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Journal Pre-proofs

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PII: S0022-1910(20)30303-6
DOI: https://doi.org/10.1016/j.jinsphys.2020.104159
Reference: IP 104159

To appear in: *Journal of Insect Physiology*

Received Date: 25 May 2020
Revised Date: 28 September 2020
Accepted Date: 23 October 2020

Please cite this article as: Sriti Murmu, M., Hanoune, J., Choi, A., Bureau, V., Renou, M., Dacher, M., Deisig, N., Modulatory effects of pheromone on olfactory learning and memory in moths, *Journal of Insect Physiology* (2020), doi: https://doi.org/10.1016/j.jinsphys.2020.104159

MODULATORY EFFECTS OF PHEROMONE ON OLFACTORY LEARNING AND MEMORY IN MOTHS

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Running title: Pheromones modulate olfactory learning and memory in moths

ABSTRACT

Pheromones are chemical communication signals known to elicit stereotyped behaviours and/or physiological processes in individuals of the same species, generally in relation to a specific function (e.g. mate finding in moths). However, recent research suggests that pheromones can modulate behaviours, which are not directly related to their usual function and thus potentially affect behavioural plasticity. To test this hypothesis, we studied the possible modulatory effects of pheromones on olfactory learning and memory in *Agrotis ipsilon* moths, which are well-established models to study sex-pheromones. To achieve this, sexually mature male moths were trained to associate an odour with either a reward (appetitive learning) or punishment (aversive learning) and olfactory memory was tested at medium- and long-term (1h or 1.5h, and 24h). Our results show that male moths can learn to associate an odour with a sucrose reward, as well as a mild electric shock, and that olfactory memory persists over medium- and long-term range. Pheromones facilitated both appetitive and aversive olfactory learning: exposure to the conspecific sex-pheromone before conditioning enhanced appetitive but not aversive learning, while exposure to a sex-pheromone component of a heterospecific species (repellent) facilitated aversive but not appetitive learning. However, this effect was short-term, as medium- and long-term memory were not improved. Thus, in moths, pheromones can modulate olfactory learning and memory, indicating that they contribute to behavioural plasticity allowing optimization of the animal's behaviour under natural conditions. This might occur through an alteration of sensitization.

Keywords: moths, olfaction, plasticity, learning and memory, olfactory conditioning, pheromone, proboscis extension response (PER).

1. INTRODUCTION

Pheromones are chemical substances acting as communication signals. They elicit stereotypical behavioural responses or physiological processes in the receiving individual(s) of the same species (Karlson and Lüscher, 1959). They are therefore crucial cues in animal communication and mediate a wide range of responses in a large spectrum of behavioural and ecological contexts. Pheromone-elicited behaviours are generally innate and do not require learning (Wyatt, 2014). For instance, new-born rabbits display a stereotyped suckling behaviour when exposed to the mother-emitted mammary pheromone (Coureaud et al., 2000). Similarly, male moths show a stereotyped upwind flight-behaviour towards females when exposed to their sex pheromone (Schneider, 1992; Hansson, 1995; Allison and Cardé, 2016). In the honey bee, the main component of the sting alarm pheromone (isopentyl acetate) causes the receiver bees to sting, attack and stop foraging (Boch, 1962). Furthermore, alarm pheromone released from a stressed rat can serve as a unconditioned stimulus and produce associative learning in a receiver rat (Carew et al., 2018). Pheromone processing in the brain is usually specialized and dedicated structures often exist, underlying the specific and stereotyped behaviours they elicit; pheromone coding pathways are generally separated from other olfactory, non-pheromonal pathways (Christensen and Hildebrand, 2002). The components of female moth pheromone blends are detected by specialized olfactory receptor neurons housed in long trichoid hairs on the male antennae (Deisig et al., 2014), and processed in a macroglomerular complex, a sexually-dimorphic region of the antennal lobe constituted of hypertrophic glomeruli dedicated to respond to sex pheromone components (Hansson and Anton, 2000; Rospars and Hildebrand, 2000; Arnold et al., 1985). Insects employ a very rich repertoire of pheromones in a wide range of behavioural contexts (Sandoz et al., 2007) including recruitment of foragers (Von Frisch, 1967), for marking pathways to resources and indicating the resource richness (Thom et al., 2007), and colony defence (Hölldobler and Wilson, 1990).Thus, pheromones have well-documented functions as communication signals in specific contexts (e.g. mate finding, foraging, aggregation, alarm).

A series of results also points to a role of pheromones in modulating behavioural plasticity, contrasting with their classical description as elicitor of stereotyped behaviours. In the European rabbit, the mammary pheromone of females promotes learning of neutral odorants paired with the pheromone in new-born pup: after only a single paired trial, an originally neutral odour elicits a typical nipple searching and grasping response in the pups (Coureaud et al., 2006). Furthermore, this pheromone-induced olfactory learning is adaptive because it rapidly extends the range of odours that predict milk reward, thus improving the ability of the pups to find a nipple (Coureaud et al., 2006). During fear conditioning, in which a tone is paired with an electric shock, exposure to a recently fear-conditioned familiar mouse impairs acquisition of conditioned fear. This effect is mediated by a putative stress-related anxiogenic pheromone emitted by the sender and perceived by the receiver mouse to be conditioned (Bredy and Barad, 2009). Similarly, in the honey bee, pheromone could modulate the response to appetitive and aversive stimuli (Baracchi et al., 2017; Rossi et al., 2018; Urlacher et al., 2010). Moreover, exposure of young workers to the queen pheromone abolished aversive learning while the ability to learn appetitive associations (stimulus-reward association) remained intact (Vergoz et al., 2007). Thus, pheromones can modulate behaviours, which are not the primary target of their action by affecting their intensity, their success or probability of occurrence and by promoting plasticity.

Some insects are excellent model systems to study the modulatory effects of pheromones on olfactory learning since they possess a rich pheromone repertoire, well-established learning capacities, and an easy access to the neural circuits of pheromone- and odour associative-learning processing (Baracchi et al., 2017; Rossi et al., 2018; Urlacher et al., 2010, Vergoz et al., 2007). Insects use olfactory learning to locate food, find mates or avoid potential danger. The ability to associate odours with positive (i.e. reward) or negative (i.e. punishment) events could facilitate insects' capability to locate and find food-sources or potential mating partners, or to avoid potential noxious situations. Olfactory learning and memory have been extensively studied in many insect

species, the pioneer being the honey bee which is an excellent learner when it comes to associating odours with a sucrose reward (appetitive conditioning) or a mild electric shock (aversive conditioning). Appetitive conditioning of the proboscis extension response (PER; Takeda, 1961; Bitterman et al., 1983; Giurfa and Sandoz, 2012) leads to the formation of an association between an odour and a sucrose reward while in aversive conditioning of the sting-extension response (Vergoz et al., 2007; Giurfa et al., 2009), bees learn to associate an odour with a mild electric shock. Ants can be trained to form an appetitive odour-reward association via conditioning of the *maxilla-labium* response (Guerrieri and d'Ettorre, 2010, Guerrieri et al., 2011). Aversive conditioning protocols in harnessed ants implicate quantification of the mandible open response (MOR), an indicator of aggression (Guerrieri and d'Ettorre, 2008). Appetitive olfactory learning has also been shown for moths (Daly et al., 2001; Daly and Smith, 2000; Cunningham et al., 2004; Riffell et al., 2013). As honey bees, moths can be conditioned to release a PER to an odour previously associated with a sucrose reward (Hartlieb et al., 1999; Hartlieb, 1996; Fan and Hansson, 2001; Fan et al. 1997; Skiri et al., 2005).

Sex-pheromones have a well-established and important role in moth biology. Moths are essential actors in the study of pheromones (Schneider, 1992; Hansson, 1995; Allison and Cardé, 2016). Our hypothesis is that sex-pheromone could be modulating moth's learning abilities. Thus, in the present study, we first studied the performance of male *Agrotis ipsilon* moths in appetitive as well as in aversive olfactory learning assignments, both protocols being new in this species; then, we investigated whether these learnings could be modulated by sex-pheromones.

2. MATERIALS & METHODS

2.1. Animals

Adult male *A. ipsilon* (Hufnagel 1766, Lepidoptera: Noctuidae) were used at five days old, when they were sexually mature. Moths always had *ad libidum* access to water, but were deprived of

sucrose solution, which is commonly used to feed moths under laboratory conditions. Animals were kept in plastic boxes in an air-conditioned room at 23-24°C, 60-70% humidity and a 16:8h light: dark photoperiod (light at 18h). Since the photoperiod was inverted, all experiments were performed under red light (invisible for the moths). Furthermore, males were never exposed to the female pheromone or tested odours before experiments. For sucrose responsiveness and appetitive conditioning assays, individual moths were restrained in 1 ml pipette tips cut at the top before onset of the scotophase (**Figure 1a**). Restrained moths were then allowed to adapt to the experimental room for 2h before the experiment.

2.2. Sugar responsiveness

Before appetitive olfactory conditioning, we tested sucrose responsiveness in restrained male moths by establishing a dose-response curve in order to determine the optimal sucrose concentration for training, following the protocol of Hostachy et al (2019a). In moths, taste receptors are present on antennae as well as on their feet (Hostachy et al 2019a). For testing sugar responsiveness, both antennae of restrained male *A. ipsilon* were briefly touched with sucrose solutions of increasing concentrations: 0%, 0.1%, 0.3%, 1%, 3%, 10%, 30% and 60% (weight/weight). The antenna to be stimulated was randomly chosen. This sucrose stimulation on the antenna elicits PER if the concentration is high enough. The interval between successive sucrose presentations (inter-trial-interval, ITI) was five minutes.

2.3. Shock responsiveness

To conduct aversive olfactory conditioning, we developed a novel experimental set-up and tested for optimal parameters of shock application to be used later for aversive olfactory conditioning. The setup consisted of a rectangular Plexiglas arena (**Figure 1b**, Length: 18 cm, Width: 15 cm, Height: 1 cm) with an electric circuit covering the floor (kindly provided by CRCA Toulouse,

France). The height of the arena was chosen to prevent moths climbing on the side walls and walking upside down, thus escaping the electric circuit. The top cover was detachable to allow placement and removal of the moths. A shock-responsiveness curve was established without odour to determine optimal shock intensity. Single moths were subjected to increasing shock intensities: 0.4 mV, 0.6 mV, 0.8 mV, 4 mV, 6 mV, 8 mV and 10 mV. The interval between successive shock intensities (ISI) was ten minutes. The shock was delivered using a Velleman® DC Power Supply (LABPS1503) and the behaviour of moths during shock application was observed and quantified.

2.4. Odour Stimulus Delivery

For olfactory conditioning experiments, geraniol (98%, Sigma-Aldrich, St. Quentin Fallavier, France) was delivered via a SYNTECH stimulus controller CS-55 (Kirchzarten, Germany). Geraniol is classically used for insect appetitive olfactory conditioning as it is a component of flower odours. One µl of pure geraniol was applied to a piece of filter paper (0.5 cm x 0.5 cm, Fisherbrand, Fisher Bioblock, Illkirch, France). Pasteur pipettes connected to two dedicated channels were inserted into a Y-shaped glass hose connector. Air passed either through a Pasteur pipette containing a filter paper without odour (control airflow), or through a Pasteur pipette containing a filter paper with geraniol (odour loaded flow in case of olfactory stimulation). Odour-containing pipette was freshly prepared every experimental day. A permanent humidified airflow (0.5 ml/s) was delivered during the entire experiment to prevent mechanical stimulation by the air puff during stimulation. For olfactory stimulation during conditioning, the airflow was thus switched from one channel (no odour) to the other (odour). An exhaust system was placed behind the animal (appetitive conditioning, see below) or at the opposite side of the arena (aversive conditioning, see below) to remove odour from inside the arena.

2.5. Appetitive Conditioning and Memory Tests

During appetitive conditioning, restrained moths were trained to associate an odour (conditioned stimulus, CS) with a 60% sucrose reward (unconditioned stimulus, US). One conditioning trial consisted of a total of 30 seconds (**Figure 2a**); a moth was positioned in front of the odour-stimulation device, allowed to habituate for approximately 15 seconds before the odour was presented for five seconds. During the last two seconds of CS presentation, the US (sucrose solution) was presented to both antennae, which in general elicits the extension of the proboscis (PER) and moth was allowed to feed for five seconds. The CS-US overlapped for two seconds. The moth was left undisturbed in the device for 5 seconds before it was removed and the next moth was placed in front of the device. In an anticipation to obtain better learning curves, we tested different floral odours (geraniol and linalool) as conditioning stimuli. Similarly, the unconditioning stimulus (i.e. sucrose) was used at two different concentrations (60% and 67%). Geraniol produced better results, and therefore, the experiments described in figure 1-3 are done using geraniol odour as CS. The results obtained by 60% sucrose were better, thus, the experiments described in figure 1-3 are carried out using 60% sucrose as US.

Each moth received ten conditioning trials with an inter-trial interval (ITI) of 5 minutes. Comparing ITIs of 5 and 10 minutes showed better acquisition for 5 minutes, allowing to train moths by groups of 10. Odour response was evaluated by quantifying the PER (0 = no response, 1 = response); no moth responded to the odour during the first learning trial. Memory was tested by stimulating the antennae with the conditioned odour for five seconds at 1,5h (mid-term memory, MTM) and 24 hours (long-term memory, LTM) post-conditioning.

2.6. Aversive Conditioning and Memory Tests

For aversive conditioning experiments, moths were placed individually in the shock-conditioning arena. To present geraniol, the arena was connected to the stimulus controller on one side while the other side was connected to an exhaust-system to eliminate the odour. The conditioning

protocol followed the same time delays as the appetitive conditioning (**Figure 2a**): the animal was allowed to adapt for 15 s in the arena in a constant clean airflow, geraniol (CS) was then applied during five seconds; during the last two seconds of CS presentation, 8 mV electric shock (US) was delivered via the electrical grid. The CS-US overlap lasted for two seconds. The total duration of the shock was 7 seconds. The ITI was 10 minutes, so that moths were trained by groups of 10. Response to odour and/or shock was evaluated by quantifying the motor activity of moths (e.g. movement of antennae, legs, wings, body etc.): absence of motor activity was coded as 0 and motor activity as 1.

Mid and long-term memory were tested 1h and 24 hours post-training. For this, moths previously conditioned aversively were transferred to a four-arm olfactometer (Laucoin s.a., Thoiry, France, 50 x 50 cm, height: 3 cm, **Figure 2d**, Saïd et al., 2006), containing four sectors. Incoming airflow (generated by a membrane pump, Cole Parmer, U.S.A.) to each sector was controlled by a flowmeter (Brooks, U.S.A.) and adjusted to 500 ml/min. The design of the olfactometer allowed to have four independent sectors (plus a neutral sector in the middle, in which the air was extracted from below), which could contain either clean air or an odour, depending on the filter paper inserted at the entry of each sector. In our test protocol, the previously aversively conditioned odour geraniol was present in one sector, while the three other sectors contained clean control air. One µl of geraniol was applied to a piece of filter paper (1 cm x 1 cm) and placed inside a glass reservoir at the entry of the respective sector. The position of the odour-containing arm was rotated between mid and long-term memory testing periods to avoid bias due to possible spatial cues. For each test, an individual moth was released at the centre of the olfactometer, allowed to habituate for 20 Sec and its trajectory was then recorded by the experimenter during a five-minute test period using a custom-written software (EVEN v. 1.0; INRA, France). Data obtained using the software allowed to quantify time spent in each of the four sectors of the olfactometer during the test period. For some experiments (i.e. testing aversive memory after pheromone exposure, figure 3), results were not taken into account if the moths spent more than 20% of the total time in the middle sector. For another experiment (i.e.

figure2), we included all moths that moved out from the central zone because most of the moths remained immobile, and we had to take into account all moths which showed some activity.

2.7. Pheromone Exposure

Immediately prior to conditioning (appetitive or aversive, respectively), moths were exposed to one of the following compounds (**Figure 3a**): i) the sex pheromone blend emitted by *A. ipsilon* females (composed of three components: Z7-12:Ac, Z9-14:Ac and Z11-16:Ac in a ratio of 4:1:4, Picimbon et al., 1997; Gemeno and Haynes, 1998, Hoffmann et al., 2020), ii) one component of the sexual pheromone emitted by the heterospecific species *A. segetum* (i.e. Z5-10:Ac, which replaces Z11-16:Ac in the *A. ipsilon* pheromone blend, Renou et al., 1996) or iii) the pheromone solvent hexane (as control) for 15 minutes. For exposure, 5 µl of solution containing either compound at the appropriate dilution (10 ng/µl) were deposited on a piece of 1 cm x 1 cm filter paper placed inside a 1 ml Eppendorf tube. Pheromone or hexane exposure was carried out by placing five moths inside a small, dark plastic box containing one of the three compounds. Pheromones were purchased from Pherobank (http://www.pherobank.com, Wijk bij Duurstede, Netherland). N-Hexane (pure) was purchased from Carlo Erba Reagents S.A.S. (Val de Reuil, France).

2.8. Data and Statistical Analysis

For statistical analysis of binary data (appetitive and aversive training, retrieval test in appetitive memory), a binomial generalized linear mixed model (GLMM) was used. For statistical comparison of non-binary data in aversive memory retrieval, linear mixed model (LMM) was used. All statistical analysis was performed using the program R 3.4 and with an α risk of 5%.

3. RESULTS

3.1. Sucrose and Shock Responsiveness in male *A. ipsilon*

In a first series of experiments, the responsiveness of five-day old naïve male *A. ipsilon* was studied by establishing stimulus intensity-response curves. Behavioural responses were quantified in response to increasing concentrations of sucrose (%) or intensities of electric shock (mV).

PER rate increased linearly with the log of sucrose concentration (**Figure 1c,** N = 22); pure water did not elicit any PER. At the lowest sucrose concentration (0.1%) 32% of moths already showed PER. At concentrations above 3% more than 70% of moths showed PER, and the maximal PER rate was observed in the highest sucrose concentration.

In the absence of shock (control), moths were mostly immobile and did not show any movement (**Figure 1d,** N = 22). However, the application of increasing shock intensities generated three distinct types of motor behaviours: i) twitching ii) jumping, and iii) running. These behaviours were phase-locked with the shock-onset and could be interpreted as 'shock avoidance behaviour'. The probability of moths showing avoidance behaviours increased with increasing shock intensities with more than 40% of moth responding to the electric shock at 8 mV.

These results on sucrose and shock sensitivity allowed us to determine optimal intensities for the US in appetitive and aversive learning experiments: i.e. 60% (w/w) for the sucrose solution and 8 mV for the mild electric shock.

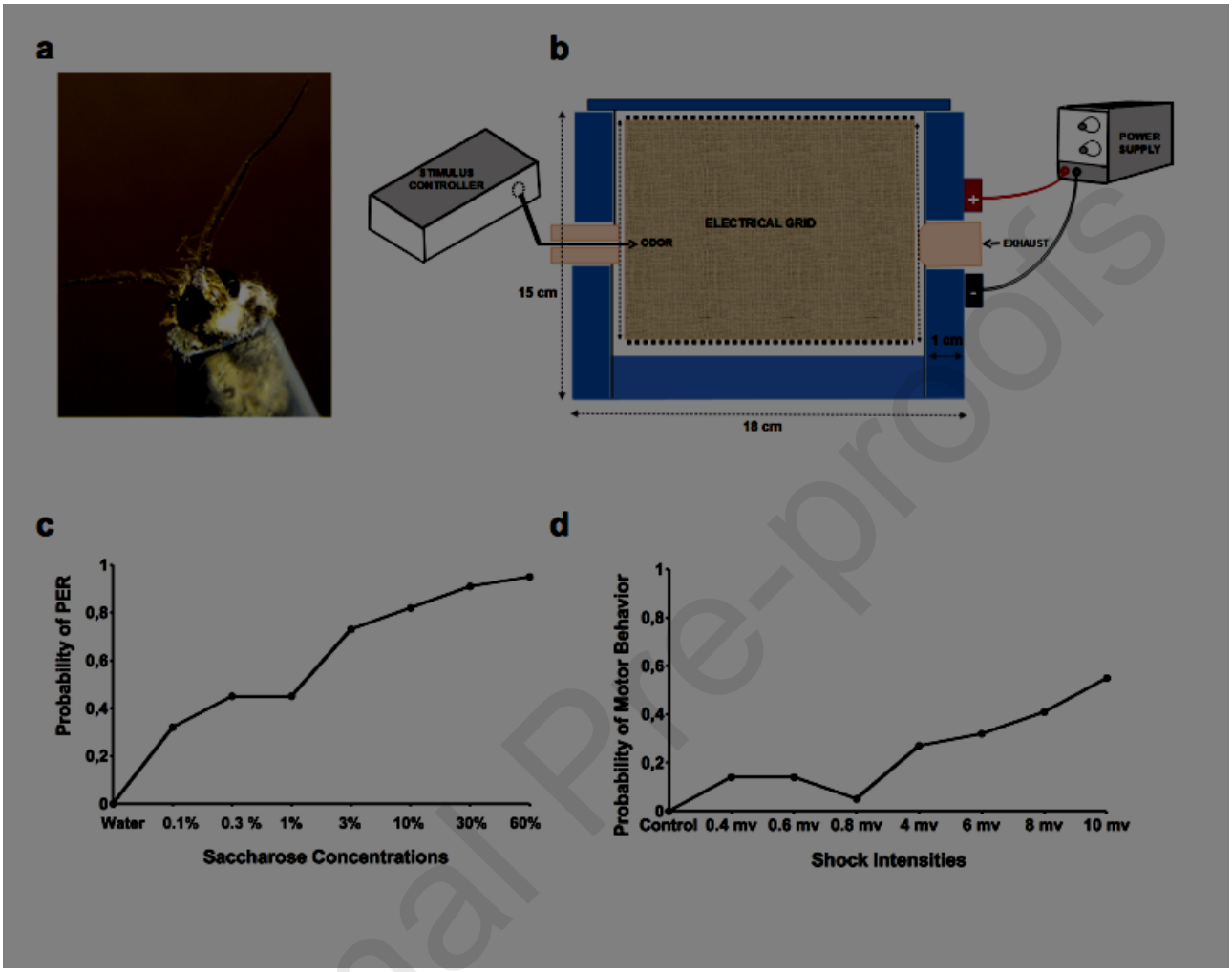

Figure 1. Testing responsiveness of *Agrotis ipsilon* males to increasing concentrations of sugar or intensities of electric shocks. a) A restrained moth. b) Experimental set-up to deliver pulses of electric shock, and to quantify shock-induced motor behaviours. c) Probability of PER to increasing concentration of sucrose. d) Probability of avoidance-like behaviour to varying intensities of electric shock.

3.2. Appetitive Olfactory Learning and Memory in Male Moths

When trained along 10 conditioning trials, some male *A. ipsilon* successfully learned to associate geraniol with a 60% sucrose reward (**Figure 2b**, GLMM binomial family: $p < 0.0001$, $N = 87$). Correct responses increased along training and maximum learning was found in the 9th conditioning trial where approximately 40% of moths showed conditioned PER to the odor. During training, even non-responding moths always released a PER in response to sucrose solution (US). A few moths formed an appetitive memory for geraniol, which lasted for 1h30 (mid-term-memory) and stayed stable up to 24 h (long-term memory, **Figure 2c**).

Further studies confirmed that our training parameters were optimal. Performance during appetitive learning was significantly higher for 5-min ITI group than for 10-min ITI group (GLMM binomial family; significant effect of interval ($p = 0.0001$) or trial ($p = 0.0001$, **Figure S1a**). In addition, moths trained to associate geraniol with 60% sucrose reward showed higher percentage of PER than the moths trained to associate geraniol with 67% sucrose reward (GLMM binomial family: significant effect of trial, $p = 0.0001$, and concentration, **Figure S1b**). Furthermore, moths trained to associate 1 µl pure geraniol (0.8 mg) with 60% sucrose reward displayed higher percentage of PER than the moths trained to associate sucrose with diluted geraniol or pure linalool (GLMM binomial family: $p = 0.012$ for lower concentration geraniol, $p < 0.001$ for linalool, **Figure S1c**). Finally, we found that the moths that were trained using the usual conditioning protocol displayed significantly higher percentage of PER than those trained using the trace conditioning protocol, i.e. a protocol during which CS and US do not overlap (GLMM binomial family: significant effect of protocol, $p = 0.06$) or trial effect ($p = 0.0001$, **Figure S1d**).

3.3. Aversive Olfactory Learning and Memory in Male Moths

When trained along 10 conditioning trials, male *A. ipsilon* did not respond to geraniol in the first trial, however, a single pairing with US (electric shock) resulted in an increased locomotor activity (avoidance-like behaviour) to the odour in the second trial, which then decreased during the

successive trials. Due to this response pattern, the learning curve was overall not significantly different from 0 (**Figure 2e**, GLMM binomial family: $p = 0.080$, $N = 30$; a generalized additive model was used to take into account the non-linear performance).

The moths did not form an aversive memory (neither MTM, nor LTM) for geraniol since they spent the same amount of time in all four sectors of the olfactometer (**Figure 2f**). These data suggest that unlike appetitive memory, medium- or long-term aversive memory was not formed as the moths failed to avoid the previously shock-associated odour. It is likely that moths have a hard time learning to associate flower odour compound (geraniol) to an artificial stimulus (electric shock).

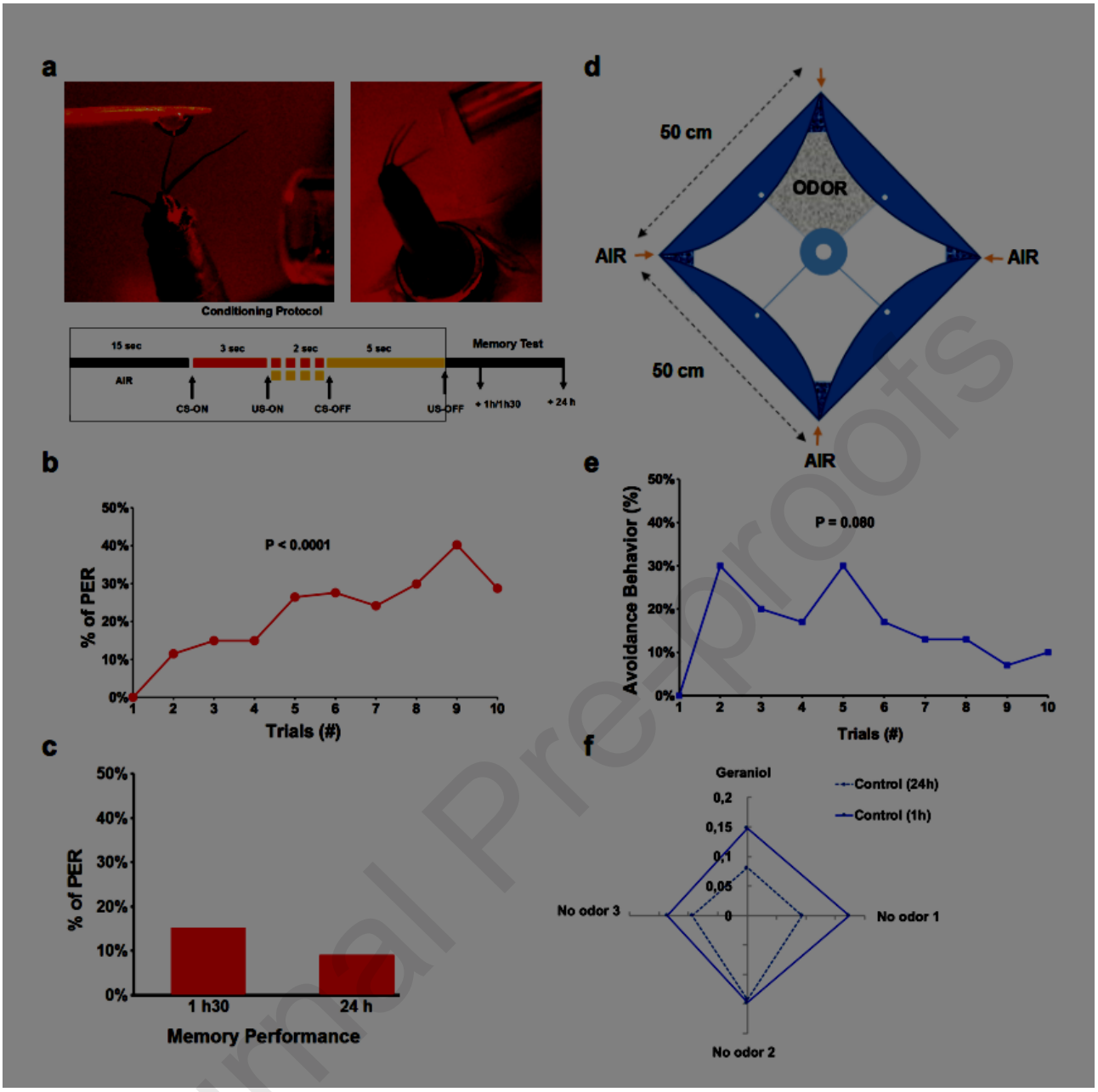

Figure 2. ***Agrotis Ipsilon*** **Males Perform Appetitive and Aversive Olfactory Tasks.** a) Moth is placed in front of the olfactometer and following odour-stimulation (CS, geraniol) receives a sucrose reward (US) (image on the left). Moth learns the CS-US association and extends its proboscis to an odour (image on the right). A conditioning pairing consisted of 10 odour/sucrose pairing, each pairing lasting 30s; the chronology of one pairing is described in the schematic. b) Percentage of animals that showed PER to geraniol during appetitive conditioning. c) The percentage of animals that extended

their proboscis to geraniol during mid (1h30) and long-term (24h) appetitive memory tests. d) Schematic of four-arm olfactometer used for aversive memory experiments. e) Percentage of animals that responded to geraniol during aversive conditioning. f) Percentage of time spent (in minutes) in each area of the olfactometer during mid (1h, N = 11) and long-term (24h, N = 11) aversive memory tests.

3.4. Pheromone Exposure and its Effect on Appetitive Olfactory Learning and Memory

Exposure to a conspecific female sex pheromone blend (Z7-Z9-Z11) for 15 minutes prior to conditioning significantly increased learning of geraniol compared to the solvent-exposed group (**Figure 3b**, red vs. blue curve, Binomial GLMM, effect of treatment: $p = 0.0001$). Exposure of males to *A. segetum* pheromone component Z5 did not modulate learning compared to the control group (**Figure 3b**, yellow vs. blue curve, Binomial GLMM, effect of treatment: $p = 0.15$). Interestingly, performance in the control group (i.e. solvent exposed) was lower in this experiment than in the previous controls (i.e. controls not exposed to any compound, **Figure 2b** and **Figure S1**).

MTM and LTM for geraniol did not differ in moths exposed prior to conditioning to the conspecific sex pheromone or the heterospecific pheromone component Z5. However, appetitive LTM for geraniol was significantly increased in moth exposed to solvent prior to conditioning compared to MTM (**Figure 3c**, blue bars, Binomial GLMM, effect of memory, $p = 0.030$), suggesting LTM performance is not correlated to previous performance (see e.g. Dacher and Gauthier, 2008); however, this is not consistent with results shown in **Figure 2c**. A similar effect was not observed for animals exposed to sex-pheromone or Z5, suggesting LTM formation is impaired in these groups. This suggests sex-pheromone improves performance during training, but prevents LTM formation; only performance during training is affected.

The facilitation in appetitive learning performance was not due to changes in appetitive motivation. Moths exposed to solvent or pheromone showed similar sucrose responsiveness during

appetitive conditioning. Likewise, appetitive pheromone does not modulate sugar responsiveness in our sugar responsiveness test (**Figure S2a**), which is consistent with the study by Hostachy et al. (2019b). Thus, we assume that the facilitation of appetitive learning induced by a conspecific pheromone is not due to changes in reward perception.

3.5. Pheromone Exposure and its Effect on Aversive Olfactory Learning and Memory

Exposure of males to heterospecific pheromone component Z5 for 15 min before training increased aversive learning of geraniol (**Figure 3d**, yellow vs. blue lines, Binomial GLMM, interaction trial*treatment: $p = 0.003$). By contrast, conspecific sex-pheromone did not improve aversive learning (**Figure 3d**, red vs. blue lines, Binomial GLMM: $p = 0.177$). The performance of the control groups is similar in Figure 2e and 3d, and as observed previously is not significant (**Figure 3d**, blue line, Binomial GLMM: $p = 0.508$).

During MTM testing, control animals did not avoid the sector with the shock associated odour (**Figure 3e**). By contrast, males exposed to the conspecific pheromone blend or to the heterospecific pheromone component Z5 seemed to avoid this sector at 1h but not 24h after training (**Figure 3e-f**); however, the difference is not significant, (except for the Z5 group, which spent more time in arm 3, GLMM, $p = 0.021$). There was no difference between the group during LTM testing.

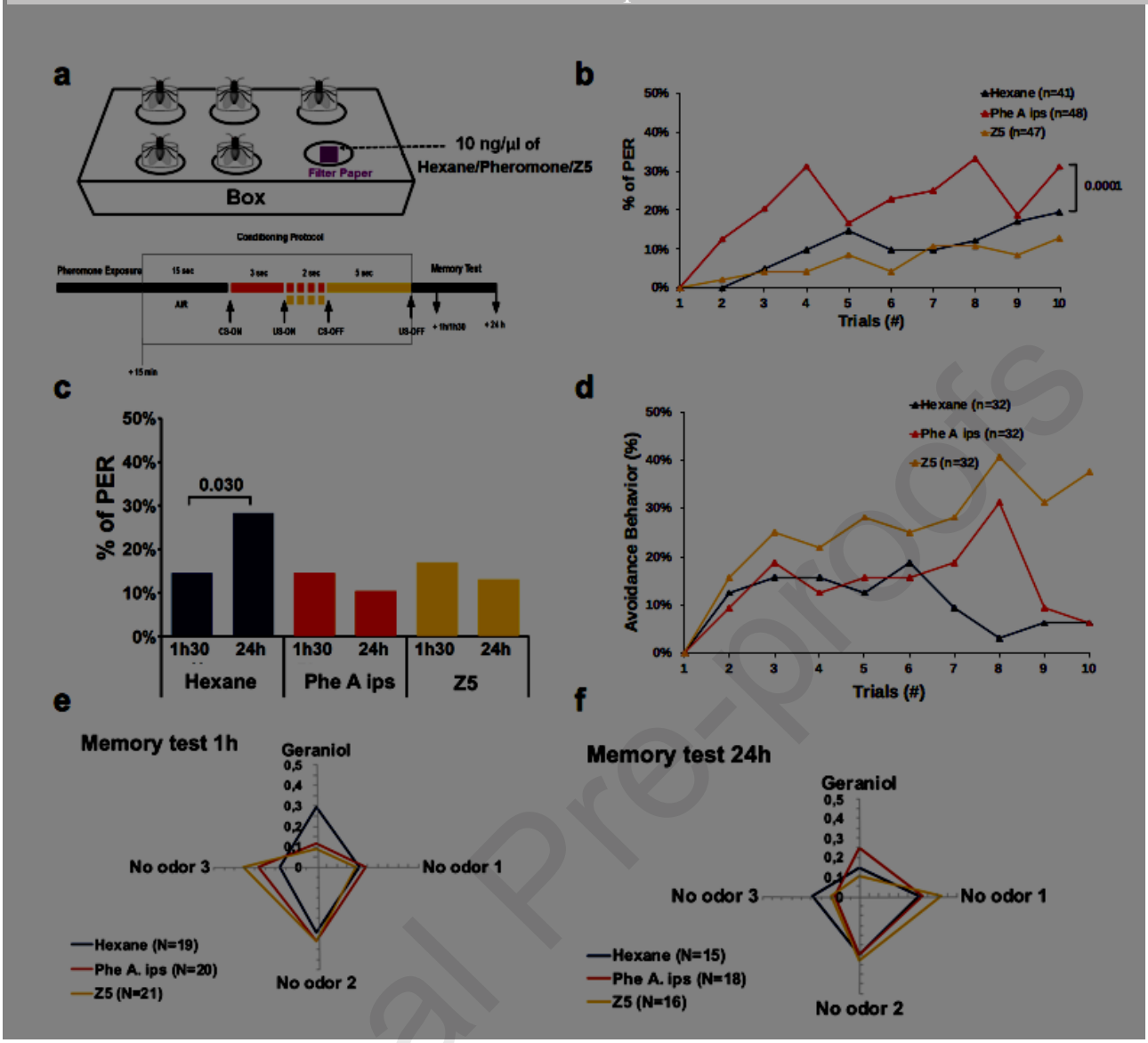

Figure 3. Pheromone Modulation of Appetitive and Aversive Learning/Memory Formation. a) Pheromone exposure set-up (picture above) and conditioning protocol (box below). b-c) The percentage of moths showing PER to CS (odour: geraniol) after hexane, conspecific female sex pheromone blend (Z7-Z9-Z11) or heterospecific pheromone (Z5) exposure during appetitive conditioning (b), and during mid (1h30) and long-term (24h) appetitive memory tests (c). d-f) The percentage of moths showing avoidance-like behaviours to CS (odour: geraniol) after hexane, conspecific female sex pheromone blend (Z7-Z9-Z11) or heterospecific pheromone (Z5) exposure

during aversive conditioning (d), and during mid (1h, e) and long-term (24h, f) aversive memory test. Time in e-f is in minutes.

4. DISCUSSION

4.1. Moth Feeding and Avoidance Behaviours

In Lepidoptera, sugars (e.g. sucrose, fructose, and glucose) stimulate feeding behaviour, and constitute the most important food source (Boggs, 1987; Gilbert and Singer, 1975). It is thus vital for these insects to detect them. It is equally important for insects to detect warning or aversive signals as they could indicate the presence of a predator, pathogen or harmful substances. In laboratory conditions, feeding behaviour in moths has extensively been studied using the proboscis extension reflex (PER) assay (Hostachy et al., 2019a). In contrast to the PER, the behavioural response elicited by aversive signals is studied to a lower extent; nevertheless, there is data to suggest that moths can detect and avoid aversive signals (Salloum et al., 2011).

Consistent with previous findings in various moth species including *Agrotis ipsilon* (Hartlieb 1996; Fan et al 1997; Daly et al 2004; Skiri et al 2005; Hostachy et al., 2019a), touching of moth's antennae with sucrose resulted in the moth extending its proboscis, which was consistent and highly reproducible among individuals and from trial to trial. Furthermore, PER was concentration-dependent: increasing the sucrose concentration increased the probability of the moth displaying a PER. These results indicate that sucrose induce robust PER and could be reliably used to characterize feeding behaviours in *Agrotis ipsilon* moths, as reported previously by Hostachy et al (2019a).

Presentation of an aversive stimulus (i.e. electric shock) produced a wide range of avoidance behaviours, which was phased-locked with the shock onset. Increasing the shock intensity increased the probability of avoidance-like behaviours. Taken together, our shock sensitivity assay set-up not only allowed us to accurately monitor behavioural responses to aversive signal in *Agrotis ipsilon* adults, but also allowed us to discover voltages that evoke the best avoidance response.

4.2. Moths Learn and Memorize Odour Cues Associated with Reward and Warning Signals

Moths use olfaction not only to locate food, find mates or oviposition sites, but also to detect potentially noxious stimuli. Learning to associate an olfactory cue with reward or warning signal is thus important for food-searching, reproduction and defensive behaviours. It is well known in honey bees that olfactory learning in laboratory conditions is relevant, as they can be transferred to free-flying foraging, and vice-versa (Gerber et al., 1996; Sandoz et al., 2000; Chaffiol et al., 2005; Gil and De Marco, 2006). Our conditioning results show that tethered *Agrotis ipsilon* male moths learn to associate a floral odour with sucrose, suggesting that like bees, they could use such information during foraging (Riffell et al., 2013).

In appetitive conditioning, CS alone did not initially induce a conditioned PER; however, after a single CS-US pairing (e.g. during the second trial), 11% of moths displayed a conditioned response (i.e. PER) to the odorant. Unlike honey bees, moths are known to require a higher number of trials (e.g. at least 8 trials to reach a maximal CS-US association, Skiri et al., 2005). This phenomenon was also observed in our appetitive conditioning experiments as the conditioned PER was found to be highest during the 9th trial. In our study, the highest proportion of moths that learned CS-US association is around 40%, which is not unusual: similar acquisition curve has been reported in other moth species (Skiri et al., 2005; Hartlieb, 1996). Lower learning performance could not be explained by the lack of motivation in moths since they systematically responded to sugar during appetitive conditioning. It is entirely possible that the moths possess inherent lower learning capabilities than social insects given their shorter life span and solitary living habits.

The attributes of the CS are critical in the associative olfactory learning process. The identity, concentration and frequency of CS regulate the animal's ability to learn CS-US association. Certain odorants result in a better learning curve than others (Skiri et al., 2005). Similarly, the ability to learn CS-US association is sensitive to the concentration, and better learning performance are observed when odour concentration is optimal (Skiri et al., 2005; Daly et al., 2001). Consistently with these

findings, we found that the moth's ability to learn an odour-sucrose association was regulated by the identity and intensity of the CS (**Figure S1c**). These results suggest that the characteristics of the CS are critical in determining whether the moths can learn an olfactory cue associated with a reward signal, how fast the moths learn an odour-reward association as well as the overall learning success rates.

Similar to the CS, the features of the US also affected the abilities of moths to learn odour-sucrose association. We found that as compared to 67% sucrose reward, a pairing of an odour (CS) with 60% sucrose (US) resulted in a higher proportion of moths (up to 32% increase) showing a conditioned PER to the odour. We also observed that trace conditioning (Conditioning protocol in which the CS and US do not overlap but are presented separately with an interval of time in between) resulted in fewer moths (-87% decrease) showing a conditioned PER to the odorant. Furthermore, we found that the CS-US association was optimal at 5-min ITI relative to 10-min ITI. These results provide important insights into associative olfactory learning mechanisms in moths.

In retrieval tests, only some of the moths were able to remember CS-US association. Re-presentation of geraniol at 1h30 resulted in approximately 34% of moths showing a conditioned PER to the odour. They did not forget information related to the rewarding stimulus 24h post-conditioning. These results imply that moths are capable of building long-term olfactory memories. In moths, learning of plant odours primarily serves self-consumption and oviposition. It is likely that the appetitive olfactory memory observed in our study is adequate to mediate foraging and oviposition purposes in moths. However, not all moths were able to learn; moths that did learn might be advantaged in nature.

In moths, aversive learning has been studied to a lower extent because of some obvious difficulties. Absence of a robust physical demonstration of defensive behaviour (such as extension of sting in honey bees) makes aversive learning in moths challenging; nevertheless, in some species of moths, aversive learning has been studied by quantifying the suppression of PER (Jorgensen et al.,

2007). We developed a new set-up to study the moth's ability to learn an odour-shock association and form aversive memories. In aversive conditioning, CS alone did not induce an avoidance response; however, after a single CS-US pairing (e.g. during the second trial), 30% of moths displayed a conditioned avoidance response to the odour. In an anticipation of punishment, the moths tried to avoid an odour previously associated with an aversive signal. In the consecutive trials, there was a significant reduction in the proportion of moths showing CS-US association. We did not investigate how attributes of the aversive stimulus, for instance, the intensity, and duration regulate the moth's ability to form odour-shock association. Future studies will address whether varying the intensity, duration or an inter-trial interval improve the overall aversive learning curves in moths.

Avoidance of an odour was not retained at mid- or long-term memory testing periods indicating that the moths seem to forget information about the aversive signal rather quickly. Alternatively, their responses during training might be supported by a non-associative learning, i.e. sensitization to the electric shock: they would initially respond to the odour because they are excited by the shock rather than because they recognize the odour. In that case, they would not learn at all. By contrast, in some moth species, associative aversive long-term memory is retained for at least 48h post conditioning (Jorgensen et al., 2007), but unlike our study, the aversive stimulus used was the bitter compound found in nectars (Barlow et al., 2017). It is possible that we did not use the most suitable memory-testing set-up, as electric shocks are not natural stimuli. Moreover, to assess MTM and LTM, moths were placed in a four-arm olfactometer where avoidance to an odour-containing arm was measured. This was carried out in anticipation to obtain better retention scores given that the aversive acquisition curve was relatively low. It is possible that changing the set-up (and thus the context) prevented the animals to fully recall the learned CS-US association (Rosas et al., 2013). Future experiments should address whether the aversive retention scores could be improved using the same set-up that was used for the aversive conditioning experiments.

We found that the few moths learning odour-shock associations did so at a much faster rate than for odour-sucrose association. After a single CS-US pairing, 30% of moths showed a conditioned avoidance response, whereas only 10% of moths showed a conditioned PER to an odour. Aversive signals represent danger and are associated with the noxious stimulus. It seems logical that the moths learn olfactory cues associated with the aversive signal faster to respond quickly and generate appropriate defensive behaviour; this is also consistent with the hypothesis that the initial response is actually sensitization rather than associative learning. Olfactory cues associated with a reward signal probably does not require an immediate response. Interestingly, although the moths learn odour-shock association at a much faster rate, the conditioned avoidance response decreased significantly during the consecutive trials. By contrast, moths learn odour-sucrose association at a much slower rate, but the conditioned PER increased during the consecutive trials.

4.3. Pheromone Modulation of Appetitive or Aversive Learning

Learning and memory is not a static process. Wide ranges of intrinsic (e.g. age, motivational state) and extrinsic (e.g. environmental, stressors) factors modulate cognitive process (Mallon et al., 2003; Iqbal and Mueller, 2007; Farooqui, 2008; Amdam et al., 2010). Among the extrinsic factors, pheromones have recently been reported to modulate the probability of performing certain behaviours including learning in several model organisms including vertebrates and insects. Pheromones act as modulators of cognitive tasks and affect the capacity of animal to learn and memorize specific information about its environment. Recent studies indicate that olfactory appetitive or aversive learning can be enhanced or impaired by pheromones depending on the valence of the task (Urlacher et al., 2010; Vergoz et al., 2007; Free, 1987; Hunt, 2007; Nieh, 2010). Our results suggest that moths exposed to a conspecific (Z7-Z9-Z11) but not heterospecific (Z5) sex-pheromone showed appetitive learning performances that were significantly higher than moths exposed to the solvent, hexane; they had the opposite effect on aversive learning. Pheromone exposure prior to conditioning leads to better

learning by affecting the success of learning. We observed that in the appetitive conditioning assay, conspecific-pheromone exposed male moths not only learn faster as compared to the solvent or heterospecific-pheromone exposed group but exhibit higher olfactory appetitive learning than the latter two. It is interesting that the pheromones emitted by the conspecific females enhance olfactory appetitive but not olfactory aversive learning while pheromones emitted by the heterospecific males (i.e. Z5) enhance aversive but not appetitive learning. These results suggest that pheromones provide contextual information rendering subsequent learning more or less relevant. Our hypothesis is that exposure to sex-pheromones prior to conditioning makes the moth more vigilant or alert allowing them to respond better to relevant stimuli while at the same time preventing them from being distracted by stimuli of secondary importance. Foraging, a collective behaviour is largely ruled by olfaction. In some insects, sex pheromones play a crucial role in foraging (Bordereau and Pasteels, 2010) and promote food-searching behaviours (Poivet et al., 2012). In the context of olfactory appetitive learning, it could be argued that the male moths might perceive exposure to conspecific sex pheromones as a signal to aggregate at a common site that could favour enhance food intake, and thus show enhanced olfactory appetitive learning. On the other hand, pheromone emitted by a heterospecific males does not signal resource richness and therefore, do not improve appetitive olfactory learning. In the context of aversive olfactory learning, pheromone emitted by heterospecific males, but not conspecific females may act as a relevant cue for unfavourable conditions (e.g. resource scarcity) resulting in higher olfactory aversive learning. A modulation of the non-associative component of learning (McSweeney and Murphy, 2009) could explain our result: conspecific sex-pheromone would facilitate sensitization to sucrose, and heterospecific pheromone would facilitate sensitization to electric shock. As sensitization and habituation are opposing phenomena (Peeke and Petrinovich, 1984; Eisenstein et al., 2001; Eisenstein and Eisenstein, 2006; Blumstein, 2016), this explanation is consistent with the impairing effect of sex-pheromones on sucrose habituation that we previously observed (Hostachy et al., 2019b; see also Baracchi et al., 2018 for honey bees).

4.4. Conclusion

To conclude, our study provides important insights into pheromonal action. Moths are agricultural pests of various plants of economic importance (e.g. tomatoes, corn). The findings that pheromones can modulate the learning capabilities of moths have a functional significance in the fields of pest control. How pheromones influence insect learning and memory is valuable for the conception and development of novel methods of pest control. A comprehensive knowledge on the olfactory modulation of moth's learning capacities by attractant (e.g. Z7-Z9-Z11) or deterrent (e.g. Z5) pheromones is useful, and could be used to develop specific traps to attract or lure moths or to deter them from specific cultures.

AUTHOR CONTRIBUTIONS

MSM, MR, MD and ND designed the experiments, which were conducted by MSM, JH, AC and VB. MSM wrote the first draft of the manuscript, and all authors contributed to manuscript revision, read and approved the submitted version.

DECLARATION OF COMPETING INTEREST

The authors declare no competing financial interests.

ACKNOWLEDGEMENTS

We thank our colleagues Lucie Conchou and Camille Meslin for their comments on this article and technicians from iEES Paris for rearing the animals used in the experiments. This work was supported by Agence Nationale de la Recherche (project PheroMod, ANR grant number ANR-14-CE18-0003).

SUPPLEMENTARY DATA

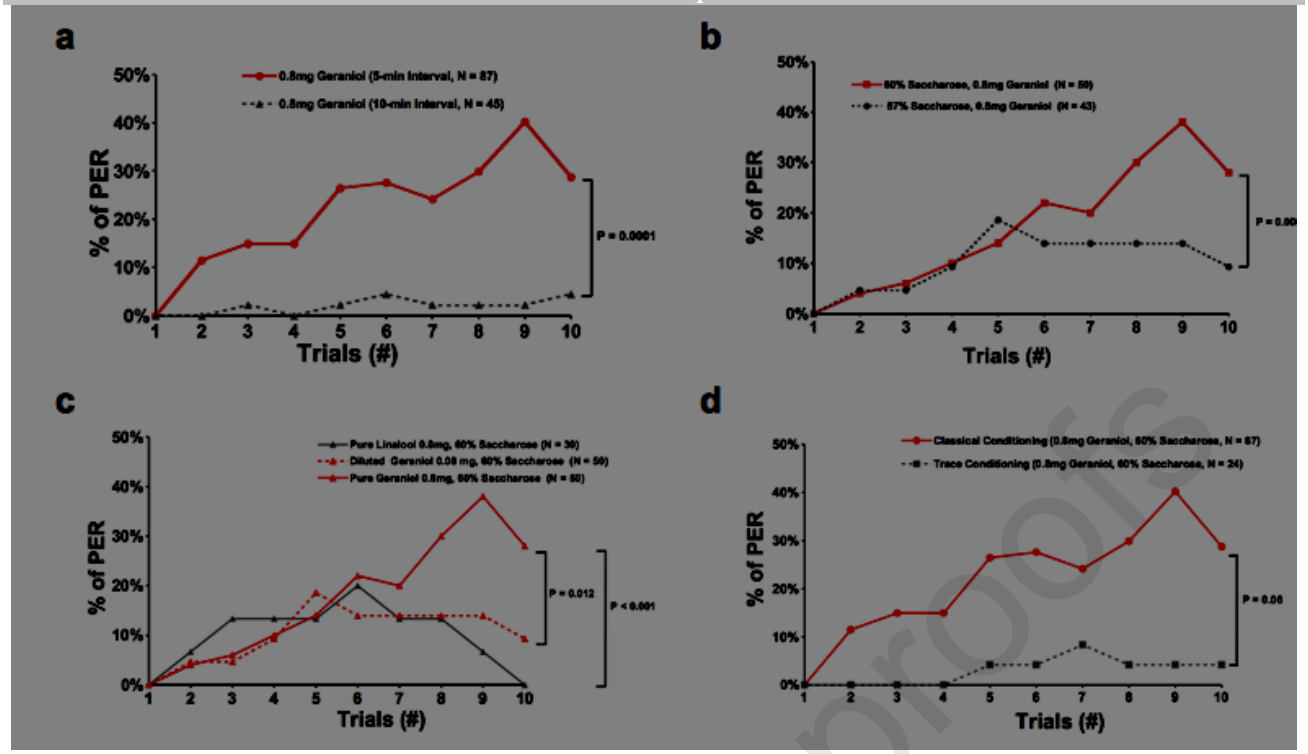

Figure S1: Factors modulating the acquisition of appetitive olfactory learning in male moths. a) The percentage of male *Agrotis ipsilon* moths showing PER to geraniol at 5 or 10-min inter-trial interval. b) The percentage of moths showing PER to geraniol in conditions where 60% or 67% sucrose was used as a reward. c) The percentage of moths showing PER to pure linalool, diluted or pure geraniol. d) The percentage of moths showing PER to CS in classical versus trace conditioning (i.e. without overlap between geraniol and sucrose).

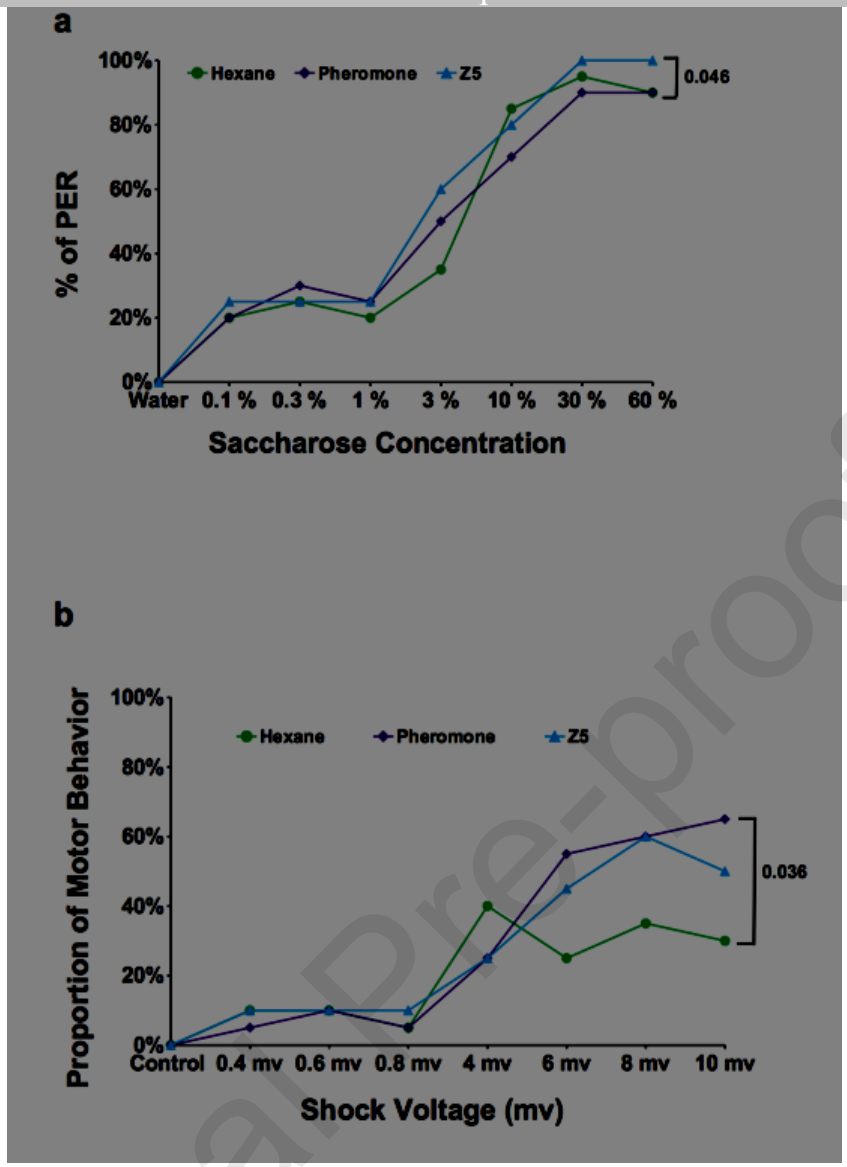

Figure S2: Effect of pheromones on sucrose (a) and shock (b) Responsiveness. a-b) Proportion of male moths showing a) PER to varying concentrations of sucrose, b) avoidance behaviour to varying intensities of shock voltage after solvent (i.e. hexane), pheromone (i.e. conspecific sex pheromone) or Z5 (heterospecific pheromone) exposure. a) A significant difference was found between the percentage of PER displayed by Z5 and pheromone-exposed moths (P = 0.046). b) Sex-pheromone exposed animals had a slightly higher response probability to the shock relative to hexane-exposed moth (p = 0.036), probably because of the highest voltage. N = 20/group.

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AUTHOR STATEMENT

Manuscript title: MODULATORY EFFECTS OF PHEROMONE ON OLFACTORY LEARNING AND MEMORY IN MOTHS

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- Drafting the manuscript: Meena Sriti MURMU, Matthieu DACHER, Nina DEISIG
- Revising the manuscript critically for important intellectual content: Meena Sriti MURMU, Matthieu DACHER, Nina DEISIG

DECLARATION OF COMPETING INTEREST

The authors declare no competing financial interests.