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ORIGINAL PAPER

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# The carrot, not the stick: appetitive rather than aversive gustatory stimuli support associative olfactory learning in individually assayed *Drosophila* larvae

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**Abstract** The ability to learn is universal among animals; we investigate associative learning between odors and "tastants" in larval *Drosophila melanogaster*. As biologically important gustatory stimuli, like sugars, salts, or bitter substances have many behavioral functions, we investigate not only their reinforcing function, but also their response-modulating and response-releasing function. Concerning the response-releasing function, larvae are attracted by fructose and repelled by sodium chloride and quinine; also, fructose increases, but salt and quinine suppress feeding. However, none of these stimuli has a nonassociative, modulatory effect on olfactory choice behavior. Finally, only fructose but neither salt nor quinine has a reinforcing effect in associative olfactory learning. This implies that the response-releasing, response-modulating and reinforcing functions of these tastants are dissociated on the behavioral level. These results open the door to analyze how this dissociation is brought about on the cellular and molecular level; this should be facilitated by the cellular simplicity and genetic accessibility of the *Drosophila* larva.

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**Abbreviations** AM: Amylacetate · EMP: Empty · FRU: Fructose · LI: Learning index · NaCl: Sodium chloride · OCT: 1-octanol · PREF: Preference · QUI: Quinine hemisulfate · SOL: Solvent · +: Positive reinforcement · −: Negative reinforcement

## Introduction

Associative plasticity is a fundamental feature of behavior. We chose to characterize associative learning between odors and "tastants" in larval *Drosophila melanogaster*. These animals offer a fortunate combination of learning ability (Aceves-Pina and Quinn 1979; Heisenberg et al. 1985; Tully et al. 1994; Dukas 1998; Scherer et al. 2003; Gerber et al. 2004) and cellular simplicity. As the chemosensory system of the larva has recently become the focus of intense investigations on the molecular and cellular level (Cobb 1999; Heimbeck et al. 1999; Scott et al. 2001; Python and Stocker 2002; Gendre et al. 2003; Liu et al. 2003), and as the larval neuromuscular junction is a much-used preparation for the study of synaptic plasticity (Koh et al. 2000), our research on the behavioral level might be a helpful contribution towards a multi-level understanding of associative plasticity.

Beyond their potential to support olfactory learning, it is clear that gustatory stimuli play many different roles in the biology of animals. To analyze these behavioral functions, it seems useful to choose an organism for which gustation and feeding play a particularly prominent role. Larvae of the fruit fly *Drosophila* meet this demand as they are the main feeding stages in the life cycle of the fly. We chose to

investigate the behavioral functions of fructose (FRU), sodium chloride (NaCl) and quinine hemisulfate (QUI) in this animal. We did so with respect to three of their potential functions: (1) response-releasing, (2) response-modulating, and (3) reinforcing function (we use the term "reinforcer" synonymous with unconditioned stimulus).

To study the reinforcing function of gustatory stimuli in the *Drosophila* larva, we used the olfactory learning paradigm of Scherer et al. (2003). For that paradigm, larvae were exposed to one odorant, for example amylacetate, in the presence of FRU (+), and to a second odorant, for example 1-octanol, in the presence of NaCl (−) (AM+/OCT−). A second group of animals was trained reciprocally (AM−/OCT+). In a subsequent test, individual animals were given a choice between AM and OCT. Associative learning was shown by higher preferences for AM after AM+/OCT− training than after the reciprocal AM−/OCT+ training. In these experiments, the authors always used two potential reinforcers in a differential conditioning procedure, one presumably appetitive (FRU), the other presumably aversive (either NaCl or QUI). It was found that both combinations of stimuli (i.e. FRU-NaCl, and FRU-QUI) support learning. It remained unclear, however, whether FRU, NaCl or QUI alone would effectively support learning and would thus qualify as reinforcers. This issue is addressed in the current study, which uses either FRU, or NaCl or QUI alone as reinforcers. As only one reinforcer is used and the alternative odor is presented simply without any such overt reinforcer, we call this procedure " absolute" conditioning.

To study a potential nonassociative, response-modulating effect of FRU, NaCl, or QUI on olfactory choice behavior, we tested whether olfactory choice is altered when tested in the presence of either of these stimuli. It was previously shown (Scherer 2002) that presentation of NaCl immediately before testing did not have any effect on olfactory choice. That is, no evidence for a nonassociative, sensitization-like effect was found; therefore, in this study we chose a yet more rigid test and assayed olfactory choice in the presence of either FRU, NaCl, or QUI to see whether these substances modulate olfactory choice.

To study the response-releasing function of these three gustatory substances, we tested the gustatory choice of the larvae between two substrates. One substrate was plain agarose (PURE), whereas the other, in addition, contained either FRU, NaCl, or QUI. We also tested whether any of these substances induces or suppresses feeding behavior.

We found that FRU supports appetitive and NaCl and QUI aversive responses. However, none of these stimuli has a response-modulating, nonassociative effect on olfactory choice. Finally, only FRU but not NaCl and not QUI has an apparent reinforcing effect.

## Methods

### General

In all cases, experimenters were blind with respect to the experimental conditions (reinforcer presence and identity), which were decoded only after the experiment. Also, the experimental groups to be statistically compared were run in strict temporal parallelity to avoid false positive differences between groups which could result from variations over time as they are typical for behavior in invertebrates. All statistical analyses were performed with Statistica 6.0 for PC and/or StatView 4.51 for the Macintosh (significance level: $P < 0.05$).

We used third instar feeding stage larvae aged 5 days (± 12 h) after egg lay. Flies of the Canton-S wild-type strain were used and kept in mass culture, maintained at 25°C, 60–70% relative humidity and a 14/10 h light/dark cycle (12/12 h for Figs. 3, 5). Experiments were performed in red light under a fume hood at 20–24°C room temperature.

Petri dishes (Sarstedt, D) of 90-mm inner diameter were used throughout, unless stated otherwise. These were filled with 1% freshly boiled aqueous agarose solution (electrophoresis grade; Roth, D), which was then allowed to solidify, covered with their lids, and left untreated at room temperature until the following day. As potentially reinforcing "tastant" stimuli, we used fructose, (FRU, purity: 99%, Sigma–Aldrich, D), sodium chloride (NaCl, purity 99.5%, Fluka, D) or quinine hemisulfate (QUI, purity 92%, Sigma–Aldrich, D) added to the agarose solution after boiling. We used 2 mol FRU or 4 mol NaCl, respectively, dissolved in 1 l of water; for QUI, we used 2 g dissolved in 1 l to obtain a 0.2% w/w solution. These values are at the upper limit for NaCl and QUI, avoiding crystallization in the petri dishes.

Immediately before experiments, we replaced the regular lids of the petri dishes with lids perforated in the center by 15 1-mm holes to improve aeration. For experiments, a spoonful of food medium containing larvae was taken from the food bottle and transferred to a glass vial. From there, larvae were picked on demand, briefly washed, and transferred into the middle of the petri dish for the start of experiments.

In our choice of olfactory stimuli, we followed Scherer et al. (2003) and used 1-octanol (OCT, purity: 99.5%; Sigma-Aldrich, D) and amyl acetate (AM, purity: 99%, diluted 1:50 in paraffin oil; Sigma-Aldrich, D); at these concentrations, we expected naïve animals to show about equal distribution between the two odors in a choice assay (Scherer et al. 2003; Fig. 1). Odorants were applied by adding 10 µl of odor substance into Teflon containers (inner diameter 5 mm) which could be closed by a perforated lid (seven holes, 0.5-mm diameter). These containers were then placed onto the agarose surface in the petri dishes.

### Detectability of odors

To test for odorant detectability, we performed olfactory choice tests (for a sketch see insets of Fig. 1). Individual larvae were put into the middle of petri dishes containing pure agarose and two odorant containers on opposite sides, approximately 7 mm from the edges. The content of the containers differed on both sides to achieve a choice situation. Specifically, we compared choices between the sides equipped with an empty (EMP) container versus a container filled with AM dissolved in paraffin, a container with that solvent (SOL) versus OCT, with SOL versus EMP or with AM versus OCT. Larvae were allowed to move freely on the test plate and their position (defined by the mouth hooks) was scored every 20 s for 5 min as ''AM'', ''OCT'', or ''neutral'' (a 7-mm-wide zone in the middle of the assay plate). On these data, we calculated an odor preference for each animal. We determined the number of times a given animal was observed on the AM side during the test minus the number of times that animal was observed on the OCT side, divided by the total number of observations (if experiments involved EMP or SOL, calculations were done analogously):

$$\mathrm{PREF} = \frac{\text{number of observations}_{\mathrm{AM}} - \text{number of observations}_{\mathrm{OCT}}}{\text{number of observations}_{\mathrm{Total}}} \quad (1)$$

Thus, positive values indicate a preference of that animal for AM, and negative values a preference for OCT. These data were tested against random by one-sample sign tests and compared between groups with Mann-Whitney *U*-tests.

### Response-releasing function of ''tastants'': testing avoidance and approach

To test the response-releasing functions of FRU, NaCl and QUI, we performed gustatory choice assays. Larvae could choose between two substrates, one consisting of pure agarose (PURE), and one of agarose with a ''tastant'' added (for a sketch see insets of Fig. 2). Petri dishes of 52-mm inner diameter were equipped with a vertical barrier in the middle. These barriers were made from overhead transparencies and fixed to the plates with small stripes of tape. Parafilm was used to tighten the barrier. Then, the respective agarose solutions were gently poured into either side of the split petri dish to yield the desired combination of substrates on either side. Shortly before the substances solidified, the barriers were torn out. This procedure leads to a smooth yet sharp border between sides. After 20 min of cooling, the plates were covered with their standard lids and left at room temperature over night to be used for experiments on the next day. As this preparation of plates is rather laborious, we chose to use an en masse assay to determine gustatory choice. Groups of ten animals were put into the middle of the plate. Then animals could move about the plate for 15 min until we determined the number of animals located on either the ''tastant'' side or the PURE side. Animals that dug into the agarose or crawled up the lids of the plates were not considered in data analysis. A preference index was calculated as

$$\mathrm{PREF} = \frac{\text{number of animals}_{\mathrm{TASTANT}} - \text{number of animals}_{\mathrm{PURE}}}{\text{number of animals}_{\mathrm{Total}}} \quad (2)$$

Thus, positive values indicate attraction to the ''tastant'' while negative values indicate repulsion; to statistically analyze these data between tastants, Kruskal–Wallis and Mann–Whitney *U*-tests were used; to test the preference values against random levels, one-sample sign tests were used. We chose to allow 15 min for choice, as in mass assays larvae typically remain in a clump for a while before dispersing (Gordesky–Gold et al. 1996).

### Response-releasing function of ''tastants'': testing for an influence on feeding

To test for an influence of the ''tastants'' on feeding, carmine red (BDH Chemical Ltd., distributed via VWR International, Mississauga, Ontario, Canada) was added to the agarose, which upon feeding leads to staining of larval guts. We dissolved 200 mg carmine red powder in 200 µl distilled water and added to 100 ml hot 1% agarose solution, leading to a 0.2% final concentration of carmine red in the agarose; then, ''tastants'', either 2 M FRU, 4 M NaCl, or 0.2% QUI, were stirred into the hot agarose solution. Pure, dyed agarose plates were used as reference. Groups of ten larvae were placed onto such dyed agar plates. After 15 min of feeding, larvae were removed from the petri dishes, gently rinsed and placed in 70–90°C distilled water for approximately 20 s to achieve full body extension. They were then placed ventral side up on a small petri dish and digital images were taken. For each animal, the percent area of food intake was measured

by counting the number of red-dyed pixels in the gut (see Fig. 3) and dividing this by the number of pixels of the entire body. This measure is used to estimate the amount of dyed agarose swallowed. Statistical analyses of these data were done between ''tastant'' conditions with Kruskal–Wallis and Mann–Whitney *U*-tests. To compare feeding levels to zero, we used one-sample sign tests. We analyzed the same data also after classifying larvae as either ''eaters'' or ''noneaters'' and compared their frequencies by $\chi^2$ tests.

### Response-modulating function

To test for an influence of ''tastants'' on olfactory preferences, we performed olfactory choice tests as described above, but in the presence of the ''tastants'' (FRU, NaCl, QUI) (for a sketch see insets of Fig. 4); olfactory preferences were measured on pure agarose for comparison. Olfactory preferences were calculated as in Eq. 1; any difference in these preference data between PURE versus FRU, NaCl, or QUI as determined in a Kruskal–Wallis test would point to a nonassociative modulation of odor responses by ''tastants''. A one-sample sign test was used to test the pooled data from these groups against zero.

### Reinforcing function as tested in ''absolute'' conditioning

In ''absolute'' conditioning, we compare individual animals which underwent either of two reciprocal training regimes (for a sketch see Fig. 5a): one received AM with e.g. appetitive reinforcement by FRU (AM+) and OCT without reinforcement (AM+/OCT); the second was trained reciprocally (AM/OCT+). Then, animals from both treatment conditions are individually tested in a choice situation for their preference between AM versus OCT. Associative learning is indicated by differences of individuals from reciprocal treatment conditions during test. This conclusion is compelling as during training individuals from the AM+/OCT group and the AM/OCT+ group have identical exposure to odorants and reinforcement. What differs between treatment conditions is solely the contingency between them. Importantly, in all cases the reciprocally trained animals were run alternatedly, which allows stringent pairing of data for the calculation of a learning index (LI; see below).

A group of eight larvae was transferred to a training plate. These plates contained either pure agarose or pure agarose plus one of the reinforcers (FRU, NaCl, or QUI). We started with pure agarose as substrate for half of the cases, and for the other half of the cases, we started with a reinforcer-containing plate (see also legend of Fig. 5a).

Immediately before a trial, two containers loaded with the same odorant were placed on opposite sides of the plate, 7 mm from the edges. We started with AM for half the animals and with OCT for the other half. Then, lids were closed and the larvae were allowed to move about the plate for 1 min. Thereafter, animals were transferred to a completely empty petri dish for a 1-min inter-trial interval. The larvae were then transferred to a plate with the alternative odorant and the respective other substrate for 1 min, followed by another 1-min inter-trial-interval. This cycle was repeated three (Fig. 5) or ten (Figs. 6, 7, 8) times. Fresh assay plates were used for each conditioning cycle.

After this training, each larva was individually tested for its odor choice; thus, animals were trained in small groups of eight, but tested as individuals. For testing, each larva was placed on a fresh, pure-agarose assay plate with a container of AM on one side and a container of OCT on the other side to create the desired choice situation; sides were changed for every other animal. Individual larvae were placed in the center of the petri dish, the lid was closed and the position of the larvae was scored for 5 min every 20 s as ''AM'', ''OCT'', or ''neutral''. After this was completed, animals from the reciprocal training group were run. In all cases, we discarded larvae that moved onto the lid or onto the odorant containers (< 5% of animals). We present test performance in three consecutive steps:

First (see Fig. 5b), for a time-resolved description of the animals' performance, we present the preference of the population of larvae by calculating for each time point the number of animals located on the AM side minus the number of animals located on the OCT side, divided by the total number of animals:

$$\mathrm{PREF} = \frac{\text{number of animals}_{\mathrm{AM}} - \text{number of animals}_{\mathrm{OCT}}}{\text{number of animals}_{\mathrm{Total}}} \tag{3}$$

Thus, a value of 1 indicates that all larvae were recorded on the AM side at that time point, whereas a value of −1 indicates that all were on the OCT side.

Second (see Fig. 5c), we calculate the odor preference for each animal as described in Eq. 1. In order to test for an associative effect of training, we took the paired PREF values from the alternatedly run, reciprocally trained animals and compared them with a Wilcoxon signed ranks test.

Third, to quantify learning, we calculated a learning index from these pairs ranging from −1 to 1 as:

$$\mathrm{LI} = \frac{\mathrm{PREF}^{\mathrm{AM+/OCT}} - \mathrm{PREF}^{\mathrm{AM/OCT+}}}{2} \quad \text{for FRU;} \tag{4a}$$

$$\mathrm{LI} = \frac{\mathrm{PREF}^{\mathrm{AM/OCT-}} - \mathrm{PREF}^{\mathrm{AM-/OCT}}}{2} \quad \text{for NACL or QUI.} \tag{4b}$$

Accordingly, in both Eq. 4a and Eq. 4b, positive LI values indicate associative learning. An exhaustive

analysis using the bootstrap technique reveals that these LIs are a reasonable basis for statistical analysis (see Appendix). The bootstrap analyses were warranted to test whether results might be distorted by a random component when assigning animals into pairs; that is, such pairing errors might lead to a changed distribution of LI values and hence to changed variances and/or medians; for small sample sizes, which is not the case in this study, the possibility exists that this can affect the outcome of statistical tests. However, the bootstrap analyses suggest that such pairing errors are negligible (see Appendix). Therefore, we use the LIs as basis for our statistical analyses. We use nonparametric statistics throughout: for comparisons of LIs against zero, we use the one-sample sign test; for multiple-group comparisons of the LIs we use the Kruskal–Wallis test; for two-group comparisons of LIs we use the Mann–Whitney *U*-test.

For the first learning experiment to be reported in the Results, we present all of the three steps outlined above (see Fig. 5b–d); for the subsequent learning experiments, we present the data only in the "condensed" format of the LIs and/or the PREF values (Figs. 6, 7, 8).

### Reinforcing function as tested in differential conditioning

In the "differential" conditioning version of this paradigm, animals received two reinforcers: both appetitive reinforcement by FRU and aversive reinforcement by either NaCl or QUI; thus, in this case we compared the test performance of animals trained AM+/OCT− to ones trained AM−/OCT+; LI values were calculated as

$$\mathrm{LI} = \frac{\mathrm{PREF}^{\mathrm{AM+/OCT-}} - \mathrm{PREF}^{\mathrm{AM-/OCT+}}}{2} \qquad (4c)$$

## Results

### Detectability of odors

Larvae show clear attraction to both odors used: that is, they choose the side of the assay plate equipped with the AM container over that side with an empty container, and they prefer the OCT side over the side with a solvent-filled container (Fig. 1; $P < 0.05$ in either case; $N = 72, 79$). The response to AM is a genuine response to AM and not to the paraffin oil used as solvent, as the paraffin oil does not elicit significant responses (Fig. 1; $P > 0.05$; $N = 48$). If signs for the OCT group are reversed, we find that the degree of preference for AM and OCT is equal (no Fig.; $U = 2632.5$; $P > 0.05$; Ns as above). Interestingly, however, if larvae are given a choice between AM and OCT, they show a preference for OCT (Fig. 1; $P < 0.05$; $N = 77$). Thus, although both odors are equally attractive when tested in isolation, in a binary choice situation OCT is preferred over AM, at least in naïve larvae (for animals which had undergone associative training, see below). In the simplest account, this implies that a relative preference is a more sensitive measure than an absolute preference. In any event, because during associative training the odors are used in isolation, we chose to use AM and OCT at the present concentrations as olfactory stimuli in the subsequent learning experiments.

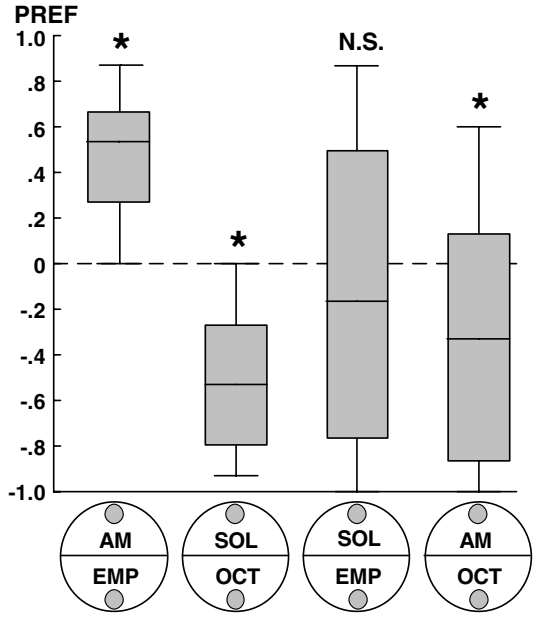


**Fig. 1** Detectability of odors: AM and OCT are well detectable. Insets below the figure depict the procedure for testing the detectability of odors. From *left to right*, groups were tested for their choice between: an empty container (EMP) and a container filled with AM dissolved in paraffin; a container with that paraffin solvent (SOL) versus OCT; with SOL versus EMP; or with AM versus OCT. Animals were observed for 5 min, and every 20 s, the position of the animals was noted as being AM, neutral, or OCT. For each animal, the odor preference is calculated by summing up the number of times it was observed on the AM side minus the number of times it was observed on the OCT side; the result was then divided by the total number of times the animal was observed. Thus, positive values indicate AM preference and negative values OCT preference. *: $P < 0.05$; NS: $P > 0.05$. The *box plots* represent the median as the *middle line* and 10 and 90, and 25 and 75% quantiles as *whiskers and box boundaries*, respectively. Ns are from *left to right* 72, 79, 48, 77

### Response-releasing function of "tastants": avoidance and approach

Larvae show attraction to fructose (FRU) and are repelled by sodium chloride (NaCl) and quinine (QUI) (Fig. 2; $P < 0.05$ in all cases; $N = 19, 21, 20$). Avoidance of NaCl is, statistically speaking, about as strong as that of QUI (Fig. 2; $P > 0.05$, $U = 150.5$; Ns as above). If one reverses signs for the aversion responses, one can ask whether the extent to which FRU, NaCl, and QUI

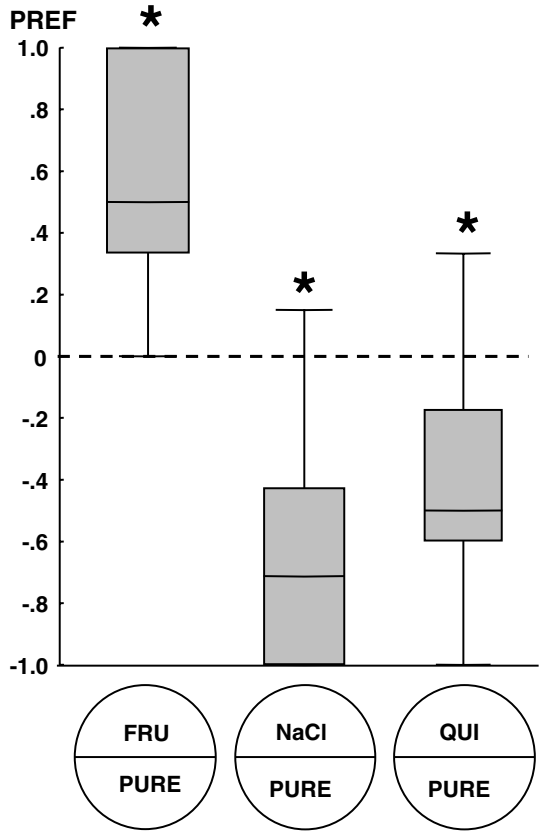


**Fig. 2** Response-releasing function: appetitive response to FRU, aversive responses to NaCl and QUI. Insets below the figure depict the procedures for the gustatory response tests. In all cases, preferences between plain agarose (PURE) versus "tastant" were measured; groups differed with respect to "tastant" used: either FRU, NaCl, or QUI. Groups of ten animals each were placed in the middle of the test plate and after 15 min the number of animals located on the PURE, or "tastant" side was determined. To calculate gustatory preferences, the number of animals located on the "tastant" side was subtracted from the number of animals located on the PURE side; that value was then divided by the total number of animals. Thus, positive values indicate attraction and negative values repulsion of a "tastant". $*P < 0.05$. For an explanation of the box plots, see legend of Fig. 1. Ns are from *left to right* 19, 21, 20

possess response-releasing functions is different. No such difference is found (no Fig.; $P > 0.05$, $H = 2.308$, $df = 2$; Ns as above). Thus, all three "tastants" are detected well and, at the concentrations used, about equally potent in releasing avoidance and approach responses.

## Response-releasing function of "tastants": influence on "appetite"

As another way of testing appetitive and aversive responses, we asked whether FRU, NaCl, and QUI would have an effect on "appetite", i.e. on feeding behavior. We find that larvae swallow agarose even without any "tastant" added (Fig. 3a; $P < 0.05$, $N = 60$). Larvae also eat when FRU is added (Fig. 3a; $P < 0.05$, $N = 60$), but not if either NaCl or QUI are present in the substrate: in none of these animals did we observe any sign of feeding (Fig. 3a; $P > 0.05$, $N = 60$ in both cases). Thus, feeding clearly depends on the kind of "tastant" present (Fig. 3a: $P < 0.05$, $H = 149.0$, $df = 3$, Ns as above); specifically, FRU increases (Fig. 3a; $P < 0.05$, $U = 1{,}172$; Ns as above), whereas NaCl and QUI decrease feeding (Fig. 3a; $P < 0.05$, $U = 600$ in both cases; Ns as above) as compared to the pure condition. These conclusions remain unaltered if larvae are scored as "eaters" and "noneaters" and the frequency of these cases is compared (FRU: 57/3; PURE: 44/16; NaCl: 0/60; QUI: 0/60) (no Fig.: $P < 0.05$, $\chi^2 = 165.6$, $df = 3$; for FRU versus pure: $\chi^2 = 4.36$, $df = 1$; for pure versus NaCl: $\chi^2 = 69.47$, $df = 1$).

Taken together, in accord with the literature (Heimbeck et al. 1999; Scherer et al. 2003), all stimuli used in this study are detected well by the larvae under our experimental conditions and at the concentrations used. Concerning "tastants", our results in particular show that FRU, NaCl, and QUI possess substantial response-releasing properties for appetitive and aversive responses, respectively.

## Response-modulating function of "tastants"?

Given that FRU, NaCl and QUI are triggering strong appetitive and aversive responses, we asked whether these same stimuli would modulate olfactory responses in a nonassociative way: would the choice between AM and OCT be different on a neutral versus a sweet, salty or bitter substrate? For example, larvae might ignore olfactory stimuli altogether if crawling on an unpleasant substrate. However, we find that larvae perform similarly on all four substrates (Fig. 4; $P > 0.05$, $H = 2.876$, $df = 3$; $N = 68, 66, 64, 66$). If the data from all these experimental groups are pooled, we find a preference for OCT (no Fig.; $P < 0.05$; $N = 264$). This OCT preference is indistinguishable from the data for the AM versus OCT condition in Fig. 1 (no Fig.; $P > 0.05$; $U = 7{,}018.0$; Ns as above) arguing that patterns of preference have reasonable stability over repetitions. Importantly, the equal olfactory choice performance on PURE, FRU, NaCl and QUI substrates suggests that FRU, NaCl and QUI, although potent response-releasing stimuli, do not lead to nonassociative modulations of olfactory choice behavior.

## Reinforcing function: Do FRU, NaCl or QUI support associative learning?

We next asked whether these "tastants" are able to act as reinforcers in associative learning. We first report in some detail on a pilot experiment performed in the Toronto laboratory using only FRU in an "absolute" conditioning experiment (Fig. 5a–d). In a next step, we report three "absolute" conditioning experiments using

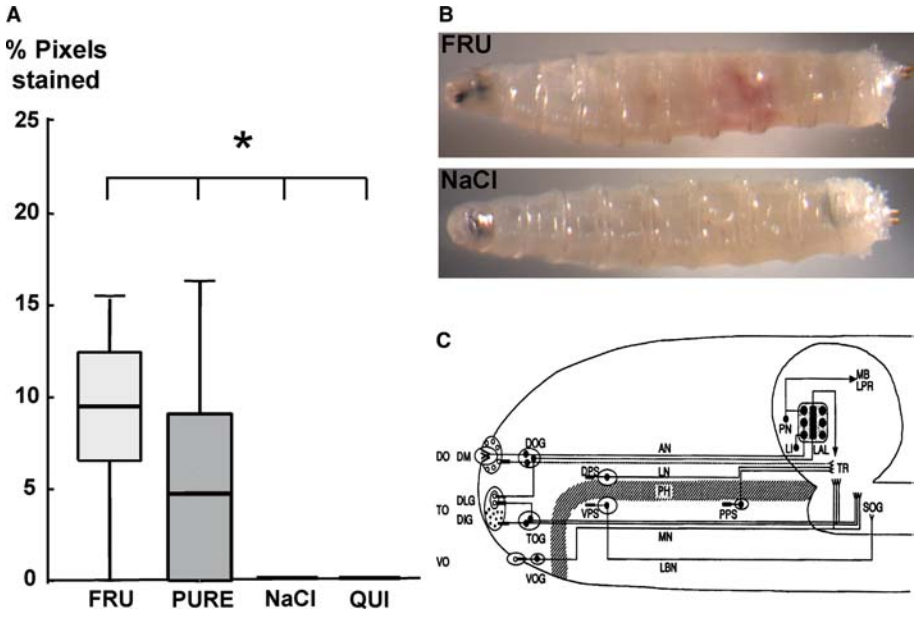


**Fig. 3** Response-releasing function: appetitive effect of FRU, aversive effect of NaCl and QUI in a feeding assay. **a** To estimate the amount of food intake, the percentage of stained pixels is shown for animals that fed on different carmine-red stained substrates: From *left to right* the results are shown for stained agarose with FRU added, without any "tastant", with NaCl and with QUI. Animals eat on pure agarose; this is increased by the presence of fructose and suppressed by the presence of sodium chloride or quinine. The conclusions remain unaltered if alternatively the frequencies of "eaters" and "noneaters" are evaluated (see text). **b** Examples of animals after opportunity to feed on stained agarose with added FRU (*upper picture*) or NaCl (*lower picture*). **c** Schematic (from Python and Stocker 2002) showing the external mixed olfactory/gustatory sense organ (dorsal organ, *DO*), and the external gustatory sense organs (terminal and ventral organ, *TO*, *VO*). The internal gustatory sensillae are situated along the pharynx (*DPS, VPS, PPS* dorsal, ventral, and posterior pharyngeal sensilla). For all these sensory structures, the central projections to the antennal lobe (*AL*), tritocerebrum (*TR*) and suboesophageal ganglion (*SOG*) are shown. From the AL, projection neurons (*PN*) relay onto the lateral protocerebrum (*LPR*) and provide collaterals into the mushroom bodies (*MB*). *AN, LN, MN, LBN* antennal, labral, maxillary, labial nerves. *DLG, DIG* dorsolateral and distal group of TO. *DOG, TOG, VOG* ganglia of DO, TO, VO. The figure does not cover other potentially chemosensitive structures on cephalic, thoracic and abdominal segments. *: $P < 0.05$. For an explanation of the *box plots*, see legend of Fig. 1. Ns are from *left to right* 60, 60, 60, 60

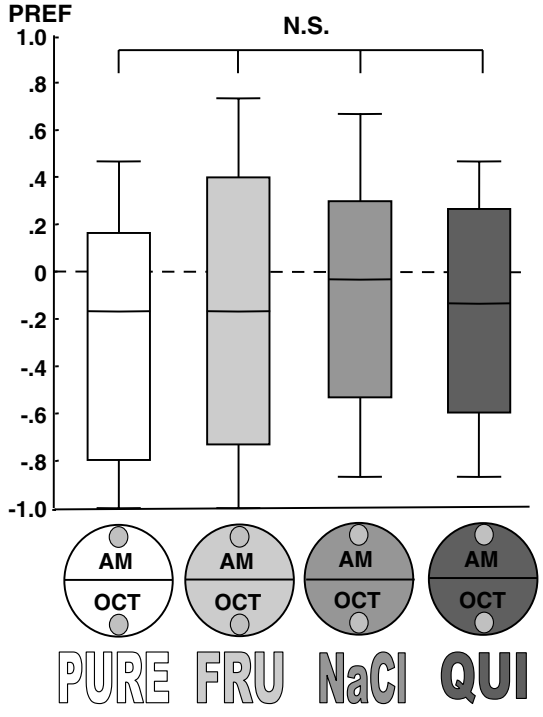


**Fig. 4** Response-modulating function: Olfactory choice is unaffected by FRU, NaCl, or QUI. Insets depict the experimental procedure. Groups differ in that the odor choice between AM and OCT was measured on different substrates: plain agarose (PURE), FRU, NaCl, or QUI. Odor preferences, calculated as explained in the legend of Fig. 2, do not differ between these groups. NS: $P > 0.05$. For an explanation of the box plots, see legend of Fig. 1. Ns are from *left to right* 68, 66, 64, 66

either only FRU as reinforcement, or only NaCl or only QUI; it will turn out that FRU, but neither NaCl nor QUI can induce learning (Fig. 6).

We compared the performance of two reciprocally trained groups (Fig. 5a): one received the reinforcer with AM and received OCT without reinforcement (AM+/OCT); the other group was trained with OCT being accompanied by the "tastant" (AM/OCT+). As shown in Fig. 5b, c animals which had received AM+/OCT training showed a higher AM preference than animals which had received AM/OCT+ training (Fig. 5b, c; $P < 0.05$, $Z = 2.71$; Ns = 46). We quantified this difference by a learning index (LI); this LI is positive in about 75% of the cases (Fig. 5d). The median LI is 0.10, which represents that LIs are significantly above chance level (Fig. 5d; $P < 0.05$; $N = 46$). This result must lead to the conclusion that individually assayed *Drosophila* larvae show associative learning between olfactory stimuli and FRU reinforcement. It should be emphasized that this

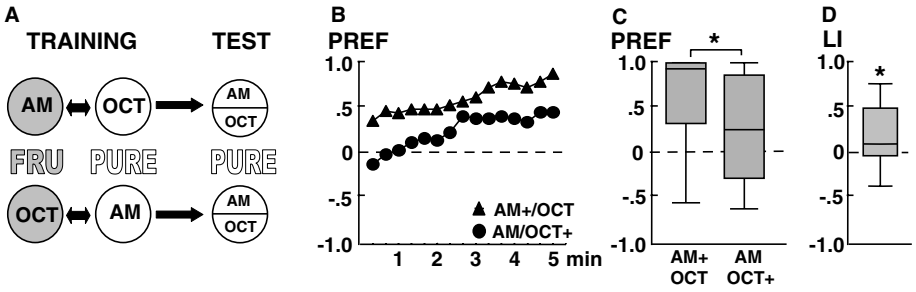


**Fig. 5** Reinforcing function: FRU supports "absolute" conditioning. **a** Diagram of the procedure for the conditioning experiment; please note that for half of the cases, the sequence of training trials within each reciprocal group was as indicted (i.e. AM+/OCT and OCT+/AM), whereas for the other half of the cases (not shown), the sequence of training trials was reversed (i.e. OCT/AM+ and AM/OCT+). **b** Time-resolved description of the odor preferences during test calculated as the number of animals located on the AM side at a given time point minus the number of animals located on the OCT side, divided by the total number of animals. Thus, positive values indicate that a majority of larvae were recorded on the AM side at that time point, whereas negative values indicate that a majority was located on the OCT side. **c** Peference values were calculated for each animal as explained in the legend of Fig. 2. Positive values indicate AM preference and negative values OCT preference. The AM preference was higher after AM+/OCT training than after AM/OCT+ training, indicating associative learning. **d** A learning index (LI) was calculated for pairs of animals which underwent either of the reciprocal training regimes, e.g. either AM+/OCT or AM/OCT+, by subtracting the PREF values of both animals and dividing the result by two. The LIs are significantly larger than zero, indicating associative learning. $*P<0.05$. For an explanation of the box plots, see legend of Fig. 1 $N=46$

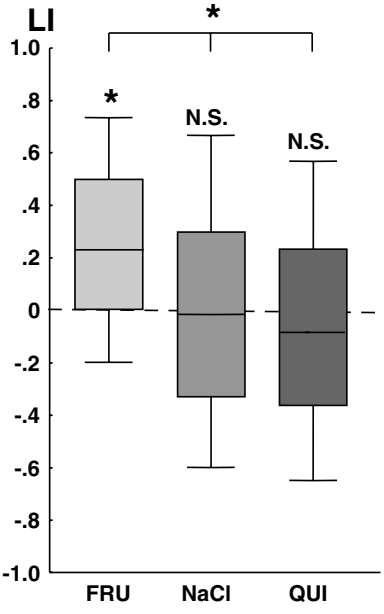


**Fig. 6** Reinforcing function: FRU but neither NaCl nor QUI supports learning. Direct comparison of the effectivity of "absolute" conditioning between the different "tastants". The LI values are significantly different from zero for FRU, but not for NaCl or QUI. In a direct comparison, the LI values differ significantly between the three reinforcers. $*P<0.05$; *NS* $P>0.05$. For an explanation of the box plots, see legend of Fig. 1. Ns are from *left to right* 115, 122, 120

conclusion is drawn from the comparisons between pairs of animals which had undergone reciprocal training regimes (AM+/OCT versus AM/OCT+). As only the relation of odors and reinforcement differs between these training regimes, only associative learning can account for differences during the test. This conclusion is unaffected by the overall preference for AM over OCT (see below); this preference merely leads to an offset of the preference values for both reciprocal groups (Fig. 5b, c) but cannot cause differences in preference values between them as measured by the learning index (Figs. 5d, 6, 8). Therefore, the conclusion that larval *Drosophila* form associations between olfactory stimuli and FRU reinforcement is compelling.

The next experiment, using ten instead of three conditioning cycles, compared the effectiveness of FRU, NaCl, and QUI in "absolute" conditioning. Concerning FRU, the results from the previous experiment nicely reproduced: preferences for AM are higher after AM+/OCT training than after AM/OCT+ training (no Fig.; $P<0.05$, $Z=6.02$; Ns = 115). This difference can be quantified by an LI of 0.23, which was significantly above chance level (Fig. 6; $P<0.05$; $N=115$). With NaCl and QUI as reinforcers, the training regime has no influence on the behavior during the test: after training with AM−/OCT and AM/OCT−, odor preferences were indistinguishable (no Fig.; for NaCl: $P>0.05$, $Z=0.31$; Ns = 122; for QUI: $P>0.05$, $Z=1.76$; Ns = 120). The corresponding learning indices were in both cases indistinguishable from zero (Fig. 6; $P>0.05$ in both cases, Ns = 122 and 120, respectively). Thus, despite the fact that both NaCl and QUI are potent in eliciting avoidance responses (Figs. 2, 3), they are apparently not potent as reinforcers in associative learning. This is in marked contrast to the effectiveness of FRU in this respect (Figs. 5, 6, 8). Importantly, a direct comparison between the experiments which used either FRU or NaCl or QUI showed a significant difference in the effectiveness across these three stimuli (Fig. 6; $P<0.05$, $H=32.28$; $df=2$; Ns as above). This directly demonstrates that reinforcer identity is a determinant for olfactory associative learning in the *Drosophila* larva.

In a further analysis of our data, we asked whether FRU reinforcement could bi-directionally modulate odor preferences. We reasoned that, as animals do not learn associatively when using NaCl and QUI, pooling the preference data from the two reciprocal NaCl groups

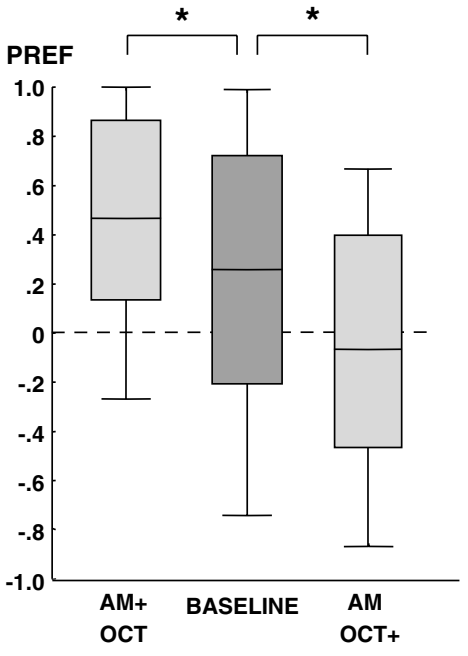


**Fig. 7** Reinforcing function: Bi-directional effects of FRU in the reciprocally trained groups. Post-hoc analysis of the experiment shown in Fig. 5, comparing the PREF values of animals after FRU training to baseline; the baseline was provided by pooling the data of the two reciprocal NaCl groups as well as the two reciprocal QUI groups. After AM+/OCT training, animals had a higher AM preference than baseline, and after AM/OCT+ training, they had a lower preference than baseline. $*P < 0.05$. For an explanation of the box plots, see legend of Fig. 1. Ns are from *left to right* 115, 484, 115

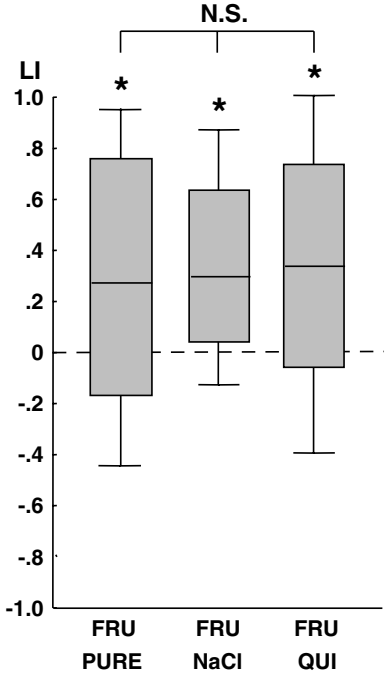


**Fig. 8** Reinforcing function: Neither NaCl nor QUI potentiates FRU reinforcement. Comparison of "absolute" and differential conditioning. Groups differed in that either only FRU was used for "absolute" conditioning (*left*) or differential conditioning was performed using FRU for positive as well as either NaCl (*middle*) or QUI (*right*) for negative reinforcement. Under all three conditions, the same amount of learning as measured by the LI was observed; in all three cases, the LIs are above chance level. $*P < 0.05$; *NS* $P > 0.05$. For an explanation of the box plots, see legend of Fig. 1. Ns are from *left to right* 59, 58, 47

as well as the two reciprocal QUI groups would result in a baseline, post-training measure of odor preference against which the performance of the FRU-trained groups (AM+/OCT and AM/OCT+) could be compared. As shown in Fig. 7, animals from the AM+/OCT group have a higher AM preference than baseline (Fig. 7; $P < 0.05$, $U = 22{,}210$; $N = 484$, 115), whereas the ones from the AM/OCT+ group are below baseline (Fig. 7; $P < 0.05$, $U = 21{,}185$; $N = 484$, 115). In the simplest account, this opposite effect of FRU in the reciprocally trained groups suggests that larvae can associate FRU with both AM and OCT.

Interestingly, the baseline performance shows a preference for AM over OCT (Fig. 7; $P < 0.05$; $N$ as above). This is in contrast to the naïve preference for OCT over AM (Figs. 1, 4). This shift in overall preference in animals that show no associative learning (for OCT before training, for AM after training) might be due to either: (1) the passage of time, affecting the animals sensory system and/or the physical or chemical properties of the odors; (2) odor exposure, leading to sensory adaptation and/or habituation; (3) handling of the larvae and the stress this might entail; (4) previous exposure to "tastants". At present, we cannot distinguish between these possibilities. Interesting and obvious as this effect is, it should be stressed that it cannot dismiss the associative effects as measured by the learning indices. This is because the reciprocally trained groups are equal with respect to all the above-mentioned parameters.

## Reinforcing function: do NaCl or QUI potentiate associative learning?

Although NaCl and QUI did not induce associative learning on their own in "absolute" conditioning (Fig. 6), they still might have a potentiating effect if used in combination with FRU in differential conditioning. Therefore, three learning experiments were compared. In one, larvae were trained in "absolute" conditioning with FRU reinforcement alone (this replicates the FRU experiment shown in Fig. 6). In the other two, animals received differential conditioning using two reinforcers: either the combination FRU–NaCl or FRU–QUI. All three procedures resulted in indistinguishable learning indices (Fig. 8; $P > 0.05$, $H = 0.38$, $df = 2$; $N = 59$, 58, 47). Within all three experiments, the learning indices were above chance level (Fig. 8; $P < 0.05$ in all cases; Ns as above). Thus, neither NaCl nor QUI had an apparent

potentiating effect on olfactory learning when used in differential conditioning together with FRU. If one compares the learning indices between repetitions of the FRU-learning experiments in Fig. 6 versus Fig. 8, no difference is found (no Fig.; $P > 0.05$; $U = 3{,}305{,}5$; Ns as above). This suggests that, with boundary conditions unchanged, the learning indices have reasonable stability over repetitions.

## Discussion

Among the behavioral functions of gustatory stimuli, we investigated their potential response-releasing, response-modulating, and reinforcing function. We found that these functions are dissociated: FRU, NaCl and QUI can all release appetitive or aversive responses, respectively (Figs. 2, 3). Still, none of these stimuli has detectable nonassociative, response-modulating effects on olfactory choice (Fig. 4). Finally, only FRU but neither NaCl nor QUI has an apparent reinforcing effect for olfactory learning (Fig. 6). Taken together, this implies that these functions are dissociated between the three tastants on the behavioral level. Clearly, such a dissociation can come about only by a dissociation of the underlying neuronal circuitry. To this end, Menzel et al. (1999) found that octopamine is sufficient to rescue the reinforcing, but not the response-releasing function of sucrose in honeybees depleted of biogenic amines by reserpine. Dopamine, on the other hand, was able to rescue the response-releasing but not the reinforcing function. This is in line with the finding of Hammer (1993) who showed that driving the identified putatively octopaminergic neuron $VUM_{mx1}$ is sufficient to substitute for the reinforcing, but not the response-releasing function of reward. In mammals, dopamine plays a role in mediating the reinforcing function of reward (Waelti et al. 2001), but not its response-releasing function (Cannon and Palmiter 2003). Also, the analyses of classical conditioning of eyeblink conditioning in the rabbit have shown that in the brainstem processing of the corneal air puff is separated out into a direct connection to the motor nuclei to release the reflex, and an extensive reinforcing cerebellar loop targeting the site of synaptic plasticity to support learned responses (Christian and Thompson 2003). Together, these data suggest that, as a general rule, processing of potentially reinforcing, unconditioned stimuli might diverge already at early processing stages into separate circuitries to on the one hand directly trigger reflexes, and on the other hand a reinforcement signal to induce learning.

### Effectiveness of FRU as reinforcer

With respect to reinforcement function, we report three "absolute" conditioning experiments and find that FRU but not NaCl or QUI can act as a reinforcer for associative olfactory learning (Fig. 6). Furthermore, neither NaCl nor QUI have a potentiating effect on learning when used together with FRU in differential conditioning (Fig. 8). The reinforcing effect of FRU was repeatedly found in the Würzburg laboratory (Figs. 6, 8) and was, even with only three conditioning cycles, replicated in the Toronto laboratory as well (Fig. 5). These results provide the first evidence of appetitive olfactory learning in *Drosophila* larvae to date. Concerning NaCl and QUI, however, an absence of proof for learning is not a proof of its absence, which, on principle grounds, is difficult to obtain.

The same differential reinforcement effectiveness for FRU was found in a companion paradigm in which larvae were trained to associate visual stimuli with the same three gustatory reinforcers as in the current study (Gerber et al. 2004). As FRU, NaCl and QUI are well detectable to larvae (Figs. 2, 3), we suggest that larvae are, in general, more susceptible to appetitive than to aversive reinforcement. The relatively low reinforcement effectiveness of aversive stimuli might be one reason why the studies of Aceves-Pina and Quinn (1979), Heisenberg et al. (1985), and Tully et al. (1994), which used electric shock for aversive reinforcement, were compromised in their reproducibility (Forbes 1993; F. Python, University of Fribourg, Switzerland, personal communication).

### A possible ultimate reason for the effectiveness of FRU reinforcement

On an ultimate level, the high effectiveness of appetitive stimuli as reinforcers in the larva might reflect their evolutionary design as a feeding stage. In other words, the larvae might be "clamped" into a feeding motivation, similar to the situation in the honeybee worker (Hammer and Menzel 1995; Menzel et al. 1999), and therefore appetitive, nutritious stimuli might be particularly rewarding to them. Interestingly, in adults the situation is reversed, in that aversive reinforcers are more effective than appetitive ones (Schwaerzel et al. 2003; see below). This reversed pattern of results might reflect the life style of adults as reproductive stages for which feeding and growth play less of a role than for larvae. A similar developmental switch, by the way, is also seen in odor and light preferences, as larvae are usually attracted by odors and repelled by light (Sawin-McCormack et al. 1995; Cobb 1999; Hassan et al. 2000), whereas adults are usually repelled by odors, at least at intermediate and high concentrations, and attracted by light (Ballinger and Benzer 1988; Ayyub et al. 1990).

### Possible proximate reasons for the effectiveness of FRU reinforcement

On a proximate level, reinforcement signals triggered by aversive gustatory stimuli might not converge with processing of olfactory and visual input, and hence no associations can possibly form. Concerning QUI, how-

ever, we were informed that if very long training trial durations are used in an en masse version of our assay (30 min, instead of 1 min as in this study), QUI is able to support aversive olfactory learning in the larva (F. Mery, University of Fribourg, Switzerland, personal communication). This is in line with the fact that also in adult flies quinine has been successfully applied in olfactory learning experiments (Medioni and Vaysse 1975; DeJianne et al. 1985; Bouhouche et al. 1995; Mery and Kawecki 2002). This implies that in both larvae and adults the neuronal circuitry to support aversive olfactory learning by QUI does, in principle, exist. With respect to the current study, it underlines the cautious conclusion (see above) that QUI is less effective than FRU as a reinforcer, but might not be totally ineffective. Concerning NaCl, prolonged training trials might eventually also reveal aversive learning. It should then be interesting to test different concentrations of NaCl, as low concentrations might be appetitive but high concentrations might be aversive reinforcers (Miyakawa 1982).

Another proximate reason for the reduced effectivity of NaCl and QUI reinforcement could be that the reinforcing effects of the gustatory stimuli depend on food intake. Larvae carry three paired groups of external gustatory sensilla on their head segment, in the so called dorsal-, terminal- and ventral organs; in addition, they also possess three paired groups of internal gustatory sensilla in the pharynx (Python and Stocker 2002; Gendre et al. 2003). These latter sensilla might be used to sample swallowed food, whereas external sensilla might be used to sample the gustatory environment. Thus, it is conceivable that the external gustatory sensillae mediate attraction and avoidance responses (Heimbeck et al. 1999) as well as the decision to swallow food or not, whereas the pharyngeal sensilla mediate the reinforcing effect of food. We found that larvae swallow crumbs of agarose under our assay conditions and are more likely to do so if FRU, but less likely if high concentration NaCl or QUI, are added to the agarose (Fig. 3). Thus, any reinforcing effect as mediated by the pharyngeal sensillae would be negligible as only minute amounts of NaCl or QUI are swallowed, whereas the large amount of swallowed FRU might be sufficient to induce a reinforcing effect. Interestingly, despite extensive reorganization of the larval nervous system during metamorphosis, the majority of the pharyngeal sensilla persist into the adult stage (Gendre et al. 2003). As most other larval sensory neurons die during metamorphosis (but see Tissot and Stocker 2000; Helfrich-Foerster et al. 2002), this might indicate that pharyngeal gustatory sensilla play a particularly important role in the life of the fly.

## Outlook

Interestingly, in adult olfactory learning, appetitive sucrose conditioning requires octopaminergic, but not dopaminergic neurons, while aversive learning with electric shock reinforcement in turn requires dopaminergic, but not octopaminergic neurons (Schwaerzel et al. 2003). This dissociation of possible reinforcement systems is, albeit with reversed sign, similar to the situation in monkeys where activations of midbrain dopamine neurons were found to be stronger and more frequent by appetitive compared to aversive stimuli (Mirenowicz and Schultz 1996). Furthermore, stimuli that predict the occurrence of reward activate these neurons in the monkey, whereas stimuli that predict the absence of reward lead to their inhibition (Tobler et al. 2003). It is of interest to determine whether similar dissociations might apply in *Drosophila* larvae, which due to their cellular simplicity, transparent cuticle and genetic as well as optophysiological accessibility (Liu et al. 2003) might be a fruitful system for such an analysis.

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## Appendix

### Is the distribution of LI values influenced by pairing? A bootstrap analysis

The LI values are calculated as the difference in odor preference between reciprocally trained animals. A statistical analysis of these LI values makes only sense if their distribution does not depend on the pairing of the preference values on which they are based. This is because different values of sample variance and sample median may be obtained depending on which particular animals are assigned into pairs (the sample mean, however, is independent of pairing). Consequently, one may argue that the outcome of statistical tests of these LIs may be due to a particular choice of pairing. In the following, we investigate whether this is indeed the case. It turns out that the distribution of the LIs is not influenced by pairing; specifically, the bootstrap method is used to test whether the distributions of sample variance and sample median are influenced by pairing. Several hundred samples of randomly taken pairs are thereby considered.

The basic idea behind bootstrapping (inventor: Efron 1979) is that it treats the empirical distribution of the data (probability mass $1/n$ on $x_1,\ldots, x_n$) as if it were the underlying true (but unknown) distribution. Thus, the sampling distribution of a certain statistic of interest, in our case variance and median, can be estimated by

generating a large number of new samples (called bootstrap samples) by drawing with replacement from the actual sample. From these many samples, bootstrap can provide us with confidence intervals for the variance (var) and the median (med) of the "original pairing". The same can then be done for LI values obtained by various (random) pairings of the preference values. Note that bootstrapping makes no assumption about the underlying distribution.

Various methods are known for using bootstrap to determine confidence intervals. In the present paper, we use the percentile method (Efron and Tibshirani 1993, Chap. 13), which is briefly described in the following. We prefer this over the standard method to approximate confidence intervals for an unknown parameter via standard errors, because standard errors are crude measures of statistical accuracy, and transformations that may improve the normal approximation (if they exist) would have to be known. Denote by $x_1,..., x_n$ a LI sample, based on the "original" or a randomly generated pairing, of size $n$. A bootstrap sample $x_1^*,..., x_n^*$ is a random sample of size $n$ drawn with replacement from

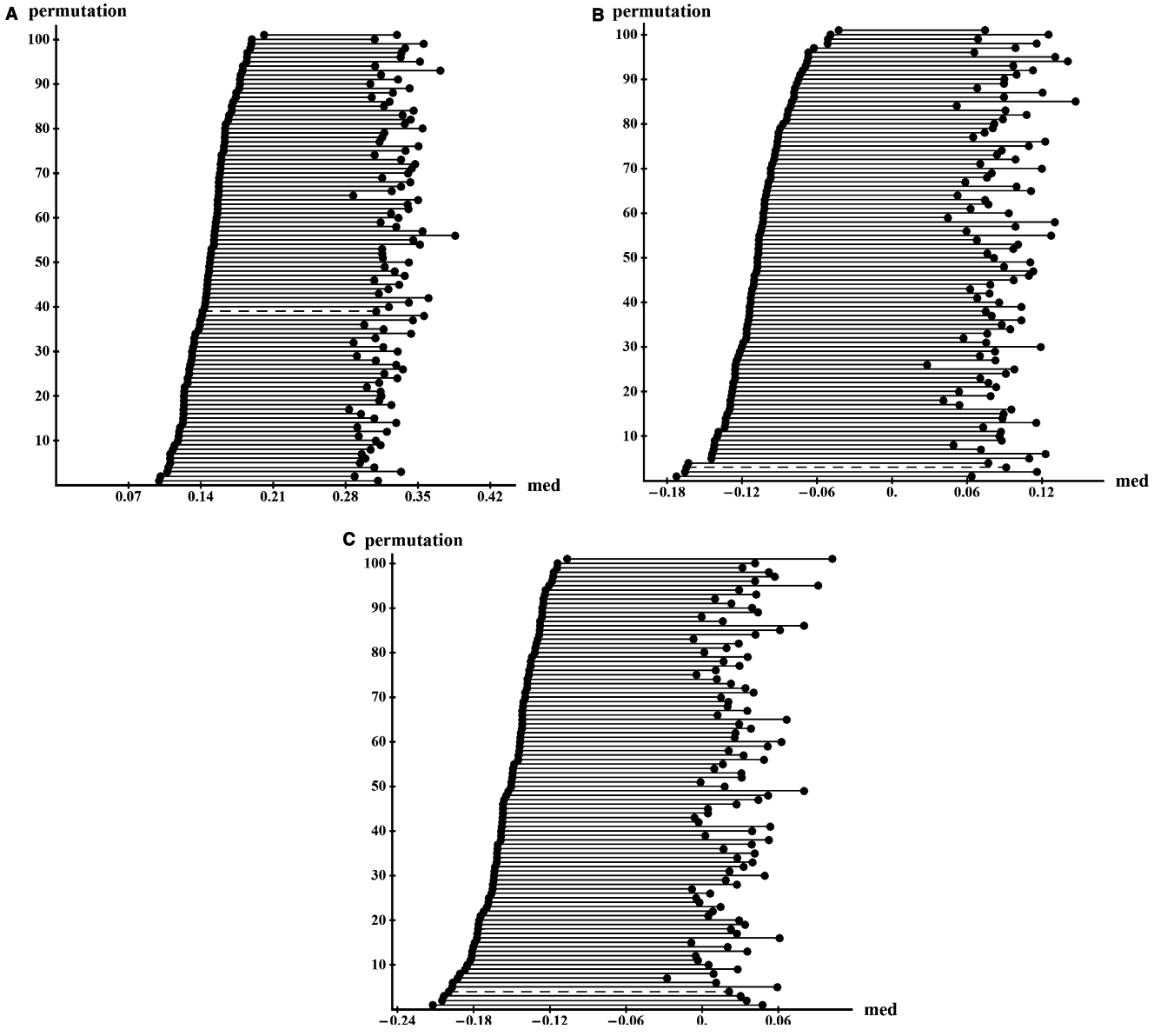


**Fig. 9** The median of the LIs is not influenced by pairing. For each of the 100 randomly permutated pairings of PREF values the corresponding LI values were calculated and the confidence interval for the median derived by the bootstrap method using that particular pairing is presented; from *bottom* to *top*, the permutations are ordered by increasing lower limits of the confidence interval. The *dashed confidence interval* labels the median for that sample which is based on the original pairing; the original pairing uses the animals trained alternately for the calculation of LIs and is the basis of data presentation and statistical analysis in the main text (Fig. 6). In all three experimental conditions (FRU, NaCl, QUI in *a*, *b* and *c*, respectively), the confidence interval for the median of the original pairing overlaps with the confidence intervals for the median of the permutated combinations. This indicates that pairing does not substantially alter the median. The same analysis was performed on the data presented in Figs. 5 and 8, leading to the same result (not shown)

the actual sample $x_1,..., x_n$. For the bootstrap sample, we evaluate the statistics of interest; here, the variance of the bootstrap sample:

$$\mathrm{var}\left(x_1^*,\ldots,x_n^*\right)=\frac{1}{n}\sum_{j=1}^{n}\left(x_j^*-\frac{1}{n}\sum_{i=1}^{n}x_i\right)^2$$

(note that $\sum_{i=1}^{n} x_i/n$ is the mean of the bootstrap sample), and median of the bootstrap sample $med(x_1^*,..., x_n^*)$.

The bootstrap algorithm begins by generating a large number, for example, $N = 20{,}000$, of independent bootstrap samples:

$$\text{Resample \# 1}, \text{Resample \# 2}, \ldots, \text{Resample \# } N.$$

For each bootstrap sample, we calculate the variance:

$$\mathrm{var}(\text{Resample \# 1}), \ldots, \mathrm{var}(\text{Resample \# } N) \quad (5)$$

and the median:

$$\mathrm{med}(\text{Resample \# 1}), \ldots, \mathrm{med}(\text{Resample \# } N). \quad (6)$$

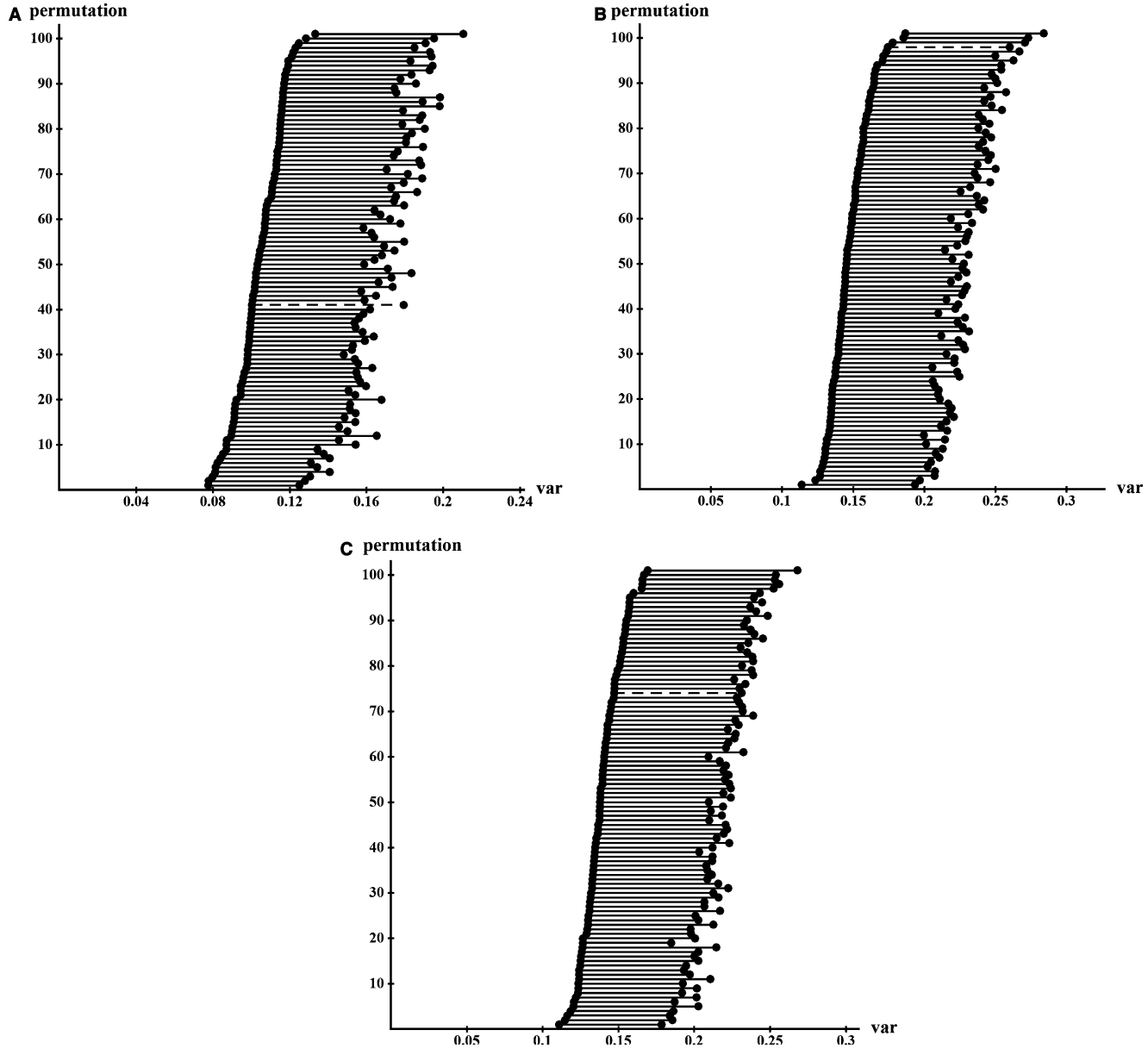


**Fig. 10** The variance of the LIs is not influenced by pairing. For each of the 100 randomly permutated pairings of PREF values the corresponding LI values were calculated and the confidence interval for the variance derived by the bootstrap method using that particular pairing is presented; from *bottom* to *top*, the permutations are ordered by increasing lower limits of the confidence interval. The dashed confidence interval labels the variance for that sample which is based on the original pairing; the original pairing uses the animals trained alternately for the calculation of LIs and is the basis of data presentation and statistical analysis in the main text (Fig. 6). In all three experimental conditions (FRU, NaCl, QUI in *a*, *b* and *c*, respectively), the confidence interval for the variance of the original pairing overlaps with the confidence intervals for the variance of the permutated combinations. This indicates that pairing does not substantially alter the variance. The same analysis was performed on the data presented in Figs. 5 and 8, leading to the same result (not shown)

We then consider the empirical distribution pertaining to the bootstrap samples Eqs. 5 and 6, respectively. The confidence interval with coverage probability 1-$\alpha$ is the interval between the $100\times\alpha/2$ and $100\times(1-\alpha/2)$ percentile of the empirical distribution of the statistic of interest. In our simulations we chose $\alpha = 0.05$. Note that in the same way we obtained bootstrap confidence intervals for randomly permutated LI data.

For the experimental data shown in Fig. 6, Figures 9 and 10 show that for all 100 randomly chosen pairings and the original pairing (dashed line), the confidence intervals overlap, showing that variance and median are not influenced by pairing. The same is true for the experimental data in Figs. 5 and 8. This indicates that the "original" LI values are a reasonable basis for further statistical analysis.

It is known that for the median an unreasonably high number of bootstrap samples would be necessary to yield reliable bootstrap estimates. In such cases, the bootstrap approach can be improved by involving a smoothed version of the sample quantile function; for a survey of smoothed bootstrap we refer to Falk and Reiss (1992). In our simulations the normal kernel was used in order to obtain such a smoothed version of the sample quantile function. The simulations were carried out with the software package *MATHEMATICA*, Version 5.0.

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