Title: Elemental and non-elemental olfactory learning in *Drosophila*

Running title: Olfactory learning in *Drosophila*

Authors: Young JM\textsuperscript{1,2}, Wessnitzer J\textsuperscript{1}, Armstrong JD\textsuperscript{2}, Webb B\textsuperscript{1} *

\textsuperscript{1} Institute for Perception, Action & Behaviour
\textsuperscript{2} Institute for Adaptive & Neural Computation

* corresponding author
Research highlights

- Flies can learn to distinguish disjoint and overlapping odour mixtures
- Flies cannot solve negative patterning or biconditional discrimination problems
- Flies do not exhibit blocking in the odour shock paradigm
- None of the standard learning models explain the data

Keywords

*Drosophila*, learning, non-elemental, model, olfactory
Abstract

Brain complexity varies across many orders of magnitude between animals, and it is often assumed that complexity underpins cognition. It is thus important to explore the cognitive capacity of widely used model organisms such as Drosophila. We systematically investigated the fly’s ability to learn discriminations involving compound olfactory stimuli associated with shock. Flies could distinguish binary mixtures (AB+ CD-), including overlapping mixtures (AB+ BC-). They could learn positive patterning (AB+ A- B-) but could not learn negative patterning (A+ B+ AB-) or solve a biconditional discrimination task (AB+ CD+ AC- BD-). Learning about the elements of a compound (AB+) was not affected by prior conditioning of one of the elements (A+ AB+): flies do not exhibit blocking in this task. We compare these results with the predictions from simulation of several well-known theoretical models of learning, and find none are fully consistent with the overall pattern of observed behaviour.

1. Introduction

Drosophila exhibit conditioned approach or avoidance to an odour that has been paired to an appetitive or aversive stimulus, such as sugar (Tempel et al., 1983) or shock (Tully & Quinn, 1985). To date, the majority of our knowledge of Drosophila associative olfactory learning is from straightforward elemental studies in which one odour is reinforced and a second odour is not reinforced (A+ B-) and the response measured as a choice between A and B. A considerable volume of work has investigated the brain regions, underlying circuitry and genetic control involved in such associations (de Belle & Heisenberg, 1994; Dubnau et al., 2001; Krashes et al., 2007; reviewed by Keene & Waddell 2007; Fiala, 2007).

Conversely, we have limited understanding of the fly’s ability to solve more complex discrimination problems. In nature it is more likely that animals are presented with combinations of stimuli (e.g. fruits or flowers combining several odours and colours) that need to be processed appropriately to extract the relevant cues. For example, a compound stimulus (AB) may be comprised of two elements (A and B), but can it be processed simply as the sum of the two components, or is the compound somehow distinct? This issue has been widely explored in vertebrate learning (usually referred to as configural learning, Pearce 1994) and has been studied to some extent using various learning paradigms in various invertebrates (particularly bees, Giurfa, 2007), as will be discussed further below. Our aim in this study is to carry out a more systematic investigation of more complex forms of learning in Drosophila.

The learning problems included in the current study can be split into three categories:

• Problems that involve compound stimuli rather than single elements. Can flies form associations when conditioned with compound stimuli (i.e. mixture learning, AB+ vs. CD-)? Can they form such associations as readily as with single stimuli?
Can they still learn if the compound stimuli overlap (i.e. overlap learning, AB+ vs. BC-)?

- Problems involving compounds whose elements are equally reinforced and not reinforced (e.g. positive patterning, AB+ A- B-). An important subset of such problems encompasses so-called 'non-elemental' paradigms, because they cannot be accounted for under the assumption that compounds are simply the sum of their elements. One such paradigm is negative patterning (A+ B+ AB-): the sum of A and B must be greater than A or B. Another is biconditional discrimination (AB+ CD+ AC- BD-).

- Problems in which prior experience with elements or compounds may affect the subsequent learning. The classic example here is blocking (Kamin, 1968) in which learning about elements presented in a compound (AB+) is affected by prior learning about one of the elements (A+), such that the association with B is reduced.

To date, the evidence for such learning capabilities in insects is very mixed. Flies can still learn when exposed to more than two odours in one training trial i.e. A+ B+ C- (we call this two element learning) or A+ B+ C+ D- (Dudai, 1977; Yin et al., 2009). They can also learn to discriminate a specific odour mixture from the same odours mixed in different proportions (Borst, 1983). Dasgupta & Waddell (2008) demonstrated that *Drosophila* could discriminate individual components (e.g. A vs C) after training with binary mixtures (AB+ CD-).

Negative and positive patterning have been demonstrated to odour combinations using the proboscis extension reflex (PER) in bees (Deisig et al., 2001; Komischke et al., 2003) and also in free-flying bees using colour-odour combinations (Couvillon & Bitterman, 1988, see also summary of other studies in Giurfa 2007). It would be interesting to know whether they display this capability in a shock conditioning paradigm (Giurfa et al. 2009). We are not aware of any other insect that has been shown to learn negative patterning.

Brembs and Wiener (2006) reported successful biconditional conditioning of flies under certain circumstances (including a purely classical conditioning procedure) using heat punishment for specific colour-pattern pairs in a flight simulator. They describe this as 'occasion setting' i.e. the background colour determines which pattern is paired with heat. Bees are also able to solve visual biconditional discrimination problems using different colours combined with line grating orientations (Schubert et al., 2002). Matsumoto and Mizunami (2004) show that crickets can solve the equivalent task in a multimodal context learning setting, where odours are rewarded or punished in opposite contingencies in the light and the dark, and Sato et al. (2006) found the same result for cockroaches. However, fly larvae trained in similar circumstances failed to learn the task (Yarali et al. 2006); and adult flies also failed to learn after receiving differential shock-odour pairings in the light and the dark (Yarali et al. 2008).

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1 This problem is particularly interesting as it corresponds to the XOR problem in computing which, famously, cannot be solved by a single layer perceptron.
Blocking reports vary for invertebrates (Smith & Cobey, 1994; Gerber & Ulrich, 1999; Hosler & Smith, 2000; Brembs & Heisenberg, 2001; Couvillon et al., 2001; Guerrieri et al., 2005) but in general no undisputed evidence for blocking in insects has been reported. Guerrieri et al. (2005) showed that honeybee groups demonstrated blocking for only 4 out of 24 sets of odours tested (and for none if the significance level is adjusted for multiple comparisons), and in one case found enhancement, concluding that blocking is not a robust occurrence, and also (contrary to the suggestion of Hosler & Smith, 2000) that it does not depend on odour similarity. Most recently, Blaser, Couvillon and Bitterman (2008) using a more powerful within-subjects paradigm failed to observe evidence of blocking in honeybees, but found evidence of facilitation.

In the current study we explore the ability of flies to solve this range of learning problems using a single modality (olfaction), and using the paradigm that is most widely used for investigating elemental learning (shock association). Much of what is known about brain mechanisms of simple learning in Drosophila has been derived from studies using this paradigm, so it is useful to assess what complexity of learning it can reveal. Assessing these learning capabilities is also critical to evaluate the applicability to flies of some widely accepted formal frameworks of associative learning of compound stimuli. We explicitly address this by implementing each framework in a learning simulation to compare their predictions to the results obtained for the flies. This modelling is kept deliberately simple, as our aim is to understand the basic predictions made under different assumptions about the nature of associative learning and the responses of the sensory system to mixtures. The simulation outcomes are interpreted in the context of plausible neurobiological mechanisms in the discussion.

2. Materials & Methods

2.1 Fly husbandry

Drosophila were reared on standard cornmeal food at 25°C on a 12:12 hour light:dark cycle in bottles in LMS cooled incubators. The wildtype strain used for all experiments was Canton S (gift from Scott Waddell, University of Massachusetts, USA). Only flies aged 2-7 days old were used for behaviour experiments. A mixed population of both male and female flies were used in all experiments.

2.2 Behavioural assay

The olfactory associative learning assay used was the Drosophila T-maze (Tully & Quinn, 1985). The T maze apparatus was purchased from Simple Behavioural Systems, Houston, USA). The apparatus consists of a separate training tube for conditioning the flies and a T maze with two arms for testing. All experiments were conducted at 21-24°C under red light.
The assay comprises first a training phase then a testing phase. Approximately 80-100 flies were aspirated into a training tube containing a copper electric grid that covered over 90% of the available surface inside the tube. The training phase varies depending on the learning test design (see next section). In general, the first odour(s) in the training sequence is delivered to the training tube in tandem with shock reinforcement for one minute. Shock reinforcement consists of an 80V pulse delivered for 1.5 seconds duration at intervals of 3.5 seconds. After the shock/odour combination the flies are given 30 seconds of fresh air, then the next odour(s) in the sequence is delivered to the training tube in the presence or absence of shock for one minute, followed by 30 seconds more of fresh air. The process continues until training is complete. For testing, flies are then transferred immediately to the neutral choice point between the two arms of the T maze and given the choice between two odours or odour combinations. The number of flies that have entered each arm is counted after two minutes.

Four odours were used in total and each is referred to in the text and figures by a single letter (O, M, H or B) shown here; DL-3-Octanol (‘O’ in the text, Sigma, 218408), 4-Methyl-cyclohexanol (‘M’, Sigma, 66360), 6-Methyl-5-hepten-2-one (‘H’, Sigma, M48805) and Benzaldehyde (‘B’, Sigma, B6259). Air was bubbled through an odour:mineral oil blend. Amounts of each odour varied between 6-20μl in 8ml of mineral oil depending on the experiment. Prior to conducting learning experiments all test odour combinations were first ‘balanced’ to eliminate any odour bias. For balancing, untrained flies were given the choice between the test odours for two minutes. If flies distributed evenly between both test arms of the T maze then they showed no preference (n ≥ 5 tests). If flies showed a preference then odour concentrations were varied accordingly until they distributed evenly. The amount of each odour used for every experiment after this balancing procedure is detailed in Supplementary table 1.

Results in all graphs show box plots of the performance indices (PI) for each set of groups. The PI is calculated as the number of flies that choose the non-reinforced odour minus the number of flies that choose the reinforced odour divided by the total number of flies, for all experiments except in blocking which is detailed below.

2.3 Experimental design

Each learning assay had a unique training and testing design as detailed below. A positive sign (+) denotes the presence of reinforcement, and a negative sign (-) the absence of reinforcement. In all cases massed training (no more than 30 second intervals between training cycles) was performed. All training and testing combinations for all types of learning are also shown in Tables 1 and 2. Where possible, the reciprocal training cycle was performed (e.g. train: A+ B-, reciprocal training: B+ A-) and the results averaged to further control for any pre-existing odour biases. Every paradigm was repeated using several different odours or odour combinations. Where learning was not obtained after one cycle we tested with repeated cycles; otherwise we held training parameters as constant as possible across all paradigms, having chosen values for these parameters that seemed optimal from the previous fly learning literature.
Elemental learning (A+ B-): Elemental learning experiments were conducted as previously described (Tully & Quinn, 1985). Flies were trained with one odour that was reinforced then a second odour that was not reinforced. They were then given the choice between the two odours during testing (A vs. B). The reciprocal test was also performed (B+ A-). This was performed for all six different combinations of the four odours used. For each testing set \( n \geq 10 \) groups.

Two element learning (A+ B+ C-) : The training phase for two element learning reinforced odour A, then odour B in turn but did not reinforce odour C. The testing phase gave groups of flies the choice of A vs C or B vs C. For each testing set \( n \geq 8 \) groups. Four groups were tested;

1. Train: B+ O+ H- Test: O vs H
2. Train: B+ O+ H- Test: B vs H
3. Train: O+ B+ H- Test: B vs H
4. Train: O+ B+ H- Test: O vs H

Mixture learning (AB+ CD-): In these experiments two binary compounds each comprised of two different odours were presented during training. The first compound was reinforced and the second was not. The training cycle was then repeated in this order. During testing the flies were then given the choice between the two compounds (AB vs CD). The reciprocal test was also performed (CD+ AB-). This was repeated for three different odour combinations. For each testing set \( n \geq 8 \) groups.

Overlap learning (AB+ BC-): In this case the two binary compounds each contained one identical odour (B) and one different odour. The flies were trained with sequential presentations of the compounds for two training cycles, then given the choice between them at the testing phase (AB vs BC). The reciprocal test was also performed (BC+ AB-). This was repeated for three different odour combinations. For each testing set \( n \geq 8 \) groups.

Positive patterning (AB+ A- B-): In these experiments each of the individual odours were not reinforced but the compound was reinforced. Three training cycles were performed and the trained flies were split into two testing sets, with half of the groups tested with the first combination (A vs AB) and the other half with the second (B vs AB). For each testing set \( n \geq 8 \) groups. This was repeated for three different odour combinations.

Negative patterning (A+ B+ AB-): The training phase for negative patterning reinforced odour A then odour B but did not reinforce their compound AB. Three training cycles were performed using this sequence. For the testing phase trained flies were split into two testing sets, half of the trained groups were tested with one odour against the compound (A vs AB) and the other half were tested with the second odour against the compound (B vs AB). For each testing set \( n \geq 8 \) groups. This was repeated for three different odour combinations.
Biconditional discrimination (AB+ CD+ AC- BD-): The training phase for biconditional discrimination involved four different binary compounds comprised of four different odours. Two of these compounds were reinforced and two were not reinforced. Each element was as equally reinforced as non-reinforced during training. Three training cycles were performed using this sequence of compound presentations. The trained flies were split into four testing sets as follows; test set 1 (AB vs AC), test set 2 (AB vs BD), test set 3 (CD vs AC) and test set 4 (CD vs BD). For each testing set n ≥ 8 groups. The reciprocal experiment was also performed; train: AC+ BD+ AB- CD- and four test sets as follows; test set 1a (AB vs AC), test set 2a (AB vs BD), test set 3a (CD vs AC) and test set 4a (CD vs BD). The performance indices for each reciprocal test set were pooled to control for odour bias (individual PI scores from test set 1 were averaged with individual scores of test set 1a, set 2 with set 2a, set 3 with set 3a and set 4 with set 4a).

Blocking (A+ AB+): Flies were trained with a reinforced odour first then a reinforced compound that included the first odour. Only a single training cycle was performed. Groups were then tested with the additional element in the compound against a novel odour (B vs C). Blocking experiments were performed with four different odour sets. For each set of blocking experiments we conducted four separate control experiments (see below). Therefore the training for experiments for each odour set was conducted thus;

1. A+ AB+ (blocking) : Previous learning of A should block learning of B
2. Air- AB+ (control) : Control to show flies can learn B if presented in mixture
3. D- AB+ (control) : Control to show previous odour does not affect learning
4. D+ AB+ (control) : Control to show learning a different odour does not block
5. A- AB+ (control) : Control to show simple exposure to A does not block.

For all experiments testing was B vs C.

The blocking and the blocking control group scores were then compared. Blocking was performed for four different odour combinations as follows; block_BO (train: B+ BO+, test: O vs M), block_MB (train: B+ BM+, test: O vs M), block_OM (train: O+ OM+, test: B vs M) and block_MO (train: M+ MO+, test: O vs H). For each testing set n ≥ 8 groups.

2.4 Statistical analysis

All graphs and statistical analysis were performed using the free statistical software R. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org.) For all odour sets in all experiments we calculated the 95% confidence intervals for the population mean of the learning indices using t-scores for a Student-t distribution with df=n-1 as shown in Tables 1 and 2. A parametric test is justified as each learning index represents the sum of many binary decisions by individual flies, hence by the Central Limit Theorem, the distribution should tend towards the normal distribution. Using confidence intervals is appropriate to control for power: as well as taking a
confidence interval for a P.I. that excludes zero as evidence for learning, we also take a confidence interval that includes zero and excludes a P.I. of more than ±0.2 as evidence against learning (Loftus, 1996). A Kruskal-Wallis test was used to assess the differences between groups in each type of learning test.

2.5 Simulation Methods

In order to compare the results of the fly experiments to several different models of associative learning of compound stimuli, we implemented a simulation in Matlab (code available on request from the authors). In the following, we assume that the role of the US in learning is simply one of reinforcement, i.e., the presence or absence of the US (shock) in training trials can increase or decrease the likelihood that the CR (avoidance) will be produced in response to the CS (odour). Although the learning rules are phrased in terms of changing the ‘value’ of the CS towards the ‘value’ of the US, we make no assumption that there is any internal correlate of ‘value’ per se (see Discussion), but rather use this as shorthand for the strength of the CR that will be produced in response to the CS.

We also assume that the shock paradigm, in which the fly is constantly exposed to an odour for a period of time while receiving intermittent shocks, is equivalent to a discrete number of pairings of odour-shock; and that the following period in which another odour is presented without shocks is equivalent to the same number of pairings of odour-no shock. This may not be a valid assumption, but it is a limitation of the experimental paradigm that ‘pairing’ is not well defined in temporal terms.

We will first describe the learning rules for an elemental encoding of the CS, and then describe the alternative encodings that were explored (also see Table 3). The elemental encoding represents the CS as a vector $\text{CS}=[A B C D]$ where each element is 1 if that odour is present in the CS, and zero otherwise. Thus $A=[1 0 0 0]$, $B=[0 1 0 0]$, etc., and $AB=[1 1 0 0]$, etc. A corresponding vector encodes the current value $V$ of each element, as determined by the learning process. For simplicity, we assume that at the start of a trial $V=[0 0 0 0]$.

2.6 Learning rules

Our first ‘basic’ learning rule updates the value of each element $i$ of the value vector as follows:

$$\Delta V_i = \lambda \cdot CS_i \cdot (V_{us} - V_i)$$

where $\lambda$ is a learning rate (fixed at 0.5 for all the following), and $V_{us}$ is 1 if the US is present, 0 if the US is absent. Thus the values of all elements present in the CS increase asymptotically towards 1 if paired with the US, or decrease towards 0 if presented without the US.

A well known alternative to this rule was introduced by Rescorla & Wagner (1972), and has the form:
\[ \Delta V_i = \lambda \cdot CS_i \cdot (V_{as} - \sum_j CS_j V_j) \]

This makes learning of the value of one element dependent not only on its own current value but also the value of any other elements also present on the trial. A central motivation for its introduction was to account for blocking phenomenon (e.g. if the value of A is already 1, then there will be no increase in the value of B when presenting AB+). We will refer to this as the 'rw' rule. Note that this rule, unlike the previous one, can also lead to conditioned inhibition (negative values): for example, if B has initial value zero, and a positive US value predicted by A does not occur after presentation of AB, B will be decreased below zero.

Balkenius et. al. (2006), modelling multimodal learning in hawkmoths, suggest an alternative rule for learning, based on the concept that the animal is attempting to form a template for the reinforced CS, whenever the US occurs, by increasing the value of elements present and decreasing the value of elements not present. It assumes:
- That learning occurs, using the Rescorla-Wagner rule, only on reinforced trials, i.e. when US=1.
- That elements not present on reinforced trials have their value reduced by a fixed decrement (down to a minimum of zero).
- That all the values are then normalised (this step is relevant to making the method sensitive to patterns rather than magnitudes but is not relevant to the current case, as we do not consider magnitudes).

This rule has several limitations, in particular that it does not allow for extinction of learning during unreinforced trials. Nevertheless it is an interesting alternative conception of the mechanism of learning and has been successfully applied to model moth learning data. We implemented a simplified version of this as the 'template' rule. In our version, we use the basic rule rather than the Rescorla-Wagner rule for increasing the value, we reduce the value in increments of 0.2, and we omit the normalisation step.

Finally we implemented a rule that corresponds more closely to some current assumptions about the underlying neural mechanisms of associative learning in invertebrates (Heisenberg, 2003; Gerber et al., 2004). That is, we assume the US acts via release of a neuromodulator to increase the value of (or likelihood of a response to) any CS that occurs at the same time. We implement this increase as a fixed increment; consequently this method has no inherent asymptote but we assume there is a fixed maximum value that can be reached (this maximum, or the increment rate, could be made proportional the value/strength of the US). Note this does not provide any mechanism by which value can be decreased, otherwise it would be effectively equivalent to a linearised version of either the basic rule (if the value of a CS not followed by a US is decreased) or the template rule (if the value of a CS that is absent when a US follows is decreased). It could also be considered as an abstracted equivalent of the reward model proposed by

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2 In fact, we tested this rule both in the form given in Balkenius and in the simplified form described here. The results are essentially similar except that using the Rescorla-Wagner rule produces a blocking effect, as expected, which is not found for the fly. The normalisation process simply removes any effect of the learning rate.
Izhikevich (2007) to connect STDP and dopamine signalling, and hence we refer to it here as the ‘neuromod’ rule.

2.7 Trials

To determine how these rules perform under the different conditions used to test the fly, we apply each rule using the corresponding training patterns. E.g. for elemental learning, A+ B-, the values are updated by pairing the pattern CS = [1 0 0 0] with US=1, then the pattern CS= [0 1 0 0] with US=0. We use three consecutive pairings, or applications of the learning rule, for each one minute pairing of stimuli in the fly paradigm. This number of pairings is somewhat arbitrary but with the high learning rate (0.5) produces an appropriately large change in the values; and changing this number does not qualitatively alter any of the results.

The outcome of the learning is then measured by calculating the values, after training, of the stimulus options offered to the flies at the choice point of the T-maze:

\[ V_{\text{pref}} = \sum_{i} CS_i V_i \text{ for } CS = \text{stimulus fly should avoid after learning} \]
\[ V_{\text{alt}} = \sum_{i} CS_i V_i \text{ for } CS = \text{alternative stimulus offered in choice} \]

and taking the difference between them (\( \text{response} = V_{\text{alt}} - V_{\text{pref}} \)) as equivalent to the flies’ performance index. We limit the maximum possible response to 1 or -1.

For each learning rule, we run this process on the same training patterns as used for the fly, i.e.:

1. Elemental: train A+, B-; test A vs. B
2. Two elements: train with A+, B+, C-; test A vs C and B vs C.
3. Mixture: train twice with AB+, CD-; test AB vs CD
4. Overlap: train twice with AB+, BC-; test AB vs BC
5. Positive patterning: train three times with AB+ A- B-; test AB vs A or B
6. Negative patterning: train three times with A+ B+ AB-; test A or B vs AB
7. Biconditional: train three times with AB+ CD+ AC- BD-; test AB vs AC; repeat with AC+ BD+ AB- CD-; test AC vs AB; and take the average of these results
8. Blocking: train A+ AB+; test B vs C
   Control: train AB+; test B vs C (note, we can easily predict that none of the other controls used for the fly experiments will give different results from this simple control, for any of the models, and so they have not been included)

2.8 Using Salience

It might be assumed that when two elements are present in a CS, each is less salient, or could receive only half the attention, and hence be learned half as well, as when each element is presented alone. While salience and attention are rather subtle concepts that have been used in many ways in learning theories, in what follows we will treat salience as a simple division factor, such that both learning and expression of the CR
in response to compounds is divided by the number of elements in the compound. For each of the learning rules described above, we test it with and without the inclusion of this saliency adjustment. This implementation is essentially equivalent to the use of salience in the 'modified unique cue theory' used to account for bee data in Deisig et al. (2003).

2.9 Alternative stimulus encodings

The encoding described so far treats the presentation of an odour mixture as equivalent to the sum of the presentation of the elements (AB=A+B). An opposite view would be to assume a compound is equivalent to a completely new and distinct odour (AB=X). We call this a 'configural' encoding, as it assumes odour mixtures form distinctive configurations. This can be encoded in our simulation by using a vector of length ten to represent the stimulus: [A B C D AB AC AD BC BD CD]. Thus A would be encoded [1 0 0 0 0 0 0 0 0 0] and AB encoded [0 0 0 0 1 0 0 0 0 0] etc. All the learning rules can be applied as before.

Another alternative to the simple sum view is that a compound is encoded both in terms of its elements and an additional 'unique cue' specific to that compound, i.e. AB=A+B+U. This can also be encoded using a vector of length ten, but this time AB = [1 1 0 0 1 0 0 0 0 0] etc.

Pearce (1994) proposes a variation on the extreme configural representation that assumes partial activation of elements that overlap with the current configuration. Rather than implementing his algorithm for the formation of such overlapping activations, we represent this by the simple assumption that any of the ten possible stimuli that overlaps with the current stimuli will be activated half as much, i.e. AB = [0.5 0.5 0 0 1 0.5 0.5 0.5 0.5 0].

A final encoding we tested is the elemental encoding with an additional 'contextual' element, as it is often argued that associations can be formed not only to the experimenter controlled variables but to the experimental apparatus itself. Thus, in this 'context' representation, a fifth element is included to represent the situation, A= [ 1 0 0 0 1], B = [0 1 0 0 1], etc.

3. Results

In this study we have investigated eight different types of learning. For each learning type the training protocol is indicated in brackets beside the title; full details can be found in the materials and methods section. The mean performance indices (PI) for all learning tests are shown, with confidence intervals, in Tables 1 and 2, which also summarises the number of training cycles and specific training/testing combinations for each learning test. Note that n refers to the number of repetitions of the assay, i.e., to the number of groups tested, where each group consists of up to 100 flies.
3.1 Elemental learning (train A+ B--; test A vs. B)

Elemental learning pairs one odour (the conditioned stimulus, CS+) with an electric shock (unconditioned stimulus, US) then tests this odour against a neutral, unpaired odour (CS-). In order to ascertain that flies could learn a variety of different odours and that they could clearly distinguish between the odours used, we first conducted simple elemental learning tests using six different odour pairings (see materials & methods 2.3). Flies were capable of forming associations with any of the four odours in all possible punished/non-punished combinations as shown in Figure 1(a). 95% confidence intervals (CIs) for the mean PIs were greater than zero for all odour combinations (see Table 1). A Kruskal-Wallis test showed no significant difference between the groups (H=1.7782, df = 5, p=0.8789).

3.2 Two element learning (train A+ B+ C--; test A vs C and B vs C)

In order to test whether learning about a second stimulus might interfere with a previous memory, or alternatively, whether learning one thing makes it harder to learn a second, we performed ‘two element learning’ experiments. We tested whether flies could learn to avoid either of two elements that had been punished (A+ B+) in preference for a third (C-). The results are shown in Figure 1(b). We split the data up into four groups which alternated the order in which each reinforced odour was presented and which odours were used for testing (see methods 2.3). Flies always avoided the punished stimuli, and PIs for both the first and second punished element were comparable to simple elemental learning scores. This result is relevant to interpreting some of the paradigms below, which also require the fly to recall punishment of several stimuli at the same time. A Kruskal-Wallis test showed no significant difference between the groups (H=0.9251, df = 3, p=0.8194).

3.3 Mixture learning (train AB+ CD--; test AB vs CD)

We investigated whether flies were capable of learning about odours that were only presented in compound mixtures. For these experiments we first presented the flies with a binary mixture (CS) paired with shock (US) and then a second mixture that was unpaired (AB+ CD-). We then tested the flies by giving them the choice between the two mixtures (AB vs. CD). Note that such learning can potentially be explained either by assuming flies learn about one or both elements of the reinforced compound, or by assuming they learn each compound as a 'gestalt'. Using the same four odours that we had tested for elemental learning, we trained flies with each of the six different binary odour combinations. We found that in order to solve the problem successfully each group had to be trained twice. Flies were capable of learning all different binary odour mixtures as shown in Figure 1(c), with all CIs greater than zero (table 1). The results of a Kruskal-Wallis test showed no significant difference between the groups (H=1.2966, df = 2, p=0.5229).

3.4 Overlap learning (train AB+ BC--; test AB vs BC)
Overlap learning also involves binary odour mixtures, but in this case one odour is the same in both mixtures. In this case, learning based only on elements would appear more difficult than in the mixture case, as B is sometimes reinforced and sometimes not. As with mixture learning, all groups were required to be trained twice to obtain significant PIs. Flies were capable of solving overlap learning tasks using all odour mixtures as shown in Figure 1(d), with all CIs greater than zero (Table 1). A Kruskal-Wallis test was performed and the results showed no significant difference between the groups (H=3.1906, df = 2, p=0.2028).

The double training required for both mixture and overlap learning suggests that solving these learning problems is not as straightforward as solving elemental problems. However, there is no evidence that overlap learning is more difficult than the learning of mixtures that do not overlap.

**3.5 Positive patterning (AB+ A- B-; test A vs AB and B vs AB)**

Knowing that flies could learn mixtures and distinguish elements of these mixtures we investigated whether flies could solve positive patterning problems. In these experiments flies were presented first with a mixture that was reinforced, then with each of the components of this mixture, which were not reinforced. Although this problem appears to be a simple reverse of negative patterning, positive patterning is not a non-elemental problem: flies might be expected to find the sum of two weakly aversive elements more aversive than either element alone. Each group was given three training cycles. Flies were capable of solving this task with all odour combinations as shown in Figure 1(e), with CIs for all groups greater than zero (Table 1). The results of a Kruskal-Wallis test showed that there was no significant difference between the groups (H=1.2238, df = 2, p=0.5423). It is worth noting, given the results for the following paradigms, that this shows that some reliable association can be formed under conditions where all elements are both reinforced and non-reinforced.

**3.6 Negative patterning (train A+ B+ AB-; test A vs AB and B vs AB)**

Next we investigated whether flies could solve negative patterning problems, using three different sets of odours. In contrast to the previous paradigms, negative patterning can only be solved using non-elemental processing. If AB is treated simply as A+B, the associative strength of the compound would always be higher than that of the elements, and the flies would (incorrectly) avoid the compound in the choice test. Each group was trained with three training cycles. The results are shown in the three box plots in Figure 1 (f): overall it appears that flies cannot solve negative patterning problems, with mean scores of 0.06 or less and the CIs for all groups falling between -0.2 and 0.2. A Kruskal-Wallis test was performed and the results showed no significant difference between the groups (H=1.6318, df = 2, p=0.4422). We also tried negative patterning using five massed training cycles with no effect on results. While the flies have not learned to avoid the elements in favour of the compound, nor do they show evidence of avoiding the compound in favour of the elements (which would have resulted in significant negative scores).
3.7 Biconditional discrimination (train AB+ CD+ AC- BD-; test AB vs AC, AB vs BD, CD vs AC, CD vs BD)

Biconditional discrimination is another paradigm which cannot be accounted for by simple elemental learning, but this time all training and testing involves compound stimuli. It is conceptually equivalent to context dependent learning, which can be specified as \( AX+ BY+ AY- BX- \), where \( X \) and \( Y \) form the context in which \( A \) or \( B \) are respectively reinforced. In this case we have used one modality with four components presented as a series of binary mixtures where each component is as equally reinforced as non-reinforced. Groups were trained three times prior to testing. Each reinforced mixture used in training was then tested against each unreinforced mixture (see Table 1). The reciprocal training cycle (AC+ BD+ AB- CD-) and tests were also performed. The performance index was calculated as an average of the two identical test groups, as shown in Table 1. Flies were unable to solve biconditional discrimination problems as shown in Figure 1(g), with mean scores of 0.06 and all CIs between −0.2 and 0.2. The results of a Kruskal-Wallis test showed that there was no significant difference between the groups (\( H=2.0915, df = 3, p=0.5536 \)).

3.8 Blocking (train A+ AB+; test B vs C)

Blocking occurs when learning about a component (B) that is presented in a compound (AB+) is reduced if there has been prior reinforcement of the other component (A+). Therefore the learning scores for blocking should be lower than those of the controls. We tested four sets of odours for blocking along with four control groups for each odour set. For each odour set we tested for blocking (train: A+ AB+ , test: B vs C) by comparison to the four controls detailed in the materials and methods. Our results are shown in Figure 2 and Table 2. For three odour sets (Figure 2a,c,d) no significant decrease in learning score was detected for the blocking group relative to the controls. The results for the fourth odour set (Figure 2b) were different because the second control group was significantly higher than the blocking group (Wilcoxon rank sum test; \( p < 0.002 \)), however the other three control groups were not.

3.9 Simulation results

We assessed the behaviour for every possible combination of four different learning rules, five different mixture encodings, and the inclusion or otherwise of a ‘saliency’ reduction in the individual effectiveness of elements presented simultaneously (see Table 3). The results after running each of the forty resulting learning models, for all eight training paradigms, is shown in Table 4. Here we summarise the main observations resulting from the simulation.

All the models are able to produce elemental learning, as expected. For two-element learning, the template rule has difficulty, as the presentation of reinforcement with the
second element in the absence of the first decreases the previously learnt value of the first. The other rules can learn about two elements successfully.

For mixture learning, flies can learn but require several cycles to do so (indicated in the table as a value of ‘<1’). All the models are also able to learn mixtures. Including the ‘saliency’ factor produces slightly lower learning scores, as this means only half as much is learnt about each element on each trial when elements appear together. A similar pattern of results occurs for overlap learning.

Flies were able to learn positive patterning, but not negative patterning. This proved the most difficult result to reproduce with any model. With the basic learning rule, only the unique cue encoding without the salience adjustment could support positive patterning and give a zero result for negative patterning. Most other encodings under the basic rule produced no learning in either paradigm. The overlap encoding without salience learnt positive patterning but had a significant negative score for negative patterning. The configural encoding produced successful learning for both (this was true for this encoding with all learning rules). Broadly similar results were found with the Rescorla-Wagner rule, except in this case the unique encoding and context encodings could learn negative patterning. For the template rule, most encodings without saliency learnt positive patterning and had negative scores for negative patterning; whereas with saliency neither was learnt. A similar pattern was seen for the neuromod rule, except that negative patterning was learnt with the unique cue encoding.

For biconditional discrimination, the task is not learnt by the flies, and is only learnt by the models with either unique cue or configural encoding. It is reduced or zero for the unique cue encoding if salience is included. Note, in particular, that the only model which matched the flies in learning positive but not negative patterning (the basic rule with unique cue encoding) fails to match the flies here as it easily learns the biconditional discrimination which flies could not.

Flies did not show blocking, performing as well in the blocking condition as in the control. For all models, the configural encoding learns nothing in either situation, as B is never presented alone in training, so can acquire no value for the test. The Rescorla-Wagner learning rule produces a consistent blocking effect, as would be expected. All the other learning rules and encodings produce equal learning in both conditions, like the flies.

4. Discussion

We have investigated the ability of flies to solve a range of problems involving the association of shock to a variety of odours and odour mixtures. In addition to elemental learning, we have found that flies are capable of mixture and overlap learning and solving positive patterning problems. In our tests they could not solve negative patterning or biconditional discrimination problems and blocking is generally not observed.
It is not clear how far this pattern of results can be generalised. It is possible that learning under different paradigms, e.g., with sugar reward rather than shock, involves different mechanisms, and may produce some differences in the pattern of results. Some variant of the trial parameters, such as inter-trial intervals, odour identities or alternative testing measures might reveal learning in cases that were not successful in our tests - we can only say this was not seen when using 'standard’ methods. Specifically for blocking, training A+ to asymptote might be necessary to see an effect on learning of AB+. Similarly, it is plausible that despite the same basic brain architecture, other insects may be capable of solving problems that the fly cannot. As mentioned in the introduction, bees have been shown to learn negative patterning for odour combinations, using the proboscis extension reflex (Deisig, 2001), but (to our knowledge) this has not been tested in a shock paradigm. Flies have shown biconditional learning for two modalities in the flight simulator (Brembs and Wiener, 2006) but not for light/odour combinations in the shock paradigm (Yarali et al. 2008). Crickets and cockroaches have shown multimodal biconditional learning of odour-taste associations (Matsumoto and Mizunami, 2004). Whether any insect has unequivocally demonstrated blocking remains debatable.

We next consider the implications of our data for current models of learning. Perhaps the simplest ‘model’ is that when an odour is paired with shock, the tendency for the fly to avoid that odour in future increases (this is the learning rule we have called ‘neuromod’); and that mixtures of odours are treated as the sum of their elements (AB=A+B, elemental encoding), with each association formed independently. Such a simple model can, in fact, account for almost all our data. It would predict that mixtures can be discriminated, except where all elements are equally reinforced (i.e. biconditional discrimination) and that learning about one element should not affect another (i.e. no blocking). However, contrary to our data, it would predict that if positive patterning occurs (flies avoid AB after training AB+ A- B-) then negative patterning should produce an equally large but negative learning score (flies trained A+ B+ AB- should avoid AB, as A+B must be greater than A or B).

A well-known alternative to such an entirely elementary and independent association rule is the Rescorla-Wagner learning rule (Rescorla & Wagner, 1972), which assumes that strengthening of an association to a stimulus depends on the current association strength of any other stimuli presented at the same time. For example, in blocking, the prior association strength of A reduces any strengthening of B if B is presented only in the combination AB. This is usually interpreted in cognitive terms: because A already 'predicts' the shock, B carries no further information and is therefore not learnt. However, as noted, this rule predicts blocking, which was not observed for the flies, so it cannot account for our data. It also predicts (for elemental encoding) that neither positive nor negative patterning will be learnt, which is not the case.

Rather than change the assumption that strengthening of the association is independent for all stimuli present on a trial, we can alternatively change the assumption that presenting mixtures of stimuli is exactly equivalent (in the flies' experience) to the sum of the elements. One alternative, originally suggested as a modification of the Rescorla-Wagner rule (Rescorla, 1973), but here assuming independent learning, is that
presentation of a compound activates its elements and a unique cue (AB=A+B+U). This has the potential to account for our finding that negative patterning produces no net learning effect, while positive patterning does. Both involve equal strengthening and weakening of the elements A and B, but the unique cue is strengthened in positive patterning (ABU+ A- B-) and is unaffected in negative patterning (A+ B+ ABU-). However, this model would also suggest that learning to discriminate mixtures should be faster or produce stronger responses than learning of single odours, which was not observed; introducing a saliency factor to try to account for this reduces the match in other paradigms. It would also predict that biconditional discrimination should be learnt by the flies.

Another alternative is to assume a compound is equivalent to a completely new and distinct odour (AB=X, configural encoding). However the results do not support this assumption, which would predict that mixtures are learned as easily as single elements, including under the negative patterning and biconditional paradigms, which our flies do not learn; and by contrast, would also predict that nothing about B should be learnt in the blocking and blocking control paradigms, as B is never presented alone in training, whereas flies learn to avoid B after experiencing AB+ conditioning. Configural encoding with generalisation (Pearce, 1994), which we have approximated with our ‘overlap’ encoding, comes closer to emulating the fly behaviour as it learns positive patterning but not biconditional, under either the basic or neuromodulation rule, and learns about B in both blocking and control paradigms. However it also predicts a negative learning score for negative patterning which was not observed. A possibility that we have not considered in our modelling is that configural learning involves two stages: first the acquisition of the configural cue through associating two elements, followed by association of that cue to the US (Pearce, 1994). There is some evidence from bees that over the course of training, initially elemental results shift to configural (Müller et al, 2000, Deisig et al 2003). It remains possible that more extended training would reveal non-elemental learning in flies, but as noted in our results, we found that five training repetitions for negative patterning appeared no more effective than using three.

In summary, no combination of learning rule, encoding method or saliency in our simulation is able to account completely for the overall pattern of results. It is worth noting that requiring a model to simultaneously account for a collection of eight paradigms is a stronger constraint than is normally applied to such models which are often tested relative to more limited data sets or with ad hoc adjustments for different conditions. In extended exploration of the models we have found no simple adjustment sufficient to produce responses consistent with the observed fly behaviour. We thus tentatively conclude that a more complex mechanism than any of those tested is involved. This is perhaps not surprising, yet it is important to have ruled out simpler models before exploring more complex ones. In particular, it is significant that the Rescorla-Wagner rule (or by extension, learning rules involving prediction error as the signal for changing

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3 This result depends on using the basic rather than the Rescorla-Wagner learning rule. If the latter is applied, ‘U’ should acquire the opposite value to that predicted by A+B on ABU- trials. This would lead to greater avoidance of A or B than AB, i.e., negative patterning could be successfully learnt.
CS values) does not provide a good match to the data. Similarly, although the fly appears to learn elemental tasks and fail to learn non-elemental tasks, it turns out that this cannot be simply explained by assuming it has a purely elemental encoding of the stimuli.

Given that the pattern of findings in the fly does not directly fit any of the algorithmic models, can we relate our observations to what is known of the processing pathways for (short-term) olfactory learning in Drosophila? Around 1500 olfactory receptor neurons (ORNs) in each fly antennae project axons to the antennal lobe, where they connect to projection and local interneurons in discrete structures called glomeruli. There are ~45 glomeruli in the Drosophila antennal lobe, and ORNs expressing the same odourant receptor converge on the same glomerulus. Thus an odour will elicit a consistent and unique spatio-temporal pattern of activation across the antennal lobe. A projection neuron receives excitatory input from one glomerulus and inhibitory inputs from other lateral glomeruli (Olsen & Wilson, 2008). Clearly this wiring produces several mechanisms by which simultaneous presentation of odours could lead to non-linear interactions: the activation patterns may overlap; odours could facilitate or suppress each other’s patterns (Deisig et al., 2006; Silberling & Galizia, 2007) through lateral connections between glomeruli. Moreover, these interactions could depend on the precise odour identities, such that certain odour combinations overlap more or less, or particular odours tend to suppress other odours.

The antennal lobe projection neurons form several tracts, connecting to the lateral horn and the mushroom bodies. The latter is believed to be the main site of aversive olfactory memory (Heisenberg et al., 1985; Heisenberg, 1998; much of the evidence is summarised in Gerber et al. 2004). Restoration of the rutabaga adenylyl cyclase in the mushroom bodies of rutabaga learning mutants revealed that the learning defect could be rescued, indicating a sufficiency for the mushroom bodies in short term memory (Zars et al., 2000). Dubnau et al. (2001) and others suggested that output from the mushroom bodies is not required for (short-term) acquisition; but more specifically targeted studies suggest that output from the α’β’ lobes is required (Krashes et al., 2007). In the mushroom bodies around 150 projection neurons diverge onto around 2500 Kenyon cells, which in turn converge onto around 50 extrinsic neurons (Tanaka et al., 2008). A common interpretation of this connectivity pattern is that the divergent connection onto the Kenyon cells serves to separate the patterns on the antennal lobe, in the manner of a linear classifier. However, such a system, like the two-layer perceptron it resembles, should be capable of solving problems such as negative patterning (equivalent to the XOR problem) and biconditional discrimination, as we have shown in a previous model (Wessnitzer et al., 2007). Given that the fly does not solve these problems, at least in the shock paradigm, we may have to reconsider the function, although the ability of other insects to do some of these tasks in other paradigms perhaps leaves this issue open.

What connections are actually changed in learning? At an abstract level, classical conditioning is most often described as the formation of an association between the CS and the US, such that the CS comes to evoke a response, the CR, which resembles the UR. In more cognitive interpretations, the CR is said to ‘anticipate’ the US, because the CS acquires a ‘value’ corresponding to or predicting the innate ‘value’ of the US.
However in the shock conditioning paradigm the CR – avoidance of the odour – does not resemble the UR – jumping and/or freezing in response to electric shock. Flies are not learning a simple CS-UR or CS-US-UR connection. In fact we tried, but were unable to condition flies to produce consistent shock-like responses to odour stimuli. An alternative view is that learning consists in the strengthening or weakening of a CS-CR connection (an odour avoidance response) when the CS is paired with a reinforcer, the US. This is a rather different concept. It implies the UR is irrelevant to learning (except perhaps as an indicator of whether a US is likely to be rewarding or punishing). It also implies that the CR is in some sense a ‘pre-wired’ potential output of CS processing. Though it is consistent with the interpretation that the CS has acquired a ‘value’, it does not necessarily require an explicit mechanism for ‘value learning’ (see further discussion of this point below). A final possibility is that flies are in fact strengthening an approach CR to the CS (B–) that is paired with the positive US of relief from the shock. Yarali (2008), in careful experiments to identify such ‘relief learning’, suggest that such an effect exists in the fly shock training paradigm, but that it is substantially weaker than the basic learned avoidance of A+.

The onward connections from the mushroom body extrinsic neurons and lateral horn that lead ultimately to the control of approach or avoidance behaviour in response to odours are essentially unknown. However, the idea that the US acts primarily to modulate such a pathway is consistent with neurophysiological and genetic studies of the role of monaminergic neurons in learning. Specifically, olfactory learning to shock is mediated by dopaminergic pathways (Schwaerzel et al., 2003). Shock produces activation of dopaminergic neurons (Riemensperger, 2005, Mao and Davis, 2009). Light-induced activation of dopaminergic neurons can be substituted for shock to induce aversive odour learning (Schroll et al. 2006, Claridge-Change et al. 2009) and a specific dopamine receptor (dDA1) in the mushroom body has been implicated in shock learning (Kim et al., 2007). Recently, aversive reinforcement signals have been mapped to a cluster of twelve dopaminergic cells (PPL1 cluster) that target parts of the vertical lobes and the heel of the mushroom body (Claridge-Chang et al., 2009). Neurons from other dopaminergic clusters have been implicated in the formation of aversive labile memory (Aso et al., 2010). The coincidence of olfactory-evoked Kenyon cell activity and the dopamine signal is thought to elicit a cAMP signalling cascade that changes the strength of Kenyon cell output synapses, and consequently, it is assumed, the behaviour of the animal (Gerber et al., 2004; Tomchik & Davis 2009; Gervasi et al., 2010). A recent review summarises what is currently known of dopaminergic systems in *Drosophila* (Waddell, 2010).

Given that this seems sufficient to explain aversive conditioning, is there also a role or mechanism for ‘value’? Value is implicit in the Rescorla-Wagner learning rule and in the generalised forms that have followed it (temporal difference learning), but the lack of evidence for blocking in insects suggests this conceptual framework may not apply. Furthermore, the dopaminergic response to the US is not reduced or shifted towards the CS after training (Riemensperger et al., 2005, Mao & Davis, 2009), so it lacks a critical characteristic of the predictive dopaminergic signal in vertebrates that underpins the ‘value’ interpretation (Schulz et al. 1997). However, several recent lines of
evidence suggest that the expression of at least some learned responses to odours are gated by motivational factors and/or the activation of dopamine/octopamine (Gerber and Hendel 2006, Krashes and Waddell 2008, Krashes et. al. 2009, Mizunami et al. 2009). Mizunami et al. (2009) propose that learning involves strengthening of two separate associations, CS-CR and CS-'value'. Allowing other stimulus conditions (such as presence of food) or internal motivational states (such as hunger) further modulate the same 'value' system, which then gates expression of a CR, seems a plausible way to unite these findings. It might also be the case that an extreme and unnatural stimulus such as electric shock does not engage the 'value' mechanism. This would be consistent with the observation that synaptic output from dopaminergic neurons is apparently not necessary for retrieval of shock-odour associations (Schwaerzel et al. 2003).

The genetic tools available in Drosophila make it a powerful system for understanding the neural mechanisms of learning. However, the fact that flies can learn should not imply a default assumption that their capabilities and mechanisms are equivalent to vertebrate models. In this study we have shown that while flies show a certain capability to learn about compound and overlapping configurations of odours, they do not appear to learn the non-elemental tasks of negative patterning and biconditional discrimination. However, our modelling shows that this pattern of results is not simply explained by the assumption that learning is purely elemental in these animals. What seems most parsimonious is that simple associations are formed between olfactory input and avoidance responses, but that the pre-processing of olfactory compounds is non-linear, and dependent on actual odour identities. The failure to observe blocking supports the conclusion that there is no higher level influence of what one odour pattern ‘predicts’ on the learning of other associations.

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References


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**Legends**

**Figure 1: Learning scores.** a) Elemental learning showing flies can learn all single odours when using six different odour pairs. b) Two element learning: Groups 1 & 2 were
trained with B+ O+ H- and groups 3 & 4 were trained with O+ B+ H-. Flies avoid both punished odours. c) Mixture learning; flies can learn binary mixtures of odours. d) Overlap learning; flies can learn to distinguish between binary odours even when one component is the same. e) Flies can solve positive patterning tasks. Each group represents a different set of odours used. Results are pooled from the two different testing sets (A vs AB and B vs AB). f) Flies cannot solve negative patterning tasks. Each group represents a different set of odours used. Results are pooled from the two different testing sets (A vs AB and B vs AB). g) Flies are not able to solve biconditional discrimination tasks. Data from each of the four test groups is pooled for both training combinations. h) Graph showing pooled data from all odour sets for each type of experiment. Odours: DL-3-Octanol (O), 4-Methyl-cyclohexanol (M), 6-Methyl-5-hepten-2-one (H) Benzaldehyde (B). Tr, train; Ts, test.

**Figure 2: Blocking.** a-d) Each graph represents one odour set. The first plot in every graph is the data for blocking and the remaining four are respective controls (see methods section). e) Pooled data from all four odour sets. Odours: DL-3-Octanol (O), 4-Methyl-cyclohexanol (M), 6-Methyl-5-hepten-2-one (H) Benzaldehyde (B). There is no consistent evidence of a blocking effect, which should result in lower scores in the blocking condition compared to the controls.

**Table 1: Olfactory shock avoidance scores.** The ‘(r)’ in column two denotes that separate groups were trained with the reciprocal (reverse the odour(s) associated with the shock) situation. ‘+’ denotes reinforcement, whereas ‘-’ denotes no reinforcement. Where the number of training cycles is >1, massed training was performed. For all odour sets in all experiments we calculated the 95% confidence intervals for the population mean of the learning indices using t-scores for a Student-t distribution with df=n-1. PI, performance index; CI, confidence interval. Odours: 3-Octanol (O), 4-Methylcyclohexanol (M), Benzaldehyde (B) and 6-Methyl-5-Hepten-2-one (H).

**Table 2: Blocking experiment scores.** Scores for all blocking experiments and subsequent control experiments performed. Column 1 shows the odour code relating to figure 2. The training for each experiment is detailed in column 2. The first row in each odour code is the blocking experiment and the following four are controls for that experiment. For all odour sets in all experiments we calculated the 95% confidence intervals for the population mean of the learning indices using t-scores for a Student-t distribution with df=n-1. PI, performance index; CI, confidence interval. Odours: 3-Octanol (O), 4-Methylcyclohexanol (M), Benzaldehyde (B) and 6-Methyl-5-Hepten-2-one (H).

**Table 3: Learning rules and mixture encodings used in the simulation.** Each of the four learning rules was applied, both with and without the saliency adjustment, to all five forms of mixture encoding, so that a total of forty different models were tested. Each was trained under the same eight paradigms as the fly, and generated corresponding learning
scores based on the difference in values, after training, of the choice stimuli presented to the fly in testing.

**Table 4: Results of simulation.** A value of 1 signifies successful learning; <1 is weaker or slower but still significant learning. A value of 0 (light grey) signifies no learning (no preference shown in test). A value of <0 (dark grey) indicates that the punished stimulus (+) was preferred in the test. None of the forty models fully replicates the results for the fly (top row).

**Supplementary Figure 1: Sensory preconditioning (AB- A+).** The training phase for sensory preconditioning involved two training cycles (AB- AB- A+ A+) first presenting a non-reinforced compound then reinforcing one element from that compound. Groups were then tested with the non-reinforced odour from the compound against a novel odour (B vs. C) and the performance index is calculated as: PI = no. of flies in tube C – no. flies in tube B / total flies. This was repeated for two different odour combinations. No learning about B was observed.

**Supplementary Table 1: Amounts of each odour used.** Every test set of odours was ‘balanced’ against each other to ensure there was no preference. Odours: 3-Octanol (O), 4-Methylcyclohexanol (M), Benzaldehyde (B) and 6-Methyl-5-Hepten-2-one (H).