Chapter II :

Stimulus Dominance in Compound Processing: Uncovering the Mechanisms of Olfactory Overshadowing

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Schubert, M., Sandoz, J.C. & Giurfa, M.

The contributions of the different authors were as follows:
I performed all the experiments and I did all the data analyses together with J.C. Sandoz and wrote the manuscript under the guidance of J.C. Sandoz and M. Giurfa.

Introduction

The question of how animals process and learn compound stimuli has inspired different conditioning protocols aimed at deciphering whether compound identity results from the simple sum of component properties or from interactions between components that confer unique properties to each compound. One of such protocols has focused on a phenomenon called *overshadowing* (1, 2, 3). In an overshadowing protocol, an animal is trained with a binary compound of two stimuli A and B (henceforth AB+, with + indicating the presence of reinforcement) and then tested with the single components A and B. Overshadowing is said to occur if, after compound conditioning, the animal responds significantly more to one component at the expense of the other (4, 5, 6). The reasons for such difference may vary: overshadowing may result from differences in perceived strength of stimuli (stimulus salience), leading to better learning of the most salient component in detriment of the less salient one (6). Salience depends on physical characteristics of stimuli and of the sensory systems of the perceiver. In this case, no competing or inhibitory interactions between components are necessary to account for overshadowing: evaluating each component through independent channels may lead to a differential perception of their salience. Alternatively, within-compound interactions may determine component dominance and thus overshadowing (7). These different hypotheses illustrate the necessity of studies addressing the causal mechanisms of overshadowing rather than simply assessing its occurrence. Appropriate control procedures (2, 3) may help deciding between these options. Together with a group trained to the compound stimulus (*OVS group*), control groups trained to either component (*Ctrl A* trained with A+ and *Ctrl B* trained with B+) are necessary for the interpretation of overshadowing performances. Within-mixture interactions can be assumed if the response to a component after compound training is lower than after single-component training.

Overshadowing has been found both in invertebrates and vertebrates trained with stimuli of different modalities [spiny lobsters (8), fruit flies (9), honeybees (10, 11, 12, 13, 14, 15), fishes (16), pigeons (17), nutcrackers (18), rats (19, 20), rabbits (21), dogs (1) and monkeys (22)]. However, no predictive rule is available to determine beforehand and based on the properties of the components whether or not overshadowing will occur. Here we studied overshadowing in the olfactory modality in honeybees *Apis mellifera*, with the aim of uncovering the mechanisms underlying this phenomenon. We chose this sensory modality because of the difficulty in deciding between component-based and holistic forms of compound processing in the case of olfactory mixtures. We used honeybees because odors

play an essential role throughout their different life stages (23) and because olfactory learning and processing in bees can be studied in the laboratory using the olfactory conditioning of the proboscis extension reflex (henceforth PER; 24, 25). Naïve, hungry bees respond with a PER to a stimulation of their antennae with sucrose solution. After pairing of an odor with sucrose delivered to the antennae and proboscis, bees learn that the odor, the conditioned stimulus (CS), anticipates the sucrose reward, the unconditioned stimulus (US), and thus respond with PER to the odor (25).

Overshadowing in the olfactory modality has been reported in honeybees trained using this procedure (15). Smith (15) presented three odors in the form of two binary mixtures and found that overshadowing depended on the odor used and on the number of mixture conditioning trials employed. He concluded that within-mixture interactions are responsible for overshadowing and that these interactions are odor and experience dependent, thus lacking of a general explanatory mechanism for overshadowing. Such limitation may be, however, due to the reduced number of odors used (three), of mixtures trained (two), and/or of bees trained per group (fifteen). Here we studied olfactory overshadowing in bees and asked whether or not it is possible to predict its occurrence beforehand, by considering the properties of the mixture components. Using 6 odors that varied systematically in their chain length and functional group, two parameters that define a putative olfactory space in honeybees (26, 27), we analyzed the occurrence of overshadowing in all possible 15 binary mixtures arising from our odorant choice. For each binary mixture trained, the performance of two control groups, each trained to one of the two odorants, was recorded. We controlled stimulus salience by equalizing the vapor pressure of the odorants used. We studied the incidence of the number of trials in the occurrence of overshadowing by comparing performances after 1- vs. 3-trial conditioning. Recording the performance of more than 2700 bees allowed us to determine that overshadowing can be predicted beforehand on the basis of component properties such as how well a component is learned when trained alone and how much it is generalized after such training. Interactions within a mixture are therefore not necessary to account for overshadowing in the olfactory modality in bees.

Material & Methods

Animals. Bees were captured at the entrance of an outdoor hive and were cooled down on ice to near immobility. They were harnessed into individual metal tubes, such that only movements of the antennae and the mouth parts were possible. After 2 undisturbed hours each subject was checked for intact PER by touching one antenna with a toothpick imbibed with 50% w/w sucrose solution. Extension of the proboscis was counted as PER. Animals that did not show the reflex were discarded.

Apparatus. Single odorants, binary mixtures or a clean air stream were delivered by odor cannon (49). Each odorant was applied on a filter paper and placed within a plastic syringe that was connected to the cannon. An air pump (Rena Air 400, Annecy, France) delivered a constant air stream to 8 identical channels controlled by electronic valves (Lee Company S.A., Voisins-le-Bretonneux, France). When opened, each valve directed the air stream through a syringe carrying the odorant from there to a common chamber with an exit hole allowing antennal stimulation. Binary mixtures were delivered by letting the air flow simultaneously through two syringes, each carrying a different chemical substance. A single odorant was delivered by letting the air flow simultaneously through two syringes, one containing a chemical substance and another containing a clean piece of filter paper. In this way air flow intensity was kept identical across stimulations. When no odorant was presented, a permanent clean air stream was directing to the bee. An air exhaust placed permanently behind the bee impeded olfactory contamination.

Stimuli. Six single odorants and their resulting 15 binary mixtures were used as CSs (see Table 1). A 50 % w/w sugar solution was used throughout as US. Odorants were obtained from Sigma Aldrich (Deisenhofen, Germany) and varied in chain length (8 and 9 carbons) and functional group (aldehydes, secondary ketones, and secondary alcohols). These odorants are present in floral volatile emissions (50). One of them, 2-nonanol, is a component of the bee sting alarm pheromone (51). Vapor pressures of all odorants were equated (see Table 1) by diluting pure substances in mineral oil (Sigma Aldrich). For stimulation, 4 µl of diluted odorant were applied onto a 1 cm^2 piece of filter paper, which was placed into a cannon syringe.

Experimental design. Three groups of bees were trained in parallel: an overshadowing group (*OVS group*) trained to a binary olfactory mixture (AB+) and two control groups, each trained to one of the respective mixture odorants (A+ and B+; *Ctrl A* and *Ctrl B*, respectively). One or 3-trial training was performed. For the latter, an inter-trial

interval of 10 min was used. At the beginning of each trial, the bee was placed at the training site and left there for familiarization during 10 sec. The CS was then delivered during 4 sec. Three seconds after CS onset (inter-stimulus interval: 3 sec), the US was delivered to the antennae by means of a toothpick soaked in sugar solution, thus leading to PER. The bee was allowed to feed the sucrose solution for 3 sec (i.e. 3 μl; see ref. 52). Thereafter, the bee was left in the same position for 17 sec and then replaced by the next bee. If a bee did not show a PER to the US in any training trial (less than 2% of the bees trained), it was discarded.

Ten min after training, bees from all three groups, *OVS*, *Ctrl A* and *Ctrl B*, were tested with the binary mixture AB and the single odorants A and B without sucrose delivery. Tests were performed in a randomized sequence with an inter-trial interval of 10 minutes. After the tests, bees were stimulated with sucrose on the antennae to check for intact PER. Bees not exhibiting PER were discarded (less than 3%). Overall, less than 1% of the bees died during the experiment. As for each mixture three groups of bees were trained (*OVS*, *Ctrl A* and *Ctrl B*) and each group included at least 30 bees, over 2700 bees were trained in the 1-trial and in the 3-trial experiment.

Data analysis. In training and test trials, we recorded PER to the olfactory stimuli. A PER was counted whenever a bees extended its proboscis to the CS presentation. Multiple responses during a CS were counted as a single PER. During training trials, we also recorded whether bees responded to the US. Three-trial learning curves were analyzed by using the Cochran test. Comparisons of test responses were performed using the χ^2-test.

To determine whether overshadowing can be predicted from the responses to the components after training to a single component (*Ctrl A, Ctrl B*), we quantified how good a component was learned and how much generalization it promoted towards the unknown component. For each odor mixture tested, we calculated i) the amount of overshadowing, ii) the difference in component learning and iii) the difference in generalization. i) Overshadowing was calculated as the difference between the response to the components after mixture training (*OVS group*), i.e. $R_{A(AB)}$ - $R_{B(AB)}$. For this calculation odor A was taken as the odor eliciting the higher amount of responses (the overshadowing odor) such that this coefficient was always $\geq$ 0. Odorant identity (A or B) as defined in the OVS groups was kept for the control groups. ii) The difference in component training was evaluated as the difference between the responses to odorant A after training to A and the responses to odorant B after training to B (i.e. $R_{A(A)}$ - $R_{B(B)}$). iii) Relative generalization from A to B was estimated as the proportion of bees responding to odor B after A training divided by the proportion of bees responding to odor A after A training ($R_{B(A)}$ / $R_{A(A)}$). Generalization from B to A was $R_{A(B)}$ / $R_{B(B)}$. The difference in

generalization was calculated as $[(R_{B(A)} / R_{A(A)}) - (R_{A(B)} / R_{B(B)})]$. Pearson correlations between these three variables were calculated to determine whether component properties (learning, generalization) account for overshadowing. To evaluate the relative contributions of component learning and generalization to the overshadowing effect, we performed multiple regression analyses. The contribution of each parameter in the best model obtained was taken from the standardized β values. Significance of the best regressions was assessed using ANOVA.

Results

Bees learned all 6 odorants conditioned (CS) both in the 1-trial (Fig. 1A) and in the 3-trial conditioning experiments (Fig. 1B). In the 1-trial experiment (Fig. 1A), bees significantly increased their response to the CS from the training to the test (Cochran test, $P < 0.0001$ in all 6 cases). Learning differed between odorants ($\chi^2 = 31.23$; df: 5; $P < 0.001$) as shown by the fact that some odorants (2-nonanol and nonanal) elicited more responses than others in the CS test. In the 3-trial experiment (Fig. 1B), bees also increased their response to the CS from the first conditioning trial to the CS test ($P < 0.0001$ in all 6 cases). Again, some odorants (2-nonanol and nonanal) were better learned than others ($\chi^2 = 63.39$; df: 5; $P < 0.001$).

All 15 binary mixtures were also learned by the bees, both in the 1-trial (Fig. 1C; $P < 0.001$) and in the 3-trial conditioning experiment (Fig. 1D; $P < 0.0001$). Learning did not differ between mixtures both in the 1-trial ($\chi^2 = 21.90$; df: 14; $0.05 < P < 0.10$) and in the 3-trial experiment ($\chi^2 = 12.67$; df: 14; $0.50 < P < 0.75$). Test values for each odorant and mixture trained can be found in Table 2 – supporting information.

Differences in odorant response after mixture conditioning. To determine the occurrence of overshadowing, we analyzed the performances of bees trained with the binary mixtures (*OVS* groups) when tested with the single components of the mixtures. We aimed at identifying those cases in which bees responded significantly more to one component than to the other. In the OVS groups we defined A as the former and B as the latter. In the 1-trial experiment, we found overshadowing in 6 out of 15 mixtures trained (Mc Nemar test, $P < 0.05$ in all 6 cases). Figure 2A shows an example of overshadowing after conditioning of the mixture of 2-octanol and 2-octanone. In this case, bees responded significantly more to 2-octanol (white bar; henceforth odor A) than to 2-octanone (gray bar; henceforth odor B) (McNemar test: $\chi^2 = 4.00$; df: 1; $P < 0.05$). In the other 5 cases (see Table 2A – supporting information, red-marked 'mix' rows), the dominant odorants were 2-nonanol (mixed with octanal or 2-octanone), 2-nonanone (mixed with octanal or with 2-octanone) and 2-octanol (mixed with 2-octanone).

In the 3-trial experiment, we found overshadowing in 9 out of 15 mixtures. Six of these 9 mixtures corresponded to those exhibiting an overshadowing-like effect in the 1-trial experiment. Such an effect was found additionally in the mixtures of 2-nonanol and nonanal, nonanal and octanal and 2-nonanol and 2-octanol (see Table 2B – supporting information, red-marked 'mix' rows). In these cases, 2-nonanol was always dominant; in the mixture of nonanal and octanal, nonanal was the dominant odorant.

We conclude that differences between responses to single odorants occur after conditioning of olfactory binary mixtures. Multiple trial conditioning induced more cases of overshadowing than single-trial conditioning. Training length did not change odorant dominance: whenever a tendency to respond more to one component was found after 1-trial conditioning, it was accentuated after 3-trial conditioning. Also, 9-carbon components dominated generally over 8-carbon components although all odorants were equated in their vapor pressure. More specifically, 2-nonanol, a component of the sting alarm pheromone, generally dominated over the other odorants.

Differences in learning and generalization after single-odorant conditioning. To elucidate the mechanisms of overshadowing, we analyzed the performance of our two control groups (*Ctrl A* and *Ctrl B*), each trained to a single odorant. Within each control group, we quantified how well an odorant was learned when trained alone, and how much was this odorant generalized to a different odorant after such training. We determined if responses to components after mixture training could be predicted based exclusively on these parameters.

Firstly, we focused on *component learning*. We compared the test responses to the odorants conditioned in each control group (*Ctrl A* and *Ctrl B*) for all 15 mixtures used. Figures 2B, C show an example of such comparison for the control groups trained to 2-octanol (Ctrl A) and 2-octanone (Ctrl B), respectively (comparison of white vs. gray CS bars in Figs. 2B, C, respectively). In this case, no difference was found in component learning (Fig. 3A: $\chi^2 = 0.60$; df: 1; P = 0.44). In the 1-trial conditioning experiment, we found 3 odor combinations in which one odorant was better learned than the other: nonanal and 2-octanone, nonanal and octanal, and 2-nonanol and octanal (P < 0.025 in all 3 cases; see Table 2A – supporting information, blue-marked odorant rows). Overshadowing was found in only 1 of these 3 cases (mixture of 2-nonanol and octanal), thus showing that differences in component learning cannot fully account for the overshadowing effect. In the 3-trial conditioning experiment, we found 2 odor combinations in which one odorant was better learned: 2-nonanol and 2-octanone, and 2-nonanol and 2-octanol (P < 0.025; see Table 2B). In both cases, the corresponding *OVS group* showed overshadowing. Thus, for both 1-trial and 3-trial conditioning experiments, whenever overshadowing and differences in component learning were related (3 cases in total), 2-nonanol was the dominant odorant.

Secondly, we focused on asymmetric *cross-generalization* between odorants after odorant learning. Cross-generalization between odors is often asymmetrical as bees can respond more to an odor B after learning odor A, than vice versa (27). We compared the generalization responses obtained within each control group, for all 15 odor combinations. An

example of generalization within control groups A and B can be seen in Figs. 2B (gray bar) and C (white bar), respectively. To provide an appropriate account of generalization, only generalization responses of those bees that learned to respond to their respective CS were considered ('*relative generalization*'). Differences in relative cross-generalization ('*cross-generalization asymmetry*') can be then compared between control groups (see Fig. 3B: cross-generalization asymmetry is $R_{B(A)} / R_{A(A)} - R_{A(B)} / R_{B(B)}$ where $R_{Y(X)}$ is the response to stimulus Y after training to stimulus X; see 2x2 χ^2 analysis in Fig. 3B for the control groups of Figs. 2B, C). In Fig. 2B, C, for instance, asymmetric cross-generalization was found between 2-octanol and 2-octanone: bees trained to 2-octanone generalized relatively more to 2-octanol than vice versa (2x2 $\chi^2 = 7.11$; df: 1; P < 0.025). In the 1-trial experiment, this odor combination was the only one yielding asymmetric cross-generalization and overshadowing (Fig. 2). In the 3-trial experiment, asymmetric cross-generalization was found in five odor combinations (P < 0.025 in all 5 cases; see Table 2B – supporting information, green-marked odorant rows). In all these combinations, overshadowing was found.

An integrative account of the overshadowing effect. We first analyzed whether overshadowing ($R_{A(AB)} - R_{B(AB)}$) can be accounted for by differences in component learning ($R_{A(A)} - R_{B(B)}$). We performed for both experiments and for 14 odor combinations a correlation analysis involving these variables. Odor combination I was excluded from these and all following correlation analysis (for the 1- and 3-trial experiments) because of an unexpected result in the 3-trial experiment. The CS responses after 2-octanone conditioning were unexpectedly very low and generalization responses to the unconditioned odor, 2-nonanone, were unexplainable higher. Such a result was neither found in the 1-trial experiment nor in any other odor combination. Because of the uniqueness of this case, we decided that this odor combination should be excluded from correlation analysis to avoid adulteration of general trends. For calculation of the overshadowing effect in the OVS groups we defined A as the odor to which bees responded more and B as the odor to which they responded less. In the 1-trial and the 3-trial experiment we found 2 cases (1-trial: odor combination XIV [nonanal mixed with 2-octanol] and XV [2-nonanol mixed with nonanal]; 3-trial: odor combination XII [2-nonanone mixed with 2-octanol] and XIV [nonanal mixed with 2-octanol]) in which responses to A and B in the OVS groups were exactly the same. Therefore, it was not possible to decide which odor should be named A and which one B. To solve this problem we performed different correlations for all possible combinations to label the odors of the two odor pairs with A and B (4 correlations in both, the 1-trial and the 3-trial experiment). Thus, we were able to calculate a mean (*r'*) from the four correlation *r* values. In

the 1-trial experiment a significant correlation between the amount of overshadowing and the amount of component learning was found (r' = 0.57 ± 0.008, mean ± s.e.m., P < 0.05, Fig. 4A shows one of the four correlations with r closest to r'). This effect was not found in the 3-trial experiment (r' = 0.47 ± 0,070, mean ± s.e.m., NS, Fig. 4B shows one of the four correlations with r closest to r') thus showing that with increasing experience, differences in component learning are not generally decisive for the overshadowing effect. Despite the significant correlations found in the 1-trial experiment, few odor combinations supporting overshadowing did not show practically differences in component learning (see, for instance, odor combination XIII in Fig. 4A) while some odor combinations in which differences in component learning were found did not always induce overshadowing (see, for instance, odor combination VIII in Fig. 4A).

        We then analyzed whether overshadowing can be accounted for by asymmetries in cross-generalization ($R_{B(A)}$ / $R_{A(A)}$ − $R_{A(B)}$ / $R_{B(B)}$). Both for the 1-trial (r' = 0.63 ± 0.003, mean ± s.e.m., P < 0.02, Fig. 4C shows one of the four correlations with r closest to r') and the 3-trial experiment (r' = 0.61 ± 0.018, mean ± s.e.m., P < 0.05, Fig. 4D shows one of the four correlations with r closest to r'), significant correlations were found. Note, however, that some odor combinations showed a tendency towards asymmetric cross-generalization without overshadowing (see odor combinations VII and VIII in Fig. 4C), while other combinations exhibited overshadowing but no asymmetric cross-generalization (see odor combination II in Fig. 4D).

        Finally, we analyzed whether overshadowing can be accounted for by a combination of both parameters, differences in component learning and asymmetric cross-generalization. We performed multiple-regression analyses to define the weight of these two parameters in a model predicting the overshadowing effect. As for the correlations between overshadowing and the single parameters, we defined A as the odor to which bees responded more and B as the odor to which they responded less in the OVS groups. Thus, we were again confronted with the problem of how to label the odor components in cases where responses to A and B in the OVS groups were exactly the same. We used the same way out as above and performed different multiple-regression analysis for all possible combinations to label the odors of the two odor pairs with A and B (4 multiple-regression analysis in both, the 1-trial and the 3-trial experiment) and calculated the mean weight of these two parameters. These analyses indicated that in the 1-trial experiment, differences in component learning contributed a relative weight of 41 ± 3% (mean ± s.e.m.) while asymmetric cross-generalization contributed 59 ± 3% (mean ± s.e.m.) to the overshadowing effect (based on the mean of standardized beta

values; $r' = 0.68 \pm 0.000068$ (mean $\pm$ s.e.m.), P < 0.05, Fig. 4E shows one of the four correlations with r closest to r'). Due to the magnified weight of asymmetric cross-generalization, the combined model does not yield a significant improvement with respect to the model that takes only asymmetric cross-generalization into account. When the same analysis was performed for the 3-trial experiment, asymmetric cross generalization still was the dominant factor as it contributed $57 \pm 8\%$ (mean $\pm$ s.e.m.) to the overshadowing effect while differences in component learning contributed $43 \pm 8\%$ (mean $\pm$ s.e.m.) (based on the mean of standardized beta values; $r' = 0.75 \pm 0.019$ (mean $\pm$ s.e.m.), P < 0.05, Fig. 4F shows one of the four correlations with r closest to r'). In this case, the combined model was better than that taking only differences in component learning ($r' = 0.47 \pm 0,070$, mean $\pm$ s.e.m.) and than that considering only asymmetric cross generalization ($r' = 0.61 \pm 0.018$, mean $\pm$ s.e.m.).

Thus, both after 1-trial and 3-trial conditioning, the overshadowing effect is well described by generalization asymmetries between odor components. This shows that this factor, which has been rarely taken into account in analyses of overshadowing, plays a dominant role in olfactory mixture learning in bees. For conditioning independent correlations and multiple-regression analyses (grouped data of the 1- and 3-trial experiment) see Appendix B.

Discussion

Our results show that the overshadowing effect, which has attracted the interest of researchers interested in compound learning and perception since Pavlov (1), can be fully predicted in the case of olfactory binary mixture learning in bees by considering intrinsic properties of the odorants that integrate the mixture. No within-mixture interactions in the form of mutual inhibition or competitive effects are necessary to describe odorant dominance upon compound learning in honeybees. Two factors account for such a dominance: 1) asymmetric cross-generalization between odorants, due to bees responding more to odorant B after learning odorant A than in the reversed situation, and 2) differences in odorant learning, due to bees learning better one of two odorants. From these two factors, asymmetric cross-generalization contributed more to the overshadowing effect as differences in odorant learning were less relevant after 3 trial-conditioning.

Asymmetries in odorant cross-generalization seem to be a frequent feature of the honeybee olfactory system (27, 28). They can be due to differences in odorant salience, which can be in turn innate and related to biologically relevant natural odors, or based on previous odorant experiences. They may also result from asymmetric changes in odorant similarity after conditioning: if, for instance, the representation of A after conditioning becomes A', which is more dissimilar from B than A was in the bee's olfactory space, and if the representation of B becomes B' after conditioning, which is closer to A than B, then bees would show less generalization from A to B than from B to A. Conditioning-dependent asymmetric changes in odorant similarity should be more evident with increasing conditioning trials. In other words, asymmetric cross-generalization should be the driving force in overshadowing after 3, instead of 1, conditioning trials. This was, however, not the case as shown by the relative contribution of asymmetric cross-generalization after 1-trial (59%) and 3-trial conditioning (57%). Asymmetries in cross-generalization were probably based, therefore, on differences in odorant salience. More salient odorants promote less generalization while less salient odorants exhibit the opposite trend. Combining a salient and a less salient odorant results, therefore, in asymmetric cross-generalization and thus in overshadowing. Differences in odorant learning also contributed to overshadowing but as mentioned above their contribution was only evident after 1 learning trial. In our experiments, molecules with nine carbons (C9, especially 2-nonanol and nonanal) were in general more salient than molecules with eight carbons (C8), as shown by their different learning levels evinced in the control groups. More importantly, generalization from C9 to C8 molecules was

less than from C8 to C9 (see Table 2A,B – supporting information), which resulted in C9 molecules promoting generally overshadowing when presented with C8 molecules. The reason why C9 molecules were more salient and promoted more asymmetric generalization than C8 molecules is unclear. All odorants had equated vapor pressures such that odorant intensity cannot account for this difference. It is noteworthy that the most dominant odor, 2-nonanol, has a biologically relevant role as alarm pheromone for honeybees (29), a fact which may underlie its particular salience. In few cases, overshadowing occurred when the compound consisted of two C9 or two C8 molecules (1-trial experiment: 2-octanol mixed with 2-octanone and 2-octanol mixed with octanal; 3-trial experiment: 2-nonanol mixed with nonanal and same combinations as in the 1-trial experiment). In these cases, alcohols overshadowed ketones and aldehydes.

In the Rescorla and Wagner model of classical conditioning (6), component salience affects, through a specific equation term, the associative strength gained along conditioning trials by a conditioned stimulus. Whenever two stimuli are conditioned in compound, the model posits that the total amount of associative strength available has to be shared by the two components, which compete for it. As conditioning progresses, the component with higher salience gains more associative strength, thus setting the basis for overshadowing. The results of the 3-trial conditioning experiment are not in line with this interpretation because after 3 learning trials differences in component learning contributed *less* to overshadowing than after 1-trial conditioning. This result underlines the importance of considering asymmetric-cross generalization as an alternative mechanistic explanation for overshadowing.

Overshadowing was more frequent after 3 than after 1 conditioning trial. As pointed out above, differences in component salience may underlie this increase. However, another learning phenomenon, the "blocking" effect (2), could also be invoked to account for the increase in overshadowing cases (30). From this perspective, components having at the beginning of conditioning different saliencies should gain different associative strengths already after the first conditioning trial. Under these circumstances, conditioned subjects may ignore the weaker component as the stronger one may be necessary and sufficient to predict the outcome of the conditioning experiment. Thus, in subsequent conditioning trials, the stronger component would block learning about the weaker component, thus leading to an enhancement of overshadowing. This kind of within-compound interaction is, however, not supported by our finding that pure elemental component properties account for overshadowing. If a blocking effect were present after 3-trial conditioning, the correlations between overshadowing and component properties would become weaker. Clearly, this was

not the case, thus underlining that within-compound interactions are unnecessary for accounting for overshadowing in the olfactory modality. Moreover, in the olfactory conditioning of proboscis extension reflex in bees, blocking seems to be an inconsistent phenomenon (31, 32; but see 33, 34), thus diminishing the potential impact of this argument in our case.

Our results demonstrate that component properties account for olfactory overshadowing and that within-compound interactions are unnecessary to this end. Honeybees thus treat a binary olfactory mixture as being the sum of its components, a result which is consistent with elemental theories of compound processing (6). Within a binary olfactory mixture, independence between odorants has to be assumed on the basis of our results. This is in contrast to conclusions by other authors (10, 35) who studied compound learning in free-flying bees and who stated that component independency is restricted to inter-modal but not to intra-modal compounds. Moreover, our results also argue against conclusions from overshadowing experiments with odor-trained honeybees (15), which suggested that binary olfactory mixtures are processed in a holistic way (rather than as the sum of their components) when the odor components are similar. In our work, no evidence for holistic mixture processing, attributing to each mixture a unique entity, was found. Such a processing was, however, proposed in other studies on olfactory compound learning by honeybees (36, 37, 38, 39). The main difference between these and our study is that in our case, bees had simply to learn to respond to the compound which was the sole stimulus trained in the overshadowing group while in the other studies, bees had to learn to differentiate a compound stimulus from its components as both had different outcomes in terms of presence or absence of reward. This difference reflects the distinction between absolute and differential conditioning, two conditioning protocols that pose different challenges to the trained animals and that, in the case of bees, result in different learning strategies (40, 41, 42). It may be thus possible that whenever bees are compelled to discriminate between compound and components, they adopt holistic forms of compound processing allowing them to treat the compounds as being different from their components, while they adopt elemental compound processing and learning when trained with a single compound stimulus.

Our study shows that asymmetric cross-generalization between components is a critical parameter that has to be contemplated for the study and prediction of overshadowing. Former analyses of this phenomenon have focused on differences in component salience and acquisition. They specifically compared responses to a component A after A conditioning with responses to the same component after AB conditioning (2, 3). Overshadowing was said

to occur whenever the response to A after AB condition was less than that after A conditioning. This argument can be partial as overshadowing may be based not on such difference but on asymmetric cross-generalization between compound components. We therefore maintain that consideration of this parameter is necessary in further overshadowing experiments. Clearly, such an analysis is only possible if both control groups, A and B, are available for each compound AB conditioned. The question of whether longer conditioning sessions promote a configural binding between compound components and whether animals are still able to identify the components in a compound when the number of components is higher, as shown in spiny lobsters (8), can now be readdressed based on these new considerations.

   Neuronal basis of asymmetric cross-generalization. Asymmetric cross-generalization could be understood if its neural basis could be unravelled. To this end, it may be worth focusing on the olfactory circuit in the honeybee brain, and particularly on the antennal lobe, the primary olfactory centre in the insect brain. Each antennal lobe is constituted of glomeruli, which are the functional units of this structure. Glomeruli constitute convergence sites for olfactory receptors, inhibitory, local interneurons connecting laterally glomeruli, and efferent projection neurons conveying the olfactory message to higher-order brain centers. Optophysiological recording of neural activity at the level of the antennal lobe showed that odors are encoded as specific glomerular activation patterns (43). Experience-induced modifications of these odor representations using different learning paradigms have already been found at the level of the antennal lobes of different insects [honeybee (44, 45), fruit fly (46), moth (47)]. If, after learning two different odorants A and B such modifications are asymmetric such that the glomerular pattern of A becomes more similar to that of B after A training, but the opposite occurs after B training (i.e. glomerular patterns become more dissimilar), then bees would exhibit more generalization from A to B than from B to A. Calcium imaging experiments have shown that inhibition between glomeruli can be asymmetric (48). In our case, glomeruli activated by odor A may inhibit glomeruli coding for odor B, while glomeruli coding for odor B may not inhibit those coding for odor A. Future physiological investigations should focus on the mechanisms of asymmetrical cross-generalization both at the level of the antennal lobe and higher-order brain structures of the olfactory circuit.
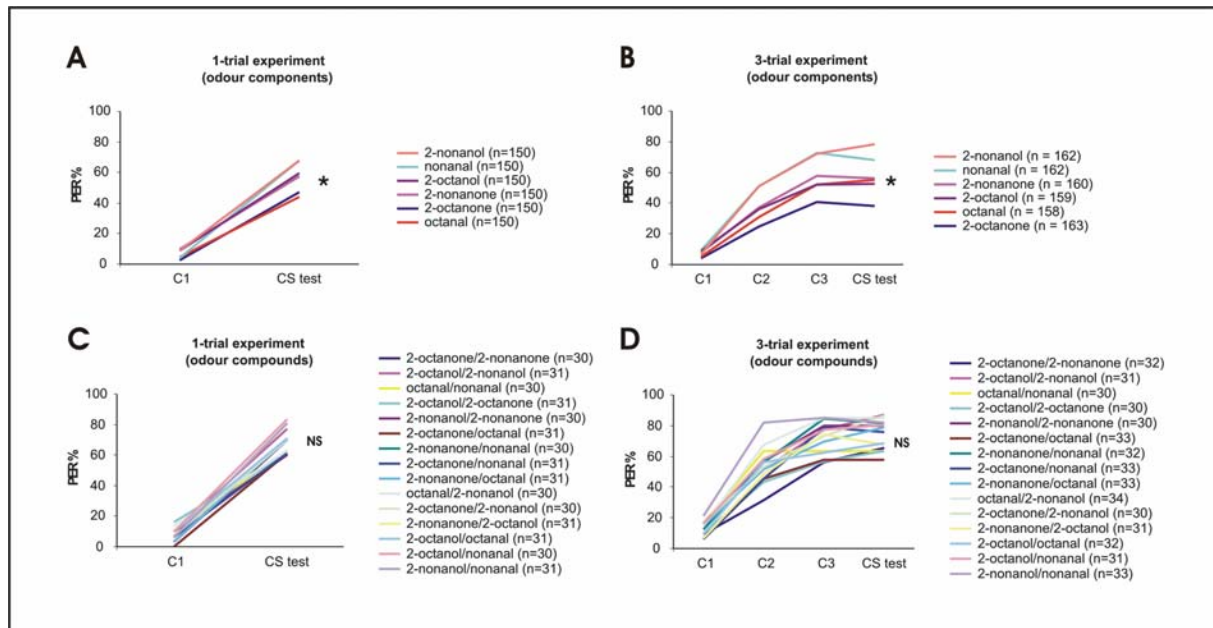
Figure 1:

Learning of single odorants and binary mixtures after 1- and 3- trial conditioning. Curves consist of one training trial (C1) and a test (CS) in the 1-trial experiment and of three training trials (C1, C2 and C3) and a test (CS) in the 3-trial experiment. The graphics show the percentage of proboscis extension reflex (PER %). A star indicates significance while NS non-significant differences between test responses. Sample size (n) is indicated in parentheses for each curve. **A**, **B)** Learning curves for single odorants conditioned in the 1- and 3-trial experiment, respectively. The curve for a given odorant results from pooling the curves obtained in control groups in which this odorant was conditioned. **C**, **D)** Learning curves for odor mixtures conditioned in the 1- and 3-trial experiment, respectively.

1-trial conditioning of 2-octanol, 2-octanone and their binary mixture

A OVS

B Ctrl A

C Ctrl B
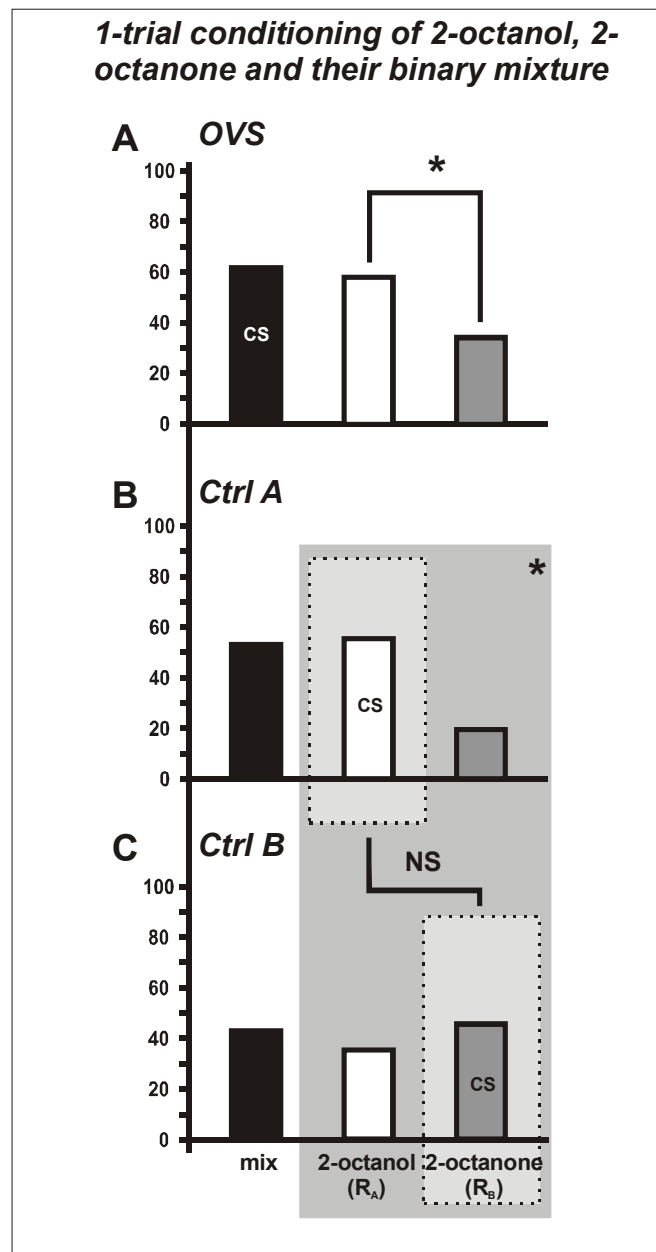
Figure 2:

Example of training to 2-octanol and 2-octanone, and to their binary mixture in a 1-trial conditioning experiment. **A)** Test results of the overshadowing group (*OVS*) after mixture training. **B**, **C)** Test results of the control groups (*Ctrl A: 2-octanol* and *Ctrl B:* 2-octanone) after single odorant training. Black bars represent responses to the mixture, white bars responses to 2-octanol (R_A) and gray bars responses to 2-octanone (R_B). CS indicates responses to the trained stimulus. A star indicates significant and NS non-significant differences between compared test responses. Light gray areas with dotted borders show CS responses which were used for computing differences in component learning between control groups. The dark gray rectangular area surrounding Ctrl A and Ctrl B responses

shows CS and generalization responses, which were used for computing cross-generalization asymmetry.

A Difference in component learning $R_{A(A)} - R_{B(B)}$

B Cross-generalisation asymmetry $\dfrac{R_{B(A)}}{R_{A(A)}} - \dfrac{R_{A(B)}}{R_{B(B)}}$

Custom Chi^2 Test Comparing proportions of generalisation only between bees which responded to the CS (100% learners)

	Ctrl A	Ctrl B
bees responding to the CS but not to the novel odorant	13	4
bees responding to the CS and to the novel odorant	4	10
Chi2 = 7.11, P < 0.025	17 $(R_{A(A)})$	14 $(R_{B(B)})$
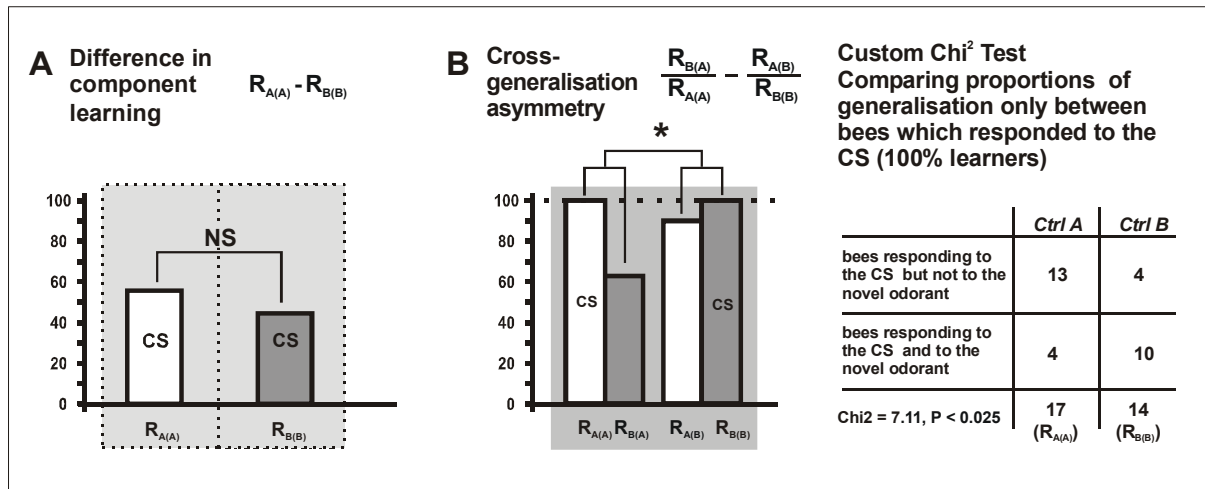
Figure 3:

Example of training to 2-octanol and 2-octanone (control groups) and computation of **A)** differences in component learning and **B)** cross-generalization asymmetry. **A)** Differences in component learning were calculated as the differences in CS responses between both control groups A and B ($R_{A(A)} -$ $R_{B(B)}$) where $R_{A(A)}$: responses to component A after A training in *Ctrl A*; and $R_{B(B)}$: responses to component B after B training in *Ctrl B*. **B)** Cross-generalization asymmetry was calculated as $R_{B(A)}/R_{A(A)} - R_{A(B)}/R_{B(B)}$ where $R_{B(A)}$: generalization responses to B after A training in *Ctrl A*; and $R_{A(B)}$: generalization responses to A after B training in *Ctrl B*. For computation of this parameter, only bees responding to the CS (A in Ctrl A and B in Ctrl B) were used, and their response levels were set to 100%. The 2x2 table used for statistical analysis depicts the number of bees responding to the CS but not to the novel odorant (1st row) and to both, the CS and the novel odorant in *Ctrl A* and *Ctrl B* (2nd row).
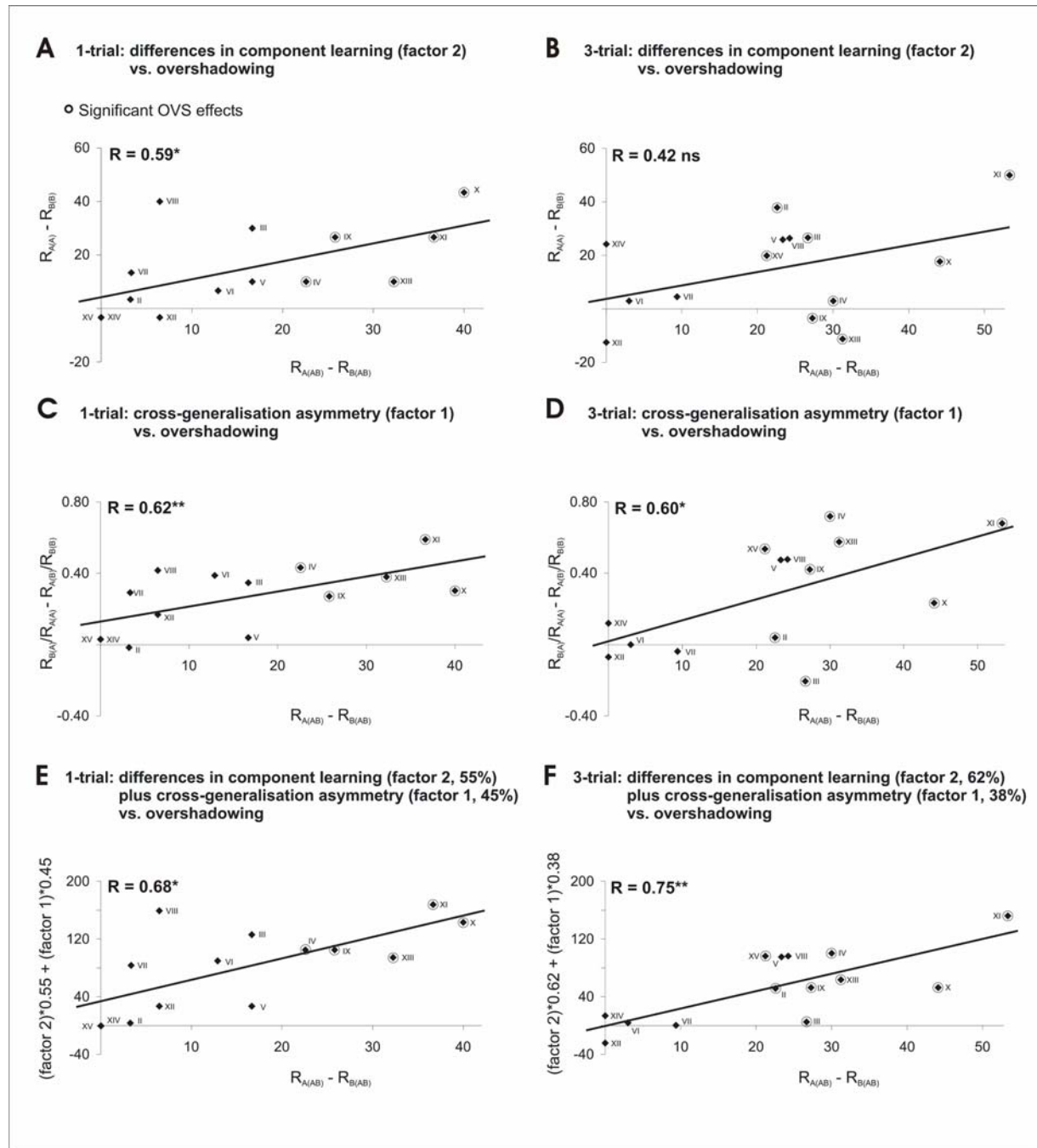
Figure 4:

Correlation analyses for the odor combinations used in our work (I – XIV; see Table 2, supplementary information for more details) between (**A,B**) overshadowing ($R_{A(AB)} - R_{B(AB)}$) and differences in component learning ($R_{A(A)} - R_{B(B)}$), (**C,D**) overshadowing and differences in asymmetric cross-generalization ($R_{B(A)}/R_{A(A)} - R_{A(B)}/R_{B(B)}$) and (**E,F**) overshadowing and a combination of both factors differentially weighted. Left column shows the correlations performed using the data from 1-trial experiment. Right column shows the same correlations using the data from the 3-trial experiment. Encircled diamonds represent odor groups in which a significant overshadowing effect was found.

Odorants	Purity (manufacturer)	VP [mmHg] 25°C	Dilution (in 1ml)		Pheromone (ref. 29)	Floral scents (ref. 50)
			Odorant in µl (1/VP*10)	mineral oil in µl	-	
octanal	100%	1.18	8.50	991.50	-	*Aglaia, Cymbidium, Hydnora, Ophrys, Rebutia, Sulcorebutia*
nonanal	95%	0.37	27.00	973.00	-	*Actaea, Aglaia, Cymbidium, Orchis, Cypripedium, Ophrys, Hydnora, Theobroma, Rebutia, Sulcorebutia*
2-octanone	97%	1.35	7.40	992.60	-	*Ophrys*
2-nonanone	99%	0.62	16.00	984.00	-	*Dendrobium, Ophrys, Rosa*
2-octanol	97,8%	0.24	41.70	958.30	-	*Ophrys*
2-nonanol	99%	0.07	147.90	852.10	*	*Ophrys*

* repels at hive entrance, releases stinging, encourages foraging activity

Table 1:

Characteristics of the 6 odorants used. The odors were listed by functional groups (aldehydes, secondary ketones and secondary alcohols). Purity [gas chromatograph measurements, GC (commercial description of the product)], vapor pressure values (VP), dilution quantities in mineral oil, pheromone characteristics (after ref. 29) and occurrence in floral scents (after ref. 50) of the odorants are given.

A 1-trial experiment

B 3-trial experiment

Overshadowing (McNemar test)

Differences in component learning (x^2 test)

Cross-generalisation asymmetry (x^2 test)

Table 2 - supporting information:

Test results obtained for all odor groups (I – XV) after training in the **A)** 1-trial and **B)** 3-trial experiment. The two tables represent in the first column on the left and in the upper row the odorants which were used in the odor groups. The table at the intersection between two odorants (e.g. Group XII: 2-octanol and 2-nonanone in **A**) gives the performance of the three groups of bees trained with this combination: one trained with the mixture (*mix*), which corresponds to the *OVS* group, and the two others trained with the single odorants, which correspond to the *Ctrl A* and *Ctrl B* groups. The values represent the % of PER responses recorded when these bees were tested after training with either the single odorants or the mixture. Red cells indicate those cases in which a significant overshadowing effect was found in the *OVS group*. Blue cells indicate significant differences between responses to the trained odorants in the *Ctrl A* and the *Ctrl B* groups. Green cells indicate significant cases of asymmetric cross-generalization.

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