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RESEARCH ARTICLE

Olfactory memories are intensity specific in larval *Drosophila*

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SUMMARY

Learning can rely on stimulus quality, stimulus intensity, or a combination of these. Regarding olfaction, the coding of odour quality is often proposed to be combinatorial along the olfactory pathway, and working hypotheses are available concerning short-term associative memory trace formation of odour quality. However, it is less clear how odour intensity is coded, and whether olfactory memory traces include information about the intensity of the learnt odour. Using odour–sugar associative conditioning in larval *Drosophila*, we first describe the dose–effect curves of learnability across odour intensities for four different odours (*n*-amyl acetate, 3-octanol, 1-octen-3-ol and benzaldehyde). We then chose odour intensities such that larvae were trained at an intermediate odour intensity, but were tested for retention with either that trained intermediate odour intensity, or with respectively higher or lower intensities. We observed a specificity of retention for the trained intensity for all four odours used. This adds to the appreciation of the richness in 'content' of olfactory short-term memory traces, even in a system as simple as larval *Drosophila*, and to define the demands on computational models of associative olfactory memory trace formation. We suggest two kinds of circuit architecture that have the potential to accommodate intensity learning, and discuss how they may be implemented in the insect brain.

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INTRODUCTION

Stimuli can differ in kind and/or intensity. At the sensory level, stimulus kind is defined by the type of receptor activated and its connectivity, while the level or latency of activation of the receptor codes for the intensity of the stimulus. Thus, processing of stimulus-kind and stimulus-intensity is entangled: one cannot conceive of a receptor that is activated, but at no particular level. In turn, a given level of activation must always be the level of activation of a particular receptor. At the perceptual level, however, the entanglement of quality and intensity can be resolved: it is possible to refer to a smell as 'fruity' without specifying the intensity of the olfactory impression, or to regard the olfactory impression of a perfume as 'too much', without specifying its kind. Clearly, such disentanglement of intensity from quality is a feature of perception, brought about by post-receptor computations. It is one of the more challenging tasks to understand these computations neurobiologically.

In this context, we decided to study intensity processing in olfactory associative function; that is, olfactory discrimination learning can rely either on intensity differences, quality differences, or both. While the coding of odour quality is often proposed to be combinatorial along the olfactory pathway (see Discussion), and although a fairly explicit working hypothesis about short-term odour quality memory trace formation is available (see Discussion), it is less obvious how odour intensity information is organized. In the present paper, we focus on the question whether odour intensity information is included in olfactory memory traces at all.

We tackle this issue using odour–sugar associative conditioning in larval *Drosophila* (Fig. 1A) (Scherer et al., 2003; Michels et al., 2005; Neuser et al., 2005; Mishra et al., 2010; Chen et al., 2011; Michels et al., 2011; Saumweber et al., 2011a; Saumweber et al., 2011b) (for reviews, see Gerber and Stocker, 2007; Gerber et al., 2009; Diegelmann et al., 2013). This is a suitable system for such a study due to its simplicity in terms of cell number, its genetic tractability and the robustness of the paradigm. Last, but not least, the circuit architecture of the olfactory pathway of the larva (as of insects in general) is functionally analogous to that in vertebrates (for reviews, see Hildebrand and Shepherd, 1997; Strausfeld and Hildebrand, 1999; Korsching, 2002; Davis, 2004; Ache and Young, 2005; Bargmann, 2006; Ramdya and Benton, 2010; Wilson, 2008; Galizia and Rössler, 2010), rendering experimental as well as computational studies of insect olfaction potentially inspiring on a broader scale.

Our approach follows the one advocated for adult flies (Yarali et al., 2009) [that paper also includes a discussion of alternative approaches (Xia and Tully, 2007; DasGupta and Waddell, 2008; Masek and Heisenberg, 2008)]. A distinguishing feature of this approach is that first the dose–effect curves of learnability are described. This allows choosing odour intensities appropriate for

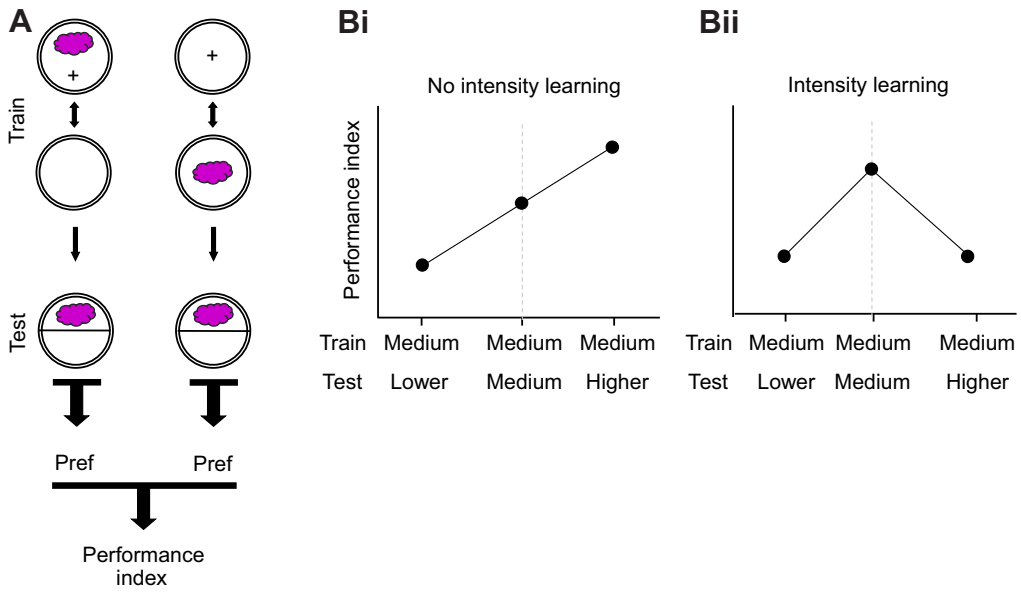

Fig. 1. Experimental design. (A) Larvae are trained and tested in cohorts of 30, using a reciprocal training regimen. At the beginning of training, odour (magenta cloud) is presented to a Petri dish containing agarose, with added fructose (+). Larvae are then removed to another dish containing no odour and filled with only agarose. This cycle of training is repeated three times. For the test, larvae are placed in the middle of a dish filled with only agarose; on one side odour is presented, and on the other side no odour is presented. After 3 min, larvae on each half of the dish are counted. Alternately, we train larvae reciprocally, by unpaired presentation of fructose and odour. This allows for subsequent calculation of a performance index (PI) comparing the preference values (Pref) between the reciprocally trained groups. The sequence of training trials within groups as well as the sidedness of placing these containers is balanced across repetitions of the experiment. (B) To test for intensity learning, we train larvae with a medium odour intensity and during the subsequent test either the same trained medium intensity, or a lower, or a higher odour intensity is presented; (i) no intensity learning: increased levels of conditioned behaviour when the test intensity is higher than in training indicate that the intensity parameter is not included in the memory trace; (ii) intensity learning: if we see the full level of conditioned behaviour only when training and testing odour intensities are matching, we conclude that the intensity parameter is included in the memory trace.

an intensity generalization type of experiment (see Fig. 1B), where we train larvae to a medium intensity, but test them with either a lower or higher intensity of the trained odour. The rationale of this experimental design is that if associative testing scores turn out to increase when the testing intensity is higher than the training intensity, this must be because a higher intensity is judged by the larvae as 'more of the trained' odour (Fig. 1Bi). If, in contrast, the larvae regard a higher intensity as 'something different', we should observe a generalization decrement for the higher testing condition (Fig. 1Bii). This latter result would imply that the memory trace established by the larvae during training is parametrically specific for the trained intensity of the odour.

MATERIALS AND METHODS

Flies

Third-instar, feeding-stage *Drosophila* larvae (5 days after egg laying) of the Canton Special wild-type strain were used. The flies were kept in mass culture under a 14 h:10 h light:dark cycle at 25°C and 60–70% relative humidity. For the learning assay, a spoonful of medium containing larvae was placed in an empty Petri dish, and 30 larvae were collected and washed in distilled water. Experiments complied with applicable law.

Petri dishes

One day prior to the experiment, Petri dishes of 85 mm inner diameter (Sarstedt, Nümbrecht, Germany) were filled either with a solution of 1% agarose (electrophoresis grade) or 1% agarose with 2 mol l^{-1} fructose (both from Roth, Karlsruhe, Germany). Once the agarose had solidified, dishes were covered with their lids and left until the following day.

Learning assay

Learning assays were performed under a fume hood at 21–26°C, under room light from a fluorescent lamp. Larvae were trained and tested in cohorts of 30, using either of two reciprocal training regimen (for a sketch see Fig. 1A). For each regimen, the sequence of training trials was balanced across repetitions of the experiment. For example, at the beginning of training, two odour-filled Teflon containers were placed at opposite sides of a Petri dish containing agarose, with added fructose (Odour +). Larvae were placed in the middle of this dish and left crawling for 5 min. They were then removed to another Petri dish containing two empty Teflon containers (EM) and filled with only agarose, where they also spent 5 min. This cycle of Odour+/EM training was repeated three times, using fresh Petri dishes each time. At the end of training, larvae were placed in the middle of a Petri dish filled with only agarose. Teflon containers were placed on opposing sides, one filled with the odour and one empty; the sidedness of placing these containers was balanced across repetitions of the experiment. After 3 min, larvae on each half of the Petri dish were counted to calculate a preference index (Pref) as:

$$\text{Pref} = (N_{\text{Odour}} - N_{\text{EM}})/N_{\text{Total}}\,. \quad (1)$$

In this formula, N is the number of larvae on the corresponding side of the dish. Pref values thus range from –1 to 1; negative values indicate avoidance of the odour, positive values reflect approach. The Pref scores for all experiments are documented in supplementary material Figs S1, S2 and S4.

Alternately, we trained larvae reciprocally, that is by unpaired presentations of odour and reward (Odour/EM+) and then tested them as described above. An associative performance index (PI)

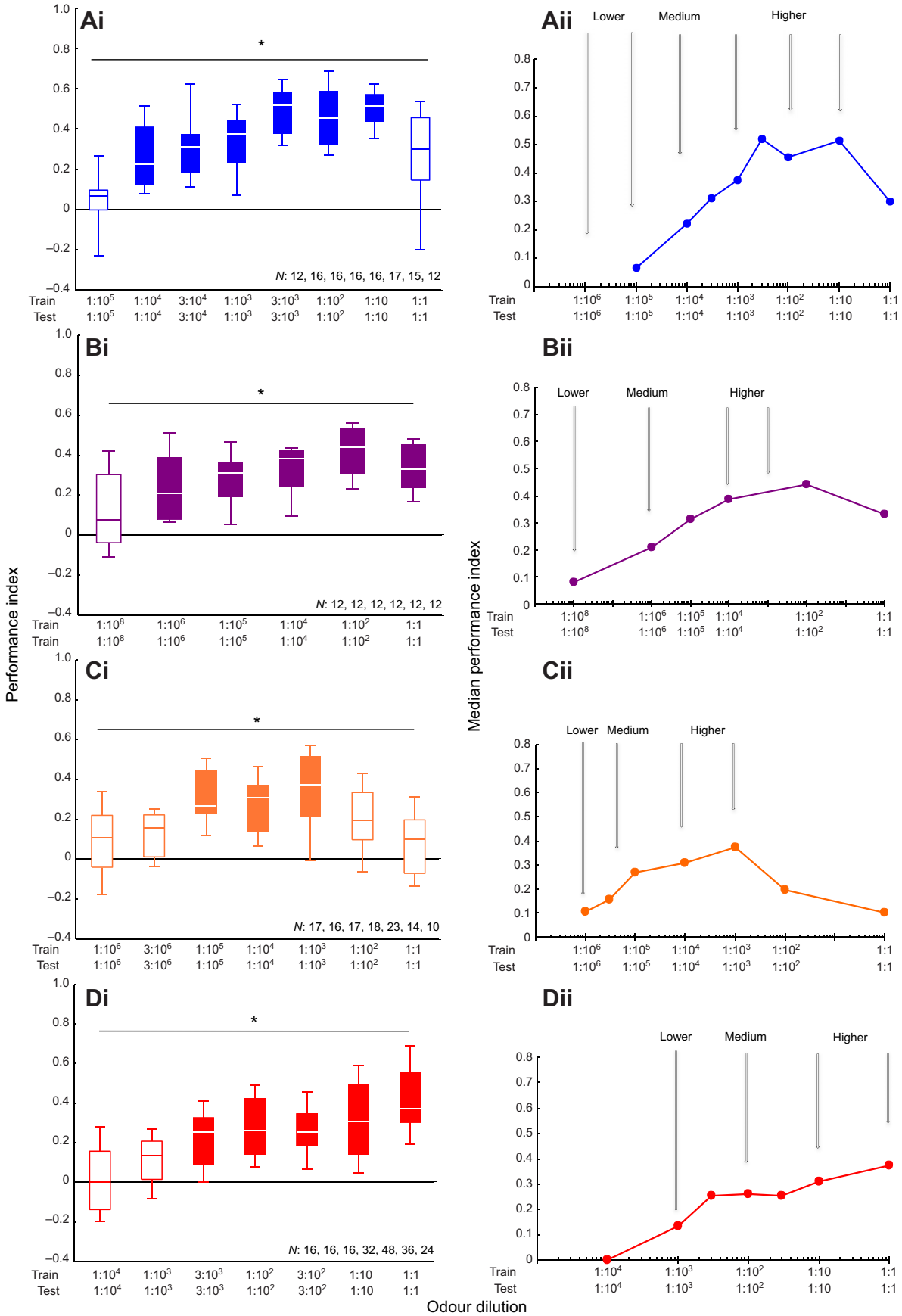

Fig. 2. See next page for legend.

Fig. 2. Dose dependency of learnability. Dose–effect curves of learnability across odour intensities for four different odours: (A) *n*-amyl acetate (AM), (B) 3-octanol (3-Oct), (C) 1-octen-3-ol (1-Oct-3-ol) and (D) benzaldehyde (BA). In the i parts, data are presented as box plots (median as the mid-line, 25/75% quartiles as box boundaries and the 10/90% quantiles as whiskers). **P*<0.05 refers to global comparisons across odour intensities in KW tests. Shading of a box indicates that the performance indices significantly differ from zero (OSS tests, Bonferroni corrected). Respective sample sizes are shown at bottom corners of the graphs. In the ii parts, the median performance indices from i are plotted over odour dilution, on a logarithmic axis. From these curves odour dilutions for the follow-up experiments (Fig. 3) are chosen such that they support about half-maximal performance indices, designated as medium intensity, as well as respectively lower and higher intensities. (Ai) For AM we find an optimum function for associative performance scores across odour intensities; from ii we designate $1{:}10^4$ as the medium intensity, $1{:}10^5$ as well as $1{:}10^6$ as lower, and $1{:}10^3$, $1{:}10^2$ as well as 1:10 as higher intensities (for statistics, see text). (Bi) For 3-Oct, associative performance indices at very low intensity are not significantly different from zero, whereas all other groups do show significant learning scores (OSS tests with *P*<0.05/6 as criterion for significance) (the KW test across groups yields *H*=13.89, d.f.=5, *P*<0.05), although one may note a trend for decreasing performance indices for the highest intensity used. From ii, we identify $1{:}10^6$ as the medium intensity, $1{:}10^8$ as a lower intensity, and $1{:}10^4$ as well as $1{:}10^3$ as higher intensities. (Ci) For 1-Oct-3-ol, we find an optimum function for associative performance scores across odour intensities (KW test: *H*=28.1, d.f.=6, *P*<0.05): at very low and very high odour intensities, performance indices are not significantly different from zero, whereas the other groups do show significant associative performance scores (OSS tests with *P*<0.05/7 as criterion for significance). From ii, we designate $5.6{:}10^6$ as the medium intensity, $1{:}10^6$ as lower, and $1{:}10^4$ as well as $1{:}10^3$ as higher odour intensities. (Di) For very low intensities of BA, associative performance indices are not significantly different from zero, whereas all other groups do show significant scores (OSS tests with *P*<0.05/7 as criterion for significance) (the KW test across groups yields *H*=43.3, d.f.=6, *P*<0.05). From ii, we designate $1{:}10^2$ as the medium intensity, $1{:}10^3$ as lower, and 1:10 as well as 1:1 as higher odour intensities.

can then be calculated based on the difference in odour preference between these two reciprocally trained groups (Saumweber, 2007; Selcho et al., 2009; Saumweber et al., 2011a; Saumweber et al., 2011b):

$$\mathrm{PI} = (\mathrm{Pref}_{\mathrm{Odour+/EM}} - \mathrm{Pref}_{\mathrm{Odour/EM+}})/2\ . \qquad (2)$$

The subscripts of Pref indicate the respective training regimen. These associative performance indices thus range from –1 to 1, positive values indicating conditioned approach (appetitive learning), and negative values indicating conditioned avoidance (aversive learning).

Odours

As odours, we used 3-octanol (3-Oct), *n*-amyl acetate (AM), 1-octen-3-ol (1-Oct-3-ol), linalool (Lin) and 1-octanol (1-Oct) (all from Merck, Darmstadt, Germany; CAS: 589-98-0, 628-63-7, 3391-86-4, 78-70-6, 111-87-5), and hexyl acetate (HA), benzaldehyde (BA) and 4-methylcyclohexanol (MCH) (from Sigma-Aldrich, Steinheim, Germany; CAS: 142-92-7, 100-52-7, 589-91-3). Odours were diluted in paraffin oil (Merck) as described in the Results section. In each case, 10 µl of odour solution was applied to custom-made Teflon containers with an inner diameter of 5 mm, and a perforated cap with seven holes of 0.5 mm diameter each.

For AM, 3-Oct, 1-Oct-3-ol and BA we describe the dose–effect functions of learnability and probe for the intensity specificity of the memory trace; for the remaining odours, we restrict ourselves to documenting the dose–effect functions of learnability and the underlying preferences (supplementary material Fig. S2), either because learnability is undesirably low (1-Oct, MCH, Lin), or in the case of HA because a high similarity, both physico-chemically and perceptually (Chen et al., 2011), seems to make HA redundant to AM. A summary of the dose–effect functions of learnability for all odours can be found in supplementary material Fig. S3.

Statistics

Data were collected in parallel for all the groups to be statistically compared, using non-parametric analyses throughout. Kruskal–Wallis (KW) tests were used to compare across multiple groups; in case of significance, we separately tested the scores of single groups against zero using one-sample sign (OSS) tests. The significance level for these tests was set to 0.05, maintaining an experiment-wide error rate of 5% by a Bonferroni correction. That is, in a case where for example five groups are to be compared individually with zero, the critical *P*-level is set to 0.05/5=0.01. The Mann–Whitney *U*-test (MWU) along with the Bonferroni correction was used to compare two groups with each other. All statistical analyses were performed with Statistica (version 8.0, StatSoft, Tulsa, OK, USA) on a PC.

Performance indices are presented as box plots with the median as mid-line, box boundaries as the 25/75% quartiles and whiskers as the 10/90% quantiles. Sample sizes are given in the figures.

RESULTS

Memory is intensity specific for *n*-amyl acetate, 3-octanol and 1-octen-3-ol

Using AM as odour, we find an optimum function for associative performance indices across odour intensities (Fig. 2Ai; KW test: H=47.4, d.f.=7, *P*<0.05). Specifically, at intermediate intensities significant associative scores are obtained, whereas the lowest intensity used is apparently not learnable; notably, also at the highest intensity performance indices do not formally differ from chance (Fig. 2Ai; OSS tests with *P*<0.05/8 as criterion for significance). This is probably because at such high intensity the relatively strong innate preference for AM hinders revealing an associative memory (see supplementary material Fig. S1A). We therefore restrict our choice of odour intensities up to the 1:10 dilution.

To probe for a possible intensity specificity of the AM memory trace, we used an intensity that supports about half-maximal associative performance indices (Fig. 2Aii), allowing us to detect both increases and decreases in scores. Specifically, we chose $1{:}10^4$ as the medium intensity for training, and then tested larvae either at lower ($1{:}10^5$, $1{:}10^6$) or higher ($1{:}10^3$, $1{:}10^2$, 1:10) intensities. It turns out that as the testing intensities deviate from the training intensity towards either higher or lower intensities, performance indices approach zero (Fig. 3A: OSS tests with *P*<0.05/6 as criterion for significance; the KW test across all groups yields *P*<0.05, *H*=29.4, d.f.=5). Thus, in order to support full retention, the testing intensity needs to match the training intensity; this follows scenario ii in Fig. 1B. Given that for 3-Oct and 1-Oct-3-ol we obtain the same results (Fig. 2B,C, Fig. 3B,C; for statistics, see legends), we conclude that as a rule olfactory associative learning establishes intensity-specific memory traces in larval *Drosophila*.

Is benzaldehyde an exception?

In the adult, BA memories do not seem to be intensity specific as assayed in an odour–electric shock associative paradigm: higher-than-trained BA intensities support higher associative performance indices than the actually trained intensity [see fig. 4D in our previous paper (Yarali et al., 2009); for a replication within the present study,

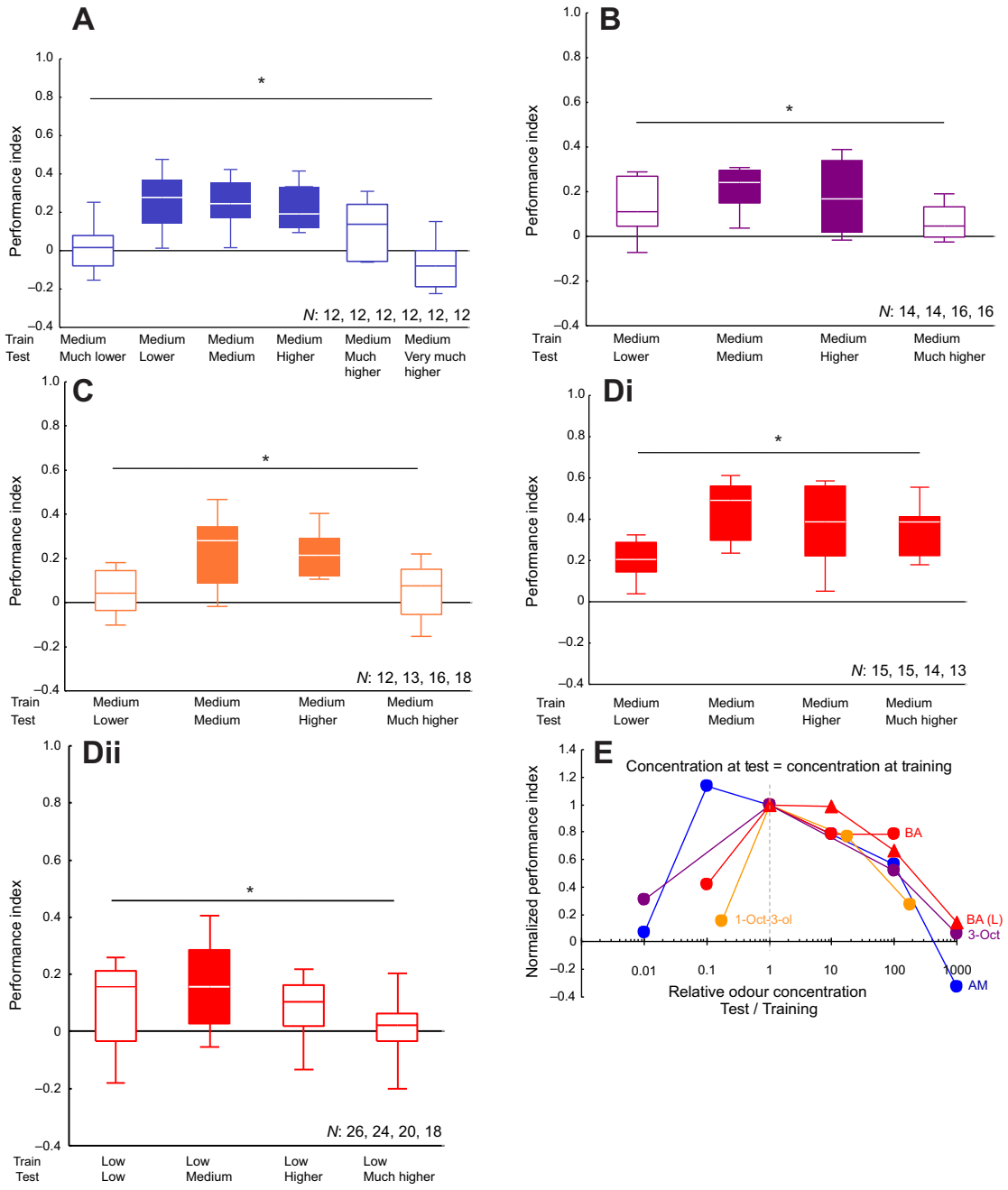

Fig. 3. Memory traces are intensity specific. Larvae are trained with a given odour intensity, but are tested for retention with either that trained odour intensity, or with higher or lower intensities. We observe a specificity of retention for the trained intensity for all four odours used: (A) *n*-amyl acetate (AM), (B) 3-octanol (3-Oct), (C) 1-octen-3-ol (1-Oct-3-ol) and (D) benzaldehyde (BA). Other details as in Fig. 2. (A) After training with a medium intensity of AM, associative performance indices degrade upon a mismatch between training and testing odour intensities (for statistics, see text). (B) For 3-Oct, larvae show the highest associative performance indices when the testing intensity matches the training intensity (OSS tests with $P<0.05/4$ as criterion for significance) (the respective KW test yields $H=8.5$, d.f.=3, $P<0.05$; pair-wise MWU tests confirm this conclusion). (C) For 1-Oct-3-ol, we also observe a loss of associative performance indices upon a mismatch between training and testing odour intensities (OSS tests with $P<0.05/4$ as criterion for significance) (the respective KW test yields $H=15.2$, d.f.=3, $P<0.05$; pair-wise MWU tests confirm this conclusion). (Di) For BA, associative performance indices decrease when the testing odour intensity is lower than the training intensity (MWU test, $U=30$, $P<0.05/3$) (scores remain significantly different from zero: OSS test: $P<0.05/4$). When testing intensity is higher or much higher than the training intensity, scores remain formally unaltered, despite an apparent trend towards decreasing scores (MWU tests: medium *vs* higher, $U=90$; medium *vs* much higher, $U=64$, $P>0.05/3$ in both cases) (the respective KW test yields $H=13.1$, d.f.=3, $P<0.05$). (Dii) When we use the low intensity of BA as the training intensity, associative performance indices decrease as the testing intensity is increased towards much higher odour intensity (MWU tests: low *vs* medium, $U=271$, $P>0.05/3$; low *vs* higher, $U=203$, $P>0.05/3$; low *vs* much higher, $U=124$, $P<0.05/3$) (the respective KW test yields $H=10.9$, d.f.=3, $P<0.05$). (E) Semi-schematic summary of the data from A–D. On the *x*-axis we use a logarithmic scale to indicate relative odour concentrations. A value of 1 indicates that testing intensity equals training intensity; all other values indicate the fold-mismatch between training and testing intensity. On the *y*-axis, for each odour we define the median associative performance index observed when training and testing intensity match as 1; all other medians regarding that odour then are plotted as normalized performance indices. For all odours used, performance scores decay upon mismatch in odour intensity between training and test.

see supplementary material Fig. S5), thus following scenario i in Fig. 1B. We therefore include BA in our analysis concerning the larva as well.

In the dose–effect description of the learnability of BA, associative performance indices increase as odour intensity is increased (Fig. 2Di; KW test: H=43.3, d.f.=6, P<0.05). We chose high, medium and low intensities from this dose–response curve (Fig. 2Dii), and trained the larvae with the medium intensity. Different groups of larvae were then tested with either the same medium, with lower, or with higher intensities, respectively. As expected, when lower intensities are used for testing, associative performance indices are lower than when the trained medium intensity is presented at test (Fig. 3Di; MWU test: U=30.0, P<0.05/3). However, associative performance indices remain unaltered if medium-trained larvae are tested with higher or even much higher intensities (Fig. 3Di; MWU tests: U=90, 64.0, P=0.51, 0.12) (the corresponding KW test yields P<0.05, H=13.11, d.f.=3). This result is not conclusive regarding the question whether BA memory traces are intensity specific (compare the data of Fig. 3Di to the two scenarios presented in Fig. 1B).

To overcome this deadlock, we trained larvae with a low intensity and tested them with either that very same trained low intensity, or the medium, or the higher odour intensity (note that in this experiment the latter two testing intensities are both higher-than-trained). Clearly, if the training intensity is relatively low, overall performance indices are lower (compare the medium-medium condition in Fig. 3Di to the low-low condition in Fig. 3Dii); more critically, we found that associative performance indices are decreased as testing intensities are strongly elevated above the trained low intensity [Fig. 3Dii; train low, test low *versus* the groups tested with medium (MWU test: U=271.0, P=0.42), higher (MWU test: U=203.0, P=0.2), or tested with much higher intensities (MWU test: U=124.0, P<0.05/3)] (the corresponding KW test yields: P<0.05, H=9.16, d.f.=3). Thus just as for all other assayed odours, these results fit the scenario in Fig. 1Bii: BA memories are intensity specific in larval *Drosophila* (Fig. 3E).

DISCUSSION

We provide an analysis of whether intensity can be a distinctly learnable parameter of an odour. Indeed, for adult flies (Xia and Tully, 2007; Masek and Heisenberg, 2008; Yarali et al., 2009) and bees (Bhagavan and Smith, 1997; Wright et al., 2005; but see Pelz et al., 1997), such intensity specificity of memory has been reported. Here, we show that in a system as simple as larval *Drosophila*, too, there is intensity learning (Fig. 3E). Interestingly, in a corresponding study in adult *Drosophila*, three of the four odours used (namely AM, 3-Oct and 4-methylcyclohexanol) support intensity learning, but apparently BA does not (Yarali et al., 2009) (for a replication of this latter result, see supplementary material Fig. S5). This discrepancy between the intensity specificity of larval and adult BA memories may be caused either by the difference in learning paradigms used (e.g. kind of reinforcer, number and duration of training trials, etc.) or by the fact that in adult *Drosophila* the genetic and neuronal basis for BA responsiveness differs from those of other odours (Helfand and Carlson, 1989; Ayer and Carlson, 1992; Keene et al., 2004; Yarali et al., 2009), while this is not apparently the case in the larvae. Also, while many investigators have found that 4-methylcyclohexanol can be learned well in adults (Yarali et al., 2009), this is not the case in larvae (supplementary material Fig. S2C). Actually, larvae seem behaviourally little responsive to 4-methylcyclohexanol (supplementary material Fig. S2C). Given that the general circuit architecture between larvae and adults is rather similar (Gerber et al., 2009), it is tempting to speculate that these discrepancies between larvae and adults may be based on different receptor repertoires of the two life stages (Kreher et al., 2005; Hallem and Carlson, 2006).

Possible circuitry underlying intensity learning

With respect to larval *Drosophila*, nothing is known as yet about the mechanisms of intensity learning. Trivially, the recognition of a particular odour intensity at test as being different from the trained one is possible only if the neuronal activity induced by a given odour intensity differs in at least some respect from the activity induced by other intensities of that same odour. At which stage along the olfactory pathway may such dissociation be found? We first briefly review the architecture of the olfactory pathway (for reviews, see Vosshall, 2000; Gerber and Stocker, 2007; Vosshall and Stocker, 2007; Stocker, 2008; Gerber et al., 2009; Masse et al., 2009; Touhara and Vosshall, 2009; Diegelmann et al., 2013) and then suggest two alternative scenarios for intensity learning.

Different odours initially activate partially overlapping subsets of olfactory sensory neurons in the olfactory organs, dependent on the ligand profile of the olfactory receptor protein expressed. In the larva, each of the 21 olfactory sensory neurons expresses only one ligand-specifying *Or* receptor gene, and in turn each receptor gene is expressed in only one sensory neuron. Each sensory neuron then innervates one of the 21 glomeruli in the antennal lobe. In analogy to the situation in adults (Wilson, 2008), the pattern of activity in the antennal lobe is probably shaped by local interneurons (Thum et al., 2011). The resulting glomerular activity pattern is picked up by typically uni-glomerular projection neurons and is relayed both to pre-motor centers as well as to the Kenyon cells of the mushroom bodies, which in turn have access to pre-motor areas as well. Thus, dependent on the ligand profiles of the receptors and the connectivity in the system, odour quality could be combinatorially encoded along the olfactory pathway.

As for odour intensity, activity patterns in sensory and projection neurons seem to broaden with increasing intensity [larva (Asahina et al., 2009); adult (Ng et al., 2002; Wang et al., 2003; Root et al., 2007)]; notably, however, at successive processing stages activity patterns become more and more intensity invariant (Voeller, 2009). Such nested representations clearly could not accommodate intensity learning. Suppose that during training a memory trace was laid down in those neurons that are activated by the particular odour intensity used. In the subsequent test, a higher intensity of the same odour would activate, among other neurons, always all these same neurons, probably even more strongly than the trained intensity does, hence inducing at least as strong conditioned behaviour as the trained intensity. It therefore seems unlikely that the traces of intensity memories are laid down at a level of processing where olfactory representations are nested, such as is probably the case for sensory or projection neurons. At the next level of olfactory processing, mushroom body Kenyon cells show different levels of intensity invariance in their responses [locust (Stopfer et al., 2003); adult *Drosophila* (Wang et al., 2004; Voeller, 2009)]; critically, the activity pattern evoked by a low intensity of an odour is not always fully nested within that evoked by a higher intensity of the same odour [e.g. for ethyl acetate, see fig. 3 in Wang et al. (Wang et al., 2004)]. It remains unclear what kind of a connectivity scheme could transform nested representations at the projection neuron level to intensity-specific representations at the Kenyon cell level. In any event, taking this scenario to its logical extreme, training with a particular intensity could lay down a memory trace in a

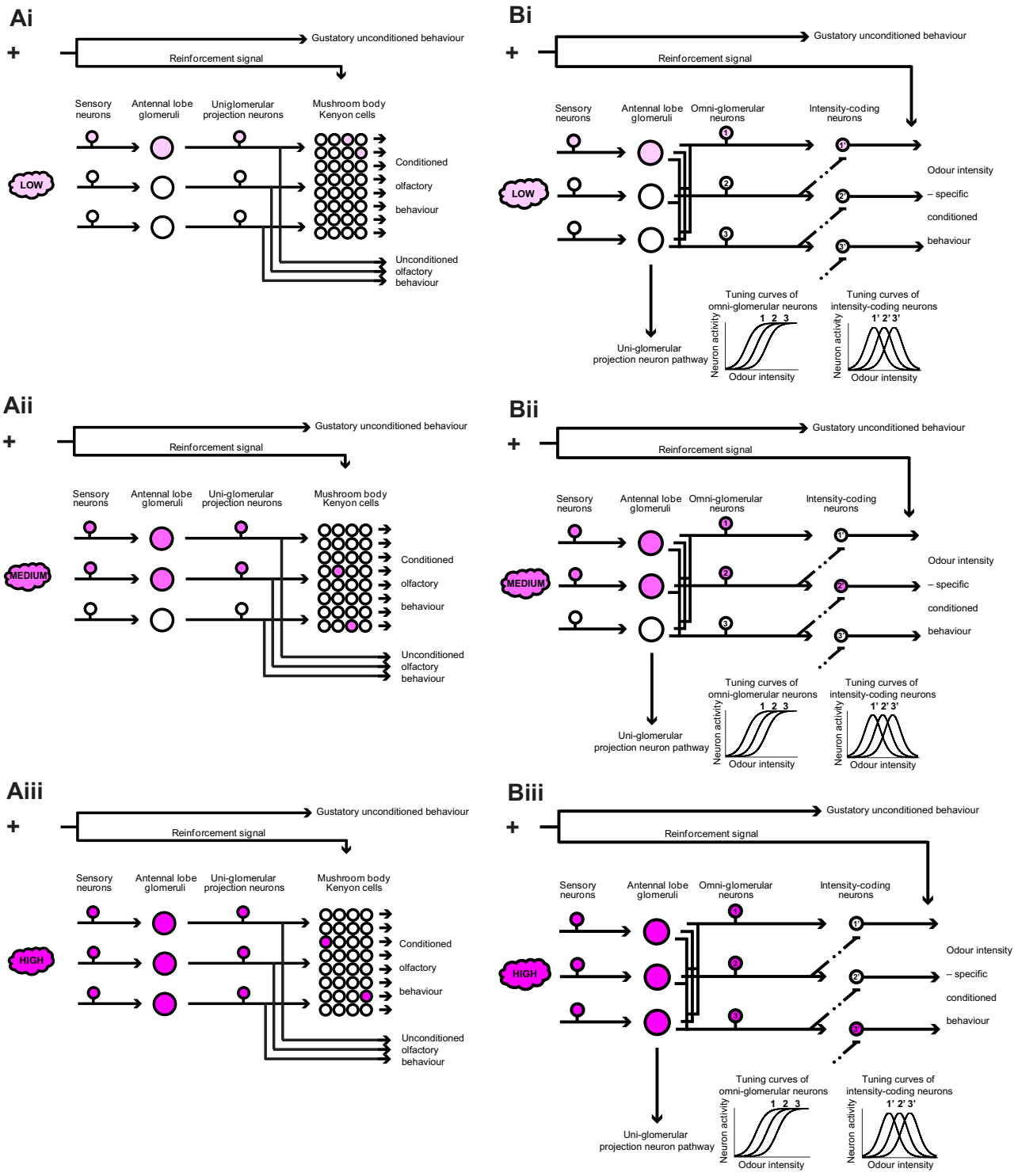

Fig. 4. Two suggested circuit architectures for intensity learning. We sketch two logical extremes as to how odour intensity may be encoded along the olfactory pathway. For simplicity, only a few units are displayed at each level of olfactory processing. We exemplify the encoding of three different intensities: (i) low, (ii) medium and (iii) high. Along the sketched olfactory pathway, those units that are activated by a particular intensity are coloured accordingly, faintest for low and strongest for high. Arrowheads indicate excitatory outputs; blunt ends represent inhibition. In either scenario (A and B), at the sensory neuron level, more units are activated with increasing odour intensity; thus the pattern of activity for the low intensity is nested within that for the medium, which in turn is nested within the pattern for the high. (A) Uni-glomerular projection neurons pick up these nested representations and relay them to the mushroom body Kenyon cells. Due to an as yet unknown scheme of connectivity from the projection neurons, non-overlapping sets of Kenyon cells are activated by different odour intensities, enabling intensity-specific memories to be laid down. (B) Omni-glomerular neurons sum up the activity over all antennal lobe glomeruli. We sketch three omni-glomerular neurons with different sensitivities, i.e. different sigmoidal tuning curves. Note that at the level of these omni-glomerular neurons, too, we obtain nested representations for different intensities as low<medium<high. This is sorted out at the next level of neurons: each of these receives excitatory input from one omni-glomerular neuron and inhibitory input from the neighbouring omni-glomerular neuron with less sensitivity, i.e. with a right-shifted tuning curve. This pattern of connectivity results in bell-shaped tuning to odour intensity at this last level of neurons, enabling intensity learning.

set of Kenyon cells that, as a set, is specifically activated only by that same odour at that same intensity. Obviously, this implies an entangled storage of quality and intensity information in the Kenyon cells (Fig. 4A).

Alternatively, quality and intensity might be encoded separately, enabling independent learning and retrieval of each (Fig. 4B). While the quality of an odour may be coded for memory formation by the unique set of Kenyon cells it activates, its intensity may be coded for example by the level of activity summed across all antennal lobe glomeruli, as previously argued with respect to adult *Drosophila* as well as the honey bee (Borst, 1983; Sachse and Galizia, 2003; Yamagata et al., 2009; Schmuker et al., 2011). Both larval and adult antennal lobes harbour inhibitory interneurons innervating various numbers of glomeruli; also, excitatory interneurons with similarly wide connectivity are found in the adult antennal lobe [larva (Python and Stocker, 2002a; Python and Stocker, 2002b; Asahina et al., 2009; Thum et al., 2011); adult (Wilson and Laurent, 2005; Olsen et al., 2007; Shang et al., 2007; Chou et al., 2010; Huang et al., 2010; Seki et al., 2010; Yaksi and Wilson, 2010); for a particularly detailed anatomical analysis, see Tanaka et al. (Tanaka et al., 2012)]. Finally, particular adult projection neurons with yet unknown response characteristics connect multiple glomeruli to Kenyon cells and premotor centers in the lateral horn (Marin et al., 2002; Lai et al., 2008; Tanaka et al., 2012). Any or all of these multi/omni-glomerular neurons could sum up the activity across broad aspects of the antennal lobe, and might thus contribute to encoding odour intensity. Note, however, that even at the level of a set of omni-glomerular neurons differing in sensitivity, the representation of a low intensity would be nested within that of a higher intensity. In order to lay down an unambiguous intensity-specific odour memory trace, one would need an additional layer of neurons. These would need to receive, for example, excitatory input from a highly sensitive omni-glomerular neuron and inhibitory input from an intermediately sensitive omni-glomerular neuron to become activated specifically at low but not at medium intensity ranges (Fig. 4B). It would be in these neurons where a memory trace for specifically a low odour intensity could be established. Note that, at its logical extreme, this scenario implies that odour intensity is encoded entirely independent of odour quality. It is as yet unclear whether such a circuit exists, and if so, whether and how such an intensity memory trace is then neuronally and behaviourally integrated with the odour quality memory trace.

To summarize, we show that in a system as simple as the one of larval *Drosophila,* olfactory memory traces are intensity specific. This reveals an unexpected richness of olfactory processing in the larva, and defines the demands on cellular accounts and computational models of associative olfactory function – indeed, the proposed kinds of circuitry may provide a useful scaffold for such an endeavour.

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AUTHOR CONTRIBUTIONS

Conception: D.M., Y.-C.C., B.G. and A.Y.; design: D.M., Y.-C.C., B.G. and A.Y.; execution: D.M., Y.-C.C. and T.O.; interpretation: B.G., A.Y., Y.-C.C. and D.M.; preparation and revision of the article: B.G., A.Y., D.M. and Y.-C.C.: D.M. and Y.-C.C. contributed equally to this work.

COMPETING INTERESTS

No competing interests have been declared.

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Fig: S1A

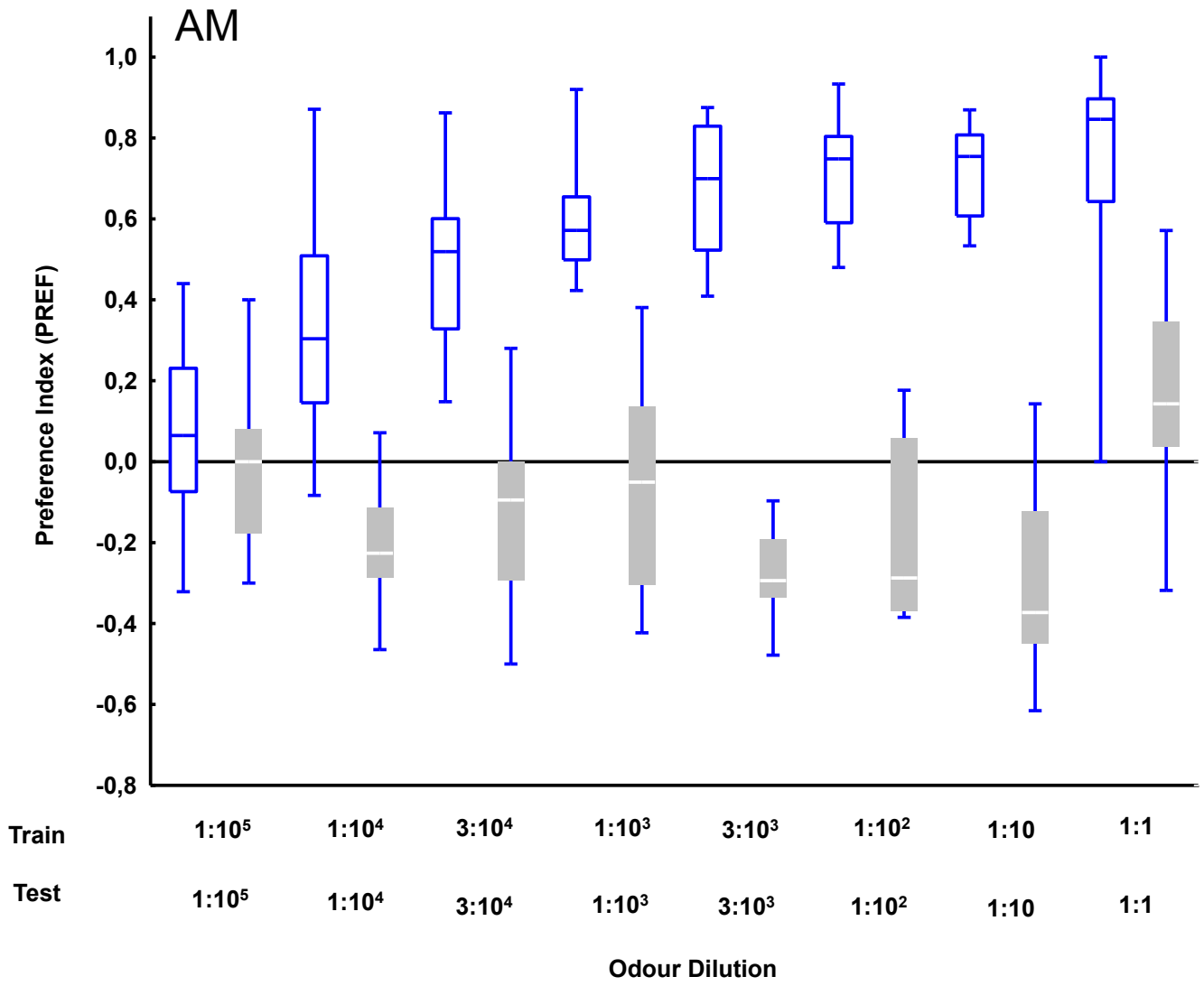
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Fig: S1B

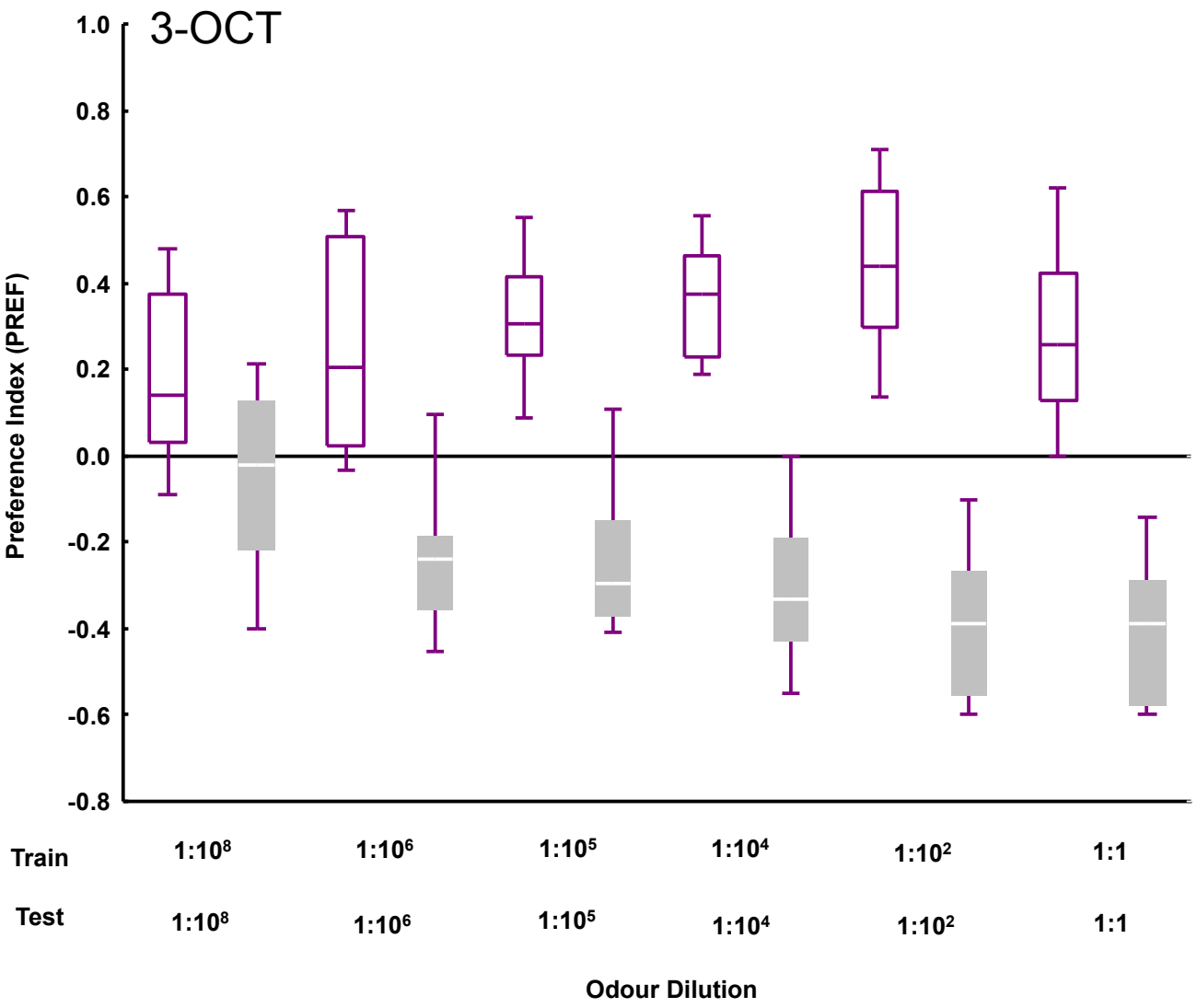
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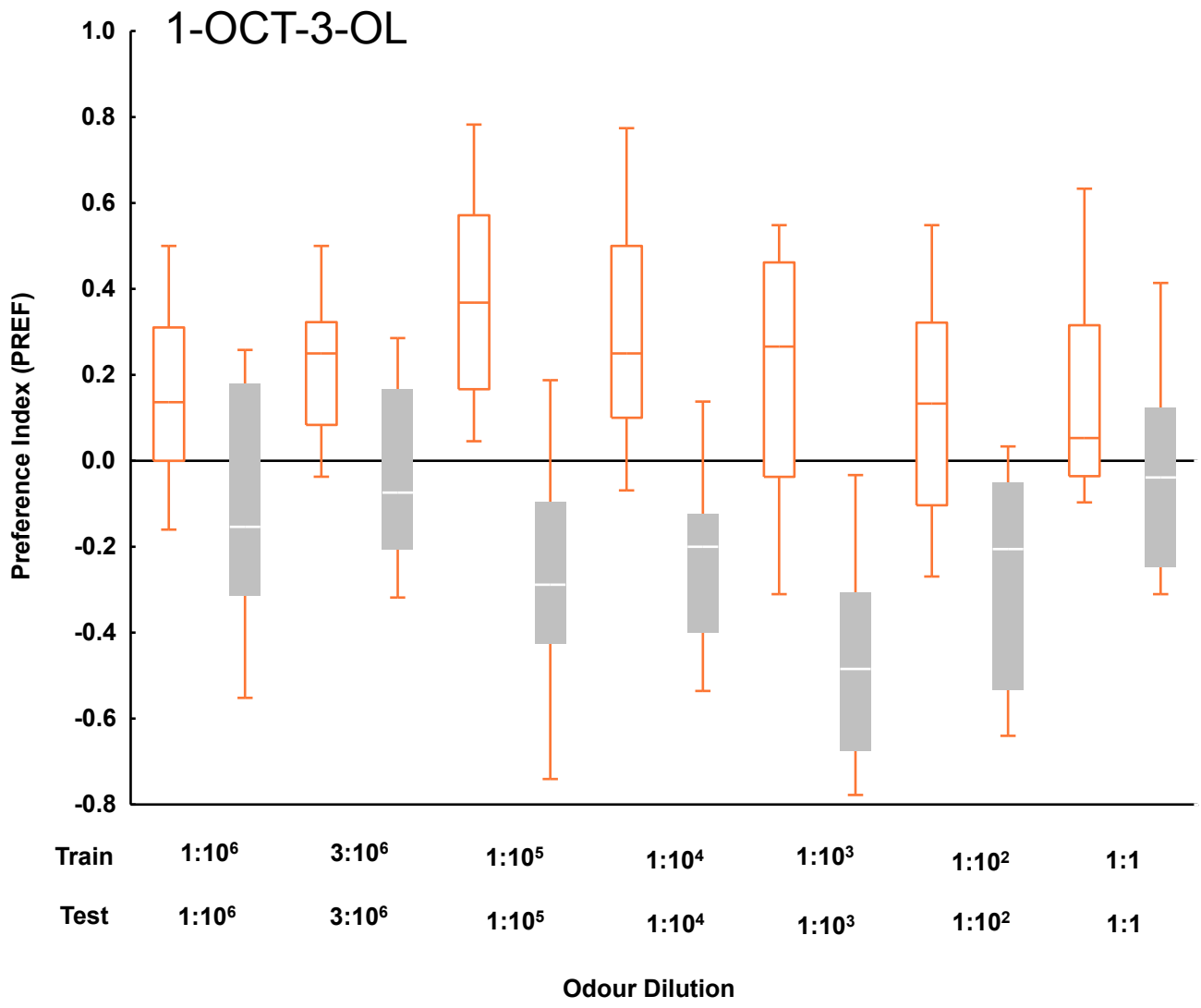
Fig: S1C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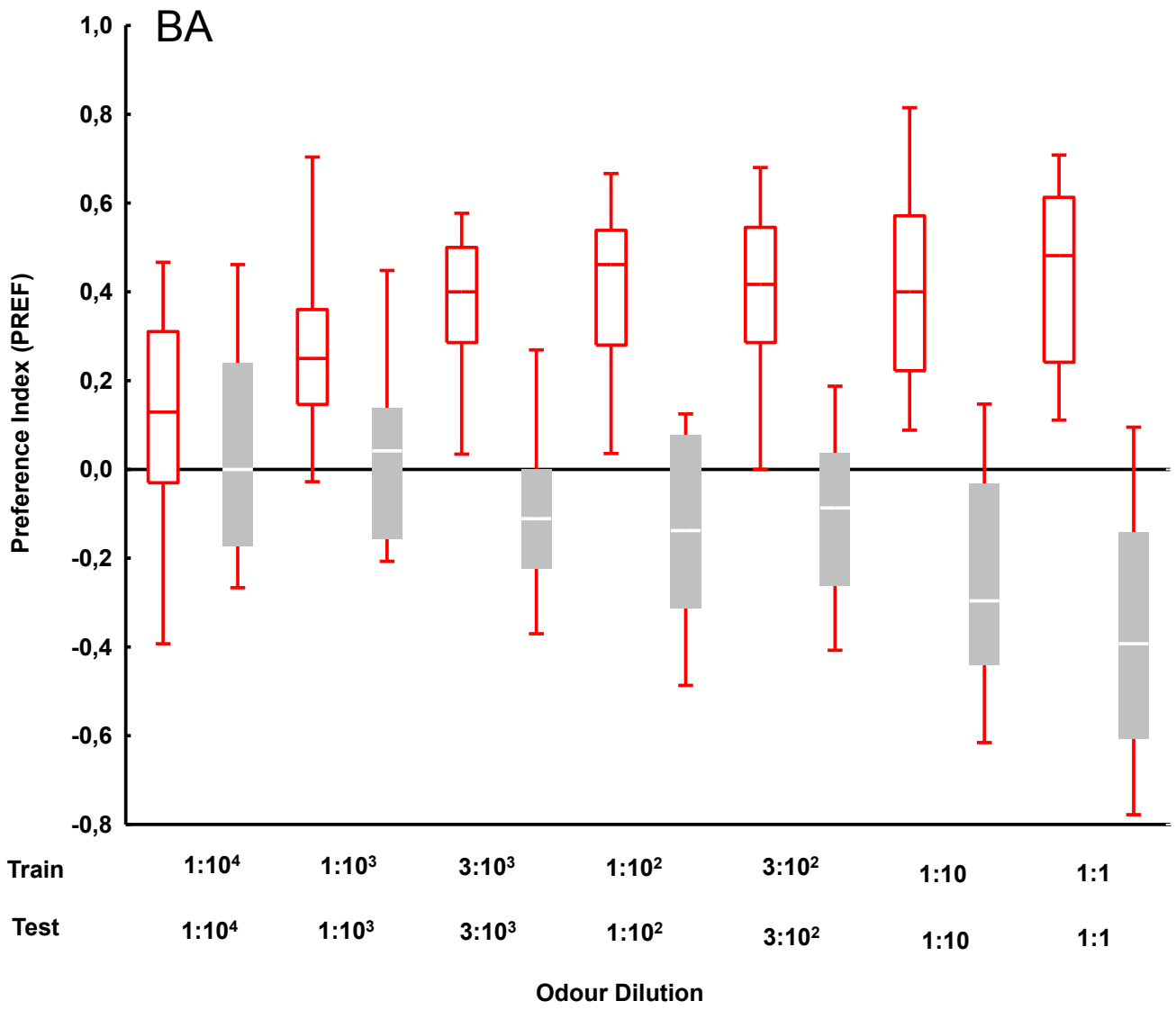
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Fig: S2A (i)

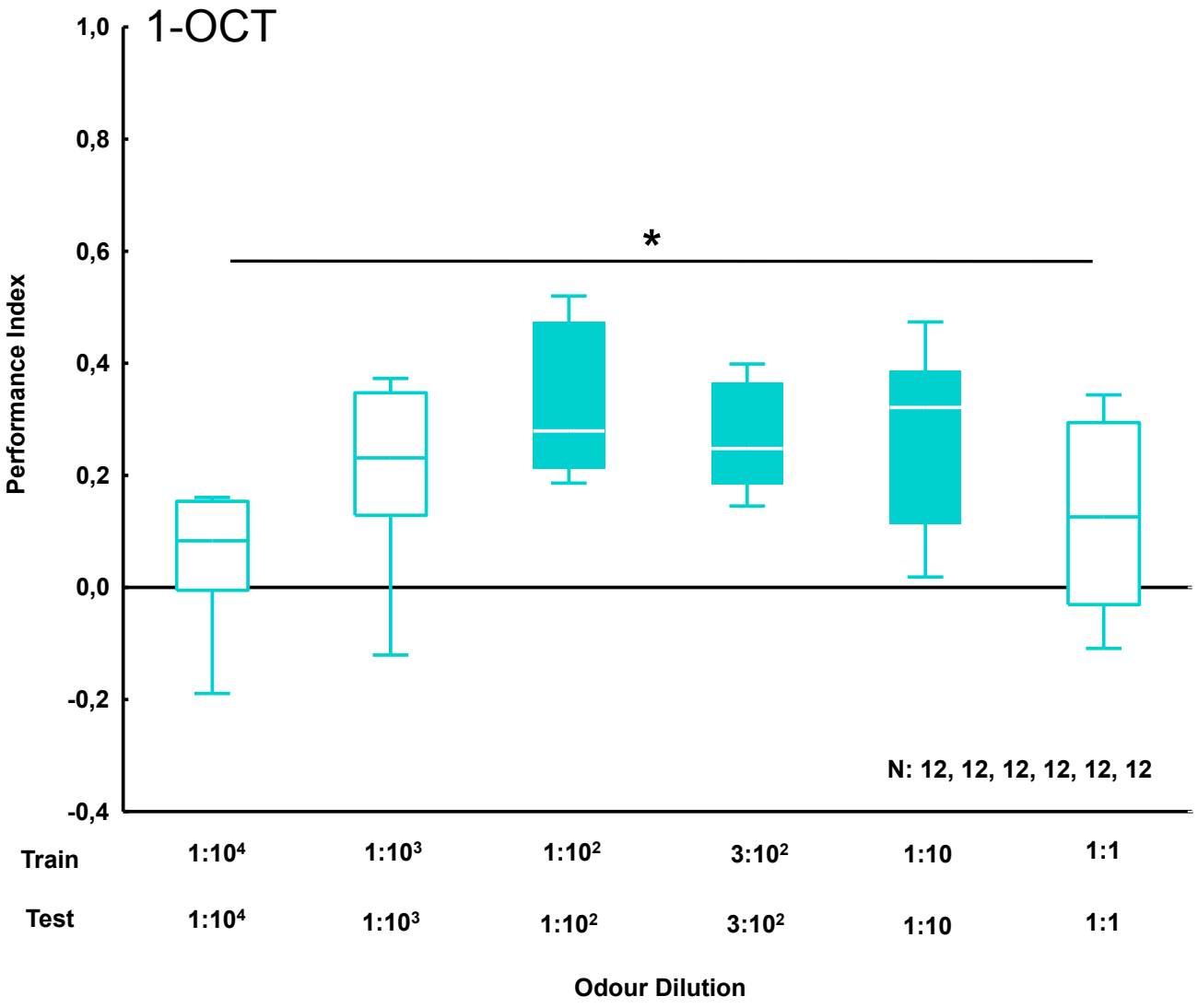
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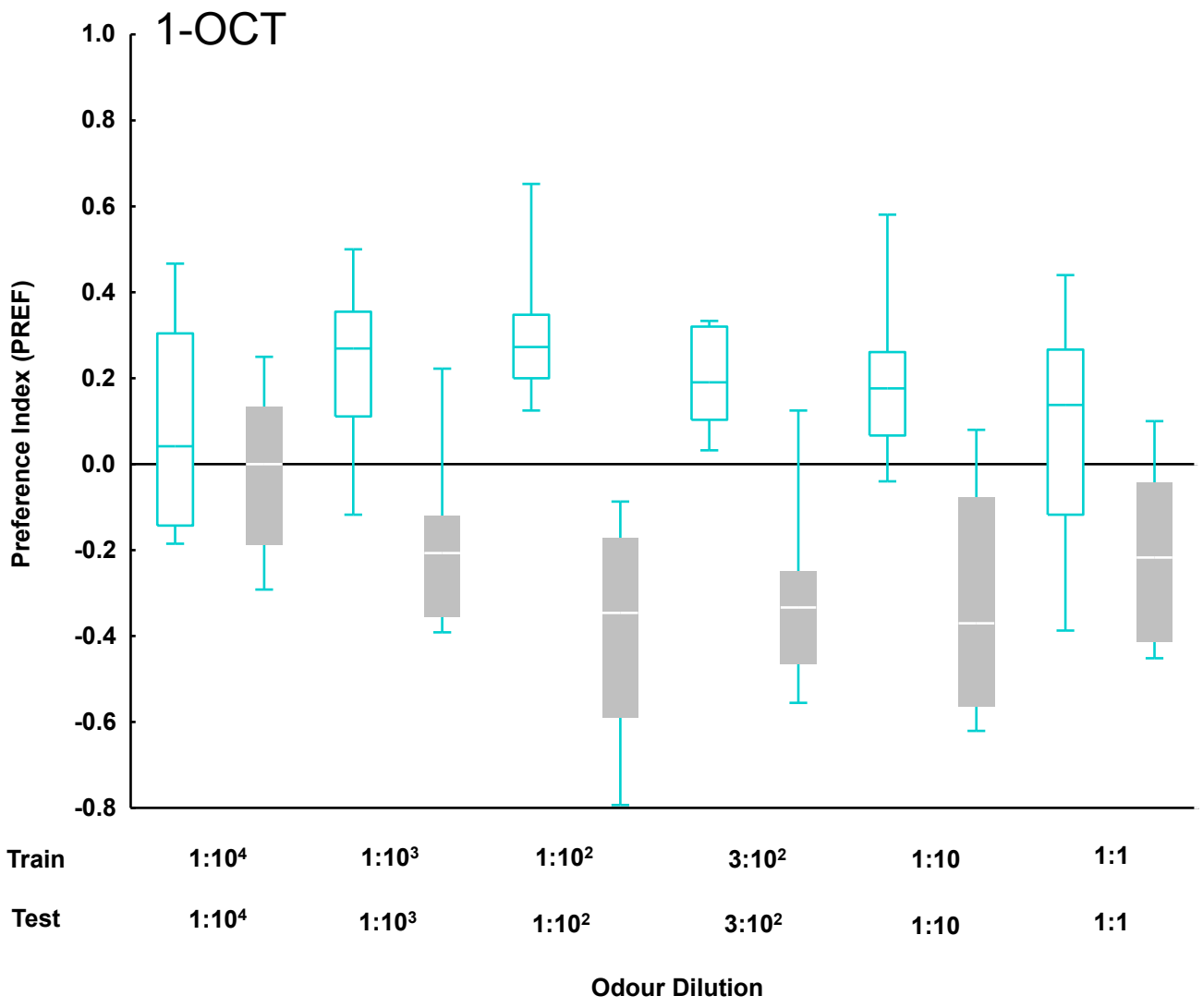
Fig: S2A (ii)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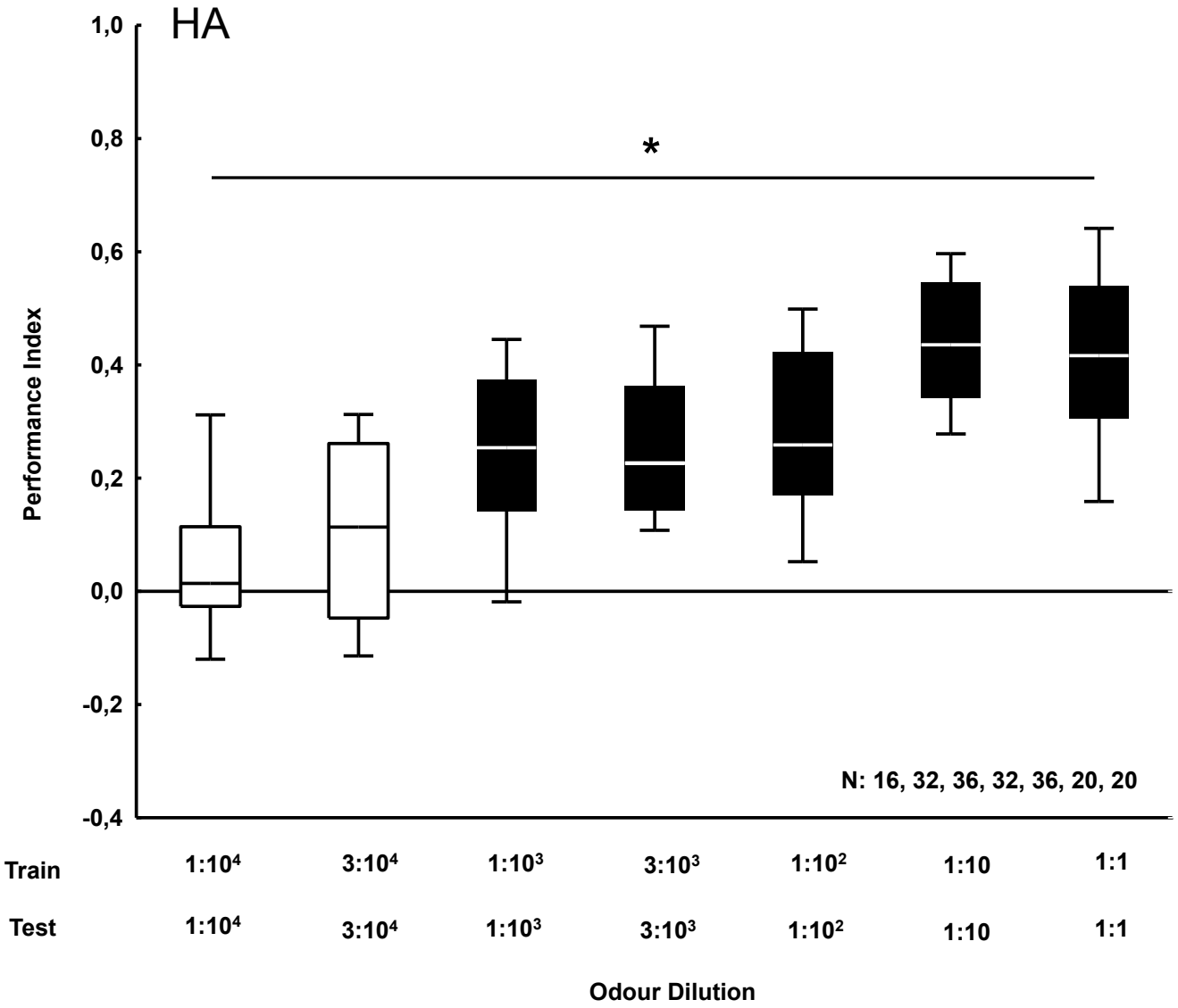
Odour Dilution

Fig: S2B (i)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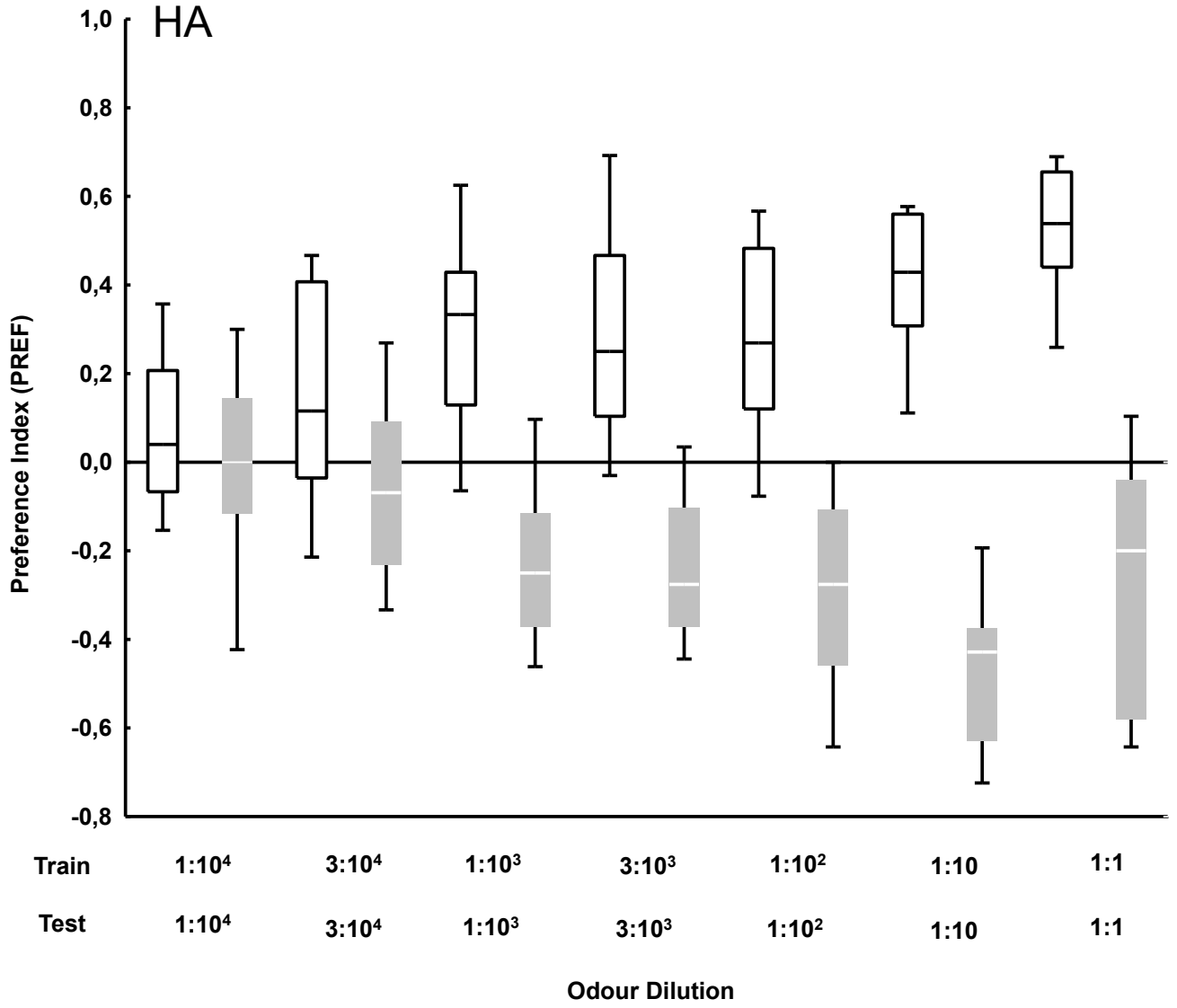
Odour Dilution

Fig: S2B (ii)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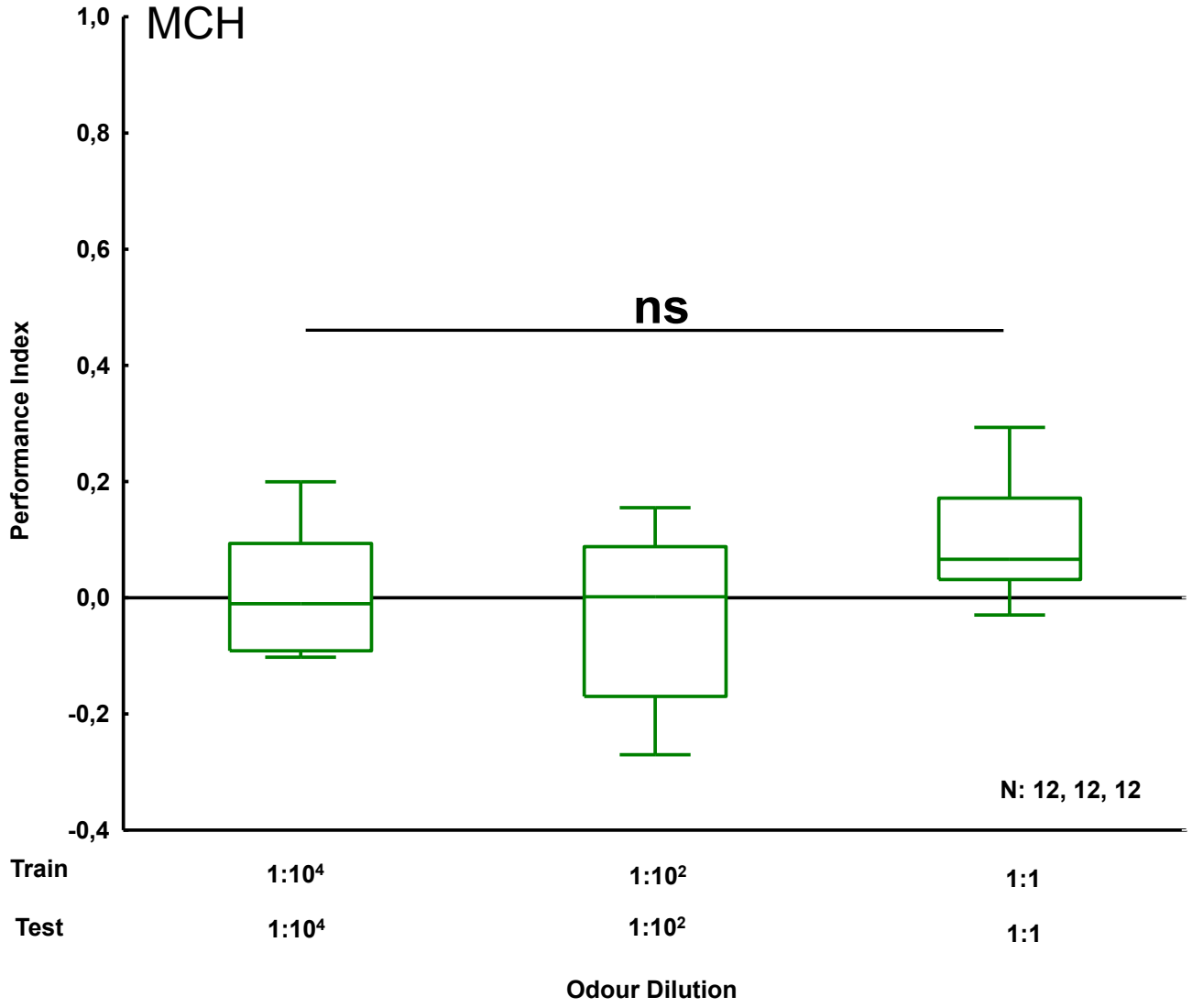
Odour Dilution

Fig: S2C (i)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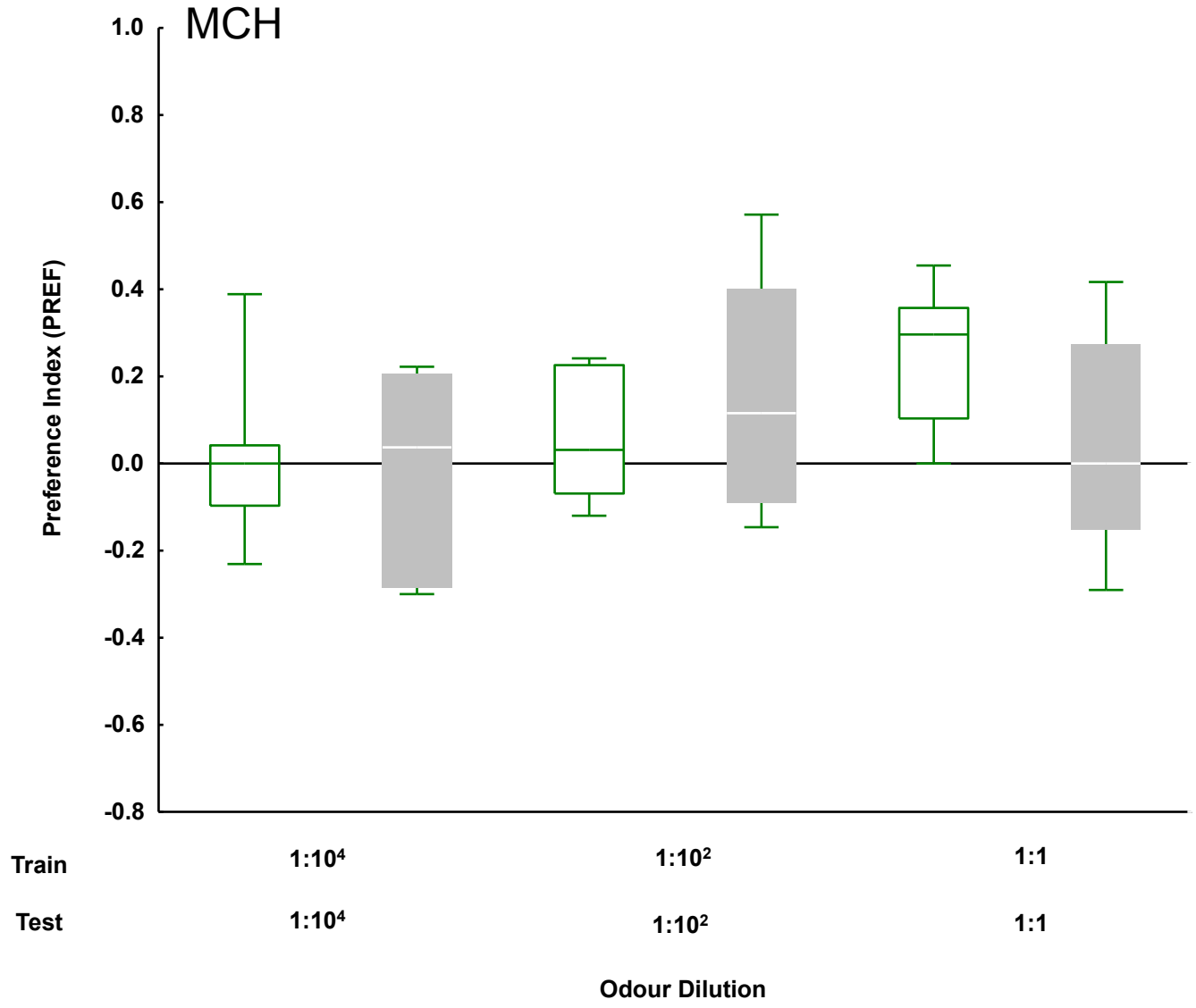
Odour Dilution

Fig: S2C (ii)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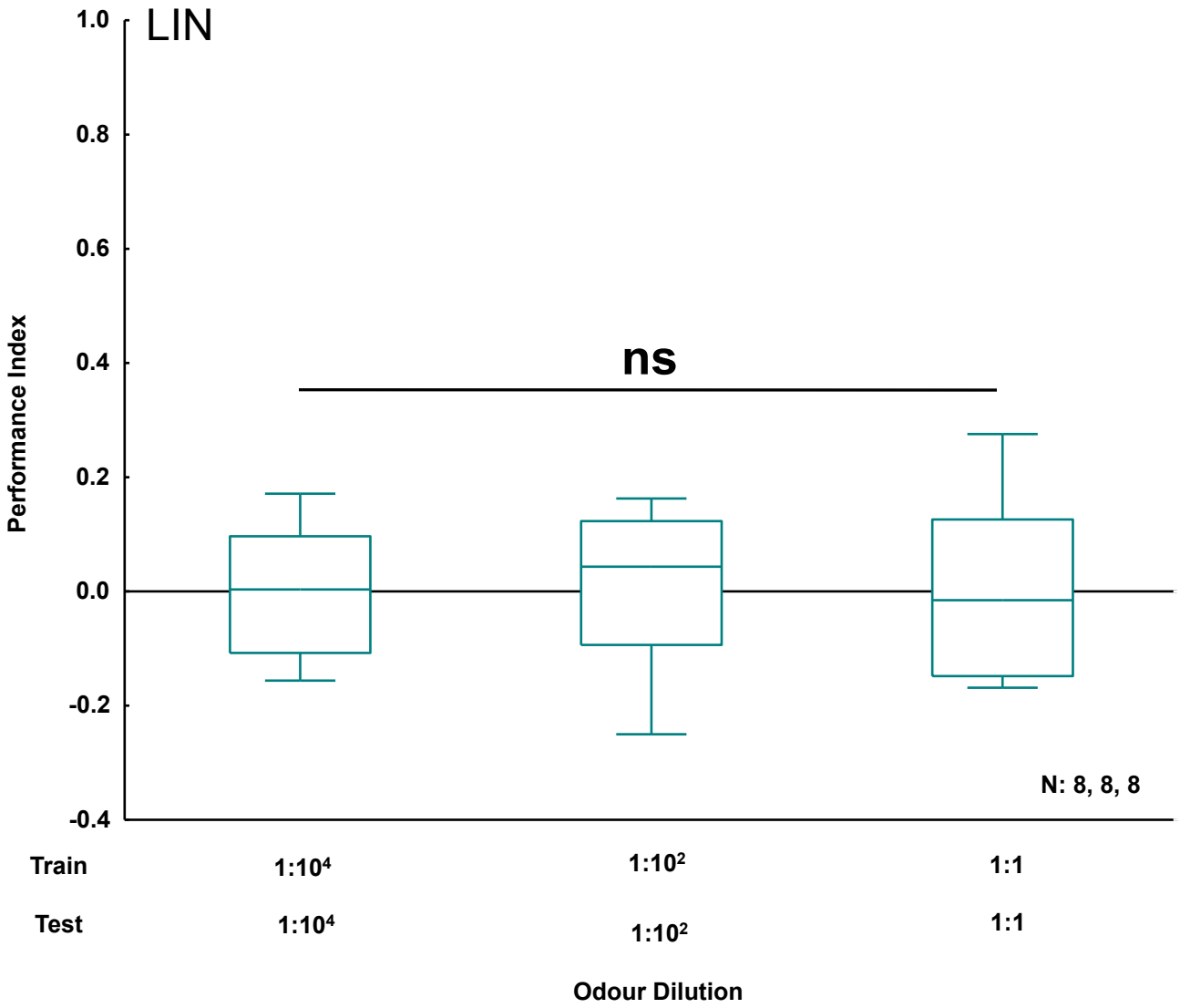
Odour Dilution

Fig: S2D (i)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Odour Dilution

Fig: S2D (ii)

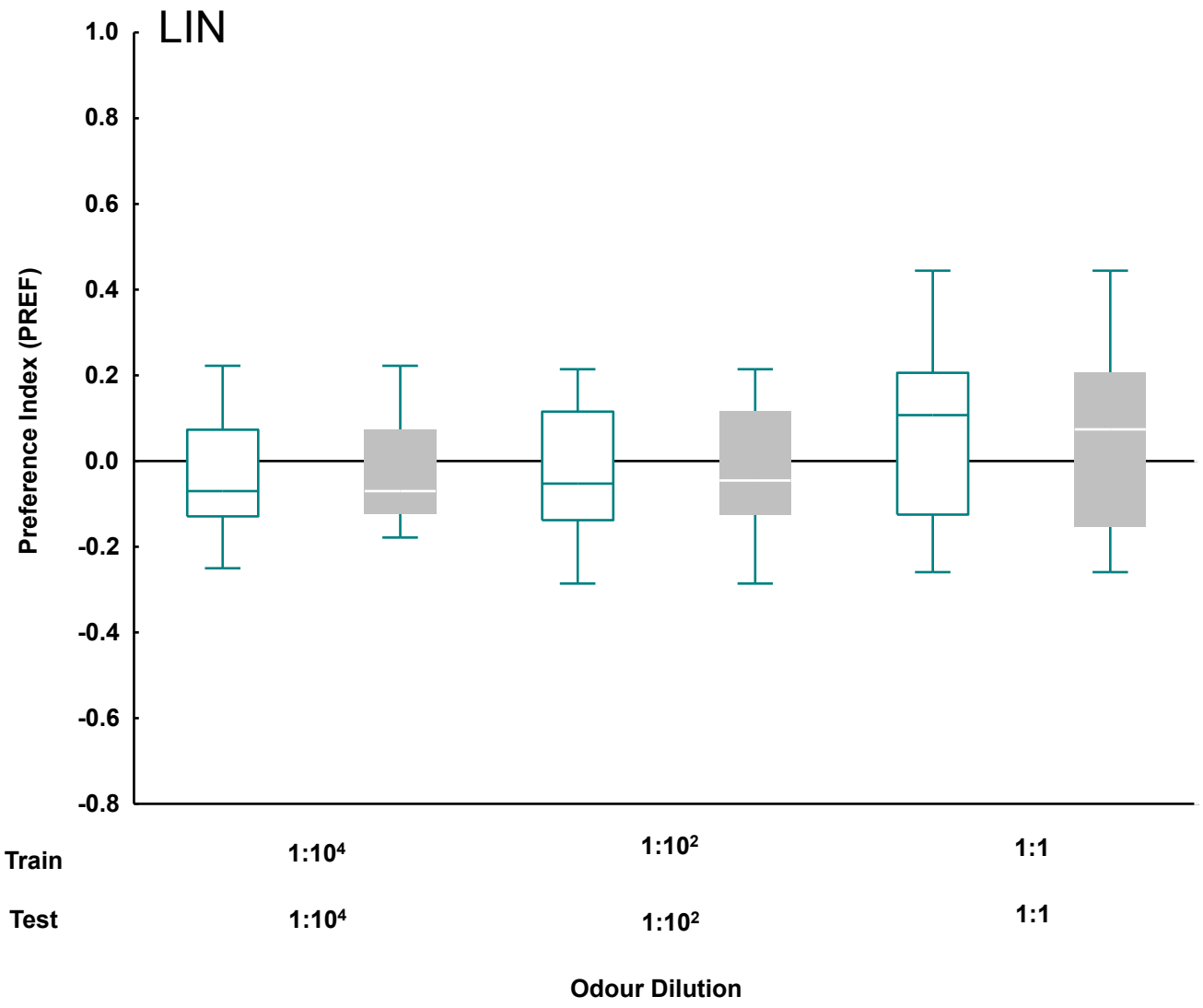
LIN
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
Preference Index (PREF)
Train
Test
1:10⁴
1:10⁴
1:10²
1:10²
1:1
1:1
Odour Dilution

Fig: S3A

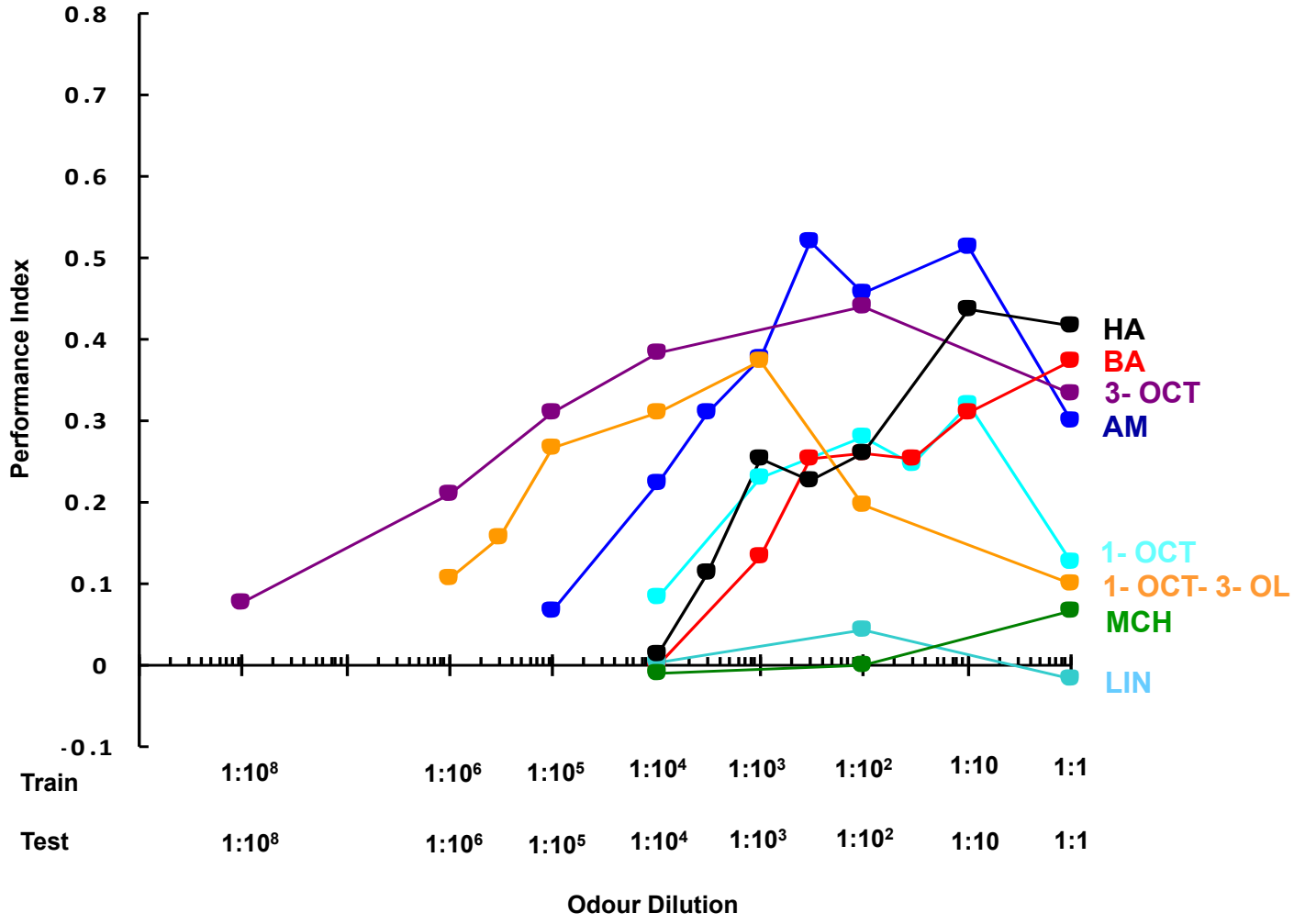
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
Performance Index
HA
BA
3- OCT
AM
1- OCT
1- OCT- 3- OL
MCH
LIN
Train
Test
1:10^8
1:10^6
1:10^5
1:10^4
1:10^3
1:10^2
1:10
1:1
Odour Dilution

Fig: S3B

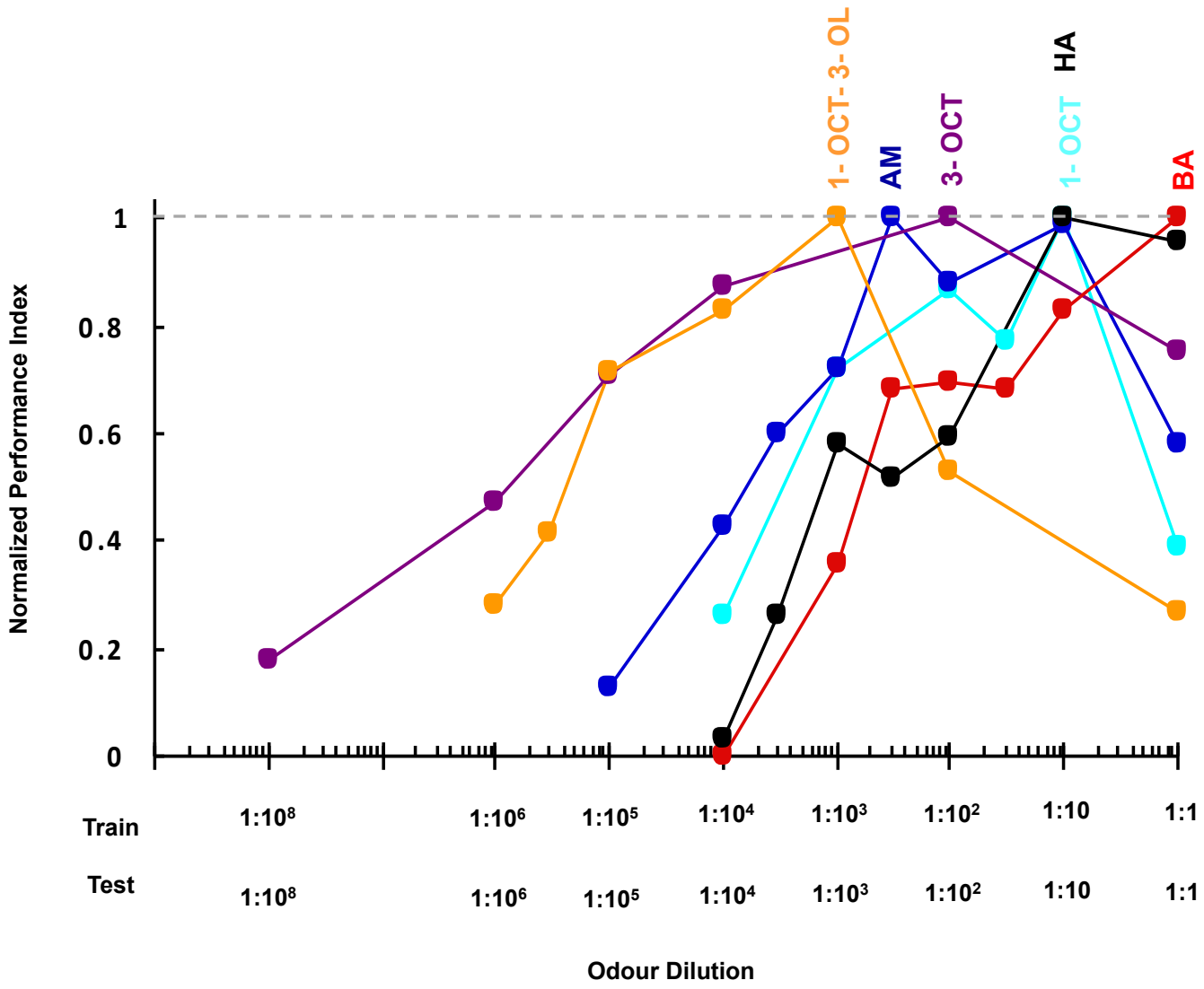
1- OCT- 3- OL
AM
3- OCT
1- OCT
HA
BA
Normalized Performance Index
1
0.8
0.6
0.4
0.2
0
Train
Test
1:10^8
1:10^6
1:10^5
1:10^4
1:10^3
1:10^2
1:10
1:1
Odour Dilution

Fig: S4A

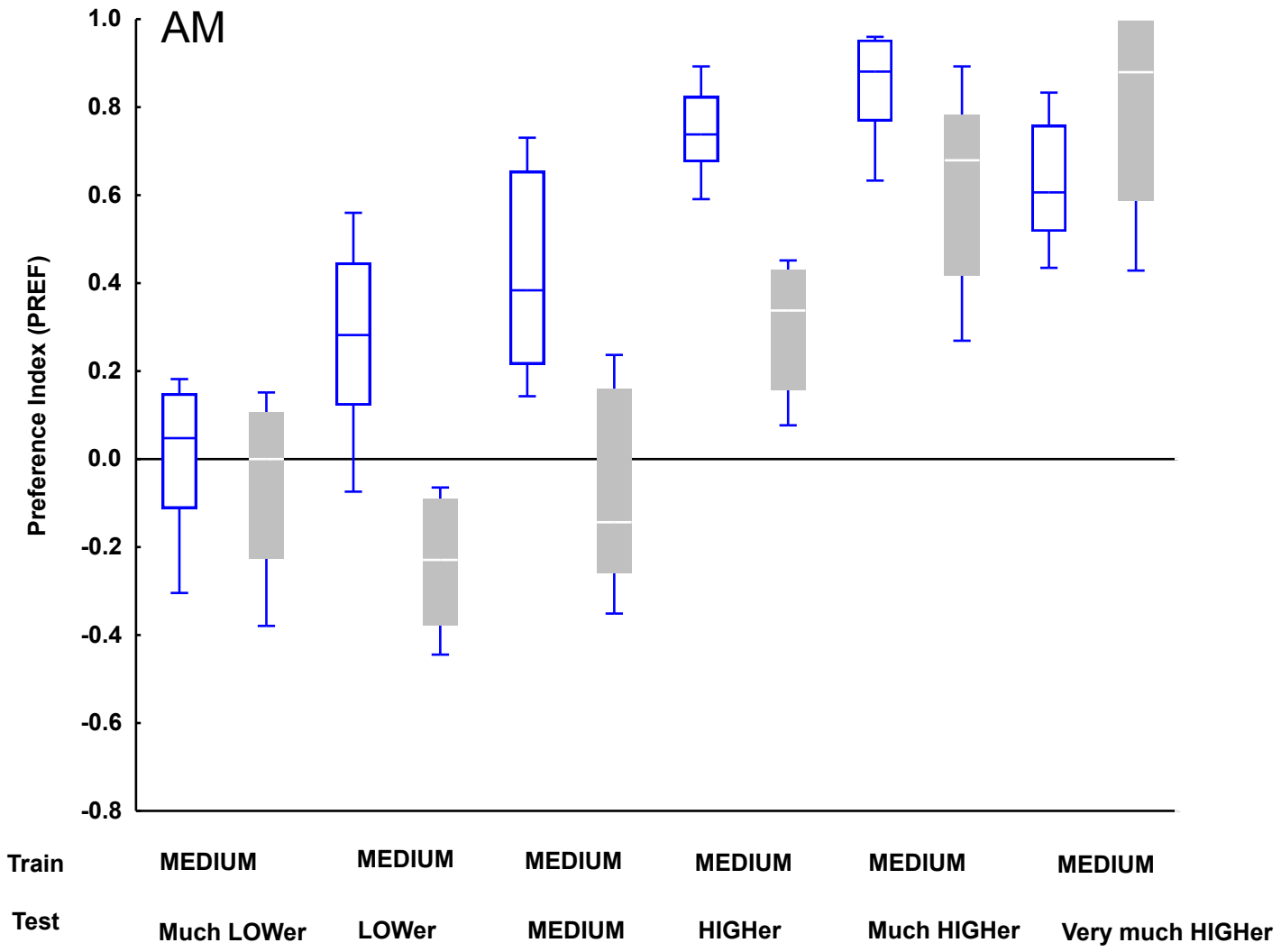
AM
Preference Index (PREF)
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
Train
Test
MEDIUM
Much LOWer
MEDIUM
LOWer
MEDIUM
MEDIUM
MEDIUM
HIGHer
MEDIUM
Much HIGHer
MEDIUM
Very much HIGHer

Fig: S4B

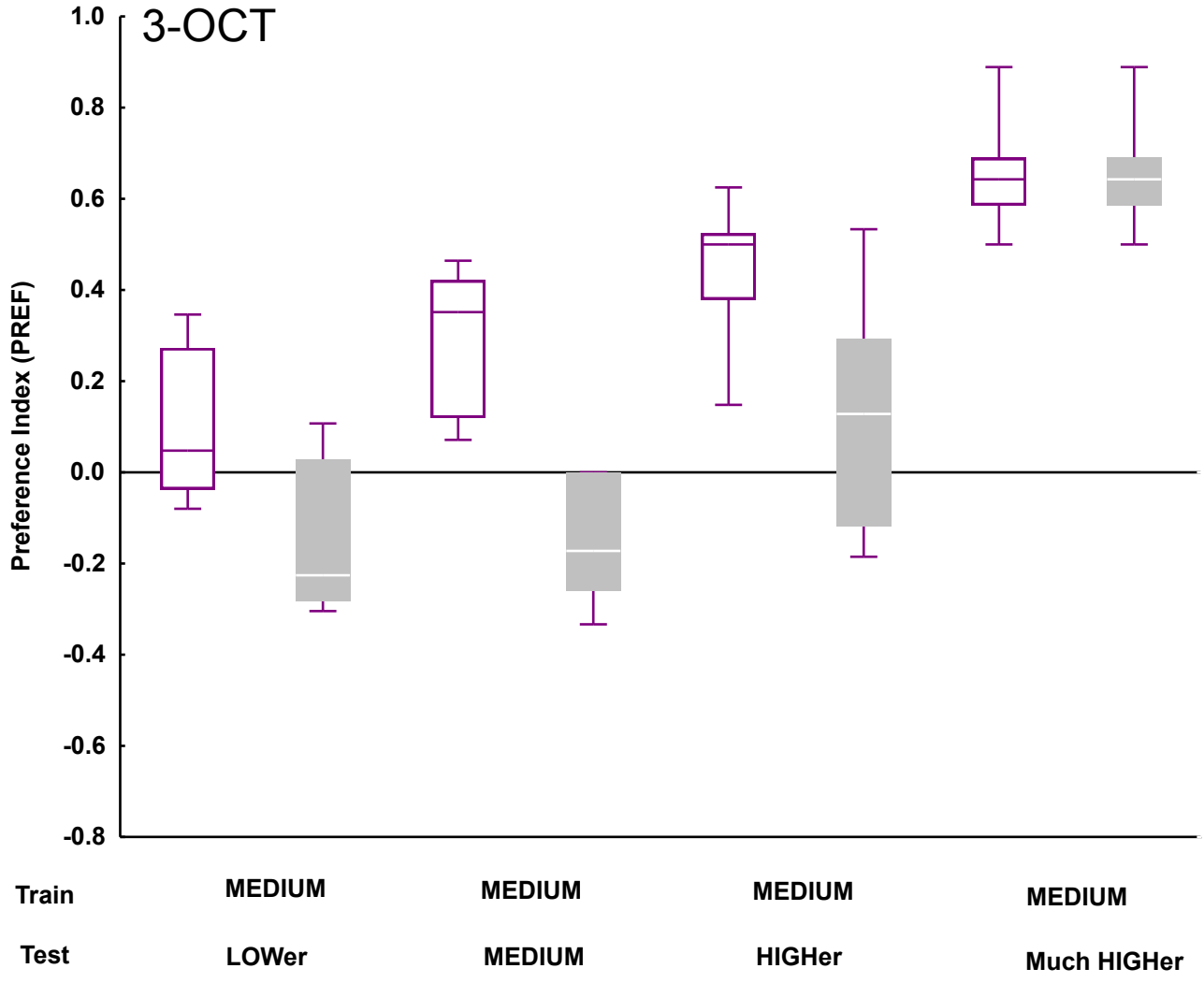
3-OCT
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
Preference Index (PREF)
Train
MEDIUM
MEDIUM
MEDIUM
MEDIUM
Test
LOWer
MEDIUM
HIGHer
Much HIGHer

Fig: S4C

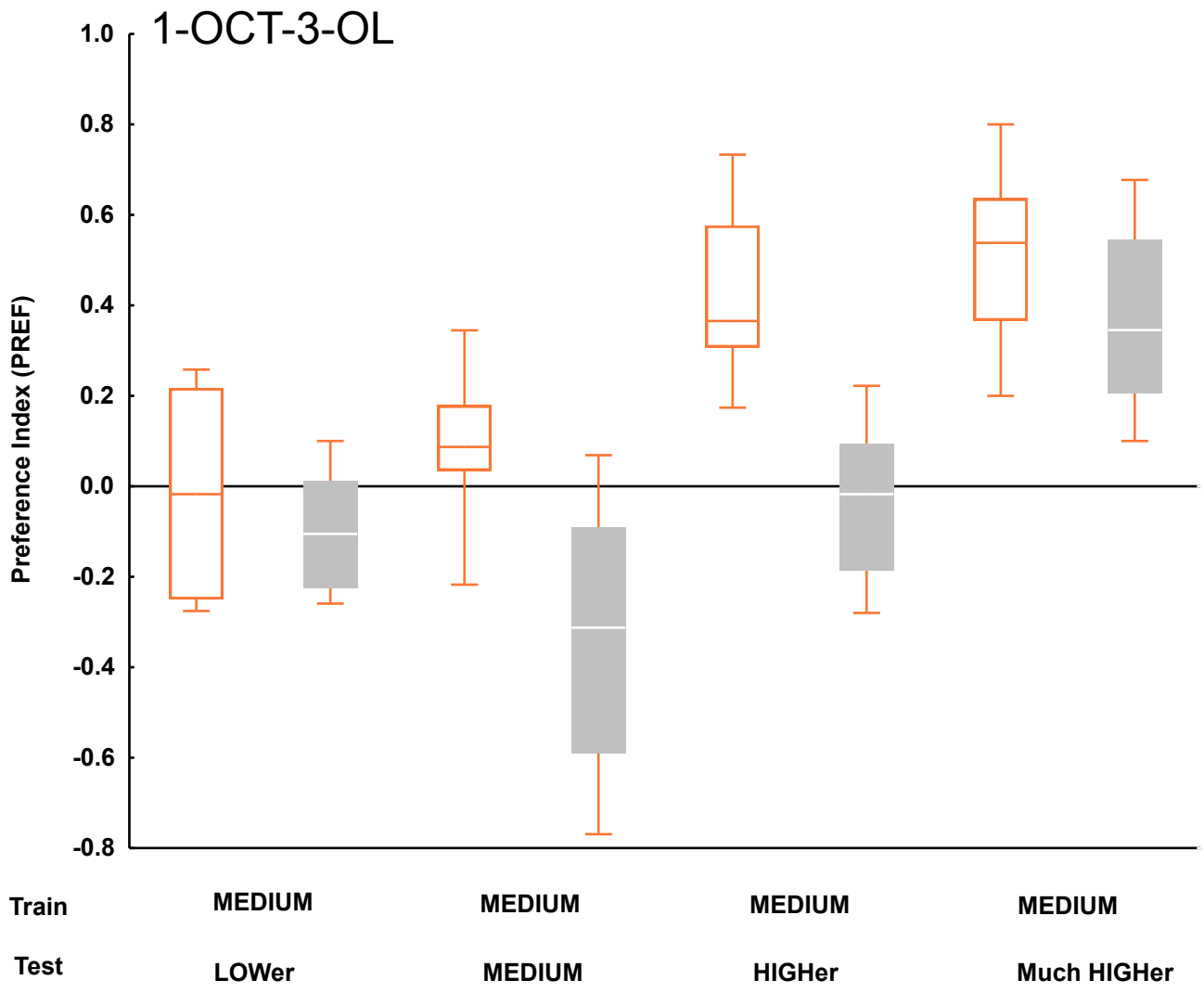
1-OCT-3-OL
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
Preference Index (PREF)
Train
Test
MEDIUM
LOWer
MEDIUM
MEDIUM
MEDIUM
HIGHer
MEDIUM
Much HIGHer

Fig: S4D (i)

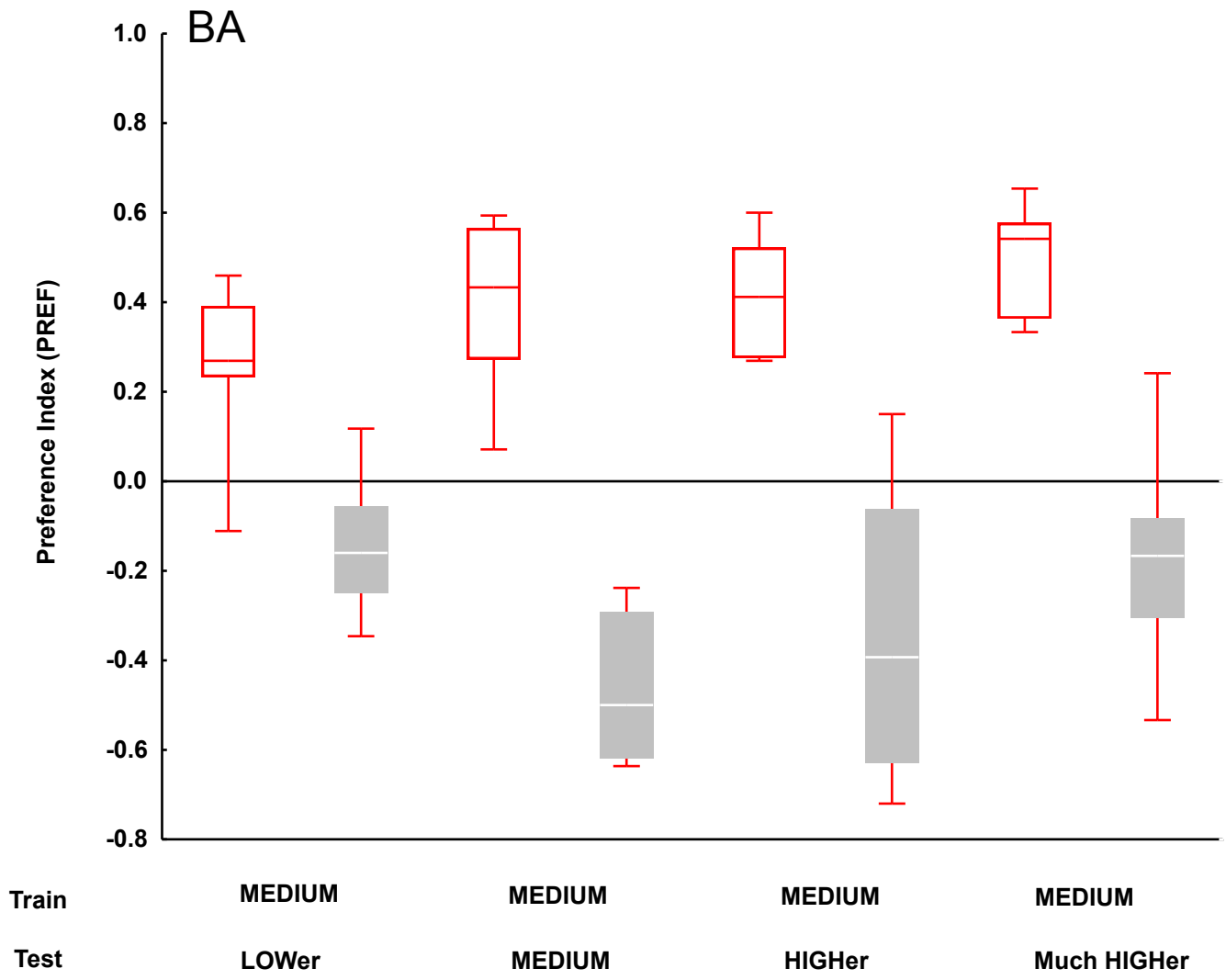
BA
Preference Index (PREF)
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
Train
Test
MEDIUM
LOWer
MEDIUM
MEDIUM
MEDIUM
HIGHer
MEDIUM
Much HIGHer

Fig: S4D (ii)

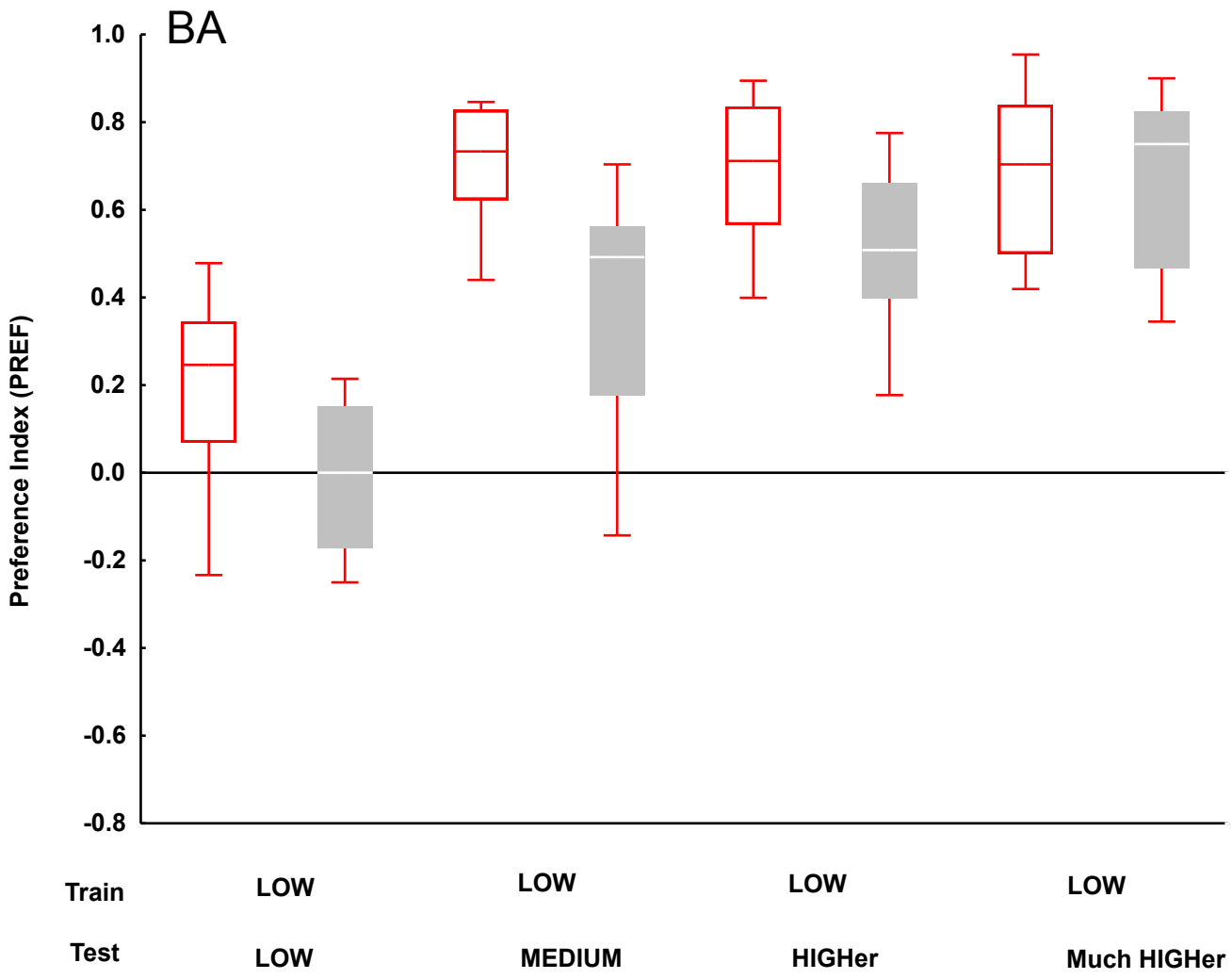
BA
Preference Index (PREF)
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
Train
Test
LOW
LOW
LOW
MEDIUM
LOW
HIGHer
LOW
Much HIGHer

Fig: S5A

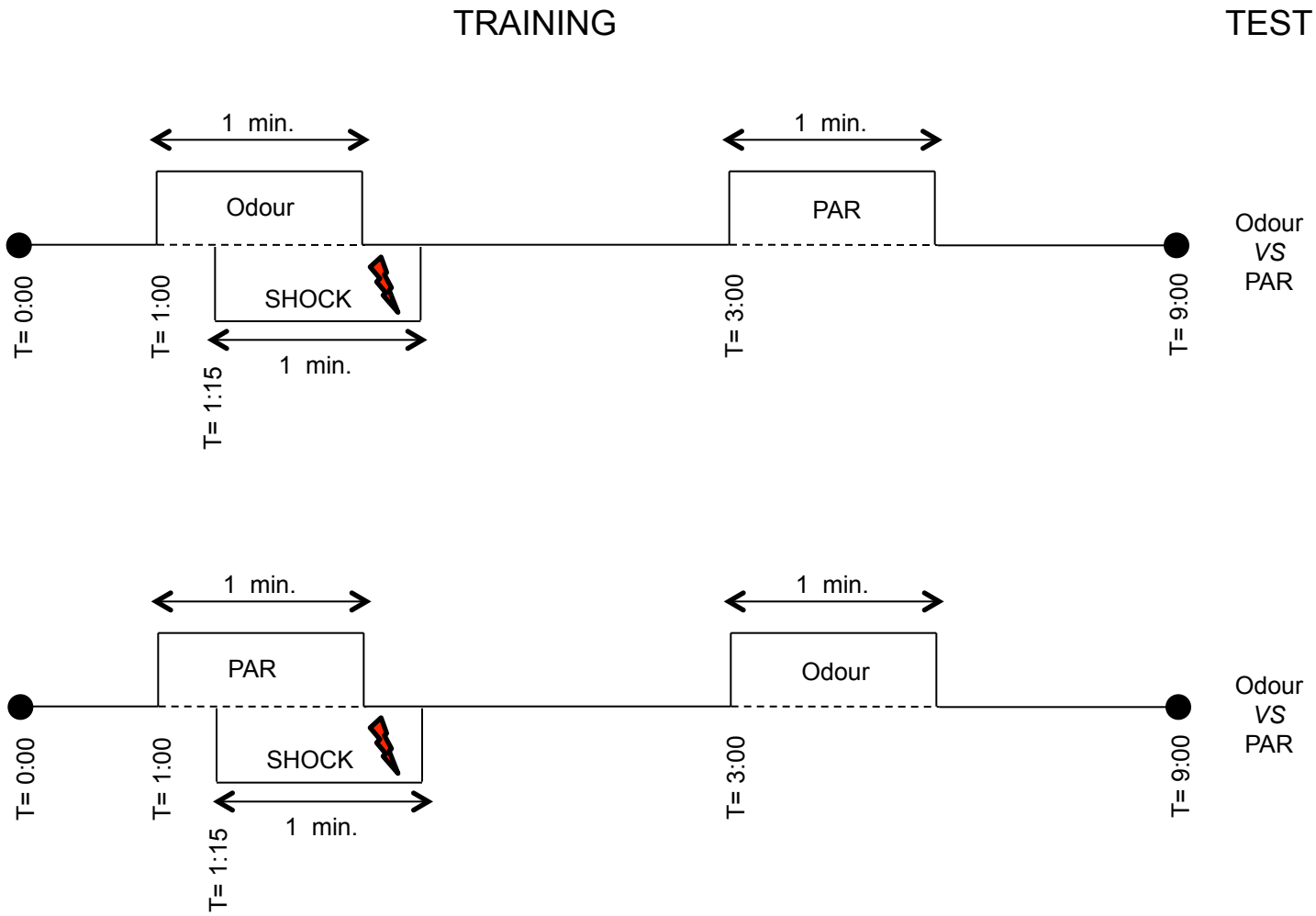
TRAINING
TEST
1 min.
1 min.
Odour
PAR
SHOCK
1 min.
T= 0:00
T= 1:00
T= 1:15
T= 3:00
T= 9:00
Odour
VS
PAR
1 min.
1 min.
PAR
Odour
SHOCK
1 min.
T= 0:00
T= 1:00
T= 1:15
T= 3:00
T= 9:00
Odour
VS
PAR

Fig: S5B

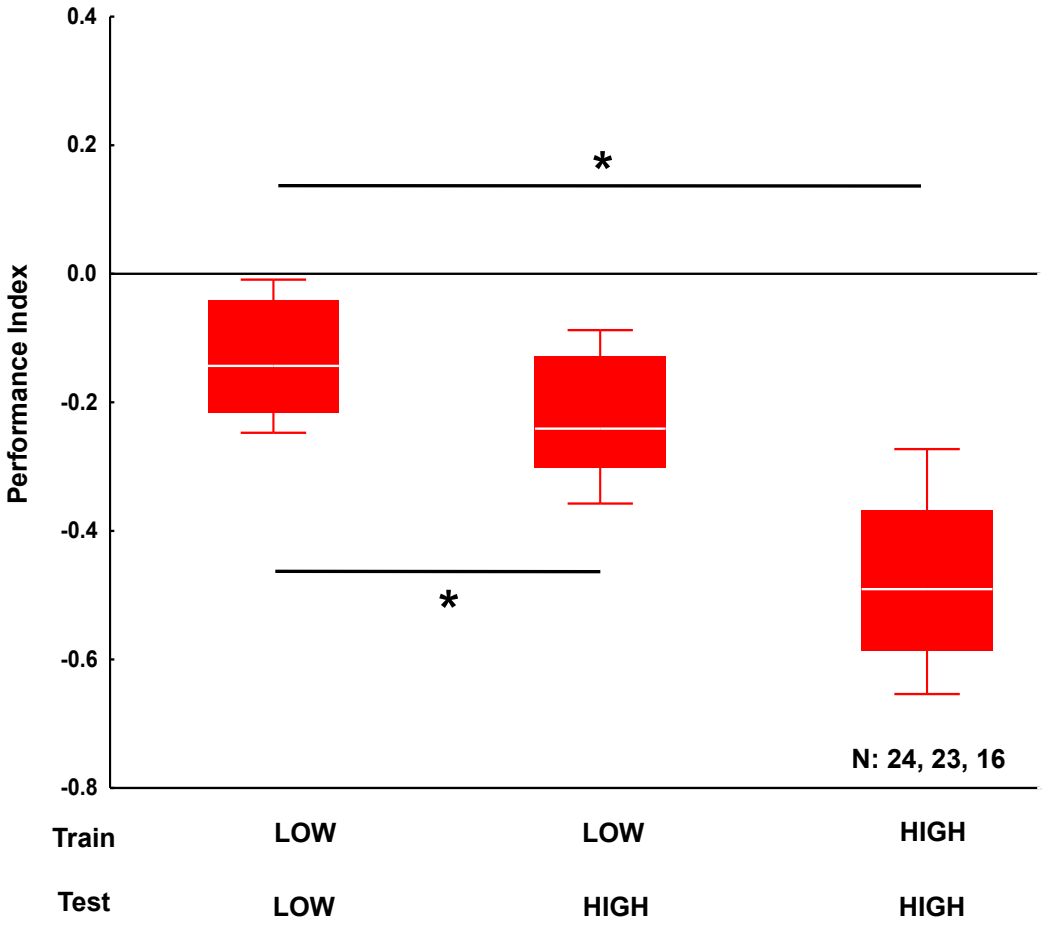
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
Performance Index
*
*
N: 24, 23, 16
Train
LOW
LOW
HIGH
Test
LOW
HIGH
HIGH

Fig: S5C

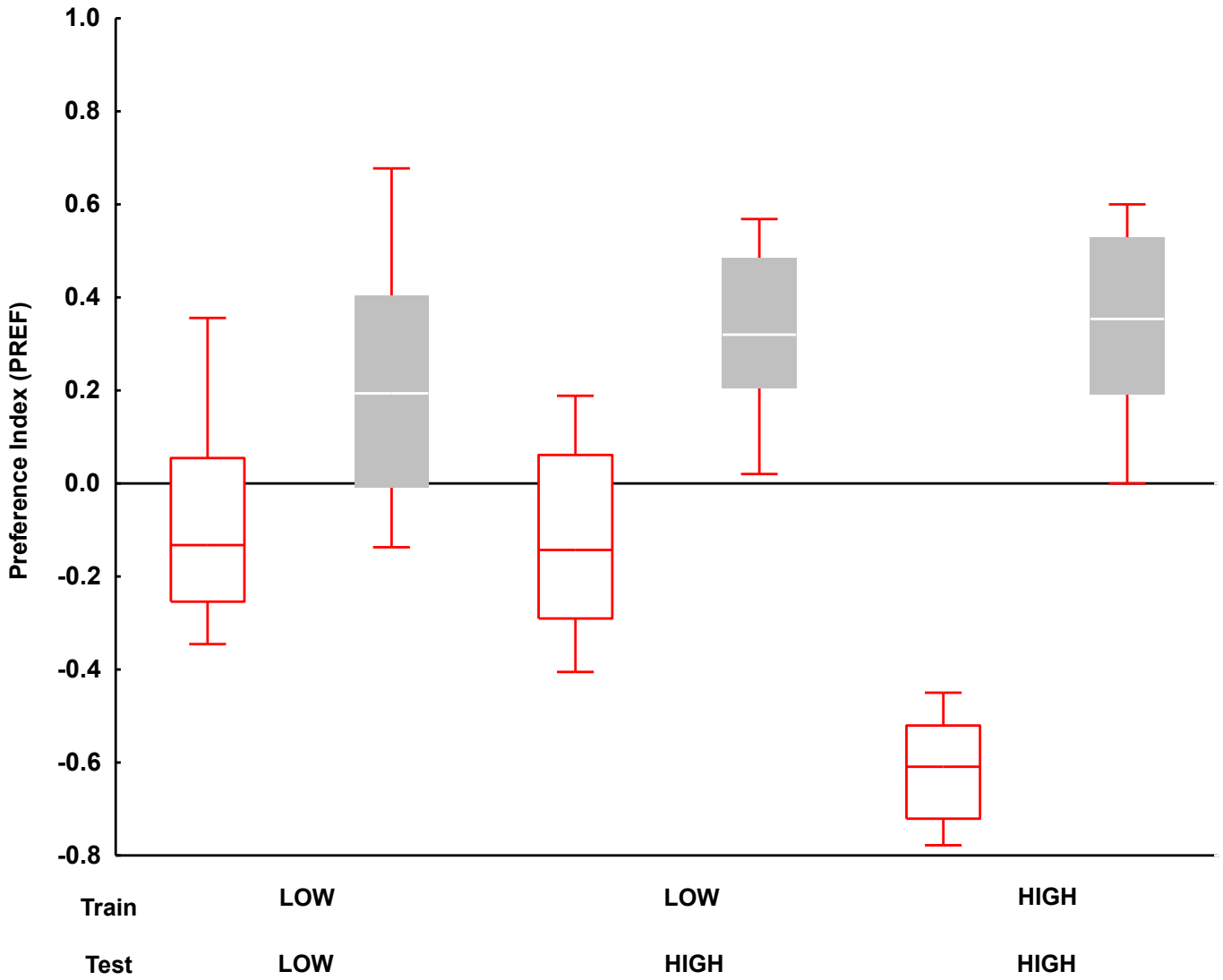
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
Preference Index (PREF)
Train
LOW
LOW
HIGH
Test
LOW
HIGH
HIGH

Fig. S1. Preference scores for AM, 3-Oct, 1-Oct-3-ol and BA as related to Fig. 2. Preference scores underlying the associative performance scores in Fig. 2A–D. Preference is measured either after the odour was rewarded (e.g. AM+/EM, open boxes) or after the odour was not rewarded (e.g. AM/EM+, grey boxes). This is displayed in A–D for AM, 3-Oct, 1-Oct-3-ol and BA, respectively. Within each panel, preferences scores are plotted across the indicated dilution of the respective odour. Positive values indicate approach, negative values indicate avoidance.

Fig. S2. Associative performance scores and the underlying respective preference values for 1-Oct, HA, MCH and Lin. (Ai) For 1-Oct we find an optimum function for associative performance scores across odour intensities. Performance scores at low ($1{:}10^3$, $1{:}10^4$) and very at high (1:1) odour intensities are not significantly different from zero, whereas all other groups do show significant learning scores, indicated by filling of the boxes (OSS tests with $P<0.05/6$ as criterion for significance). The KW test across groups yields H=18, d.f.=5, $P<0.05$. For this odour as well as the others displayed in this figure, we did not probe for intensity learning. (Aii) Preference scores of the two reciprocally trained groups (1-Oct+/EM, open boxes; 1-Oct/EM+, grey boxes) underlying the associative performance scores displayed in Ai. (Bi) At very low intensities of HA, performance indices are not significantly different from zero, whereas the other groups do show significant learning scores, indicated by filling of the boxes (OSS tests with $P<0.05/7$ as criterion for significance). The groups are significantly different from each other (KW test H=60.1, d.f.= 6, $P<0.05$). (Bii) Preference scores of the reciprocally trained groups (HA+/EM, open boxes; HA/EM+, grey boxes) underlying the associative performance scores displayed in Bi. (Ci) For MCH, we do not find any appreciable associative performance scores across odour intensities, indicated by the lack of filling of the boxes (OSS tests at $P>0.05/3$); 'ns' refers to lack of between-group significance (KW test: H=4.1, d.f.=2, $P>0.05$). (Cii) Preference scores of the reciprocally trained groups (MCH+/EM, open boxes; MCH/EM+, grey boxes) underlying the associative performance scores displayed in Ci. (Di) For Lin, we do not find any appreciable performance scores across odour intensities, indicated by lack of filling of the boxes (OSS tests at $P>0.05/3$); 'ns' refers to lack of between-group significance (KW test: H=0.06, d.f.=2, $P>0.05$). (Dii) Preference scores of the reciprocally trained groups (Lin+/EM, open boxes; Lin/EM+, grey boxes) underlying the associative performance scores displayed in Di.

Fig. S3. Semi-schematic summary of the dose–effect functions. (A) For eight different odours [*n*-amyl acetate (AM), 3-octanol (3-Oct), 1-octen-3-ol (1-Oct-3-ol), benzaldehyde (BA), 1-octanol (1-Oct), linalool (Lin), 4-methylcyclohexanol (MCH) and hexyl acetate (HA)], we plot the dose–effect curves of learnability, displaying odour dilution along the *x*-axis on a logarithmic scale and the median values of associative performance indices along the *y*-axis. (B) Same data as in A, normalized according to the highest median associative performance index obtained for the respective odour, excluding Lin and MCH, as they give no appreciable scores.

Fig. S4. Preference scores for AM, 3-Oct, 1-Oct-3-ol and BA as related to Fig. 3. Preference scores underlying the associative performance scores in Fig. 3A–D. Preference is measured either after the odour was rewarded (e.g., AM+/EM, open boxes) or after the odour was not rewarded (e.g. AM/EM+, grey boxes). This is displayed in A–D for AM, 3-Oct, 1-Oct-3-ol and BA, respectively. Within each panel, preference scores for the respective odours are plotted dependent on the training–testing regime explained in the legend of Fig. 3. Positive values indicate approach, negative values indicate avoidance.

Fig. S5. In an odour shock paradigm for adult *Drosophila* BA-memories are not intensity specific. In adult *Drosophila*, BA-memories have been reported to be not intensity specific as assayed in an odour–electric shock associative paradigm: higher-than-trained BA intensities support higher associative performance indices than the trained intensity [Yarali et al., 2009 (*loc. cit.* fig. 4D)]. The current experiment replicates this result. At 1–4 days after adult hatching, flies are collected in fresh food vials and maintained under culture conditions until they are used for experiments on the following day. Experiments are performed at 21–24 °C and 65–80% relative humidity, under white fluorescent light, in groups of ~50. (A) Training starts (T=0.00 min) by loading the flies into the set-up as described in Schwaerzel et al. (Schwaerzel et al., 2003). The odour (benzaldehyde: BA; Merck Schuchardt OHG, Hohenbrunn, Germany; CAS: 100-52-7) is presented at 1:00 for 1 min and electric shock is applied from 1:15 on as 12 pulses of 96–100 V; each pulse is 1.2 s long and is followed by the next pulse with an onset-to-onset interval of 5 s. At time 3:00 min, a blank stimulus with the solvent (paraffin: PAR; Fluka, Steinheim, Germany; CAS: 8002-74-2) is presented for 1 min. After this BA-shock/PAR training, at 9:00 min, flies are transferred to a T-maze, where they are given the choice between one arm scented with the odour and the second arm supplied with PAR. After 2 min, the arms of the maze are closed and the numbers of flies (#) in the respectively scented arms are determined. A preference index (Pref) is calculated as: Pref = (#BA – #PAR)/#total. These Pref scores are documented in C. BA-shock/PAR, represented by open red boxes. Another group of flies is trained reciprocally as PAR-shock/BA represented in C by filled grey boxes. Half the difference between the Pref values of these two reciprocally trained groups gives the associative performance index (PI): $PI = (Pref_{BA\text{-}shock\ /PAR} - Pref_{PAR\text{-}Shock/BA})/2$. The PI thus ranges from –1 to 1, negative values indicating conditioned avoidance from the odour (aversive learning), positive ones meaning conditioned approach towards the odour (appetitive learning). Half of the experiments were performed as explained above, whereas in the other half, flies were trained with the respectively reversed sequence (i.e. PAR/BA-shock or BA/PAR-shock) to balance for possible sequence effects. BA is diluted in PAR to the final concentrations given in B; in all cases, 250 µl of the BA solution or of PAR are placed in custom-made Teflon containers of 15 mm diameter. (B) Flies are trained and tested with either a Low (left; $1{:}10^4$) or with a high (right; $1{:}10^3$) BA intensity. In replication of Yarali et al. [Yarali et al., 2009 (*loc. cit*. fig. 3D)], these intensities support significantly different associative performance indices (MWU test: U= 18.0, $P<0.05/2$). Critically, we trained another group of flies with the low BA intensity and tested it with the high intensity (middle). This group, despite the training intensity not matching the test intensity, shows stronger associative performance scores than the group that was both trained and tested with the low intensity (MWU test: U=155.0, $P<0.05/2$). This replicates the result by Yarali et al. [Yarali et al., 2009 (*loc. cit.* fig. 4D)], confirming that BA-memories, at least in an odour shock paradigm using a single training trial, are not intensity specific. This contrasts with the intensity specificity of memory for 3-Oct, AM and MCH found by Yarali et al. (Yarali et al., 2009) in that paradigm, and with the intensity specificity of BA-memories in a larval odour sugar learning paradigm (see main text). (C) Documentation of the Pref scores underlying the associative performance indices in B.