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RESEARCH ARTICLE

An amino-acid mixture can be both rewarding and punishing to larval *Drosophila melanogaster*

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ABSTRACT

Amino acids are important nutrients for animals because they are necessary for protein synthesis in particular during growth, as well as for neurotransmission. However, little is known about how animals use past experience to guide their search for amino-acid-rich food. We reasoned that the larvae of *Drosophila melanogaster* are suitable for investigating this topic because they are the feeding and growth stages in the life cycle of these holometabolous insects. Specifically, we investigated whether experiencing an odour with a 20 amino-acid mixture as a semi-natural tastant during training establishes odour–tastant associative memories. Across a broad concentration range (0.01–20 mmol l^{-1}), such an amino-acid mixture was found to have a rewarding effect, establishing appetitive memory for the odour. To our surprise, however, manipulation of the test conditions revealed that relatively high concentrations of the amino-acid mixture (3.3 mmol l^{-1} and higher) in addition establish aversive memory for the odour. We then characterized both of these oppositely valenced memories in terms of their dependency on the number of training trials, their temporal stability, their modulation through starvation and the specific changes in locomotion underlying them. Collectively, and in the light of what is known about the neuronal organization of odour–food memory in larval *D. melanogaster*, our data suggest that these memories are established in parallel. We discuss the similarity of our results to what has been reported for sodium chloride, and the possible neurogenetic bases for concentration-dependent changes in valence when these tastants are used as reinforcers.

KEY WORDS: Reinforcement, Fruit fly, Appetitive learning, Aversive learning, Associative conditioning, Valence

INTRODUCTION

All organisms depend on the uptake of appropriate nutrients for their wellbeing, and at the same time they must be selective in their uptake to prevent poisoning, infection or intoxication. Animals can furthermore seek out nutritive foods and stay away from potentially harmful foods, a faculty that can be further refined by experience and the ensuing memories that make better choices possible in the future. The present study investigates such learning in the case of amino acids in larval *Drosophila melanogaster*.

Amino acids are important nutrients because they are necessary for protein synthesis, in particular during phases of bodily growth, and for neurotransmission. For *D. melanogaster*, 10 amino acids are regarded as essential, i.e. are required in their food for female flies to maintain egg production (Sang and King, 1961). Toshima and Tanimura (2012) found that adult *D. melanogaster* enhance their feeding preference for amino acids when they are deprived of amino acids, and recent findings have shown that deprivation of even a single essential amino acid can induce an enhanced preference for yeast, *D. melanogaster*'s favourite 'prey' (Leitão-Gonçalves et al., 2017); this is arguably through changes in specific dopaminergic neurons in the brain (Liu et al., 2017) and modification of sensory sensitivity to yeast (Steck et al., 2018; for pioneering work in the locust, see Simpson and Simpson, 1992). These findings suggest that *D. melanogaster* foraging behaviour for proteinogenic food is regulated according to the animal's internal needs (see also Simpson and Raubenheimer, 1993). Compared with other nutrients such as carbohydrates, however, much less is known about the sensory and central-brain processing of amino acids in *D. melanogaster* (Toshima and Schleyer, 2019; see also Discussion), and about how these animals learn about them. Indeed, in adult *D. melanogaster*, to the best of our knowledge, no studies have been published using amino acids as tastant reinforcers.

In larval *D. melanogaster*, all 20 amino acids have been reported to be rewarding when assayed individually (Kudow et al., 2017). However, it is unlikely that animals ever meet a food containing only single amino acids under natural conditions. We therefore decided to investigate the effect of a pseudo-natural 20 amino-acid mixture on the associative learning of *D. melanogaster* larvae. We decided that it made sense to use an 'egalitarian' mixture with all the individual amino acids present at the same concentration, given that previously used non-egalitarian recipes have each been found to have distinct advantages and disadvantages (e.g. Piper et al., 2014, 2017; Bjordal et al., 2014). The total amino acid concentration was chosen on the basis of our previous learning experiments (Kudow et al., 2017). Of note is that, unlike adult flies, which are able to survive without obtaining amino acids, larvae continuously require proteinogenic food for growth, and thus are more likely to be motivated to learn about amino acids. Surprisingly, we found that the AA mixture has not only a rewarding but also a punishing effect. Both of these effects will be characterized in detail at the behavioural level.

MATERIALS AND METHODS

Larvae

Drosophila melanogaster Meigen 1830 were maintained on standard medium at 25°C and under a 12 h:12 h light:dark cycle. Canton-Special (CS) was used as a wild-type strain. Third-instar larvae (5 days after egg laying) were collected from their culture vials, briefly rinsed with tap water and used for behavioural tests.

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Chemicals

We used *n*-amyl acetate (AM; CAS: 628-63-7; Merck, Darmstadt, Germany) as the odour. The AM was diluted 1:20 in paraffin oil (CAS: 8042-47-5; AppliChem, Darmstadt, Germany), filled into custom-made Teflon containers (10 µl) and used for learning and innate odour preference experiments.

To prepare a 19 amino-acid mixture stock solution, L-alanine (CAS: 56-41-7), L-arginine (CAS: 74-79-3), L-asparagine (CAS: 70-47-3), L-aspartic acid (CAS: 56-84-8), L-cysteine (CAS: 52-90-4), L-histidine (CAS: 71-00-1), L-isoleucine (CAS: 73-32-5), L-leucine (CAS: 61-90-5), L-lysine (CAS: 56-87-1), L-glutamine (CAS: 56-85-9), L-glutamic acid (CAS: 56-86-0), L-glycine (CAS: 56-40-6), L-methionine (CAS: 63-68-3), L-phenylalanine (CAS: 63-91-2), L-proline (CAS: 147-85-3), L-serine (CAS: 56-45-1), L-threonine (CAS: 72-19-5), L-tryptophan (CAS: 73-22-3) and L-valine (CAS: 72-18-4) were dissolved in distilled water at concentrations of 5 mmol l^{-1} for each amino acid (95 mmol l^{-1} in total) and stored in a freezer at −21°C until being used for the experiments. This 19 amino-acid mixture stock solution plus L-tyrosine (CAS: 60-18-4, at the same final concentration as the other 19 amino acids) was added to agarose (Roth, Karlsruhe, Germany) solution to prepare 1% agarose substrate with a 20 amino-acid mixture (AA mixture). L-tyrosine was added separately because of its low solubility. Amino acids were obtained from Sigma-Aldrich (Seelze, Germany).

Training and testing for appetitive memory

Experiments followed a standard associative learning paradigm (Gerber et al., 2013; Michels et al., 2017), with modification. We here describe our general procedure; deviations are mentioned in the Results.

In short, Petri dishes of 90 mm diameter were prepared with either pure 1% agarose or 1% agarose with AA mixture as a tastant substrate. For one experimental group, cohorts of approximately 30 larvae were collected from their culture vials and trained to associate the AA mixture as the tastant (+) with AM as the odour; i.e. AM was presented in a Petri dish with the AA mixture as substrate for 2.5 min, followed by a blank trial with an empty odour container (EM) presented in a Petri dish with only agarose, also for 2.5 min (paired training; AM+/EM). For the second experimental group, cohorts of larvae were trained reciprocally, i.e. they experienced the AA mixture tastant and the odour AM during separate trials (unpaired training; AM/EM+) (the sequence of training trials was reversed in half of the cases, namely EM/AM+ for paired training and EM+/AM for unpaired training).

After three such cycles of training, the larvae were transferred to the middle of a test Petri dish with pure agarose. An odour container with AM was placed on one side, and an empty (EM) container on the other side of the test Petri dish (sidedness was alternated across repetitions of the experiment). After 3 min, the number of larvae on the AM side (N_{AM}), on the EM side (N_{EM}) and in a 10 mm wide middle zone was counted and the preference for AM was calculated as:

$$\text{Pref} = \frac{N_{AM} - N_{EM}}{N_{Total}}. \quad (1)$$

Thus, positive Pref scores indicate attraction to the odour, whereas negative Pref scores indicate aversion to the odour.

To quantify associative memory, the performance index (PI) was calculated from the Pref scores of the reciprocally trained cohorts of larvae:

$$\text{PI} = \frac{\text{Pref}(\text{AM}+/\text{EM}) - \text{Pref}\,(\text{EM}+/\text{AM})}{2}. \quad (2)$$

Thus, PI scores can take values between −1 and 1. Positive PI values indicate appetitive and negative PI values indicate aversive associative memory.

Training and testing for aversive memory

The experimental procedure to test for aversive memory was as above except that the test Petri dish contained the AA mixture instead of pure agarose. This is because aversive memory in larvae is behaviourally expressed in the presence rather than the absence of the aversive reinforcer that was used during the training, as demonstrated for high-concentration salt as well as for quinine (Gerber and Hendel, 2006; Schleyer et al., 2011). Arguably, this is because learned odour avoidance is a form of escape behaviour that is adaptive only if the test situation does indeed warrant escape.

Odour preference experiments

To measure innate odour preference, i.e. odour preference in experimentally naive larvae, the animals were collected from their culture vials, directly followed by testing their preference for the odour AM as described above, using test Petri dishes featuring either pure 1% agarose or 1% agarose with an AA mixture as tastant.

Analyses of locomotion

Learning experiments were performed as described above, except that the number of larvae in a cohort was approximately 15, and the test Petri dishes were placed under a camera (Basler acA2040-90 µm) to video-track the larval behaviour. These videos were analysed offline by custom-written software (Schleyer et al., 2015b; Paisios et al., 2017). From the tracking data, we determined the time the animals spent on the odour side (T_{AM}) and the no-odour side (T_{EM}) throughout the 3-min test duration, and calculated the odour preference as:

$$\text{Pref}(\text{Tracked}) = \frac{T_{AM} - T_{EM}}{T_{Total}}. \quad (3)$$

We further sought to describe larval locomotion in more detail in order to reveal the microbehavioural 'footprint' of memory during the test. *Drosophila melanogaster* larvae navigate through odour gradients by a sequence of runs and lateral head movements that we call head casts (HCs). Following the analysis by Paisios et al. (2017), we focus on the modulations of HC behaviour (for more details on the definition and detection of HCs, see Paisios et al., 2017). Based on the number of HCs (N_{HC}) performed while the larva was either heading away from the odour source or heading towards it, the modulation of HC rate was calculated as:

$$\text{HC rate modulation} = \frac{N_{HC,away} - N_{HC,towards}}{N_{HC,away} + N_{HC,towards}}. \quad (4)$$

This score will be positive if larvae make more HCs when they are heading away from the odour source than when heading towards it, a behaviour that would take them towards the odour source.

Further, we asked whether the larvae direct their HCs towards rather than away from the odour source. To measure this, we determined the absolute heading angle (absHA) – i.e. the orientation of the larva's head segment relative to the odour source – before an HC and after it, and calculated the reorientation brought about by that HC as:

$$\text{Reorientation per HC} = \text{abs}(\text{HA before HC}) - \text{abs}(\text{HA after HC}). \quad (5)$$

This score will be positive when an HC gets the larva 'on target', i.e. modulates its heading angle towards the odour source rather than away from it.

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Statistics

Non-parametric statistics were applied throughout, using Statistica 13.0 (StatSoft software, Hamburg, Germany). For multiple-group comparisons, Kruskal–Wallis tests were applied, and if significant, were followed by pairwise comparisons with Mann–Whitney *U*-tests. One-sample sign tests were applied to test significant differences from zero. When multiple tests were applied in one experiment, Bonferroni–Holm corrections were used to maintain error rates below 5%. Data are presented as box plots (the median as the middle line; 25%, 75% quantiles as the box boundaries; 10%, 90% quantiles as the whiskers).

RESULTS

A 20 amino-acid mixture can have both a rewarding and a punishing effect

A previous study showed that when employed individually, all 20 common amino acids have a rewarding effect in *D. melanogaster* larvae, and that the reward strength does not differ between amino acids (Kudow et al., 2017). To test whether a pseudo-natural mixture composed of equal concentrations of all these 20 amino acids also has a rewarding effect for larval *D. melanogaster*, we performed odour–taste associative learning experiments.

Different groups of larvae received either paired or unpaired training of the odour AM with a 10 mmol l^{-1} AA mixture as tastant reinforcer. The associative PI was determined as the difference in odour preference between the paired–trained and the unpaired–trained larvae (see Materials and Methods). Larvae showed positive PI scores, i.e. appetitive memory, when they were tested on a pure agarose substrate (Fig. 1A); these appetitive memory scores were about as strong as those previously observed for individual amino acids (Kudow et al., 2017). In contrast, when the larvae were tested in the presence of the 10 mmol l^{-1} AA mixture, negative PIs were observed, indicating aversive memory. While these results appear surprising at first sight, we note that according to previous studies, appetitive memory can be viewed as a search behaviour for the reward, which is only expressed as long as the sought-for reward is absent, whereas aversive memory can be viewed as an escape behaviour, which is only expressed as long as the test situation indeed warrants escape (Craig, 1918; Gerber and Hendel, 2006). The common denominator of such learned search/escape is thus that the larvae show learned behaviour only if this promises an improvement in their situation: that is, the gain of a reward in the appetitive case, and relief from punishment in the aversive case. Accordingly, our present results indicate that a 10 mmol l^{-1} AA mixture has both a rewarding and a punishing effect during training and that during the test, the larvae behaviourally express the resulting appetitive and aversive memories selectively in accordance with the conditions of that test. Critically, this effect of the test conditions pertains to learned but not to innate olfactory behaviour (Fig. 1B).

We note that these results are reminiscent of what Niewalda et al. (2008) observed for sodium chloride. While low concentrations of sodium chloride are rewarding and high concentrations are punishing, their results suggest that at intermediate concentrations sodium chloride is not unvalenced, but rather that both appetitive and aversive memories may be established (as with the present results, innate olfactory behaviour was not affected by the presence of sodium chloride). This prompted the question of whether the rewarding and punishing effects of the 10 mmol l^{-1} AA mixture are likewise concentration dependent.

Concentration dependency of AA mixture learning

To test for the dependency of appetitive memory on the concentration of the AA mixture, we trained larvae using different AA mixture concentrations and tested them on a pure agarose substrate. The larvae showed appetitive memory at all the tested concentrations, with memory scores increasing with increasing concentrations of the AA mixture (Fig. 2A).

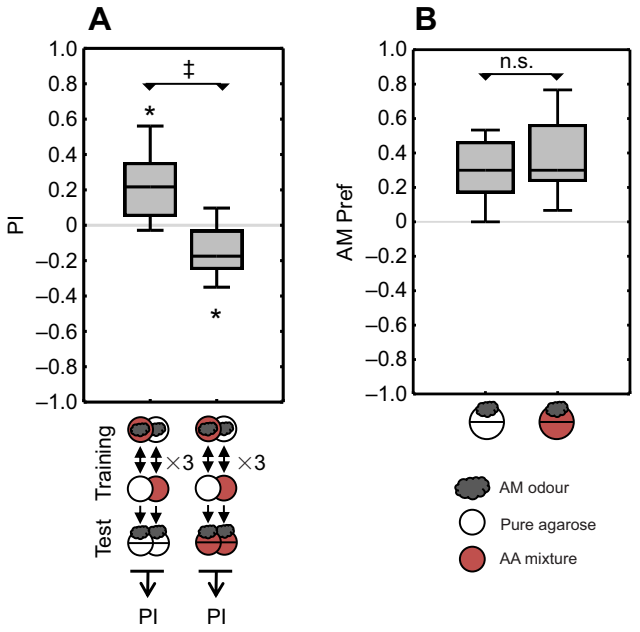

Fig. 1. A 20-amino-acid mixture (AA mixture) can have both a rewarding and a punishing effect. (A) Wild-type third-instar *Drosophila melanogaster* larvae were trained and tested for associative memory as shown below the graph. The clouds indicate odour presentation, the circles Petri dishes with either pure agarose as substrate (white circles) or agarose with a 10 mmol l^{-1} AA mixture as tastant in addition (red circles). The larvae either received paired presentations of odour with the AA mixture as tastant reinforcement, or the odour was presented unpaired from the AA mixture. The performance index (PI) was then calculated based on the difference in odour preference after paired versus unpaired training (see Materials and Methods). Positive PI scores indicate appetitive associative memory, whereas negative PI scores indicate aversive associative memory. When the larvae were tested on a pure agarose Petri dish, appetitive memory was behaviourally expressed (left), whereas in the presence of the AA mixture, aversive memory was expressed (right) (‡P<0.000001, U=135, Mann–Whitney U-test; *P<0.05, one-sample sign tests, Bonferroni–Holm corrected, exact P-values are given in Table S1; N=35, 35). The preference values underlying the PI scores are given in Fig. S1 and Table S1. (B) Odour preferences of experimentally naive larvae, determined in either the absence or the presence of the AA mixture, showing that innate odour preference is not affected in the presence of the AA mixture (n.s.: P=0.53, U=142, Mann–Whitney U-test, N=18, 18). Data are presented as box plots (the median as the middle line; 25%, 75% quantiles as the box boundaries; 10%, 90% quantiles as the whiskers).

To test likewise for aversive memory, the experiment was performed as above, yet testing was carried out in the presence of the AA mixture at the respective training concentration. The higher the concentration of the AA mixture, the more negative the memory scores; indeed, aversive memory was observed only at concentrations of 3.3 mmol l^{-1} or higher (Fig. 2B). We note that AA mixture concentrations higher than 20 mmol l^{-1} could not be tested because of the low solubility of at least some of the individual amino acids.

These results reveal the dose-dependency of both appetitive and aversive memory for a pseudo-natural AA mixture (summarized in Fig. 2C). Of note is that at concentrations of around 10 mmol l^{-1}, the AA mixture establishes robust appetitive and aversive memories, which are behaviourally expressed by the larvae depending on the test conditions. On the basis of these results, we focused on studying the

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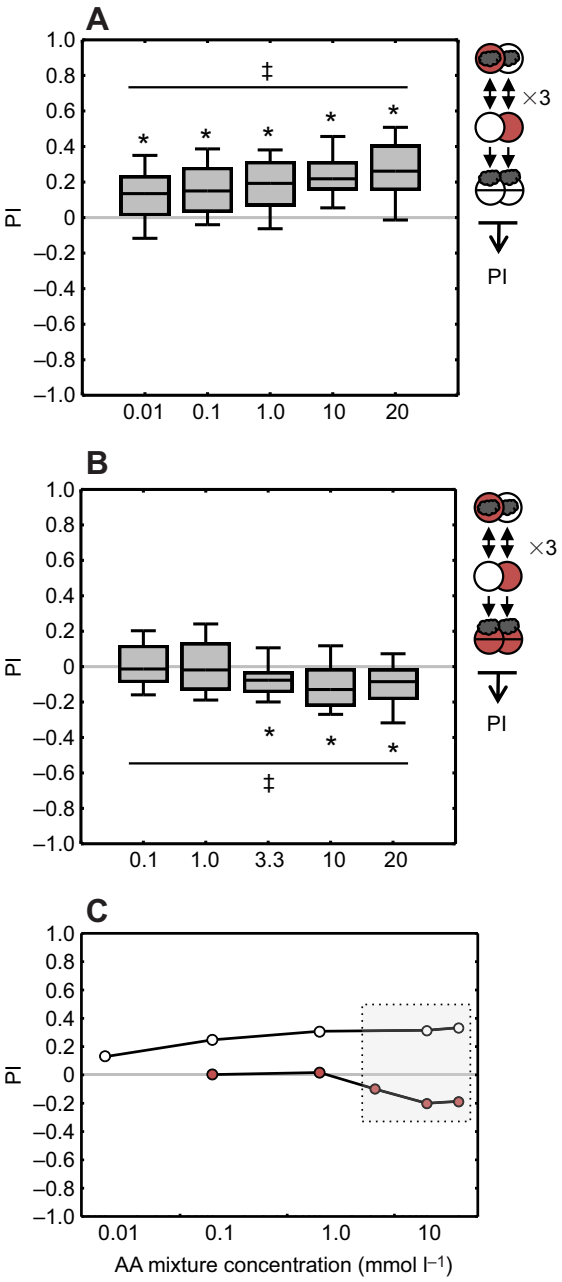

Fig. 2. Concentration dependency of AA mixture learning. (A) Odour–AA mixture learning experiments were performed as described in Fig. 1A, at the indicated concentrations of the AA mixture. The testing aimed to reveal appetitive associative memories and was therefore carried out on a pure agarose substrate. Appetitive memory, quantified by positive PIs, increased with AA mixture concentration ($^{‡}P$=0.025, H=11, Kruskal–Wallis test) and was observed for all AA mixture concentrations (*P<0.05, one-sample sign tests, Bonferroni–Holm corrected, exact P-values are given in Table S1; N=32, 31, 30, 30, 30). The preference values underlying the PI scores are given in Fig. S2A and Table S1. (B) Odour–AA mixture learning experiments were performed as described in A, but in this case the aim was to reveal aversive memory. Therefore, the testing was carried out on a substrate including the AA mixture at the respective training concentration. Aversive memory, quantified by negative PIs, increased with AA mixture concentration ($^{‡}P$=0.0028, H=16, Kruskal–Wallis test) and was observed for AA mixture concentrations of 3.3 mmol l^{-1} and higher (*P<0.05, one-sample sign tests, Bonferroni–Holm corrected, exact P-values are given in Table S1; N=32, 30, 30, 30, 30). The preference values underlying the PI scores are given in Fig. S2B and Table S1. (C) Summary of the results from A and B, plotting the median PI scores across AA mixture concentration (white circles represent data from A, red circles data from B). Both appetitive and aversive memories can be revealed when larvae are tested after training with high concentrations (shaded area). Further details as in the legend of Fig. 1.

'Janus-faced' reinforcing effects of the 10 mmol l^{-1} AA mixture in more detail.

Appetitive and aversive memory scores for the AA mixture increase with the number of training trials

It was recently found that aspartic acid as an individual amino acid is effective as a reward when three training trials are used (as in the above experiments), but not when only one training trial is carried out (Weiglein et al., 2019). We therefore asked whether the AA mixture is effective in bringing about appetitive/aversive memory when either 1, 3 or 6 training trials are used. It turned out that neither appetitive memory (Fig. 3A) nor aversive memory (Fig. 3B) was observed after 1 training trial, whereas both appetitive and aversive memory were detectable after 3 or 6 training trials. As PI scores reached a plateau with 3 training trials, we chose to continue the following experiments with this protocol.

Appetitive memory for the AA mixture is slightly more stable over time than aversive memory

Memory typically decays over time, but how quickly this decay occurs differs characteristically across species, paradigms, reinforcers and valence domains. We were therefore curious about how temporally stable appetitive and aversive memory for the AA mixture are. To investigate this for appetitive memory, we trained the larvae as described above, using 3 training trials, and tested them on a pure agarose substrate after retention periods of 0, 5, 30 or 60 min. Appetitive memory decayed across this time period, yet remained detectable until at least 30 min (Fig. 4A).

For aversive memory, we trained the larvae the same, yet tested them in the presence of the AA mixture, after retention periods of 0, 5, 10, 20, 30 or 60 min. Aversive memory decayed across this time period, and was detectable only for 5 min or less after training (Fig. 4B).

To allow for a direct comparison of the strengths (irrespective of valence) of memory at the 30 min retention period, the PI scores obtained from testing in the presence of the AA mixture were sign-inverted. This comparison revealed a small yet significant difference in memory strength between appetitive and aversive memories (Fig. 4C). This result suggests to us that appetitive memory for the AA mixture is slightly more stable over time than aversive memory for the mixture. We note that if training induced a single, unvalenced 'amino-acid memory' that could be alternatively expressed as appetitive or aversive depending on the test conditions, the memory scores based on such a memory should have decayed by the same time. Taken at face value, our results therefore rather suggest an alternative working hypothesis, namely that appetitive and aversive memories for the AA mixture decay independently of each other. As will be argued in the Discussion, this scenario is also more plausible in the light of what is known about the division of labour between dopaminergic neurons in mediating either reward- or punishment-related signals.

Starvation leaves appetitive memory for the AA mixture intact but reduces aversive memory

In adult flies, starvation leads to particularly strong appetitive associative memory scores (Krashes and Waddell, 2008; Gruber et al., 2013), whereas strong aversive memory can be observed without starvation. Therefore, we wondered whether, in the larvae,

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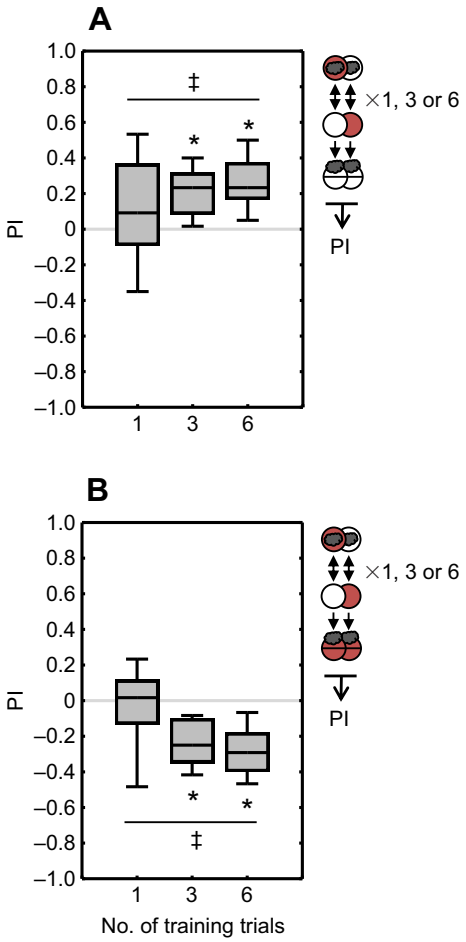

Fig. 3. Appetitive and aversive memory scores for the AA mixture increase with the number of training trials. (A) Odour–AA mixture learning experiments were performed as described in Fig. 1A, with the indicated number of training cycles, using a 10 mmol l^{-1} AA mixture. The testing was aimed at revealing appetitive associative memories and was therefore carried out on a pure agarose substrate. Appetitive memory increased with the number of training trials ($^{‡}P$=0.036, H=6.7, Kruskal–Wallis test) and was observed after 3 and 6 cycles of training (*P<0.05, one-sample sign tests, Bonferroni–Holm corrected, exact P-values are shown in Table S1; N=32, 32, 32). The preference values underlying the PI scores are shown in Fig. S3A and Table S1. (B) Odour–AA mixture learning experiments were performed as described in A, but in this case aimed at revealing aversive memory. Therefore, the testing was carried out on a substrate including a 10 mmol l^{-1} AA mixture. Aversive memory increased with AA mixture concentration ($^{‡}P$=0.0072, H=9.9, Kruskal–Wallis test) and was observed after 3 and 6 cycles of training (*P<0.05, one-sample sign tests, Bonferroni–Holm corrected, exact P-values are given in Table S1; N=18, 18, 18). The preference values underlying the PI scores are given in Fig. S3B and Table S1. Further details as in the legend of Fig. 1.

stronger appetitive and/or aversive memory scores would be observed for the AA mixture if a 60 min period of starvation was introduced before training. In a 3-fold divergence from the aforementioned case of adult flies, it was (i) appetitive memory that was not measurably affected (Fig. 5A), whereas (ii) aversive memory was clearly affected by pre-training starvation, and specifically was (iii) reduced by it (Fig. 5B). This reduction in aversive memory scores does not reflect a general, valence-independent detrimental effect of starvation on memory function (or on olfactory or motor function, for that matter), because appetitive memory is not measurably decreased. Equally, the reduction in aversive memory scores does not come about by a hidden, starvation-induced increase in how rewarding the AA mixture is to the larvae – indeed, appetitive memory is not increased, either. These inferences are confirmed by direct comparison of memory strength after 60 min starvation (Fig. 5C). Given that the compromised formation of an unvalenced 'amino-acid memory' through pre-training starvation should reduce appetitive and aversive memory scores equally, our results rather suggest the parallel establishment of appetitive and aversive AA-mixture memories. Again, we note that this is also the neurobiologically more plausible scenario (see Discussion).

Shared and inverse locomotor footprint of appetitive and aversive AA mixture memories

In larval *D. melanogaster*, appetitive and aversive memories have been found to modulate the same parameters of locomotion, with inverse sign, to bring about appetitive and aversive learned behaviour, respectively (Paisios et al., 2017). As these results were obtained for fructose as reward and quinine as punishment, we wondered whether the same is true for the appetitive and aversive memories established by one and the same tastant, namely, the AA mixture. We therefore trained the larvae and video-tracked their behaviour for offline analysis with the custom-written script from Paisios et al. (2017) (Fig. 6A,B).

For appetitive memory, we first confirmed that for the video-tracked data, which consider the complete test period rather than just end-point counting, preferences are also higher after paired training of odour and the AA mixture than after unpaired training (Fig. 6C). This difference between paired- and unpaired-trained animals is brought about by a stronger tendency of the paired-trained animals to head-cast more when heading away from the odour than when heading towards it (Fig. 6D); in other words, after paired training, the larvae are more strongly biased to run straight towards the odour than after unpaired training. Furthermore, those HCs that are performed by the larvae are more strongly biased to align them in the direction of the odour source after paired than after unpaired training (Fig. 6E). This corresponds to what has previously been described when fructose was the reward (Paisios et al., 2017).

For aversive memory established by the AA mixture, these same aspects of locomotion were modulated, with opposite sign (Fig. 6F–H), matching what was previously reported with quinine as the punishment (Paisios et al., 2017).

We note that the results from Fig. 6, showing shared memory-modulated features of locomotion, do not argue against the parallel organization of appetitive and aversive memory for the AA mixture. Rather, they suggest that the same 'final stretch' in the organization of locomotion is modulated by these memories, with opposite sign (also see Paisios et al., 2017).

DISCUSSION

Appetitive and aversive memories for a 20 amino-acid mixture

To the best of our knowledge, this study is the first to systematically analyse the reinforcing effect of a pseudo-natural 20 amino-acid mixture, in any animal. It shows that the valence of reinforcement by such a mixture depends on its concentration (Fig. 2). Significantly, for an intermediate concentration, robust appetitive and aversive memories are observed, which are somewhat weaker in strength compared with various other rewarding or punishing tastants or with reinforcement through optogenetic stimulation of dopaminergic neurons (Schroll et al., 2006; Gerber and Hendel, 2006; Niewalda et al., 2008; Schleyer et al., 2015a; Rohwedder et al., 2016; Saumweber et al., 2018; Eschbach et al., 2019 preprint). Observing both appetitive and aversive memory for the AA mixture is possible in

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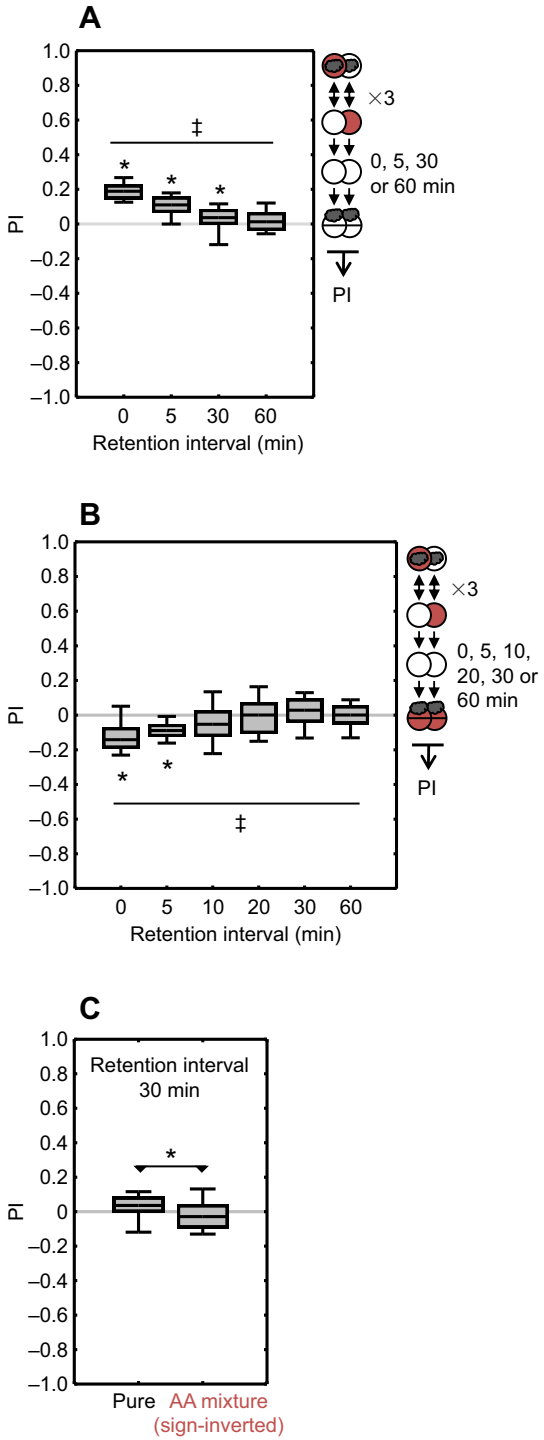

Fig. 4. Appetitive memory for the AA mixture is slightly more stable over time than aversive memory. (A) Odour–AA mixture learning experiments were performed as described in Fig. 1A, with the indicated retention period between training and test. For the retention interval, the larvae were transferred to a pure agarose plate. Appetitive memory decreased with retention interval (‡P<0.0001, H=62, Kruskal–Wallis test) and was observed until 30 min after training (*P<0.05, one-sample sign tests, Bonferroni–Holm corrected, exact P-values are given in Table S1; N=30, 30, 30, 30). The preference values underlying the PI scores are given in Fig. S4A and Table S1. (B) Odour–AA mixture learning experiments were performed as described in A with testing carried out on a substrate including a 10 mmol l^{-1} AA mixture to reveal aversive memory. Aversive memory decreased with retention interval (‡P<0.0001, H=61, Kruskal–Wallis test) and was no longer observed from 10 min after training onwards (*P<0.05, one-sample sign tests, Bonferroni–Holm corrected, exact P-values are given in Table S1; N=68, 30, 38, 38, 67, 30). The preference values underlying the PI scores are given in Fig. S4B and Table S1. The display in B combines data from two experiments shown in Fig. S5A,B. (C) To compare the strengths of appetitive and aversive memories at the 30 min retention interval irrespective of their valence, the PI scores when tested on the AA mixture were sign-inverted and then the Mann–Whitney U-test was applied (*P=0.0035, U=682, Mann–Whitney U-test, Bonferroni–Holm corrected; N=30, 67). Further details as in the legend of Fig. 1.

practice because they can be separately revealed by manipulating the test conditions (Figs 1–6) (Craig, 1918; Schleyer et al., 2011). Appetitive memory is observed as a learned search, which is expressed only as long as the sought-for reward is absent during the test and the search is therefore still adaptive. Conversely, aversive memory is observed as a learned escape, which is adaptively expressed only when the punishment is present during the test and escape is actually warranted. Indeed, pre-training starvation reduces the punishing effect of the AA mixture but leaves its rewarding effect intact (Fig. 5). In other words, when in need, search-related processing is left intact but escape-related processing is adaptively curbed.

Our results not only show a difference between the appetitive and the aversive memories for the AA mixture in terms of the aforementioned case of susceptibility to pre-training starvation (Fig. 5), but also suggest a difference in terms of their temporal stability (Fig. 4). These differences are not consistent with a unitary reinforcement process. That is, such differences would not be observed if, during training, just a single, unvalenced memory were established for the association of odour and the AA mixture, which was then retrieved as either appetitive or aversive. Indeed, we are not aware of any case of unvalenced reinforcement; neurons activated by both rewarding and punishing stimuli would be assumed to mediate attention rather than reinforcement. Without further assumptions, our results therefore suggest the alternative working hypothesis, namely that the AA mixture simultaneously establishes two parallel memories for the odour that are of opposite valence, and that these can subsequently be retrieved, or not, independently of each other. Does the peripheral or the central-brain representation of amino acids in *D. melanogaster* offer clues about exactly how such ‘Janus-faced’ reinforcement by the AA mixture could come about?

Peripheral and central processing of amino acid taste in *D. melanogaster*

Peripherally, different subsets of the larva’s gustatory sensory neurons express different members of the *Ir* gene family (Croset et al., 2016). These contribute to the sensing of, the innate preference for, and the feeding-modulating effects of individually assayed amino acids, conceivably in a combinatorial manner (Croset et al., 2016; for adults: Ganguly et al., 2017; Steck et al., 2018). Members of the *Gr* receptor family (Robertson et al., 2003) have so far not been implicated in amino acid sensing. Interestingly, in adult s-type labellar sensilla, some amino acids including tryptophan activate sensory neurons that are also activated by bitter substances (Park and Carlson, 2018); if such cells existed in larvae, too, they would certainly be candidates to mediate the punishing effects of amino acids. In contrast, sugar cells housed in adult l-type labellar sensilla are not activated by amino acids (Dahanukar et al., 2007; Park and Carlson, 2018), whereas *Ir*-expressing neurons in the adult tarsi respond both to amino acids and sugar (Ganguly et al., 2017). Even so, the exact relationships of amino acids (or their mixtures) and the various behaviours affected by them to *Ir* function

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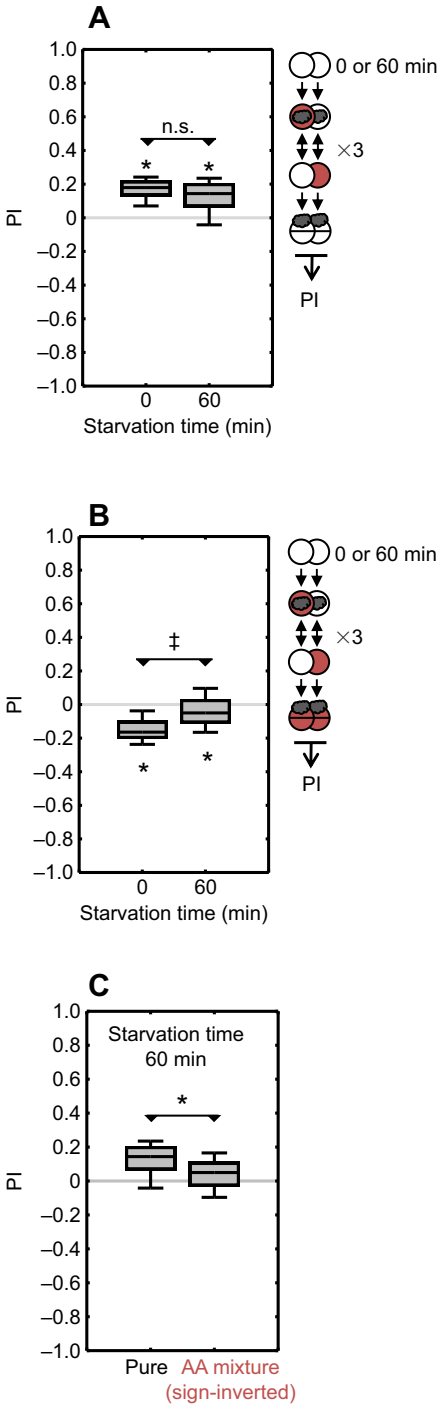

Fig. 5. Starvation leaves appetitive memory scores for the AA mixture intact but reduces aversive memory scores. (A) Odour–AA mixture learning experiments were performed as described in Fig. 1A, either with or without starvation for 60 min before training. For the starvation period, the larvae were transferred to a pure agarose plate. Appetitive memory was observed in both cases (*P<0.05, one-sample sign tests, Bonferroni–Holm corrected, exact P-values are given in Table S1), and it was not altered by starvation (n.s.: P=0.068, U=326, Mann–Whitney U-test) (N=30, 30). The preference values underlying the PI scores are given in Fig. S6A and Table S1. (B) Odour–AA mixture learning experiments were performed as described in A, with testing carried out on a substrate including a 10 mmol l^{-1} AA mixture to reveal aversive memory. Aversive memory was observed in both cases (*P<0.05, one-sample sign tests, Bonferroni–Holm corrected, exact P-values are given in Table S1), yet memory scores were reduced upon starvation (‡P=0.00004, U=171, Mann–Whitney U-test) (N=30, 30). The preference values underlying the PI scores are given in Fig. S6B and Table S1. (C) To compare the strengths of appetitive and aversive memories after 60 min starvation irrespective of their valence, the PI scores when tested on the AA mixture were sign-inverted and then the Mann–Whitney U-test was applied (*P=0.0044, U=242, Mann–Whitney U-test, Bonferroni–Holm corrected; N=30, 30). Further details as in the legend of Fig. 1.

and sensory neuron physiology remain to be revealed. In other words, from the relatively little that is known to date about the organization of the afferent processing of amino acids in *D. melanogaster*, no sound argument can be derived about exactly how the parallel formation of appetitive and aversive reinforcement through the present 20 amino-acid mixture comes about (we note that in *Grammia geneura* caterpillars, methionine activates both feeding-stimulatory and -deterrent sensory neurons: Bernays and Chapman, 2001; see also Lim et al., 2019 reporting a novel amino-acid receptor in bees). Behavioural experiments in *D. melanogaster* larvae have so far only revealed that all 20 amino acids in the mixture can be rewarding when used individually (Kudow et al., 2017), but it is not known whether any of them can likewise have a punishing effect, or whether such a punishing effect can result from any specific combination of amino acids. A systematic analysis of the latter possibility would require testing for the concentration dependency of reinforcement for all 20 amino acids, with testing carried out in a way that can reveal either appetitive or aversive memory (20 amino acids×5 concentrations×2 testing conditions=200 experimental groups), possibly followed by iterating this 5×2 experimental design for up to 1,048,554 possible mixtures.

In the central brain, distinct sets of aminergic neurons mediate either reward (Schroll et al., 2006; Rohwedder et al., 2016; Eichler et al., 2017; Saumweber et al., 2018) or punishment signals (Schroll et al., 2006; Eichler et al., 2017; Eschbach et al., 2019 preprint) to be associated with olfactory activation at their distinct target compartments in the mushroom body (Thum and Gerber, 2019). Neither in larval nor adult *D. melanogaster*, nor in any other insect, have such neurons been described as being activated by both rewards and punishments; such an activation profile, as noted above, would be suggestive of a role in attentional rather than in reinforcement processing. However, it remains unknown which mushroom body input neurons mediate the rewarding effects and which mediate the punishing effects of the AA mixture, or whether there is a role for central-brain amino-acid sensors in this respect (Bjordal et al., 2014). Of note is that within the appetitive domain, silencing the dopaminergic mushroom body input neuron DAN-h1 impairs associative learning through fructose, but not through aspartic acid reward (Saumweber et al., 2018). This fits with behavioural data showing that fructose and aspartic acid memories can be distinct (Schleyer et al., 2015a). In any event, given that the specific neurogenetic basis for how the present 20 aa mixture can confer both a rewarding and a punishing effect remains enigmatic, the question arises of whether there is any precedent for such ‘Janus-faced’ memory formation in *D. melanogaster*.

Learning about good and bad taste

In larval *D. melanogaster*, Niewalda et al. (2008) have shown that low concentrations of sodium chloride have a rewarding effect, whereas high concentrations are punishing. Their data suggest that at intermediate concentrations, both of these processes take place. This could be based on the activation of both low- and high-threshold sensory neurons at intermediate concentrations (Hiroi et al., 2004; Jaeger et al., 2018). However, whether and how low- and high-threshold sensory neurons map salt information separately to

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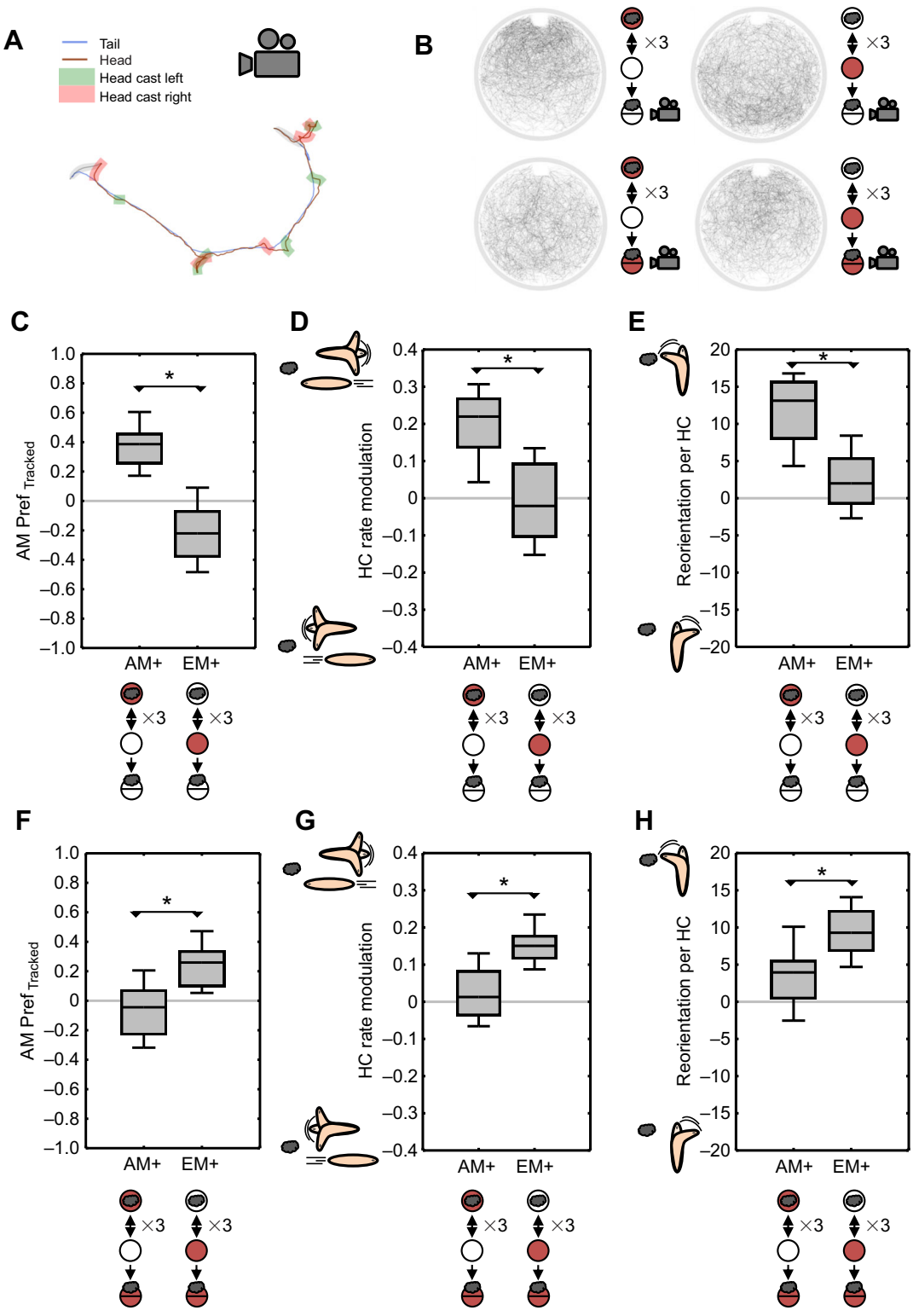

Fig. 6. See next page for legend.

central-brain reward and punishment pathways remains unknown. Notably, acetic acid, which is both a nutritious and a harmful substance, activates both sweet and bitter taste neurons, and apparently confers opposite valence in adults, depending on their hunger state; specifically, in starved flies, acetic acid induces proboscis extension, whereas in fed flies, acetic acid added to a sugar solution prevents the proboscis extension otherwise elicited by the sugar (Devineni et al., 2019; also see König et al., 2014). How acetic acid information is relayed onto internal reinforcement pathways and whether mnemonic processing is likewise affected remain unknown.

In adult *D. melanogaster*, Das et al. (2014) found that after training with a mix of sugar and the bitter substance DEET, flies showed aversive memory when tested immediately after training; however, appetitive memory was observed after longer retention

Fig. 6. Shared and 'inverse' locomotor footprint of appetitive and aversive AA mixture memories. Odour–AA mixture learning experiments were performed as described in Fig. 1A. After AA mixture training, either a pure test substrate (C–E) or a substrate including the AA mixture (F–H) was used. In all cases, the animals were video-tracked and the data were analysed offline by custom-written analysis software (Schleyer et al., 2015b; Paisios et al., 2017). AM+ represents paired training of the AM odour and the AA mixture, and EM+ represents presentation of the AM odour unpaired from the AA mixture. (A) An example of a track of a single larva showing the locomotion of the larva through relatively straight runs and lateral head movements (head cast, HC). (B) Superimposed trajectories of all larvae tested on a pure agarose substrate after paired training (upper left), and after unpaired training (upper right). Tracks of larvae tested on a substrate containing the AA mixture after paired training (lower left) and after unpaired training (lower right) are likewise shown. (C–E) Results for the larvae tested on a pure substrate, revealing appetitive memory and the underlying modulations of locomotion. (C) Based on the video-tracked data, the preference scores for the AM odour are shown, revealing higher preference after paired than after unpaired training (**P*<0.000001, *U*=2.00, Mann–Whitney *U*-test; *N*=24, 24). (D) The modulation of the HC rate is shown. The scores are positive if larvae make more HCs when they are heading away from the odour source than heading towards it, a behaviour that would bring the larvae towards the odour source; this is more pronounced after paired than after unpaired training (**P*<0.000001, *U*=39.0, Mann–Whitney *U*-test; *N*=24, 24). (E) The modulation of HC direction is shown as the reorientation per HC. The scores are positive when, through an HC, the larvae modulate their heading angle towards rather than away from the odour source, a behaviour that would bring the larvae towards the odour source; this is more pronounced after paired than after unpaired training (**P*=0.000002, *U*=59.0, Mann–Whitney *U*-test; *N*=24, 24). (F–H) Results for the larvae tested on a substrate containing the AA mixture, revealing aversive memory and the underlying modulations of locomotion. (F) Based on the video-tracked data, the preference scores for the AM odour are shown, revealing lower preference after paired than after unpaired training (**P*=0.000019, *U*=80.0, Mann–Whitney *U*-test; *N*=24, 24). (G) Modulation of the HC rate. The score is lower after paired than after unpaired training (**P*=0.000002, *U*=59.0, Mann–Whitney *U*-test; *N*=24, 24). (H) The modulation of HC direction is shown as the reorientation per HC. The score is lower after paired than after unpaired training (**P*=0.000025, *U*=83.0, Mann–Whitney *U*-test; *N*=24, 24). The data are given in Table S1. Further details as in the legend of Fig. 1.

intervals. This suggests that when trained with a sweet–bitter compound, flies do not establish an averaged unitary memory, but simultaneously establish parallel, oppositely valenced memories for sugar and DEET, differing in their rate of consolidation. Although it seems clear that sensory processing for sugars and bitter substances is distinct (Thorne et al., 2004; Wang et al., 2004; but see Meunier et al., 2003; König et al., 2014), how sweet and bitter sensory input is relayed to internal reinforcement circuits has not been revealed in detail (but see Yagi et al., 2016 for adults; Hückesfeld et al., 2016; Miroschnikow et al., 2018 for larvae).

In tackling the issues discussed above, *D. melanogaster* larvae will be a versatile study case. This is because of the richness of the available genetic tools, and because analyses can draw on an electron microscopy atlas of its olfactory and gustatory pathways including the mushroom body (e.g. Eichler et al., 2017) and on a light microscopy atlas of most of the neurons in its central nervous system (e.g. Li et al., 2014). Last but not least, specific transgenic drivers allow many of these neurons to be manipulated one at a time (e.g. Saumweber et al., 2018; Eschbach et al., 2019 preprint).

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: N.T., B.G.; Validation: N.T., M.K.W.; Formal analysis: N.T., A.W., F.B.; Investigation: N.T., M.K.W.; Writing - original draft: N.T., B.G.; Writing - review & editing: N.T., A.W., B.G.; Visualization: N.T.; Supervision: N.T., B.G.; Project administration: N.T.; Funding acquisition: N.T., B.G.

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Supplementary information
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