellence (see LA+DEET trained group; Fig 5).

In all these experiments, the observed memory was retained during at least 24 h. In fruit flies the memory observed 24 h post training is from the combination of genetically distinct and functionally independent memory components: anaesthesia resistant memory (ARM) and long term memory (LTM) (Tully *et al.*, 1994, 2003; Margulies *et al.*, 2005). When focusing on memory in fruit flies, Tully *et al.* (1994) showed that: 1) anaesthesia

sensitive memory (ASM) could be disrupted by cold anaesthesia, 2) ARM was resistant to hypothermic disruption and insensitive to protein synthesis inhibitor and 3) LTM could be disrupted by ingestion of cycloheximide (CXM), a protein synthesis inhibitor. By applying similar memory-disrupting treatments (cold shock and CXM) at different times before and after training, we were able to shed light on the consolidated nature of the formed mnesic trace in mosquitoes submitted to a two trials training procedure. Results revealed the progressive consolidation of a form of memory that is resistant to cold induced anaesthesia. Similar results have been observed in fruit flies, where a 2 min cold shock delivered immediately after training significantly impaired flies performances in a 3 h memory retention test, whereas shock delivered later after training (from 30 min to 2 h) indicated the progressive consolidation of this ARM (Tully *et al.*, 1994). In the present work, mosquitoes that were treated with the protein synthesis inhibitor CXM also showed disrupted performances 24 h post-training. These results could suggest the formation of a CXM-sensitive long-term memory, but future work is needed to definitely characterize the different memory forms on which mosquitoes' performances relied after training.

In addition, we found that two training trials were sufficient for mosquitoes to form a consolidated form of long-term memory trace. This would actually appear highly adaptive for mosquitoes to be able to remember an experience with a defensive host (or a successful feeding experience) after a limited number of encounters, and to remember these unique experiences in a reliable way, i.e. on the long term and in a consolidated stable way, would then directly contribute to their fitness.

Consequences of learning

In nature, mosquitoes rely on their innate responses to CO_2 (e.g. Acree *et al.*, 1968; Dekker *et al.*, 2005) in combination with other host-related odours such as L-lactic acid and

1-octen-3-ol (Takken *et al.*, 1997; Geier and Boeckh, 1999, Dekker *et al.*, 2002). These responses can be modulated by their individual experience, but mosquitoes can also associate odorants that were initially neutral, or non-attractive, with a successful feeding event. As a consequence, host-vector interactions can be modulated by learning, which may underlie the observed heterogeneous distribution of vectors in host populations (Kelly and Thompson, 2000; McCall and Kelly, 2002), leading to high reproductive rates and increases in the transmission of disease in these subpopulations (Dye and Hasibeder, 1986; Hasibeder and Dye, 1988).

Taken together, results from this study provide the first characterisation of consolidated olfactory memory in mosquitoes, and the demonstration that certain odorants have differing valences in their ability to be associated with a blood reward. In addition, as discussed above, these results suggest that mosquitoes' learning ability might also play a crucial role in the efficiency of odorant based insect repellents. Finally, it is worth mentioning that this is only the third study to investigate associative learning in *Ae. aegypti* (Alonso *et al.*, 2003; Menda *et al.*, 2013) despite its potential vectorial importance. This work also sets the bases for future work on mosquitoes that would unravel what stimuli are adequate as CS or US, characterize their learning abilities, and determine the effects of physiological state (ie, age-, appetitive-, reproductive-, and circadian-related effects) on mosquito learning abilities. Last, these methods and results provide strong impetus for identifying the neurobiological substrates and mechanisms of blood host-odour learning.

MATERIAL AND METHODS

Insects

Wild type *Ae. aegypti* (line F21 MRA-726, MR4, ATCC® Manassas Virginia) were used all along the experiments. Groups of 200 larvae were reared on a diet of Hikari Tropic First Bites (Petco, San Diego, CA, USA) in a 26×35×4cm covered pan containing 1 cm of water, at 25˚C, 60±10% relative humidity, and under a photoperiod of 12:12 h (L:D). Adults were transfer in mating cages, maintained on 10% sucrose and blood-fed on weekdays on bovine heparinised blood (Lampire Biological Laboratories, Pipersville, PA, USA), using an artificial feeder (D.E. Lillie Glassblowers, Atlanta, Georgia; 2.5 cm internal diameter).

For the mosquitoes used in learning bioassays, approximately 200 same-age animals (both males and females) were separated from the colony at pupation and maintained on 10% sucrose after emergence. Emerged males and females were kept in a collapsible cage (20×20×20cm; BioQuip Products, Rancho Dominguez, CA, USA) for 6 days to allow for mating to occur (random dissection of females revealed that 95% of them had ovocytes present). After this time period, female mosquitoes were either captured individually using a mouth aspirator for individual training, or chilled until immobile at 10˚C for group training, and transferred to either individual or group containers (300mL clear plastic cups, Solo Cup Company, Lake Forest, IL, USA), which tops were covered by a piece of fabric mesh. Females were used in experiments the day following their isolation.

Preliminary experiments

In *Ae. aegypti*, it has been shown that various behavioural activities follow cyclic patterns (Haddow & Gillett, 1957; McClelland, 1960; Boorman, 1961; Gillett *et al.*, 1962; Jones *et al.*, 1972; Trpis *et al.*, 1973). In order to train mosquitoes during periods of the day at which they are responsive to host associated cues, and determine the best amount of blood meal that maintained motivational states, a series of preliminary experiments were performed. First, behavioural responses to artificial feeding of 6 days old starved females were tested at

four different time of mosquitoes' subjective day. The results of these preliminary experiments revealed that mosquitoes displayed higher levels of responses a few hours after the onset of the lights and a few hours before the offset of lights (see supplementary results, Fig. S1). Consequently, the experiments presented here were performed during the two activity peaks displayed by female *Ae. aegypti* (Trpis *et al.*, 1973). In addition, the volume of blood provided during training is an important component for the learning paradigm – a large enough volume is necessary to provide a reward, but the volume needs to be small enough to maintain a high motivational state in the behaving insect. To assess the ingested volume we exposed mosquitoes to blood for short durations during training and then compared the mass of females to unfed and fully engorged females. For individually and group trained mosquitoes, the amount of blood ingested during training represents approximately 38.57% and 72.51% of a full meal, respectively. For CXM treated mosquitoes, the amount of blood ingested during training represents 16.38% of a complete blood meal (i.e. 2.82±0.57 mg; n=20 females).

Experimental apparatus

Artificial feeder

Female mosquitoes were handled in the plastic containers described above and trained 24 h after their isolation. The tops of the containers were covered with a fabric mesh, allowing the insects to fly and access the artificial feeder by landing on the surface of the mesh. The artificial feeder (Fig. 1A) was composed of a glass feeder (D.E. Lillie Glassblowers, Atlanta, Georgia; 3.8 cm internal diameter, 6 cm height) which bottom was sealed with Parafilm® through which mosquitoes were able to bite. The feeder was filled with 10 ml of bovine heparinized blood (Lampire Biological Laboratories, Pipersville, PA, USA) and connected to

a water bath maintaining the temperature of the blood at 36±1°C, which roughly corresponds to human blood temperature. A volatile-delivery system, via a constant, charcoal-filtered, airstream (5 cm.sec^{-1}), could be connected to insect containers to deliver the CS (Fig.1A). Similar to the stimulus delivery system of the olfactometer (detailed below), the airflow was split in two circuits, each circuit being made of teflon® tubing (3 mm internal diameter), conducting the air flow through 20 mL glass bottles filled with 8 mL of either the test solution or the same volume of the corresponding solvent. During training, the choice of the circuit was controlled manually by connecting the tubes into the individual container; this enabled us to submit the mosquitoes to streams of either clean ambient air or air loaded with the CS at the same temperature, flow rate and relative humidity.

Olfactometer

To compare the responses of controlled, untrained and trained mosquitoes to different odours, an olfactometer was used. It consisted of an enclosed Y-maze made of Plexiglas® (Cooperband *et al.*, 2008; 110 cm long, 10 cm internal diameter, Fig. 1B). Fans (Rosewill, Los Angeles, CA, USA) were connected to two of the arms of the olfactometer (choice arms) to generate airflows (air speed approximately 20 cm.sec^{-1}). Airflow generated by the fans first went through an air filter (to remove odour contaminants; C16x48, Complete Filtration Services, Greenville NC USA) and a series of mesh screens and a honeycomb (10 cm long) to create a laminar flow before entering the Y-maze (Fig. 1B). As for the artificial feeder, odour delivery was achieved via a charcoal-filtered air stream that was split in two circuits and adjusted via flowmeters equipped with needle valves. Each circuit was made of teflon® tubing (3 mm internal diameter) conducting the airflow (5 cm.sec^{-1}) through a 20 mL glass bottle containing 8 mL of either the test odour or the control solution (i.e. MilliQ water). To avoid contamination, tubing and bottles were cleaned with ethanol and changed for each

odour. Ends of the tubes were placed in the arms of the olfactometer, 4 cm from the fans, and in the center of the olfactometer's arm. The bottles containing the test and control stimuli were replaced every 15 to 30 minutes to control for any change in odorant intensity.

To prevent the accumulation of odours in the experimental room, both artificial feeding and olfactometer experiments were conducted in a well-ventilated environmental chamber (Environmental Structures, Colorado Springs, CO, USA), at a temperature (25±2°C) and relative humidity (40–50%) that remained constant throughout all experiments.

In order to avoid environmental biases, the stimulus and control treatments were randomly exchanged in the olfactometer arms between experiments. In addition, the positions of the different parts of the olfactometer (i.e. choice tubes and fans) were also randomized. Data analysis did not reveal a preference for the left or right side of the olfactometer ($p=0.86$). After each experiment, the olfactometer was cleaned with ethanol (50%, 70%, and 95% ethanol) to remove odorant contamination.

Training protocols

Two conditioning paradigms were adapted in the present work to assess the ability of mosquitoes to learn the association between an olfactory stimulus and a blood reward. The first involved training individual mosquitoes using conditioning protocols adapted from classical insect models to haematophagous insects (Vinauger *et al.*, 2011a). This permitted detailed control of the experimental treatments to determine whether mosquitoes learned the association under Pavlovian conditioning and to investigate the nature of the involved memory form. In a second set of experiments, group training allowed rapid training and efficient testing of different olfactory stimuli as CS.

Individual training

For individual training experiments, single mosquitoes were exposed to L-(+)-lactic acid (LA; Sigma, ≥ 98% purity), at a concentration of 22 mM in milliQ water. This concentration is similar to that emitted by human skin (Eiras and Jepson, 1991; Cork and Park, 1996; Geier *et al.*, 1996).

Before the training session began, and before each trial, mosquitoes were allowed to acclimate for 1 min in the absence of stimulation, except for the delivery of a clean air current. After this time, a trial begun when the airflow loaded with LA was delivered for 2 min. The artificial feeder was then placed over the containers for 2 further minutes during which LA stimulation was maintained.

From this moment, mosquitoes that did not feed during this period of time were considered as not motivated and discarded from analysis. Those that landed on the mesh and started biting through the membrane of the feeder were allowed to feed for 20 sec before the artificial feeder was removed from the individual container. Only females that fed during the two trials were kept for the analysis. Trials were separated by 20 min. During this inter- trial interval (ITI) mosquitoes were maintained in the same experimental room, only exposed to a clean air current. Conditioned mosquitoes were submitted to two trials before being tested in the olfactometer, 24 h after the end of the training session.

In order to discard potential effects of CS or US on the performance of mosquitoes during the test, specific control groups were performed where mosquitoes were exposed only to the CS or to the US during the first session and tested 24 h later. Another group was exposed to both the CS and the US in an unpaired way during the first session, i.e. in the absence of contingency (Rescorla, 1988), and then tested 24h later. Additional untrained mosquitoes were tested in the olfactometer while having to chose between two clean air currents, a clean air stream versus CO_2 (positive control, $[CO_2]$= 2300 ppm above ambient level) or a clean air current versus LA (Barrozo and Lazzari, 2004).

Group training

For group training experiments, the following odorants were tested: LA, 1-octen-3-ol (OCT; Aldrich, ≥ 98% purity; enantiomeric ratio: ≥99:1 (GC)), *Z*-3-hexen-1-ol (Z3H; Sigma, > 98% purity; 92% of the *Z* isomer) and β-myrcene (MYR; Fluka, 95% purity). LA was dissolved in milliQ water at the same concentration as for individual training experiments (22 mM). In order to provide similar level of humidity – a strong activator of behaviour in mosquitoes –, OCT (14 mM) and Z3H (91 mM) were also diluted in MilliQ water. These concentrations were chosen to match the same volatility as odours used successfully in behaviour assessment paradigms similar to our own (Cooperband *et al.*, 2008). For MYR treatments, we used a 1/10,000 odorant dilution (0.58 mM) as higher concentrations caused avoidance behaviours resulting in no insects leaving the starting chamber.

In a preliminary experiment, solid phase micro-extraction fibers (SPME) were placed in the two arms of the olfactometer while delivering Z3H in water in one arm and Z3H in mineral oil in the other arm. This allowed us to quantify (via GCMS) the emission rate of Z3H diluted in water or mineral oil. The analysis revealed that emission rates between the two arms were not statistically different (0.98 ± 0.33 ng/minute for mineral oil; 1.09 ± 0.13 ng/minute for water).

Similarly to individual training, the air-delivery system was connected to insects' containers to deliver the odorant or the 'no odour' (MilliQ water) control (Fig. 1A). The training session began when the odorant was perfused into the container for 2 min. The group was then exposed to the blood-feeder for 20 min while still delivering the odorant. This succession of events represented one training trial. As for individually trained mosquitoes, groups were submitted to two training trials spaced by a 20 min ITI.

One hour before testing, groups were chilled and females were transferred into individual containers. Mosquitoes that did not feed during the training session (determined by the absence of blood in the abdomen or by the absence of abdominal distension) were considered as not motivated and thus discarded from analysis. Tests were then performed similarly to the individual training experiments, one mosquito being tested at a time. The testing session was performed 24 h post-training. To determine whether the mosquitoes exhibited any innate preference for the test odorants, control groups were also tested. Insects of these groups were naïve and had not been exposed to the odorant prior to the test.

Effect of learning on DEET repellency

We performed additional series of training experiments in order to test whether mosquitoes' ability to learn the association between odorants and blood feeding could interfere with the action of insect repellents such as DEET (N,N-diethyl-meta-toluamide; Supelco, ≥ 95% purity). We thus performed a series of experiments using pre-exposed, DEET-trained and DEET-naive animals. In these experiments, 100 µL of either 10% DEET/90% ethanol (DEET) or 100% ethanol (solvent) was loaded on to a filter paper (Whatman) that was placed in a 20 mL scintillation vial (DeGennaro *et al.*, 2013).

Different procedures were tested:

a) females trained to LA were tested for their response to LA+DEET *versus* control (clean air + solvent).

b) females trained to LA+DEET were tested for their response to LA+DEET *versus* control (clean air + solvent).

c) females pre-exposed to DEET 1h before training were then trained to LA and tested for their responses to LA+DEET *versus* control (clean air + solvent).

d) females pre-exposed to DEET 1h before training were trained to LA+DEET and tested for their response to LA+DEET *versus* control (clean air + solvent).

Memory characterization

The observed performance of trained animals relies on the formation of a mnesic trace that can last for different durations, depending on its consolidated nature (Tully *et al.*, 1994; Tully *et al.*, 2003). Since short term (STM) and intermediate term (ITM) memories last from a few to several hours (Tully *et al.*, 2003), we expect the memory that we observed 24 h post-training to belong to longer lasting forms of memory. Among them, the anaesthesia resistant memory (ARM) formed after massed training and spaced training is not disrupted by cold induced anaesthesia but insensitive to the protein synthesis inhibitor cycloheximide (CXM), while the long-term memory (LTM) is formed after spaced training only and is sensitive to CXM (see Tully *et al.*, 1994 for detailed review).

Additional treatments were thus performed to investigate the nature of the memory formed during conditioning experiments. Four groups of individually trained mosquitoes were submitted to a 15 min cold shock (2.6° C) either 1 h before training, 20 min after training, 2 h or 6 h after training. The cold shock was delivered by placing the individual container in an ice filled Styrofoam box (30×35×20 cm). Two additional groups were constituted and individuals from these groups were starved for 14-18h and then fed with either 17 or 35 mM of CXM (Tully *et al.*, 1994) in a 10% sucrose solution during 16-18 h previous to the training session. All these groups were tested 24 h post training. An additional group was fed on 35 mM CXM before its response to CO_2 was tested (CXM positive control group). For this group, insects were fed on 35mM CXM in 10% sucrose fed for the same duration as the experimental groups and tested for their response to CO_2 (positive control, [CO_2]= 2300 ppm above ambient level) versus ambient air.

It is worth mentioning that the CXM treated groups displayed a higher mortality rate than the sugar fed groups (34.6% of mortality being observed 8 days after training compared to 12% for sugar fed females). Interestingly, the increased mortality induced by the ingestion of CXM appears to be due to the ingestion of a warm blood meal, since CXM-fed mosquitoes that were not blood-fed had similar mortality rates as sucrose-fed (control) mosquitoes (16% of mortality). These results suggest that the drug might also impair their ability to deal with the heat stress generated by the ingestion of warm blood (Benoit *et al.*, 2011).

To make sure CXM effects were not affecting the mosquitoes' flight motor responses or olfactory perception, tracking of flight pathways were performed by video recording (Logitech Quickcam pro, 2MPixel; Newark, CA, USA) of the experiments in the olfactometer and subsequent analysis of the data in Matlab (v7.02, The Mathworks, Natick, MA) using the DLT toolbox (DLT DigitizingTools; Hedrick, 2008). Three treatment groups were tested: naïve mosquitoes fed on 10% sucrose solution (naïve control); trained mosquitoes fed on 10% sucrose solution (trained treatment); and trained mosquitoes and fed on 35 mM of CXM in 10% sucrose solution (CXM treatment).

Testing protocols

The testing sessions began when one single mosquito was placed in the starting chamber located at the extremity of the starting arm of the olfactometer and closed by a transparent Plexiglass® door (Fig. 1B). After a 30 s of familiarisation period, the door was opened. Led by its positive anemotaxis and optomotor responses (Kennedy, 1940; Takken and Knols, 1999), the insect flew along the starting arm and, at the bifurcation, could choose to follow one of the olfactometer arms, one bearing the stimulus and the other only clean air (plus the associated solvent), by entering into one of the two choice arms. We considered the first choice made by mosquitoes when they crossed the entry of an arm. Females that did not

choose or did not leave the starting chamber were considered as not responding and discarded from the preference analyses. On average, 42% of females were motivated to leave the starting chamber of the olfactometer and chose between the two choice arms. For both individually-trained and group-trained experiments, more females were active in the trained groups (52% on average) than in the corresponding control groups (33%) (χ^2-test: $p<0.001$).

Data analysis

For both individual and group experiments, binary data collected in the olfactometer were analysed and all statistical tests were computed using R software (R Development Core Team, 2012). Comparisons were performed by means of the exact binomial test ($\alpha=0.05$). For each treatment, the choice of the mosquitoes in the olfactometer was either compared to a random distribution of 50% on each arm of the maze or to the distribution of the corresponding control when appropriate. For binary data, the standard errors (SE) were calculated as (Le, 2003):

$$SEM = \left(\frac{p(1-p)}{n} \right)^{\frac{1}{2}} \quad (1).$$

For each experimental group a preference index (PI) was computed the following way: PI = [(number of females in the test arm – number of females in the control arm) / (number of females in the control arm + number of females in the test arm)]. A PI of +1 indicates that all the motivated insects chose the test arm, a PI of 0 indicates that 50% of insects chose the test arm and 50% the control arm, and a PI of -1 indicates that all insects chose the control arm of the olfactometer (adapted from Schwaerzel *et al.*, 2003). Means of instantaneous flight speeds were analysed in Excel and flight speed comparisons were made in R, by means of Student *t*-test ($\alpha=0.05$).

ACKNOWLEDGEMENTS

We thank B. Nguyen for mosquito care, and P. Chhabra for assistance in behavioural experiments. J.Z. Parrish, C.R. Lazzari and C. Lahondère provided helpful suggestions and critical reading of the manuscript, and comments from three anonymous reviewers substantially improved the manuscript.

FUNDING

This work was supported by a National Institutes of Health grant to JAR (01R01DC013693), and the University of Washington's Royalty Research Fund.

AUTHOR CONTRIBUTIONS

CV, EKL, and JAR conceived the study; CV and EKL conducted the experiments and analyzed the data; and CV, EKL, and JAR prepared the manuscript.

AUTHOR COMPETING INTERESTS

The authors have no competing interests.

REFERENCES

Acree, F., Jr, Turner, R. B., Gouck, H. K., Beroza, M. and Smith, N. (1968). L-Lactic acid: a mosquito attractant isolated from humans. *Science.* **161**, 1346-1347.

Alonso, W. J. and Schuck-Paim, C. (2006). The 'ghosts' that pester studies on learning in mosquitoes: guidelines to chase them off. *Med. Vet. Entomol.* **20**, 157-165.

Alonso, W. J., Wyatt, T. D. and Kelly, D. W. (2003). Are vectors able to learn about their hosts? A case study with *Aedes aegypti* mosquitoes. *Mem. Inst. Oswaldo Cruz.* **98**, 665-672.

Andrade, E. H. A., Maia, J. G. S. and Zoghbi Md G. B. (2000). Aroma volatile constituents of brazilian varieties of mango fruit. *J. Food Comp. Anal.* **13**, 27-33.

Barrozo, R. B. and Lazzari, C. R. (2004). Orientation behaviour of the blood-sucking bug *Triatoma infestans* to short-chain fatty acids: synergistic effect of L-lactic acid and carbon dioxide. *Chem. Senses.* **29**, 833-841.

Benoit, J. B., Lopez-Martinez, G., Patricka, K. R., Phillips, Z. P., Krausea, T. B. and Denlinger, D. L. (2011). Drinking a hot blood meal elicits a protective heat shock response in mosquitoes. *Proc. Natl. Acad. Sci. USA.* **108**:19, 8026–8029

Bock, G. R. and Cardew, G. (1996). Olfaction in mosquito-host interactions. *Ciba Foundations 200.* Wiley, New York.

Bohbot, J. D. and Dickens, J. C. (2010). Insect repellents: Modulators of mosquito odorant receptor activity. *PLoS ONE.* **5**, e12138.

Boorman, J. P. T. (1961). Observations on the habits of mosquitos of Plateau Province, Northern Nigeria, with particular reference to *Aedes (Stegomyia) vittatus* (Bigot). *Bull. Entomol. Res.* **52** (04), 709-725.

Bouyer, J., Pruvot, M., Bengaly, Z., Guerin, P. M. and Lancelot, R. (2007). Learning influences host choice in tsetse. *Biol. Lett.* **3**, 113-116.

Chilaka, N., Perkins, E. and Tripet, F. (2012). Visual and olfactory associative learning in the malaria vector *Anopheles gambiae* Sensu Stricto. *Malaria J.* **11**:27.

Choochote, W., Chaithong, U., Kamsuk, K., Jitpakdi, A., Tippawangkosol, P., Tuetun, B., Champakaew, D. and Pitasawat, B. (2007). Repellent activity of selected essential oils against *Aedes aegypti*. *Fitoterapia*. **78**:5, 359–364.

Clements, A. N. (1999). The biology of mosquitoes: sensory, reception, and behavior (Volume 2). Chapman & Hall, London. 752pp.

Cooperband, M. F., McElfresh, J. S., Millar, J. G. and Cardé, R. T. (2008). Attraction of female *Culex quinquefasciatus* Say (Diptera: Culicidae) to odors from chicken feces. *J. Insect. Physiol*. **54**, 1184–1192.

694 **Cork, A. and Park, K. C.** (1996). Identification of electrophysiologically-active compounds
695 for the malaria mosquito, *Anopheles gambiae*, in human sweat extracts. *Med. Vet. Entomol.*
696 **10**, 269-276.
697

698 **DeGennaro, M., McBride, C. S., Seeholzer, L., Nakagawa, T., Dennis, E. J., Goldman,**
699 **C., Jasinskiene, N., James, A. A. and Vosshall, L. B.** (2013). *orco* mutant mosquitoes lose
700 strong preference for humans and are not repelled by volatile DEET. *Nature.* **498,** 487–491.
701

702 **Dekker, T., Steib, B., Cardé, R. T. and Geier, M.** (2002). L-lactic acid: a human-signifying
703 host cue for the anthropophilic mosquito *Anopheles gambiae*. *Med. Vet. Entomol.* **16**:1, 91-98.
704

705 **Dekker, T., Geier, M. and Cardé, R. T.** (2005). Carbon dioxide instantly sensitizes female
706 yellow fever mosquitoes to human skin odours. *J. Exp. Biol*. **208**, 2963-2972.
707

708 **Dye, C. and Hasibeder, G.** (1986). Population dynamics of mosquito-borne disease – effects
709 of flies which bite some people more frequently than others. *Trans. R. Soc. Trop. Med. Hyg.*
710 **80**, 69-77.
711

712 **Eiras, A. E. and Jepson, P. C.** (1991). Host location by *Aedes aegypti* (Diptera, Culicidae) –
713 a wind-tunnel study of chemical cues. *Bull. Entomol. Res.* **81,** 151-160.
714

715 **Fradin, M. S.** (1998). Mosquitoes and mosquito repellents: a clinician's guide. *Ann. Intern.*
716 *Med.* **128**(11), 931-940.
717

718 **Geier, M., Sass, H. and Boeckh, J.** (1996). A search for components in human body odour
719 that attract females of *Aedes aegypti*. In *Mosquito Olfaction and Olfactory- mediated*
720 *Mosquito–Host Interactions* (ed. G. Cardew and J. Goode), pp. 132-148. *Ciba Foundation*
721 *Symposium 200*. New York: John Wiley & Sons Ltd.
722

723 **Geier, M. and Boeckh, J.** (1999). A new Y-tube olfactometer for mosquitoes to measure the
724 attractiveness of host odours. *Entomol. Exp. Appl.* **92**:1, 9-19.
725

726 **Gillett, J. D., Haddow, A. J. and Corbet, P. S.** (1962). The sugar-feeding-cycle in a cage-
727 population of mosquitoes. *Entomol. Exp. Appl*. **5**:3, 223-232.
728

729 **Guerenstein, P. G. and Lazzari, C. R.** (2009). Host-seeking: how triatomines acquire and
730 make use of information to find blood. *Acta Trop.* **110**, 148-158.
731

Haddow, A. J. and Gillett, J. D. (1957). Observations on the oviposition-cycle of *Aedes (Stegomyia) aegypti* (Linnaeus). *Ann. Trop. Med. Parasit*. **51**(2), 159-169.

Hasibeder, G. and Dye, C. (1988). Population dynamics of mosquito-borne disease-persistence in a completely heterogeneous environment. *Theor. Popul. Biol*. **33**, 31- 53.

Hedrick, T. L. (2008). Software techniques for two- and three-dimensional kinematic measurements of biological and biomimetic systems. *Bioinspir. Biomim.* 3, 034001.

Jones, M. D. R., Cubbin, C. M. and Marsh, D. (1972). The circadian rhythm of flight activity of the mosquito *Anopheles gambiae*: the light-response rhythm. *J. Exp. Biol.* **57**, 337-346.

Kaur, J. S., Lai, Y. L. and Giger, A. D. (2003). Learning and memory in the mosquito *Aedes aegypti* shown by conditioning against oviposition deterrence. *Med. Vet. Entomol.* **17,** 457-460.

Kelly, D. W. and Thompson, C. E. (2000). Epidemiology and optimal foraging: modelling the ideal free distribution of insect vectors. *Parasitology* **120**, 319-327.

Kelly, D. W. (2001). Why are some people bitten more than others? *Trends Parasitol.* **17**, 578-581.

Kennedy, J. S. (1940). The visual responses of flying mosquitoes. *Proc. Zool. Soc. Lond.* **109**:4, 221-242.

Klowden, M. J. (1990). The endogenous regulation of mosquito reproductive behaviour. *Experientia*. **46**:7, 660-670.

Klowden, M. J., and Lea, A. O. (1978). Blood meal size as a factor affecting continued host-seeking by *Aedes aegypti* (L.). *Am. J. Trop. Med. Hyg.* **27**:4, 827-831.

Lazzari, C. R. (2009). Orientation towards hosts in haematophagous insects: an integrative perspective. *Adv. Insect Physiol.* **37**, 1-58.

Le, C. T. (2003). Introductory Biostatistics. John Wiley & Sons, Inc., Hoboken, New Jersey. USA.

Lehane, M.J. (2005). *The Biology of Blood-Sucking in Insects.* New York: Cambridge University Press. 321pp.

Liu, C., Pitts, R. J., Bohbot, J. D., Jones, P. L., Wang, G. and Zwiebel, L. J. (2010). Distinct olfactory signaling mechanisms in the malaria vector mosquito *Anopheles gambiae*. *Plos Biol*. **8**(8), e1000467.

Margulies, C., Tully, T. and Dubnau, J. (2005). Deconstructing memory in *Drosophila*. *Curr. Biol*. 15:17, R700-R713.

McCall, P. J. and Eaton, G. (2001). Olfactory memory in the mosquito *Culex quinquefasciatus. Med. Vet. Entomol*. **15**:2, 197-203.

McCall, P. J. and Kelly, D. W. (2002). Learning and memory in disease vectors. *Trends Parasitol.* **18**:10, 429-433.

McCall, P. J., Mosha, F.W., Njunwa, K.J. and Sherlock, K. (2001). Evidence for memorized site-fidelity in *Anopheles arabiensis*. *Trans. R. Soc. Trop. Med. Hyg*. **95**:6, 587-590.

McClelland, G. A. H. (1960). Observations on the mosquito, *Aëdes (Stegomyia) aegypti* (L.), in East Africa. I.—The biting cycle in an outdoor population at Entebbe, Uganda. *Bull. Entomol. Res.* **50**:2, 227-235.

McMeniman, C. J., Corfas, R. A., Matthews, B. J., Ritchie, S. A., and Vosshall, L. B. (2014). Multimodal Integration of Carbon Dioxide and Other Sensory Cues Drives Mosquito Attraction to Humans. *Cell*, **156**:5, 1060-1071.

Menda, G., Uhr, J. H., Wyttenbach, R. A., Vermeylen, F. M., Smith, D. M., Harrington, L. C. and Hoy, R. R. (2013). Associative learning in the dengue vector mosquito, *Aedes aegypti*: avoidance of a previously attractive odor or surface color that is paired with an aversive stimulus. *J. Exp. Biol.* **216**:2 , 218-223.

Mwandawiro, C., Boots, M., Tuno, N., Suwonkerd, W., Tsuda, Y. and Takagi, M. (2000). Heterogeneity in the host preference of Japanese encephalitis vectors in Chiang Mai, northern Thailand. *Trans. R. Soc. Trop. Med. Hyg.* **94**:3, 238-242.

Pellegrino, M., Steinbach, N., Stensmyr, M. C., Hansson, B. S. and Vosshall, L. B. (2011). A natural polymorphism alters odour and DEET sensitivity in an insect odorant receptor. *Nature*, **478**:7370, 511-514.

R Development Core Team (2013). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL: http://www.R-project.org.

Reddy, G. V. P., Holopainen, J. K. and Guerrero, A. (2002). Olfactory responses of *Plutella xylostella* natural enemies to host pheromone, larval frass, and green leaf cabbage volatiles. *J. Chem. Ecol.*, **28**:1, 131-143.

Rescorla, R. A. (1988). Behavioral studies of Pavlovian conditioning. *Annu. Rev. Neurosci.* **11**:1, 329-352.

Schwaerzel, M., Monastirioti, M., Scholz, H., Friggi-Grelin, F., Birman, S. and Heisenberg, M. (2003). Dopamine and octopamine differentiate between aversive and appetitive olfactory memories in *Drosophila*. *J. Neurosci.* **23**:33, 10495-10502.

Sfara, V., Mougabure-Cueto, G., Zerba, E. N. and Alzogaray, R. A. (2011). Adaptation of the repellency response to DEET in *Rhodnius prolixus*. *J. Insect Physiol.* **57**:10, 1431-1436.

Stanczyk, N. M., Brookfield, J. F., Field, L. M. and Logan, J. G. (2013). *Aedes aegypti* mosquitoes exhibit decreased repellency by DEET following previous exposure. *PloS ONE.* **8**:2, e54438.

Syed, Z. and Leal, W. S. (2008). Mosquitoes smell and avoid the insect repellent DEET. *Proc. Natl. Acad. Sci. USA.* **105**:36, 13598-13603.

Takken, W. and Knols, B. G. (1999). Odor-mediated behavior of Afrotropical malaria mosquitoes. *Annu. Rev. Entomol.* **44**:1, 131-157.

Takken, W., Dekker, T. and Wijnholds, Y. G. (1997). Odor-mediated flight behavior of *Anopheles gambiae* (Giles) Sensu Stricto and *An. stephensi* (Liston) in response to CO2, acetone, and 1-octen-3-ol (Diptera: Culicidae). *J. Insect Behav.* **10**:3, 395-407.

Tomberlin, J. K., Rains, G. C., Allan, S. A., Sanford, M. R. and Lewis, W. J. (2006). Associative learning of odor with food-or blood-meal by *Culex quinquefasciatus* (Say) (Diptera: Culicidae). *Naturwissenschaften*. **93**:11, 551-556.

Trpis, M., McClelland, G. A. H., Gillett, J. D., Teesdale, C. and Rao, T. R. (1973). Diel periodicity in the landing of *Aedes aegypti* on man. *B. World Health Organ.* **48**:5, 623-629.

Tully, T., Preat, T., Boynton, S. C. and Del Vecchio, M. (1994). Genetic dissection of consolidated memory in *Drosophila*. *Cell.* **79**:1, 35-47.

Tully, T., Bourtchouladze, R., Scott, R. and Tallman, J. (2003). Targeting the CREB pathway for memory enhancers. *Nat. Rev. Drug Discov.* **2**:4, 267-277.

Vinauger, C., Buratti, L. and Lazzari, C. R. (2011a). Learning the way to blood: first evidence of dual olfactory conditioning in a blood-sucking insect, *Rhodnius prolixus*. I. Appetitive learning. *J. Exp. Biol.* **214**:18, 3032-3038.

Vinauger, C., Buratti, L. and Lazzari, C. R. (2011b). Learning the way to blood: first evidence of dual olfactory conditioning in a blood-sucking insect, *Rhodnius prolixus*. II. Aversive learning. *J. Exp. Biol.* **214**:18, 3039-3045.

Vinauger, C., Pereira, M. H. and Lazzari, C. R. (2012). Learned host preference in a Chagas disease vector, *Rhodnius prolixus*. *Acta Trop.* **122**:1, 24-28.

Vinauger, C., Lallement, H. and Lazzari, C. R. (2013). Learning and memory in *Rhodnius prolixus:* habituation and aversive operant conditioning of the proboscis extension response. *J. Exp. Biol.* **216**:5, 892-900.

Woolhouse, M. E., Dye, C., Etard, J. F., Smith, T., Charlwood, J. D., Garnett, G. P., Hagan, P., Hii, J. L., Ndhlovu, P. D., Quinnell, R. J. *et al.* (1997). Heterogeneities in the transmission of infectious agents: implications for the design of control programs. *Proc. Natl. Acad. Sci. USA.* **94**, 338-342.

FIGURE LEGENDS

Figure 1. Illustration of methods for olfactory training and testing. A) **Left**: Artificial feeder used in the appetitive conditioning procedure. It allows the pairing of the presentation of an odorant [Conditioned Stimulus (CS)] with a heated [Unconditioned Stimulus (US)] blood reward (associative conditioning). a, plastic insect container; b, glass bottle containing the CS and connected to an air pump via silicone tubing; c, glass artificial feeder; d, silicone tubing conducting warm water (37°C) from a water bath to the artificial feeder. **Right**: Miniaturized photoionization detector (miniPID) characterisation of the stimulus delivery, using a low molecular weight volatile (ethanol) as a tracer. The probe of the miniPID was positioned at the junction between the central box and the olfactometer arms.
B) **Left**: Olfactometer designed for the analysis of the olfactory orientation of mosquitoes. a, releasing chamber; b, decision arms of the olfactometer; c, computer fans, filters and screens;

d, glass bottles containing either the CS or the solvent-control solution; e, flowmeter equipped with a valve needle; f, charcoal filter; g, air pump. **Right**: Visualisation of the stimulus plume structure using a smoke plume. The series of flow straighteners and mesh screens in the olfactometer provided a unidirectional airflow that rapidly created a filamentous plume when the odour left the nozzle from the odour-line. For all experiments the air speed of the stimulus line represented 25% of the air velocity generated by the olfactometer fans.

Figure 2. Sequence of event delivery (i.e. US, CS and inter-trial interval) during training sessions of the different experimental groups. LA: L-lactic acid; OCT: 1-octen-3-ol; MYR: myrcene; Z3H: hexenol; DEET: N,N-diethyl-meta-toluamide; CXM: Cycloheximide; CS: Conditioned Stimulus; US: Unconditioned Stimulus; ITI: Inter-Trial-Interval; Pre-exp: Pre-exposed; BT: Before Training; PT; Post Training.

Figure 3. Preference of individually trained *Aedes aegypti* females. Female mosquitoes were individually tested in the olfactometer and given a choice between two stimuli: clean air *versus* air loaded with either clean air (white bar) CO_2 (2300ppm above ambient level; grey bar) or L-lactic acid (black bars). Preference is represented as the preference index computed from the distribution of insects in the olfactometer and error bars represent the standard errors of the binary distribution. Each bar represents an experimental group: *clean air*, neutral control group; *CO_2*, positive control group; *LA*, L-lactic acid naïve control group; *CS only*, CS- only group; *US only*, US-only group; *unpaired US–CS*, unpaired US–CS group; *paired US–CS*, appetitive conditioning group. Asterisks indicate distributions that are significantly different from random ($p<0.05$). Bars are the mean ± SEM.

Figure 4. Preference of group trained and individually tested *Aedes aegypti* females to different odorants. Once placed in the olfactometer, mosquitoes were given a choice between two stimuli: clean air *versus* air loaded with either: L-lactic acid, LA (black bars), 1-octen-3-ol, OCT (grey bars), myrcene, MYR (black hatching on grey background bars) or *Z*-3-hexen-1-ol, Z3H (grey hatching on white background bars). Preference is represented as the preference index computed from the distribution of insects in the olfactometer and error bars represent the standard errors of the binary distribution. Each bar represents an experimental group: *Naïve*, untrained groups; *Trained*, appetitive-conditioning groups. Asterisks indicate distributions that are significantly different from random ($p<0.05$). Bars are the mean ± SEM.

Figure 5. Preference of group trained and individually tested *Aedes aegypti* females to LA and DEET. Once placed in the olfactometer, mosquitoes were given a choice between two stimuli: clean air *versus* air loaded with a mixture of L-lactic acid (LA) and 10% DEET (black bars). Preference is represented as the preference index computed from the distribution of insects in the olfactometer and error bars represent the standard errors of the binary distribution. Each bar represents an experimental group: *Naïve*, untrained groups; *Trained LA*, trained with LA only; *Trained LA+DEET*, trained with LA plus DEET; *Pre-exp DEET, Trained LA*, pre-exposed to DEET 1h before training and then trained to LA only; *Pre-exp DEET, Trained LA+DEET*, pre-exposed to DEET 1h before training and then trained to LA+DEET. Asterisks indicate distributions that are significantly different from random ($p<0.05$), and hash signs indicate distributions that are significantly different from the distribution of naïve, untrained, insects when tested for their response to LA+DEET ($p<0.05$). Bars are the mean ± SEM.

Figure 6. Preference of individually trained *Aedes aegypti* females that were individually tested in the olfactometer and given a choice between two stimuli. Insects exposed to cold

shock (white dots on black background bars) were tested for their preference between clean air and air loaded with L-lactic acid. Insects fed on cycloheximide (CXM) were tested for their preference between clean air and LA (grey hatching on white background bars) or between clean air and CO_2 (2300ppm above ambient level; grey hatching on grey background bar). Preference is represented as the preference index computed from the distribution of insects in the olfactometer and error bars represent the standard errors of the binary distribution. Each bar represents an experimental group: *Cold shock 1h BT*, trained insects exposed to a cold shock 1h before training; *Cold shock 20min PT*, trained insects exposed to a cold shock 20min post training; *Cold shock 2h PT*, trained insects exposed to a cold shock 2h post training; *Cold shock 6h PT*, trained insects exposed to a cold shock 6h post training; *CXM 35mM*, trained insects fed on 35mM CXM in 10% sucrose solution during 16-18h previous to the training session; *CXM 17mM*, trained insects fed on 17mM CXM in 10% sucrose solution during 16-18h previous to the training session; *35mM CXM CO2*; naïve insects fed on 35mM CXM in 10% sucrose solution, and tested for their response to CO_2 as a positive control. Asterisks indicate distributions that are significantly different from random ($p<0.05$). Bars are the mean ± SEM.

SUPPLEMENTARY FIGURE

Figure S1. Percentage of response to an appetitive stimulation (35°C) of *Aedes aegypti* females, when tested at four different time windows. Groups of 10 six days old females were placed in plastic containers and exposed to the artificial feeder described in Fig. 1 during 5min. The number of females responding to a close range thermal stimulation was recorded for each group and the mean percentage of response was calculated for each time frame. *8-10h*, early morning (63.6%; n=11 repetitions); *12-14h*, noon (42.5%; n=16 repetitions); *16-18h*, late afternoon (59.2%; n=14 repetitions); *01-03h*, night (43.3%; n=9 repetitions). Different letters indicate significant differences (Pearson's Chi-squared test with Yates' continuity correction; $p<0.05$).

Figure S2. Distribution of group trained *Aedes aegypti* females that were group tested in the olfactometer and given the choice between an olfactometer arm bearing clean air (white bars) versus an arm loaded with either: 1-octen-3-ol, OCT (grey bars), nonanol, NON (black hatching on light-grey background bars); hexenol, Z3H (grey hatching on white background bars); benzyl alcohol, BEA (black vertical stripes on grey background bars) and myrcene, MYR (black hatching on dark grey background bars). Preference is represented by the percentage of mosquitoes choosing each of the two test arms. Each bar represents an experimental group: *US Only*, untrained groups, exposed to the US alone during the first session; *Trained*, appetitive-conditioning groups. The relatively low number of repetitions in these preliminary experiments did not allow any reliable statistical analysis.

Figure S3. Flight tracks of individually treated *Aedes aegypti*. Female mosquitoes were individually tested in the olfactometer and given the choice between two stimuli: clean air (white arrows) versus L-lactic acid, LA (black arrows). Thin arrows indicate the starting position of mosquitoes. a, naïve untrained insects (42% LA; n=12); b, trained untreated females (75% LA; n=12); c, trained females treated with 35mM CXM (54% LA; n=11). Each track represents one individual mosquito.

Figure S4. Average flight speed of *Aedes aegypti* females tested in an olfactometer and confronted with two air currents: clean air versus L-lactic acid. *Naïve*, naïve untrained insects

(n=12); *Trained*, trained untreated females (n=12); *CXM*, trained females treated with 35mM CXM (n=11). Asterisks indicate significant differences between groups (*t*-test, $p<0.05$).

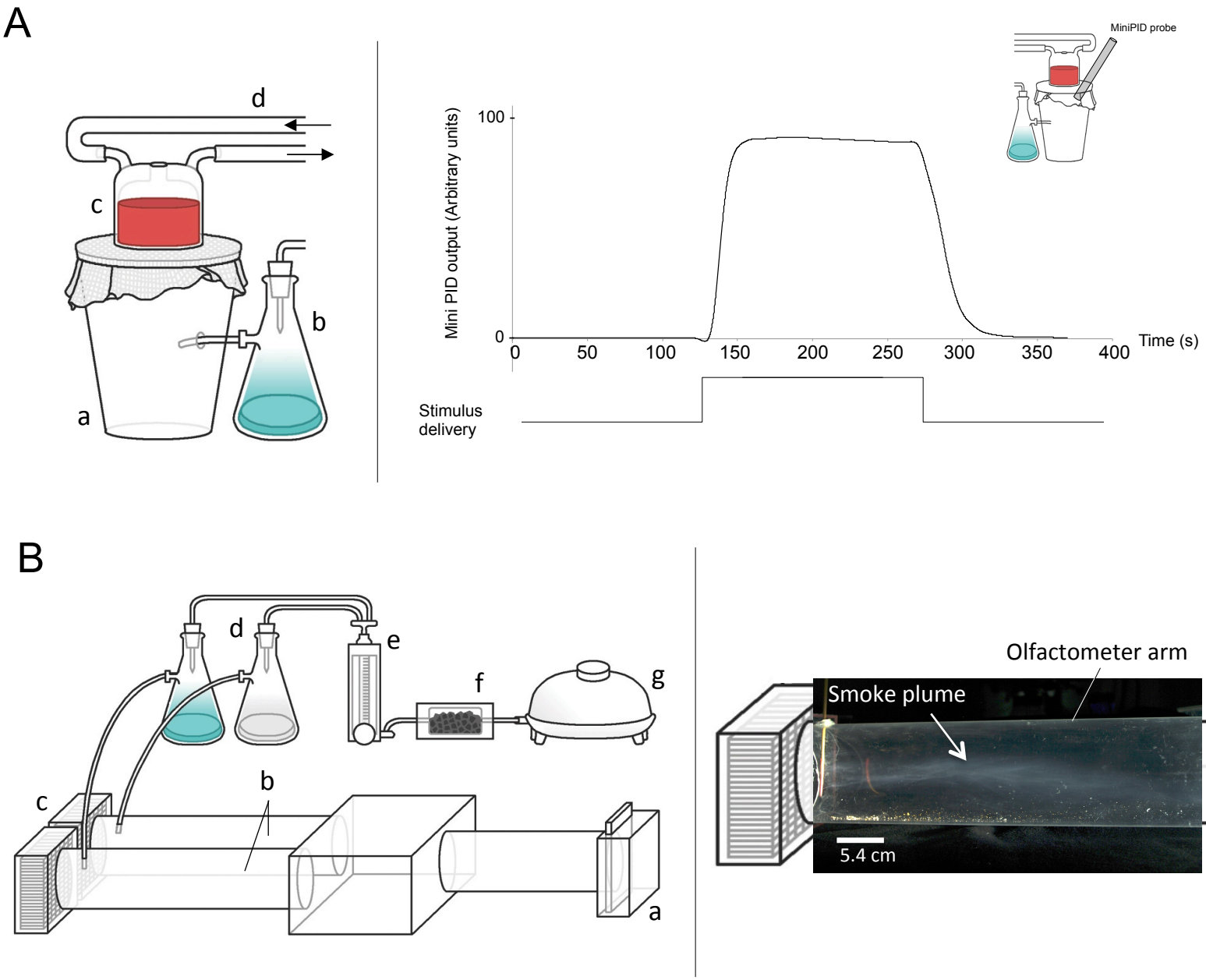
A
d
c
b
a
MiniPID probe
Mini PID output (Arbitrary units)
100
0
0
50
100
150
200
250
300
350
400
Time (s)
Stimulus
delivery
B
d
e
f
g
c
b
a
Olfactometer arm
Smoke plume
5.4 cm

Figure 1

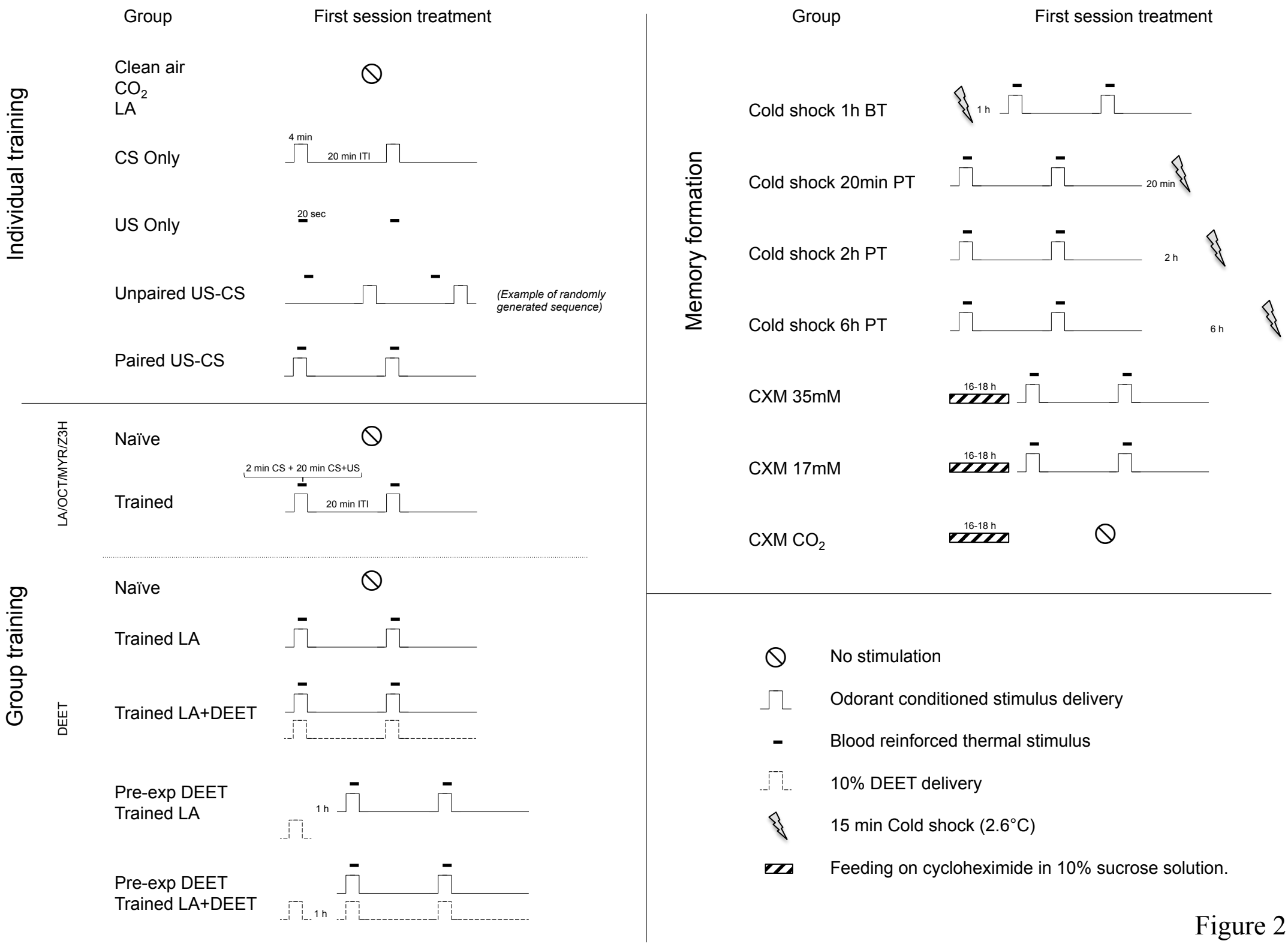
Group
First session treatment
Individual training
Clean air
CO_2
LA
CS Only
4 min
20 min ITI
US Only
20 sec
Unpaired US-CS
(Example of randomly generated sequence)
Paired US-CS
LA/OCT/MYR/Z3H
Naïve
Trained
2 min CS + 20 min CS+US
20 min ITI
Group training
DEET
Naïve
Trained LA
Trained LA+DEET
Pre-exp DEET
Trained LA
1 h
Pre-exp DEET
Trained LA+DEET
1 h
Group
First session treatment
Memory formation
Cold shock 1h BT
1 h
Cold shock 20min PT
20 min
Cold shock 2h PT
2 h
Cold shock 6h PT
6 h
CXM 35mM
16-18 h
CXM 17mM
16-18 h
CXM CO_2
16-18 h
No stimulation
Odorant conditioned stimulus delivery
Blood reinforced thermal stimulus
10% DEET delivery
15 min Cold shock (2.6°C)
Feeding on cycloheximide in 10% sucrose solution.

Figure 2

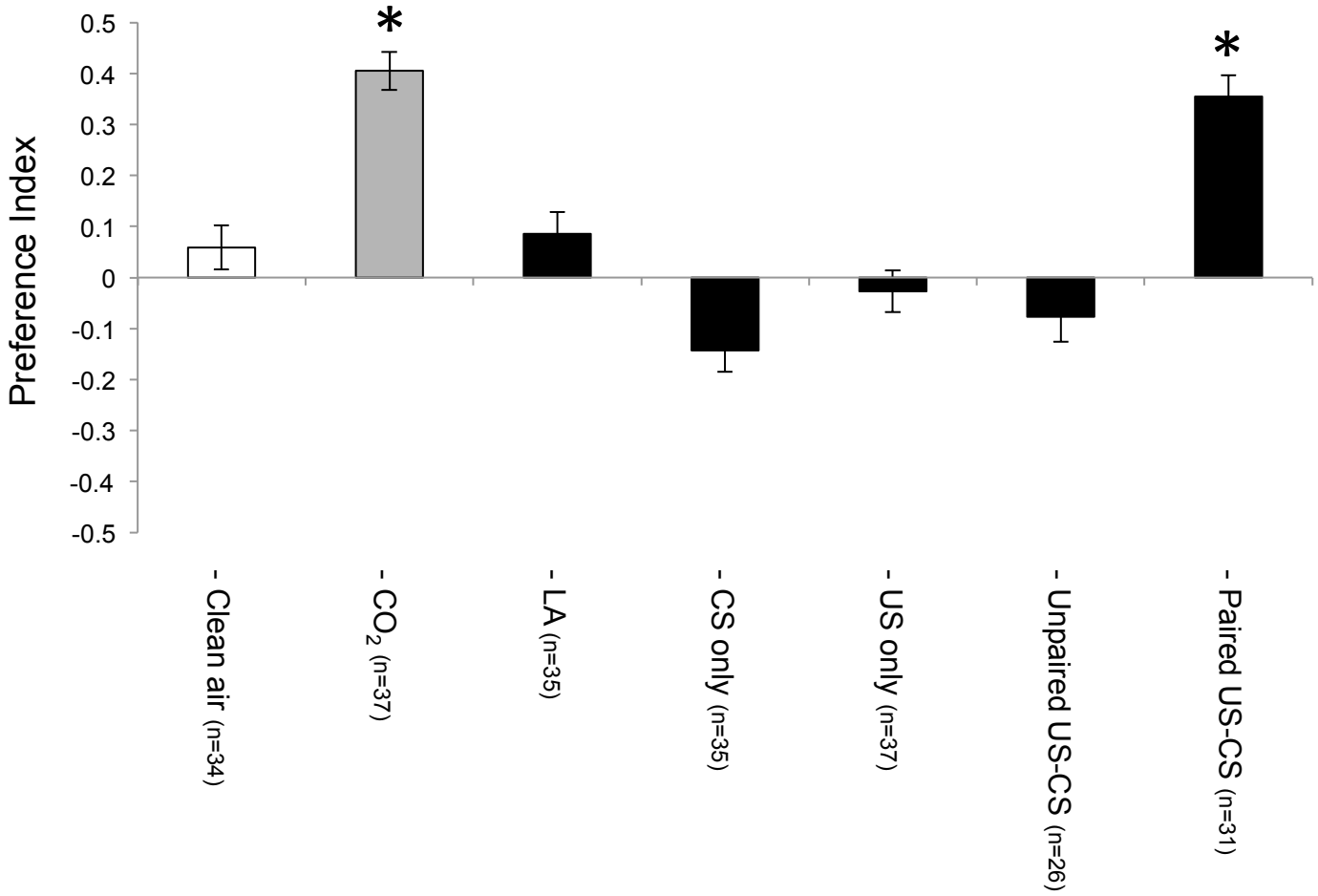
Preference Index
0.5
0.4
0.3
0.2
0.1
0
-0.1
-0.2
-0.3
-0.4
-0.5
*
*
- Clean air (n=34)
- CO2 (n=37)
- LA (n=35)
- CS only (n=35)
- US only (n=37)
- Unpaired US-CS (n=26)
- Paired US-CS (n=31)

Figure 3

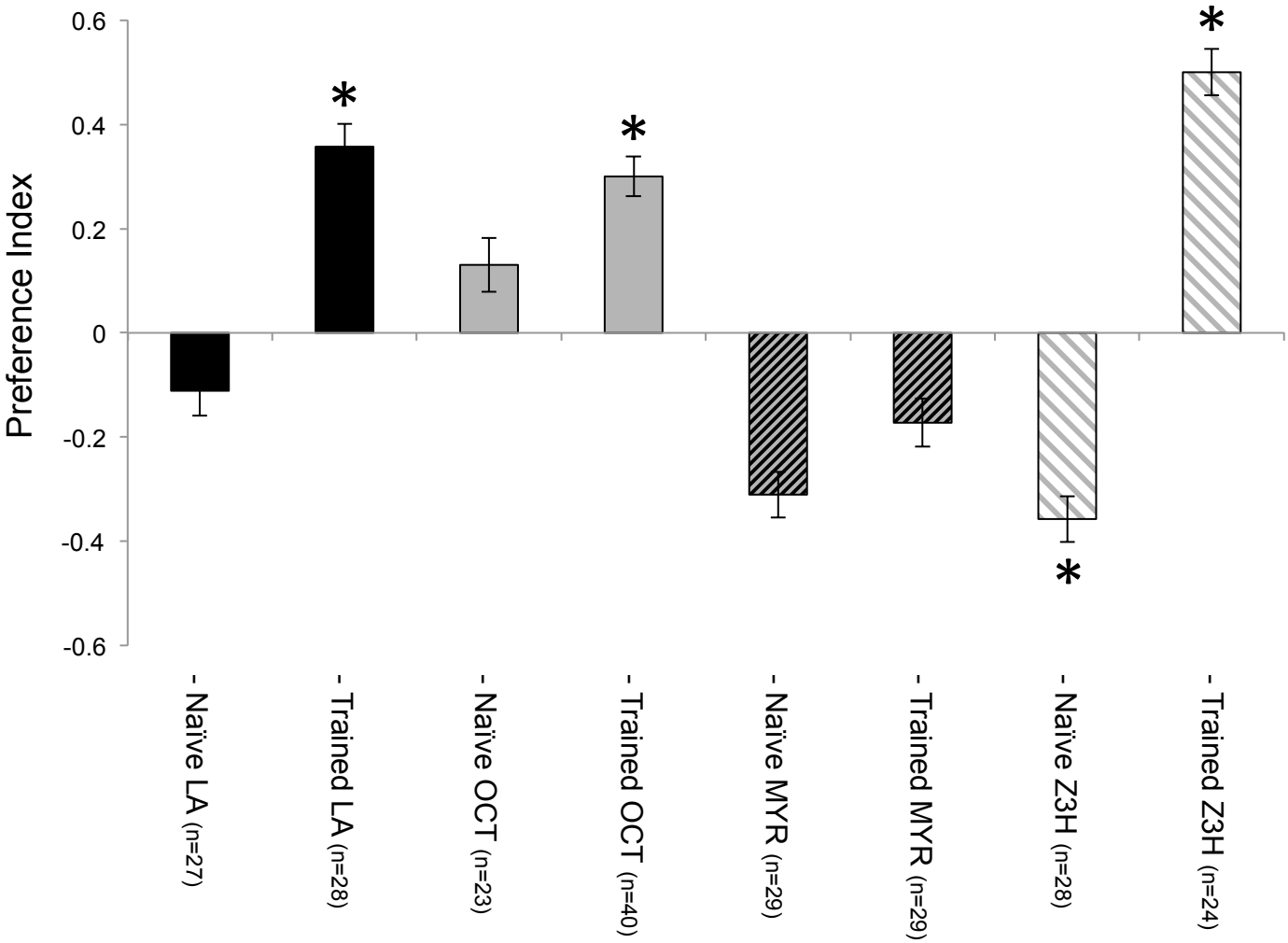
Preference Index
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
*
*
*
*
- Naïve LA (n=27)
- Trained LA (n=28)
- Naïve OCT (n=23)
- Trained OCT (n=40)
- Naïve MYR (n=29)
- Trained MYR (n=29)
- Naïve Z3H (n=28)
- Trained Z3H (n=24)

Figure 4

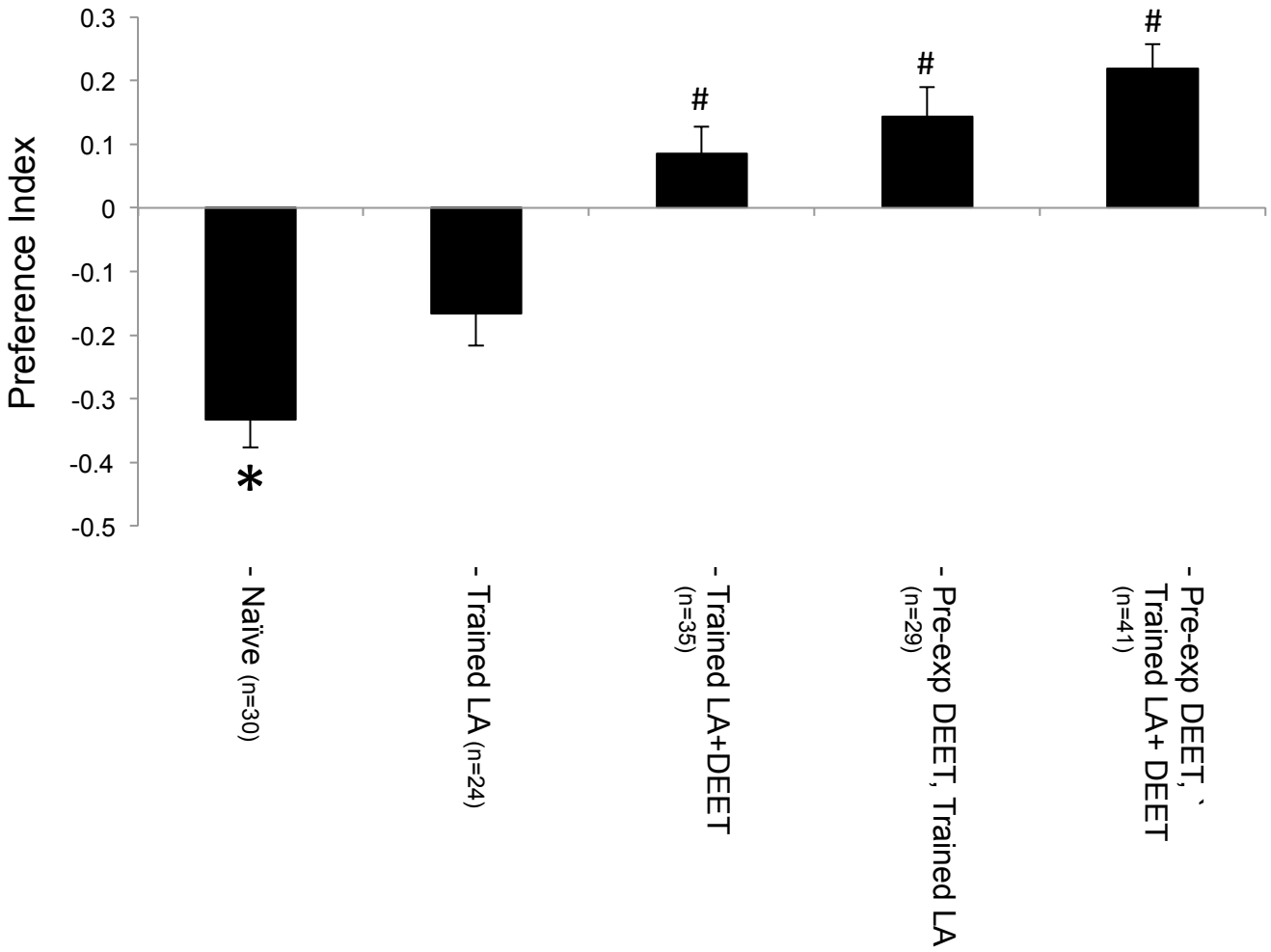
Preference Index
0.3
0.2
0.1
0
-0.1
-0.2
-0.3
-0.4
-0.5
*
#
#
#
- Naïve (n=30)
- Trained LA (n=24)
- Trained LA+DEET (n=35)
- Pre-exp DEET, Trained LA (n=29)
- Pre-exp DEET, ` Trained LA+ DEET (n=41)

Figure 5

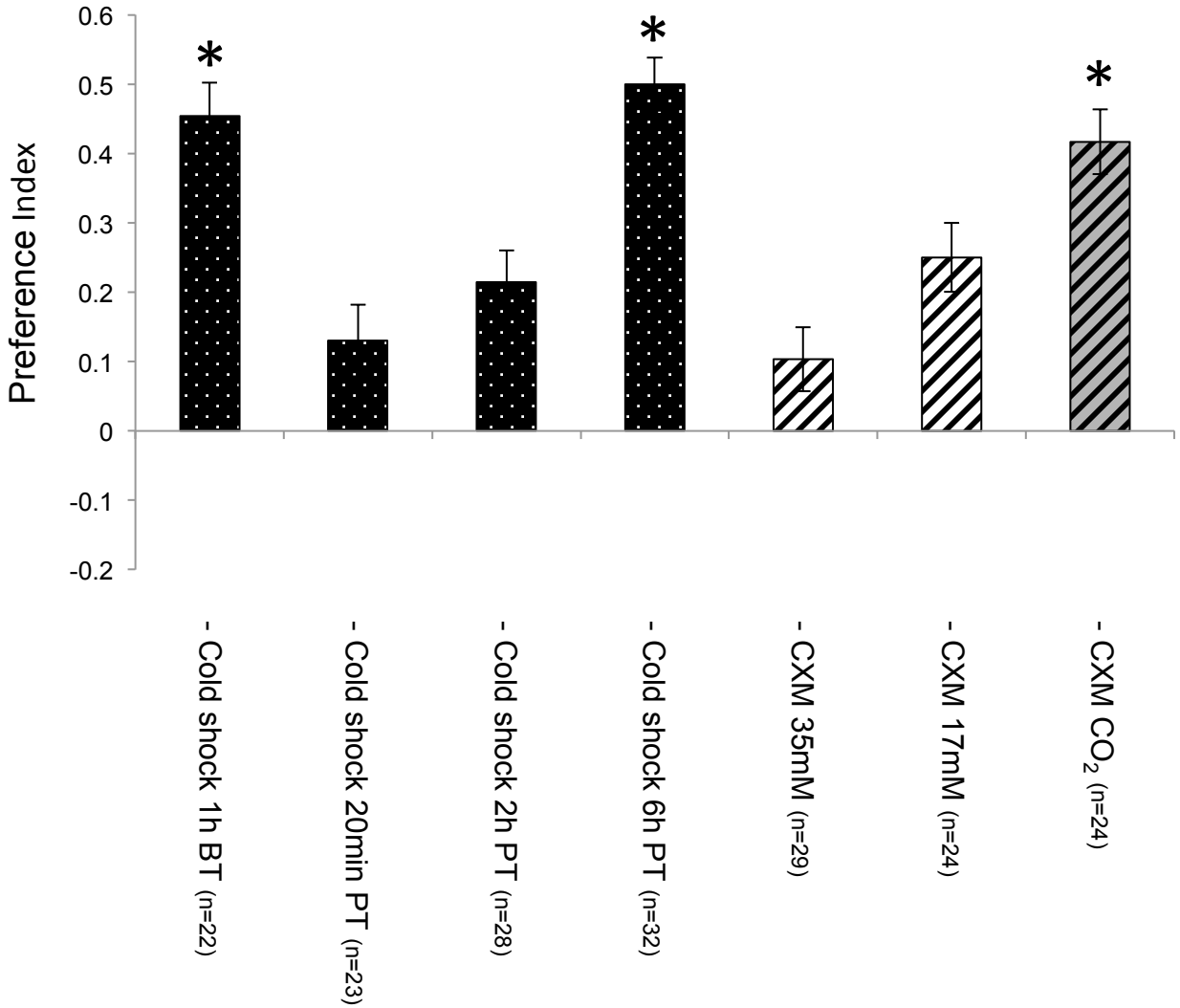
Preference Index
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
-0.2
*
*
*
- Cold shock 1h BT (n=22)
- Cold shock 20min PT (n=23)
- Cold shock 2h PT (n=28)
- Cold shock 6h PT (n=32)
- CXM 35mM (n=29)
- CXM 17mM (n=24)
- CXM CO_2 (n=24)

Figure 6