General Discussion

To understand how olfactory systems accomplish odour discrimination, it is essential to find out which properties of an odour molecule are relevant to determining the degree of perceived similarity between odour molecules. Odours are multidimensional entities whose chemical structures are known. The question I raised in the first part of this study was whether odours which are similar in chemical structure are also perceived as similar by honeybees (Chapter I). I showed that odour learning and generalization to new odours (a measure for odour similarity) in these animals depended critically on chemical molecule structures, the functional group and the carbon-chain length. Both features turned out to constitute orthogonal inner dimensions of a putative olfactory space of honeybees. Perceptual distances in such a space correlate well with physiological distances determined from optophysiological recordings performed at the level of the primary olfactory centre, the AL (Sachse et al. 1999). Therefore differences in olfactory neural activity for different odours corresponded well to the amount of behavioural generalisation between these odours. Thus the results show that chemical molecule characteristics represent relevant perceivable dimensions to the honeybee, making odour differentiation possible, which finally leads to proper behavioural responses.

Cross-generalization between two odours in some cases was found to be asymmetric. This phenomenon was neither strictly linked to chain length nor to the functional group, but depended on particular odorants. These results were confirmed by the outcome of the experiments in Chapter II and III, where asymmetric cross-generalization was found to predict dominance of an odour in a mixture and therefore could predict overshadowing. Vapour pressure seems to be no essential dimension to asymmetric cross-generalization because the effect occurred in both situations, between vapour pressure equalized (mineral oil dilutions, Chapter II) and un-equalized (pure odours, Chapter I and III) binary odour mixtures, although an altering odour concentration effect is most likely. High generalization responses to novel odours seem to reflect "odour saliency" for honeybees. Previous studies have observed such a phenomenon, but it was mostly related to olfactory compounds with pheromonal value (aggregation pheromone citral [Smith & Menzel 1989, Getz & Smith 1991)] and alarm pheromones 2-heptanone and isoamyl acetate [Sandoz & Menzel 2001]). Here I extended the field in which asymmetric cross-generalization can occur to natural floral odours. Thus generalisation asymmetries could be due to innate or experience-dependent differences in the salience of odours for honeybees, such that more salient odours would induce higher generalisation than less salient odours (for an example see the chemical group of aldehydes

[hexanal, heptanal, and octanal] in Chapter I). The studies mentioned above found asymmetrical cross-generalization in absolute conditioning procedures (like in my work) as well as in differential conditioning experiments. In differential conditioning experiments an odour A+ (paired with a reward) and an odour B- (unrewarded) were presented during the conditioning phase in separated trials. During conditioning phase responses to A+ normally increase while responses to B- decrease after a short increase (generalization effect between odour A and B) back to zero. Nevertheless, the increases to the rewarded stimulus and decrease to the unrewarded one are stimulus-dependent. For example, while the separation of A+ and B- could be very clear, separation in the inverted situation where odour B is rewarded and odour A is not (B+ and A-) could be less clear. Analysis of such a result can show that the separation between B+ and A- is less significant than the separation between A+ and B-, representing an asymmetric cross-differentiation between those odours depending on the rewarded or unrewarded odour. It is not known if the mechanisms of asymmetric cross-generalization and asymmetric cross-differentiation are the same, and there is no experimental proof showing that both phenomena are correlated or not (further experiments and comparisons should easily answer this question). Additionally, asymmetries could be the result of the conditioning procedure. As shown in Chapter II the number of asymmetric cross-generalizations increased, comparing generalization tests after 1-trial and 3-trial conditioning procedures. These results suggest that conditioning can modify odour representation in an asymmetric way. Unfortunately, I did not find any experience-induced modifications of odour representations at the level of AL projection neurons (for a similar result see Peele 2005) for the odour pair 2hexanol - hexanal in Chapter III. Behavioural experiments revealed strong asymmetric cross-generalization, while neuronal activity did not show any propagation of pattern similarity/distances for the two odours related to conditioning, thus indicating that such modifications should be found further upstream to the PNs in more central regions like the mushroom bodies or the lateral protocerebral lobes (keeping in mind the experimental limits discussed in Chapter III). Nevertheless, optical imaging experiments have shown that inhibition between glomeruli may be asymmetric (Sachse 2002). Glomeruli activated by odour A may inhibit glomeruli coding for odour B, while glomeruli coding for odour B might <u>not</u> inhibit those coding for odour A. Such asymmetric connections would give an ideal medium for asymmetric cross-generalization and if, in addition, the strength of lateral inhibitions between glomeruli can be modified by learning - as proposed by Linster & Smith (1997) - then asymmetrical cross-generalisation would come from the fact that inhibitory lateral connections are asymmetrically modified. Further physiological investigations of

conditioning effects on glomerular activation patterns and inhibitory networks in the AL are needed to finally reach a confident result. Such experiments should include a higher number of antennal lobe glomeruli (all, if possible), reveal the nature of inter-glomerular connections and show whether these connections are sensitive or insensitive to experience. Furthermore, investigations should cover a wider range of odours, odour mixtures and odour concentrations to take into account the sensitivity of the network and to gain a complete picture of the AL capacities.

Elemental processing of odour mixtures in overshadowing experiments

After the investigation of single-odour processing in the first part of my work I took the next logical step and continued to study binary odour mixture processes. The overshadowing effect has long attracted the attention of researchers interested in the learning and processing of sensory compounds (Pavlov 1927; Kamin 1968, 1969; Staddon 1983; Gallistel 1990). Overshadowing occurs when a subject trained with a binary compound of two stimuli responds significantly more to one component at the expense of the other. The mechanism of overshadowing is still unclear. Within-mixture, inhibitory interactions between components as well as differential, independent processing of mixture components have been proposed to account for it. Here I clearly showed that component properties revealed after single-component conditioning can predict an overshadowing effect found after mixture conditioning. For the prediction of an overshadowing effect asymmetric cross-generalization seems to constitute a more relevant factor than the difference between conditioning levels of the single mixture odours. At least this result holds true for binary odour mixtures; experiments with more complicated mixtures with three and more odours should reveal more insights into the importance of both factors, asymmetry in cross-generalization and differences in conditioning levels. Although the two factors taken together show significant correlations with an overshadowing effect, asymmetric cross-generalization seems to be more important for the prediction of overshadowing, since the beta values given by multiple regression analysis showed a higher level. Nevertheless, using both factors (weighted differently by multiple regression beta values) correlation with the overshadowing effect resulted in more significant results than were found for the single factor correlations.

The relation and dependency between both single factors is unclear, since correlations between them show insignificance in the 1-trial and the 3-trial conditioning. Although not significant, much higher correlation results for the 1-trial experiment were found than for the 3-trial experiment, indicating that with conditioning the relationship between them seems to

diverge. It is plausible that with more conditioning trials the association between CS and reward becomes stronger, but apparently the conditioning level is not positively linked to the overshadowing effect, since the number of overshadowing cases increased and correlations between this factor and the overshadowing effect decreased significantly with additional conditioning trials. Asymmetric cross-generalization, on the contrary, kept the same correlation level with the overshadowing effect after one as well as after three conditioning trials. Therefore asymmetric cross-generalization seems to be more resistant to a conditioning effect and could be a possible indicator for odour salience (innate or based on previous extended odour experience in the field).

For the analysis of the overshadowing effect in the OVS groups we defined A as the odour to which bees responded more (stronger or dominant odour), and B as the odour to which they responded less (weaker or dominated odour). When comparing A, B and AB responses clear relationships between them were found. After AB conditioning test responses to A (dominant odour) show similar response levels as to AB and after A or B conditioning test responses to AB always showed high generalization levels. Thus if a bee was conditioned to A and tested with A and an added odour B, the bee responds to the mixture just as if it was A (with some scatter). We can think of this in terms of active glomeruli: if the animal is trained to respond to a pattern of active glomeruli it will respond to this pattern even when it occurs only as a sub-pattern of a pattern with more active glomeruli. Or: if one adds an additional odour to a flower, this flower still remains attractive, because the bee can detect that there is still the flower odour in the mixture. This is a very important finding, since it shows that the component A and the component B are accessible for the bee in the mixture AB, and if either A or B were conditioned, than the bee responds to AB. Thus a bee not only accepts an additional component, but also accepts some missing components (i.e. the lack of B in the mixture AB). It does not accept the absence of an odour when it is a very important part of the pattern (i.e. A missing in the mixture AB). Overall, my study shows very well the high degree in robustness of the honeybee olfactory system, which a bee needs to forage in a fluctuating environment, where flower odours can vary in the same flower when it grows in a different type of soil, but maintain a core scent that is common to all of a blossom's variations.

The odour 2-nonanol, which plays an important role as an alarm pheromone for honeybees (Free 1987), was easily learned as a predictor when it was paired with a reward in an appetitive conditioning procedure and showed its high salience as the most dominant odour in the mixtures used in overshadowing experiments. Pheromones not only release specific

behavioural sequences, they can be learned in other contexts, showing the flexibility of associative centres of the honeybee brain. Also, pheromones rarely occur alone; they usually come in mixtures with specific ratios and concentration of the components. Thus, only exact ratios and concentrations of mixture components (with varying specificity between species) should release specific behavioural sequences and act as pheromones (Hansson & Anton 2000).

Here I want to state that overshadowing effects in binary odour mixtures cannot be judged as a within-mixture effect or as an effect based on the intrinsic properties of the single odours without taking into account a test for asymmetric cross-generalization. To my knowledge, such a test was until now never applied in an overshadowing experiment. Until now overshadowing effects were thought to arise from within-mixture interactions when responses to the overshadowed odour after mixture conditioning were lower than they were after a single-odour conditioning of this odour. Obviously, I found such cases, but the overshadowing effect was, nevertheless, correlated to another intrinsic odour property, the amount of cross-generalization between two odours, thus showing that common methods used to investigate overshadowing effects are not sufficient to determine the origin of these effects: from within-mixture effects or from single odour properties. Only if one were to find no difference in response levels between odours after they were conditioned alone and if one found no asymmetric cross-generalization between them, could an overshadowing effect after mixture conditioning arise from within-mixture interactions. Such cases were not found in the binary odour mixtures that I used in this work.

Overshadowing, generalization and conditioning effects in AL odour representations

In a third step I tried to find the neuronal origin of the behavioural effects that I found in the second part of my work. The neuronal correlates for overshadowing and asymmetric cross-generalization are not known; neither are their sites in the olfactory pathway. Also, conditioning-related neuronal modulation of glomerular odour representations is strongly debated (Faber et al. 1999; Sandoz et al. 2003; Yu et al. 2004; Peele 2005). Unfortunately, my experiments in Chapter III on ALs projection neuron activities revealed no neuronal correlates for asymmetric cross-generalization or overshadowing effects, neither in naïve nor in conditioned honeybees. The odours that I used, 2-hexanol and hexanal, have very similar glomerular activation patterns. It is believed that inter-glomerular inhibition is proportional to the similarity in glomerular odour response patterns (Linster et al. 2005) and that modulation of olfactory representation within the AL (Sachse & Galizia 2003) are most likely responsible

for contrast enhancement between similar odour representations (Linster & Cleland 2004). Nevertheless, I found no such contrast enhancement between the two odours or modulation of the odour representation after conditioning. These results are astonishing, since the inhibitory network of the AL (Sachse & Galizia 2002) constitutes an ideal structure for modulation (Linster & Smith 1997). A critical issue may be the precision and resolution of calcium-imaging techniques that I used here to investigate modulation of odour representations. Nothing is known about the amount of neuronal activation modulation that leads to a change of behavioural response in the animal and whether such a correlation is proportional. Thus minor (not detectable for the technique used here) neuronal modulation could result in significant behavioural changes and, therefore, could explain why I did not find neuronal correlates for overshadowing and conditioning-related changes, while I did find an increase in the amount of overshadowing from the behavioural 1- to 3-trial conditioning experiment in Chapter II. A computational model by Linster et al. (2005) for the inhibitory network of the AL suggests response properties rather than spatial neighbouring as responsible for inhibitory connectivity between glomeruli. Thus, connectivity and probable modulation should not be limited to neighbouring glomeruli but could be extended to more distant glomeruli. These analyses were done on only 20 glomeruli situated on the surface of the AL. The experiments I did concentrated on a similar set of glomeruli, but there is a high probability that critical connections and glomeruli important for mixture perception and overshadowing have not been included in the analysis. Of course my work investigated the ALs only as one possible site for neuronal correlates of the studied effects and modulation after conditioning, thus other olfactory centres could be responsible and hold the correlates that I was looking for.

Deisig et al. (2006) found, as I did in Chapter III, that odour mixture representations constitute the added representations for the single odour components of the mixture. Glomerular mixture representations with two, three and four components revealed simple additivity of component representations and, therefore, elemental processing of mixture perception in the AL of the honeybee. Interestingly, calculated arithmetic sums of component responses revealed much higher amplitudes than measured mixture responses, indicating global inhibitory effects. Odours were ranked as strong and weak according to the number of activated glomeruli in their glomerular representation. Comparisons between response patterns for mixtures and components always showed higher similarity between mixture activity patterns and the more salient components. This result corresponds to the outcome of my behavioural overshadowing experiments (Chapter II) in which responses to the dominant component after mixture conditioning revealed similar levels as response levels to the

conditioned mixture. Both results constitute strong indications, on neuronal and behavioural levels, of elemental processing in mixture perception in the honeybee.

In summary, this comprehensive study of olfactory coding and processing of single odours and odour mixtures, combining behavioural and physiological techniques, revealed a putative olfactory space in which perceptual distances correlate well with physiological distances. In this space the molecular odour properties, carbon chain-length and functional group, were found most relevant for odour identification by honeybees. I showed that odour salience is expressed by the amount of generalization between odours and that salience is responsible for dominance of one odour within a binary mixture. Also, I demonstrated neuronal modulation resistance of odour representations after conditioning within the AL. I could confirm elemental processing of binary odour mixtures on the level of glomerular representation and on a behavioural level and, unfortunately, I could not show a neuronal correlate for asymmetric cross-generalization at the physiological level and an overshadowing effect in the AL of the honeybee.

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