

RESEARCH ARTICLE

A long-latency aversive learning mechanism enables locusts to avoid odours associated with the consequences of ingesting toxic food

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SUMMARY

Avoiding food that contains toxins is crucial for the survival of many animals, particularly herbivores, because many plants defend themselves with toxins. Some animals can learn to avoid food containing toxins not through its taste but by the toxins' effects following ingestion, though how they do so remains unclear. We studied how desert locusts (*Schistocerca gregaria*), which are generalist herbivores, form post-ingestive aversive memories and use them to make appropriate olfactory-based decisions in a Y-maze. Locusts form an aversion gradually to an odour paired with food containing the toxin nicotine hydrogen tartrate (NHT), suggesting the involvement of a long-latency associative mechanism. Pairing of odour and toxin-free food accompanied by NHT injections at different latencies showed that locusts could form an association between an odour and toxic malaise, which could be separated by up to 30 min. Tasting but not swallowing the food, or the temporal separation of odour and food, prevents the formation of these long-latency associations, showing that they are post-ingestive. A second associative mechanism not contingent upon feeding operates only when odour presentation is simultaneous with NHT injection. Post-ingestive memory formation is not disrupted by exposure to a novel odour alone but can be if the odour is accompanied by simultaneous NHT injection. Thus, the timing with which food, odour and toxin are encountered whilst foraging is likely to influence memory formation and subsequent foraging decisions. Therefore, locusts can form specific long-lasting aversive olfactory associations that they can use to avoid toxin-containing foods whilst foraging.

Key words: aversive conditioning, classical conditioning, decision-making, insect, memory, olfaction.

INTRODUCTION

Feeding, though necessary for obtaining energy and nutrients, often entails a risk of ingesting toxins. Learning to avoid certain foods is thus essential to reduce exposure to toxins; its importance is reflected in the diversity of animal groups in which learned food aversions have been described (Garcia et al., 1974; Gelperin, 1975; Logue, 1985; Bernays, 1993; Manteifel and Karelina, 1996; Zhang et al., 2005).

In insects, food selection is regulated by gustatory systems (Chapman, 2003; Yamolinsky et al., 2009). The taste of nutrients or toxic substances within the food detected by gustatory receptors can be associated with other environmental stimuli and used in subsequent feeding decisions (Bernays, 1993; Chapman, 2003; Gerber et al., 2009; Yamolinsky et al., 2009). Insects, like vertebrates (Garcia et al., 1974; Gelperin, 1975; Longue, 1985; Manteifel and Karelina, 1996), are also able to learn and make decisions based upon the consequences of eating toxic food (Bernays, 1993). Although non-associative mechanisms can account for some instances of food avoidance behaviour (Bernays, 1993; Simpson and Raubenheimer, 1993), insects can form aversive associations between post-ingestive inputs elicited by poisoning with the taste or odour of food (Bernays and Lee, 1988; Lee and Bernays, 1990; Wright et al., 2010). Associative food aversion learning is characterised by a relatively long delay between food intake and visceral noxious effects (Bernays, 1993), though fast-acting post-

ingestive toxic signals have also been identified (Glendinning, 1996). Appetitive associations can also be formed between the tastes or odours of food and specific nutrients detected after ingestion (Burke and Waddell, 2011; Fujita and Tanimura, 2011).

Because not all toxins can be tasted to deter ingestion, learning from post-ingestive feedback can provide an additional means by which insects can avoid a particular toxin-containing food. Generalist herbivores, like locusts, are known to use non-associative as well as pre- and post-ingestive associative learning mechanisms to evaluate food and decide whether to ingest it (Szentesi and Bernays, 1984; Blaney and Simmonds, 1985; Blaney et al., 1985; Bernays and Lee, 1988; Lee and Bernays, 1990; Chapman et al., 1991; Behmer et al., 1999; Simões et al., 2011). Pre- and post-ingestive learning processes may involve separate neuroaminergic pathways, suggesting they are mechanistically distinct (Wright et al., 2010). However, how aversive associations with post-ingestive inputs are made by insects, particularly at the level of temporal dynamics, and the nature of their relationship to other learning mechanisms remains unclear.

We studied the mechanisms by which aversive associations are formed in desert locusts (*Schistocerca gregaria*). Using a Y-maze paradigm (Simões et al., 2011), we show that the olfactory preference of locusts can be aversively conditioned by pairing a naively preferred odour with a nicotine hydrogen tartrate (NHT)-enriched diet. A single trial was sufficient to produce a robust aversive

memory that lasted for 24 h. This association was concentration dependent. However, odour aversion was not observed immediately, but rather increased gradually until 4 h after training. Injection of NHT demonstrated that this long-latency odour aversion was due to an olfactory association with the post-ingestive effects of the toxin, which could be separated by up to 30 min. However, the post-ingestive aversive association was conditional upon the ingestion of food simultaneously with exposure to the odour. We also observed a second, food-independent, olfactory associative mechanism, which operates only when the conditioned odour was simultaneous with the NHT microinjection, with no long-latency association observed. The action of this second mechanism can, under certain conditions, interfere with the acquisition of the post-ingestive aversive response.

MATERIALS AND METHODS

Animals

Gregarious adult desert locusts (*Schistocerca gregaria* Forskål 1775) of either sex were taken 5 days after their final moult from crowded colonies maintained either at the Department of Zoology, University of Cambridge, UK or at LiveFoods Direct™, Sheffield, UK. Batches of approximately 40 adult locusts were transferred to a heated holding tank (24×30×20 cm, 35°C) and starved for 24 h. The locusts remained in the holding tank to maintain crowding except during training and testing.

Associative conditioning of odour aversion

Locusts were secured in Plasticine® and allowed to rest for 5 min prior to training. The aversive unconditioned stimulus (US) was composed of NHT (NS260, Sigma-Aldrich, St Louis, MO, USA) mixed in a blank artificial diet. The blank diet was composed of cellulose, which is indigestible to locusts (Dadd, 1960; Simpson and Abisgold, 1985). The diet was mixed with an equal volume of water and delivered to the locust's mouth on a small metal spatula.

Aversive olfactory conditioning was designed to work against the locusts' naive preference for vanilla over lemon odour (Simões et al., 2011). Therefore, the conditioned stimulus (CS) was 25 µl of pure vanilla extract (Dr Oetker, Thorpe Park, Leeds, UK) on 1 cm² of filter paper inside a plastic tube connected to an air pump. The end of the tubing was directed towards the locusts' antennae and placed approximately 5 cm away. A single training trial consisted of a 5 s of CS presentation followed by 20 s of simultaneous CS/US presentation. A 20 s pre- and post-trial resting time was given. Animals that refused to feed in any of the trials were excluded. Each experimental group comprised 44 locusts. After training, the locusts were removed from the Plasticine® and returned to the holding tank to await testing.

Microinjections

Locusts were injected with 8.5 µl of 0.4% NHT in physiological saline per gram of body mass using a Hamilton Microliter® Syringe (Bonaduz, Switzerland) that penetrated the cuticle at the wing insertion. An equivalent dose has been used previously in food aversion learning studies in *Schistocerca americana* (Bernays and Lee, 1988; Lee and Bernays, 1990). Control animals were injected with an equivalent volume of saline without NHT. NHT injection typically caused one or more of the following symptoms: unusual leg stretching, difficulty in locomotion, regurgitation, increased ventilation, prostration and 'knockdown'. These symptoms persisted for approximately 5–10 min. No increase in mortality was observed after 24 h in pilot tests. Some locusts exhibited no apparent symptoms. Locusts that refused the US during conditioning were excluded.

Locusts were injected before, during, or after the training trial. For injections during the training trial, the syringe was positioned at the wing insertion of the restrained locust ready for injection 2 min before the start of the trial, and the solutions were injected in the last 5 s of the training trial. At other injection times, unrestrained locusts were taken from the holding tank, injected and returned to the tank. The percentage of animals discarded did not differ significantly across experiments, except for those in which NHT was injected 15 min before conditioning, when the rejection percentage was higher, though still below 25%.

Y-maze arena and odour preference test

Locusts were submitted to a single odour preference test in a Y-shaped arena containing a raised wooden Y-shaped rod on which the locusts had to walk. The two alternative test odours were presented in the two decision arms: vanilla extract (the CS), and lemon extract (Holland & Barrett, Nuneaton, Warwickshire, UK), the untrained odour. The arena, odour delivery setup and test procedure are described elsewhere (Simões et al., 2011). Half of the animals were tested with vanilla odour in the right arm and the other half with vanilla in the left. A locust was placed inside a holding tube positioned on the stem of the Y-bar (Simões et al., 2011) and left undisturbed for a few seconds before the air flow inside the Y-maze was turned on. Each locust was allowed 5 min to exit the tube and make a choice. A choice was made when a locust walked to the end of an arm and touched the wall with either the front legs or the antennae. Locusts that failed to make a decision within 5 min, or that fell off or jumped from the rod were discarded. After each test, the locust was removed from the Y-maze and the wooden rod was wiped with 70% alcohol to disperse any pheromone cues and left to dry.

Statistical analysis

The locusts' odour preference in the Y-maze test was recorded. In some experiments, the occurrence of regurgitation and mortality was also recorded. The statistical tests used reflected the binary nature of these response variables. In each experimental group, both the locusts' sex and the position of the odour relative to the decision arms were balanced. *G*-tests for independence were used to determine whether sex affected the locusts' behaviour. *G*-tests for goodness of fit were used to determine whether there was divergence from an expected 50% decision for each arm side. No significant sex differences or side biases were found in any of the experiments; consequently, the data were pooled with respect to sex and odour position in the Y-maze's arms. The odour preference after aversive conditioning was compared against that of naive locusts as an extrinsic null hypothesis in *G*-tests for goodness-of-fit. For experiments involving microinjections, the odour preference of non-injected conditioned locusts was used as the extrinsic null hypothesis. All other statistical comparisons between experimental groups were by *G*-tests for independence. Yate's correction for continuity was used to avoid overestimation of significance when response counts are low (Sokal and Rohlf, 1998). The Dunn–Sidak correction was applied to determine significance in multiple comparisons tests (Sokal and Rohlf, 1998). The standard error of the locusts' odour preference was calculated as $\sqrt{p(1-p)/n}$, where *p* is the proportion of locusts that avoid the CS and *n* is the number of locusts that were tested (Collett, 2002).

RESULTS

Associative conditioning of odour aversion

Naive locusts prefer vanilla odour when placed in a Y-maze in which one arm contains lemon odour and the other vanilla odour (Simões

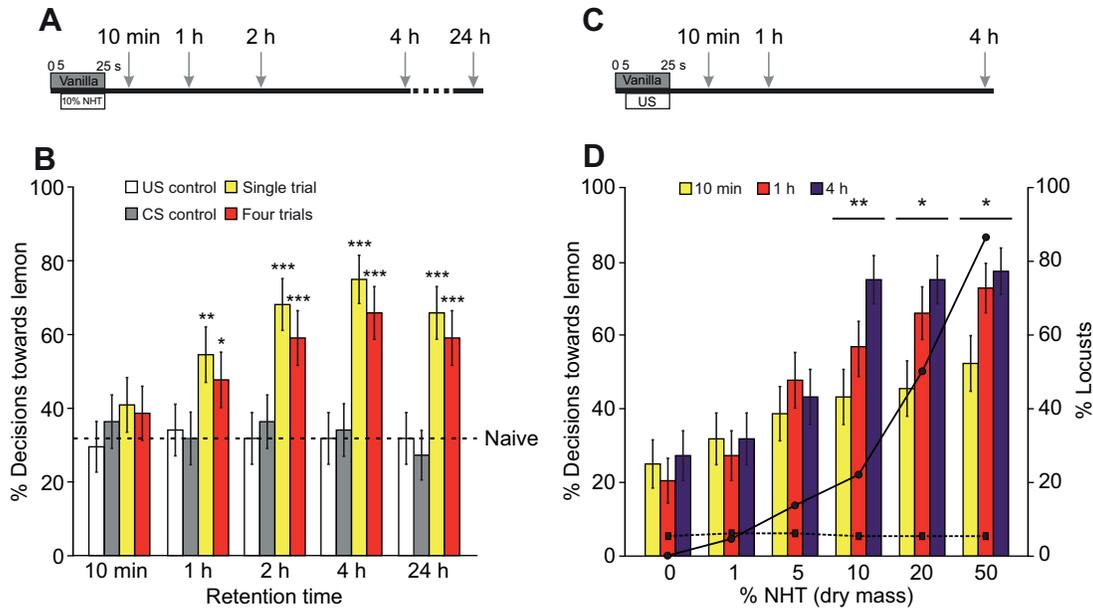


Fig. 1. Aversive associative conditioning of the desert locusts' odour preference. (A) Schedule for aversive conditioning with vanilla odour as the conditioned stimulus (CS) and blank artificial diet containing 10% nicotine hydrogen tartrate (NHT) as the unconditioned stimulus (US). (B) Odour preference of aversively conditioned locusts, measured as the percentage of decisions towards the arm with lemon odour in the Y-maze. Paired CS/US training caused a significant increase in the preference for lemon compared with that of naive locusts (dashed line). Asterisks indicate a significant difference from the naive preference (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). (C) Single-paired trial aversive conditioning using one of six different NHT concentrations as the US. (D) Toxin concentration, defined as percent of NHT dry mass in the blank diet, influenced odour preference of locusts. For NHT concentrations of 10% or more, locusts' preference for lemon odour increased significantly with time after training up to 4 h (bars and left y-axis; * $P < 0.05$; ** $P < 0.01$). The higher NHT concentrations led to an increase in the percentage (pooled across all three time points; right y-axis) of locusts regurgitating within 5 min after the training trial (solid line), but not in mortality (dashed line). Error bars represent \pm s.e.m.

et al., 2011). To determine whether aversive olfactory memories modify their operant behaviour in the Y-maze, we conditioned locusts against their naive preference using a single paired presentation of vanilla odour as the CS and blank diet containing 10% NHT as the US (Fig. 1A). If locusts were to associate vanilla odour with the noxious effect of the NHT and use this memory in an operant task, the proportion of individuals choosing the arm of the Y-maze containing lemon should be greater than in naive locusts, indicating an aversion to vanilla. Control locusts should be unaffected, however, retaining an odour preference similar to that of naive locusts.

Conditioned locusts were tested 10 min, 1, 2, 4 or 24 h after training and their odour preference was compared with that of the naive locusts (Fig. 1B). The odour preference of locusts tested 10 min after training did not differ significantly from the naive preference ($G_1=1.61$, $P=0.21$). By contrast, locusts tested 1 h later showed a significantly higher preference for lemon odour ($G_1=9.65$, $P < 0.01$). The preference for lemon was also significantly higher at 2, 4 and 24 h (2 h, $G_1=24.39$; 4 h, $G_1=34.52$; 24 h, $G_1=21.44$; all $P < 0.001$). Thus, a single paired training trial was sufficient to significantly increase the locusts' preference for lemon odour. This effect did not manifest immediately after training but instead increased gradually up to 4 h after training ($G_4=13.27$, $P=0.01$).

Training with four paired trials likewise caused the locusts' preference for lemon odour to increase significantly when tested after 1, 2, 4 and 24 h (1 h, $G_1=4.81$, $P=0.03$; 2 h, $G_1=13.80$, $P < 0.001$; 4 h, $G_1=21.44$, $P < 0.001$; 24 h, $G_1=1.80$, $P < 0.001$; Fig. 1B). After 10 min, however, there was again no significant difference compared with the naive preference ($G_1=0.91$, $P=0.34$; Fig. 1B). At all tested

times, the preference for lemon odour of the locusts trained with four trials was similar to that of locusts conditioned with a single trial (10 min, $G_1=0.05$; 1 h, $G_1=0.42$; 2 h, $G_1=0.79$; 4 h, $G_1=0.88$; 24 h, $G_1=0.44$; all $P > 0.35$). Although it was not significant ($G_4=8.37$, $P=0.08$), the preference for lemon odour of locusts trained with four trials appeared to follow the same trend over time as that of locusts trained with a single trial, increasing from an initial low preference until reach a maximum preference 4 h after the training (Fig. 1B).

We tested locusts at all retention times after a single trial of either only vanilla odour (CS-only control) or only diet containing 10% NHT (US-only control; Fig. 1B). Neither of the two control groups showed a significant deviation from the naive preference (CS only: 10 min, $G_1=0.41$; 1 h, $G_1=0$; 2 h, $G_1=0.41$; 4 h, $G_1=0.10$; 24 h, $G_1=0.43$; US only: 10 min, $G_1=0.11$; 1 h, $G_1=0.10$; 2, 4 and 24 h, $G_1=0$; all $P > 0.50$). The odour preferences of both control groups were also unaffected by the time after conditioning at which they were tested (CS only, $G_4=1.17$; US only, $G_4=0.21$; both $P > 0.88$; Fig. 1B). Thus, the odour preference of locusts assessed in an operant task was aversively conditioned by the association of an odour with an NHT-enriched diet. A single training trial was sufficient to evoke a change in odour preference that persisted for at least 24 h. Importantly, however, there was no detectable change in the locusts' preferences 10 min after training, with the aversion to the CS increasing gradually over the following 4 h. This slow accumulation of learning is consistent with a long-latency mechanism of associative aversion.

NHT concentration affects odour aversion

How is the observed gradual increase of aversion to the CS affected by the NHT concentration in the US? To explore this, we trained

locusts with a single associative trial in which vanilla, the CS, was paired with blank diet containing 0, 1, 5, 10, 20 or 50% NHT per dry mass as the US (Fig. 1C). Only locusts that ingested the US during training were included in the analysis and no differences between the different NHT concentrations were observed in the percentage of animals that refused to feed. At each NHT concentration, locusts were tested in the Y-maze 10 min, 1 or 4 h after training.

The proportion of locusts that chose lemon odour over the CS in the Y-maze increased with higher NHT concentrations in the diet (Fig. 1D). After 1 or 4 h, this increase was highly significant (1 h, $G_5=40.48$; 4 h, $G_5=50.50$; both $P<0.001$). However, there was no significant increase in the proportion of locusts choosing lemon after 10 min regardless of NHT concentration ($G_5=9.06$, $P=0.11$). Locusts conditioned with blank diet (0% NHT) had the lowest preference for lemon odour. This preference was similar across all three testing times ($G_2=0.59$, $P=0.75$). Locusts trained with 1 or 5% NHT also did not increase the proportion of choices for lemon odour over time (1% NHT, $G_2=0.29$; 5% NHT, $G_2=0.74$; both $P>0.69$). However, when compared with locusts trained with the blank diet, the 5% NHT-trained locusts showed significantly higher preference for lemon irrespective of the time at which they were tested (10 min, $G_1=3.97$; 1 h, $G_1=16.27$; 4 h, $G_1=5.12$; all $P<0.04$). Locusts trained with 10, 20 or 50% NHT showed a significant increase in the preference for lemon odour with time (10% NHT, $G_2=9.47$; 20% NHT, $G_2=8.57$; 50% NHT, $G_2=6.99$; all $P<0.03$). In all three treatments, locusts tested 10 min after training always showed the lowest preference for lemon, whereas those tested after 4 h showed the highest (Fig. 1D). Thus, increasing NHT concentrations in a single associative trial elicits gradually stronger aversive responses to the CS when the NHT concentration is above a threshold, whereas lower NHT concentrations elicit no or a weak aversive response.

Ingestion of NHT may cause locusts to regurgitate the food or, at high concentrations, possibly even die. It is conceivable that locusts form aversive memories only upon regurgitation, or if they are so adversely affected that their survival is compromised. Therefore, we assessed the proportion of locusts regurgitating food 5 min after training, and their mortality 24 h after training, in relation to the CS aversion. No locust regurgitated when trained with a blank diet, and the proportion of locusts that regurgitated after training increased significantly with the NHT concentration ($G_5=378.57$, $P<0.001$; Fig. 1D). The highest concentration of NHT (50%) in the blank diet caused 86% of locusts to regurgitate. However, regurgitation was unnecessary for subsequent avoidance of the CS in the Y-maze (0% NHT, not calculated because regurgitation did not occur; 1%, $G_1=1.07$, $P=0.30$; 5%, $G_1=0.84$, $P=0.36$; 10%, $G_1=0.66$, $P=0.46$; 20%, $G_1=3.23$, $P=0.07$; 50%, $G_1=0.22$, $P=0.64$). Mortality 24 h after training was approximately 6% in all experimental groups, and was not significantly affected by NHT concentration ($G_5=0.19$, $P=0.99$; Fig. 1D). Olfactory preference in the Y-maze was unrelated to the subsequent mortality at any concentration of NHT (0%, $G_1=0.07$; 1%, $G_1=1.49$; 5%, $G_1=0.11$; 10%, $G_1=0.004$; 20%, $G_1=0.28$; 50%, $G_1=0.06$; all $P>0.22$).

Long-latency associative conditioning of odour aversion

The acceptance of the NHT-containing diet during training and the delayed and gradually increasing aversion to the CS after conditioning both suggest that the latter is mediated by a long-latency taste-independent associative mechanism. To test this hypothesis, NHT or saline solutions were injected into the locusts before, during or after a single associative training trial. The trial consisted of the CS (vanilla)

paired with blank diet as the US. Injections were given 60, 30 or 15 min before training, during the last 5 s of training, or 15, 30, 60 or 120 min after training (Fig. 2A). Four hours after training, the locusts were tested in the Y-maze and their observed odour preference was compared with that of non-injected conditioned locusts. In this latter group, only 27% of locusts preferred the Y-maze arm containing lemon odour (Fig. 2B). If aversive learning had occurred, a higher proportion of NHT-injected locusts would be expected to select the arm containing lemon odour (avoiding the CS) relative to those injected with saline. An aversion to the CS that is still observable when NHT is injected after training would indicate that a long-latency associative mechanism for odour is involved.

Approximately 80% of locusts that received a NHT injection during training selected the arm containing lemon odour, a proportion significantly higher than in non-injected locusts ($G_1=52.10$, $P<0.001$; Fig. 2B). This preference for lemon was still observed when NHT was injected 15 or 30 min after training (15 min, $G_1=28.49$; 30 min, $G_1=16.89$; both $P<0.001$). The proportion of locusts that preferred lemon during testing declined with increasing delays between training and injection, but this trend was not significant ($G_2=5.50$, $P=0.07$). NHT injection 1 or 2 h after training, however, did not significantly affect the proportion of locusts preferring lemon compared with those that were not injected ($G_1=2.67$, $P=0.10$ and $G_1=0.44$, $P=0.51$, respectively; Fig. 2B). Likewise, NHT injections before training had no significant effect on the preference for lemon (–1 h, $G_1=0.11$; –30 min, $G_1=1.73$; –15 min, $G_1=2.67$; all $P>0.10$; Fig. 2B). The preferences of saline-injected locusts did not differ from those of non-injected locusts (–1 h, $G_1=0.44$; –30 min, $G_1=0.12$; –15 min, $G_1=0.99$; 0 min, $G_1=0.44$; 15 min, $G_1=0.11$; 30 min, $G_1=0.44$; 1 h, $G_1=0.12$; 2 h, $G_1=0.48$; all $P>0.32$; Fig. 2B). Thus, locusts learn to associate the effects of injected NHT with the odour presented during associative conditioning. This aversive association was made without the odour and the NHT-related toxic effects being simultaneous and occurred even when the two events were separated by up to 30 min. These results indicate that locusts have a long-latency associative mechanism for odour aversion learning.

Post-ingestive mechanism for olfactory associative aversion

The ability of locusts to associate an odour with detecting a toxin or its effects after a long delay suggests a post-ingestive mechanism, where the formation of long-latency aversive associations is contingent upon the tasting or ingestion of food. However, it is also conceivable that aversive associations across long periods occur without ingestion taking place when the odour is encountered. To differentiate between these two hypotheses, we repeated the previous experiment without presenting the US (blank diet) (Fig. 2C). This no-US group was compared at each injection time with the locusts that had received paired conditioning. If the olfactory aversion observed in the paired-conditioned locusts was mediated by a post-ingestive mechanism, then it should be dependent upon food ingestion, and there should be no increase in lemon preference in the no-US group.

More than 86% of the locusts conditioned only with the CS (vanilla odour) preferred the lemon odour in the Y-maze test when NHT was injected during training. This strong preference towards lemon was not significantly different from that observed in the paired-conditioned locusts ($G_1=0.73$, $P=0.39$; Fig. 2D). This indicates that the formation of an aversive odour memory is not contingent upon simultaneous ingestion of food and NHT injection. When NHT was injected 15 or 30 min after the CS trial, however, only 36 and 27% of the locusts showed a preference for lemon odour, respectively, significantly less than the 66 and 57% observed when

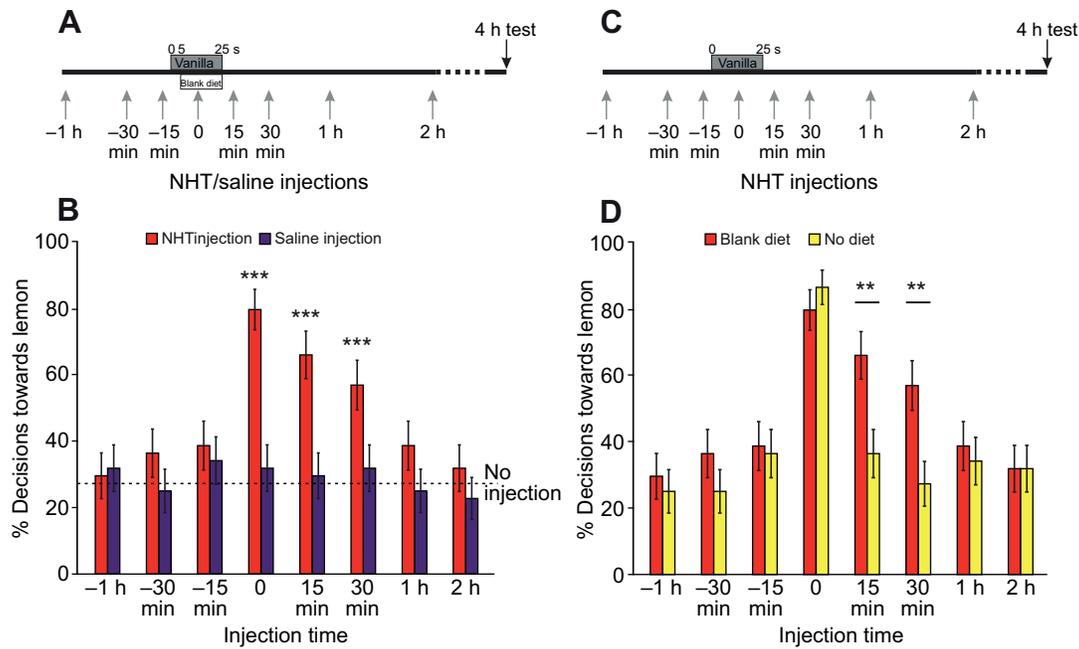


Fig. 2. A post-ingestive mechanism mediates the association between the toxic effects of NHT microinjection with the odour presented during a paired conditioned trial in locusts. (A) Single-paired trial conditioning (CS: vanilla odour; US: blank diet) combined with a microinjection of NHT or locust saline solution before, during or after the trial. (B) Odour preference of the microinjected locusts, measured as the percentage of decisions towards the arm with lemon odour in the Y-maze. NHT-injected locusts showed a higher preference for lemon than non-injected conditioned locusts (dashed line; *** $P < 0.001$) when injection occurred during or up to 30 min after the paired trial. NHT injection at other time points had no significant effect on odour preference; saline injections had no effect at any time point. (C) A single CS-only trial combined with a NHT injection before, during or after the CS onset. (D) Odour preference of NHT-injected locusts 4 h after a CS-only or a paired trial (these data are duplicated from B). Locusts that did not receive the US showed a significantly lower preference for lemon than locusts conditioned with paired-trials when injection occurred 15 or 30 min after the CS onset (** $P < 0.01$). Both groups of locusts had a similarly high preference for lemon odour when NHT microinjection was concurrent with the CS onset. Error bars represent \pm s.e.m.

the CS was paired with food (15 min, $G_1=7.80$; 30 min, $G_1=8.02$; both $P < 0.01$; Fig. 2D). When NHT was injected 1 or 2 h after the trial, both paired- and CS-conditioned groups showed a similar low preference for lemon (1 h, $G_1=0.20$; 2 h, $G_1=0$; both $P > 0.66$; Fig. 2D). Additionally, when NHT was injected before training, the preference towards lemon was also equally low regardless of whether the CS was presented alone or with food (-1 h, $G_1=0.23$; -30 min, $G_1=1.34$; -15 min, $G_1=0.05$; all $P > 0.36$; Fig. 2D). These results indicate the presence of two distinct mechanisms mediating olfactory associative aversion: an ingestion-dependent mechanism that allows the formation of aversive associations between the CS and the toxic effect of NHT up to 30 min later; and a second, ingestion-independent, mechanism that operates only when the CS is simultaneous with the toxic effect of NHT.

The formation of the long-latency aversion between the CS and the delayed toxic effects requires the ingestion of food. However, prior to ingestion, food is detected by gustatory receptors on the locust's mouthparts and it is possible that the gustatory inputs are sufficient for the formation of a delayed aversive memory without the involvement of the gut. To investigate this possibility, we conditioned locusts by pairing vanilla odour (CS) with stimulation of their palps, which allowed them to taste but not ingest the blank diet (US). The palps play an important role in the locusts' food selection (Blaney and Chapman, 1970; Chapman and Sword, 1993) and possess contact chemoreceptors that respond to several chemical classes (Blaney, 1974).

The locusts were injected with NHT solution during or 15 min after the conditioning trial. The locusts' odour preference 4 h after training was compared with that of a second group of locusts that had also

tasted the US during the CS onset but had not been injected with NHT solution (Fig. 3A). NHT injection given during conditioning was sufficient to evoke a significant preference for lemon odour ($G_1=18.46$, $P < 0.001$; Fig. 3B), indicating the formation of an aversion to the CS. This was expected from earlier experiments that showed that aversive memory formation occurs independently of food ingestion when the CS and NHT injection coincide (Fig. 2D). However, the odour preference of locusts that tasted the US and were injected with NHT solution 15 min later was not significantly different from that of locusts that were not injected ($G_1=0.41$, $P=0.52$; Fig. 3B). This shows that gustatory inputs from the palps are not sufficient to elicit an aversive memory, suggesting that ingestion is necessary for the formation of long-latency aversive memories.

Does the olfactory aversion mediated by the post-ingestive mechanism require food ingestion to be temporally coupled with the CS? To assess this, we varied across three groups of locusts the time relative to the CS at which the locusts were presented with blank diet (Fig. 3C). In all groups, the locusts were presented with the CS, injected with NHT 15 min later, and tested in the Y-maze 4 h after training. The first group received the blank diet (US) 5 min before CS presentation, the second received the blank diet as part of a paired CS/US trial, and the third received the blank diet 5 min after CS presentation. The blank diet was presented to the restrained locusts for 20 s.

The locusts' odour preference was significantly different across the three US presentation times (Fig. 3D; $G_2=15.03$, $P < 0.001$). The group of locusts trained with paired presentation of the CS and US showed a significantly higher preference for lemon odour than the groups that received the US temporally uncoupled from the CS

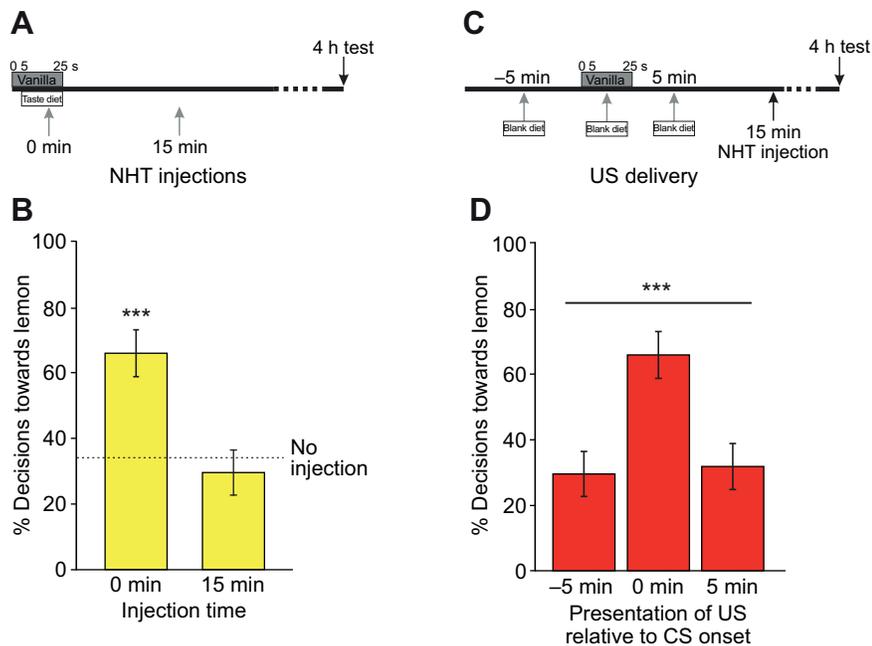


Fig. 3. Ingestion and the temporal coupling of food with odour onset are necessary for the formation of long-latency aversive memories via the post-ingestive mechanism. (A) A single-paired conditioning trial in which the CS (vanilla odour) was paired with the gustatory stimulation of the palps with blank diet. Microinjection of NHT solution was delivered during or 15 min after the trial. (B) Odour preference of the microinjected locusts 4 h after the conditioning trial. Odour preference of locusts injected 15 min after the trial was similar to that of non-injected conditioned locusts (dashed line), whereas concurrent injection elicited a higher preference for lemon odour ($***P<0.001$). (C) Delivery of the US (blank diet) relative to the onset of a single CS-only trial combined with a NHT injection 15 min after. (D) Odour preference of locusts 4 h after the CS onset. Locusts that received a paired CS/US trial had a significantly higher preference for lemon odour ($***P<0.001$) than locusts where the US was presented before or after the CS onset. Error bars represent \pm s.e.m.

(-5 min, $G_1=11.94$; 5 min, $G_1=10.44$; both $P<0.01$; Dunn–Sidak correction $\alpha'=0.017$). Additionally, odour preference was similar between the two groups conditioned with an unpaired US ($G_1=0.05$, $P=0.82$, $\alpha'=0.017$). These results show that an association between the CS and the delayed toxic effects of NHT is formed only if the ingestion of food is simultaneous with the CS onset.

Effect of exposure to a second odour on the post-ingestive mechanism for odour aversion

One problem that may conceivably arise with long-latency associative mechanisms is if a distinct second odour stimulus is encountered either before or during the subsequent effects of the toxin. In this event, the aversive association may be formed with the last odour detected before the onset of the toxic malaise, or with the odour detected concurrently with food ingestion. To determine how a second odour affects the formation of a post-ingestive association, locusts were first trained with a single paired CS₁/US trial (vanilla odour/blank diet) and 15 min later were presented with 25 s of lemon odour only (CS₂). NHT or saline injections were given to the locusts during the paired trial (0 min), during the CS₂ onset (15 min) or after the CS₂ onset (20 or 30 min; Fig. 4A). All locusts were restrained throughout the conditioning, including during injections. Odour preference was tested in the Y-maze 4 h after the paired CS₁/US trial. Injections given after the paired trial fall within the temporal window in which the post-ingestive mechanism operates (Fig. 2D). If the lemon odour presentation (CS₂) did not interfere with the post-ingestive mechanism, then NHT injections would be expected to increase the proportion of locusts preferring lemon during the test. A low preference for lemon odour in the test, however, would suggest that the second odour interferes with the post-ingestive association.

The proportion of locusts that preferred lemon odour was significantly affected by the timing of NHT injection, whereas those injected with saline showed no change in their preference for lemon (NHT, $G_3=20.70$, $P<0.001$; saline, $G_3=1.45$, $P=0.69$; Fig. 4B). Locusts injected with NHT during or 20 min after the CS₁/US trial showed a significantly higher preference for lemon than locusts injected with saline (0 min, $G_1=7.84$; 20 min, $G_1=6.76$; both $P<0.01$). Although not statistically significant, the same trend was observed

when injection was applied 30 min after the CS₁/US trial ($G_1=3.81$, $P=0.05$). Crucially, locusts injected with NHT during the presentation of lemon odour (i.e. 15 min after the CS₁/US trial) showed a reduced preference for lemon during testing, not significantly different from that of the saline-injected locusts ($G_1=0.24$, $P=0.62$; Fig. 4B). These results indicate that locusts relate toxic effects to the odour they experienced during ingestion independent of odours they may experience in the intervening period, unless the second odour experience temporally coincides with the onset of the toxic effects.

DISCUSSION

We have shown that desert locusts can learn to associate an odour with food containing NHT and use this olfactory memory to make appropriate decisions by avoiding the odour in a Y-maze. A single paired presentation of odour and food containing NHT was sufficient to produce an aversive memory that lasted at least 24 h in our experiments. This may underestimate the retention time, however, because previous studies have shown that locusts can retain single-meal aversive memories for 2 days (Lee and Bernays, 1990). The aversive association between odour and toxin was not formed immediately upon ingestion but increased gradually until reaching a maximum 4 h later. Locusts tested 10 min after training showed no greater aversion to the conditioned odour than did controls. No such delay occurs when locusts learn the association between an odour and an appetitive food reward (Simões et al., 2011). Multiple trial conditioning did not elicit a stronger aversive memory in the locusts, a single trial being sufficient to saturate the aversive response during the odour preference tests. This lack of summation of the aversive conditioning trials may be due to the relatively high concentration used as the US and the rapid behavioural habituation to nicotine showed by locusts (Szentesi and Bernays, 1984).

The delay in learning the association between an odour and a toxin-containing food argues against a role for gustatory inputs during ingestion, and instead suggests a long-latency mechanism for associating post-ingestive feedback with odour cues detected during ingestion. Analysis of the individual olfactory decisions showed that these were not influenced by whether the conditioned locusts had regurgitated the toxin-containing food, indicating that

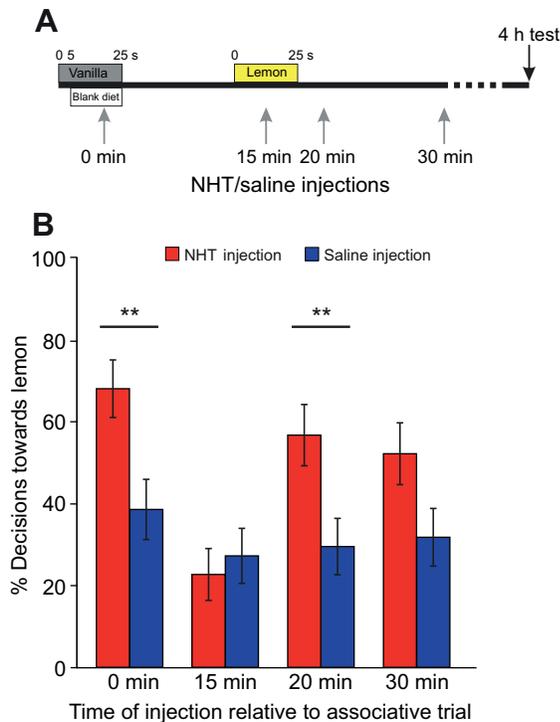


Fig. 4. Odour aversion learning mediated by the post-ingestive mechanism is suppressed when NHT injection coincides with the onset of a second odour. (A) An initial conditioning trial using vanilla odour as CS₁ and blank diet as the US was followed 15 min later by presentation of lemon odour (CS₂) without the US. Locusts were injected with NHT or saline during the CS₁/US paired trial (time=0 relative to CS₁/US), during CS₂ (time=15 min) or after CS₂ (time=20 or 30 min). (B) Odour preference of locusts 4 h after the paired trial. NHT-injected locusts had a significantly stronger preference for lemon odour than saline-injected locusts (** $P < 0.01$) when injection was given during the CS₁/US paired trial or 5 min after the CS₂ onset (20 min after CS₁/US), but not when it coincided with the CS₂. Error bars represent \pm s.e.m.

the association was made not with regurgitation, but rather with a noxiously evoked internal signal. The most likely explanation, then, for the delay is that it takes time for NHT to pass through the gut and reach sufficient concentrations in the haemolymph to have an effect. A post-ingestive mechanism would also account for the concentration dependency of aversive memory formation that we observed because higher NHT concentrations in food will lead to NHT reaching higher concentrations in the haemolymph earlier.

To confirm and characterize this long-latency post-ingestive mechanism, we uncoupled ingestion from NHT toxicity by presenting locusts with NHT-free food and injecting the NHT directly into the haemolymph instead, to simulate its accumulation after feeding. By shifting the timing of the injections relative to the co-presentation of odour and food, we revealed a period of up to 30 min during which an aversive association could be formed. This also confirms that gustatory cues from NHT in the food are unnecessary for associating an odour with the subsequent toxic effects. In the absence of food or when food was only tasted by the palps, locusts failed to learn to associate an odour with NHT toxicity unless odour presentation and NHT microinjection were simultaneous. Thus, food ingestion is necessary to initiate the extended period over which an odour can be associated with NHT toxicity. Moreover, this extended period is triggered only when

feeding is temporally linked to odour presentation; shifting food intake before or after odour presentation prevents the formation of the long-latency associations, though how precise the temporal association must be remains unclear.

In the absence of feeding, locusts presented with an odour learned to associate it with the effects of NHT only if odour presentation and microinjection were applied simultaneously. Therefore, our experiments demonstrate the presence of two separate classes of association between an odour and aversive effects in locusts: (1) an ingestion-dependent mechanism that enables them to form an association between an odour and the effects of a toxin experienced within an extended time window after feeding; and (2) an ingestion-independent mechanism that operates in the absence of feeding but only when the odour is immediately followed by the onset of the toxic malaise.

Memories formed when odour presentation was synchronous with NHT injection were equally strong regardless of whether the locusts had ingested food. Thus, the strength of the memories formed provides no clue as to whether in locusts these two forms of aversive memory are independent of one another. It is conceivable that a single mechanism of aversive acquisition operates and that food ingestion extends the period over which an association can be made. However, it remains unclear how bridging between the odour and the delayed post-ingestive inputs might be accomplished. One possibility is that the negative post-ingestive feedback updates the value of an initially appetitive labile memory between the odour and feeding. The presence of water in the diet used for conditioning, ingestion of any sort of matter or the feeding behaviour itself may provide this initial positive reinforcement. This putative initial positive reinforcement is unlikely to have been strong because it ultimately led to an aversive response. This is consistent with previous experiments in locusts (Bernays and Lee, 1988; Lee and Bernays, 1990) and vertebrates (Eitcorn, 1973) that have shown that the acquisition of an aversive response through post-ingestive mechanisms is stronger when toxic malaise is associated with less preferred foods, and weaker with highly palatable foods. Our results show that this hypothetical initial appetitive memory was not formed with the sensory inputs of the locusts' palps, which are sensory structures known to play an important role in food selection (Blaney and Chapman, 1970; Chapman and Sword, 1993). However, it is possible that such an association is formed with inputs from internal taste receptors and integrated with output from gut stretch receptors and other changes associated with feeding (Abisgold and Simpson, 1987).

The ability to learn associations over an extended period raises the possibility that odours encountered during this period could be incorrectly associated with the NHT. Remarkably, however, a second odour interposed between the co-presentation of odour with food and the NHT microinjection did not affect the association and the most recently encountered odour was ignored. This suggests not only that the memory of the odour encountered whilst feeding maintained up to 30 min but also that it is not disrupted by coding other odours in the intervening period. The neural substrates of this extended window of memory formation (labelling of the memory) and of the robustness to additional odours are unknown, but the results are compatible with the hypothesis of an initial labile appetitive association, where the requirement for ingestion and odour to be simultaneous safeguards against 'erroneous' attribution of the toxic malaise to the second odour.

An association between NHT and the second odour was formed, however, if the second odour was accompanied by NHT microinjection; this scenario engages the ingestion-independent

mechanism. However, formation of this memory may depend on the sharp rise in NHT concentration following microinjection. In the field, where locusts may encounter a succession of different odours, 'causally correct' associations through the ingestion-dependent mechanism may be even more robust if the gradual rise in the concentration of toxin in the haemolymph occurs after eating contaminated food does not engage the ingestion-independent mechanism. The odour specificity of food avoidance learning has also been investigated in honeybees (Wright et al., 2010). When subjected to a differential conditioning paradigm, where a sucrose solution laced with an almost tasteless toxin was paired with one odour and toxin-free sucrose solution with a second odour, the bees were found to form aversive memories to both odours. This lack of discrimination between the odours was not due to generalization but rather because the bees repeatedly experienced both odour–food pairings before and during the gradual onset of the malaise; in this paradigm there is no cue that would permit the bees to relate their malaise to one of the two odours specifically. This suggests that the gradual accumulation of a toxin in the haemolymph may lead to odours being incorrectly associated with the toxin were an insect to sample different food sources in succession before the toxic effects manifest.

In many insect species, acceptance of toxic food declines markedly with experience. Food aversions can arise from diverse mechanisms, making it unclear whether learning accounts for the observed behavioural change (Bernays, 1993). The ability of insects, like vertebrates (Garcia et al., 1974; Logue, 1985; Manteifel and Karelina, 1996), to associate gustatory cues (or possibly other inputs) with subsequent post-ingestive consequences caused by toxic compounds was first demonstrated in locusts using toxin injections following feeding (Bernays and Lee, 1988; Lee and Bernays, 1990). The confirmation that, in insects, Pavlovian associations can be made between an odour and the consequences of ingesting toxin was demonstrated recently in honeybees (Wright et al., 2010). In contrast to previous studies, our experiments separated the four functional components involved in the acquisition of a learned aversion to food: odour, food, toxin and time.

Post-ingestive mechanisms are also known to be used by locusts to acquire aversion to nutritionally deficient foods (Lee and Bernays, 1988; Champagne and Bernays, 1991). Similarly, locusts can make associations between olfactory, gustatory or visual cues with specific nutrients, including sterols, proteins or carbohydrates (Simpson and White, 1990; Raubenheimer and Tucker, 1997; Behmer et al., 1999). Moreover, post-ingestive feedback was shown to contribute to the acquisition of an aversive association towards unsuitable sterols (Behmer et al., 1999). Recent studies of associative learning in fruit flies have disambiguated the role of taste inputs and have shown that, as in vertebrates (de Araujo et al., 2008), post-ingestive inputs from particular nutrients are sufficient to act as positive reinforcers upon appetitive associations (Burke and Waddell, 2011; Fujita and Tanimura, 2011).

Locusts possess taste receptors that respond to NHT and other alkaloids (White and Chapman, 1990; Chapman et al., 1991), and the degree to which they are activated is correlated with the feeding deterrence, as was shown in a free-moving meal test (Chapman et al., 1991). Yet when forced to feed on NHT-contaminated food, desert locusts show a rapid behavioral habituation to the toxin and increase its ingestion on the subsequent exposures (Szentesi and Bernays, 1984). We observed that during conditioning, restrained locusts did not reject the NHT-enriched diet, even at very high concentrations. Tethered honeybees (Ayestaran et al., 2010; Wright et al., 2010) and

confined crickets (Matsumoto and Mizunami, 2002) exhibit the same lack of rejection behaviour, ingesting considerable amounts of toxic or bitter substances during associative conditioning. Thus, it is possible that restraint disrupts the behavioral sequence that free-moving locusts use to select or reject food (Blaney and Simmonds, 1985; Blaney et al., 1985), leading to the acceptance of toxic substances even when they are registered by taste receptors.

Locusts are polyphagous herbivores that must decide which amongst a wide range of plants to feed upon. Each new food source requires evaluation that should be informative for subsequent encounters (Bernays, 1993; Simpson and Raubenheimer, 2000). Not all plant toxins can be tasted, making post-ingestive associative mechanisms advantageous because they allow locusts to modify their feeding based upon a toxin's effect, provided they survive. Moreover, by forming associations between post-ingestive toxic malaise and an odour rather than a taste, a locust encountering the same species of plant again would not have to taste it (thereby risking ingestion) to avoid it. Thus, aversive learning through post-ingestive inputs may act, in conjunction with peripheral processes, to reduce the locusts' risk of toxic poisoning and improve their survival. Given the advantages they confer by allowing toxins to be avoided, post-ingestive olfactory associations are likely to be particularly developed in polyphagous herbivores.

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REFERENCES

- Abisgold, J. and Simpson, S.** (1987). The physiology of compensation by locusts for changes in dietary protein. *J. Exp. Biol.* **129**, 329-346.
- Ayestaran, A., Giurfa, M. and de Brito Sanchez, M. G.** (2010). Toxic but drank: gustatory aversive compounds induce post-ingestive malaise in harnessed honeybees. *PLoS ONE* **5**, 1-14.
- Behmer, S. T., Elias, D. O. and Bernays, E. A.** (1999). Post-ingestive feedbacks and associative learning regulate the intake of unsuitable sterols in a generalist grasshopper. *J. Exp. Biol.* **202**, 739-748.
- Bernays, E. A.** (1993). Aversion learning and feeding. In *Insect Learning: Ecological and Evolutionary Perspectives* (ed. D. R. Papaj and A. C. Lewis), pp. 1-17. New York: Chapman and Hall.
- Bernays, E. A. and Lee, J.** (1988). Food aversion learning in the polyphagous grasshopper *Schistocerca americana*. *Physiol. Entomol.* **13**, 131-137.
- Blaney, W. M.** (1974). Electrophysiological responses of the terminal sensilla on the maxillary palps of *Locusta migratoria* (L.) to some electrolytes and non-electrolytes. *J. Exp. Biol.* **60**, 275-293.
- Blaney, W. M. and Chapman, R.** (1970). The functions of the maxillary palps of Acrididae (Orthoptera). *Entomol. Exp. Appl.* **13**, 363-376.
- Blaney, W. M. and Simmonds, M. S. J.** (1985). Food selection by locusts: the role of learning in rejection behaviour. *Entomol. Exp. Appl.* **39**, 273-278.
- Blaney, W. M., Winstanley, C. and Simmonds, M. S. J.** (1985). Food selection by locusts: an analysis of rejection behaviour. *Entomol. Exp. Appl.* **38**, 35-40.
- Burke, C. J. and Waddell, S.** (2011). Remembering nutrient quality of sugar in *Drosophila*. *Curr. Biol.* **21**, 746-750.
- Champagne, D. E. and Bernays, E. A.** (1991). Phytosterol unsuitability as a factor mediating food aversion learning in the grasshopper *Schistocerca americana*. *Physiol. Entomol.* **16**, 391-400.
- Chapman, R. F.** (2003). Contact chemoreception in feeding by phytophagous insects. *Annu. Rev. Entomol.* **48**, 455-484.
- Chapman, R. F. and Sword, G.** (1993). The importance of palpation in food selection by a polyphagous grasshopper (Orthoptera: Acrididae). *J. Insect Behav.* **6**, 79-91.
- Chapman, R. F., Ascoli-Christensen, A. and White, P. R.** (1991). Sensory coding for feeding deterrence in the grasshopper *Schistocerca americana*. *J. Exp. Biol.* **158**, 241-259.
- Collett, D.** (2002). *Modeling Binary Data*, 2nd edn. London: Chapman and Hall.
- Dadd, R. H.** (1960). The nutritional requirements of locusts – I. Development of synthetic diets and lipid requirements. *J. Insect Physiol.* **4**, 319-347.

- de Araujo, I. E., Oliveira-Maia, A. J., Sotnikova, T. D., Gainetdinov, R. R., Caron, M. G., Nicolelis, M. A. L. and Simon, S. A. (2008). Food reward in the absence of taste receptor signaling. *Neuron* **57**, 930-941.
- Etscorn, F. (1973). Effects of a preferred vs a non-preferred CS in the establishment of a taste aversion. *Physiol. Psychol.* **1**, 5-6.
- Fujita, M. and Tanimura, T. (2011). *Drosophila* evaluates and learns the nutritional value of sugars. *Curr. Biol.* **21**, 751-755.
- Garcia, J., Hankins, W. G. and Rusiniak, K. W. (1974). Behavioral regulation of the milieu interne in man and rat. *Science* **185**, 824-831.
- Gelperin, A. (1975). Rapid food-aversion learning by a terrestrial mollusk. *Science* **189**, 567-570.
- Gerber, B., Stocker, R. F., Tanimura, T. and Thum, A. S. (2009). Smelling, tasting, learning: *Drosophila* as a study case. *Results Probl. Cell. Differ.* **47**, 139-185.
- Glendinning, J. I. (1996). Is chemosensory input essential for the rapid rejection of toxic foods? *J. Exp. Biol.* **199**, 1523-1534.
- Lee, J. and Bernays, E. A. (1988). Declining acceptability of a food plant for the polyphagous grasshopper *Schistocerca americana*: the role of food aversion learning. *Physiol. Entomol.* **13**, 291-301.
- Lee, J. and Bernays, E. A. (1990). Food tastes and toxic effects: associative learning by the polyphagous grasshopper *Schistocerca americana* (Drury) (Orthoptera: Acrididae). *Anim. Behav.* **39**, 163-173.
- Logue, A. W. (1985). Conditioned food aversion learning in humans. *Ann. N. Y. Acad. Sci.* **443**, 316-329.
- Manteifel, Y. B. and Karelina, M. A. (1996). Conditioned food aversion in the goldfish, *Carassius auratus*. *Comp. Biochem. Physiol.* **115A**, 31-35.
- Matsumoto, Y. and Mizunami, M. (2002). Temporal determinants of long-term retention of olfactory memory in the cricket *Gryllus bimaculatus*. *J. Exp. Biol.* **205**, 1429-1437.
- Raubenheimer, D. and Tucker, D. (1997). Associative learning by locusts: pairing of visual cues with consumption of protein and carbohydrate. *Anim. Behav.* **54**, 1449-1459.
- Simões, P., Ott, S. R. and Niven, J. E. (2011). Associative olfactory learning in the desert locust, *Schistocerca gregaria*. *J. Exp. Biol.* **214**, 2495-2503.
- Simpson, S. J. and Abisgold, J. D. (1985). Compensation by locusts for changes in dietary nutrients: behavioural mechanisms. *Physiol. Entomol.* **10**, 443-452.
- Simpson, S. J. and Raubenheimer, D. (1993). The central role of the haemolymph in the regulation of nutrient intake in insects. *Physiol. Entomol.* **18**, 395-403.
- Simpson, S. J. and Raubenheimer, D. (2000). The hungry locust. *Adv. Study Behav.* **29**, 1-43.
- Simpson, S. J. and White, P. (1990). Associative learning and locust feeding: evidence for a 'learned hunger' for protein. *Anim. Behav.* **40**, 506-513.
- Sokal, R. and Rohlf, F. (1998). *Biometry: the Principles and Practice of Statistics in Biological Research*. New York: W. H. Freeman and Company.
- Szentesi, A. and Bernays, E. A. (1984). A study of behavioural habituation to a feeding deterrent in nymphs of *Schistocerca gregaria*. *Physiol. Entomol.* **9**, 329-340.
- White, P. R. and Chapman, R. F. (1990). Tarsal chemoreception in the polyphagous grasshopper *Schistocerca americana*: behavioural assays, sensilla distributions and electrophysiology. *Physiol. Entomol.* **15**, 105-121.
- Wright, G. A., Mustard, J. A., Simcock, N. K., Ross-Taylor, A. A. R., McNicholas, L. D., Popescu, A. and Marion-Poll, F. (2010). Parallel reinforcement pathways for conditioned food aversions in the honeybee. *Curr. Biol.* **20**, 2234-2240.
- Yarmolinsky, D. A., Zuker, C. S. and Ryba, N. J. P. (2009). Common sense about taste: from mammals to insects. *Cell* **139**, 234-244.
- Zhang, Y., Lu, H. and Bargmann, C. I. (2005). Pathogenic bacteria induce aversive olfactory learning in *Caenorhabditis elegans*. *Nature* **438**, 179-184.