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Doctoral Dissertation

Relative value learning in *Drosophila melanogaster* larvae

（キイロショウジョウバエ幼虫における相対的価値学習）

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A dissertation submitted for the degree of Doctor of Philosophy in Life Science

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Hokkaido University, Japan

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Dedicated to

My beloved father, whom I lost during my PhD journey

Statement of Original Authorship

All the research described in this thesis is my own original work.
This work has not previously been submitted for a degree or diploma at any university.

RAHMAN SADNIMAN

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The Author

Abstract of Doctoral Dissertation

Degree requested: Doctor of Life Science Applicant's name: RAHMAN SADNIMAN

Title of Doctoral Dissertation

Relative value learning in *Drosophila melanogaster* larvae

（キイロショウジョウバエ幼虫における相対的価値学習）

The ability to learn from past experiences to inform future decision-making is crucial for humans and animals alike. One question with important implications for adaptive decision-making is whether we learn by comparing the values of different options and associate the outcome of this comparison with stimuli (called relative value learning) or whether we learn about values irrespective of their alternatives (called absolute value learning). Many studies in humans and vertebrates demonstrated relative value learning even when it leads to suboptimal decisions. While some studies in insects have demonstrated that insects do discriminate values of different rewards or punishments and can learn accordingly, none of them clearly showed whether the value associated with one option changes because another option with a different (better or worse) value had been present. Thus, it has remained unclear whether organisms with simple nervous systems (such as insects) learn by the comparison of different values and thus whether the principles of value-based learning are shared across evolutionarily distant species.

To answer this question, I investigate whether and how insects learn by comparing the values of rewards and punishments during classical conditioning using *Drosophila melanogaster* larvae. I carefully designed associative odour-taste learning experiments to distinguish absolute and relative value learning. At first, larvae were trained using two odours, n-amylacetate (AM) and 1-octanol (OCT), each paired alternately with reward or punishment substrates of two different strengths. Neutral training conditions were provided using tasteless agar. Larvae underwent three alternating training cycles in which each odour was paired with its assigned substrate. Only AM was given during the test which allowed a direct evaluation of the associative value attributed to that odour alone. The numbers of animals selecting each side of the test dish were analyzed using quasibinomial generalized linear models (GLMs), followed by Wald tests to evaluate the significance of predictors, and the Quasi-Akaike's Information Criterion (QAIC) was used to compare model fits. In four independent experiments, I observed that the larvae's behaviour was mostly determined by the comparison of the reward or punishment strengths during the training, which corresponds to the definition of relative value learning as it is used in human research.

To test whether learning by comparing reward values requires the presence of two odours during training, I performed one-odour training experiments where larvae experienced the to-be-tested odour paired with either the reward or the neutral substrate, while the alternative training phase included no odour. The results of three experiments, each using another odour, suggested that learning by comparing reward values does not require the presence of two odours during the

training. However, the results differed depending on the odours being used. For AM, I found evidence suggesting a potential dominance of the reward value paired with the odour whereas when using OCT and Benzaldehyde (BA), the larvae's behaviour was best explained by a simple subtraction of the two reward values.

In addition, to know whether relative value learning depends on the sequence of training experiences, I performed one-trial training experiments in which larvae were trained with one training cycle with OCT or AM being presented first in separate experiments. I found that while the training sequence had a significant effect, in both sequences the larvae were able to learn by comparing the two reward values.

In summary, this work using *D. melanogaster* larvae strongly suggests that insects can learn by comparing reward or punishment values during training, equivalent to what is called relative learning in humans. These findings reveal that relative value learning is not a high-level cognitive feature restricted to complex brains but an evolutionarily adaptive process which can be observed even in a nervous system with only ~10,000 neurons. Given the great potential of *D. melanogaster* as a model organism for in-depth neurobiological analyses, this study opens up the opportunity to reveal the mechanism underlying relative value learning in unprecedented detail. In my thesis, I discuss one simple and plausible possibility for such a neuronal mechanism, based on the rich existing literature about the mushroom body, the memory centre of insects.

Introduction

One of the most fundamental functions of our memory is to guide adaptive decision-making (Fellows, 2018; St-Amand *et al.,* 2018; Biderman *et al.,* 2020). From what to eat for dinner to which work project to prioritize, decision-making pervades our daily lives. A wealth of evidence suggests that choice options are evaluated in a relative rather than absolute manner, that is, we rate an option according to how it compares with other options (Seymour & McClure, 2008; Vlaev *et al.,* 2011; Rangel & Clithero, 2012). Critically, our previous experiences help us to correctly assess the value of each option and to select the best choice. However, how exactly value is assigned to a given option during such previous experience is the subject of intensive research.

A common approach to studying this question is associative learning. In the last decade, several studies have shown convincingly that humans apply relative evaluation of rewards and punishments during associative learning. This means that humans associate stimuli with the value of a reward or punishment relative to other rewards or punishments that were available in a particular training context (how much better or worse?) rather than an "absolute" value (how good or bad?) that would be independent from the training context. This process is known as relative value learning, adaptive value coding, value normalization or range adaptation (Palminteri *et al.,* 2015; Klein *et al.,* 2017; Palminteri & Lebreton, 2021; Juechems *et al.,* 2022; Bavard & Palminteri, 2023; Hayes & Wedell, 2023a; Hayes & Wedell, 2023b). Following the common definitions in the human literature (e.g., Klein *et al.,* 2017; Hayes & Wedell, 2023a), for the rest of this thesis, I will use the term "relative value learning" for a learning process that associates a stimulus with the outcome of a comparison between alternative values during training, whereas "absolute value learning" refers to a learning process that associates the stimulus with the value of the training situation paired with it irrespective of any alternatives during training. Thus, after "absolute value learning", a subject's behaviour towards a trained stimulus will depend only on the value of the situation that had been paired with this stimulus, whereas after "relative value learning", the subject's behaviour will depend on how much better or worse the situation paired with the stimulus was compared to the training alternatives.

The discovery that humans employ relative value learning is important for our understanding of economic, value-based decisions because relative value learning can lead to suboptimal choices. For example, an option that had been paired with an objectively low value, but in a context of even lower values, is preferred over another option that had been paired with an objectively higher value, but in a context of even higher values. In other words, humans tend to choose options that have been better than the alternatives in previous experiences, even

if this implies choosing the objectively worse of the options at hand (Klein *et al.,* 2017; Bavard *et al.,* 2018; Bavard *et al.,* 2021).

The last observation raises the question of the adaptive significance of relative value learning. Proposed advantages include a higher efficiency of saving relative value differences rather than absolute values (Louie & Glimcher, 2012; Bhui & Gershman, 2018) and an increased sensitivity to the most likely outcomes in a given environment (Burke *et al.,* 2016). Similar proposals have been made in the context of the optimal foraging and decision-making theory (Stephens & Krebs, 1986; Dukas, 1998). In stable environmental conditions in which the availability of different options is known to the foragers and does not change over time, learning absolute values of food sources is advantageous and foragers should accept food sources based on a fixed, absolute threshold. In contrast, in fluctuating environments, foragers profit from learning constantly updated values relative to their internal state and previously encountered food sources because it provides flexibility and improves cost efficiency and allows them to maximize food intake (Charnov, 1976; McNamara, 1982; McNamara & Houston, 1986; Real, 1991; Evans *et al.,* 2013; McNamara *et al.,* 2013; Fawcett *et al.,* 2014; Hemingway *et al.,* 2024).

Considering the adaptive advantages of relative value learning, it is an interesting question whether it is shared across the whole animal kingdom. Relative value learning has been found in mammals and birds, suggesting that the principles of value coding are shared across vertebrates (Flaherty *et al.,* 1994; Waite, 2001; Tobler *et al.,* 2005; Pompilio & Kacelnik, 2010; Freidin & Kacelnik, 2011; Webber *et al.,* 2015). The evidence is less clear, however, for invertebrates. Compared to vertebrates, invertebrates like insects have very differently structured brains that are much smaller and often regarded as too simple to allow for complex tasks. Although associative learning has been studied in insects like bees, crickets and flies for decades (Giurfa, 2015; Modi *et al.,* 2020; Mizunami, 2021), the question of whether insects evaluate rewards or punishments relatively has rarely been asked. Nevertheless, several insect species, including flies, bees and ants, have been demonstrated to be capable of discriminating values of different rewards or punishments and forming memories of different strengths accordingly (Gerber & Hendel, 2006; Yin *et al.*, 2009; Riveros & Gronenberg, 2012; Price *et al.,* 2016; Nityananda & Chittka, 2021; Villar *et al.*, 2022). A study in grasshoppers showed that insects can associate a stimulus with a reward value relative to their current internal state when being trained at different hunger states (Pompilio *et al.*, 2006). Honeybees have been found to anticipate increasing and decreasing rewards and thus to associate a stimulus with a reward value relative to previous instances of the same reward (Gil *et al*., 2007). Still, the

question remains whether insects can learn by comparing values of different rewards and punishments that are present during training, even if this leads to suboptimal decisions, like vertebrates do.

Specifically, none of the previous findings were able to differentiate between two different explanations of the animals' behaviour: (1) Insects form separate memories for each stimulus-reward (or punishment) pair, with the strength of the memory depending on the strength of the reward but independent of any other rewards during the training (this would correspond to absolute value learning as the term is used in human research). These memories then can be compared in the moment of decision-making to choose the best option. (2) Insects compare the different reward alternatives during the training and associate the outcome of this comparison with the stimuli (this would correspond to relative value learning as the term is used in human research). Thus, the conceptual difference between these hypotheses is whether the association formed during the training already reflects a comparison between the available options, or whether each option is "saved" independently and only compared during the test.

In the previous *Drosophila* studies (Gerber & Hendel, 2006; Yin *et al.*, 2009; Villar *et al.*, 2022), the memory test, or moment of decision-making, gave the animals the choice between the stimuli that had been trained before. Such a test cannot discriminate between the two hypotheses because a comparison during the training and a comparison and choice during the test both lead to the same outcome.

Thus, in this thesis, I aim to disentangle these two hypotheses and reveal whether insects can learn by comparing available options and associating the outcome of this comparison with a stimulus, like it had been demonstrated in vertebrates. This can help answer the question of whether indeed the same principles of value coding and learning are shared across all animals, even across the vertebrate-invertebrate divide.

In my thesis, I use the larval stage of the fruit fly *Drosophila melanogaster*. In the past decades, the *D. melanogaster* larva has established itself as an extremely useful model organism for neurobehavioural research that often pioneers and inspires research in animals with more complex brains (Sokolowski, 2001; Schroll *et al.,* 2006; Eichler *et al.,* 2017; Michels *et al.,* 2018; Winding *et al.,* 2023). It combines a simple brain with only about 10,000 neurons, almost unlimited possibilities for transgenic manipulations of individual cells, and a surprisingly complex behavioural repertoire given its simplicity. This includes appetitive and aversive classical as well as operant conditioning, long-term memories, decision-making and adaptive outcome expectations (Scherer *et al.,* 2003; Gerber & Hendel, 2006; Schleyer *et al.,*

2011; Ohyama *et al.,* 2015; Lihoreau *et al.,* 2016; Gepner *et al.,* 2018; Mancini *et al.,* 2019; Eschment *et al.,* 2020; Rahman *et al.,* 2020; Croteau-Chonka *et al.,* 2022; Sen *et al.,* 2024).

In this study, I designed a behavioural paradigm suitable to distinguish absolute and relative value learning in classical conditioning. Based on established odour-taste associative experiments (Scherer *et al.,* 2003; Saumweber *et al.,* 2011; Michels *et al.,* 2017; Schleyer *et al.,* 2018), I performed a series of associative learning experiments in which two situations of different value were presented alternately, each of them paired with a different olfactory stimulus. Subsequently the larvae were tested for their preference of only one of the trained stimuli. This is the critical difference from previous studies in insects (see above), as it allows us to assess whether the stimulus has been associated with the value it was paired with (absolute value learning) or with the outcome of the comparison between the two values (relative value learning). Using this behavioural strategy, I asked four specific questions:

Research question 1: Do larvae compare between different rewards during training and associate the outcome of this comparison with a stimulus? To address this question, I performed three experiments in which I trained the larvae by pairing each of two different odours with different concentrations of a sugar as rewards and tested their subsequent preferences for one of the trained odours under different testing conditions. By comparing quasibinomial GLMs with different combinations of the reward strengths predictive factors, I asked whether the animals' choice is explained best by the reward strength that had been paired with the tested odour, or the alternative reward strength, or the outcome of a comparison between both reward strengths.

Research question 2: Do larvae compare between different punishments during training and associate the outcome of this comparison with the stimulus? Towards answering this question, I performed an analogous experiment with different concentrations of a bitter chemical as punishment instead of sugar, followed by an analogous statistical analysis.

Research question 3: Does the capability of larvae to compare the values of the different training rewards depend on the presence of two different odour cues? I hypothesized that the presence of two different odours that can be associated with the values of each training situation facilitates a comparison between the situations and thus the association of each odour with the outcome of the comparison. To test this hypothesis, instead of using two different odours during the training, I used only one odour. To test whether the outcome of this experiment depends on the specific olfactory stimuli used, I performed three experiments, each employing another odour.

Research question 4: Does the capability of larvae to compare the values of the different training rewards depend on the sequence in which they are presented? One relatively simple way how the larvae could compare between the values during training is to monitor the relative change of reward strength over time and associate this change with the stimuli currently present. Thus, the comparison between different rewards would always occur between the current reward and the previous reward, and the outcome would be associated with the currently present stimulus. To test this hypothesis, I switched to a one-trial version of the learning experiment (Weiglein *et al.*, 2019) in which each odour-reward combination is presented only once and which allows for a precise control of the training sequence without potential complications of repeated training cycles. I trained and tested the animals in two conditions: either the preference towards the odour that had been presented second is tested after the training, or the preference towards the odour that had been presented first. The hypothesis above predicts that only the second odour can be associated with the outcome of the value comparison because only at that time point there is a previous reward experience that the animals can use for the comparison. Thus, the hypothesis predicts that when the first odour is tested, the behaviour reflects only the value paired with this odour but not the outcome of a comparison. I applied the same statistical analysis as described above to test this hypothesis.

Materials and Methods

2.1 Animals

Throughout the study, I used 4 days old, third instar, feeding-stage *Drosophila melanogaster* larvae of the Canton-S wild-type strain. This is the last developmental stage before entering the metamorphosis. I maintained them on standard fly food at 25°C with a 12:12 h light–dark cycle. The standard fly food recipe contains, per 1 L water, 100 g glucose, 50 g cornmeal, 31.65 g wheat germ, 40 g dry yeast, 8 g agar, 2 ml propionic acid (Kanto-kagaku, Tokyo, Japan) and 5 ml 10% butyl p-hydroxybenzoate (Fujifilm Wako Pure Chemical, Japan) in ethanol as described in detail previously (Sugime *et al.,* 2017). Following international and Japanese laws as well as the rules of Hokkaido University on animal welfare, experiments with insects do not require specific permission of ethics committees.

2.2 Reinforcements and odours

In nature, adult flies lay eggs on ripe fruits and vegetables, and larvae live in the ripe, fermenting fruits. Fructose is a natural sugar found in many ripening fruits, which are the primary food and oviposition substrates for *Drosophila* in the wild (McCoy, 1962; Jaenike, 1983). In the laboratory setup, instead of using natural sugar sources, the pure chemical form of fructose has widely been established as rewarding reinforcement (Scherer *et al.*, 2003; Michels *et al.*, 2017; Weber *et al.*, 2023). Fructose has been shown to be innately attractive and rewarding to larvae depending on its concentration in the substrate, based both on its peripherally sensed sweetness and its nutritional benefits (Schipanski *et al.*, 2008; Rohwedder *et al*., 2012; Mishra *et al.,* 2013a). On the other hand, while living in the natural environment, animals are often exposed to potentially harmful or toxic food that is sensed as bitter (French *et al.,* 2015). While quinine itself is not abundant in the typical fruit diet of *Drosophila*, it is traditionally used in laboratory experiments as a well-established bitter-tasting stimulus. In larvae (the feeding state of the fruit fly's life cycle), it has been shown to be innately aversive and punishing in associative conditioning experiments depending on its concentration (Gerber and Hendel, 2006; El-Keredy *et al.*, 2012; Apostolopoulou *et al.*, 2014). Thus, in my study, I used fructose (CAS: 127-02765, Fujifilm Wako Pure Chemical) and quinine (CAS: 177-00462, Fujifilm Wako Pure Chemical) as rewarding and punishing situations respectively.

My associative learning experiments (see section 2.5) require the use of several rewards and punishments that induce sufficiently discriminable behaviour in the memory test. I initially picked three concentrations of each fructose (20, 200 and 2000 mM) and quinine (50, 500 and 5000 μM) that should induce appetitive and aversive associative memories, respectively, that in turn should result in experimentally discernible behaviour based on previous studies

(Schipanski *et al.*, 2008; El-Keredy *et al.*, 2012). I used these concentrations in initial learning experiments to decide the final concentrations of my main experiments.

I used three different odours, *n*-amylacetate (AM; CAS: 628-63-7, Tokyo Chemical Industry Co., Ltd, Japan), 1-octanol (OCT; CAS: 111-87-5, Tokyo Chemical Industry) and Benzaldehyde (BA; CAS: 025-12206, Fujifilm Wako Pure Chemical) as different olfactory stimuli.

AM is an ester found in various fruits and fermenting substrates, such as bananas and other overripe fruits that *Drosophila* encounter in nature, BA is an aromatic aldehyde found naturally in many plants and fruits, whereas OCT, a volatile alcohol, is not a dominant fruit odour. Importantly, all three odours are well-established for being used in olfactory associative learning experiments, chosen not because they are the most ecologically important odours for flies, but because the animals can easily discriminate them and learn their association with rewards and punishments (Chen *et al.*, 2011; Saumweber *et al.*, 2011; Mishra *et al.*, 2013b).

Thus, in my study, I choose to use AM diluted 1:49 in paraffin oil (CAS: 128-04375, Fujifilm Wako Pure Chemical), OCT diluted 1:1 in paraffin oil, and undiluted BA as olfactory odour cues. These concentrations are based on previous studies (Saumweber *et al.*, 2011; Mishra *et al.*, 2013b; Michels *et al.*, 2017; Weber *et al.*, 2023).

2.3 Training and test Petri dishes

During training, I trained larvae with different rewarding, punishing and neutral situations. For different rewarding situations, I prepared training Petri dishes (9 cm inner diameter, order #3-1491-01, AS ONE, Japan) by adding 20, 100, 200, or 2000 mM of fructose to 2% agar (CAS: 016-11875, Fujifilm Wako Pure Chemical). For different punishing situations, I prepared training Petri dishes using 50, 100, 500, and 5000 µM quinine mixed in 2% agar. Pure agar without taste reinforcement was used as a neutral situation.

The memory test was performed on test Petri dishes. To make test Petri dishes, 5000 µM quinine was mixed in 2% agar. Previous studies showed that quinine memory is only expressed in the presence of quinine (Gerber & Hendel, 2006; El-Keredy *et al.*, 2012; Schleyer *et al.*, 2015), while the expression of fructose memories is independent of the absence or presence of quinine (Schleyer *et al.,* 2011). As in this study I used fructose and quinine in different experiments, to maintain comparability across experiments, all experiments in this study used quinine test Petri dishes unless explicitly mentioned otherwise.

2.4 Taste preference experiment

In the learning experiments in this study, the larvae are often exposed to a highly concentrated tastant (fructose or quinine) followed by a much lower concentration of the same taste. It is conceivable that after being exposed to a high concentration, the animals' sensing and/or motivation towards the lower concentration is impaired. In order to test whether the detection of the low concentration is still intact, I placed approximately 20 larvae on a Petri dish with either pure agar or agar containing the highly concentrated tastants (2000 mM fructose or 5000 µM quinine) for 2.5 minutes. Afterwards, the larvae were transferred to a split Petri dish with pure agar on one side and the low concentration of the same tastants (100 mM fructose or 100 µM quinine) on the other side. After 3 minutes, the number of the animals on either side was counted and the taste proportion was calculated:

Fru Proportion = n_Fru / (n_Fru + n_Pure)

Qui Proportion = n_Qui / (n_Qui + n_Pure)

In each case, I checked whether the proportion of larvae choosing the taste-containing half was different from the chance level, that is, from 0.5. If that were the case, I concluded that the larvae were still able to detect the lower concentration and still motivated to choose, even after being exposed to the higher concentration.

2.5 Associative learning experiment

The associative learning experiments used in this study are based on a commonly used experimental paradigm (Scherer *et al.*, 2003; Michels *et al.*, 2017; Weber *et al.*, 2023), with some deviations. In the basic experiment, I trained larvae with two odours, odour A and B, associated with substrates of different value. For example, one of them contained a particular fructose concentration as a reward, and the other one was pure agar as a neutral situation. After typically three training cycles, the animals' preference for one of the two odours is tested (see below). A typical procedure, using *n*-amylacetate (AM) as odour A and 1-octanol (OCT) as odour B, is displayed in Fig. 1a. Importantly, throughout this thesis, I refer to the odour that is tested after the training as odour A and the odour that is not tested as odour B.

At first, approximately 20 larvae were taken from the food vials, gently washed with tap water and placed at the centre of a training Petri dish (9 cm inner diameter, order #3-1491-01, AS ONE), filled with one substrate. Two custom-made teflon odour containers filled with the same odour had been placed on opposing sides of the Petri dish just before the start of the experiment. The larvae were allowed to move freely over the dish for 2.5 minutes. After that, they were gently transferred to another training dish filled with another substrate with the

respective other odour placed and stayed there for another 2.5 min. I repeated this cycle 3 times. For example, the larvae were placed in a training dish featuring two odour containers filled with AM and a fructose reward. Subsequently, the larvae were transferred to another training dish featuring OCT and pure agar. In half of the repetitions of the experiment, the sequence was reversed such that the training started with OCT followed by AM and fructose. After three training cycles, I immediately tested the proportion of larvae choosing odour A (for example, AM) by placing them at the centre of the test Petri dish which featured on one side an odour container filled with odour A and on the opposite side an empty container (EM). After 3 min, the number of larvae on the odour A side (n_A) and on the EM side (n_EM) was counted. For visualisations, I calculated the proportion of larvae choosing odour A using the following formula. Larvae on the lid were excluded from the analysis.

Proportion = n_A / (n_A + n_EM)

Thus, the proportion is limited between 0 and +1, with scores greater than 0.5 indicating approach to odour A.

I used variations of this basic experiment first to find suitable fructose and quinine concentrations and then to answer my four main research questions. In the following, I describe the details of the experiments.

2.6 Finding suitable reward and punishment strengths

At first, I used this experiment with three different concentrations of fructose and quinine as rewarding and punishing substrates, respectively (see section 2.3), to find suitable conditions for my follow-up experiments (see below). Specifically, I required several concentrations of fructose and quinine that were potent rewards and punishments, respectively, and sufficiently different in their value for the larvae. My only insight into the perceived values of each substrate for the animals was their behaviour. Therefore, the larvae were trained with AM as odour A and OCT as odour B and with one of the three fructose or one of the three quinine concentrations along with pure agar as a neutral situation. Importantly, always two cohorts of larvae were trained in a reciprocal fashion, that is, with opposite odour-reinforcement contingencies. For example, one cohort received training with AM paired with fructose of a given concentration and OCT with pure agar (AM+/OCT), and the other one, trained at the same time, received training with OCT paired with fructose instead (AM/OCT+). As both cohorts have been trained identically apart from which odour had been paired with the reward, the difference in proportion of larvae choosing AM after each training can be used as a proxy for the rewarding strength of the trained fructose concentration. This follows the established

logic of a “Learning index” or “Performance index” as it has been established in the field for decades (Tully & Quinn, 1985; Scherer *et al.,* 2003). Specifically, I calculated a Δ Proportion as follows:

$$\Delta \text{ Proportion} = \{\text{Proportion (AM)}_{AM+/OCT} - \text{Proportion (AM)}_{AM/OCT+}\}/ 2$$

The division by 2 ensured that the Δ Proportion was bound between 0 and 1, with values above and below 0.5 indicating the behavioural expression of appetitive and aversive memories, respectively. I used this Δ Proportion as a proxy for the rewarding or punishing strength of a given fructose or quinine concentration, respectively, and thus as a proxy for the value of each concentration as perceived by the larvae. Based on the results of these experiments, I selected suitable concentrations of fructose and quinine that should support sufficiently strong and sufficiently different appetitive and aversive memories, respectively.

2.7 Main research questions: General statistical analysis

For the statistical analysis, I applied General Linear Models (GLM) using the lme4 package and the glm() function in R (R Core Team, 2013). The raw data in my experiments are the number of animals on either side of the test dish after three minutes. As this is a binary choice for each of the animals on the Petri dish, these numbers follow a binomial distribution. Therefore, at first, I applied binomial GLMs with a logit link function. However, as I observed over- or underdispersion in most of the cases (that is, the observed variance in the data did not match the assumed variance used in a binomial regression), I decided to use quasibinomial GLMs instead, which allow for a correction of the dispersion. I report the dispersion ratio for each model along with the results.

As factors of my GLMs, I used the reward (or punishment) strengths during the experiment. As previous studies had shown that substantial differences in the animals’ behaviour required differences in the orders of magnitude of the used rewards or punishments (Gerber & Hendel, 2006; Niewalda *et al.,* 2008; Schipanski *et al.,* 2008; El-Keredy *et al.,* 2012), I used the $\log_{10}$ of the fructose concentration as a proxy for the reward strength, henceforth called reward level (RL). When there was no reward (pure agar), the reward level was set to 0. For the quinine experiment, the punishment levels (PL) were defined analogously.

For most of my main research questions (see below), I performed several experiments that varied in specific experimental parameters. In these cases, those experimental parameters were used as additional factors for the GLM. In addition, the interaction terms between the parameters and the reward (or punishment) levels were included. If an experimental condition had a significant interaction with the reward levels, that is, if this condition changed the way

how the reward levels determined the animals' choice, I performed the following analyses separately for the different conditions. Otherwise, I continued with the combined data. In any case, the following analysis always applied four models with different combinations of reward (or punishment) levels as factors. I will explain the logic and details of each model in the next section. The models were compared by the Quasi-Akaike Information Criterion (QAIC), which extends AIC to quasi-likelihood models by accounting for over- or underdispersion (Akaike, 1974; Anderson *et al.,* 1994). Models with lower QAIC values were considered to provide a better balance between model fit and complexity. In addition, to evaluate the contribution of each factor, Wald tests based on quasi-likelihood were used to assess whether estimated regression coefficients differed significantly from zero in the models. Standard errors were adjusted by the estimated dispersion parameter to account for over- or underdispersion. A significant Wald test therefore indicates rejection of the null hypothesis that the factor has no effect on the response variable (Hauck & Donner, 1977). I reported the QAICs as well as the outcome of the Wald tests for each model, both in summary tables and in the results figures.

In the figures, data showing the proportion of larvae choosing odour A during the test are displayed as individual raw data points. In overview figures, the mean as the central line and the 95% confidence intervals as error bars are shown instead of the raw data points to allow for simple comparisons between experimental conditions.

2.8 Research question 1: Experimental details

First, I asked whether larvae compare between different rewards during training and associate the outcome of this comparison with an odour. To answer this question, I trained the larvae with two odours, AM and OCT paired with different concentrations of fructose. Specifically, I used 100 mM (+) and 2000 mM (++) mixed in 2% agar as weakly and strongly rewarding substrates, respectively, as well as pure, tasteless agar as a neutral situation (∅). Every cohort of larvae was trained with each odour being paired with one of the three different substrates. A total of nine combinations were tested in this experiment, summarized in Table 1. Each cohort of larvae, after three repetitions of the respective training cycle, was tested for their preference for odour A as described in section 2.5.

In total, I performed this experiment three times, always following the same experimental design with 9 combinations of neutral substrate, weak and strong reward, and always testing for the preference of one odour as described. In the first experiment, AM was used as odour A and OCT as odour B. The test was performed on a Petri dish with 5000 µM quinine as described

in section 2.3 and the preference for AM was tested. The second experiment was identical except that the test Petri dish was pure, neutral agar. Thus, the comparison between these experiments asked whether the test substrate mattered for the animals' behaviour. In the third experiment, the test Petri dish contained quinine as in experiment 1, but the odour identities were swapped: odour A was OCT and odour B was AM, and thus, the preference for OCT was subsequently tested. Thus, the comparison between experiments 1 and 3 asked whether the odour identity mattered for the animals' behaviour.

For the statistical analysis, data from all three experimental variants were pooled and analyzed jointly. I fitted a generalized linear model to the number of larvae choosing odour A versus the alternative (EM), using a quasibinomial distribution with a logit link function to account for overdispersion in binomial choice data.

The model included as fixed effects the reward level paired with odour A (RL_A) and odour B (RL_B), the type of test Petri dish (pure or quinine), and the identity of odour A (AM or OCT). To test whether reward effects depended on experimental context, I additionally included interaction terms between each reward level (RL_A and RL_B) and the test dish, as well as between each reward level and the identity of odour A. For clarity, I provide a simple overview of the main parameters of the model:

- Response variable: numbers of larvae choosing odour A versus EM
- Quasibinomial distribution (logit link function)
- Fixed effects: RL_A, RL_B, odour A identity, test dish
- Interaction terms: RL_A:test dish, RL_B:test dish, RL_A:odour A identity, RL_B:odour A identity

The main purpose of this model was to reveal whether the two experimental parameters of the test dish and the odour identities make a difference for the behaviour. This would be indicated by significant interaction terms between the respective parameter and the reward levels. If these interaction terms remained non-significant, I performed further analysis for the combined data, otherwise, I split the data. In either case, I next applied four models as follows.

Model 1 was identical to the model above except without considering the experimental parameters and their interaction as factors:

- Response variable: numbers of larvae choosing odour A versus EM
- Quasibinomial distribution (logit link function)
- Fixed effects: RL_A, RL_B

It can thus be used to test whether either reward level has a significant effect on the animals' choice behaviour.

Next, to test the effects of the reward strengths paired with each odour separately, I applied Model 2 and Model 3, with only the reward level paired with odour A (RL_A) or odour B (RL_B) as fixed effects:

- Response variable: numbers of larvae choosing odour A versus EM
- Quasibinomial distribution (logit link function)
- Fixed effects: RL_A (Model 2) or RL_B (Model 3)

Importantly, if the larvae would learn by associating a reward value with an odour irrespective of the alternative training situation (equivalent to absolute value learning in human experiments), in Model 1, only the RL_A should have a significant effect, as it represents the value of the reward paired with the odour that subsequently was tested. In addition, I would expect that Model 1 and Model 2 would have the same (or a very similar) QAIC, as the RL_A would fully explain the animals' behaviour and RL_B should be irrelevant. In contrast, if the larvae were to learn by comparing the two reward values during training, both reward levels should have a significant effect in Model 1, and the QAIC of both Model 2 and 3 should be significantly higher than that of Model 1, as each of them would explain the animals' behaviour only partially.

In the event that the animals take both reward values into account, I asked whether a simple subtraction between the two reward levels would describe the animals' behaviour best, or whether a potentially more complex integration would occur. To test this, I applied Model 4, using the Δ Reward level (RL_A – RL_B) as a factor:

- Response variable: numbers of larvae choosing odour A versus EM
- Quasibinomial distribution (logit link function)
- Fixed effects: Δ Reward Level = RL_A - RL_B

If this simple subtraction of the reward levels fully captures the larvae's odour choice, I predict that Model 4's QAIC would be equal or very similar to the one of Model 1. Table 2 summarises the predicted outcomes of all models and their respective interpretation.

2.9 Research question 2: Experimental details

Next, I asked whether the larvae learned by associating the outcome of a comparison between different punishments with an odour. In this case, only one experiment was performed, in the same way as described above, except using two concentrations of quinine, 100 µM (-) and

5000 µM (--) as weakly and strongly punishing situations (Table 3). AM was used as odour A and OCT as odour B. During the test on a quinine-containing Petri dish, the proportion of larvae choosing AM was calculated as mentioned earlier.

For the statistical analysis, I applied the analogous four models mentioned in section 2.8. As quinine was used as punishment, I used the punishment level (PL) as a proxy of punishment strengths instead of reward strengths, calculated in an analogous way by the $\log_{10}$ of the quinine concentration and defining the pure, neutral agar as 0. The interpretation of the models was analogous to the reward experiments described above.

2.10 Research question 3: Experimental details

Next, I asked whether learning by comparing reward values would require the presence of two different odours during the training. I performed three experiments similar to the one in section 2.8, but using only one odour during training. Such one-odour training is well established (Saumweber *et al.,* 2011; Schleyer *et al.,* 2011; Widmann *et al.,* 2016; Eichler *et al.,* 2017; Paisios *et al.,* 2017; Saumweber *et al.,* 2018; Eschbach *et al.,* 2020; Schleyer *et al.,* 2020; Eschbach *et al.,* 2021; Sen *et al.,* 2024; Toshima *et al.,* 2025). I did three separate experiments using three different odours, AM, OCT and BA. The training and testing procedures were the same except for replacing odour B with empty containers (EM). For example, instead of training the animals A+/B++, they were trained A+/EM++. The test was performed with the same odour as during the training (odour A), and the proportion of larvae choosing that odour was calculated.

For the statistical analysis, data from all three experimental variants were pooled to test whether the identity of odour A (AM, OCT, or BA) influenced animals' choice behaviour. I fitted a GLM with a quasibinomial distribution and a logit link function to the proportion of individuals choosing odour A.

The model included as fixed effects the reward level paired with odour A (RL_A), the reward level paired with the empty odour container (RL_EM) and the identity of odour A. To assess whether the effects of reward level depended on odour identity, I additionally included interaction terms between odour identity and each reward level.

- Response variable: numbers of larvae choosing odour A versus EM
- Quasibinomial distribution (logit link function)
- Fixed effects: RL_A, RL_EM, odour A identity
- Interaction terms: RL_A:odour A identity, RL_EM:odour A identity

If the interaction terms between the odour identity and the reward levels remained non-significant, I performed further analysis for the combined data, otherwise, I split the data. In either case, I next applied and interpreted the analogous four models as described above.

2.11 Research question 4: Experimental details

Finally, I asked whether the sequence of the odours being presented during the training mattered for the animals' ability to compare between the different reward situations. To answer this question, I performed the similar experiment with the experimental groups mentioned in Table 1, except that during training, each substrate-odour pairing was only presented a single time before testing for the proportion of larvae choosing odour A in an immediate test (Weiglein *et al.*, 2019). This allowed me to compare two possible sequences: either odour B was presented first and odour A (the odour that subsequently was tested) was presented second (e.g., B++ → A+) or odour A was presented first and odour B second (A+ → B++). In the performed experiments, AM was always odour A and OCT was odour B.

For the statistical analysis, data from both experimental variants were pooled to test whether the training sequence influenced animals' choice behaviour. I fitted a GLM with a quasibinomial distribution and a logit link function to the proportion of individuals choosing odour A.

The model included as fixed effects the reward level paired with odour A, the reward level paired with odour B, and the training sequence. To examine whether the effects of reward level depended on the training sequence, I additionally included interaction terms between training sequence and each reward level.

- Response variable: numbers of larvae choosing odour A versus EM
- Quasibinomial distribution (logit link function)
- Fixed effects: RL_A, RL_B, training sequence
- Interaction terms: RL_A:training sequence, RL_B:training sequence

If there would be no significant interaction terms between the training sequence and the reward levels, I would conclude that the training sequence would not affect the animals' capability to learn by comparing the two training situations. If there were significant interaction terms, I would split the data and analyse both sequences separately. Importantly, I predicted that in the first sequence (odour A was presented second), the animals should in principle behave as during the three-trials experiment (section 2.8). That is, they should be able to compare the value of the current reward with the value of the previous reward and associate the outcome of

this comparison with the currently present odour A. In contrast, I predicted that in the other sequence (odour A was presented first), this should not be possible, because at the moment when odour A was presented, there was no previous reward value available for a comparison. Thus, I predicted that in that sequence, odour A would be associated with the currently present reward value without considering the alternative.

For the eventuality that my prediction was wrong and my results would suggest that the larvae can associate the outcome of the comparison between reward values with odour A even if it was presented first, I performed a control experiment. When, for example, odour A had been paired with a weak reward, followed by a strong reward (A+ → B++), it is possible that the simple exposure to the strong reward would increase the animals' subsequent preference for any odour by a non-associative process. In the previous experiment, such a non-associative process could be mistaken for an associative learning based on the comparison between the reward values. Therefore, I performed a variation of the experiment with the same nine combinations of zero, weak and strong reward, but without any odours during training. Thus, the animals were exposed to different fructose concentrations (e.g., + → ++) before being tested for their proportion choosing odour A just as described above.

Because no odours were present during training in this experiment, the predictors included in the GLMs differed from those used in the odour-conditioning experiments. I therefore fitted two alternative GLMs with a quasibinomial distribution and a logit link function to the proportion of individuals choosing odour A.

In the sequence model, odour choice was predicted by the difference between the reward levels experienced in the first and second training phases (Δ Reward level = RL_first − RL_second). This factor corresponds to the Δ Reward level used in the previous experiment in which odour A was presented first.

- Response variable: numbers of larvae choosing odour A versus EM
- Quasibinomial distribution (logit link function)
- Fixed effects: Δ Reward level = RL_first − RL_second

In the sum model, odour choice was predicted by the sum of the reward levels experienced across both training phases (RL_first + RL_second).

- Response variable: numbers of larvae choosing odour A versus EM
- Quasibinomial distribution (logit link function)
- Fixed effects: RL_first + RL_second

This model tests the possibility that the total amount of fructose exposure changed subsequent odour preference. The sum of the reward levels of both training situations was used as a proxy for the total amount of fructose exposure.

Results

3.1 Choosing reward and punishment concentrations

In order to identify suitable rewards and punishments for my experiments, I first performed associative learning experiments during which I trained larvae with two odours, AM and OCT, and three different concentrations of fructose (20, 200 and 2000 mM) or quinine (50, 500 and 5000 µM) as rewarding and punishing substrates, respectively (see section 2.6).

The proportion of larvae choosing AM increased with increasing fructose concentrations when AM had been paired with the fructose rewards (Fig. 2a, left), but decreased when OCT had been paired with fructose rewards (Fig. 2a, right). The difference in the proportion between reciprocally trained groups which is indicative of the rewarding strength of each fructose concentration showed that all three concentrations supported appetitive associative memories, confirming previous findings (Fig. 2b) (Schipanski *et al.*, 2008). However, the level of appetitive memory induced by the lowest fructose concentration was deemed too low, and the contrast between the middle and highest concentrations was deemed too small to be suitable for the following experiments. In order to maximize the contrasts between the different training situations for the following experiments, I decided to use only two fructose concentrations. As the stronger of the rewards, I picked 2000 mM fructose, and as the weaker one, I aimed for a concentration that supported a memory level between those of the lowest and middle concentrations and decided to use 100 mM fructose. I ensured that the larvae could detect and process the lower fructose concentration immediately after being exposed to the higher concentration by performing a taste preference experiment, as seen by a positive preference for the low fructose concentration even after high-fructose exposure (Fig. 2c).

The experiment with different concentrations of quinine as punishment showed that the proportion of larvae choosing AM decreased with increasing quinine concentrations when AM had been paired with quinine (Fig. 2d, left), but increased when OCT had been paired with quinine (Fig. 2d, right). The difference in the proportion between reciprocally trained groups which is indicative of the punishing strength of each quinine concentration showed that all three concentrations supported aversive associative memories, confirming previous findings (Fig. 2e) (El-Keredy *et al.*, 2012). Similar to the fructose case, in order to maximize the contrasts between the different training situations for the main experiment, I decided to use only two quinine concentrations. As the stronger of the punishments, I picked 5000 µM quinine, and as the weaker one, I decided to use 100 µM quinine. Again, I ensured that the larvae could detect and process the lower quinine concentration immediately after being exposed to the higher concentration by performing a taste preference experiment (Fig. 2f).

3.2 Research question 1: Do larvae learn by comparing different reward options?

To investigate whether larvae can learn by comparing different rewards during the training, I performed associative learning experiments with two odours, AM and OCT, and nine different combinations of the weak reward (100 mM fructose), the strong reward (2000 mM fructose) and a neutral situation (pure agar) (Table 1). I performed the same experiment three times, using two different substrates during the memory test (pure agar or 5000 µM quinine) and two different odours as odour A (AM or OCT) (Fig. 3a).

For the first three groups, odour A was paired with the same situation (pure agar) while odour B was paired with different reward concentrations (Fig. 3a, left three groups). Subsequently, the larvae were tested for their preference for odour A. If the larvae would consider only the situation paired with odour A, then for all three groups the proportion of larvae choosing odour A would be the same. However, for all three experiments I found that the proportion was decreasing with increasing reward strength that had been paired with odour B during the training, even though odour B was not being presented in the test.

Also, when odour A was paired with either the weak reward (Fig. 3a, middle three groups) or the strong reward (Fig. 3a, right three groups) but odour B featured different reward strengths, the proportion declined along with the reward strength that had been paired with odour B during training. In each case, the result indicated that the animals' behaviour was determined by both the reward strength paired with odour A and odour B, even though odour B was not presented during the memory test. This was confirmed by the GLM analysis, which indicated a significant effect of the reward levels paired with each odour (Table 4).

Importantly, the substrate during the memory test had no significant effect on how the reward levels determined the animals' preference for odour A, as indicated by non-significant interactions between the test dish and the two reward levels (Table 4). Thus, as predicted based on previous work (Schleyer *et al.*, 2011), the presence or absence of quinine had no effect on the behaviour after training. In contrast, there were significant interactions between the identity of odour A and the reward levels (Table 4). Thus, for the further statistical analysis, I separately analysed the data for each tested odour, either AM (Fig. 3b-d) or OCT (Fig. 3e-g).

For the case of AM as odour A, I first confirmed that both the reward levels paired with AM (RL_A) and OCT (RL_B) were important to determine the animals' odour choice. The proportion of larvae choosing AM increased with increasing reward levels paired with AM (Fig. 3b) and decreased with increasing reward levels paired with OCT (Fig. 3c). This was confirmed by significant effects of both factors in the respective model (Model 1 in Table 5). I further scrutinized this result by running models with only one of the two reward levels as

factors (Model 2 and 3 in Table 5). Both models obtained QAICs that were much higher than that of Model 1, indicating that neither model sufficiently explained the results and confirmed that both reward levels were important for determining the animals' choice.

Finally, I tested specifically whether the difference in reward level (Δ Reward level, RL_A – RL_B) was a good descriptor of my results. The animals' proportion choosing AM was negative when the Δ Reward level was negative, that is, when the reward level paired with AM was lower than that paired with OCT, and increased along with the Δ Reward level (Fig. 3d). The QAIC of the corresponding Model 4 was slightly lower than that of Model 1, indicating that the difference in the reward levels explained my results as good, or even slightly better, than the two reward levels as independent factors (Table 5).

For OCT as the odour A, I obtained comparable results (Fig. 3e-g, Table 6), indicating that also in this case, both reward levels were important to determine the animals' choice. However, Model 4's QAIC, although much lower than those of Model 2 and 3, was still higher than that of Model 1. This suggests that, although the larvae considered both reward levels during training for their choice, the simple subtraction between the reward levels was not the best descriptor of the animals' behaviour. It is thus possible that the precise way in which the reward levels affect the behaviour may depend on the stimuli present during the test.

In summary, I concluded that *D. melanogaster* larvae can learn by comparing the values of the available rewards during training and that the outcome of this comparison determines their behaviour towards the stimuli associated with the rewards.

3.3 Research question 2: Do larvae learn by comparing different punishment options?

To investigate whether the larvae learn by comparing different punishment strengths during training, I performed the analogous experiment as before, with nine experimental groups being trained with different concentrations of quinine along with pure agar (Table 3). AM was used as odour A and OCT as odour B.

When AM was paired with the same situation and OCT was paired with increasing punishing situations, the proportion of larvae choosing AM increased, indicating that the punishment levels in the absence of AM were important for the larvae's decision in the subsequent memory test (Fig. 4a).

Indeed, the proportion of animals choosing AM decreased with increasing punishment levels paired with AM (PL_A) (Fig. 4b) and increased with increasing punishment levels paired with OCT (PL_B) (Fig. 4c). Statistically, this was confirmed by significant effects of both punishment levels in the respective model (Model 1 in Table 7) and by the fact that the models featuring only one of the punishment levels (Model 2 and 3 in Table 7) had much higher QAICs than the model with both punishment levels.

The proportion of larvae choosing AM was positive when the Δ Punishment level was negative, that is, when AM had been paired with the lower of the two Punishment levels during training and decreased with an increasing Δ Punishment level (Fig. 4d). I observed that the QAIC of Model 4 was much lower than those of Model 2 and 3 but still higher than that of Model 1, indicating that the Δ Punishment level does not completely explain my data.

Thus, I concluded that larvae can learn by comparing the values of the available punishments during training and that the outcome of this comparison determines their behaviour towards the stimuli associated with the punishments. A simple subtraction of the punishment values, however, cannot fully explain my results.

3.4 Research question 3: Are two different odours required for comparing between rewards?

To investigate whether the presence of two odours during the training would be required for comparing the different reward strengths during training, I performed a set of experiments in which I used the same nine combinations of rewards as before (Table 1) but trained the larvae by pairing the various substrates with only one odour as odour A, either AM, OCT, or BA (Fig. 5a).

When OCT or BA had been paired with the same situation (Fig. 5a, no reward for the leftmost three groups, low reward for the middle three groups and high reward for the rightmost three groups) while the alternative situation featured different reward strengths, the proportion of larvae choosing OCT or BA decreased along with the reward strengths unpaired with these odours, suggesting that the larvae indeed compared the different values during the training. However, in the case of AM, no or only a weak decrease of the animals' preference was observed. Across the three experiments, the statistical analysis indicated that both reward levels were significant factors for the animals' preference and that the training odours had significant interactions with the reward levels (Table 8). This confirmed the visual impression that the training odours affected the way the reward levels determined the animals' choice. Therefore, I analysed the results for each odour separately.

In the case of AM, the proportion of larvae choosing the odour was increased with increased reward levels paired with AM (Fig. 5b) and decreased with increased reward levels unpaired with AM (Fig. 5c). This was confirmed by significant effects of each reward level in the respective model (Model 1 in Table 9) and by the fact that the QAICs of the models featuring only one reward level (Model 2 and 3 in Table 9) were much higher than that of Model 1. Finally, the proportion of larvae choosing AM increased with an increasing Δ Reward level (Fig. 5d). However, the QAIC of the respective model (Model 4 in Table 9) was much higher than that of Model 1. Moreover, different from all other experiments so far, Model 2, only featuring the reward level paired with AM, had a lower QAIC than Model 4, featuring the Δ Reward level as a factor. This may suggest that, although the larvae considered both reward levels during training, the reward level paired with AM played a dominant role in determining the animals' odour choice.

For OCT (Fig. 5e-g) and BA (Fig. 5h-j) as training odours, the results were overall similar to each other and similar to the previous experiments with two odours. In both cases, both reward levels were required to explain the results (compare Models 1, 2 and 3 in Tables 10 and

11), and the Δ Reward level explained the results best, with the lowest QAICs of all models (Model 4 in Tables 10 and 11).

In summary, I concluded that *D. melanogaster* larvae can learn by comparing the values of the available rewards during training even when only one odour was present during training. However, the extent to which this comparison determines the animals' subsequent behaviour was different for different training odours.

3.5 Research question 4: Do larvae learn by comparing rewards independent of the training sequence?

To investigate whether the sequence of the odours being presented during training affected the animals' ability to learn by comparing reward strengths, I switched to a one-trial version of the associative learning experiment and performed two variations of this experiment, with either OCT or AM being presented first. Subsequently, I tested for the proportion of larvae choosing AM.

For either sequence, when the reward strength paired with AM was kept constant but the reward strength paired with OCT was different, the proportion of larvae choosing AM decreased with increasing reward strengths paired with OCT (Fig 6a). Overall, the results were similar but not identical between both sequences, with significant effects of both reward levels and significant interactions between the training sequence and the reward levels in the GLM (Table 12). Thus, the training sequence mattered for the subsequent animals' behaviour. I therefore analysed both sequences separately (Fig. 6b-g).

For both sequences, the proportion of larvae choosing AM was increased with increased reward levels paired with AM (Fig. 6b for OCT as first odour and Fig. 6e for AM as first odour) and decreased with increased reward levels paired with OCT (Fig. 6c for OCT as first odour and Fig. 6f for AM as first odour). This was confirmed by significant effects of each reward level in the respective model (Model 1 in Tables 13 and 14) and by the fact that the QAICs of the models featuring only one reward level (Model 2 and 3 in Tables 13 and 14) were much higher than that of the model with both reward levels. For both sequences, the proportion of larvae choosing AM increased with an increasing Δ Reward level (Fig. 6d for OCT as the first odour and Fig. 6g for AM as the first odour). However, the QAIC of the respective model (Model 4 in Tables 13 and 14) was slightly lower than that of Model 1 when OCT had been the first odour, but higher than that of Model 1 when AM had been the first odour.

This means that for both sequences, larvae learned by comparing the two reward levels, contrary to my initial hypothesis. However, when AM had been the first odour, a simple

subtraction of the reward levels did not sufficiently explain the animals' behaviour. This may suggest a different mode of integrating the reward values in this case.

As a control to test whether any potentially non-associative mechanism (sugar exposure) had any effect, I repeated the last experiment, but without presenting any odors during training, that is, I exposed the animals to different combinations of fructose rewards and subsequently calculated the proportion of larvae choosing AM in the test (Fig. 7).

The proportion of larvae choosing AM increased with increasing Δ Reward level (Fig. 7a, Table 15). This indicates that the proportion after such sugar exposure indeed had an effect, suggesting non-associative processes. But this effect was much weaker than in the previous experiment with odors during training (Fig. 6g), indicating that such a non-associative process alone cannot explain my results. The proportion slightly but significantly decreased when the sum of the reward level (∑ Reward level) increased (Fig. 7b, Table 15), indicating that also the total amount of reward exposure affected the subsequent odour preference.

Discussion

4.1 General discussion

In this study, I asked whether *D. melanogaster* larvae learn associatively by comparing different situations during training and associating the outcome of this comparison with a stimulus. To answer this question, I designed an experiment in which I paired each of two odours with rewards or punishments of different strengths, followed by testing the proportion of larvae choosing one odour alone to assess what the animals had associated with it. In a first step, I differentiated between two potential hypotheses: that the animals' behaviour would be determined solely by the value paired with the tested odour, or by both the value paired with the tested odour and the alternative value (see Table 2). As a second step, I tried to determine in which specific way the two values would be integrated. Specifically, I tested whether a simple subtraction of the values of both training situations would capture the animals' behaviour best.

For my first experiments using two different odours paired with rewards (research question 1, Fig. 3) or punishments (research question 2, Fig. 4), my results suggest that the values of both training situations indeed together determined the animals' behaviour, and that the animals' behaviour was best explained by the hypothesis that the larvae compared the two values and associated the outcome with one odour. Regarding the question of how specifically the two values were integrated, I found mixed results. In some experiments, the simple subtraction indeed turned out to be the best-fitting model (e.g., Fig. 3b-d). In other experiments, while the subtraction explained the data better than either of the two training values alone, it was still a less good fit than a model with both values as independent factors (Fig. 3e-g, Fig. 4). Overall, these results suggest that the larvae indeed did learn by comparing the values during the training, but the way how the outcome of this comparison was associated with the odour and determined the animals' behaviour was probably more complicated than a simple subtraction of both values. Many potential factors could plausibly affect the integration of the two values, like different pre-training preferences towards some of the used stimuli or the specific sequence in which the stimuli were presented. Some of these potential factors I addressed with the following experiments.

First, I asked whether the presence of two different odour cues is necessary for larvae to compare the values of different reward situations during training (research question 3). Initially, I hypothesized that the presence of two different odours that can be associated with the values of each training situation facilitates a comparison between the situations and thus the learning about the outcome of the comparison. To test this hypothesis, instead of using two different odours during the training, I used only one odour, AM (Fig. 5b-d), OCT (Fig. 5e-g) or BA (Fig.

5h-j) in separate experiments. For OCT and BA, I observed that the presence of only one odour is enough for larvae to compare the different training reward situations and that the animals' behaviour is best explained by the animals associating the result of a subtraction of the two reward values with the odour (Fig. 5e-g for OCT and Fig. 5h-j for BA).

However, for AM, although both the reward values paired and unpaired with AM, as well as their subtraction, were significant factors, each of these models fitted the data much worse than the model with both reward levels as independent factors (Fig. 5b-d). Moreover, this was the only experiment in my study for which the reward value paired with AM provided a relatively better fit to the results than the difference between the two reward values (Fig. 5b and d). It seemed that during decision-making, the reward value paired with AM played a dominating role, even if the other reward value was still relevant. Taken together, these results indicate that for associations with AM, the presence of an alternative odour during the training matters: if such an odour (in my case, OCT) was present, AM was associated with the outcome of the comparison between both reward values (Fig. 3b-d); without the second odour, the value paired with AM seemed to gain dominance in the associative process (Fig. 5b-d). For associations with OCT, in contrast, the presence or absence of an alternative odour during training seemed to play no major role: in either case, OCT was associated with the outcome of the comparison between both reward values (Fig. 3e-g, 5e-g).

The actual reason for this discrepancy in odour-reward associations for different odours is yet to be understood. To address this question in future experiments, many more odours should be tested in a similar manner as I did in this study to systematically investigate potential underlying reasons for the observed differences, such as the chemical structure of the odours, their ecological meaning for the animals, or different innate responses to the odours. Furthermore, I expect that following up on investigating the neuronal mechanism of this learning process (see below) will help to explain my behavioural results.

Second, I asked whether the sequence in which the cues are presented during training is important for the larvae's ability to compare the different training reward situations (research question 4). To answer this, I switched to a one-trial version of the learning experiment that allowed me better control over the training sequence without potential complications of repeated training cycles. I trained larvae with two different reward values paired with two odours in two different sequences: either OCT was presented first and AM second, or the other way around. In either case, I subsequently observed the proportion of larvae choosing AM and thus assessed what the animals had associated with AM (Fig. 6). My initial hypothesis was that the comparison between different rewards always occurs between the current reward and the

previous reward, and the outcome is associated with the currently present cues. This hypothesis predicted that the larvae should only be able to associate AM with the outcome of the value comparison if AM was the second odour. If AM was the first odour, there was no previous reward available for the comparison. I found that the training sequence had a significant effect, and the reward values seemed to affect the animals' odour choice to a smaller extent when AM was the second odour (Fig. 6a). However, in either sequence, both reward levels were important to explain the animals' behaviour, which did not match my initial hypothesis (Fig. 6b-g). I concluded that the larvae associated AM with the outcome of the comparison between both values in either training sequence. This implies that an initial odour-reward association can be changed by later experienced rewards even if the odour is not present. My neuronal working model was specifically conceived to also capture this surprising result (see below).

4.2 Alternative explanations

Although my data overall fit to the hypothesis that larvae can learn by associating stimuli to the outcome of a comparison between training values, I was considering alternative explanations.

Previous exposure to sugar rewards may change the animals' motivation and evaluation of further sugar rewards. Indeed, I observed a decrease in the proportion of larvae choosing a low sugar concentration when they had experienced a higher concentration before (Fig. 2c). Also, I found that the proportion of larvae choosing an odour was reduced after sugar exposure (Fig. 7b). Thus, it seems plausible that non-associative processes like sugar exposure also influence the observed behaviour in my associative learning experiments.

Moreover, the learning processes in the two training phases may disrupt each other, a phenomenon called memory interference in psychology (Hardt *et al.,* 2013; Alvares & Do-Monte, 2021). Theoretically, the memory formed about sugar in the first training phase may inhibit learning about the odour-sugar association in the second phase (Jacob *et al.,* 2021). Likewise, learning about the sugar in the second phase may impair the subsequent retrieval of the memory from the first phase (Felsenberg *et al.,* 2017). Both processes could lead to differences between my training regimens.

However, neither a reduced motivation nor impaired learning or retrieval capabilities can explain an inversion of memory valence, that is, that the animals form an aversive memory about the odour with the weaker of two rewards, as I observed it repeatedly (Fig. 3a, 5a, 6a, sixth group from the left).

In contrast, this result is in line with one of the experimental hallmarks of relative value learning in humans: that it can lead to objectively suboptimal decisions. Humans tend to choose options that had been better than alternatives in previous experiences over options that predict a higher total value (Klein *et al.,* 2017; Bavard *et al.,* 2018; Bavard *et al.,* 2021). The larvae avoided an odour that had been paired with a potent reward if the alternative had been even better, although no better option was available in the moment of the choice test (Fig. 3a, 5a, 6a, sixth group from the left). This prevented the larvae from finding an otherwise positively rated sugar source. Thus, it seems that the way brains evaluate rewards or punishments during learning is shared across the animal kingdom, from insect larvae to humans. I therefore conclude that my results, showing that insect larvae learn by associating a stimulus to the outcome of a comparison between reward values, correspond to relative value learning as it was found in humans.

4.3 My results are consistent with previous studies

To my knowledge, this is the first clear demonstration that *D. melanogaster* larvae can learn by comparing the different reward or punishment values that were presented during training. Previous studies in both larvae and adult flies had used different punishment strengths during training, each paired with a given odour, and then had tested the animals' choice between these odours (Gerber & Hendel, 2006; Pompilio *et al.,* 2006; Villar *et al.*, 2022). These studies demonstrated that the animals could learn how bad a given punishment is but did not clearly show whether the value of one option changed because of the value of the alternative options. It remained possible that the animals learned the value of different options paired with each odour and then compared during the choice test which of the odours predicted the better outcome. The critical advantage of my design is that I tested only a single odour and therefore could directly assess what the animals had learned about it.

A number of studies used an experimental paradigm similar to mine with two odours during the training but only one of them during the test (Galili *et al.,* 2014; Schleyer *et al.,* 2018; Jacob & Waddell, 2020; Zhao *et al.,* 2021). In addition, a one-odour training version is widely used in larvae (Saumweber *et al.,* 2011; Schleyer *et al.,* 2011; Widmann *et al.,* 2016; Eichler *et al.,* 2017; Paisios *et al.,* 2017; Saumweber *et al.,* 2018; Eschbach *et al.,* 2020; Schleyer *et al.,* 2020; Eschbach *et al.,* 2021; Sen *et al.,* 2024; Toshima *et al.,* 2025). In these studies, a single reward or punishment was paired with one odour, while the other training situation was neutral and therefore had a value of zero. This makes their results non-conclusive but nevertheless consistent with the hypothesis of relative value learning.

For example, one-odour experiments with rewards always consisted of two types of training regimens: either the odour was paired with the reward while the other training situation featured no odour on a neutral substrate (so-called paired training), or the odour was paired with the neutral substrate while the reward was presented without an odour (so-called unpaired training). For paired training, whether the animals associate the odour only with the training situation paired with it or whether they compare both training situations would lead to the same outcome, an increased proportion of larvae choosing that odour compared to the baseline. For unpaired training, in contrast, the two hypotheses predict different outcomes. The preference for the tested odour would be the same as the baseline if the animals only considered the training situation paired with the odour because during training this odour had not been paired with any reward. On the other hand, the preference for the tested odour would decrease relative to the baseline if the animals learned by comparing the two training situations as the odour had been paired with the less rewarding of the two substrates. The latter is precisely what had been found in several previous studies and was confirmed in this study (Fig. 5) (Saumweber *et al.,* 2011; Paisios *et al.,* 2017; Schleyer *et al.,* 2018; Sen *et al.,* 2024). Analogous results have been observed when using punishments instead of rewards (Paisios *et al.,* 2017; Schleyer *et al.,* 2018) as well as when using a training with two odours (Schleyer *et al.,* 2018). Using a different experimental design, similar results have been found in adults, too. Here, the resulting memories were referred to as "CS+" and "CS-" memories (Jacob & Waddell, 2020).

These observations gave rise to the proposal of two separate learning processes after paired and unpaired training, leading to memories of opposite valences and with separate neurobiological mechanisms (Saumweber *et al.,* 2011; Schleyer *et al.,* 2018; Jacob & Waddell, 2020; Zhao *et al.,* 2021; Sen *et al.,* 2024). However, relative value learning, where animals learn by comparing different situations, provides a unified framework to explain the results of both kinds of training: the memory's valence depends on whether the trained odour had been paired with the better or worse of the training situations. This new perspective should be incorporated into the current theoretical, computational and neuronal working models of associative learning in *D. melanogaster*. Before I briefly discuss one plausible neuronal working model, I first want to consider the limitations of the current study.

4.4 Limitations of the study

One limitation of the current experimental design was that I used only two different reward and two different punishment values. This was a deliberate decision to maximize the contrasts between the values and thus to increase the likelihood that the larvae recognized the difference between the training situations. However, this limited the resolution of the value differences and may be partially responsible for the unclear results regarding the mode of how the different values were integrated (as a simple subtraction or not). To compensate for the lacking additional reward and punishment values, I used a neutral situation with value 0. However, this raised another concern, as a comparison between zero and any other value may be evaluated as a qualitative rather than a quantitative difference by the animals and may lead to fundamentally different behaviours. As my experiments yielded very strong and robust differences in the animals' behaviour, future studies can use more different reward and punishment values with smaller differences between them for a clearer picture with higher resolution.

In addition, following the established basic classical conditioning protocols in larvae, I exposed the animals to only two different training situations. It would be very interesting to observe how the value associated with an odour change when the animals are exposed to further different values. However, the larvae's memory capacities are limited, and experiments of my lab members showed that, for example, fructose memories are forgotten within a few minutes (master's degree project of Ayaka Fukushima, Schleyer lab). Thus, a too-complex training protocol with too many training situations may have to deal with the animals forgetting earlier training situations before the training is even finished.

The established experimental protocols in the field, used with little change for many decades (Tully & Quinn, 1985; Scherer *et al.*, 2003; Michels *et al.*, 2017), give rise to another very fundamental limitation that also affects the present study: the experiments cannot reveal how population-based odour preferences relate to learning at the level of individual larvae. Specifically, the proportion of larvae choosing a reward- or punishment-associated odour does not allow direct inference about the memory formation in single individuals. It is well established that larvae form associative memories and that these memories influence navigational decisions and lead to changes in the number of animals found at a given part of the test dish (Gerber & Hendel, 2006). However, it is not clear whether such population-level changes are caused by changes within each animal (i.e., the training induces neurobiological processes that cause each individual to gradually change its preference towards the odours), or by switch-like (all-or-none) changes in some animals (i.e., the training induces neurobiological

processes that cause some individuals to strongly change their preference towards the odours). A study in honeybees found that a switch-like learning process with a variable timing across individuals can yield gradual learning curves at the population level (Pamir *et al.,* 2011). Within this framework, differences in preference are best explained by changes in the proportion of animals expressing learned behaviour, rather than uniform changes in choice probability across all individuals. Disambiguating these possibilities would require repeated testing of the same individuals; however, as already mentioned earlier, memories in larvae are short-lived and labile, and repeated testing can itself modify memory expression through extinction or relearning processes (e.g., Felsenberg *et al.,* 2018), which limits the feasibility of repeated testing in individual larvae. A recent study provided a completely new experimental paradigm for associative learning in larvae that allows for rapid repeated testing of individual animals and indeed found evidence suggesting a switch-like learning process (Lesar *et al.*, 2021). However, this new experimental protocol relies on providing virtual rewards and punishments via optogenetic tools, which limits its usefulness for my current research question.

An additional consideration in this study is that the behavioural testing was performed in groups, which may complicate the interpretation of individual learning effects due to potential social interactions among larvae. Indeed, previous studies found that larvae employ some forms of social interactions, including joint feeding (Durisko & Dukas, 2013). Notably, previous work using similar larval olfactory learning paradigms as used in my thesis found no evidence that the odour choice of an individual larva is influenced by the behaviour of surrounding conspecifics (Niewalda *et al.,* 2014). This supports the assumption that individual decisions are largely independent under standard assay conditions and underlies the widespread use of population-based approaches in larval learning research over several decades. Nevertheless, I cannot exclude that some interactions occurred in my experiments that affected my results. Future studies should try to establish an experimental setup for assessing individual larvae, with repeated choices and optimized to minimize extinction. Such experimental designs, although very challenging within the biological limits of the larva, will be necessary to further reduce remaining ambiguities.

4.5 A potential neuronal circuit for relative value learning

In *D. melanogaster*, olfactory associative memories are established in the mushroom body, a major insect memory centre (Fig. 8a) (Thum & Gerber, 2019; Modi *et al.,* 2020). In brief, odours and other stimuli are coded by specific combinations of Kenyon cells (KCs), the intrinsic neurons of the mushroom body. The KCs are intersected by (mostly) dopaminergic modulatory input neurons (DANs) and mushroom body output neurons (MBONs). The innervations of DANs and MBONs compartmentalize the mushroom body into microcircuits between DANs, KCs and MBONs that act as functional units. As a rule, DANs conveying reward signals pair up with avoidance-promoting MBONs, and DANs conveying punishment signals pair up with approach-promoting MBONs. DAN signals are received by the axonal terminals of all KCs and modulate the KC-MBON synapses. The synaptic weights of the KC-MBON synapses thus contain the associative strengths of all stimuli coded by the KCs. Crucially, studies in adults found that the same DAN signal can have opposite effects on internal KC signalling when a KC is active versus inactive (Handler *et al.,* 2019) and that the KC-MBON synapses can be depressed or potentiated depending on the training contingency (Cohn *et al.,* 2015).

The MBONs gather input from all KCs and on the one hand, convey this information towards motor control, tilting the behaviour of the animals towards approach or avoidance, depending on the memory (Aso *et al.,* 2014; Owald & Waddell, 2015; Eschbach *et al.,* 2021). On the other hand, they feed this information back either directly or via one and two step feedback relays to the DANs. The DANs that receive the feedback may innervate the same or other compartments and may even code for the opposite valence (Eschbach *et al.,* 2020).

The predominant working model for associative learning in *D. melanogaster* proposes that when a DAN reward signal is received by a KC while it is active (i.e., while the respective odour is present), its output synapses are depressed to an extent matching the reward strength (Fig. 8b, left). This reduces the activity of the avoidance-promoting MBON while leaving the activity of approach-promoting MBONs unchanged and thus shifts the mushroom body's net output towards approach (Aso *et al.,* 2014; Owald & Waddell, 2015; Eschbach *et al.,* 2021). This process can be seen as the basis for learning without considering any alternative values: an odour is associated with the reward value it coincides with (Villar *et al.*, 2022).

For relative value learning, the crucial question is how the reward strengths can be compared and a comparative or relative value can be computed. In mammals, DANs have been found to compute an associative prediction error that can be interpreted as the difference between the current and previously learned reward strengths and thus as a computation of relative reward

value (Rescorla & Wagner, 1972; Van Hamme & Wasserman, 1994; Schultz, 2015). Many computational models assume a similar mechanism in *D. melanogaster*, driven via the feedback from the MBONs to the DANs, but hard evidence for such a mechanism is lacking (Felsenberg *et al.,* 2017; Eschbach *et al.,* 2020; Bennett *et al.,* 2021; Zhao *et al.,* 2021; Jürgensen *et al.,* 2023; Toshima *et al.,* 2025). I therefore decided to not propose an analogous neuronal mechanism in this thesis, although I cannot exclude that the DANs play a role for computing the comparison on reward values in my case.

In this thesis, I rather propose that the computation of the relative value can occur in the KCs themselves: when a DAN signal is received by an inactive KC (i.e., the respective odour is not present), its output synapses are potentiated to an extent matching the reward strength, leading to a shift in the mushroom body's net output towards avoidance (Fig. 8b, right) (Cohn *et al.,* 2015; Schleyer *et al.,* 2018; Villar *et al.*, 2022). If rewards of different strengths are presented in the presence and absence of an odour, like in my experiments, the final strength of the KC-MBON synapses reflects an integration, in the simplest case a sum, of all modulations during training (Fig. 8c). In other words, the relative reward value is computed within the KC-MBON synapse as an integration of all positive and negative molecular modulations of the synapse strength. The precise molecular mechanism responsible for an accurate integration of all the values in the synapse is not known yet.

This working model captures my results regarding all experiments that used two odours during the training. In each case, the final strength of the KC-MBON synapse reflects an integration of all experienced values that were presented paired with the odour that activates the respective KC, depressing the synapse, and all values that were presented in the absence of the odour, potentiating the synapse. This principle works in the same way for rewards (as in Fig. 3) and punishments (as in Fig. 4) and is independent of the sequence in which the different values were presented (as in Fig. 6).

Interestingly, the proposed model predicts that KC-MBON synapses get potentiated whenever a reward is encountered while a KC is not active. This should decrease the preference of larvae for any odour that was not present at that moment. Indeed, I found that exposing the animals to a sugar reward alone decreases their proportion of choosing a novel odour, although the effect was quite small (Fig. 7b).

The model also predicts that a second odour during training would not make any difference for the process. This is what I found for OCT (Fig. 3e-g, Fig. 5e-g), but not for AM (Fig. 3b-d, Fig. 5b-d). Although I cannot explain this discrepancy with the current working model, I hope

that this model can help future studies to explore why different odours, which are represented by different sets of KCs, follow different rules for the associative process.

Although the proposed working model is very simple, it can be easily expanded to account for additional considerations. For example, humans have been found to compare reward strengths within each learning session (Klein *et al.,* 2017), and mammals often learn in a context-dependent manner (Maren *et al.,* 2013; Izquierdo *et al.,* 2016; Roberts, 2019). Possibly, also the KCs that get potentiated are limited, e.g., to those that code for stimuli that had been encountered recently or in the same experimental context. Such a mechanism would prevent an unnecessary potentiation of all KC-MBON synapses whenever a reward is encountered. In adult flies, reward-signalling DANs have been found to be required for memories regarding the punishment-unpaired odour, but not regarding the punishment-paired odour (Jacob & Waddell, 2020). Another study found an MBON pathway that encodes a "better-than-before" signal and activates a reward-signalling dopaminergic pathway, leading to a secondary reward memory in a different mushroom body compartment (Villar *et al.*, 2022). In other words, when an odour was paired with the weaker of two rewards or punishments, the modulations of the KC-MBON synapses I propose here may establish a memory of the opposite valence via the opposite-valence DANs. Future research will elucidate these and other pending questions regarding the detailed mechanism of how brains integrate the relative values of options into associative learning, in larvae as well as in other animals. The *D. melanogaster* larva, with its small brain and excellent tools for transgenic manipulations, is perfectly suited to contribute to this endeavour.

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Tables

Table 1: Experimental design. AM: *n*-amylacetate, OCT: 1-octanol, ∅: neutral substrate, +: weak reward, ++: strong reward.

		Reward paired with odour A		
		∅	+	++
Reward paired with odour B	∅	A∅/B∅	A+/B∅	A++/B∅
	+	A∅/B+	A+/B+	A++/B+
	++	A∅/B++	A+/B++	A++/B++

Table 2: Summary of the statistical models and their interpretation. RL_A: reward level paired with odour A, RL_B: reward level paired with odour B, QAIC: Quasi-Akaike Information Criterion.

Significant factors	QAIC comparison	Interpretation
Only RL_A	Model 1 = Model 2, Models 3 and 4 much higher	Learning by associating a reward with an odour irrespective of training alternatives
Both RL_A, RL_B	Model 1 = Model 4, Models 2 and 3 much higher	Learning by associating the outcome of the subtraction between the reward values with an odour
Both RL_A, RL_B	Model 1 lower than all other models	Learning by associating the odour with a reward value that considers both reward values during training, but not by a simple subtraction

Table 3: Experimental design of the quinine-only experiment. For more details, see Table 1.

		Punishment paired with AM		
		∅	-	--
Punishment paired with OCT	∅	AM∅/OCT∅	AM-/OCT∅	AM--/OCT∅
	-	AM∅/OCT-	AM-/OCT-	AM--/OCT-
	--	AM∅/OCT--	AM-/OCT--	AM--/OCT--

Table 4. Summary of the combined GLM results, combining three variations of the experiment with two odours and different rewards. Factors were the reward levels paired with each odour (RL_A and RL_B), the substrate of the test dish, the identity of odour A, and their interactions (indicated with ":"). The dispersion factor of the model indicates underdispersion. P values were not corrected for multiple testing.

Factor	Coefficient	P value	Dispersion factor
Intercept	0.193	0.141	0.775
RL_A	0.525	< 0.001	
RL_B	-0.504	< 0.001	
test dish	0.009	0.920	
odour A	-0.355	< 0.001	
RL_A:test dish	-0.073	0.030	
RL_B:test dish	0.067	0.052	
RL_A:odour A	-0.245	< 0.001	
RL_B:odour A	0.131	< 0.001	

Table 5. Summary of GLM results of four models when the animals were trained with two odours and different rewards, and AM was odour A. To compare between models, the Quasi-Akaike Information Criteria (QAIC) is displayed. The dispersion factor of each model is reported, with values < 1 indicating underdispersion and > 1 overdispersion.

Model	Factor	Coefficient	P value	QAIC	Dispersion factor
Model 1	Intercept	0.205	< 0.001	2015.075	0.545
	RL_A	0.425	< 0.001		
	RL_B	-0.414	< 0.001		
Model 2	Intercept	-0.486	< 0.001	2693.072	1.764
	RL_A	0.391	< 0.001		
Model 3	Intercept	0.878	< 0.001	2751.400	1.856
	RL_B	-0.378	< 0.001		
Model 4	Intercept	0.226	< 0.001	2013.372	0.544
	RL_A – RL_B	0.420	< 0.001		

Table 6. Summary of GLM results of four models when the animals were trained with two odours and different rewards, and OCT was odour A.

Model	Factor	Coefficient	P value	QAIC	Dispersion factor
Model 1	Intercept	-0.143	0.073	1151	1.270
	RL_A	0.205	< 0.001		
	RL_B	-0.306	< 0.001		
Model 2	Intercept	-0.653	< 0.001	1342	1.966
	RL_A	0.196	< 0.001		
Model 3	Intercept	0.214	0.002	1234.452	1.577
	RL_B	-0.298	< 0.001		
Model 4	Intercept	-0.320	< 0.001	1160	1.302
	RL_A – RL_B	0.257	< 0.001		

Table 7. Summary of GLM results of four models when both odours were presented and associated with punishments during training.

Model	Factor	Coefficient	P value	QAIC	Dispersion factor
Model 1	Intercept	0.414	< 0.001	1119.525	0.984
	PL_A	-0.511	< 0.001		
	PL_B	0.439	< 0.001		
Model 2	Intercept	1.120	< 0.001	1576.770	2.562
	PL_A	-0.456	< 0.001		
Model 3	Intercept	-0.469	< 0.001	1742.193	3.137
	PL_B	0.377	< 0.001		
Model 4	Intercept	0.277	< 0.001	1124.633	1.011
	PL_A – PL_B	-0.475	< 0.001		

Table 8. Summary of the combined GLM, combining three variations of the experiment with only odour A (AM, OCT or BA) and different rewards. Other details are as in Table 4.

Factor	Coefficient	P value	Dispersion factor
Intercept	0.373	< 0.001	0.609
RL_A	0.398	< 0.001	
RL_EM	-0.152	< 0.001	
Odour A=BA	-0.112	0.166	
Odour A=OCT	-0.229	0.002	
RL_A:odour A=BA	0.007	0.821	
RL_A:odour A=OCT	-0.200	< 0.001	
RL_EM:odour A=BA	-0.283	< 0.001	
RL_EM:odour A=OCT	-0.071	0.008	

Table 9. Summary of GLM results of four models when only one odour (AM) was presented and associated with rewards during training.

Model	Factor	Coefficient	P value	QAIC	Dispersion factor
Model 1	Intercept	0.373	< 0.001	2061.432	0.749
	RL_A	0.398	< 0.001		
	RL_EM	-0.152	< 0.001		
Model 2	Intercept	0.099	0.016	2142.070	0.894
	RL_A	0.396	< 0.001		
Model 3	Intercept	1.023	< 0.001	2662.304	1.856
	RL_EM	-0.148	< 0.001		
Model 4	Intercept	0.803	< 0.001	2172.989	0.948
	RL_A – RL_EM	0.279	< 0.001		

Table 10. Summary of GLM results of four models when only one odour (OCT) was presented and associated with rewards during training.

Model	Factor	Coefficient	P value	QAIC	Dispersion factor
Model 1	Intercept	0.144	0.003	1108.943	0.415
	RL_A	0.198	< 0.001		
	RL_EM	-0.222	< 0.001		
Model 2	Intercept	-0.244	< 0.001	1225.658	0.812
	RL_A	0.194	< 0.001		
Model 3	Intercept	0.484	< 0.001	1201.112	0.729
	RL_EM	-0.219	< 0.001		
Model 4	Intercept	0.100	< 0.001	1107.695	0.415
	RL_A – RL_EM	0.210	< 0.001		

Table 11. Summary of GLM results of four models when only one odour (BA) was presented and associated with rewards during training.

Model	Factor	Coefficient	P value	QAIC	Dispersion factor
Model 1	Intercept	0.262	< 0.001	891.249	0.534
	RL_A	0.405	< 0.001		
	RL_EM	-0.435	< 0.001		
Model 2	Intercept	-0.467	< 0.001	1223.147	1.925
	RL_A	0.372	< 0.001		
Model 3	Intercept	0.918	< 0.001	1180.188	1.726
	RL_EM	-0.404	< 0.001		
Model 4	Intercept	0.207	< 0.001	890.144	0.533
	RL_A – RL_EM	0.420	< 0.001		

Table 12. Summary of the combined GLM, for experiments with one-cycle-training and two training sequences. Other details are as in Table 4.

Factor	Coefficient	P value	Dispersion factor
Intercept	0.413	< 0.001	1.189
RL_A	0.334	< 0.001	
RL_B	-0.261	< 0.001	
Training sequence	0.173	0.046	
RL_A:Training sequence	0.085	0.007	
RL_B:Training sequence	-0.152	< 0.001	

Table 13. Summary of GLM results of four models of one-cycle-training when odour B (OCT) was presented first during training.

Model	Factor	Coefficient	P value	QAIC	Dispersion factor
Model 1	Intercept	0.586	< 0.001	939.991	1.269
	RL_A	0.418	< 0.001		
	RL_B	-0.41	< 0.001		
Model 2	Intercept	-0.132	0.076	1194.186	2.430
	RL_A	0.389	< 0.001		
Model 3	Intercept	1.236	< 0.001	1213.500	2.491
	RL_B	-0.383	< 0.001		
Model 4	Intercept	0.596	< 0.001	938.015	1.263
	RL_A – RL_B	0.416	< 0.001		

Table 14. Summary of GLM results of four models of one-cycle-training when odour A (AM) was presented first during training.

Model	Factor	Coefficient	P value	QAIC	Dispersion factor
Model 1	Intercept	0.413	< 0.001	1914.176	1.150
	RL_A	0.334	< 0.001		
	RL_B	-0.261	< 0.001		
Model 2	Intercept	-0.045	0.298	2131.190	1.628
	RL_A	0.322	< 0.001		
Model 3	Intercept	0.954	< 0.001	2285.517	1.944
	RL_B	-0.246	< 0.001		
Model 4	Intercept	0.543	< 0.001	1921.409	1.168
	RL_A – RL_B	0.298	< 0.001		

Table 15. Summary of the GLM results of one-cycle-training with no odours during training.

Model	Factor	Coefficient	P value	QAIC	Dispersion factor
Sequence Model	Intercept	1.074	< 0.001	1138.565	1.003
	RL_first – RL_second	0.127	< 0.001		
Sum Model	Intercept	1.267	< 0.001	1182.805	1.143
	RL_first + RL_second	-0.057	0.002		

Figures

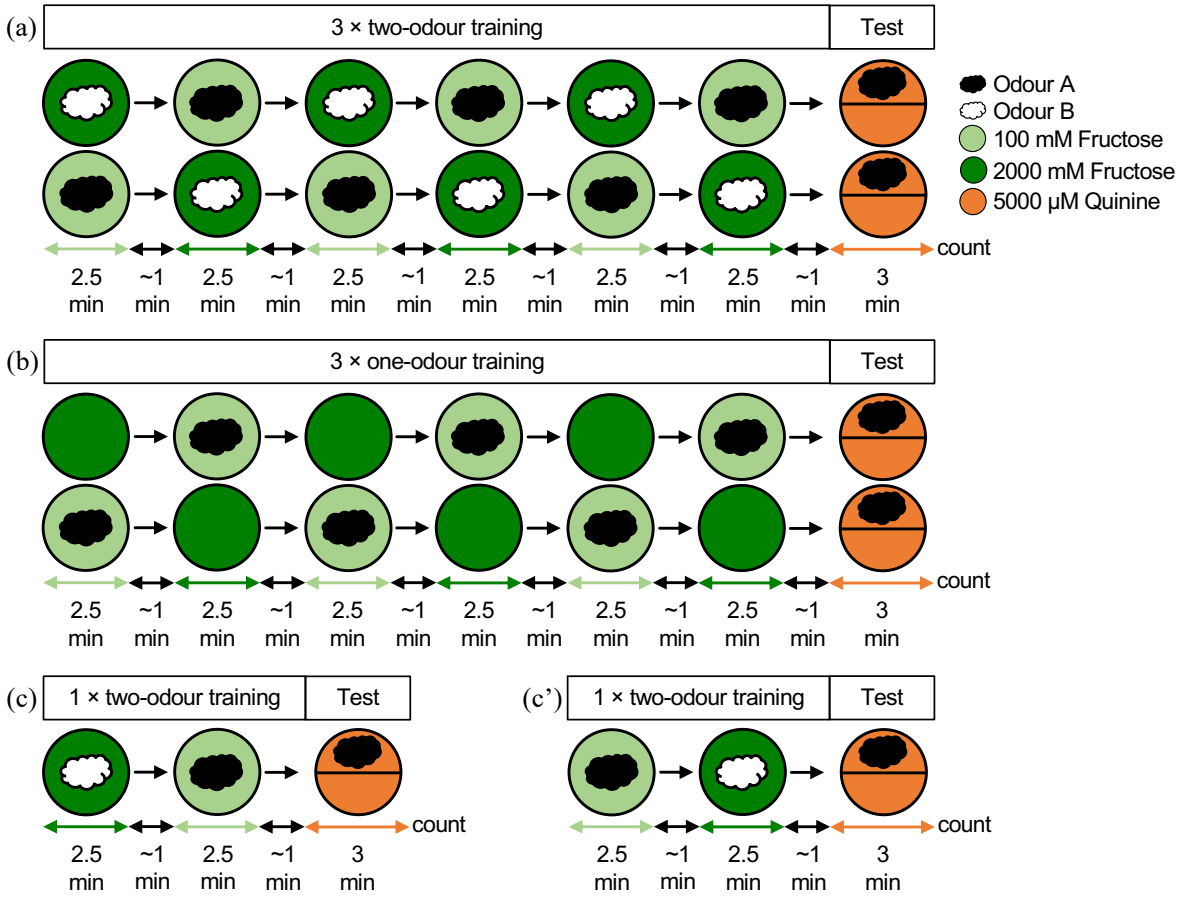

(a)
3 × two-odour training
Test
Odour A
Odour B
100 mM Fructose
2000 mM Fructose
5000 µM Quinine
count
2.5 min
~1 min
2.5 min
~1 min
2.5 min
~1 min
2.5 min
~1 min
2.5 min
~1 min
2.5 min
~1 min
3 min
(b)
3 × one-odour training
Test
count
2.5 min
~1 min
2.5 min
~1 min
2.5 min
~1 min
2.5 min
~1 min
2.5 min
~1 min
2.5 min
~1 min
3 min
(c)
1 × two-odour training
Test
count
2.5 min
~1 min
2.5 min
~1 min
3 min
(c')
1 × two-odour training
Test
count
2.5 min
~1 min
2.5 min
~1 min
3 min

Figure 1

Figure 1. Training procedures in this study. (a) Larvae were trained for 3 cycles with two odours, A and B, and different concentrations of fructose. Depicted is a group as an example, exactly as it was performed for Fig. 3b-g, with AM as odour A and OCT as odour B. The larvae spent 2.5 minutes on each dish and were transferred manually with a brush between dishes, which took about 1 minute. After 3 complete cycles, the animals were transferred to a test dish covered with quinine and with only one odour on one side, and after 3 minutes the number of larvae on either side was counted. Each displayed training sequence was used in half of the repetitions of the experiment (samples). For Fig. 3e-g, the identities of odour A and B were swapped; for Fig. 4, different quinine concentrations were used during training instead of fructose. (b) One-odour version of the experiment, as it was performed for Fig. 5. Here, odour A is AM for Fig. 5b-d, OCT for Fig. 5e-g and BA for Fig. 5h-j. (c) One-trial version of the experiment as it was performed for Fig. 6b-d. (c') One-trial version of the experiment as it was performed for Fig. 6e-g.

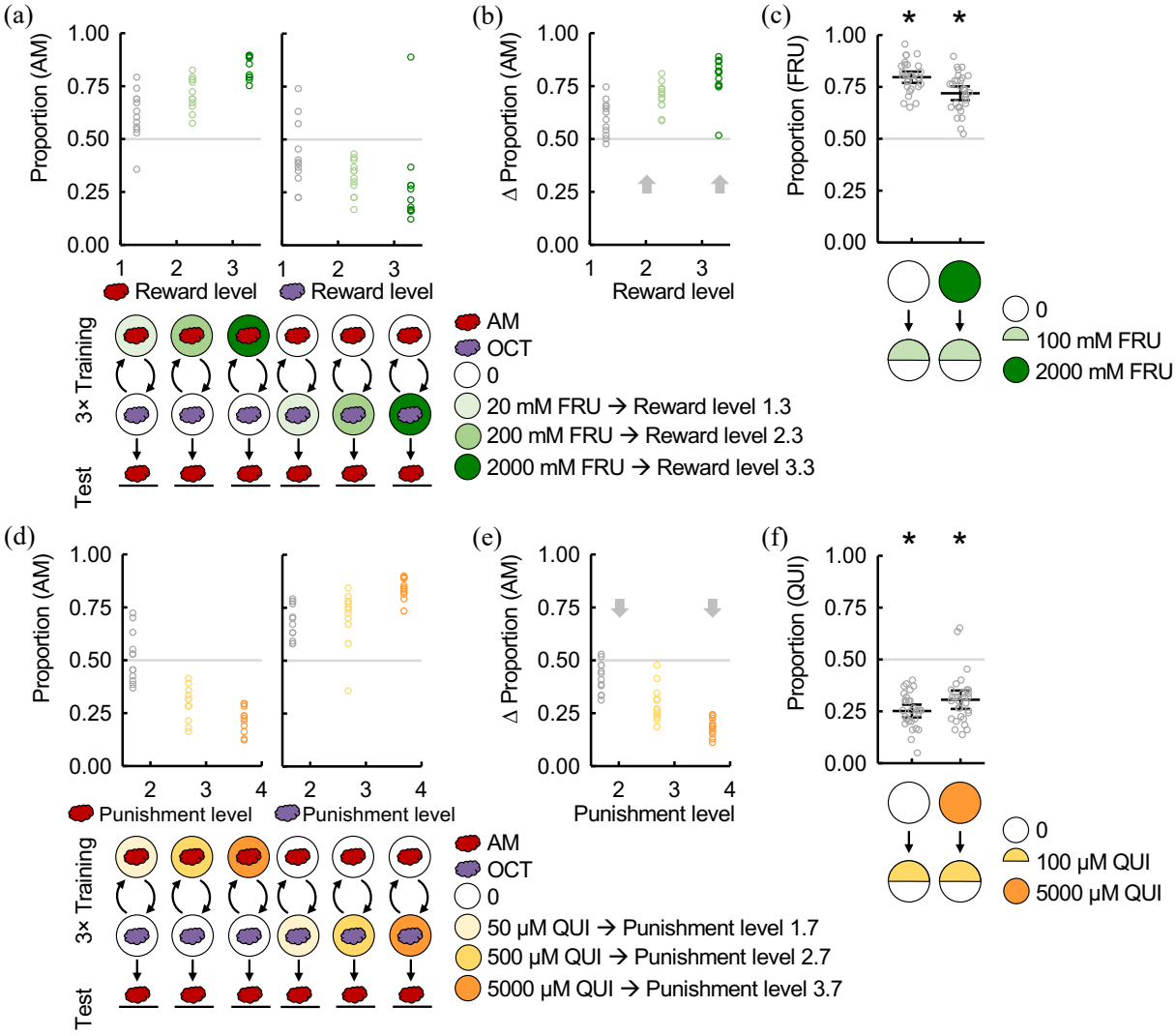

(a)
Proportion (AM)
1.00
0.75
0.50
0.25
0.00
1 2 3
Reward level
1 2 3
Reward level
(b)
Δ Proportion (AM)
1.00
0.75
0.50
0.25
0.00
1 2 3
Reward level
(c)
Proportion (FRU)
1.00
0.75
0.50
0.25
0.00
*
*
0
100 mM FRU
2000 mM FRU
3× Training
Test
AM
OCT
0
20 mM FRU → Reward level 1.3
200 mM FRU → Reward level 2.3
2000 mM FRU → Reward level 3.3
(d)
Proportion (AM)
1.00
0.75
0.50
0.25
0.00
2 3 4
Punishment level
2 3 4
Punishment level
(e)
Δ Proportion (AM)
1.00
0.75
0.50
0.25
0.00
2 3 4
Punishment level
(f)
Proportion (QUI)
1.00
0.75
0.50
0.25
0.00
*
*
0
100 µM QUI
5000 µM QUI
3× Training
Test
AM
OCT
0
50 µM QUI → Punishment level 1.7
500 µM QUI → Punishment level 2.7
5000 µM QUI → Punishment level 3.7

Figure 2

Figure 2. Choosing different fructose and quinine concentrations to be used in this study as rewards and punishments. (a) Larvae were trained with two odours, AM and OCT, and different concentrations of fructose (20, 200 and 2000 mM). When AM had been paired with the fructose reward, the proportion of larvae choosing AM increased with increasing fructose concentration. When OCT had been paired with the fructose reward, the proportion decreased with increasing fructose concentration. (b) The Δ Proportion of larvae choosing AM for these three fructose concentrations, indicating their rewarding strength. Based on this result, I picked two fructose concentrations for all future experiments (grey arrows). (c) Larvae were exposed to either pure agar or agar mixed with 2000 mM fructose for 2.5 minutes. Subsequently, they were tested for their proportion of a 100 mM fructose substrate in a split Petri dish assay. In both cases, larvae chose the fructose half. This demonstrates that larvae can sense the low concentration of reward even after exposure to a high concentration, as it is used in the learning experiments. (d) As in (a), but with different concentrations of quinine (50, 500 and 5000 µM). (e) The Δ Proportion of larvae choosing AM for these three quinine concentrations, indicating their punishing strength. Based on this result, I picked two fructose concentrations for all future experiments (grey arrows). (f) Larvae were exposed to either pure agar or agar mixed with 5000 µM quinine for 2.5 minutes. Subsequently, they were tested for their proportion of a 100 µM quinine substrate in a split Petri dish assay. In both cases, larvae avoided the quinine half. This demonstrates that larvae can sense a low concentration of punishment even after exposure to a high concentration, as it is used in the learning experiments. Sample size is 12 each for (a), (b), (d), (e) and 30 each for (c) and (f). In (c) and (f), raw data are displayed with the mean ± 95% confidence interval and * indicates a significant Wald test of the corresponding GLM.

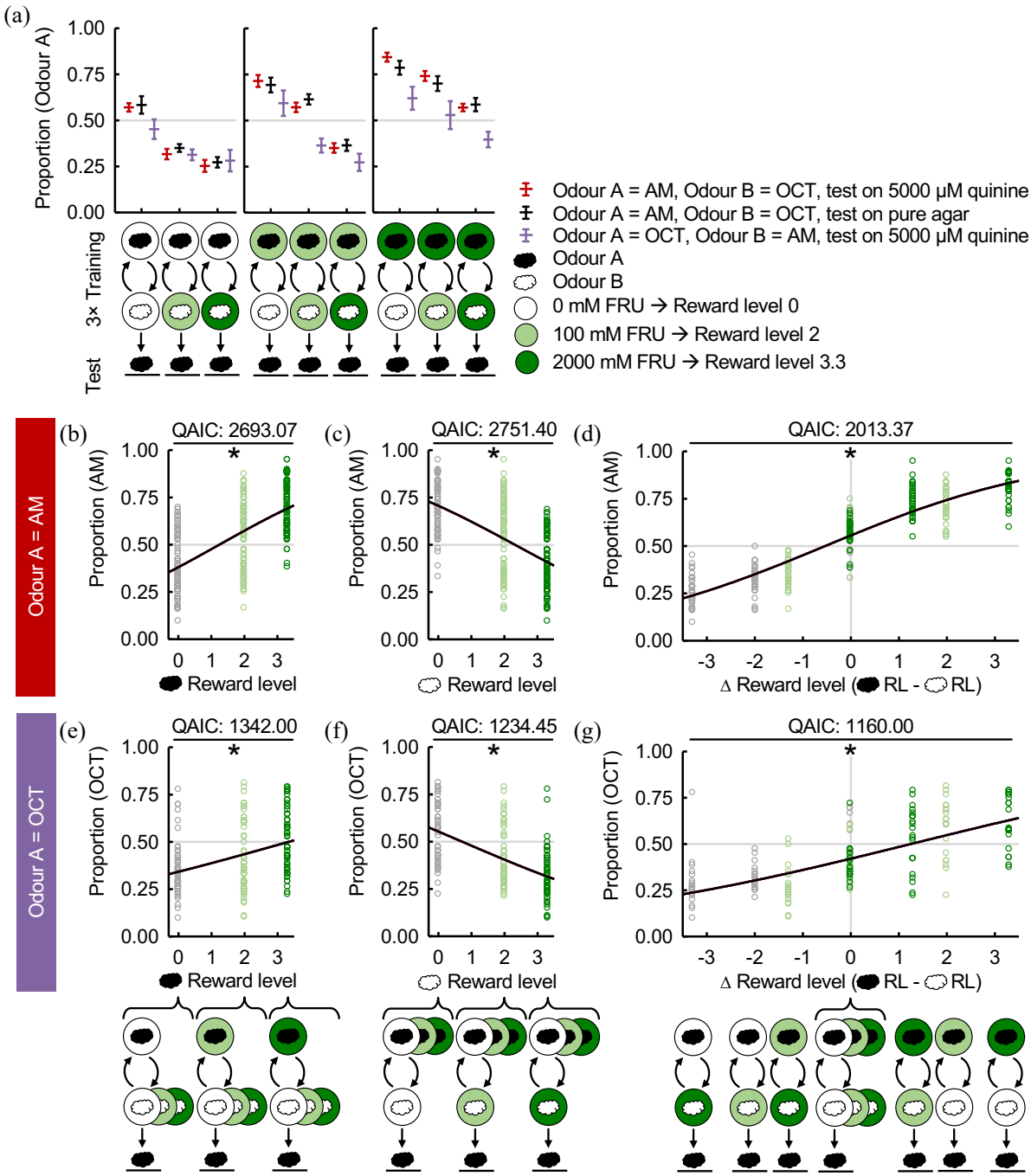

(a)
Proportion (Odour A)
1.00
0.75
0.50
0.25
0.00
3× Training
Test
Odour A = AM, Odour B = OCT, test on 5000 µM quinine
Odour A = AM, Odour B = OCT, test on pure agar
Odour A = OCT, Odour B = AM, test on 5000 µM quinine
Odour A
Odour B
0 mM FRU → Reward level 0
100 mM FRU → Reward level 2
2000 mM FRU → Reward level 3.3
Odour A = AM
(b)
QAIC: 2693.07
(c)
QAIC: 2751.40
(d)
QAIC: 2013.37
Proportion (AM)
Reward level
Δ Reward level (RL - RL)
Odour A = OCT
(e)
QAIC: 1342.00
(f)
QAIC: 1234.45
(g)
QAIC: 1160.00
Proportion (OCT)

Figure 3

Figure 3. Larvae compare two different reward situations and associate the outcome with an odour. (a) Comparison of three variations of the experiment where larvae were trained with odour A and B paired with 0, 100 or 2000 mM fructose reward (0, 2 and 3.3 Reward levels respectively). The x-axis shows the nine experimental groups arranged in the order listed in Table 1. Red and black bars indicate the results of the experimental variations when odour A was AM, B was OCT and the test was performed on the quinine or pure Petri dishes, respectively. Purple bars indicate the results of the variation where odour A was OCT and B was AM, with the test being performed on quinine Petri dishes. (b-d) Results of the combined two experimental variations where the proportion of larvae choosing AM (odour A) was tested. (b) The nine groups were positioned from left to right based on the Reward level paired with AM (RL_A), along with the regression line from Model 2 of the GLM analysis. The proportion of larvae choosing AM increased with increasing Reward levels paired with AM. (c) The nine groups were positioned from left to right based on the Reward level paired with OCT (RL_B), along with the regression line from Model 3. The proportion of larvae choosing AM decreased with increasing Reward levels paired with OCT. (d) The nine groups were positioned from left to right based on the Δ Reward level, that is, the Reward level paired with AM minus the Reward level paired with OCT, along with the regression line from Model 4. Here, the proportion of larvae choosing AM increased along with the increased Δ Reward level. (e-g) As in (b-d), but with OCT as odour A. The QAIC of each model is shown on the top of each figure. Sample size is 30, 16 and 22 each for the three variations, respectively in (a), 46 each for (b-d) and 22 each for (e-g). * indicates significant Wald tests of the corresponding GLMs, respectively.

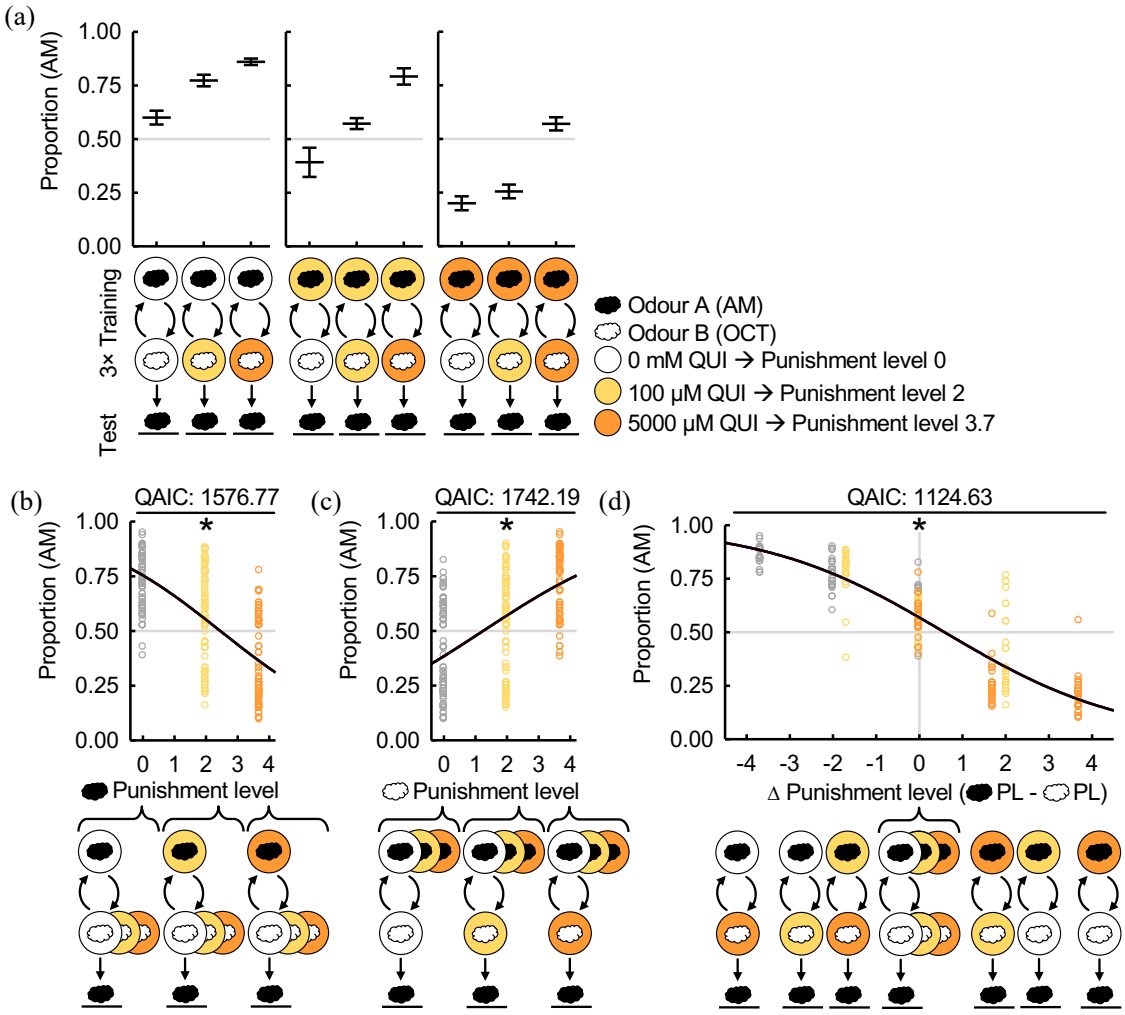

(a)
Proportion (AM)
1.00
0.75
0.50
0.25
0.00
3× Training
Test
Odour A (AM)
Odour B (OCT)
0 mM QUI → Punishment level 0
100 µM QUI → Punishment level 2
5000 µM QUI → Punishment level 3.7
(b)
QAIC: 1576.77
(c)
QAIC: 1742.19
(d)
QAIC: 1124.63
Punishment level
Δ Punishment level (PL - PL)

Figure 4

Figure 4. Larvae compare two different punishment situations and associate the outcome with an odour. (a) Larvae were trained with AM (odour A) and OCT (odour B) paired with 0, 100 and 5000 μM of quinine (0, 2 and 3.7 Punishment levels respectively). The x-axis shows the nine experimental groups arranged in the order listed in Table 3. (b) The nine groups were positioned from left to right based on the Punishment level paired with AM (PL_A) along with the regression line from Model 2 of the GLM analysis. The proportion of larvae choosing AM decreased along with the increased Punishment level paired with AM. (c) The nine groups were positioned from left to right based on the Punishment level paired with OCT (PL_B) along with the regression line from Model 3. The proportion of larvae choosing AM increased with the increasing Punishment levels paired with OCT. (d) The nine groups were positioned from left to right based on the Δ Punishment level, that is, the Punishment level paired with AM minus the Punishment level paired with OCT, along with the regression line from Model 4. Here, the proportion of larvae choosing AM was positive when AM had been paired with the lower punishment level of the two options and decreased with an increasing Δ Punishment level. The QAIC of each model is shown on the top of each figure. Sample size is 30 each. * indicates significant Wald tests of the corresponding GLMs, respectively.

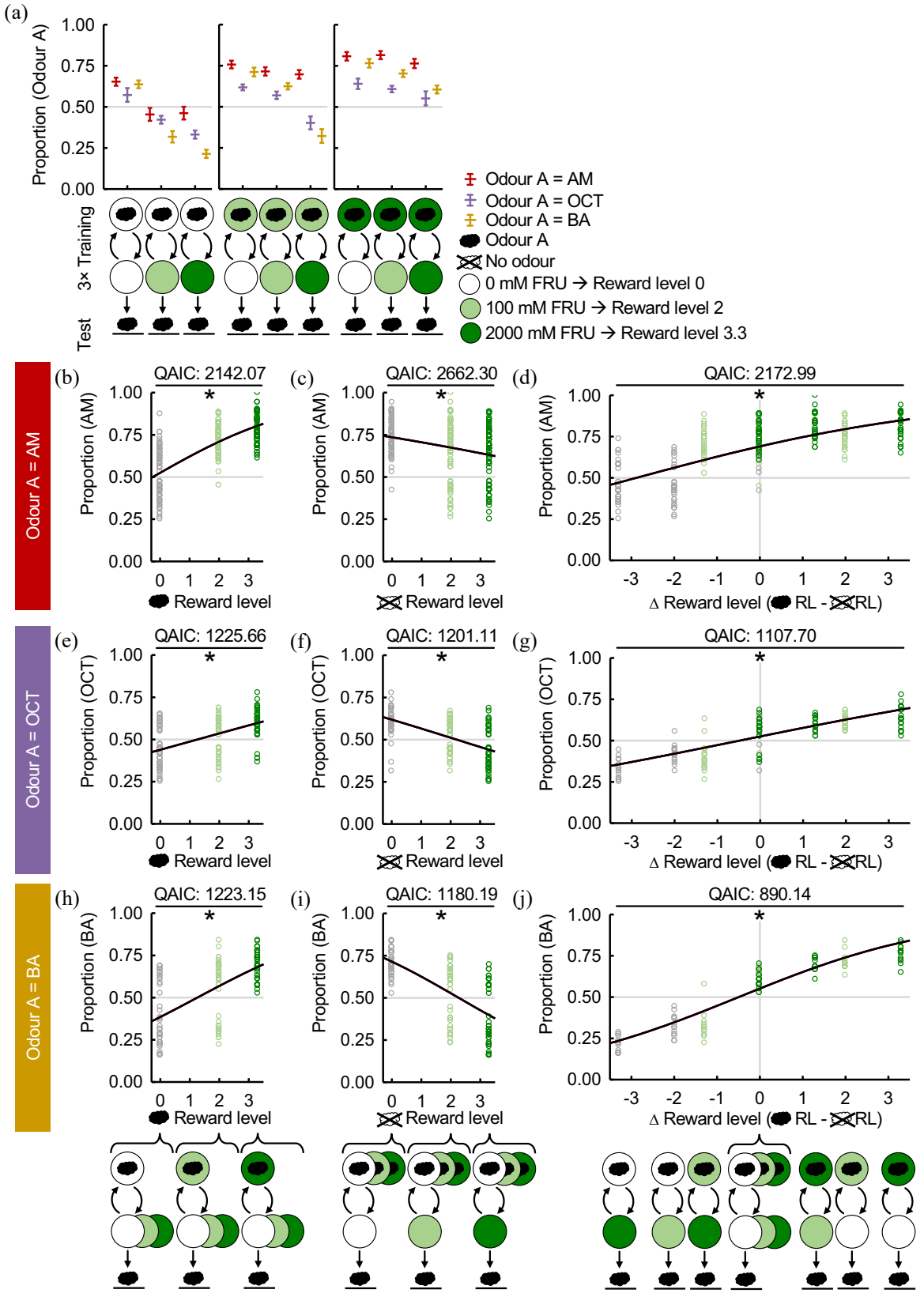

(a)
Proportion (Odour A)
1.00
0.75
0.50
0.25
0.00
3× Training
Test
Odour A = AM
Odour A = OCT
Odour A = BA
Odour A
No odour
0 mM FRU → Reward level 0
100 mM FRU → Reward level 2
2000 mM FRU → Reward level 3.3
Odour A = AM
(b)
QAIC: 2142.07
(c)
QAIC: 2662.30
(d)
QAIC: 2172.99
Proportion (AM)
Reward level
Δ Reward level (RL - RL)
Odour A = OCT
(e)
QAIC: 1225.66
(f)
QAIC: 1201.11
(g)
QAIC: 1107.70
Proportion (OCT)
Odour A = BA
(h)
QAIC: 1223.15
(i)
QAIC: 1180.19
(j)
QAIC: 890.14
Proportion (BA)

Figure 5

Figure 5. Larvae compare the two different reward situations with one-odour training. (a) Comparison of three variations of the experiment where larvae were trained with only odour A, paired with 0, 100 or 2000 mM fructose reward (0, 2 and 3.3 Reward levels respectively). The x-axis shows the nine experimental groups arranged in the order listed in Table 1; all groups were trained with one odour. Red, purple and yellow bars indicate the results of the experimental variations when odour A was AM, OCT or BA respectively. (b) The nine groups were positioned from left to right based on the Reward level paired with AM (RL_AM) along with the regression line from Model 2. The proportion of larvae choosing AM increased along with the increased Reward level paired with AM. (c) The nine groups were positioned from left to right based on the Reward level paired with the alternative situations without any odour (RL_EM, indicated by a crossed-out odour symbol) along with the regression line from Model 3. (d) The nine groups were positioned from left to right based on the Δ Reward level, along with the regression line from Model 4. The proportion of larvae choosing odour A increased with an increasing Δ Reward level. (e-g) As in (b-d), but with OCT as odour A. (h-j) As in (b-d), but with BA as odour A. The QAIC of each model is shown on the top of each figure. Sample size is 18 each for (b-d), 20 each for (e-g) and 16 each for (h-j). * indicates significant Wald tests of the corresponding GLMs, respectively.

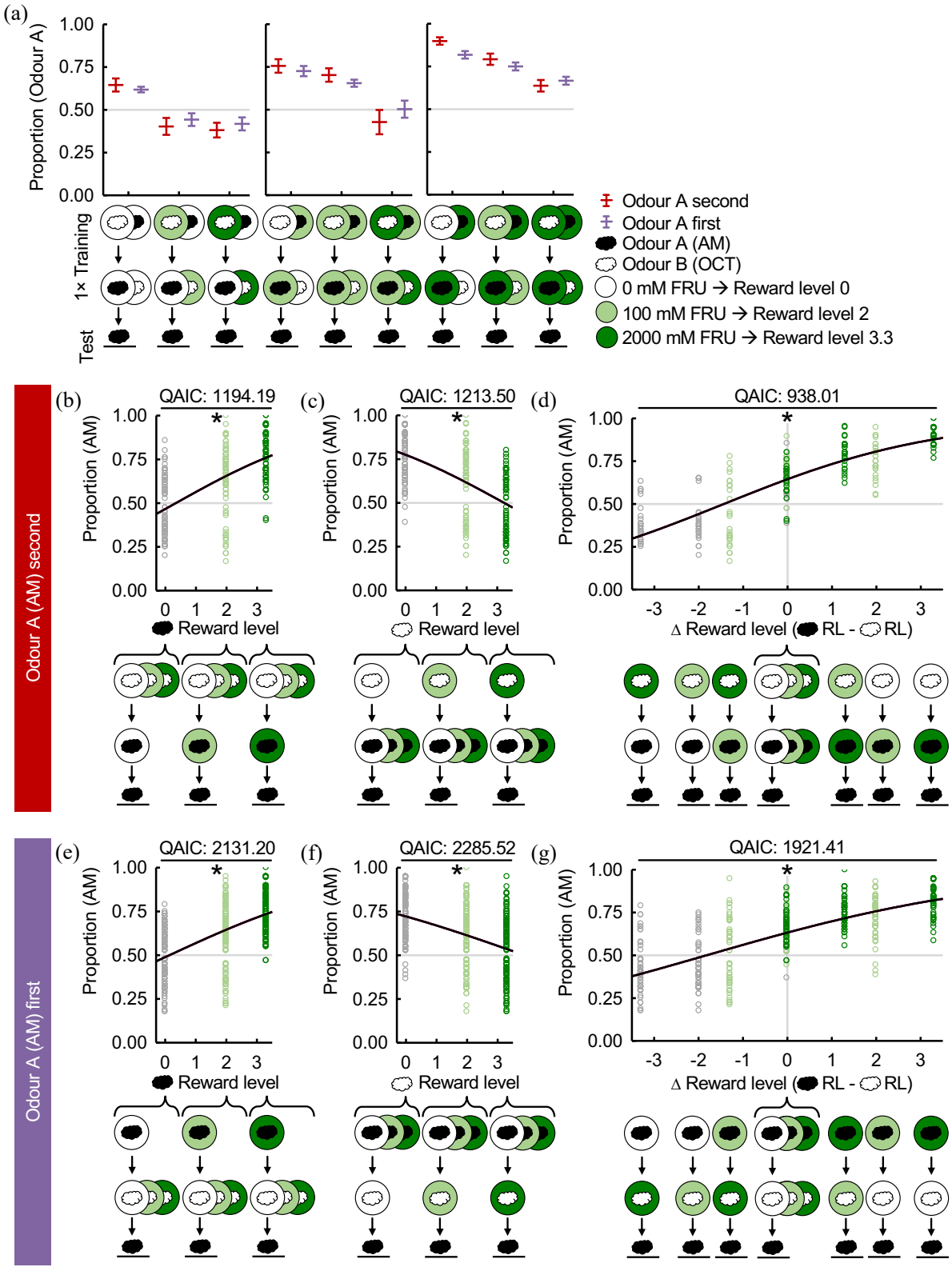

(a)
Proportion (Odour A)
1.00
0.75
0.50
0.25
0.00
1× Training
Test
Odour A second
Odour A first
Odour A (AM)
Odour B (OCT)
0 mM FRU → Reward level 0
100 mM FRU → Reward level 2
2000 mM FRU → Reward level 3.3
Odour A (AM) second
(b)
QAIC: 1194.19
(c)
QAIC: 1213.50
(d)
QAIC: 938.01
Proportion (AM)
Reward level
Δ Reward level (RL - RL)
Odour A (AM) first
(e)
QAIC: 2131.20
(f)
QAIC: 2285.52
(g)
QAIC: 1921.41

Figure 6

Figure 6. Larvae compare the two different reward situations in both training sequences. (a) Comparison of two variations of the experiment where larvae were trained with two odours, AM and OCT paired with 0, 100 or 2000 mM fructose reward (0, 2 and 3.3 Reward levels respectively). Only a single training trial was performed. Red and purple bars indicate the results of the experimental variations when OCT is presented first or AM is presented first during training, respectively. (b-d) Results when OCT was presented first during training. (b) The nine groups are positioned from left to right based on the Reward level associated with AM (RL_A) along with the regression line from Model 2 of the GLM. (c) The nine groups are positioned from left to right based on the Reward level associated with OCT (RL_B) along with the regression line from Model 3. (d) The nine groups are positioned from left to right based on the increased Δ Reward level along with the regression line from Model 4. (e-g) As in (b-d), but when AM was presented first during training. The QAIC of each model is shown on the top of each figure. Sample size is 28 each for (b-d) and 58 each for (e-g). * indicates significant Wald tests of the corresponding GLMs, respectively.

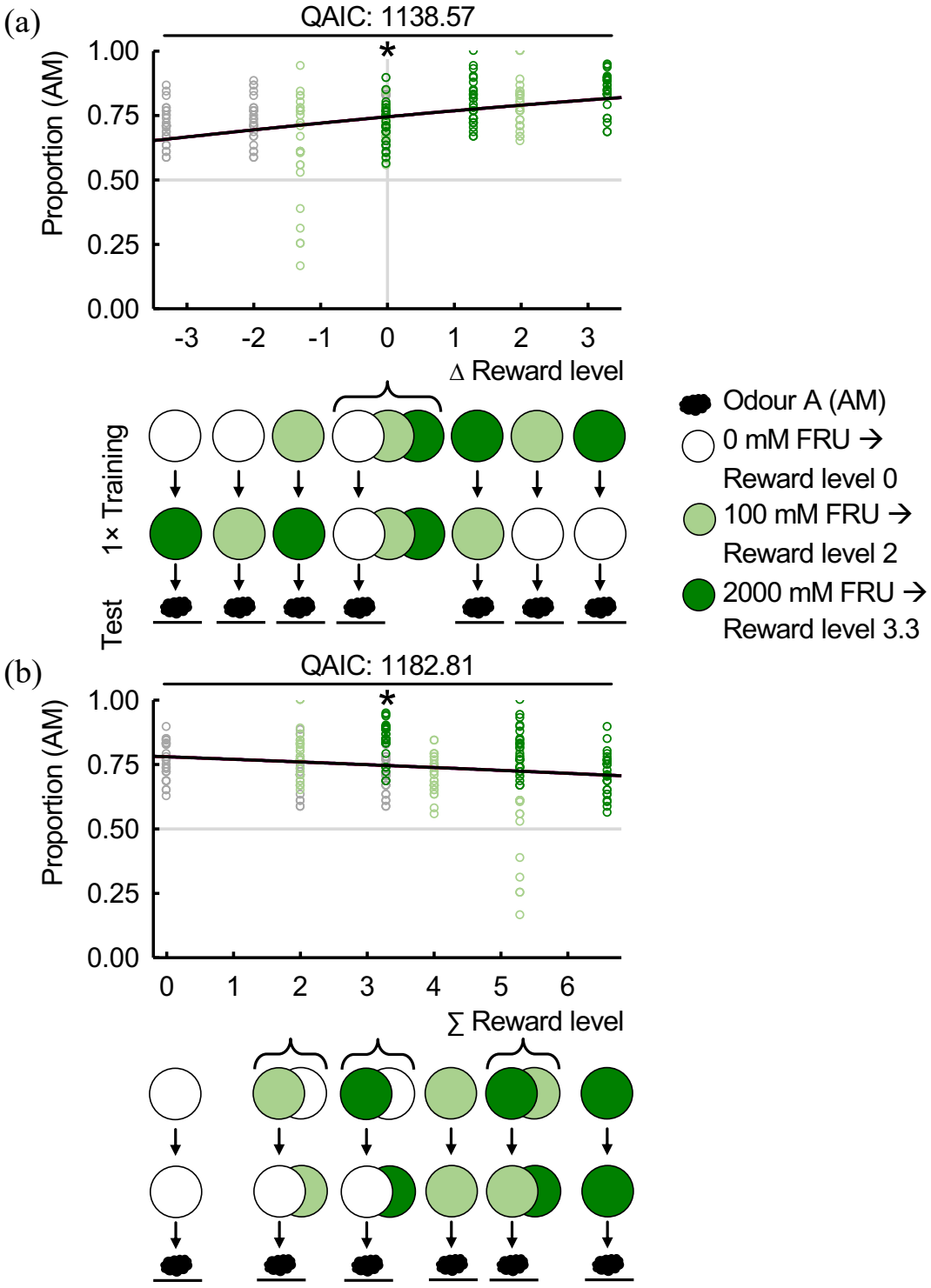

(a)
QAIC: 1138.57
Proportion (AM)
1.00
0.75
0.50
0.25
0.00
-3
-2
-1
0
1
2
3
Δ Reward level
1× Training
Test
Odour A (AM)
0 mM FRU →
Reward level 0
100 mM FRU →
Reward level 2
2000 mM FRU →
Reward level 3.3
(b)
QAIC: 1182.81
Proportion (AM)
1.00
0.75
0.50
0.25
0.00
0
1
2
3
4
5
6
∑ Reward level

Figure 7

Figure 7. Sugar exposure affects subsequent odour choice. Larvae were trained in the same manner mentioned in Fig. 6 (e-g) but without presenting any odour during training. After a single training cycle, the proportion of larvae choosing AM was calculated. (a) The nine groups were arranged from left to right based on the increased Δ Reward level along with the regression line of the GLM, calculated in this case as the Reward level of the first dish minus that of the second dish. Here, the proportion of larvae choosing AM increased with the increased Δ Reward level. The effect was significant but much weaker than in the experiments with odour (see Fig. 6g). (b) The nine groups were positioned from left to right based on the increased ∑ Reward level (the sum of the Reward levels) along with the regression line of the respective GLM. Here, the proportion of larvae choosing AM decreased along with the increased ∑ Reward level. The QAIC of each model is shown on the top of each figure. Sample size is 30 each. * indicates significant Wald tests of the corresponding GLM.

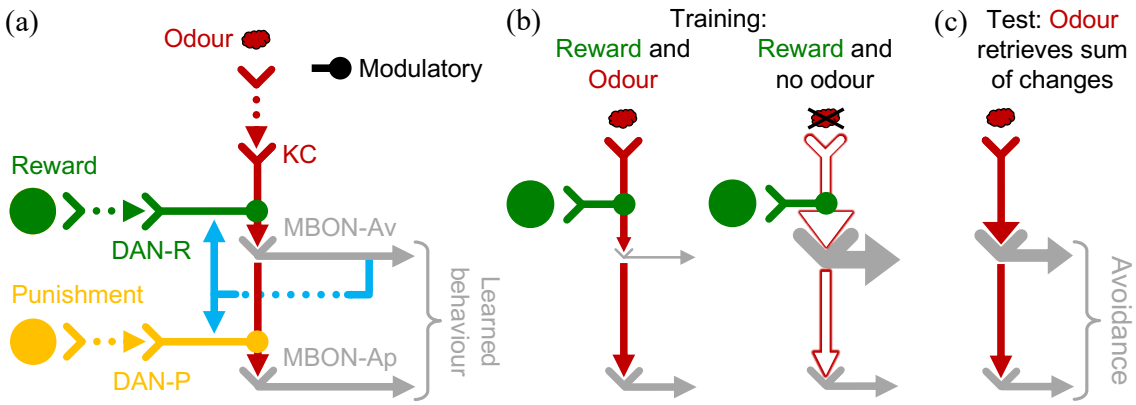

(a)
Odour
Modulatory
KC
Reward
MBON-Av
DAN-R
Punishment
MBON-Ap
DAN-P
Learned behaviour
(b)
Training:
Reward and Odour
Reward and no odour
(c)
Test: Odour retrieves sum of changes
Avoidance

Figure 8

Figure 8. Simplified potential circuitry underlying relative value learning. (a) Mushroom body circuit for associative learning. DAN: dopaminergic neuron; KC: Kenyon cell; MBON: mushroom body output neuron. Two sample compartments are shown, one receiving reward, the other one punishment input. The feedback from MBONs to DANs (blue) can be direct or indirect and excitatory or inhibitory (adapted from Eichler *et al.,* 2017; Saumweber *et al.,* 2018; Eschbach *et al.,* 2020). (b) Simple working model of absolute and relative value learning. Left: coincidence of odour and reward depresses the synapses of odour-activated KCs and an avoidance-promoting MBON. Right: a reward received by an inactive KC potentiates the KC-MBON synapses. (c) The KC-MBON synapses integrate the modulations by previous rewards in the presence and absence of the odour. In the shown example, the net effect is a potentiated synapse and thus odour avoidance.

Research List

Name: RAHMAN SADNIMAN

1. Papers (related to Doctoral Dissertation)

(1) **Rahman S**, Tanaka NK and Schleyer M. 2026. Relative value learning in *Drosophila melanogaster* larvae. *Proc. R. Soc. B*, 293(2064): 20252263.

2. Papers (Other than the above)

(1) **Rahman S**, Terao K, Hashimoto K and Mizunami M. 2024. Independent operations of appetitive and aversive conditioning systems lead to simultaneous production of conflicting memories in an insect. *Proc. R. Soc. B*, 291(2031): 20241273.

(2) **Rahman S**, Modak C, Akter M and Alam MS. 2020. Role of microRNA genes *miR-1000* and *miR-375* in forming olfactory conditional memory in *Drosophila melanogaster*. *MicroRNA*, 9(4).

(3) **Rahman S**, Roy DK and Alam MS. 2020. First record of *Zaprionus indianus* (Gupta, 1970) (Diptera Drosophilidae) from Bangladesh. *Biodiv. Jour*. 11(3): 757–760.

(4) Alam MS, Poly FS, **Rahman S** and Begum RA. 2019. Larval locomotion behavior in three species of *Drosophila. Bangladesh J. Zool.* 47(2): 367-371.

3. Conference (related to Doctoral Dissertation)

(1) **Rahman S** and Schleyer M. Relative associative value learning in *Drosophila* larvae. Presented at: IGP symposium 2024 supported by the International Graduate Program (IGP) of the Graduate School of Life Science, Hokkaido University; 4 December 2024, Japan.

(3) **Rahman S**. Tiny minds, big lessons: Exploring learning and memory in insects. Presented as an invited speaker at: "The Ecological Turn of Learning" symposium at the 84th Annual Meeting of the Japanese Society for Animal Psychology, 25 October 2024, Japan.

(3) Rahman S, Schutte J and Schleyer M. Neuronal and behavioral analyses of unpaired associative learning in *Drosophila* larvae. Poster presented at: NEURO2024, the joint meeting of the 47th Annual Meeting of the Japan Neuroscience Society (JNS), the 67th Annual Meeting of the Japanese Society for Neurochemistry (JSN), and the 46th Annual Meeting of the Japanese Society of Biological Psychiatry (JSBP); 2024 July 24-27, Japan.

4. Conference (Other than the above)

(1) **Rahman S** and Mizunami M. Roles of octopamine and dopamine neurons in the formation and retrieval of parallel appetitive and aversive memories in Pavlovian conditioning in the cricket *Gryllus bimaculatus*. Poster presented at: the 44th Annual Meeting of the Japanese Society for Comparative Physiology and Biochemistry (JSCPB 2022); 2022 November 21-27, Japan.

(2) **Rahman S**, Modak C, Akter M and Alam MS. Association of microRNA gene *miR-375* with neuronal gene *ATPalpha*: Transcript level analysis. Poster presented at: 2nd Annual BioProcessing (Virtual Event) Conference organized by Labroots (virtual); 2020 April 08.