The Similarity between Odors and Their Binary Mixtures in *Drosophila*

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Abstract

How are odor mixtures perceived? We take a behavioral approach toward this question, using associative odor-recognition experiments in *Drosophila*. We test how strongly flies avoid a binary mixture after punishment training with one of its constituent elements and how much, in turn, flies avoid an odor element if it had been a component of a previously punished binary mixture. A distinguishing feature of our approach is that we first adjust odors for task-relevant behavioral potency, that is, for equal learnability. Doing so, we find that 1) generalization between mixture and elements is symmetrical and partial, 2) elements are equally similar to all mixtures containing it and that 3) mixtures are equally similar to both their constituent elements. As boundary conditions for the applicability of these rules, we note that first, although variations in learnability are small and remain below statistical cut-off, these variations nevertheless correlate with the elements' perceptual "weight" in the mixture; thus, even small differences in learnability between the elements have the potential to feign mixture asymmetries. Second, the more distant the elements of a mixture are to each other in terms of their physicochemical properties, the more distant the flies regard the elements from the mixture. Thus, titrating for task-relevant behavioral potency and taking into account physicochemical relatedness of odors reveals rules of mixture perception that, maybe surprisingly, appear to be fairly simple.

Key words: *Drosophila*, mixture, memory, olfaction, perception, recognition

Introduction

The discovery of the *Or* family of olfactory receptors of *Drosophila* (Clyne et al. 1999; Vosshall et al. 1999; see also Benton et al. 2009 regarding the *Ir* receptor family) and ensuing neurogenetic analyses have led to a reasonably detailed picture of how different odors can cause different neuronal activity patterns along the olfactory pathway of insects (reviewed in Stocker 1994; Strausfeld and Hildebrand 1999; Galizia and Menzel 2000; Hallem et al. 2006; Vosshall and Stocker 2007; Gerber et al. 2009; Masse et al. 2009). Also, the short-term memory trace for olfactory associations with electric shock punishment has been localized to the mushroom bodies (reviewed in Heisenberg 2003; Gerber et al. 2004; Krashes et al. 2007), a third-order "cortical" (Tomer et al. 2010) brain region of the insects, and the molecular nature of this trace is being characterized (reviewed in Davis 2004; Zars 2010). However, many questions remain, including how mixtures are processed, which is particularly relevant when consider-

ing that under natural conditions, animals always encounter volatile chemicals within mixtures or at least within substantial olfactory background.

On the physiological level, Silbering and Galizia (2007) compared patterns of calcium activity evoked by odors and their binary mixtures between the input and the output neurons (olfactory sensory neurons and projection neurons, respectively) of the *Drosophila* antennal lobe, the first relay of the olfactory pathway of the insects. The authors suggested both a global lateral inhibition acting as a gain control mechanism and specific inhibitory and likely also excitatory lateral connectivity, together leading to nonadditive processing of mixtures (a corresponding approach in honeybees also suggested that while on the level of olfactory sensory neurons there is little if any mixture interaction [Deisig et al. 2006], the projection neurons carry an olfactory representation that is not readily predictable by the activity patterns evoked by its components

[Deisig et al. 2010]). Recent progress in the characterization of local interneurons in the antennal lobe is now shedding light on exactly how these effects may come about (Chou et al. 2010; Huang et al. 2010; Yaksi and Wilson 2010). Although such analyses of the transfer functions within the microcircuit of olfactory sensory neurons, local interneurons, and the projection neurons certainly are indispensable to understand the physiology of mixture processing, it remained unclear how flies actually perceive mixtures relative to their component odors. Here, we take a behavioral approach toward this question.

We ask how strongly flies would avoid a mixture after punishment training with one of its constituent elements and how much, in turn, flies avoid an odor if it had been a component of a previously punished mixture. That is, we perform associative recognition experiments where a given single odor "X" is paired with an electric shock; then, conditioned avoidance of the flies toward a mixture containing X plus another odor "1" is measured. In independent sets of flies, the reverse is probed for, namely flies are trained with the mixture X1 and are tested with X. A distinguishing feature of our approach is that we adjust the dilutions of the used odors (benzaldehyde [B], 3-octanol [O], 4-methylcyclohexanol [M], and n-amylacetate [A]) for task-relevant behavioral potency, that is, for equal learnability (Niewalda 2010), rather than merely choosing odor dilutions that are physically the same or by adjusting for preference in experimentally naive animals (indeed, adjusting for equal behavioral effect of 2 odors in a given behavioral paradigm, such as naive preference behavior, does not necessarily entail equal behavioral effect in another paradigm such as learning [Saumweber et al. 2011]). We specifically ask:

1. Is generalization between an odor and a binary mixture containing it symmetrical, that is, is conditioned avoidance equal if X is trained and the X1 mixture is tested, as when X1 is trained and X is tested?
2. Is an odor equally similar to different mixtures containing it, that is, is X equally similar to X1, X2, and X3?
3. Is a mixture equally similar to its constituent odor elements, that is, is X1 equally similar to X as it is to 1?

Material and methods

Flies

Wild-type Canton-S flies are raised in groups of approximately 200, at 25 °C, 60–70% relative humidity and a 14:10 h light:dark cycle. We collect flies 1–5 days after hatching from the pupal case and keep them overnight at 18 °C until 24 h before the start of the experiment.

Stimuli and apparatus

We use 4 odors and their respective binary mixtures: benzaldehyde (B), 3-octanol (O), 4-methylcyclohexanol (M), and n-amylacetate (A) (CAS: 100-52-7, 589-98-0, 589-91-3, 628-

63-7; all from Fluka, except A, which is from Merck). Odors are diluted in paraffin oil (symbolized henceforth by Θ) (Merck) such that all odors support statistically undistinguishable conditioned avoidance after odor-shock associative learning (Niewalda 2010) (B: 1:66; O: 1:1000; M: 1:25; A: 1:1000); this equal learnability was confirmed within this study (Figure 1A,A′).

The training apparatus, modified from Tully and Quinn (1985), has been described in detail elsewhere (Schwaerzel et al. 2003; Yarali et al. 2008). In short, flies are transferred into 90-mm long and 15-mm inner-diameter plastic tubes, covered with an electrifiable copper grid to administer electric shocks during training (see section Principle of training and testing). These tubes are connected to Teflon containers for odor delivery by means of a suction pump that draws fresh air across the tube and ensures removal of odor-saturated air from the training apparatus. For single-odor presentation, 130 μL of odorant is applied in a 7-mm diameter Teflon cup. For the presentation of binary mixtures, twin cups are used which allow separately loading 2 such volumes such that the total surface for evaporation is doubled.

Principle of training and testing

Training is performed in dim red light, testing in darkness. In the following, we refer to Table 1 and use group 9a as an example: At $t = 0$ min, approximately 100 flies are loaded to the training tube. At $t = 2$ min, odor O is applied for 60 s. At $t = 2$ min 15 s, electric shock is applied for 60 s (90 V, 12 pulses to 1.2 s, with an onset–onset interval of 5 s). At $t = 4$ min, Θ is presented for 60 s. Flies are left in the training tubes for recovery until at $t = 9$ min, when they are transferred back to food vials for 13 min until the next of a total of 3 such training cycles starts.

Once this O_{Shock}/Θ training is complete, the regular 13 min break is given until animals are loaded again to the apparatus for testing. After an accommodation period of 4 min, animals are transferred to the choice point of a T-maze, where they can escape toward either BO or Θ. After 2 min, the arms of the maze are closed and the number of animals within each arm (denoted # in the following) is counted. A preference score (PREF) is calculated as

$$\text{PREF } O_{Shock}/\Theta = (\#BO - \#\Theta)/\#\text{Total}. \quad (1a)$$

After one such score has been obtained, a second set of flies is trained reciprocally such that electric shock is applied upon presenting Θ but not upon presenting odor O (O/Θ_{Shock} training; Table 1, group 9b). Again, choice between BO and Θ is measured and a preference score determined:

$$\text{PREF } O/\Theta_{Shock} = (\#BO - \#\Theta)/\#\text{Total}. \quad (1b)$$

All PREF scores are documented in Supplementary Figure S1. From the PREF scores of the 2 reciprocally trained sets of flies, a learning index (Figure 1A, group 9) is calculated as

Table 1 Summary of experimental groups

	Group	Punished[a]	Not punished[a]	Test	Group	Punished[a]	Not punished[a]	Test
A	1a	B	Θ	BB vs Θ	17a	A	Θ	BA vs Θ
	1b	Θ	B	BB vs Θ	17b	Θ	A	BA vs Θ
	2a	BB	Θ	B vs Θ	18a	BA	Θ	A vs Θ
	2b	Θ	BB	B vs Θ	18b	Θ	BA	A vs Θ
	3a	O	Θ	OO vs Θ	19a	O	Θ	OA vs Θ
	3b	Θ	O	OO vs Θ	19b	Θ	O	OA vs Θ
	4a	OO	Θ	O vs Θ	20a	OA	Θ	O vs Θ
	4b	Θ	OO	O vs Θ	20b	Θ	OA	O vs Θ
	5a	M	Θ	MM vs Θ	21a	B	Θ	BM vs Θ
	5b	Θ	M	MM vs Θ	21b	Θ	B	BM vs Θ
	6a	MM	Θ	M vs Θ	22a	BM	Θ	B vs Θ
	6b	Θ	MM	M vs Θ	22b	Θ	BM	B vs Θ
	7a	A	Θ	AA vs Θ	23a	M	Θ	BM vs Θ
	7b	Θ	A	AA vs Θ	23b	Θ	M	BM vs Θ
	8a	AA	Θ	A vs Θ	24a	BM	Θ	M vs Θ
	8b	Θ	AA	A vs Θ	24b	Θ	BM	M vs Θ
					25a	B	Θ	BO vs Θ
B	9a	O	Θ	BO vs Θ	25b	Θ	B	BO vs Θ
	9b	Θ	O	BO vs Θ	26a	BO	Θ	B vs Θ
	10a	BO	Θ	O vs Θ	26b	Θ	BO	B vs Θ
	10b	Θ	BO	O vs Θ	27a	M	Θ	MA vs Θ
	11a	O	Θ	OM vs Θ	27b	Θ	M	MA vs Θ
	11b	Θ	O	OM vs Θ	28a	MA	Θ	M vs Θ
	12a	OM	Θ	O vs Θ	28b	Θ	MA	M vs Θ
	12b	Θ	OM	O vs Θ	29a	A	Θ	OA vs Θ
	13a	B	Θ	BA vs Θ	29b	Θ	A	OA vs Θ
	13b	Θ	B	BA vs Θ	30a	OA	Θ	A vs Θ
	14a	BA	Θ	B vs Θ	30b	Θ	OA	A vs Θ
	14b	Θ	BA	B vs Θ	31a	M	Θ	OM vs Θ
	15a	A	Θ	MA vs Θ	31b	Θ	M	OM vs Θ
	15b	Θ	A	MA vs Θ	32a	OM	Θ	M vs Θ
	16a	MA	Θ	A vs Θ	32b	Θ	OM	M vs Θ
	16b	Θ	MA	A vs Θ				

Description of all different training and test regimens. We use benzaldehyde, 3-octanol, 4-methylcyclohexanol, and *n*-amylacetate at single amounts (B, O, M, A), double amounts (BB, OO, MM, AA), or as binary mixture (BO, BM, BA, OM, OA, MA). In all cases, 2 reciprocal groups are trained, 1 receiving the shock in association with the odor, (e.g., odor B: group 1a) and presentation of the solvent (denoted as Θ) without shock, while the reciprocal group experiences the reverse contingency (odor B is applied alone, and the shock is delivered with the solvent: group 1b). A learning index is calculated as the difference in odor avoidance between these reciprocally trained groups (see Material and methods). Note that within all groups the sequence of trial types is as indicated in half of the cases (e.g., first B-shock, then Θ), whereas in the other half of the cases it is reversed (e.g., first Θ then B-shock).
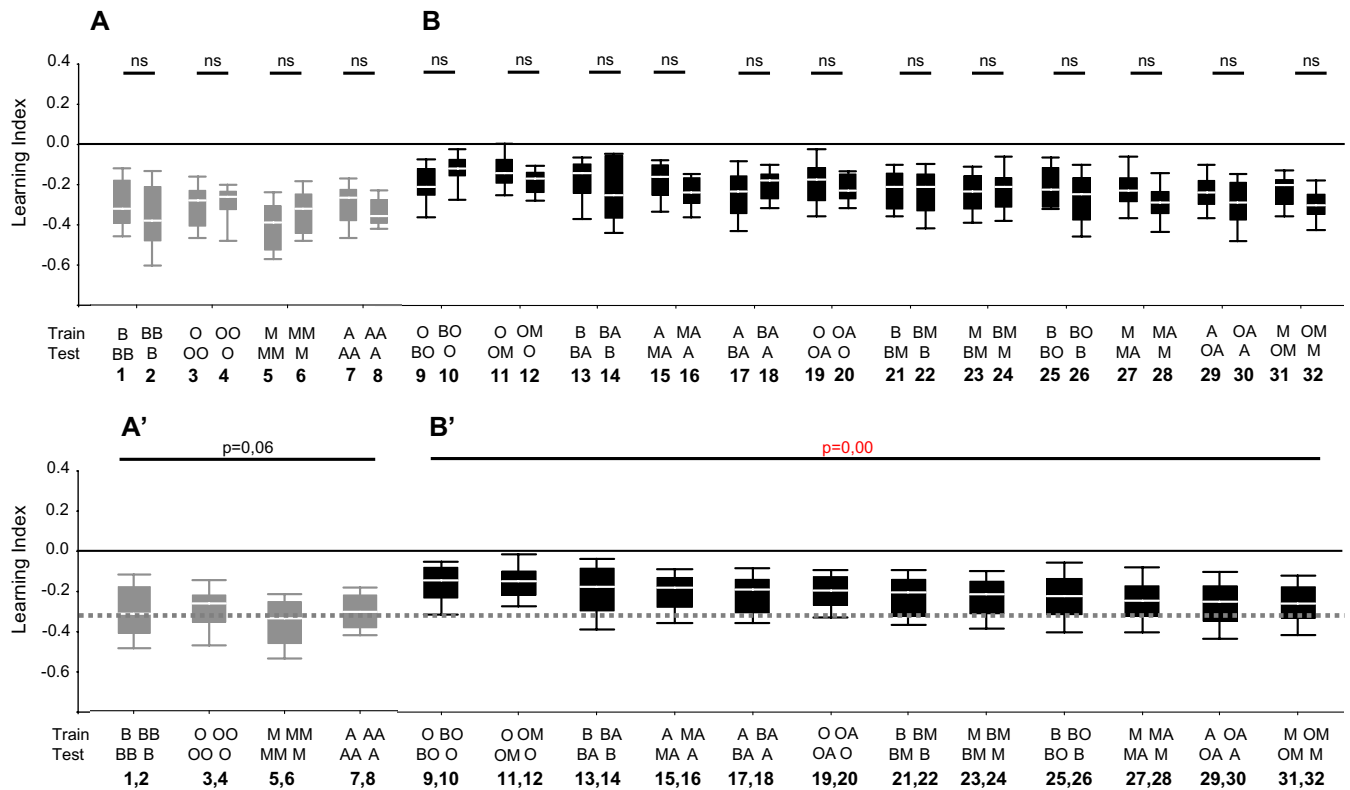[a]The sequence of trial types is balanced within each group.

Figure 1 Symmetry of perceived distances. Learning indices (LIs) dependent on the combination of odors for training and test (B: benzaldehyde, O: 3-octanol, M: 4-methylcyclohexanol, A: n-amylacetate). The numbers in the bottom row refer to the group numbers listed in Table 1. **(A)** Complementary groups of flies are either trained with an element and tested with a double quantity of this element, or vice versa. In all cases, the resulting learning scores are equal between these complementary groups (Mann–Whitney U tests, $P > 0.05/4$). Sample sizes are from left to right: 24, 19, 22, 25, 22, 20, 21, 22. **(B)** Complementary groups of flies either are trained with an element and tested with a binary mixture containing it or are trained to the mixture and are tested with one of its constituent elements. In all cases, scores are equal between these complementary groups (Mann–Whitney U tests, $P > 0.05/12$), arguing for symmetry in perceived distance between element and mixture. Sample sizes are from left to right: 24, 20, 22, 23, 23, 22, 23, 21, 20, 20, 28, 23, 24, 22, 23, 20, 24, 24, 24, 21, 22, 26, 22, 21. **(A′ and B′)** The pooled scores of the complementary groups from **(A)** and **(B)** are presented. Note that learnability in **(A′)** is statistically equal across the data set (Kruskal–Wallis test, $P > 0.05/2$) such that the stippled line, representing the median of the pooled data from **(A′)**, can serve to indicate baseline learnability of the odors used. The significant difference of the scores in **(B′)** (Kruskal–Wallis test, $P < 0.05/2$) argues that perceived distance between elements and mixture is different, depending on which odors are employed. All 12 odor pairs considered in **(B′)** show scores different from zero (one-sample sign tests at $P < 0.05/12$) and have a score lower than the baseline (Mann–Whitney U test, $P < 0.05/12$), except the score obtained from A and OA (Mann–Whitney U test, $P > 0.05/12$); this argues for a usually partial generalization between mixtures and their elements. This figure appears in color in the online version of *Chemical Senses*.

$$LI = (PREF\ O_{Shock}/\Theta - PREF\ O/\Theta_{Shock})/2. \quad (2)$$

Thus, positive LIs indicate conditioned approach, negative LIs conditioned avoidance. Note that across independent measurements, the sequence of events is either as indicated during all 3 training cycles (e.g., first O-shock and then Θ) or is reversed (i.e., first Θ and then O-shock) (in the reciprocally trained sets of flies either first shock-Θ and then O, or first O and then shock-Θ). For all other groups listed in Table 1, experiments are performed and analyzed accordingly.

Data are presented as box plots with the middle line showing the median and box boundaries and whiskers the 25/75% and 10/90% quantiles, respectively, and are analyzed with nonparametric statistics (Statistica; Statsoft). In cases of multiple comparisons, we use a conservative approach by employing a Bonferroni correction to maintain the experiment-wide error rate below 5%. That is, we divide $P = 0.05$ by the number of comparisons made such that if, for example, 3 comparisons are made, $P < 0.05/3$ is used for each individual comparison. The respectively employed cut-off is indicated in the legends. Flies are trained and tested only once.

Experimental rational

To test how similar flies regard a binary mixture to one of its elements, we train flies with an element X and test them with a mixture containing it (X1) or train them with a mixture X1 and test them with one of its elements (either with X or with 1). The more similar the flies regard the trained and tested olfactory stimulus, the higher the obtained score should be. From the 4 odors we use, we can thus draw 32 experimental groups (Table 1).

Given that in this approach we compare behavior toward a mixture with behavior toward an element contained in it, we first need to see whether 2-fold differences in the total amount of odor between training and test would confer any asymmetry to this comparison. Therefore, in the case of the first 8 experimental groups listed in Table 1, we train groups of flies with a single quantity of odor—that is, using single odor cups as mentioned above—and test them with the double quantity—that is, using twin odor cups—of that same odor (e.g., train B, test BB: Table 1, group 1); or we train flies with a double quantity of odor and test them with a single quantity (e.g., train BB, test B: Table 1, group 2).

For the following 24 experimental groups (Table 1, groups 9 to 32), we either train flies with an element and test them with a mixture containing it (e.g., train O, test BO: Table 1, group 9; or train B, test BO: Table 1, group 25); or we train flies with the mixture and test them with one of its elements (e.g., train BO, test O: Table 1, group 10, or train BO, test B: Table 1, group 26).

Physicochemical distance

We used the 184 physicochemical properties that have been calculated previously (Schmuker and Schneider 2007) using MOE, the Molecular Operating Environment (Chemical Computing Group). Because the exact 3D conformation of the odorant that is required to elicit receptor responses is not known, we included only those properties that are independent of conformation (2D features). The features were scaled to a mean of zero and a variance of one ("unit variance") with respect to the original data set used in Schmuker and Schneider (2007). Specifically, we calculated the mean of each feature, such as the number of bonds in the longest chain, over all 836 monomolecular compounds from the 2004 Sigma-Aldrich *Flavors and Fragrances* catalog and subtracted this mean from the value of each individual compound. This was done separately for each of the 184 features such that the average for each feature over the 836 compounds was zero. Similarly, we calculated the variance of each feature and divided the values of each individual compound by it such that the variance of each feature was one. We used the same scaling factors also to scale the features of M, which had not been included in the original data set. Physicochemical distances between odorants were then calculated using the L_1 distance measure ("manhattan distance": sum of absolute coordinate differences).

Results

Generalization between element and mixture is partial and symmetrical

We ask for the perceptual difference between binary mixtures and their constituent elements. We test flies' behavior toward a mixture after having been trained with one of its elements or their behavior toward an element after having been trained with the mixture. Given that each element is presented at its elemental quantity in the mixture, such that the total amount of odorant is doubled in the mixture, we first test whether the same learning scores are obtained after training with a single quantity of odor (e.g., B) and testing with the double quantity (e.g., BB) or if we use the double quantity for training and the single quantity for test (Figure 1A). We find that scores are equal in all cases (Figure 1A; Mann–Whitney U tests, $P > 0.05/4$ for groups 1–8) (this is nontrivial because over wider ranges at least, odor concentration is a major determinant for associative recognition [Yarali et al. 2009]). We thus can pool the respective pairs of groups for further analysis. Using these pooled data, a comparison across all 4 odors does not reveal significant differences in learnability (Figure 1A'; Kruskal–Wallis test, $H = 7.35$, $P > 0.05/2$, df = 3), allowing us to estimate the baseline level of learning scores for the olfactory stimuli in this experimental series by the stippled gray line in Figures 1–3. We can thus ask whether, under such conditions of adjusted learnability, the similarity between element and mixture is symmetrical.

The same learning scores are found when training flies with, for example, B and testing them with BO or when training them with BO and testing them with B (Figure 1B; group 25 vs. group 26); the same is found for all other element–mixture pairs (Figure 1B; Mann–Whitney U tests, $P > 0.05/12$ for groups 9–32). After pooling the respective element–mixture scores, it turns out that learned avoidance is observed to a significant extent (Figure 1B'; one-sample sign tests against zero, $P < 0.05/12$); this generalized learned avoidance, however, is partial, as in almost all cases (A and AO being the exception: Figure 1B', groups 29 and 30) scores are reduced as compared with baseline learning scores (Figure 1B'; Mann–Whitney U tests: $P < 0.05/12$ in all cases excepted for A and AO). Thus, the flies regard the mixture as similar to its elements—rather than regarding it as absolutely identical or as totally different from it. Notably, the level of generalized learned avoidance varies across the considered element–mixture pairs (Figure 1B'; Kruskal–Wallis test, $P < 0.05/2$, $H = 41.45$, df = 11).

An element is equally similar to all binary mixtures containing it

We compare the perceived distances between an element X and the 3 binary mixtures containing it (X1, X2, X3) (Figure 2A,B). We do not see any significant difference regarding any of the 4 odors (Figure 2A; Kruskal–Wallis tests, $P > 0.05/4$ in all cases). In other words, adding any of the 3 odors to the "center odor" X results in perceptually displacing the mixture to about the same extent (denoted as radius r in Figure 2B). Note, however, that the particular distance the mixtures have from X can be different depending on odor: The element O is perceived as more distant from all mixtures containing it than the other elements (Figure 2C; Kruskal–Wallis test $P < 0.05$, $H = 26.92$, df = 3; all pairwise Mann–Whitney U tests $P > 0.05/6$, except for the ones involving O, where $P < 0.05/6$). In other words, O has less
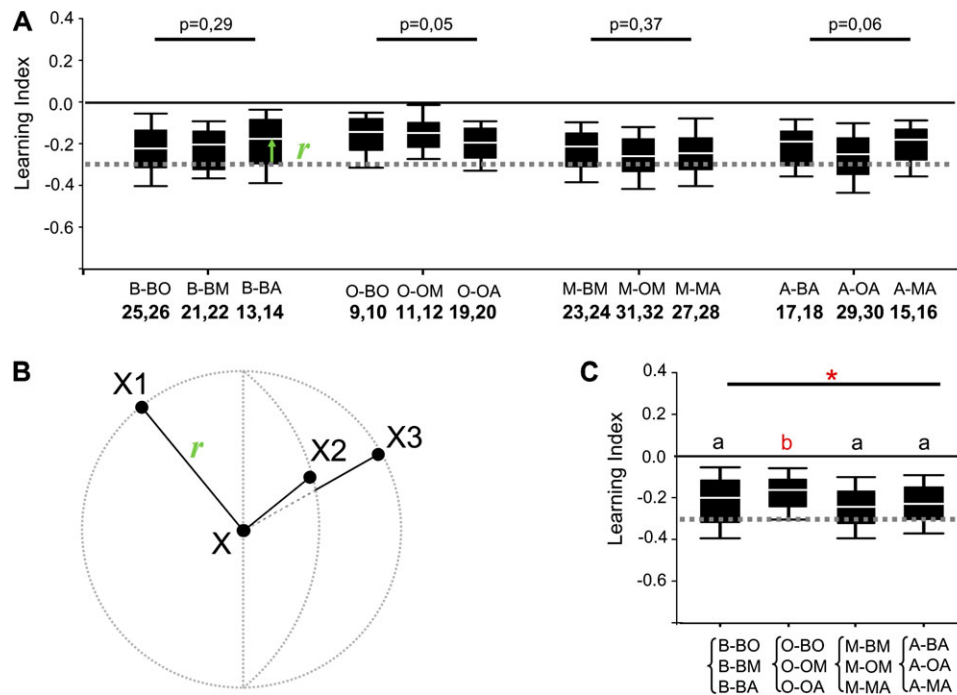
Figure 2 Perceived distances between an element and the mixtures containing it. **(A)** Data from Figure 1B' are rearranged to test for differences in perceived distance between a given element and the 3 possible binary mixtures containing it. In all cases, these distances are not significantly different (Kruskal–Wallis tests, $P > 0.05/4$), arguing that the impact of a given element is similar regardless of its companion element in the mixture. This is represented by the sketch in **(B)**. **(C)** Data from **(A)** are pooled to allow testing whether the distance between elements and mixture (the size of the sphere in **B**) differ between odors. It turns out that O is more distant to the mixtures containing it than any of the other elements (Kruskal–Wallis test $P < 0.05$; shared or different lettering indicates Mann–Whitney U tests with $P >$ or $< 0.05/6$). For sample sizes, see legend of Figure 1A,B; other details as in Figure 1. This figure appears in color in the online version of *Chemical Senses*.

impact on mixture perception than the other odors, an effect which is also seen in the following analysis.

A binary mixture is equally similar to both constituent elements

Next, we ask for the distance between the mixture X1 and its constituent elements (i.e., X and 1) (Figure 3). We find that in all cases, except for OM as a mixture, the elements are at about equal distance (denoted as r' in Figure 3B) to the mixture (Figure 3A; Mann–Whitney U tests in all cases $P > 0.05/6$, except OM where $P < 0.05/6$). In other words, as a rule, both elements contribute about equally to mixture perception (Figure 3B). The exceptional case of OM, as well as the corresponding trends for the cases of BO and OA which just fall short of the Bonferroni-corrected statistical cut-off, prompts the question whether the learnability of an element accounts for its weight in the mixture. Specifically, we ask: If a given odor is more learnable than the other, would that more learnable odor also have the higher weight in the mixture? To this end, we first calculate the difference in learnability (Δl) between any pair of odors as the difference between the median learning index for the less learnable element minus the median learning index for the more learnable element; in the case of O and M, for example, the median learning index for M is more negative than for O (Figure 4A).

Second, we correspondingly calculate the difference in weight in the mixture (Δw) (Figure 4B); for the example of O and M, this reveals than the weight of the more learnable element M in the OM mixture is higher than the weight of O (Figure 4B). After doing so for all cases, we find that these differences correlate (Figure 4C; Spearman rank correlation: $r = 0.94$, $t_{N-2} = 5.66$, $P = 0.005$) with fairly steep slope. This suggests that even small increases in learnability of an odor can fairly strongly increase its impact in a mixture containing it. The better learnability is being adjusted, however, the more do differences in weight disappear.

Discussion

This study, based on associative recognition experiments, provides 3 relatively simple rules for the processing of binary mixtures in *Drosophila*. If the odor elements X, 1, 2, and 3 are adjusted for equal learnability, we find that 1) generalization between a binary mixture and either of its elements is symmetrical (Figure 1B) and partial (Figure 1B'), that 2) the element X is equally similar to the mixtures containing it (Figure 2B), and that 3) the mixture X1 is equally similar to both its constituent elements (Figure 3B). These results do not provide evidence for mixture-unique effects in *Drosophila* olfactory perception.
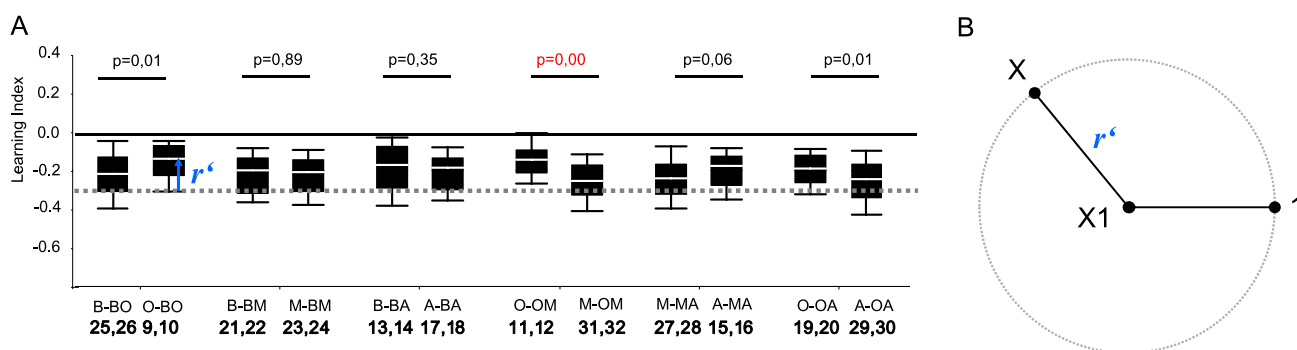
Figure 3 Perceived distances between a mixture and its constituent elements. **(A)** Data from Figure 1B' are rearranged to compare the distances between a mixture and either of its constituent elements. As a rule, the distances of either element to the mixture are equal (Mann–Whitney U tests, $P > 0.05/6$); this rule is sketched in **(B)**. The exceptional case is that the distance between O and OM is larger than the distance between M and OM (Mann–Whitney U test, $P < 0.05/6$); in other words, the weight of M in the mixture is higher than the weight of O in the mixture. For sample sizes and other details, see legend of Figure 1. This figure appears in color in the online version of *Chemical Senses*.
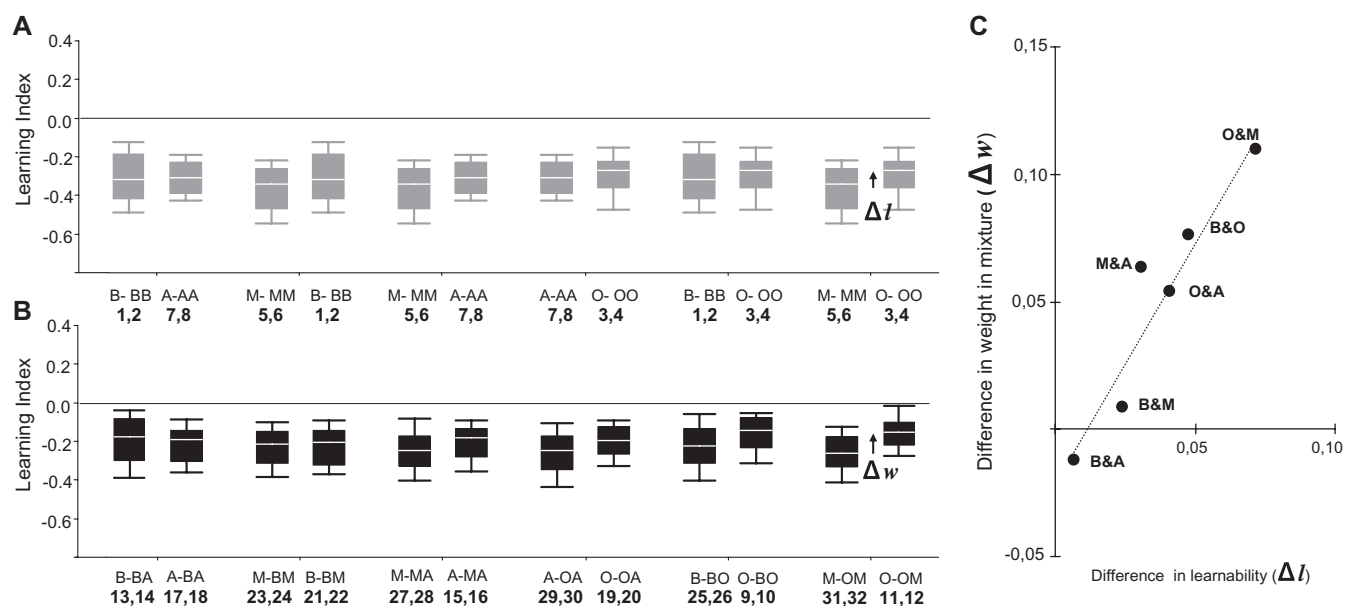
Figure 4 Small differences in learnability of the elements can entail differences in weight in the mixture. **(A)** On the basis of the learnability data in Figure 1A', the difference in learnability between a given pair of elements (Δl) is determined as the median learning index of the respectively less learnable element minus the median learning index of the more learnable element. Data are arranged according to increasing differences in learnability. **(B)** On the basis of the generalization data from Figure 1B', the differences in the weight of the elements within a mixture (Δw) are determined. The "polarity" for this subtraction is according to **(A)**. **(C)** Correlation plot of the difference in learnability of the elements (Δl; x axis) and their difference in weight within a mixture (Δw; y axis). Considering the odor pairs as indicated by the letters, the observed correlation (Spearman rank correlation, $P < 0.05$) argues that the better learnable an odor is, the heavier its weight in a mixture. For sample sizes and other details, see legend of Figure 1.

We would like to stress, though, that the boundary condition for the applicability of these rules, namely that learnability indeed is adjusted, is important. That is, although it appears as if there is more generalization between M and the OM mixture than between O and the OM mixture (Figure 3A), this can be accounted for by the slightly lower learnability of O (Figure 1A'). Indeed, although variations in learnability across all 4 odors formally remain below statistical cut-off using the warranted Bonferroni correction (Figure 1A'), we do observe a correlation between differences in learnability

and differences in weight of an element in the mixture (Figure 4C). Thus, "imperfections" in learnability adjustment uncover that even small differences in learnability may be able to feign asymmetries in mixture processing.

Interestingly, on the physiological level asymmetries in the weight of odors in a mixture can be accounted for by the signal intensities evoked by the odor elements in the projection neurons (for the honeybee: Deisig et al. 2010, loc. cit. Figure 4). Correspondingly, Lapid et al. (2008) found that human judgments of the pleasantness of an odor mixture follow

a linear model taking into account the pleasentness judgments of its constituent elements—weighted by their respective perceived intensities.

We were further wondering whether the similarity between the mixture X1 and its elements X and 1 depends on the physicochemical similarity between X and 1 (Figure 5A). Consider as an extreme case that X and 1 were practically identical in terms of their physicochemical properties; then the flies would regard the X1 mixture effectively as XX, leading to a small perceptual distance between X and what comes across to the flies as "XX." Taking advantage of the physicochemical descriptions of odors according to Schmuker and Schneider (2007) (for a related approach see Haddad et al. 2008), we find that the more distant X and 1 are in terms of their physico-

A

B

$\rho^{Physico-Chemical}$
(Schmuker and Schneider, 2007)

Figure 5 Mixture-to-element distances correlate with element-to-element distances. **(A)** Sketch to illustrate mixture–element distances (r′) and element–element distances (ρ). **(B)** The perceived distance between a mixture and its constituent elements (r′; y axis; data from Figure 3A using the median of the pooled learning indices for each odor pair considered) can be predicted by the physicochemical distance between the elements ($\rho^{Physicochemical}$: x axis, data according to Schmuker and Schneider (2007)) (Spearman rank correlation: r = 0.9, P < 0.04). Data for O and M (open symbols) cannot be considered in the correlation analysis as O and M are not equally learnable, leading to apparently asymmetric scores for r′ when considering O–OM and M–OM (Figure 3A). This figure appears in color in the online version of Chemical Senses.

chemical properties, the more distant the flies regard X1 from its elements X and 1 (Figure 5B; Spearman rank correlation: r = 0.9, t_{N-2} = 3.58, P = 0.04). This could partially account for the variations seen in the distances between element and the different mixtures containing it as seen in Figure 2A, which, however, remain below statistical cut-off when using the warranted Bonferroni correction.

Contemplating once more that generalization between element and mixture depends on the odor pairs considered (Figure 1B), what are the determinants of this generalization? As argued above, both minor learnability differences and differences in physicochemical relatedness apparently are of influence and may explain at least some variations in generalized conditioned avoidance. We further note that we do not find cases of no-generalization, that is, in no case is the mixture something totally different from the elements nor do we typically see full generalization. This latter observation is not trivial. Suppose recognition were determined by the mere presence of a learned element (for a discussion, see Pearce 1994), such that when testing with X1 the previously trained element X is recognized as such. Taken to its logical extreme, such a scenario could predict full generalization between elements and mixture, which is not observed (Figure 1B′). This may imply that either after training with X this trained element X is recognized upon testing with X1, but its impact is scaled down because it is part of a mixture (as total odor amount does not have a systematic influence [Figure 1A], scaling down according to total odor amount does not seem likely). In turn, during training with X1, the elements may accordingly share into the training effects. This process would be independent of the kinds of odor elements employed and thus also of their element–element similarity but would require information about the number of monomolecular elements comprising the mixture being available to the olfactory system. Alternatively, with regard to the memory trace (which arguably is localized to the mushroom body Kenyon cells, a third-order cortical brain region of the insects: Heisenberg 2003; Gerber et al. 2004; Krashes et al. 2007), the neurons processing X may be overlapping with rather than being nested within the ones processing X1 such that depending on the kinds of odors employed only a fraction of neurons housing the memory trace can be activated. This would require lateral inhibition between the neurons activated by X and 1 at some point in the circuit upstream of the memory trace, potentially within the antennal lobe.

In conclusion, our results provide the first systematic account of mixture perception in *Drosophila*. We derive 3 rules of mixture perception: 1) mixture–element generalization is symmetrical and partial; 2) elements are equally similar to different mixtures containing it, and 3) a mixture is equally similar to its elements. Importantly, we identify 2 boundary conditions for the applicability of these rules: First, the dilutions of the odors need to be adjusted for task-relevant behavioral potency, and second, the physicochemical distances between the elements should be about equal.
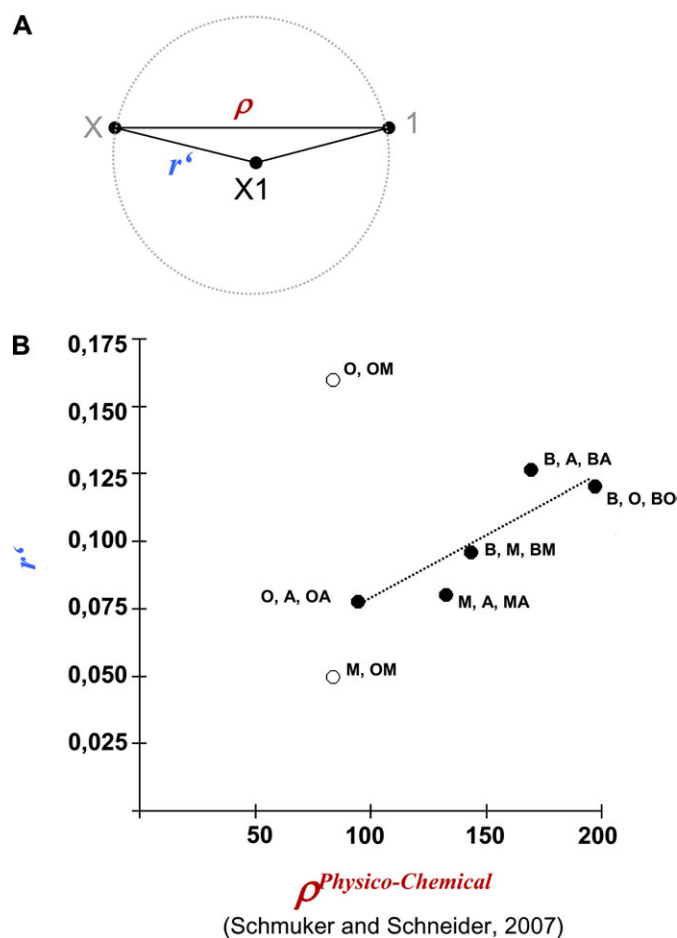
Supplementary material

Supplementary material can be found at http://www.chemse.oxfordjournals.org/.

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