Brain-behavioural olfactory asymmetries in Apoidea

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“An ecologist from Mars who visited the Earth would observe that in the United States people drive their cars on the right hand side of the road while in the United Kingdom they drive on the left. He would then perhaps make lots of measurements in an attempt to find ecological correlates to explain the adaptive significance of the difference. In fact, driving on the right and driving on the left may just be equally good alternatives for preventing accidents (Dawkins, 1980).”

From ‘An Introduction to Behavioural Ecology’ (Krebs & Davies)

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Abstract

Lateralization of the nervous system enhances optimization of neural circuitry and parallel processing in individual organisms. Over groups of individuals, brain-behavioural asymmetries might present a direction in the occurrence of the bias (the majority of the individuals showing the same direction at the population level) that has been mathematically demonstrated to be an evolutionarily stable strategy in social groups, thus optimizing coordination and cooperation. The superfamily Apoidea represents a group in which both the study of the appearance of population-level asymmetries and advantages in individual organisms (e.g., in the *A. mellifera* model) can be exploited. Here I described a study on olfactory lateralization in a primitively eusocial species of Apoidea, *B. terrestris*. I reported here that this species showed a direction in the behavioural asymmetry of short-term odour memory, but only individual-level differences in odour detection at the periphery of the nervous system. Moreover, *B. terrestris* showed a morphological difference at the level of the population in the number of structures where olfactory neurons are housed.

In the same subfamily Apoidea, the perennial eusocial honeybee, *A. mellifera,* is a good candidate for assessing neural correlates of odour asymmetries. Lateralization in olfactory memory was reported in this species in the past; here I performed for the first time a study of anatomical and functional asymmetries within the brain, in the first olfactory neuropils, the antennal lobes. I measured a subset of glomeruli in naïve individuals and found symmetrical volumes between the sides for those glomeruli that are mainly activated by odours that show lateralization in behvaiour. Furthermore, I performed single-antenna recall tests, conditioning bees to extend their proboscis (in the so-called PER paradigm) in association with those odours that more strongly activated functional responses in the selected glomerular subset. The behavioural tests showed an odour dependency in the capacity of bees to recall compounds with the two antennae. A broader subset of glomeruli was measured after long-term memory formation and symmetrical volumes were confirmed in all glomerular classes revealing also memory-dependent shrinkage effect. At the functional level, I performed *in vivo* calcium imaging data of the bee antennal lobes. Odor-evoked activity maps were recorded with two-photon microscopy allowing for better spatial and temporal resolution compared to conventional fluorescence microscopy. A first comparison between sides

from wide-field fluorescence microscopy data showed a left/right difference in distance between odour representations and different mixture interactions within each lobe.

In the same social species, *A.mellifera,* I reported the results of experiments measuring social interactions between pairs of bees with only one antenna in use, revealing that animals tested with only their right antenna in use exhibited better social context-dependent behaviours.

Overall, these results provide new evidence for the occurrence of behavioural lateralizations at the population level, and identify some of their possible anatomical and functional correlates. Finally, in relation to previous studies these results tighten the link between the occurrence of population-level asymmetries and their evolution in a social context.

CHAPTER 1 GENERAL INTRODUCTION

Brain asymmetry, or lateralization, is apparent when one side of the brain is structurally different from the other and/or performs a different function (Rogers, 2002). Humans show clear examples of this phenomenon: the left-dominance of the brain during speech production and right-handedness in motor skills, for instance, are the most well known evidence of its occurrence (Toga & Thompson, 2003). How brain asymmetries are widespread among different taxonomic groups has started to be crucial to understand the evolution of this phenomenon and its biological relevance. During the last 40 years hundreds of studies supported the idea that functional brain lateralization is a widespread strategy extended throughout vertebrates (Rogers & Andrew, 2002; Vallortigara *et al.*, 2011; Tommasi, 2009; Ocklenburg and Gunturkun, 2012). Further evidences showed that invertebrate species as well present this trait both in sensory detection and motor performances (reviewed in Frasnelli *et al*., 2012a and in Rogers *et al.*, 2013).

1.1 UNDERSTANDING THE BIOLOGICAL RELEVANCE OF BRAIN-BEHAVIOURAL ASYMMETRIES

1.1.1 Why do brain asymmetries exist?

Considering brain asymmetry, a crucial point is about their evolutionary explanation in terms of benefits given by single-side specializations of the brain.

The general advantage that could have brought to their evolution might be to optimize neural circuitry increasing its efficiency (see Vallortigara and Rogers, 2005; Vallortigara, 2006). Each side being specialized for a specific task, in fact, might avoid useless duplication of functions with a consequent net gain in the neural network (Levy, 1977; see also Vallortigara & Rogers, 2005). In particular, in order to coordinate and establish neural basis for new complex behaviours, the evolution of one more circuit (parallel factor) might have arisen as a more cost-effective strategy compared to increasing brain size (Mutha *et al.*, 2012). Two examples in support of this theory might reveal how this phenomenon is widespread and biologically relevant. In *C. elegans*, only individuals with asymmetric expression of chemoreceptors between sides can perceive specific

class of compounds and discriminate two different stimuli (Wes and Bargman, 2001). In humans, a correlation between the degree of anatomical asymmetry in parieto-frontal connections and speed in visual detection has been demonstrated (Schotten *et al*., 2011).

A better performance among lateralized individuals compared to not lateralized ones has been demonstrated also in other cognitive tasks, such as reorientation in a geometric environment (Sovrano *et al*., 2005), schooling behaviour in fish (Bisazza & Dadda, 2005), thermite fishing in chimpanzees (McGrew *et al*., 1999), and in memory retrieval in flies (Pascual *et al*, 2004), supporting the view that asymmetrical biases are linked to fitness advantages. One of the most outstanding evidence of brain asymmetries and behavioural advantages was published in 2004 on invertebrate species. Pascual and others (2004) were able to prove that morphological asymmetry in the brain was correlated with the formation and retrieval of long-term odour memory in *Drosophila melanogaster*. Individuals having symmetrical brains, conversely, showed only short-term memory recall ability, lacking any long-term memory (Pascual *et al*, 2004).

It has been assumed that lateralization might has arisen as an advantage for processing two tasks at the same time, keeping the two circuits separated, and avoiding functional incompatibility (Vallortigara & Rogers, 2005). It has been shown that having a cerebral asymmetry significantly improve domestic chick efficiency in a double-task test (Rogers *et al.*, 2004). The authors demonstrated that lateralized chicks showed an advantage over non-lateralized ones in discriminating food from pebbles on the ground while performing at the same time detection of (simulated) predators. Additional support for the double-tasking advantage hypothesis comes from a study in marmoset by Piddington and Rogers (2012). These authors demonstrated a correlation between handedness and latency to detect a predator while foraging. Curiously, this advantage disappeared when the groups of lateralized and non-lateralized individuals were compared in predator's detection alone. Comparable results have been reported on a similar task with the fish *G. falcatus*: lateralized line of fish showed significant faster velocity at catching shrimps compared to non-lateralized ones only in the presence of predators (Dadda & Bisazza 2005).

Breaking the symmetrical structure in bilateria might be seen as a further step of evolution in the way of division of labour, i.e. compartimentalization without volume increase. Nevertheless, individual efficiency cannot explain the occurrence of a general

direction of asymmetries in the evolution of a population; asymmetrical individuals, in fact, might share or not the side of their specialization with other asymmetrical individuals within a population (Vallortigara and Rogers 2005).

1.1.2 Why do brain asymmetries persist aligned in a population?

Population level asymmetries and individual level lateralization can be distinguished on the basis of consistence of the asymmetry in a population. Population-level asymmetry is apparent when more than 50% of the individuals in a population are lateralized in the same direction. Individual-level lateralization is apparent when right/left asymmetries are equally common in a population (Rogers & Andrew, 2002). One of the most intriguing open issues in brain and behaviour asymmetries is the evolution of the directional asymmetries within a species. The above-mentioned advantages of being lateralized can in fact account for individual asymmetries but cannot per se explain the persistence of directional asymmetries. Recently, a mathematical model based on game-theory has been put forward suggesting that population-level asymmetries might have arisen as an evolutionarily stable strategy (ESS) in populations where social traits are apparent (Ghirlanda and Vallortigara, 2004). It could be an advantage for individuals belonging to the same group to have the same direction in asymmetry in order to better cooperate among each other in a context where individual fitness strictly depends on what the rest of the group does (Ghirlanda & Vallortigara, 2004; Ghirlanda *et al.*, 2009 and see Vallortigara, 2006 for a review).

According to this hypothesis, therefore, lateralization in behaviour should be more likely to occur in populations with at least some degree of social interactions. Bisazza and others (2000) provided evidence for this studying teleost fish, and showing that the most social and gregarious species of fishes showed population-level biases in the right/left detour behaviour, whereas solitary species showed individual-level lateralization. Moreover, a better performance in schooling behaviour (both in cohesion and alignment) has been demonstrated in lateralized lines of fish over non-lateralized ones, demonstrating a correlation between lateralization and cooperation (Bisazza & Dadda, 2005). Other studies have linked population level asymmetries with high degree of cooperation. For instance, the stronger is the visual lateralization in chicks at the level of the population, the more stable is the group cohesion in terms of social hierarchies (Rogers & Workman 1989). Very recently, Abrams and Panaggio (2012) confirmed the

ESS model evaluating the persistence of handedness polymorphism in relation to the balance between cooperation and competition in human sports.

Numerous examples of correlation between alignment of asymmetry and sociality have been reported also among invertebrates (reviewed in Frasnelli *et al*., 2012a). In the sub-social species *Periplaneta americana*, for instance, a population level bias in turning right in a Y tube olfactometer has been revealed (Cooper *et al.*, 2010). Within the order of Hymenoptera, ants *L. niger* have shown a population bias in choosing the path during foraging (Heuts *et al.*, 2003) and in the highly social ant *F.rufa* a right dominance in the antennal contact during throphallaxis has been demonstrated (Frasnelli *et al*., 2012b). Indeed, the study of invertebrate species might be of special advantage for studying brain-behavioural asymmetries. In particular, species with complex social traits such as Hymenoptera may provide key insights for linking sociality with aligned asymmetries.

1.1.3 The superfamily Apoidea as a model for ESS theory

Comparing strictly related species of bees (Superfam. Apoidea) with different levels of intraspecific social interactions may provide important evidence in order to evaluate the hypothesis that population-level asymmetries are more likely to occur among social species. Anfora *et al*. (2010) recently reported that two different species of bees, *Apis mellifera*, the most sophisticated eusocial species, and *Osmia cornuta*, a solitary species, showed different olfactory asymmetry behaviours. The eusocial species appeared to be lateralized at the population level, whereas the solitary species appeared to be lateralized only at the individual level. The reported study is of particular interest because the authors tested species that are evolutionarily strictly related and whose sociality is a significant trait for mapping their phylogeny (Cardinal & Danforth, 2011). Considering this, it could be even more interesting to investigate population-level asymmetries considering all the tribes of the so-called corbiculate bees, species belonging to the subfamily of Apinae. The subfamily Apinae is particularly interesting to study in relation to evolution of asymmetries linked with social behaviours. It is represented by four tribes whose phylogeny is still controversial (Noll 2002; Cardinal & Danforth, 2011): i) the eusocial Meliponini (stingless bees), ii) the eusocial Apini (honey bees); iii) the primitively eusocial Bombini (bumble bees) and iv) the mostly solitary Euglossini (orchid bees). Furthermore they have the advantage to preserve their social

features during ontogeny (Michener, 2000). It would be crucial to elucidate whether or not these species showed to be lateralized, at which level and in what specific behaviours (whose sociality might or might not be a necessary trait).

Very recently, a population-level lateralization has been revealed in the eusocial Meliponini species (Frasnelli *et al.*, 2011) providing evidence for the mathematical model of evolution on population level asymmetries in Apinae. It would be worthwhile, though, to explore lateralization in all different tribes characterized by completely different social behaviours. Bumblebees, *Bombus terrestris* L. (Hymenoptera: Apidae), for instance, exhibit primitive eusocial behaviour as they have an annual cycle with single queens founding new annual nests. Therefore, they can represent one of the last evolutionary steps in the taxonomic group of Hymenoptera towards the complete development of eusociality (Michener, 1974; Goulson, 2003).

1.2. NEURAL CORRELATES OF LATERALIZED BEHAVIOURS

1.2.1 Are we able to trace behavioural asymmetries back to their morpho-physiological correlates?

A further crucial point in studying behavioural asymmetries is to disentangle their neurophysiological correlates. One of the most challenging issues in this field, in fact, is being able to trace specific maps of on how asymmetries are encoded within neural circuits. Once again, invertebrates with their relatively simple (in terms of cell number and circuitry) and - in some cases - well-known nervous systems are becoming the most suitable models to address this question. The nematode *C. elegans,* has been for years an outstanding model to understand functional asymmetries, their neural architectures and genetic development (Hobert *et al.*, 2002). It has been shown, for instance, that olfactory specializations between sides are specifically triggered by a different expression of G-protein coupled-receptors in two symmetrically head neurons, whose specialization is randomly distributed between left/right neurons in the population. *C. elegans* showed also a functional lateralization in taste receptors between left/right neurons that in this case brings about a lateralized gustatory perception invariant inside a population (Horbert *et al.*, 2002). Recently it has been

shown how this gustatory lateralization is genetically regulated during development throughout a lateralized pattern of gene activations, as well as a difference in neuron volumes between sides (Goldsmith *et al.*, 2010). It has to be noted how the same functional lateralization can be genetically established through differences in the expression of taste receptors and in voltage-dependent signal transductions (deriving from differences in cell size).

Another well-established invertebrate model, *Drosophila melanogaster,* has been recently exploited to study left-right brain behavioural asymmetries, both in odour coding and in olfactory driven behaviours, but the mechanisms and the neural correlates are far from being understood. In 1988, A lateralization in odour coding was demonstrated in the *D. melanogaster* brain for the first time in 1988, by the finding that avoiding odours elicited higher responses on the right antennal lobe while attractive odorants where mainly encoded on the left olfactory neuropile (Rodrigues, 1988). On the other hand, though flies required bilateral olfactory inputs to orient their flight up to an odour plume, the left/right antenna triggered behaviours are not equivalent. Duistermars and colleagues (2009) in fact, showed that odour information coming from the left antenna contributed significantly more than the right one in steering *D. melanogaster* towards an odour (Duistermars *et al*., 2009).

Recently, Jozet-Alves and co-authours were able to demonstrate an interesting correlation in the strength of turning left and increasing volumes of the contralateral optic lobe in single individuals of *Sepia oficinalis* (Jozet-Alves *et al*., 2012).

Other remarkable examples of asymmetries in sensory detection among invertebrates are apparent even though no direct evidence of anatomical differences in the neural system has been provided so far. *Octopus vulgaris* shows a lateralization in the eye preferred to watch a stimulus outside the tank (Byrne *et al.*, 2002). Naïve individuals of the common American cockroach, *Periplaneta americana*, displayed a right-bias in turning behaviour in a Y tube olfactometer that is dependent on the amount of antennal peripheral detection (Cooper *et al.*, 2010).

Nonetheless, though we hypothesised (e.g. as shown in *C. elegans*) how neural asymmetries might optimize sensory detection, the neurophysiological background of functional asymmetries in complex behaviours such as learning and memory is far from being unravelled. Unfortunately, few studies on functional asymmetries were able to show unilateral central pathways involved in lateralized complex behaviours and their mechanisms. The afore-mentioned work by Pascual and colleagues (2004) is one of

these. Long-term memory retrieval was in fact impaired in those organisms that did not exhibit the presence of the asymmetrical body, a spherical structure expressing the neural protein fasciclin II (Pascual *et al*., 2004). Similarly, the terrestrial slug *Limax* showed a typical long-term odour aversion behaviour that is necessarily dependent on the presence of the protocerebrum (PC), the secondary olfactory centre in the slug brain (Kasai *et al.*, 2006); the information stored is lateralized in only the left or right PC with a right/left incidence equally distributed in the population (Matsuo *et al.*, 2010). Moreover, in another mollusc, *Helix licorum*, it has been shown that aversive learning is related with increased levels of MAP-Kinase in specific neurons on the right side of the brain belonging to the aversion motor pathway (Kharachenko *et al.*, 2010). The expression of this protein is significantly linked with learning behaviour and with the withdrawal performance in presence of the conditioned stimulus and was not observed either in the contralateral neurons or in control individuals that did not undergo to paired associative learning (Kharachenko *et al.*, 2010).

The few examples reported here are the only cases where a direct link between functional asymmetries and a central neurophysiological correlation can be demonstrated. Nonetheless, the occurrence of brain asymmetries in invertebrates (reviewed in Frasnelli *et al.*, 2012a) has stressed how it is widespread among distant taxa and has raised the possibility to in dept investigation of its neuro-physiological correlates.

For this reason comparative studies throughout all steps of a functional lateralized sensory pathway in a “well-known” animal model are needed. Honeybee, *Apis mellifera* L. (Hymenoptera: Apidae) might be the key species for addressing this aim.

1.2.2 Honeybee as a model for studying brain-behavioural asymmetries

With less than one million neurons, but exploiting a complex repertoire of behaviours in term of learning and memory processes, honeybee has been considered for decades a model for studying coding, integration and output elaboration along the olfactory and visual pathways (see for review see Menzel, 2001; Giurfa, 2007). Moreover, the accessibility of the nervous system together with the possibility to reproduce pattern of behaviours in laboratory conditions, through appetitive training, showcase their importance and advantages as a model. Bees, in fact, can be trained to extend their proboscis when a specific odour is presented in association to a sugar

reward. This is the so-called proboscis extension reflex paradigm (PER, Bittermann *et al.*, 1983) that can be easily exploited to study classical conditioning. When an odour is presented immediately before a sugar reward, bees can associate the stimuli being able to extend the proboscis when the odour alone is presented in a test phase. Taking advantage of this paradigm, several studies have been conducted in the last decades that have shed light on the critical issue of learning behaviours and their neural, cellular and molecular mechanisms (reviewed in Matsumoto *et al.*, 2012). In honeybee, in fact, multiple training trials separate by a 7-10 minute inter-trial interval lead to a long-last memory trace inducing protein synthesis and long-term memory formation (see Menzel, 2001).

Using the PER paradigm, Letzkus and colleagues (2006) were able to show for the first time a significant right bias in the bee olfactory learning task (extension of the proboscis with odour presentation alone), in particular, between bees trained with only their left antenna and bees conditioned with only their right antenna in use (the other antenna being covered with a silicon compound). The latter group showed a significant better performance in the memory test than the group of bees with only their left antenna free to perceive odours (Letzkus *et al.*, 2006). Similar results revealing a right dominance in memory task were obtained in visual learning experiments training different groups of bees with only their right or only their left eye in use (Letzkus *et al.*, 2008).

Different studies confirmed the presence of odour asymmetries in this species both when bees were trained with different odour compounds and in more natural context, i.e. when bees were trained with both antenna in use and then tested with lateral (left or right) stimulus presentation without covering the antennae (Anfora *et al.*, 2010; Rogers & Vallortigara, 2008, Frasnelli *et al.*, 2010a).

Letzkus *et al.* (2006) investigated where this asymmetry may possibly take place along the olfactory processing route, showing that at the level of the antennae there was a significant difference in the number of *sensilla placodea*, the more abundant structures over the antennae where olfactory receptors neurons are housed. To extend the investigation further, Frasnelli and co-authors analysed all olfactory *sensilla* classes on a larger sample and were able to confirm a consistent morphological asymmetry favouring the right antenna (Frasnelli *et al.*, 2010a). Remarkably, the asymmetry in the morphology at the peripheral level does not *per se* allow answering whether asymmetries are apparent in olfactory detection, i.e. at the peripheral level. It has been

shown, in fact, that in each *sensilla* the number of olfactory receptor neurons is highly variant. Kelber and colleagues demonstrated that neurons in *sensilla placodea* can range from 7 to 23 and they project to different units, the so called glomeruli, in the first olfactory neuropil of the brain, the antennal lobe (AL) (Kelber *et al.* 2006). This non-linear relation between the *sensilla* and the olfactory neurons, suggests that the single *sensillum* type is far from standing for the representation of an odour code. The functional units of the olfactory system, in fact, seem to be the glomeruli of the AL that receive synapses from the olfactory neurons that are activated through the same odorant class along the antenna (Galizia and Szyszka 2008).

In this context, Anfora *et al.* (2010) further demonstrated a difference in the electroanneographic responses between the antennae in bees with unknown experience. They recorded the sum of olfactory receptors' activity from right and left antennae and revealed the right antennae to be more sensitive, i.e. showing higher electrophysiological responses (Anfora *et al.*, 2010). This could explain the right dominance in olfactory recall tests, but it has to be pointed out that, again, the activity of the entire antennal nerve might (or might not) hide an independent asymmetrical pattern in the single functional units, i.e. the first olfactory code that is primarily formed in the antennal lobes. For these reasons a study on differences between the sides of central olfactory neuropils could be worthwhile.

It is also apparent that difference in odour detection cannot be the only source of behavioural asymmetries in honeybees. In 2008, in fact, it has been showed that when bees were conditioned with both antennae in use, long-term olfactory memory (from 6h to 24h after training) was better recalled through the left antenna (Rogers and Vallortigara 2008). Considering the right antenna being specialized for short-term memory task (Rogers and Vallortigara, 2008; Frasnelli *et al.*, 2010a; Anfora *et al.*, 2010) and the left one for long-term odour memory, a shift of memory specialization has been hypothesized. This observed shift in time enhances the needs to investigate where and when olfactory asymmetries take place in the honeybee brain. Untill now, only a study by Biswas *et al.* (2010) has pointed out a difference between sides at the central level in honeybees, in particular, on the expression of *neuroligin 1 (NLG1)*, a protein related with memory formation. 24 hs after amputation of one antenna, in fact, *Neuroligin 1* appeared differently expressed in only left/only right antenna bees, with bees with only their left antenna showing a decreased expression of NLG1 (Biswas *et al.* 2010).

Considering the studies described so far, honeybee might be a promising model both for: a) unravelling central mechanisms of asymmetries and b) looking for advantages they could bring at the individual level and/or at the level of the colony.

As proposed by Rogers and Vallortigara (2008), it might be advantageous for honeybees to have the first incoming information biased toward the right side (primarily involved in short-term olfactory memories), with the left side being specialized for storing long-term olfactory associations. Foraging activities in natural context may represent a good test for this model. Bees show so-called flowerconstancy, the tendency of pollinator to visit the same flower species during foraging trips, even bypassing other valuable nectar sources (Chittka *et al.*, 1999). While the left side might be specialized for this long-term odour association, the right one could be free to perform and establish new associations with new input odorants coming from the complex foraging environment.

1.3 THE NEURAL OLFACTORY PATHWAY IN HONEYBEE (*A. MELLIFERA*)

A simplified model of the olfactory pathway in the honeybee, *Apis mellifera* L. (Hymenoptera: Apidae) is provided in fig.1.1 (from Sandoz 2011).

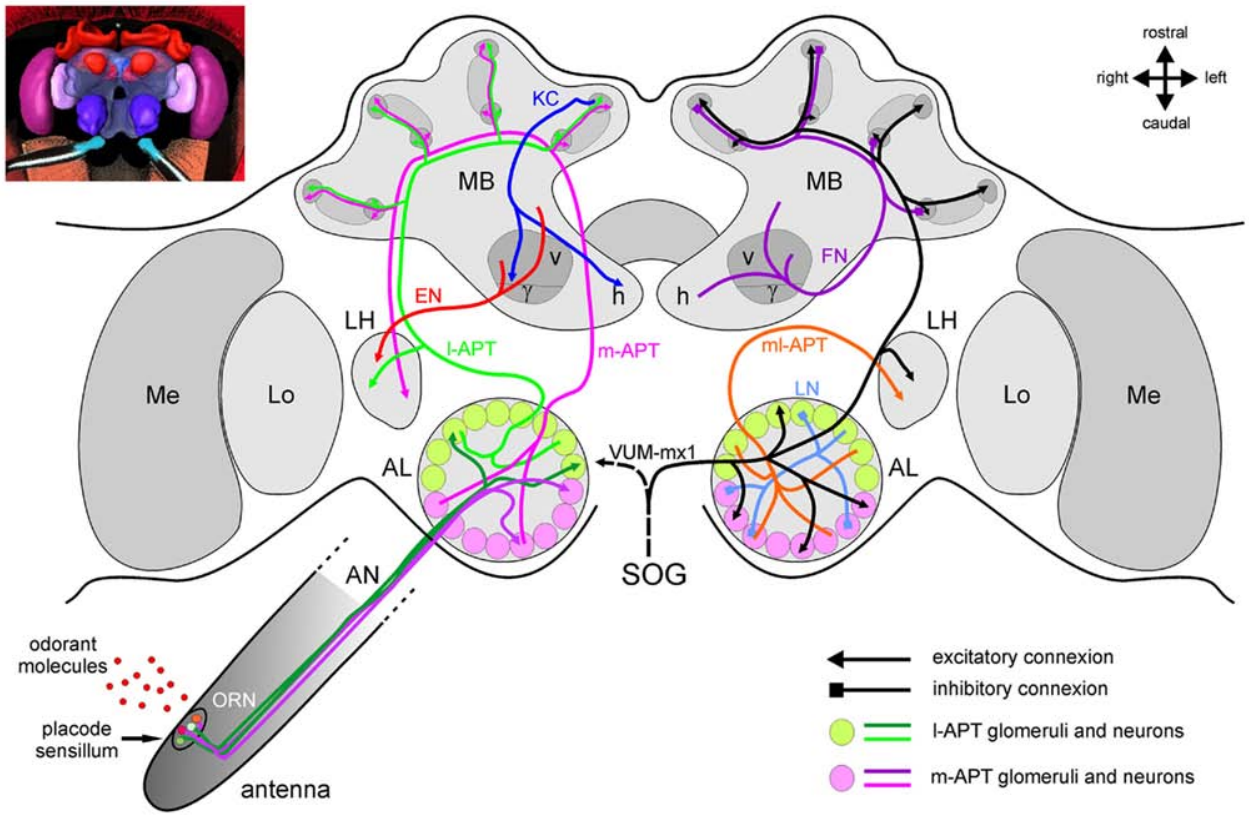

Figure 1.1 The honeybee brain and the olfactory pathway (from Sandoz 2011). See the text for a description of the olfactory pathway

At the very periphery, at the level of the antennae, odour stimuli are detected by olfactory receptor neurons (ORNs) housed in olfactory *sensilla*. Odorant moleculae enter the pores of the *sensilla*, into the *sensilla* emolymph where they may be transported via Olfactory Binding Proteins (OBPs) towards the ORNS dendrites where odorants match their equivalent odour receptor proteins (reviewed in Sandoz 2012). Through chemical transduction the activated ORNs' axons run via the antennal nerves to the first olfactory information processing centres of the insect brain, the antennal lobes (ALs). Right and left AL are bilaterally symmetrical structures formed by ~160 subunits, so called glomeruli, each supposed to be invaded by only one to two specific olfactory receptor classes. Within the AL, the glomeruli are linked by ~4000 local

interneurons (LNs) that are inhibitory neurons with some acting inside mainly one glomerulus (homo-LNs) and others interconnecting most of the glomeruli (hethero-LNs).

Each odour elicits a specific spatio-temporal pattern of glomerular activation coming from the ORNs potentials (Galizia *et al.*, 1999b). In each glomerular neuropil, the peripheral olfactory signal undergoes subsequent modulation due to LNs and to descending centrifugal neurons from the deuterocerebrum. The final output is a species-specific fine tuned combinatorial coding pattern forwarded by ~800 projection neurons (PNs) to higher order brain areas (reviewed in Galizia and Szyszka, 2008). PNs forms two main tracts (*antenno-cerebralis tracts*, APT) the lateral (l-APT) and the medial tract (m-APT) each of them differently innervating the protocerebrum. The l-APT relays its information to the lateral horn and subsequently to the mushroom bodies (MBs, see below), while the m-APT synapses firstly joint the MB and secondly the lateral horn of the protocerebrum.

MBs, the brain structures mainly involved in higher cognitive feats, receive input at the level of the calyces where the Kenyon cells (KCs) dendrites receive PNs output. Differently from the other olfactory centers, in the MBs, the ~800PNs project diverging into ~ 170000 KCs. In the MB olfactory information are integrated with other sensory modalities inputs and different classes of MBs neurons (Extrinsic Neurons, EN) project in several parts of the brain either ipsi- and contro-laterally (Rybak and Menzel, 1993), not showed in the figure. The output regions of the MB are the vertical and horizontal lobes, formed by two collaterals of each KC axon. Within the MBs, feedback neurons (FN) project from the pedunculus and lobes back to the calyces, providing inhibitory feedback to the MB input regions. The figure also presents a single identified octopaminergic neuron, VUM-mx1, which was shown to represent reinforcement during appetitive conditioning. This neuron projects from the suboesophageal ganglion (SOG), where it gets gustatory input from sucrose receptors, to the brain and converges with the olfactory pathway in three areas, the AL, the MB calyces, and the LH.

There are reports of few neurons form the MBs projecting back ipsilaterally to the ALs (Kirschner *et al*., 2006), not showed in the figure.

1.4 Aims of the thesis

The experiments presented in the following chapters aim to contribute to better understand the open-questions on brain and behavioural asymmetries previously discussed, using the superfamily Apoidea as a model.

Firstly, we were interested in defining the extent of behavioural lateralization and in providing new evidence for population-level asymmetries in Apoidea. We consequently investigated olfactory asymmetries in an annual-social species, *Bombus terrestris* (Chapter 2). We wanted to assess whether any olfactory asymmetry was present, if it was expressed at the population-level, and in case of a positive answer, to which extent it was apparent, *i.e.* whether it was strongly correlated with a population bias in odour detection at the level of the antennae. We chose *B.terrestris* also because the only study showing handedness in a natural environment was conducted in species belonging to the *Bombus* gender. In particular, a bias for rotation in the same direction (either clockwise or counter-clockwise) was shown during visits to florets in three of the four species of bumblebees investigated (Kells & Goulson, 2001).

Secondly, we wanted to investigate the neural correlates of behavioural asymmetry in Apoidea species, focusing on the *Apis mellifera* model. We choose the first olfactory integration centre in the honeybee brain, the Antennal Lobe, to look for differences both in morphology and neural coding. As a first morphological approach we wanted to see whether the volumes of glomeruli in naïve bees differ between sides (Chapter 3.1). Considering the significant difference both in number of olfactory *sensilla* and antennal nerve activation discussed above, we hypothesized a difference in volume of the structural unit of the antennal lobe. Moreover, we also performed a first comparison between sides in glomerular volumes after long-term memory (Chapter 3.2). Our starting point was that long-term memory dependent plasticity in volume has been demonstrated at the level of antennal lobes' glomeruli (Hourcade *et al.*, 2009). We wondered whether any difference in glomerular size would have been linked with the olfactory memory biases at the behavioural level (Letzkus *et al.*, 2006; Rogers and Vallortigara, 2008).

Furthermore, we wanted to search for differences in the antennal lobe also at the functional level, i.e. in the coding of glomerular activity. We focused on the AL's final

output, i.e. Projection Neurons' responses to odorants (Chapter 4.1) and we compared them for the first time between sides (Chapter 4.2).

Finally, the last chapter will focus on the possibilities for olfactory asymmetries in bees to be apparent in social interactions between pairs of conspecifics of the same or different hives. We use single-antenna tests to assess the contributions of the left and right antenna during social interactions among dyads of honeybees in an arena (Chapter 5).

CHAPTER 2 Left-right asymmetry of olfaction in bumblebee, *Bombus terrestris*

Summary

Behavioural asymmetries in a population may present a direction (consistency of the same side bias among the majority of the individuals). It has been mathematically shown that this alignment might have evolved in social context. Evidence for this hypothesis has been collected in Hymenoptera: eusocial honeybees showed olfactory lateralization at the population level, whereas solitary mason bees only individual-level olfactory lateralization. In this chapter we investigated the olfactory asymmetry in a primitively eusocial species of Apoidea, *Bombus terrestris*. We studied single side odour memory tasks and compared odour sensitivities of the antennae both at the physiological and morphological level. Data fit interestingly with the theoretical model of the evolution of population-level asymmetries as bumblebees present a directional lateralization at the behavioural level but only individual-level asymmetry at the periphery.

Introduction

As introduced in Chapter 1, research on anatomical and functional side-related specializations of the brain has mainly focused on vertebrates until now (Rogers and Andrew 2002; Rogers *et al*., 2013). Nevertheless, evidence of brain and behavioural lateralization in invertebrates have been reported, opening the field to investigations with a wider comparative view (see for a review Frasnelli *et al.*, 2012a). A seminal work in this respect has been the first demonstration of an olfactory asymmetry of memory retrieval in honeybees (*Apis mellifera* L.) (Letzkus *et al*., 2006). When conditioned using the proboscis extension reflex paradigm (PER) (Bitterman *et al*., 1983) with only one antenna in use, bees showed better learning with their right rather than their left antenna. This evidence raised interesting questions about the occurrence of brain-behavioural lateralization. First, it stressed the existence and the advantage of brain asymmetry in relatively small brains (~960000 neurons) compared to vertebrates. Second, considering that bees are a strongly eusocial species, it drew attention to the sociality as a relevant key to explain the shared direction of bias among individuals in a group (Rogers and Vallortigara 2008; Frasnelli *et al*., 2010a). In natural conditions,

asymmetries may, in fact, occur at the population-level when more than 50% of the individuals are lateralized in the same direction, whereas lateralization at the individual level occurs when most of the individuals are lateralized, but left- and right- bias are equally distributed in the population (Rogers and Andrew, 2002). The advantages for an individual of being bound into directional behavioural asymmetries common to the population has been recently reviewed (Vallortigara and Rogers, 2005). Ghirlanda and Vallortigara (2004) showed, using mathematical game theory, that in a prey-predator ecological context, population-level lateralization might represent an evolutionary stable strategy (ESS) driven by social pressures (i.e. cooperative behaviours) (see chapter 1.1.2). A well-fitting example might be the turning behaviour to escape from a predator in shoaling fish species. In a large number of teleost fishes the shoaling species appear to be lateralized at the population level, while the majority of non-shoaling species are lateralized at the individual level (Bisazza *et al*., 2000).

Studies on closely phylogenetically bee species (Superfam. Apoidea) with different levels of intraspecific social interactions may shed light on the link between population-level asymmetries and cooperative behaviours (see chapter 1.1.3). To date, eusocial honeybees and three species of the eusocial stingless bees have been shown to possess a population-level asymmetry in odour memory recall (Anfora *et al*., 2012; Frasnelli *et al*., 2011). On the other hand, the solitary species *Osmia cornuta* revealed no olfactory asymmetry in odour memory (Anfora *et al*. 2012). Furthermore, when odour detection at the level of the antenna was investigated, honeybees revealed a population-level bias also in olfactory peripheral responses, while solitary bees displayed only individual-level asymmetries in most individuals (Anfora *et al*., 2012). These studies support the ESS theory, but additional studies are required to better explore the link between sociality and population-level asymmetries.

Here we studied an annual social species of Apoidea, *Bombus terrestris* L. (Hymenoptera: Apidae), the bumblebees. This species exhibits primitive eusocial behaviour; its individuals in fact, form relatively smaller colonies with simpler caste-differentation compared to honeybees (Michener 2000; Noll *et al*., 2002). The nests of *B. terrestris* are formed of hundreds of individuals showing size-dependent division of labour, and these bumblebees have an annual cycle with single queens founding new annual nests (Goulson 2003; Beshers and Fewell, 2001). For these reasons, *B. terrestris* might be a crucial species to address how behavioural asymmetries occur within populations in a comparative context. Bumblebee, in fact represent a step

backward from the highly eusocial organization of honeybees, but features all what a population might present to evolve directional lateralization within a group. Therefore, we tested olfactory learning in bumblebees with only one antenna in use, exploiting the PER paradigm. In addition, considering that behavioural lateralization in olfactory learning in honeybees has been associated with peripheral anatomical and electrophysiological asymmetries at the peripheral level in the olfactory neural pathway (Letzkus *et al*., 2006; Anfora *et al*., 2010; Frasnelli *et al*., 2010a), we also measured the number of putative olfactory *sensilla* in the left and the right antennae using scanning electron microscopy, as well as the electrophysiological responses of the two antennae when stimulated by odours behaviourally relevant to bumblebees.

Materials and Methods

Insects

For all the experiments, bumblebee foragers were collected from the same colony of *B. terrestris*, supplied by Bioplanet s.c.a., Cesena, Italy. We used female foragers of similar size (mean body size: 1.7 cm) in order to minimize naturally occurring antennal sensitivity variations (Spaethe *et al*., 2007).

Test compounds

The test synthetic chemicals were two odours behaviorally relevant to bumblebees: isoamylacetate (Sigma-Aldrich, Milano, Italy; >99.7% purity), a component of their pheromone blends and a floral compound, and (-)-linalool (Sigma-Aldrich, >98.5% purity), a common floral compound (Fonta and Masson, 1984; Laloi *et al*., 1999).

Behavioural experiments

Behavioural methods made use of the experimental procedures developed in honeybees (Letzkus *et al*. 2006; Bitterman *et al*., 1983; Rogers and Vallortigara 2008) and bumblebees (Laloi *et al*., 1999) for studying olfactory memory retrieval. After 12 hours of food deprivation, bumblebees were cooled in 75 ml containers until immobilized and secured in metal holders. The insects were randomly assigned to three different groups; with the left (N=10), or the right (N=10) antenna coated with a two-component silicon compound (Silagum-Mono, DMG, Germany), or with both antennae uncoated (N=10). Training started one hour after the antennae had been coated. Each

animal in its holder was in turn placed in front of an exhaust fan and trained using (-)-linalool, plus 1M sucrose solution (reward) as a positive stimulus (10 µl of (-)-linalool dissolved in 3 ml of the sugar solution). The negative stimulus was an unscented saturated NaCl solution. Three learning trials were given every 6 min. During the first trial, a drop of the positive stimulus solution at the end of a 23 gauge needle was held 1 cm above the antennae, and lowered to touch the antennae after 5 s, which led to PER. The bumblebee was then allowed to ingest the drop of (-)-linalool sugar solution as reward. The procedure was immediately repeated with the saline solution, which did not trigger PER, but rather avoidance by an antennae movement away from the negative stimulus. The same procedure was repeated in the two subsequent trials, with PER usually occurring with no need to touch the antennae.

Odour retention was tested 1 hour after the end of training. Both (-)-linalool, dissolved in distilled water at the same concentration used for training, and saturated salt solution were presented holding a drop of these solutions over the bumblebee's antennae for 5 s, being careful not to touch them. Each animal was tested in a total of 10 such paired trials, presenting the stimuli in random order and separated by an inter-trial interval of 60 s. Every time the bumblebee extended the proboscis was recorded. The percentage of correct responses was calculated as number of proboscis extensions in response to the (-)-linalool over the total (-)-linalool presentations per animal (no proboscis extensions to the salt solution occurred).

Data were analyzed by analysis of variance (ANOVA) with antenna in use as a between-subjects factor.

Electroantennography (EAG)

Absolute EAG responses (mV) were recorded from right and left isolated antennae of *B. terrestris* foragers (N=20) with a standard EAG apparatus (Syntech, Hilversum, The Netherlands). Animals were anaesthetized, antennae were cut at the level of the scape and the uppermost part of the antennal tip was removed. The base of the antenna was placed inside a glass micropipette filled with Kaissling saline solution (Bjostad 1998) and the tip put into the recording glass micropipette electrode. The order in which antennae were tested was random, and the animal was kept alive until the both antennas had been recorded.

Test synthetic compounds were isoamylacetate and (-)-linalool. For each compound, 25 µl of five decadic steps hexane solutions (ranging from 10^{-2} to 10^{2} µg/µl) were adsorbed

on 1 cm^2 pieces of filter paper, inserted into individual Pasteur pipettes and put into the constant air flow tube directed to the antenna (50 cm^3/s). Stimuli of 500 ms were presented in ascending order of dosage with 30 s inter-stimuli intervals, using a stimulus controller (CS-55, Syntech). Control pipettes (loaded with 25µl hexane and an empty pipette) were used before and after each series of stimuli. Data were log transformed to account for the heterogeneity of variances and analyzed by analysis of variance (ANOVA) with antenna, scent and dose as within-subject factors.

Scanning Electron Microscopy (SEM)

Bumblebees (N=14) were cooled till immobility and their left and right antennae were cut at the base of the pedicel. The basal segments of each pair of antennae were attached to a circular stub by double-sided conductive tape (TAAB Laboratories Equipment Ltd. Aldermaston, UK). All samples were gold coated to guarantee electrical conductivity and scanned with a XL 30, Field Emission Environmental Scanning Electron Microscope (FEI-Philips, Eindhoven, The Netherlands). Each antenna was imaged from four different viewpoints: ventral (holder at 0°), right (sample tilted at -75°), left (sample tilted at +75°) and dorsal (after removing antenna from stub and turning it upside down). Because of the lack of olfactory *sensilla* on the first two segments of the flagellum of *B. terrestris*, only segments from 3rd to 10th were scanned. Each segment from 3rd to 9th was scanned longitudinally at a magnification of 600 (Figure 2.1a), while a magnification of 800 was used for the 10th segment (apex). For each segment four images were collected according to the different viewpoints.

Both putative olfactory *sensilla* (i.e. *sensilla placodea* (Figure 2.1b-c), *trichodea* type A (Figure 2.1b), *coeloconica* (Figure 2.1c), and *basiconica* (Figure 2.1d)), and non-olfactory *sensilla*, (i.e. *sensilla trichodea* type B (Figure 2.1b), and *ampullacea* (Figure 2.1c)), were identified according to their specific morphological characteristics, as described in Frasnelli *et al*. (2010a) and in Ågren and Halberg (1996). Each type of *sensillum* was then tagged and counted on all acquired images by using image analysis software (UTHSCSA ImageTool Version 3.0). Data were clustered according to the four viewpoints, eight antennal segments, two antennae and six *sensillum* types. Data were analyzed by analysis of variance with antenna, segment and type of *sensilla* as within-subjects factors. Each *sensillum* type was analyzed by analysis of variance (ANOVA) with antenna and segment as within-subjects factors.

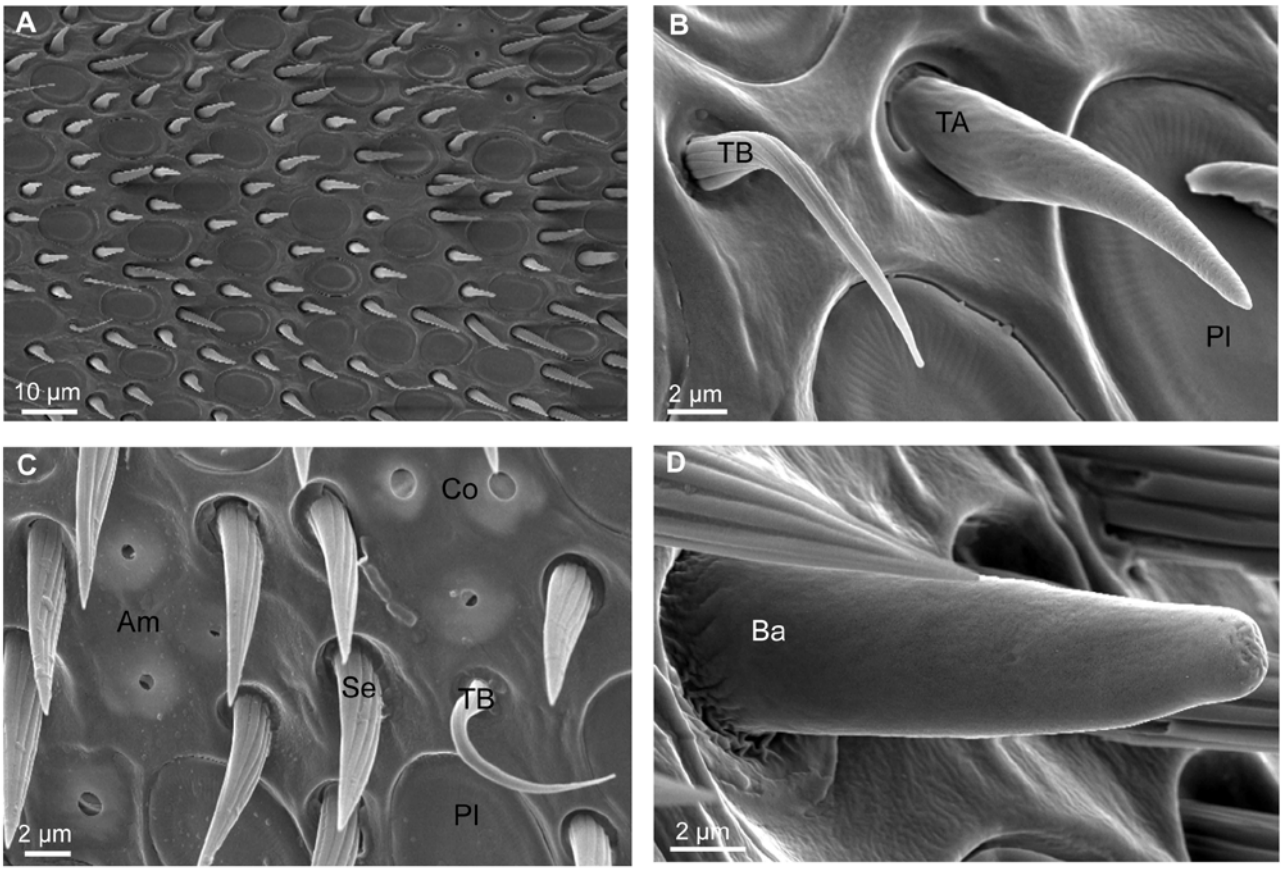

Figure 2.1. Scanning electron micrographs of *Bombus terrestris* foragers. (a) ventral view of a medial segment of the flagellum; (b) details of *sensillum trichodeum* type A, type B and *sensillum placodeum*; (c) details of *sensillum coeloconicum, ampullaceum, trichodeum* type B and *setae*; (d) detail of *sensillum basiconicum*. Am, *sensillum ampullaceum*; Ba, *sensillum basiconicum*; Co, *sensillum coeloconicum*; Pl, *sensillum placodeum*; Se, *seta*; TA, *sensillum trichodeum* type A; TB, *sensillum trichodeum* type B.

Results

Behavioural experiments

In a first series of experiments, we used a behavioural test to determine whether odour memory retention displayed side asymmetries. To this purpose bumblebees were subdivided in 3 groups, enabled to use only their left (Group1, N=10), their right (Group 2, N=10) or both antennae (group 3, N=10) in both training and test conditions. The results, showed a significant asymmetry, as illustrated in Figure 2.2, where the mean and SE of correct responses are shown for each group. The analysis of variance revealed a significant effect of the antenna in use ($F_{2,27}$=80.86, p<0.001). Post hoc comparison using Tukey HSD test revealed a significant difference between bees using their right and their left antenna (p<0.001), between bees using their left antenna and those using both antennae (p<0.001) and also between bees using their right antenna and bees using both antennae (p<0.01).

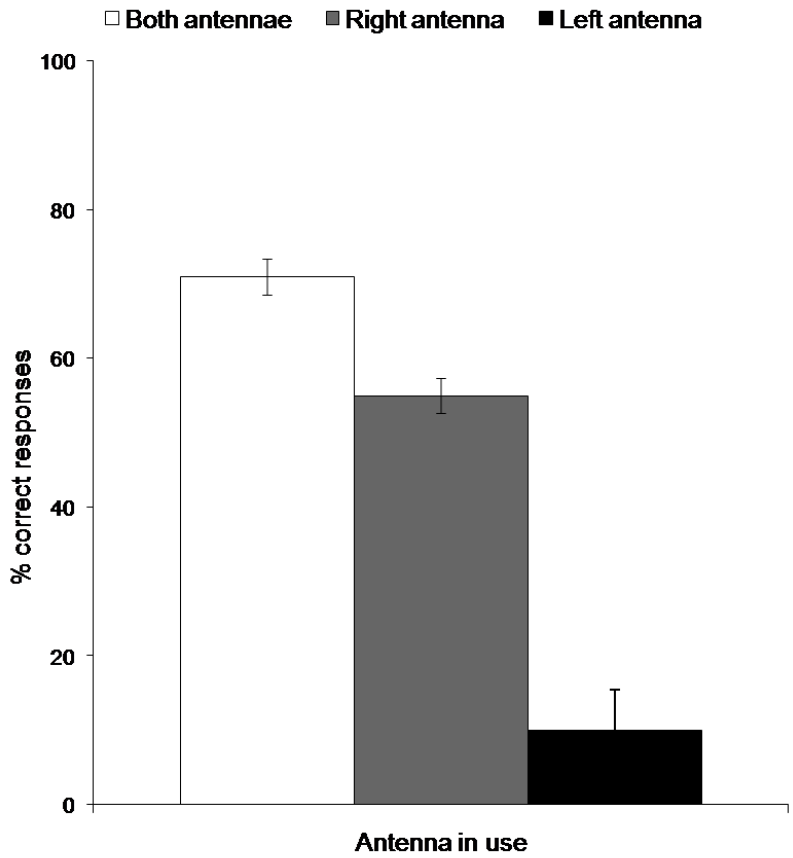

Figure 2.2 Behavioural asymmetry during recall in *Bombus terrestris* foragers, after trained on the proboscis extension reflex. Mean percent correct responses ± SE 1h after (-)-linalool conditioning with both antennae in use (white bars), right antenna in use only (grey bars), or left antenna in use only (black bars).

Electroantennography (EAG):

To measure any difference in the peripheral detection of odorants, we measured the electroantennographical recordings of the left and right antennae in 20 bumblebees. Each antennae was recorded after stimulation of 5 decadic steps of both (-)-linalool and isoamylacetate. The results of electroantennography are shown in Figure 2.3. The EAG responses elicited by the tested odours were not significantly different between the right and the left antenna ($F_{1,19}$=2.72, p=0.12). Though not lateralized at the population level, 12 out of 20 individual bumblebees showed significantly stronger responses (estimated by a two-tailed binomial test, p<0.05) either with the right (9 animals) or the left (3 animals) antenna (two-tailed binomial test, p=0.054). The Anova also revealed a significant increase in EAG responses with increasing doses of both tested odours ($F_{4,16}$=42.52, p<0.001), a significant effect of the type of odours ($F_{1,76}$=107.61, p<0.001), and a significant interaction between type of odours and dose ($F_{4,76}$=20.49, p<0.001).

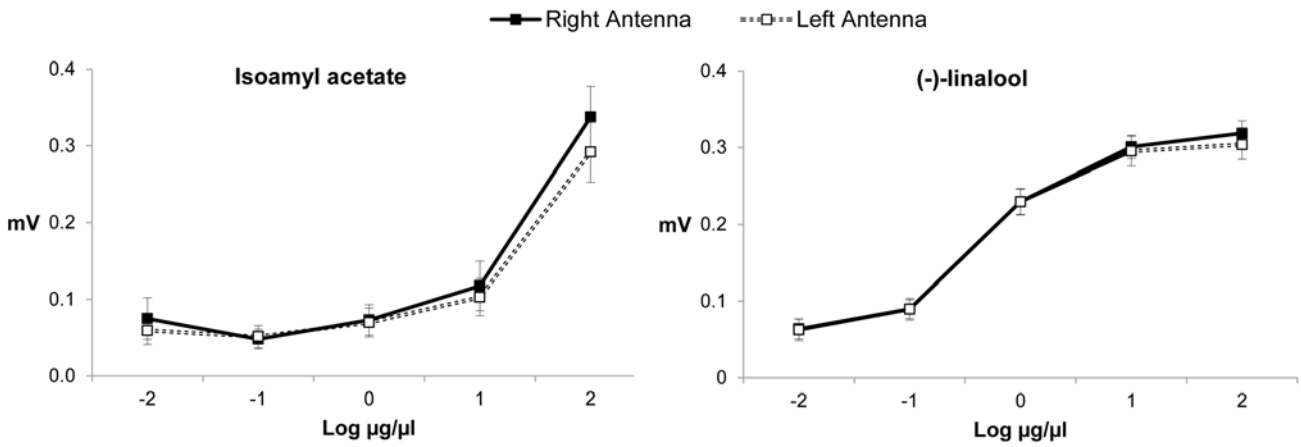

Figure 2.3. Mean EAG ± SE absolute responses (mV) of right (unbroken lines with black squares) and left (dotted lines with empty squares) antenna of *Bombus terrestris* foragers to isoamyl acetate (left) and (-)-linalool (right) at five different doses (Log_{10} µg/µl).

Scanning Electron Microscopy (SEM)

We finally investigated whether any difference in number of olfactory *sensilla* was apparent between left and right antennae. SEM analysis showed that the overall number of *sensilla* analyzed was higher on the right than on the left antenna (Figure 2.4; $F_{1,13}$=22.56, p<0.001). The analysis of variance also revealed significant effect of segment ($F_{7,91}$=43.2, p<0.001), *sensillum* type ($F_{5,65}$=396.40 p<0.001), and antenna per *sensillum* type interaction ($F_{5,65}$=17.89, p<0.001). Separate analyses for each *sensillum* type revealed a significant right antenna dominance in the number of olfactory *sensilla trichodea* type A ($F_{1,13}$=21.26, p<0.001); no significant antenna effects were found in the number of *sensilla basiconica* ($F_{1,13}$=1.47, p=0.247), *sensilla coeloconica* ($F_{1,13}$=3.61, p=0.08), and *sensilla placodea* ($F_{1,13}$=0.972, p=0.342). Analyses of non-olfactory *sensilla* did not reveal any significant difference between right and left antennae in the number of *sensilla trichodea* type B ($F_{1,13}$=3.45, p=0.086) and *sensilla ampullacea* ($F_{1,13}$=0.101, p=0.755).

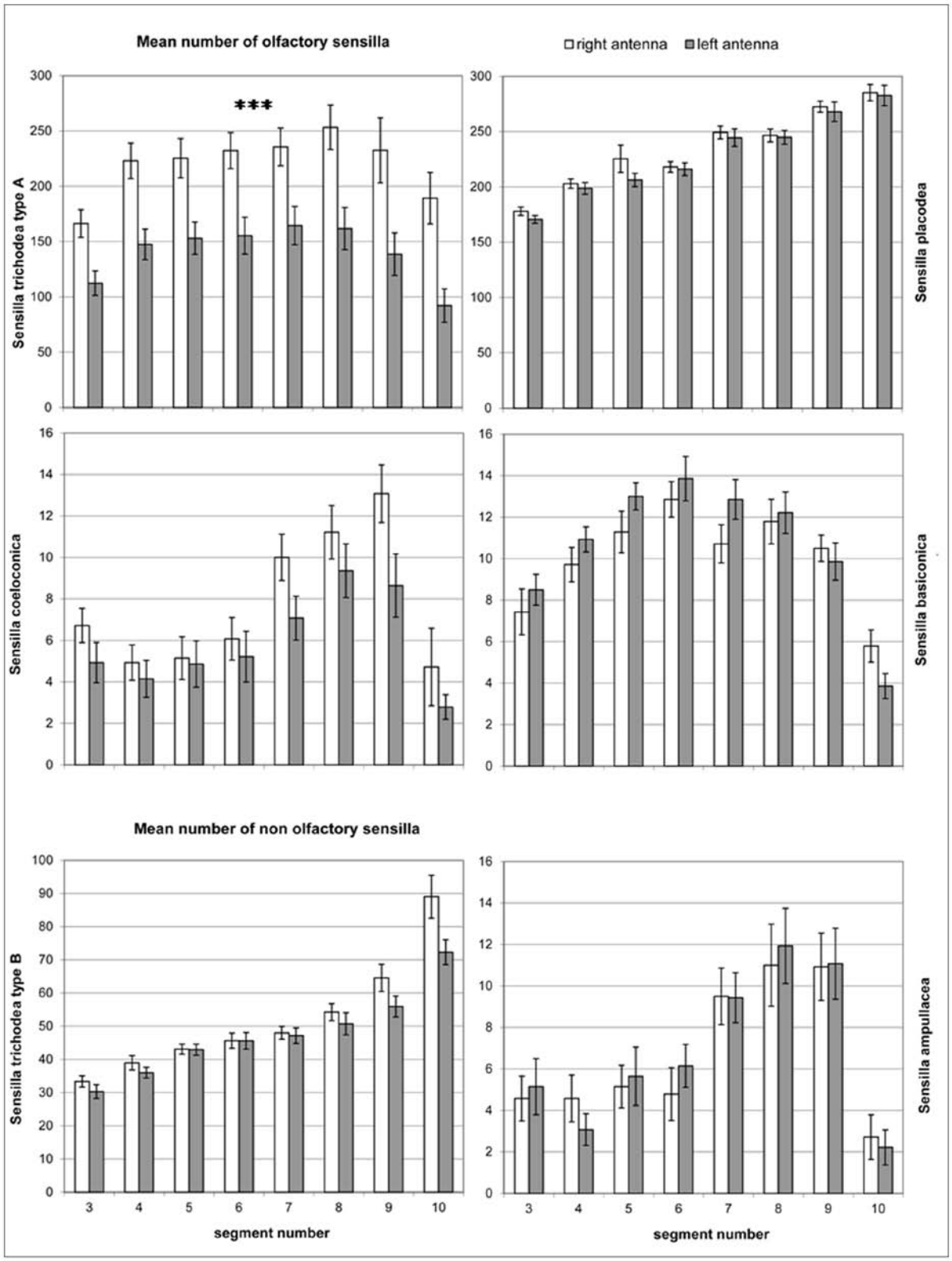

Figure 2.4. Mean number ± SE of *sensilla* for the right antenna (white bars) and for the left antenna (grey bars) of *Bombus terrestris* foragers in function of the segment number. Putative olfactory *sensilla: placodea, trichodea type A, basiconica, coeloconica* (upper graphs). Non-olfactory *sensilla*: *trichodea type B, ampullacea* (lower graphs).

Discussion

The present results extend previous findings on olfactory asymmetries in hymenopteran insects (Letzkus *et al*., 2006; Anfora *et al*., 2010), by showing a right side dominance in short-term recall of olfactory memory in another Apoidea species, *B. terrestris*. Bumblebees conditioned to extend their proboscis (PER) revealed better learning performance when trained with their right rather than their left antenna, with a magnitude comparable to that previously found in *A. mellifera* (Letzkus *et al*., 2006; Anfora *et al*., 2010).

In honeybees, lateralization of olfactory learning is associated with morphological and electrophysiological asymmetries: the number of olfactory *sensilla* and the electroantennographic responses have been shown to be higher in the right than in the left antenna (Letzkus *et al*., 2006; Anfora *et al*., 2010; Frasnelli *et al*., 2010a). In the present study no significant differences in EAG responses between the right and the left antenna of bumblebees were observed (though there was a trend when considering the number of individuals showing significant lateralization). Since electroantennography records the sum of responses of all olfactory receptor neurons housed in the *sensilla* of a single antenna, the results obtained using SEM might explain the difference with the data obtained in honeybees. Only one class of bumblebee olfactory *sensilla*, *trichodea* type A, exhibited an anatomical asymmetry, being more abundant on the surface of the right antenna than on the left one, and a slight tendency emerged for a second class, i.e. *sensilla coeloconica*. On the other hand, *sensilla placodea*, the most common olfactory organs in Apoidea species, did not show any considerable asymmetrical distribution in *B. terrestris*. This can explain why no overall asymmetry was observed in EAG responses in bumblebees.

Other factors may have also contributed to the species difference, i.e. the number of receptor neurons in each *sensillum* category and the number of receptor sites in each olfactory neuron, that could be independently associated with the gain or loss of asymmetry in the mechanisms of peripheral perception. The nematode *Caenorhabditis elegans* provides a striking example of the multiple factors contribution of lateralized odour detection in invertebrates. In this species it has been observed that a symmetrical distribution of olfactory sensory neurons hides an asymmetrical pattern on their surface of the G-protein-coupled olfactory receptors responsible for functional odour lateralization (Hobert *et al*., 2002)

Kells and Goulson (Kells and Goulson, 2001) noticed that three species of bumblebees, *Bombus lapidarius*, *Bombus lucorum,* and *Bombus pascuorum*, showed preferences in the directions of circling when they visited florets arranged in circles around a vertical inflorescence. Interestingly, they did not observe any lateralization in *B. terrestris*. It could be that lateralization in circling is mainly due to antennal asymmetries (and not to higher level mechanisms associated with learning and memory recall). Even in honeybees the evidence suggests that peripheral asymmetries in receptors density and EAG antennal responses could not entirely account for asymmetries in memory recall as evinced from PER responses. Rogers and Vallortigara (Rogers and Vallortigara 2008) showed that 1-2 hour after training using both antennae, recall was possible only when the honeybees used their right antenna, but by 6 hours after training the memory could be recalled better when the left antenna is in use. Clearly, asymmetries in receptor density could not account for this time-dependent shift in lateralization associated with memory consolidation (Frasnelli *et al*., 2010a).

The asymmetry in the olfactory learning behaviour in bumblebees corroborates the hypothesis of a link between high synergistic interactions and direction of lateralized behaviours in a population. Mathematical models of the evolution of population-level asymmetries based on game theory (Ghirlanda and Vallortigara 2004) pointed out that shared directionality in a population might be evolutionary driven by living in a social group, where lateralized individuals have to coordinate their asymmetric behaviours. Ghirlanda *et al*. (2009) extended the mathematical model examining intraspecific interactions, with antagonistic-synergistic behaviours. They showed that the consistency of direction of asymmetries in a population should arise from the most relevant of the two interactions, in term of fitness contribution. Populations with high-rate of synergistic interactions were shown to be more strongly lateralized in the same direction.

Thus, the involvement of these inter-individual interactions could have been a crucial factor for the evolution of lateralization in the olfactory associative learning also in *B. terrestris*. With respect to honeybees, the bumblebees annual society represents a less developed system in individuals exchanging information but the communication between colony members play a key role in the nest. As a matter of fact, although lacking trophallaxis, the recruitment of the bumblebee foragers is driven by the olfactory information flow carried by the incoming bees in the honey pots and the inter-individual contacts significantly increase the success of recruitment (Renner and Nieh, 2008)

In conclusion, the data described here add to increasing evidence that lateralization of the nervous system at the population level is widespread in invertebrate species. Future studies on other species of bumblebees (*Bombus* spp.), or other Apoidea species exhibiting by different social or pre-social behaviours, such as gregarism, may provide additional insights to understand how strategic inter-individual interactions in a population have been powerful forces in the evolution of asymmetries.

CHAPTER 3 Olfactory lateralization and the bee Antennal Lobe: Morphology

Summary

In this chapter we focus on the honeybee brain to search for anatomical correlates of bee's olfactory asymmetries. The antennal lobe (AL) is the first olfactory neuropil into the brain; neural fibers coming from the antennae cluster into functional units within the AL, the glomeruli. We specifically reconstructed, measured and compared between sides the volume of a subset of glomeruli in naïve bees and in bees that underwent a training for long-term odour memory formation. In chapter 3.1 we showed symmetry in glomerular morphology in naïve individuals and, conversely, an odour-dependent behavioural asymmetry when odours activating those specific glomeruli were used. Chapter 3.2 revealed a broader symmetry in different classes of glomeruli even after odour learning with a odour- and glomerular-specific plasticity observed after long-term memory.

3.1 Searching for anatomical correlates of olfactory lateralization in the honeybee antennal lobes:

A morphological and behavioural study

Introduction

The honeybee (*Apis mellifera* L., Hymenoptera: Apidae) has been used for decades as a key model for understanding the neural correlates of sophisticated cognitive skills (see Menzel, 2012 for a review). Olfaction, in particular, represents the most suitable candidate in this species for addressing cognitive issues. The honeybees olfactory pathway is a well-known model of coding, storing, and recalling information (see Chapter 1.3 and see Sandoz, 2011). The first-order olfactory brain areas, the Antennal Lobes (ALs), are located in the deutocerebrum of the honeybee brain. Each AL is ipsi-laterally connected with its antennal nerve thus receiving the neural input from olfactory receptors neurons (ORNs). From ORNs, in fact, olfactory signals are compartmentalized into the highly-organized structures of the ALs, the 165 glomeruli. Here the ORNs' axons synapse with local interneurons, that contribute in shaping the

odour information and transfer this to the ALs output, the Projection Neurons (PNs). Before leaving the first neuropil, each glomerulus processes the signal via a complex network that includes also synaptic integration between LNs, ORNs and PNs (Hansson and Christensen, 1999). The final output (PNs activity), is a fine tuned combinatorial coding pattern and represents a key source for understanding the encoding of olfactory signals. PNs transmit the output of this first olfactory processing towards higher brain areas in the protocerebrum (see Chapter 1.3).

Therefore, within the AL, each glomerulus carries unique information acting as a functional unit in the codification of olfactory information inside the neuropil (Galizia and Menzel, 2001). The size and arrangement of the AL glomeruli is highly species specific so that several anatomical and functional atlases of the honeybee ALs have been created in the past years (Flanagan and Mercer, 1989; Galizia *et al*., 1999a; Galizia *et al*., 1999b; Sachse *et al*., 1999). They allow individual glomerular identification through ALs arrangements and the single glomerular role in the ALs odour-evoked response maps.

Experience-dependent changes in the glomerular volume have been shown to take place during the bee's lifetime and to be highly specific to some glomeruli (Sigg *et al*., 1997; Winnington *et al*., 1996; Brown *et al*., 2002; Brown *et al*., 2004). A posterior glomerulus undergoes, for example, a significant increase in volume with foraging activity (Winnington *et al*., 1996). The glomerular plasticity is both strongly activity- and age- dependent and can be induced manipulating hives such as inducing precocious foraging in younger bees (Winnington *et al*., 1996; Brown *et al*., 2004).

Moreover, a striking volume increase after single odour learning tasks has been recently described (Hourcade *et al*., 2009). Bees, conditioned to extend their proboscis in response to a particular odour stimulus using the proboscis extension reflex paradigm (PER) (Bitterman *et al*., 1983), showed significant volume increase of specific glomeruli linked to the positive performance in learning retention tests 3 days after odour training.

The PER paradigm has been widely used as a behavioural learning method over the years, and it has been recently applied to describe a form of lateralization in honeybee olfactory learning. When conditioned using PER, bees showed better learning with only their right rather than only their left antenna in use (Letzkus *et al*., 2006; Frasnelli *et al*., 2010a; Anfora *et al*., 2010). Morphological analyses found a significantly higher number of olfactory *sensilla* on the right antenna (Letzkus et a., 2006; Frasnelli *et al*., 2010a). However, lateralization of olfactory learning in bees is unlikely to be

explained by morphological asymmetries in the antennae only, for experiments have shown, that after training with both antennae in recall tests 1-2h after conditioning bees performed better with only their right antenna than with only their left antenna in use. But 6h after training the memory had performed a lateral shift, being now better retrieved by the left than by the right antenna (Rogers and Vallortigara, 2008). Possible anatomical asymmetries within the brain have not been systematically investigated so far. A single rough comparison in (Winnington *et al*., 1996) has not shown any symmetry breaking.

In order to improve our understanding of the olfactory lateralization in honeybees, in this study we precisely measured the volume of specific glomeruli in left and right AL. Considered the difference between left and right antenna in number of olfactory *sensilla* and electrophysiological responses we might expect a difference in the morphology of olfactory glomeruli between the sides as well. Considering that we chose a subset of readily identifiable glomeruli, we replicated the learning PER paradigm with odours that evoke activity in these specific glomeruli to make sure about their role in behavioural asymmetries.

Methods

Insects

Italian forager honeybees, *A. mellifera ligustica* Spin., were collected during summer 2009 and 2010 in San Michele all'Adige and Mattarello (Trento, Italy).

Optical Imaging

For the imaging studies of the antennal lobes, bees (*N*=12) have been prepared in accordance to a well-established protocol (Galizia and Vetter, 2004). After chilling until immobility, insects were placed into custom made imaging stages and held in place using soft melting wax (Kerr, Sybron Dental Specialties). To expose the antennal lobes, a window was cut into the cuticle, and glands and trachea were gently removed. The neural sheath was digested by immersion in a 1% solution of Protease Type XIV (Sigma-Aldrich) for 5 min at ~40°C. The bee brain was then stained by bath-application of a 50 µM solution of the membrane-selective dye RH795 (Invitrogen) for 3 h. After rinsing with Ringer's solution, the bees were ready to be imaged.

Antennal lobes were volumetrically imaged without extracting the brain in order to prevent artefacts due to tissue isolation, fixation, and dehydration (Bucher *et al*., 2000). This was realised using two-photon microscopy (Denk *et al*., 1990; Zipfel *et al*., 2003), which offers enhanced penetration depth and a higher axial resolution than conventional fluorescence microscopy and which was recently demonstrated to allow for whole antennal lobe imaging. In this experiment a two-photon microscope (Ultima IV, Prairie Technologies) was used in combination with an ultra-short pulsed laser (Mai Tai Deep See HP, Spectra-Physics) as excitation source, tuned to the wavelength of 1040nm, corresponding to the maximum of the dye's two-photon cross section within our tuning range. The beam was focused on the sample with a water immersion objective (Olympus, 40x, NA=0.8), which provides a field of view of approximately 300µm. The system's resolution was measured to be diffraction limited, resulting in a point spread function of Gaussian width $\sigma_{x,y}$=230nm transversally and σ_z=1.1µm axially. The dye's fluorescence is epicollected by the same objective, separated from the backscattered excitation light with a dichroic beam-splitter, filtered by a 70nm bandpass filter centred at around 525nm (both Chroma Technology), and finally detected by a photomultiplier tube (Hamamatsu Photonics). Average laser powers were around 10mW on the sample.

Volumetric measurements were obtained by collecting stacks of AL image slices by varying the focal plane in steps of 3µm along the antero-posterior axis. The imaging depth was mostly limited by the diffusion depth of the bath-applied dye, and was found to be around 150µm (Fig. 3.1.1).

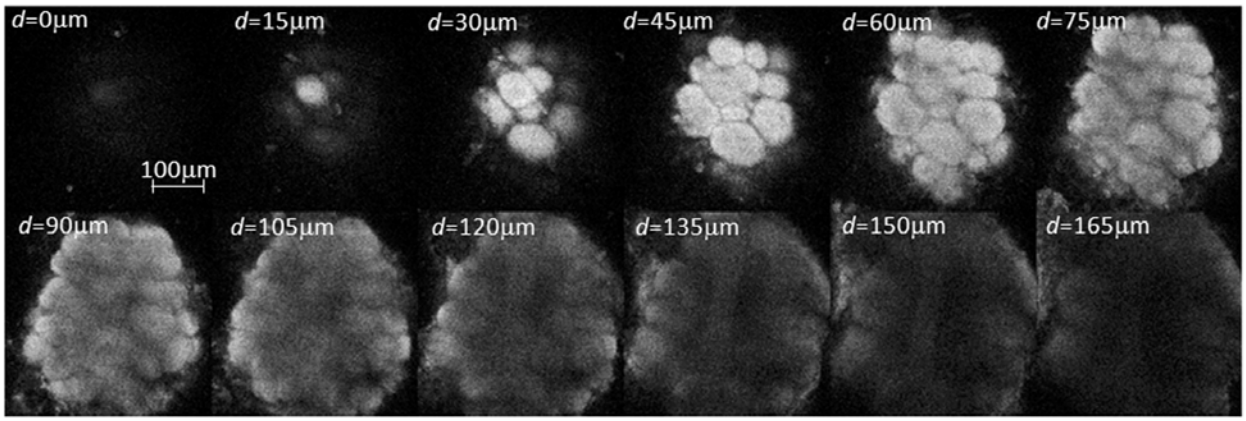

Fig. 3.1.1 Stack of two-photon microscopy images of a left antennal lobe of *Apis mellifera* foragers. The tissue is bath-stained with membrane-selective RH795 dye. The field of view of the used 40x objective is 0.3mm. Total imaging depth is 165µm.

We chose a subset of easily identifiable glomeruli: T1-17, T1-28, T1-33, T1-42 and T1-48. This subset of glomeruli show very diverse activation pattern in functional imaging studies (Galizia *et al*., 1999b; Sachse *et al*., 1999), making them they candidates to magnify a possible odour-dependence bias in volumes between sides. Moreover, T1-48 is strongly linked in response to both linalool and isoamylacetate (Galizia *et al*., 1999b), i.e. those odours that have been showed the right antenna to be more sensitive to (Anfora *et al*., 2010). Finally, we also chose those glomeruli which had shown plastic rearrangements of their volumes after odour experience in previous studies (Hourcade *et al*., 2009). We selected Glomerulus T1-17 showing significant increased volume in both odour conditioning experiments in (Hourcade *et al*., 2009), and T1-33 and T1-48 both significantly increased in one out of the two tests. We added Glomeruli T1-28 and T1-42 to our subset for their opposing odour response maps, the first having a rather broad-band, the second a rather sharp odour response bandwidth (Galizia *et al*., 1999b; Galizia and Menzel 2001) with a strongest response to e.g. 2-octanone, one of the substances used in our behavioural tests.

Image segmentation for the volumetric reconstruction was performed using the software Amira (Visualization Science Group). A semi-automatic protocol was defined, where single glomeruli were traced in the principal planes using the watershedding method “magic wand”. Then the volume images were reconstructed by the program’s wrapping interpolation method (Fig.3.1.2).

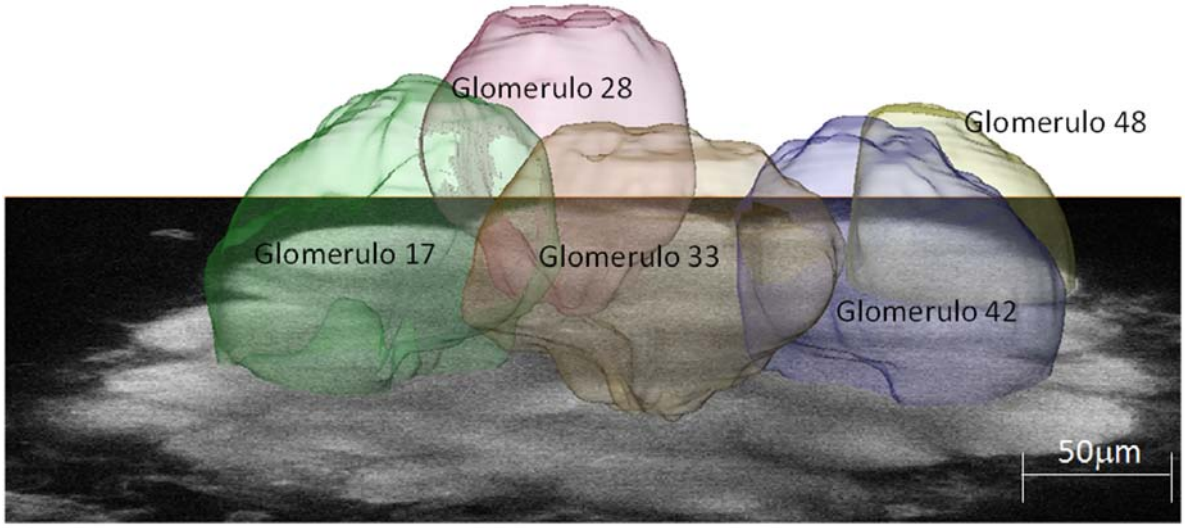

Fig. 3.1.2 Single image of the left antennal lobe of *Apis mellifera* at an imaging depth of approximately 80µm, superimposed with the reconstructed volume images of the analysed glomeruli.

The robustness of this method was checked by slight variation of initial parameters in the reconstruction procedure. If the outcome varied too strongly, the image quality was classified insufficient and the data were discarded. This was the case mostly due to poor dye diffusion or shadows from the remaining trachea. Because the absolute volumes of the single glomeruli were fluctuating among different individuals, the data of left and right side were directly compared for each bee, quantifying the left-right asymmetry by the lateralization index $L=V_R/(V_R+V_L)$, ranging from 0 to 1 around the symmetry point 0.5, where V_R and V_L denote the right and left volume, respectively. Glomerular volumes were analyzed by analysis of variance (ANOVA) with glomerular type and side as within-subjects factors.

Behaviour

Bees were cooled in 150 ml containers until immobilised and secured in holders (Bitterman *et al*., 1983; Rogers and Vallortigara, 2008). They were then assigned randomly to groups for the occlusion of one antenna, and 1h later all bees were trained in the same way. The experiment was carried out on three groups of bee referring to the antenna in use. The bees in one group (*N*=65 honeybees) had their left antenna coated with a silicone compound (Silagum-Mono, DMG), those in the second group (*N*=63) had their right antenna coated, while both antennae of the bees in the third group were left uncoated (*N*=66). Each group of bees was subdivided into three training groups on the basis of the odour compound used as a conditioned stimulus

One hour after the antennae had been coated each bee was placed in front of an exhaust fan and trained using as positive conditioned stimulus (CS+) the odour compounds 1-octanol (*N*=25 both antennae (CTRL); *N*=23 only right antenna in use (RA); *N*=22 only left antenna in use (LA)), 2-octanone (*N*= 19, CTRL; *N*=22, RA; *N*=20, LA) (both Fluka, purity >95%), or (-)-linalool (*N*= 22, CTRL; *N*=18, RA; *N*=23, LA) (Sigma-Aldrich, >98.5% purity) together with 1M sucrose solution as a food reward (unconditioned stimulus, US).

By electing these specific odours, we connected the behavioural tests to our optical study, since these odours stimulate a very diverse response pattern in the measured glomerular subset of the AL (Galizia *et al*., 1999b; Peele *et al*., 2006) and seem therefore good candidates to manifest a possible odour dependence of the test results.

10µl of each odour compound were dissolved in 3ml of the sucrose solution. The negative stimulus was a saturated saline solution (CS-). 3 trials were performed spaced

6min apart. In the first trial a droplet of the CS+/US solution at the end of a 23 gauge needle was held 1cm above the bee's antennae, after 5s the antennae were touched, which led to PER. The bee was then allowed to ingest the drop of the odour-sugar solution. The procedure was repeated with the saline solution, which did not trigger PER but avoidance by moving the antennae away from the droplet. In trial 2 and 3 the procedure of trial 1 was repeated. Usually in trial 3 the CS+/US stimulus triggered PER without the need of touching the antennae.

Retention was tested 1h later by presenting the odour dissolved in distilled water or the saline solution and holding the droplet 1cm from the antennae while moving it slightly without touching the antennae. These CS+ and CS- solutions were presented for 5s. Each bee was tested a total of 10 paired trials, where CS+ and CS- were presented in a random order with 60s between every odour presentation. Every time the bee extended the proboscis was recorded. The percentages of success were scored as extensions of the proboscis to odours and no extension to saturated salt solution (PER(CS+)-PER(CS-))/(# paired trials).

As the measurements did not meet the normality assumption of an ANOVA, data of each odour were analyzed by Kruskal-Wallis one-way analysis of variance, with the 3 different experimental conditions (left antenna/right antenna/both antennae in use) as independent groups. Within the groups of different antennae in use, performance in the various odour tests was analyzed by Kruskal-Wallis one-way analysis of variance with the 3 different odour compounds as independent groups. When a statistically difference was found, a Mann-Whitney *U* test was used for looking at the differences between the means of all groups.

Results

The optical imaging experiment allowed determination of the volume of single glomeruli (Fig. 3.1.3a). Measured absolute volumes were about twice the size of those found in previous studies (Winnington *et al*., 1996; Hourcade *et al*., 2009) where dehydrated samples had been used. This shows the order of magnitude of the shrinkage effects avoided here. From the reconstructed volume images we determined the relative volume asymmetry between left and right AL. For the five glomeruli that have been chosen, the mean values and the standard errors of the lateralization index (*N*=12) are shown in Figure 3b: for T1-17: 0.51±0.01, for T1-28: 0.49±0.02, for T1-33: 0.50±0.01,

for T1-42: 0.50±0.01, and for T1-48: 0.49±0.01. Analysis of variance revealed a significant difference in volume among different glomerular types ($F_{4,44}$=117.27, p<0.001) (Fig. 3.1.3a), but no significant glomerular volume size differences between sides ($F_{1,11}$=0.26, p=0.617). No significant effect of the interaction between glomerular type and side was found ($F_{4,44}$=0.83, p=0.51).

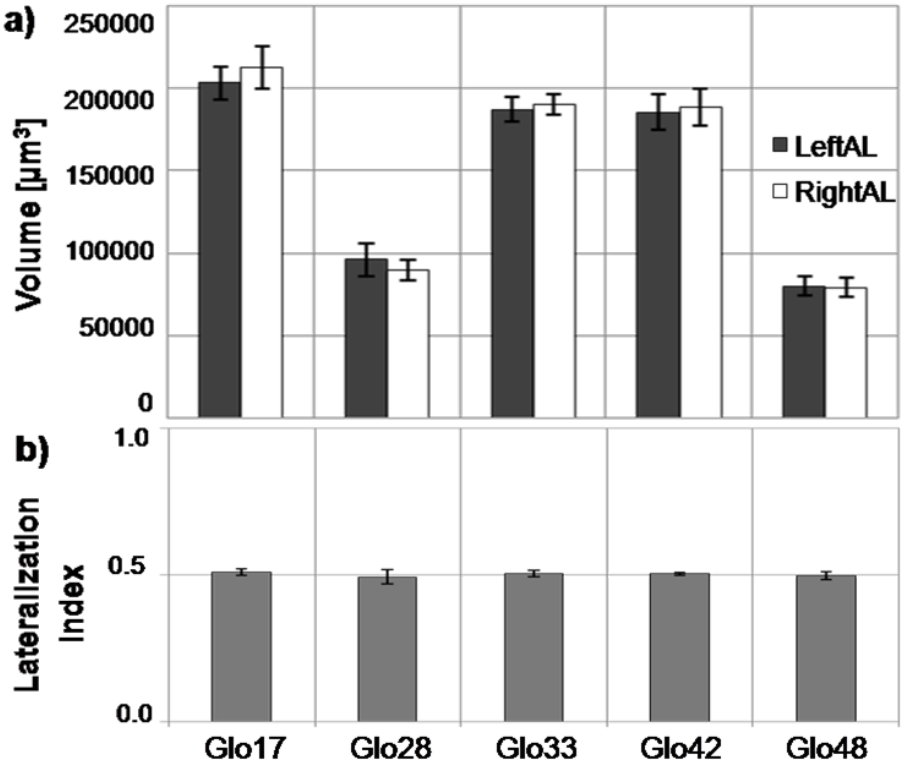

Fig. 3.1.3 Right and left absolute volumes (a) and their correspondent lateralization index (b) of the 5 investigated T1 glomeruli of the honeybee antennal lobes. Mean values are shown together with their standard errors (N=12).

The results of the behavioural tests are shown in Figure 3.1.4. The analysis of variance, using the Kruskal-Wallis test, revealed no significant differences in recall tests among the three groups of bees for 1-octanol (χ^2=1.02, p=0.60, N=70) and 2-octanone (χ^2=0.97, p=0.62, N=61), bees showed no differences in recall test either with only their right antenna in use or with only their left or with both antenna in use. In contrast, bees trained with (-)-linalool showed a significant effect of the antenna in use (χ^2=9.91, p<0.01, N=63). The Mann-Whitney U test revealed a significant difference between bees trained with both antennae in use and individuals with only their left antenna in use (U=128.5, p<0.01, N=45). A similar difference was found comparing bees with their right antenna in use and bees with their left antenna in use (U=112.5, p<0.05, N=41). No

differences were found between bees using both antennae and bees using only their right antenna (U=190.0, p=0.83, N=40). The Kruskal-Wallis test revealed significant odour effect in the performance of bees trained only with the left antenna in use (χ^2=6.17, p<0.05, N=65); Mann-Whitney U test performed inside the left antenna's group, showed significant differences both between (-)-linalool and 1-octanol (U=160.0, p<0.05, N=45) and between (-)-linalool and 2-octanone (U=143.0, p<0.05, N=43). No difference was found between 1-octanol and 2-octanone (U=215.5, p=0.91, N=42). Within the groups with only the right or with both antennae in use the Kruskal-Wallis test revealed no significant differences among different odour compounds: (χ^2=0.41, p=0.81, N=63) and (χ^2=0.20, p=0.90, N=66), respectively.

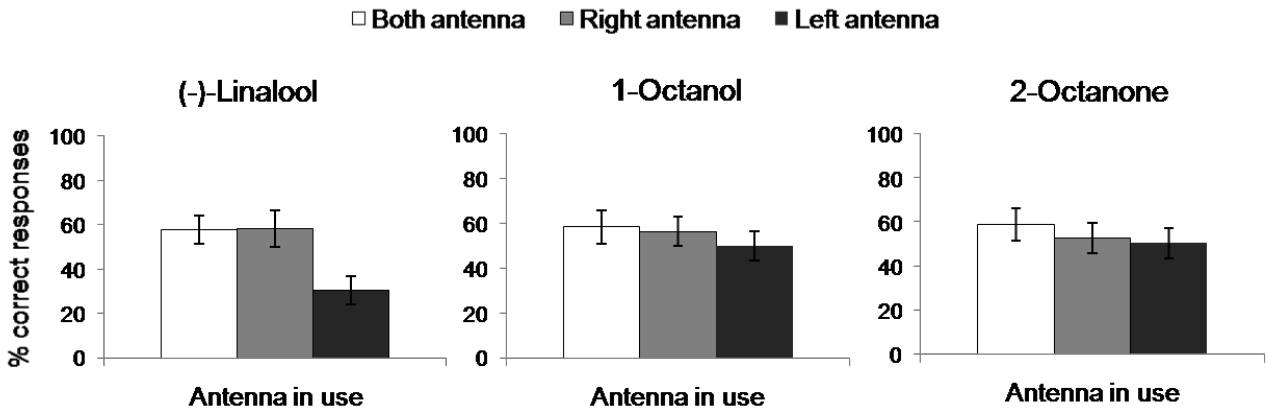

Fig. 3.1.4 Mean ± SEM of correct responses of *Apis mellifera* foragers (N=194) in the recall of olfactory memory 1h after training to associate (-)-linalool, 1-octanol, or 2-octanone with sugar rewards. For each odour tested, honeybees were separated in three groups, with both antennae (white columns), only the right (gray columns), or only the left in use (black columns).

Discussion

The two-photon imaging experiments allowed for the first time precise volume measurements in the honeybee antennal lobe, without extraction and fixation of the brain. This improved the spatial resolution by almost an order of magnitude with respect to the only previously published work comparing the AL morphology in the two brain hemispheres (Winnington *et al*., 1996), based on histological slices at distances of 25 µm. Moreover, it avoided artefacts due to anisometric shrinkage and diffraction-index mismatch (Bucher *et al*., 2000) occurring during fixation and clearing in experiments on extracted brains which are usually used for morphological imaging of the bee brain

(Galizia *et al*., 1999b; Hourcade *et al*., 2009). The measurement accuracy is limited by the decreasing contrast at higher imaging depth, causing problems in defining precisely the border between single glomeruli.

Within this newly established accuracy limits, our study showed that the chosen subset of glomeruli does not differ in volume between the right and left side of the brain in honeybee foragers without controlled experience.

At the behavioural level, previous investigations on olfactory lateralization in bees with unknown experience showed a significant lateralization towards the right antenna (i.e. higher percentage of success in PER 1h after conditioning by the right antenna (Letzkus *et al*., 2006; Frasnelli *et al*., 2010a), as well as in peripheral detection of odorants measured with electroantennography (Anfora *et al*., 2010). Moreover olfactory *sensilla* showed to be higher in number on the right antenna rather than on the left (Frasnelli *et al*., 2010a). This peripheral lateralization, found in bees without controlled experience, did not show a correspondence in the morphology of the measured set of glomeruli or, alternatively, we were not able to detect it under our experimental conditions. Even focusing on the mere anatomical view, it might be likely that the distinctly different number of olfactory *sensilla* between the antennae could not be sufficient by themselves to cause consequent volume impairment in the antennal lobe glomeruli, since the connection between *sensilla* and AL activation pattern is highly nonlinear (Kelber *et al*, 2006).

Moreover, with changing behavioural tasks and foraging experiences antennal lobes and specific glomeruli undergo a significant volume size modification that is age and odour-exposure dependent (Winnington *et al*., 1996; Sigg *et al*., 1997). Such a volume plasticity of first olfactory centres has been also described in other species (Devaud *et al*., 2006; Harvey *et al*., 1984; Sachse *et al*., 2007). For this reason, it is likely that any significant difference in volumes between the ALs in bee foragers with no controlled experience might be hidden under bigger volume fluctuations of specific glomeruli, influenced by both short-term and long-term odour experience. In addition, it has to be noted that at the behavioural level, olfactory asymmetry in bees was demonstrated to be dependent on the time interval between odour conditioning and odour retrieval (Rogers and Vallortigara 2008). In particular, short-term memory (STM) recall tests seem to be better performed with the right rather than with the left antenna, but starting from 6 hours after training, bees showed to better retain odours when they have only their left antenna in use compared with bees with only their right antenna in

use, which might be associated to a laterally displaced long-term memory (LTM). Due to this lateral shift of unilateral memories (or the access to unilateral memories) we might observe a corresponding shift in neuronal modelling both in the ALs and in higher brain centres. In forager bees without controlled experience, the absence of lateralization in the glomerular volume might by due to these competing memory processes in the two sides of the brain occurring on different time scales. Only the amplification of one of these memory processes in controlled conditioning experiments will give a definite answer. Our results serve more as a baseline for future measurements, showing the volumetric symmetry being the long-term steady state.

To connect the behavioural STM recall tests to our optical study of a subset of glomeruli in the AL, we chose to test odour compounds which, in a previous morphological imaging study (Hourcade *et al*., 2009), induced in bees the highest volumetric plasticity in the same glomerular subset and which, in a previous functional imaging studies (Galizia *et al*., 1999b; Galizia and Menzel 2001; Peele *et al*., 2006), showed very diverse activity pattern in these glomeruli. Although no difference in specific glomeruli has been found, this should have help to detect possible odour dependence in the results. One form of odour dependence asymmetry has been demonstrated already in Frasnelli *et al*., (2010b) based on retroactive interference between STM and LTM. Our behavioural experiments add a new aspect to these results, showing that in recall test 1h after conditioning, different type of plant odour volatiles being able to drive asymmetries or not, might depend on the biological relevance of the plant compound.

The elected odour stimuli were 1-octanol, an alcoholic compound, and 2-octanone, a ketone. For both these odours we found no significant differences in STM recall tests between animals trained with only their left antenna and those with only their right antenna in use. We then trained and tested another set of bees with (-)-linalool, a monoterpene floral compound, for which previous studies had shown a clear right asymmetry in odour learning recall tests (Frasnelli *et al*., 2010a) and our results confirmed the significant right-side dominance. These results suggest that STM induced lateralization in bees might be odour-specific, or that lateral shift (Rogers and Vallortigara, 2008) associated with the transition from STM to LTM occurs at different time scales for different types of odours. (-)-linalool is one of the most common derivates of floral scents playing a crucial role as cue for pollinators (Knudsen *et al*., 1993; Knudsen *et al*., 2006). It was demonstrated that honeybees were able to learn

complex odour mixtures through a subset of key odours such as (-)-linalool (Reinhard *et al*., 2010) and that (-)-linalool elicited higher levels of response when it was presented after conditioning to a mixture in respect to others components of the mixture (Laloi *et al*., 2000). Instead, 1-octanol, and 2-octanone are unspecific and ubiquitous volatiles released from the green organs of the plants and thus of minor importance in pollinator plant interaction. So this strikingly different biological relevance of the odour compounds might be a reason for the observed difference in lateralization.

Regarding a possibly different time scale for the lateral shift from STM to LTM, Rogers and Vallortigara (2008) found the balance point between left and right side dominance in memory recall tests to be 3h for lemon as odour stimulus. For the unspecific and ubiquitous volatiles tested here, this point might be shifted to shorter times causing the symmetric behaviour after 1h which was observed by us. So an important next experimental step will be the extension of memory recall tests to different points in time to measure the time-course in the lateralized odour-storage for these compounds.

To deeper address this aspect, conditioning experiments with a large range of different odours are needed. At the same time, the volumetric comparison of left and right antennal lobes has to be performed on conditioned bees (see Chapter 3.2) at distinct times after conditioning to be able to better compare these data with the results from corresponding behavioural experiments (Rogers and Vallortigara, 2008).

3.2

Searching for anatomical correlates of olfactory lateralization in the honeybee antennal lobes:

Do olfactory glomeruli

change in volume between sides after conditioning?

Introduction

As described in Chapter 1.3 and 3.1, along the honeybee olfactory pathway, the Antennal Lobes (AL) are the first crucial relay station, receiving inputs from olfactory neurons and forwarding them to higher brain areas (see Chapter 1.3). This first brain centre along the odour pathway is not simply a transmission unit. Despite bringing odour information (i.e. odour quality, quantity and temporal complexity) to more central areas of the brain (see Christensen and Hansson, 1999), insects ALs are involved in processing complex blend information (see Capurro *et al*., 2012).Furthermore, ALs participate in odour-dependent plasticity, as there is substantial evidence in honeybees that both age- and activity-dependent changes occur at the level of the glomeruli, the AL functional units (Arenas *et al*., 2009; Sandoz *et al*., 2009; Denker *et al*., 2010, Fernandez *et al*., 2009; Rath *et al*., 2011). Even more surprisingly ALs are loci where olfactory learning is supposed to take place in the bee brain (see Hammer and Menzel 1998; Grünbaum & Müller, 1998, Müller, 2000). Not only odour learning (i.e. memory acquisition) occurs in the AL the whole AL has been shown to play a role in odour memory retrieval (Erber *et al*., 1980; Müller 2012). Moreover, changes in the AL functional maps have been documented after odour learning (Faber *et al*., 1999; Fernandez *et al*., 2009; Denker *et al*., 2010; Rath *et al*., 2011) revealing in particular increased distances between odours that enhances discriminative power after associative conditioning (Rath *et al*., 2011). Alongside functional changes, also morphological reshaping has been documented along the entire bee lifespan (Sigg *et al*., 1997; Winnigtion *et al*., 1996) which is strictly activity dependent (Sigg *et al*., 1997; Winnigtion *et al*., 1996). This reshaping causes specific glomeruli to increase in volume after foraging activities, which might or might not be related with an increase in number of synapses (Brown *et al*., 2002; Brown *et al*., 2004). Interestingly, a significant change

in volumes of specific glomeruli has been shown in different species as odour-exposure effect (Sachse *et al*., 2007; Devaud *et al*., 2001; Guerrieri *et al*., 2012; for a review in Diptera and Hymenoptera see Groh & Meinertzhagen, 2010). In honeybees, a striking change in glomerular volumes has been correlated with paired olfactory conditioning (Hourcade *et al*., 2009). Only bees that underwent a paired elemental conditioning revealed an increased glomerular volume that was specific for those glomeruli that were less inhibited within the AL network (Hourcade *et al*., 2009). Taken together, these results shed light on the prominent role in experience-dependent storing and non-linear coding of the environment occurring in the ALs and not only in second-order integration centres. In this context, side-dependent morphological changes after learning have not been explored so far. In naïve bees, the antennal lobe of non-conditioned individuals has been demonstrated to be symmetrical between the right and left side of the bee brain (Winnington *et al*., 1996). However, despite the asymmetrical sensibility for odorants between the two antennae and the different number of the olfactory *sensilla* (Anfora *et al*., 2010; Frasnelli *et al*., 2010a), no differences at the level of glomerular volume have been observed (see Chapter 3.1).

Although no correlates of peripheral lateralization have been found in the AL, one may wonder whether a crucial changing in volume might be related to asymmetrical odour learning. In honeybees, olfactory memory has been showed to be biased between sides (Letzkus *et al*., 2006); in particular, bees showed to recall better a learned odour with their left antenna during a long-term memory task (Rogers and Vallortigara 2008; Frasnelli *et al*., 2010a). Here we wanted to investigate whether honeybees' ALs show any difference in volume-dependent plasticity after long-term odour memory formation between the left and the right side. We perform elemental long-term odour conditioning either with 1–hexanol for which an odour dependent plasticity in volume for specific glomeruli has been demonstrated (Hourcade *et al*., 2009) and with (-)-linalool, a floral odour that has demonstrated to trigger asymmetrical responses both in peripheral detection and memory retrieval (see Chapter 3.1; Anfora *et al*., 2010; Frasnelli *et al*. 2010a).

Methods

Insects

Honeybee foragers (*Apis mellifera* L.) were collected at the entrance of an outdoor hive at about h 9-10 in the morning in Mattarello (Trento, Italy). Experiments were conducted from March to July 2012. Bees were harnessed individually leaving the antennae and the head free to move (Bitterman, 1983), fed with 10µl of 50% sugar solution (w:w) and left for 3 hous in the dark at 25°C and ~70% humidity.

Conditioning Protocol

All the behavioural experiments were recorded with a video-camera (Sony Handycam dcr-sr87). Honeybees were left with both antennae intact and were divided randomly into two groups. One group underwent an elemental conditioning paradigm that allows long term memory formation described in Hourcade *et al*., 2009 (Paired group), the other group underwent the same protocol but the presentation of the odour (Conditioned stimulus, CS) and the sugar reward (Unconditioned Stimulus,US) was unpaired (Unpaired group). In particular, for the Paired group, each bee was placed for 30sec in front of (1 cm) a plexiglass tube with continuous controlled airflow (~30 mL/s) connected with a stimulus controller (CS-55, Syntech). 10 µl of pure odorant were absorbed in a piece of filter paper (1cm^2), inserted in a pasteur pipette and introduced in the main tube directed to the bee. Odour was presented with a stimulus controller (CS-55, Syntech) for 4 s while the total airflow remained constant to avoid mechanical stimuli. 3 sec after odour presentation, a droplet of sugar (50% w:w) at the end of a syringe was presented to the bee antennae and the bee left to feed for 3 sec (1sec overlap). 5 trials of pairing were replicated for the same bee, with an inter-trial interval of 10 min. To balance context-exposition with the bees of the unpaired group (see below) bees were left in front of the airflow for 1 min in between each paired trial (5 mins final interval of the bees in front of the experimental set up). As a control (unpaired group) bees underwent the same paradigm but differently from the paired group, the CS was temporarily unpaired with the US. Therefore, when left in front of the airflow each bee was either presented with the odour (for 4 sec) or with a droplet of sugar solution (for 3 sec) with an inter-trial interval of 5 mins. A total of 10 unpaired trials were performed for each bee. The CS was either 1-hexanol or (-)-linalool, so that within each group (paired/unpaired) two groups of bees were trained in parallel with different odours.

At the end of training bees were left in the dark, 25°C , ~70% humidity. Bees were fed each evening around h18.00 till satiation with 50% sugar solution (w:w).
After three days bees underwent test session: CS odour, a novel odour and an empty pipette (blank) were presented to the bee in a random order. The novel odours were 2-octanone and 1-nonanol for bees trained with (-)-linalool and 1-hexanol respectively. The novel odours were chosen on the basis of the generalization matrix (see Guerrieri *et al*., 2005) in order to control for their perceptual similarity (≤40% between conditioned and novel odorants). At the end of the test bees were controlled for intact PER reflex testing their antennae with a drop of sugar solution (50% w:w). Bees that could discriminate between the two odours and had an intact PER reflex were taken for the staining and imaging procedures. For the latter, heads were cut at the base of the neck and left overnight at 4°C in 4% formaldehyde solution (in PBS, DIAPATH SpA, Italy).

Staining Technique

Brains were carefully dissected in PBS and washed 3x in PBS with 0.25%Triton-X 1(PBS-TX). Brains were then dehydrated and re-hydrated through a graded series of ethanol (40, 50, 60,70, 80, 90, 96 and 99.9%, each for 10min), washed 3x10min in PBS-TX and then incubated for 2 days at 4°C in a solution of 3% α-synapsin (Hybridoma, University of Iowa, Iowa City, IA, USA) and 3% phalloidin Alexa Fluor 546 (Invitrogen) diluted in PBS-TX with 1% normal goat serum (NGS). After washing 3x10min in PBS-TX, brains were incubated overnight at 4°C with a secondary antibody, 3% a-mouse Alexa Fluor 546 (Invitrogen) (diluted in PBS-TX, 1% NGS). After washing in PBS-TX for 3x10min, brains were imaged using a two-photon microscopy.

Imaging

Antennal lobes were imaged using a two-photon microscopy (Ultima IV, Prairie Technologies) in combination with an ultra-short pulsed laser (Mai Tai Deep See HP, Spectra-Physics) as excitation source, tuned to the wavelength of 800 nm. The beam was focused on the sample with a water immersion objective (Olympus, 20x, NA=1). For each brain left and right antennal lobes were acquired in a Z-series made of a collection of 2D images (512x512; 1.41 zoom), 3µm inter-stack interval, imaging depth: ~200-250 µm.

Data Analysis

Behavioural Data

Percentages of responses were calculated and presented in Figure 3.2.2. Behavioural responses were codified as dichotomous data and analyzed using non-parametric statistic. Training performance was assessed among groups on the sum of responses along the trials. At test, bees that were able to correctly solve the task (PER response to the learned odour and not to the novel odour) were compared among groups. Both training and test were first analyzed separately by Kruskal-Wallis one-way analysis of variance, with the 4 different experimental conditions as factors (Unpaired-linalool; Unpaired-hexanol; Paired-linalool; Paired-hexanol) for assessing general differences. If a significant general difference was revealed, a Mann-Whitney test was conducted between each group.

Volume measurements

10 glomeruli of the Tract 1 (T1) were chosen on the basis of activity related responses specific for the odours we used (see Galizia and Menzel, 2001) and on the basis of their changes after learning (Hourcade *et al*., 2009). We also measured for the first time 4 glomeruli of the Tract 3 (T3). Those glomeruli have been described in the atlas but have never been investigated regarding learning or odour activity.

The chosen glomeruli were reconstructed (Fig. 3.2.1) and measured using segmentation protocol in Amira software package (Visualization Sciences Group, see Chapter 3.1). As we were not able to measure in each individual the all 14 glomeruli at both sides, i.e. we had unbalance repeated measurements, we analyzed the whole dataset using a Linear Mixed Model with learning, odour, side, and glomerulo as fixed factors and subjects as a random factor.

The analysis was performed using either SPSS (IBM Statistic 19) or the R software version 2.15.2 (www.r-project.org).

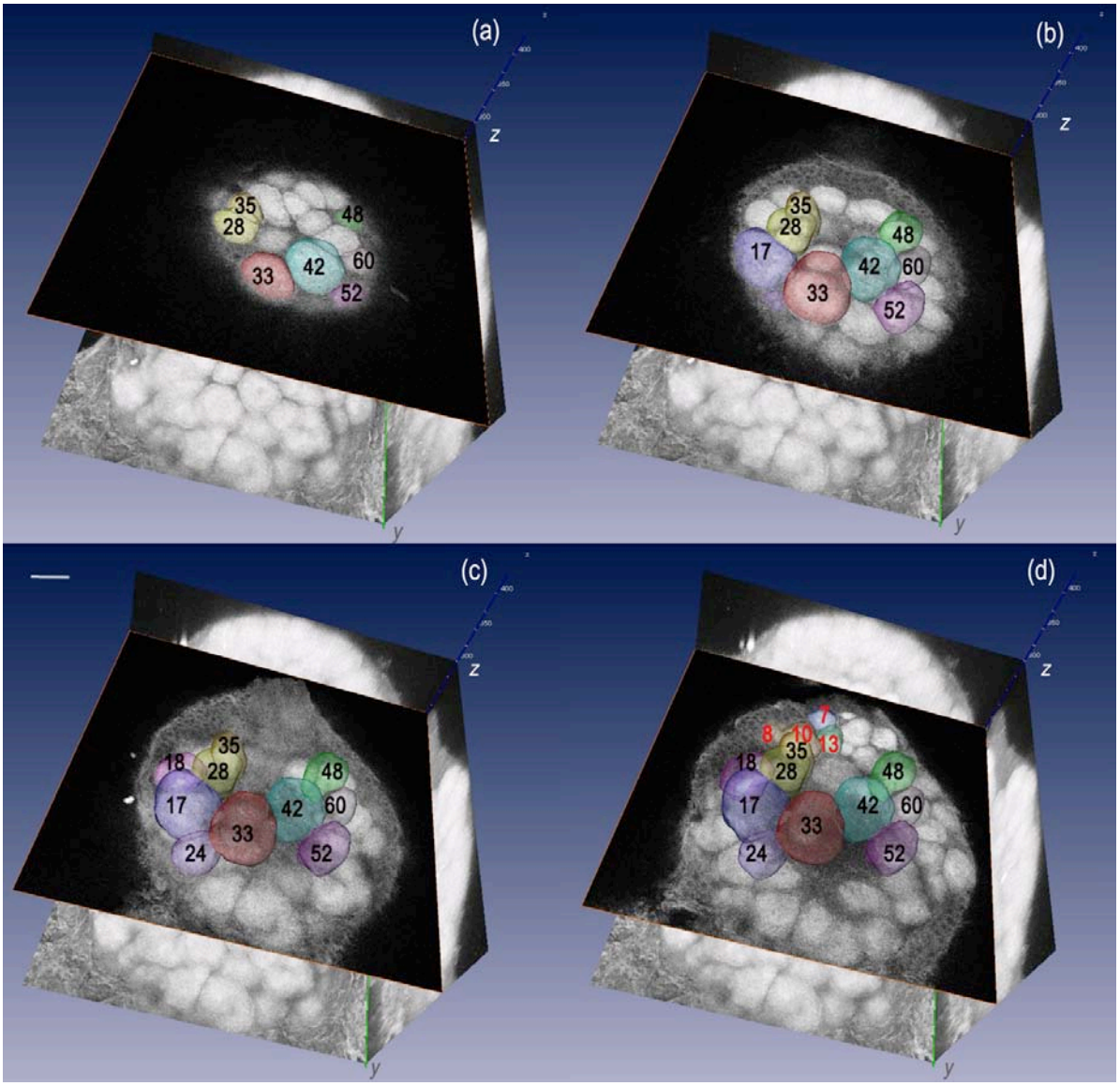

Fig.3.2.1 Transversal slices of a left antennal lobe at different depths along the z-axis. X-Y-Z planes represent the projection views; single slices of the antennal lobe at specific depth are presented (central plane) and the 3D volumes of the 14 measured glomeruli are showed. Black-tagged glomeruli belong to the T1 tract; red ones to the T3. (a) two-dimensional slice and 3D reconstruction of glomeruli at 30 µm depth; (b) 60 µm depth ; (c) 90 µm depth; (d) 120 µm depth. White Bar: 50 µm.

Results

Behavioural Tests

We compared both odour acquisition (training) and odour long-term memory (test: 3 days after olfactory conditioning) in bees trained to associate an odour (either (-)-linalool or 1-hexanol) with a sugar reward (paired-group) and in bees in which the stimuli were unpaired (unpaires-group). We analyzed both the effect of the training and of the odour used.

A difference between the paired and unpaired group was revealed by Kruskall-Wallis test both during training (χ^2=72.81, p<0.001, N=129) and in the test 3 days after training (χ^2=21.90, p<0.0015, N=129) (Fig. 2). Within the training, when paired and unpaired groups were compared, a significant difference was observed for both odours ((-)-linalool group (U=2.00, p<0.001, N=48); 1-hexanol group (U=1.0, p<0.001, N=81). In the test group, the same difference between groups was found again in each class of odour used (linalool: U=123.0, p<0.001, N=48; hexanol: U=269.5, p<0.05, N=81). On the contrary, still within the training groups, no differences were found when comparing odours within unpaired-group individuals (U=100.5, p=0.866, N=30) nor within paired-group individuals (U=919.5, p=0.430, N=99). When comparing test performance for odour, no difference was revealed among bees of the control (unpaired) group exposed to different odours (U=104.5, p=1.000, N=30), while a significant difference was observed in the performance at test after paired learning, with bees trained with linalool performing significantly better than bees trained with hexanol (U=759.5, p<0.05, N=99).

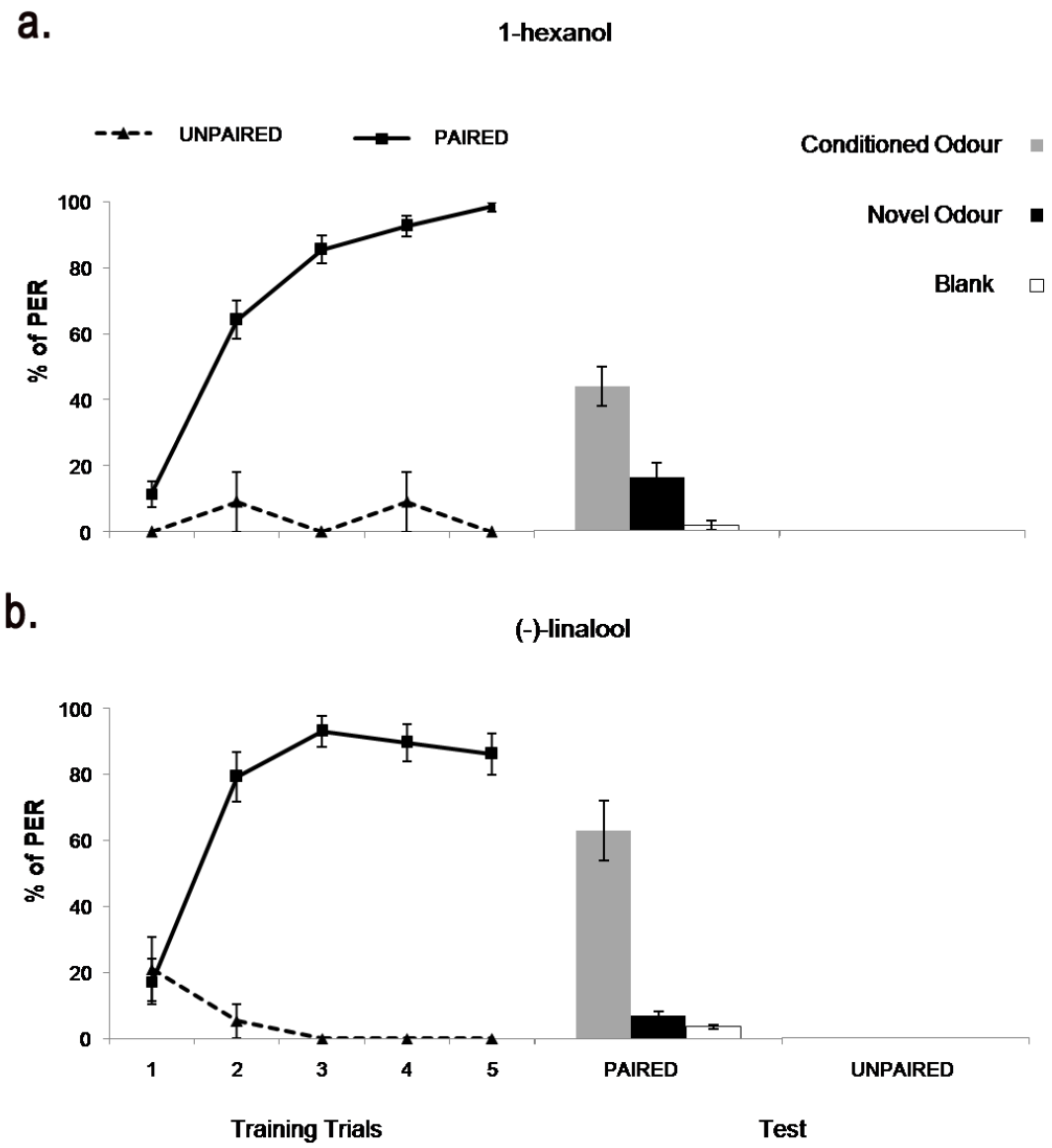

Fig. 3.2.2 On the left: mean percentages of PER responses ±SEM during training are plotted along the 5 trials (dotted lines: unpaired group; unbroken line: paired group). On the right: Mean percentages of odour responses ±SEM to the conditioned odour (grey bar); to the novel odour (black bar) and to the blank pipette (white bar) are presented. (a) Groups of bees trained and tested (either paired or unpaired protocols) with 1-hexanol. (b) Samples trained and tested (unpaired/paired) with (-)-linaloool.

Volumetric analysis

To assess morphological differences after long-term memory and possible morphological asymmetries between sides, we reconstructed and measured the volumes of 14 representative glomeruli of the bee antennal lobe. We were interested in

the morphological plasticity after learning and in any side-biases, both of them with regard to glomerular specificity.

Volumetric measurements of left and right antennal lobes are plotted in Fig. 3.2.3 for unpaired animals and in Fig. 3.2.4 for paired animal, while, in Fig. 3.2.5 paired and unpaired samples are compared (pooling left and right measurements together). We were not interested in a glomerular effect but only an interaction of glomeruli with other factors. The model revealed only a trend for a learning effect in glomerulo T1-33 (t=1.886; p=0.0595); a specific learning effect only when linalool is used in glomerulo T1-24 (t=2.128; p= 0.0336) and a trend for learning effect in glomerulo T1-17 specific for bee trained with 1-hexanol (t=-1.763; p=0.0782) and a specific trend for odour effect in glomerulo T3-8 (t=-1.880; p=0.0604) and in glomerulo T1-48(t= -1.761; p=0.078) (see Fig. 3.2.5).

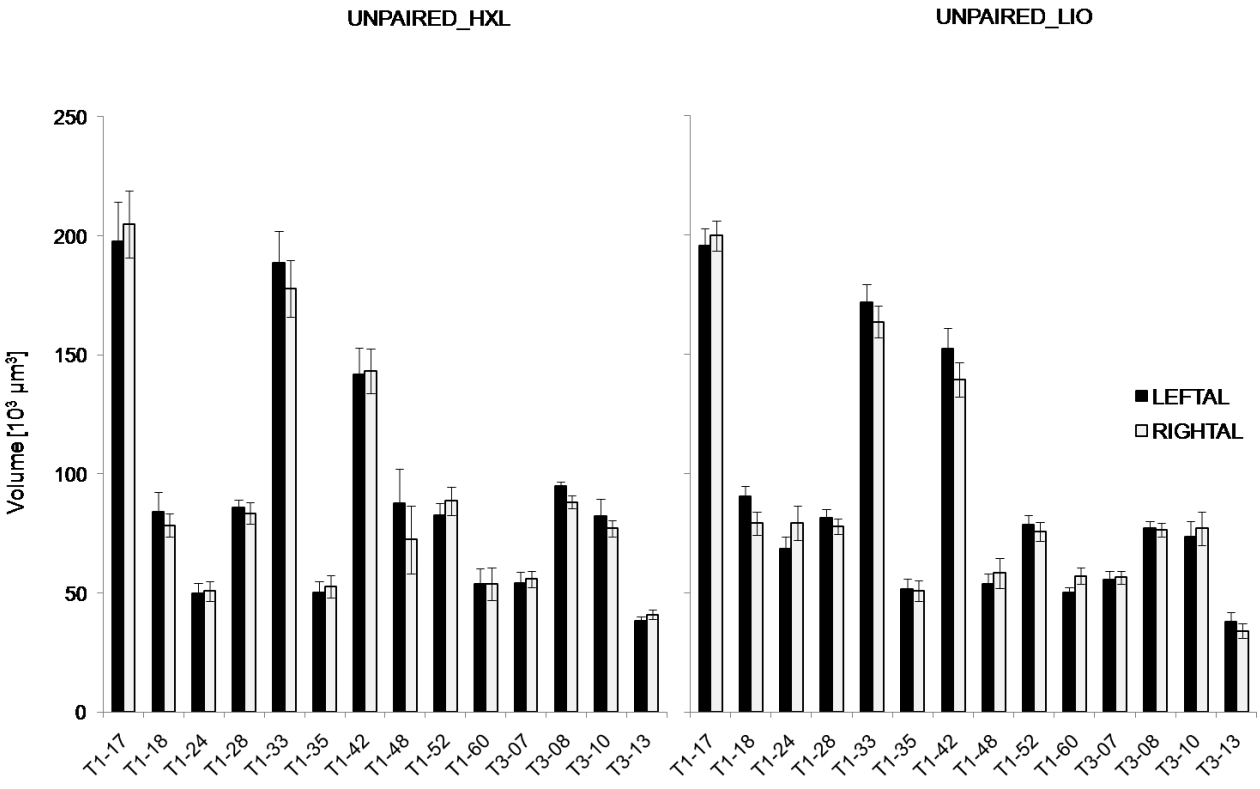

Fig. 3.2.3 Mean volumes ±SEM of the 14 glomeruli measured in the left and right antennal lobes of naïve bees that underwent an unpaired conditioning either with 1-hexanol (N=6, left panel) or (-)-linalool (N=14, right panel). Black and white bars represent the measurements of the glomeruli belonging to the left and right antennal lobe, respectively. Each glomerulo is labelled by Tract and number (Tract 1: T1; Tract 3: T3) on the basis of Galizia 1999b.

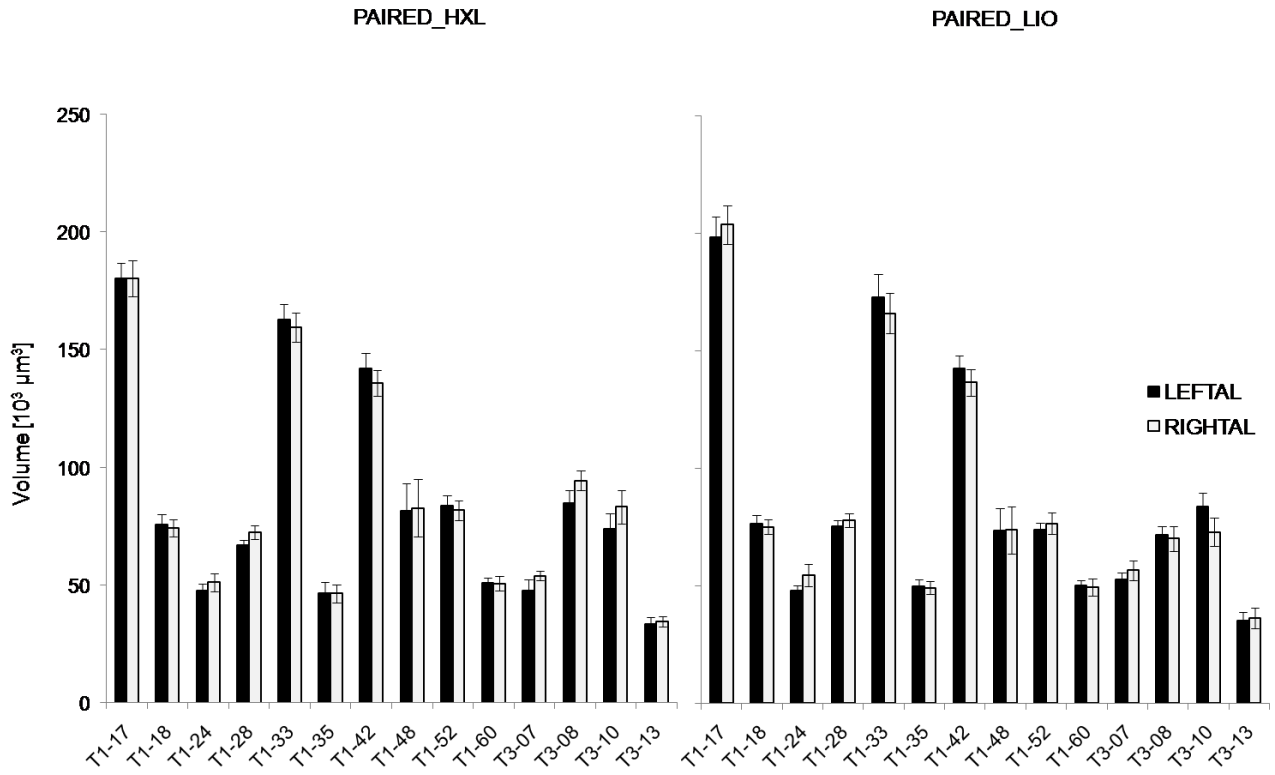

Fig. 3.2.4 Mean volumes ±SEM of the 14 glomeruli measured in the left and right antennal lobes of bees that underwent a paired conditioning either with 1-hexanol (left panel; *N*=13) or (-)linalool (right panel, *N*=14). Black and white bars represent the measures of the glomeruli belonging to the left and right antennal lobe, respectively. For each glomerulo a number indicating tract is reported (Tract 1: T1; Tract 3: T3) with the identity number of each glomerulo on the basis of Galizia 1999b.

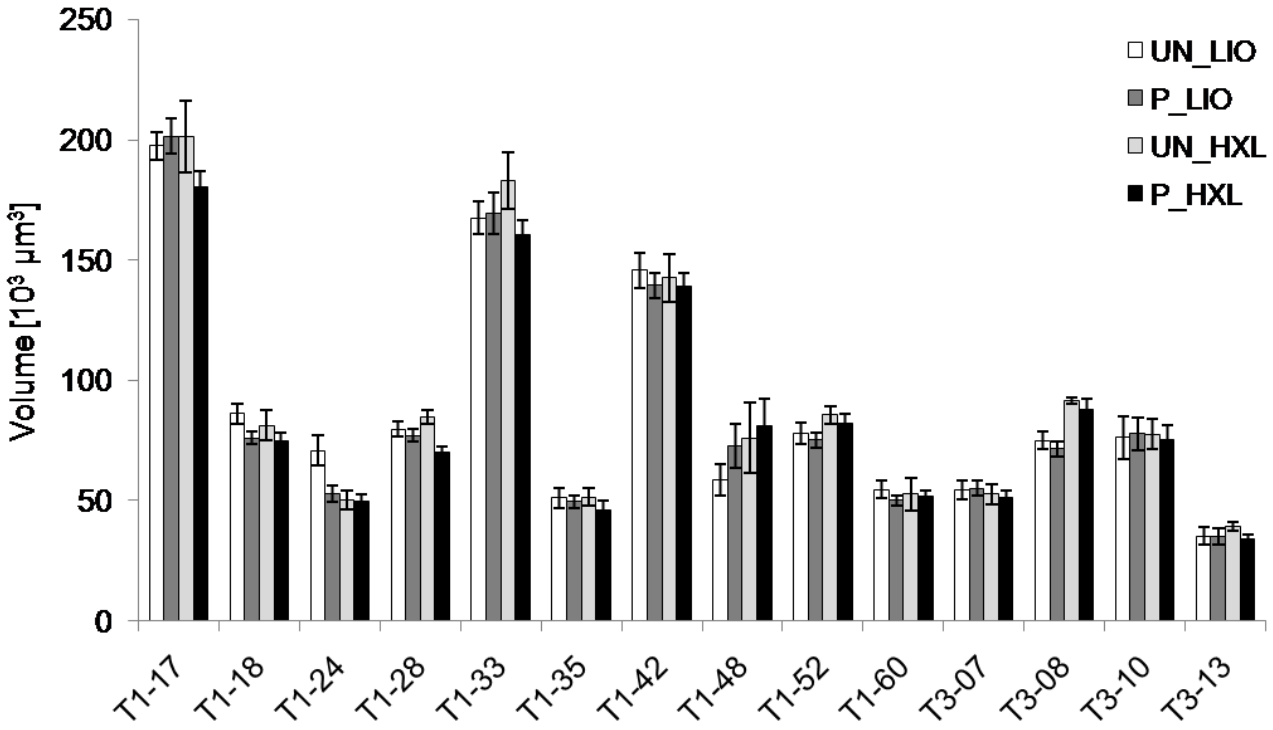

Fig. 3.2.5 Mean volumes ±SEM of the 14 glomeruli measured in paired and unpaired bees for each odour (calculated as averaged of the left and right in each individual). White and light-grey represent the volume in Unpaired individuals exposed either to linalool (UN_LIO, *N*=14) or 1-hexanol (UN_HXL, *N*=6), respectively. Dark-grey and black bars represent the measures of the glomeruli belonging to the Paired individuals trained either with (-)-linalool (P_LIO, *N*=14) or with 1-hexanol (P_HXL, *N*=13), respectively. For each glomerulo a number indicating tract is reported (Tract 1: T1; Tract 3: T3) with the identity number of each glomerulo on the basis of Galizia 1999b.

Discussion

We were unable to replicate the learning-dependent volumetric changes detected by Hourcade and colleagues (Hourcade *et al*., 2009) when bees were trained for an associative learning task with 1-hexanol. Using both the same paradigm and the same odour as in Hourcade *et al*., 2009, we found a trend for a decrease in volume for glomerulo T1-33 and for glomerulo T1-17 after learning. On the other hand, (-)-linaool did not trigger a significant modification of glomerular volumes 3 days after learning except for a specific effect in glomerulo T1-24. No differences between sides were revealed from the model, in neither of the two conditions.

<u>No significant learning-induced increase in volume after long-term memory formation was found, rather a specific-glomeruli volume reduction:</u>

While Hourcade *et al*. (2009) found an increment in glomerular volume after associative learning using 1-hexanol, no increase in volume 3 days after conditioning characterized our samples (Fig 3.2.2).

Considering our model, probably specific measures for each glomerulo in both sides in each individual, could have yield a more consistent effect which might not be detected by our model. In the lack of any volume increase, the only significant learning-dependent change we found was significant decrease in volume for specific glomeruli, namely glomerulo T1-33 and T1-17 (when conditioning is done with 1-hexanol) and T-24 (when bees are trained with (-)-linalool). However, all these glomeruli are specifically related to the odour that triggers their morphological reshaping. Glomerulo T1-33, in fact, is slightly activated during odour presentation both at the input (ORNs level) (see Galizia and Menzel, 2001) and at the output level (see Chapter 4.1; Franke, 2009; Sachse and Galizia 2002, 2003). Glomerulo T1-24, instead, was shown to be slightly activated by both odours at the input level (Galizia and Menzel, 2001), while the output data in literature are controversial. Glomerulo T1-24, in fact, has shown not to be activated during presentation of 1-hexanol when the output cells are recorded (see Sachse and Galizia 2002, Chapter 4.1, Franke, 2009) while responses to linalool have never been published at the output level but we were able to record it twice in our laboratory and we recorded an activation from GloT1-24 (see Appendix) In Franke (2009) 3 samples did not show any response in glomerulo T1-24 when linalool was presented. It should be noted that the majority of the studies used the right stereoisomer or a raceme (Franke 2009) when puffing linalool to the bee antennae. It has been demonstrated that stereoisomers can trigger different odour responses in the antennal lobe (Reisenman *et al*., 2010), so that our recordings for (-)-linalool might be more reliable for mapping the glomerular activation for this specific odour. Glomerulo T1-17, as well, is activated at the input by 1-hexanol (Galizia 1999b; Hourcade *et al*., 2009) but only slightly at the output (Chapter 4.1). It may be possible that the glomeruli that are less activated at the level of the PNs during odour responses are those to be stronger effected by glomerular volume reduction in our study.

The only study that recorded from PNs before and after odour paired conditioning in the same animal, was conducted by Rath and colleagues (Rath *et al*, 2011). Interestingly, they were able to predict glomerular dependent plasticity after associative learning on the basis of the activity recorded before long-term learning (Rath *et al*., 2011). In particular, they showed a glomerular decrement in the activity strength for

those glomeruli that are more likely to be activated by various odours (i.e. that are not strongly odor-specific). A decreased activity after learning might be linked to a consequent volume reduction found in our data (see fig. 3.2.5). In particular, despite our model, detecting statistically significant effects only in the above cited glomeruli, all the glomeruli presenting more evident volume decrease (glo T1-17; T1-33, T1-28 for 1-hexanol, see Fig. 3.2.5) are effectively slightly involved during PNs responses to 1-hexanol (with 28 being quite strongly activated (see Chapter 4.1, Galizia and Sachse 2002) and show a rather common activation for other odours (Franke 2009). Looking at PNs responses to (-)- linalool the same scenario is apparent with glomerulo T1-24 being slightly activated (see Appendix) while the T1-48 being highly activated. It is interesting to note that T1-48 showed opposite direction of volume-modification in our data (increase in volume after learning) and following Rath and colleagues' model the highly odour-specific glomeruli should in fact increase the glomerular activity strength after learning. Hourcade and colleagues found that glomeruli specificity correlated better with those that were less inhibited during odour dependent activity (on the basis of glomerular connection strength, see Linster *et al*, 2005) rather than more responsive at the input level (see Hourcade eta al. 2009). Although the olfactory receptor neurons responses on large samples of odorants have been investigated and statistically summarized in atlases (see for instance Galizia *et al* 1999b), the projection neurons' activity is far to be understood and characterized for single glomeruli (see Deisig *et al*., 2006; Sachse and Galizia, 2003). On the basis of the development of new techniques that allowed more feasible recordings from PNs, it would be interesting to check for a correlation between the model proposed by Rath and collegues (2011) and the reshaping measured by Hourcade and co-authors (2009).

For the first time in the present study, 4 glomeruli belonging to the Tract 3 in paired and unpaired bees were imaged, measured and compared between sides. The role of these glomeruli in parallel processing information is still debated and far from being understood (Müller *et al*., 2002; Yamagata *et al*., 2009; Brill *et al*., 2013). Following our results, they seem not to be implicated in reshaping after memory or in differences between sides, though a trend for odour specificity in T3-8 should be deeper analyzed.

Finally, differences in staining and processing of the fixed brain have to be considered interpreting the different outcomes between our and Hourcade's study (which are in detail) as follows: (i) differences in staining technique used; a general dye

(neutral red) was used by Hourcade and colleagues while we used antibodies and fluoropore specific for synapses and microtubules already used in literature for obtaining a well-defined pattern of glomeruli's morphology (Trona *et al*. 2010). A coincidence between antisinapsin and neutral red was anyway demonstrated (see supplemental materials in Hourcade *et al*., 2009); (ii) a difference in timing of incubation when processing fixed brain.

Morphological lateralization is not apparent in a subset of T1 and T-3 glomeruli:

In the subset of glomeruli we imaged, no differences between sides were evident, neither in the unpaired-group individuals nor in the paired-group ones. These data extends previous results shown in Chapter 3.1. The symmetrical pattern between the antennal lobes, in fact, was not only true for the 5 specific glomeruli we chose in the first analysis (see Chapter 3.1) rather for a larger sample of 14 glomeruli, including the four of the T3 tract that have never been measured before (namely T3-glomerlui). It has to be noted that the confirmed results seem to be independent from the technique used (fixed-samples presented here compared to *in-vivo* staining in Chapter 3.1). Moreover, a symmetrical morphology between left and right correspondent glomeruli is consistent after associative memory formation.

Considering the left side dominance in olfactory recall showed after long-term memory (Rogers and Vallortigara, 2008), we might have expected an asymmetrical pattern of glomerular plasticity between sides after odour memory. However, even in those glomeruli that showed specific volume reshaping (see above) paired individuals did not show any specific asymmetrical volumes between sides, though the paired conditioning paradigm was specific for long-term memory formation (involving also protein synthesis, see Menzel *et al*., 2001) and after 3 days bees were able to recall CS odour specifically. Forager bees with unknown experience were used in this study, therefore, strong individual-dependent long-lasting modification on the basis of previous experience might have added possible side-dependent noise. Again, functional plasticity or changes in substructures other than glomeruli might account for behavioural lateralization without affecting structures at a gross scale. Although the honeybee antennal lobe is implicated in memory formation (Grünbaum & Müller, 1998; Hammer & Menzel, 1998), lateralized morphological modifications might take place after associative learning in locations different from the antennal lobe neuropil, like in the lateral protocerebrum or the mushroom bodies. To shed light on these possible scenarios, further studies are

needed. Both specific staining in higher areas of the whole brain and optical recordings of the two sides before and after learning are potential methods to tackle the fascinating quest of the structural and functional correlates of olfactory lateralization in the bee brain.

Chapter 4 Olfactory lateralization and the bee Antennal Lobe: Functional Data

Summary

In this chapter results from *in-vivo* calcium imaging of the honeybee Antennal Lobes are presented. The output cells of the AL, the projection neurons, were stained and the odour-evoked activity recorded when bees' antennae were stimulated with different odours. The chapter reported the application of two-photon imaging technique in the honeybee neuropil allowing for a better resolution compared to conventional microscopy. Moreover a first comparison of odour dependent activities between left and right antennal lobes is reported, showing a first functional difference between sides in coding a bilateral symmetrical stimulus.

4.1 *In-vivo* two-photon imaging of the honeybee antennal lobe

Introduction

Since half a century, the honeybee brain is one of the best-known model in neurobiology for studying neuroanatomical correlates of learning and memory (for a review Menzel, 2012; Giurfa and Sandoz, 2012). In particular, the first olfactory relay station in the bee brain, the Antennal Lobe, is probably the most studied honeybee's neural correlate in terms of olfactory neurochemistry, circuits, and odour representation (see Sandoz 2011). Neural functions in the AL have been exploited quantitatively at the single cell level using electrophysiological recording allowing to trace the temporal olfactory response profile of intra/extra cell units. Nevertheless, the glomerular structure of ALs with each glomerulo acting as a functional entity (reviewed in Hansson and Christensen 1999) has been suitable for technique that record the ensemble activity of AL both in space and time. During the last few decades, in fact, optical imaging has opened the doors to understanding the olfactory code within the neuropil (Galizia and Vetter, 2004). Staining the AL with either voltage- or calcium-sensitive dyes allowed to show that different odours elicit spatio-temporal pattern of activated glomeruli that are consistent among individuals (Galizia *et al*., 1999b; Sachse *et al*., 1999). Calcium imaging (Grynkiewicz *et al*., 1985) moreover has been demonstrated to faithfully

indicate the electrical variations of neural evoked activity (Galizia and Kimmerle, 2004). Bath application of permeable dyes allows to record the whole activity of the bee brain without selecting for a specific class of neurons, though in the AL signal, the ORNs seem to dominate (Galizia and Vetter, 2004). Selective backfill staining with membrane-impermeable dyes (Gelperin and Flores, 1997) has instead allowed to record the AL's output signal from the Projection Neurons (PNs) (Sachse and Galizia, 2002). In particular, when used in combination with two-photon laser scanning microscopy (Denk *et al*., 1990) this opens up the possibility for *in-vivo* real-time monitoring of complex neural circuits down to several hundred micrometers within the specimen (Svoboda *et al*.,1997). While linear macro-and microscopy imaging techniques have been proven to be extremely successful in order to characterize this complex neuronal system, their intrinsic limitations have become more and more obvious (Galizia and Menzel 2001). Full-field microscopy does not offer sufficient axial resolution to resolve the exact origin of functional signals from deeper glomeruli and lacks the temporal resolution to determine whether valuable information might be encoded in the temporal structure of the recorded odor-evoked signals. Whereas confocal microscopy due to its intrinsic photo-damaging properties poses severe time constraints to *in-vivo* imaging sessions and has therefore only be used for morphological studies of the extracted brain. Herein we report on the development and new finding of a neural imaging platform for functional imaging of the honeybee antennal lobe's glomeruli. Our system permits to overcome the imaging impediments currently faced in this field. It enables us to acquire both *in-vivo* functional and morphological data of the ALs. Besides the well investigated T1 glomeruli which are projecting into the lateral antenno-cerebralis tract (l-ACT), the intrinsic two-photon optical penetration is deep enough to study the further classes of glomeruli in the ALs yet, namely the T2, T3, and T4 glomeruli, projecting into the median antenno-cerebralistract (m-ACT, see chapter 1.3). These glomeruli have been investigated through electrophysiological studies (Müller *et al*., 2002) and imaging of their axon terminals (Yamagata *et al*., 2009) suggest distinctive functional differences with respect to the l-ACT glomeruli. Moreover though recent studies showed two new methods for imaging concealed area of the ALs (Carcaud *et al*., 2012; Galizia *et al*., 2012), a direct method is lacking.

Methods

Bees have been collected from outdoor hives and prepared in accordance to a well established protocol (Galizia and Vetter, 2004). After chilling to immobility, bees were fixed to a custom made imaging stage using dental wax (Kerr; Siladent). Then a small window was cut into the head's cuticula above the mushroom body, glands and trachea were gently moved aside, and a solution of calcium sensitive dye (fura2-dextran, Invitrogen) dissolved in 2% Bovine Serum Albumin (Sigma-Aldrich) was injected by dye-coated micro-tips into the antenno-cerebralis tracts below the α-lobe. Finally, the cuticula was carefully closed and the animals were stored for 20h in a dark, cool, and humid place in order for the dye to diffuse into the AL. Before the imaging session, the cuticula, the glands, and the trachea above the AL were removed.

The experimental setup consists of a two-photon microscope (Ultima IV, Prairie Technologies) combined with an ultra-short pulsed laser (Mai Tai, Deep See HP, Spectra-Physics)(Fig.4.1.1)

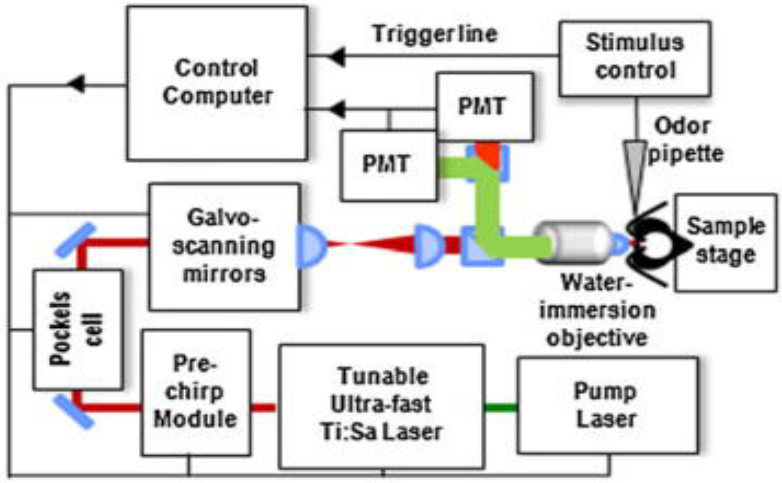

Fig. 4.1.1 Schematic setup of the two-photon microscope: A tunable ultra-short pulsed laser (Mai Tai Deep See HP, Spectra-Physics) is dispersion-compensated in a pre-chirp module. A Pockels cell controls the light intensity, and galvo-mirrors allow for fast and variable scanning. The beam is strongly focused onto the sample by a water immersion objective (40×, NA 0.8, Olympus). Fluorescence is collected by the same objective, separated from the backscattered excitation light by a dichroic beam-splitter, split into *green* and *red* detection channels by dichroics and band-pass filters (Chroma Technology), and detected by Photomultiplier tubes (PMT, Hamamatsu Photonics). A computer controls all microscope parameters and synchronises imaging with an odour stimulus generator.

The laser was tuned to 800nm for fura-2 excitation. Galvo-mirrors allowed for fast and variable scanning. All images were acquired with a water immersion objective (40x, NA 0.8, Olympus). Fluorescence is collected in reflection, separated by a dichroic beam-splitter (Chroma Technology), filtered by a 70nm band-pass filter centered at around 525nm, and detected by Photomultiplier tubes (Hamamatsu Photonics). A point spread function measurement verified the microscope's resolution to be diffraction limited to a full width at half maximum of 0.55μm in the plane and 2.6μm axially. Optimal signal-to-noise ratio was achieved with laser powers of about 10mW on the sample without observing any induced photo-damage. Temperature of the experimental environment was stabilized to 29°C.

A high functional temporal resolution of about 15ms was obtained by laser scanning along one-dimensional custom-defined traces, crossing the glomeruli of interest through an arbitrary horizontal plane (Figure 4.1.2). All acquired data have been corrected for photo-bleaching, while 2D running-average filtering was used to reduce the noise level. Spatial averaging was performed over a typical glomerulus size of 30μm, while temporal averaging was applied over 80ms preserving all main dynamic features of the data.

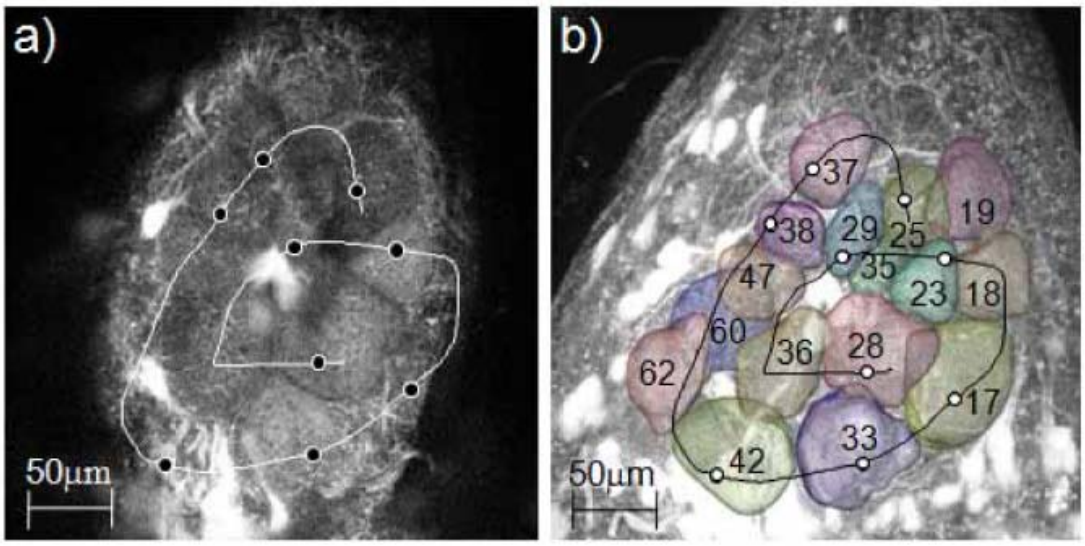

Fig. 4.1.2 a) Image of a right antennal lobe at 25μm depth: The line indicates the laser scanning trace, the dots label the measurement's reference positions corresponding to the vertical lines in Figure 3. (b) Axial projection view of the AL volume image stack, superimposed by the reconstructed surface plots of the involved T1 glomeruli, identified and labeled according to (Galizia *et al*., 1999b).

A stimulus controller (CS-55, Syntech) delivered odor stimuli to the bee's antennae without changing the total air flux to avoid mechanical stimuli. The odor stimuli come

from pasteur pipettes in which 10μL of an odor dissolved in mineral oil (0.1μg/μL) are deposited on a piece of filter paper. All command signals and acquisitions were controlled by a common gate which allows precise synchronization of the involved pulses. The experimental cycle began by starting the image acquisition. After 3s the stimulus generator received a trigger releasing an odor puff of 2s length. The exact arrival time of the odor at the bee antenna was measured and found to stable within 10ms, which allows e.g. accurate measurements of the neuronal response delay. After 9s image acquisition stopped and automatic data evaluation started. Thanks to the reduced photo-damage characteristics of the two-photon microscopy approach, due to the very limited absorption volume in the sample, the imaging sessions could be extended until 5 hours before we notice an essential drop in the brain activity. Data analysis was automatically executed during the experiments by Matlab (Mathworks) scripts, while post-processing for 3D reconstruction, image segmentation, and volumetric measurements was performed using the software Amira (Visage Imaging).

Results

We first recorded the spatio-temporal functional activity in the AL by measuring the two-photon calcium response signal along the line traces indicated in Fig 4.1.2 triggered by odor stimuli from three different floral components: 1-Hexanol, 1-Octanol, and 1-Nonanol. Enhanced neural activity, leading to an increasing intra-neuronal calcium concentration, causes a drop in the measured two-photon fluorescence intensity, producing dark bands in the scanlines-over-time maps at the positions of the corresponding glomeruli. We detected response signals of up to 20% intensity change, which is about 4 times higher than in comparable experiments using wide field imaging. The recorded maps are shown in Figure 4.1.3 and reproduce features which have already been observed by conventional single-photon fluorescence microscopy (Galizia *et al*., 1999b), such as the very broad response of glomeruli T1-28, T1-33, and T1-17 to all tested odors. Likewise, 1-Hexanol has been found to produce responses in several of the monitored glomeruli. Strikingly different from previously published data obtained with full-field microscopy (Peele *et al*., 2006) are the quite strong responses of glomeruli T1-29 and T1-37 for both 1-Hexanol and 1-Octanol. We have then exploited the larger penetration depth and the higher axial resolution offered by our setup, in order to obtain functional spatio-temporal odor response maps at different axial positions within the AL.

Figure 4.1.4 shows the calcium response maps to an 1-Octanol odor stimulus at a depth of 25μm Figure 4.1.4a and 50μm Figure 4.1.4d respectively. The high axial resolution allows to clearly resolve the functional activity at the different depths. In particular the responses of the upper surface glomeruli T1-37 and T1-29, clearly visible at the imaging depth of 25μm, disappear at 50μm, while the weak response of the glomerulus T1-17 at 25μm becomes more pronounced at 50μm. The temporal traces of the single glomeruli data allow to analyze possible temporal components of the olfactory code, like response delay or oscillatory responses as reported in other animals (Laurent *et al*., 1996).

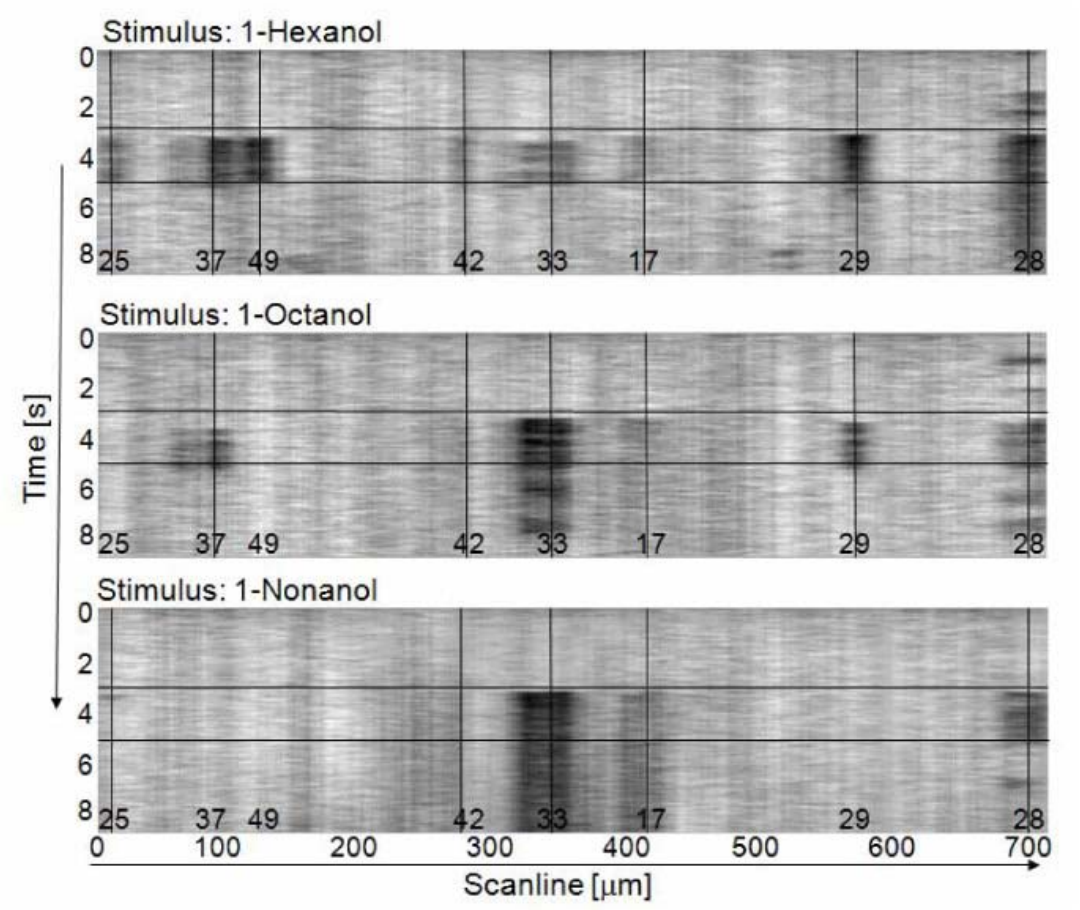

Fig 4.1.3 Calcium response maps for three different odors (1-Hexanol above, 1-Octanol middle, 1-Nonanol below), recorded along the scanning trace in Figure 4.1.2. The stimulus period is enclosed by the horizontal lines, responding glomeruli centers are marked by vertical lines, numbers label the identified glomeruli.

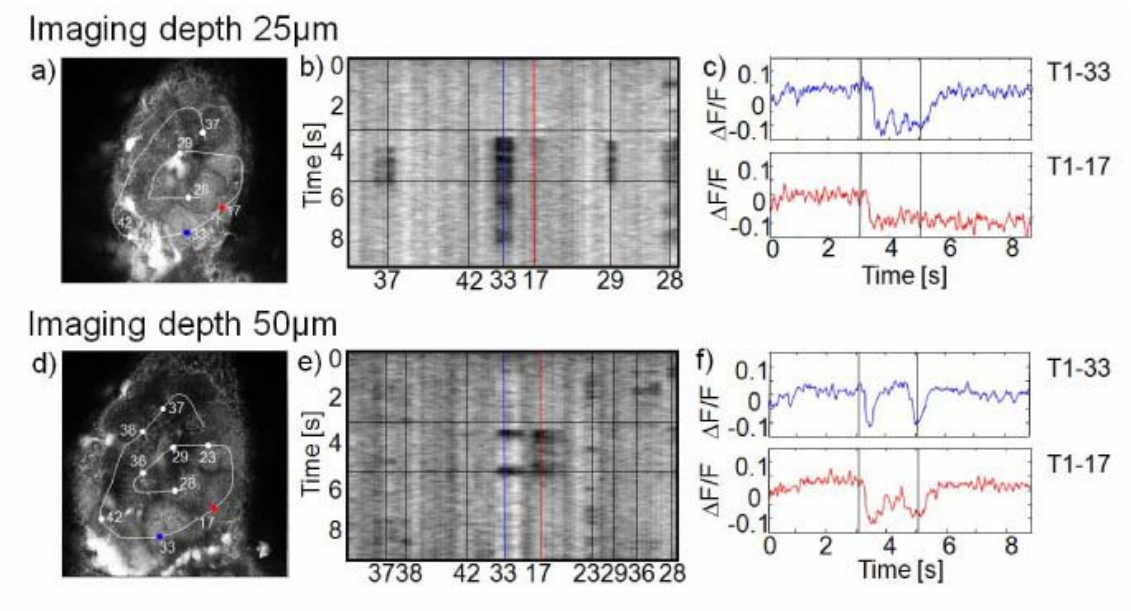

Fig.4.1.4 Odor response maps to 1-Octanol at depths of 25μm and 50μm within the AL. (a) and (d) show the 2D images of the corresponding focal plane together with the line scan traces. In (b) and (e) the activity signal is plotted as a function of position along the line trace (x-axis) and as a function of time (y-axis). The stimulus period is enclosed by horizontal lines, the single responding glomeruli are marked by vertical lines. (c) and (f) show single temporal traces for the strongest responding glomeruli T1-33 and T1-17 at the two corresponding depths.

The maximum penetration depth for functional imaging was found to be 150μm, while morphological data could be acquired from the complete ALs down to a depth of 400μm. We restrict the presented data to a depth of 200μm, to which we were so far able to identify glomeruli with certainty. In Figure 5 we present an image stack of 200μm representing the morphology of the glomeruli within the AL (Fig. 4.1.5a), together with the volumetric reconstruction of the glomeruli (Fig. 4.1.5b). Green colored glomeruli belong to the T1 sensory tract projecting into the l-ACT axonal tract, while blue and red colored glomeruli belong to the deeper laying T2 and T3 tracts, respectively, both projecting into the m-ACT.

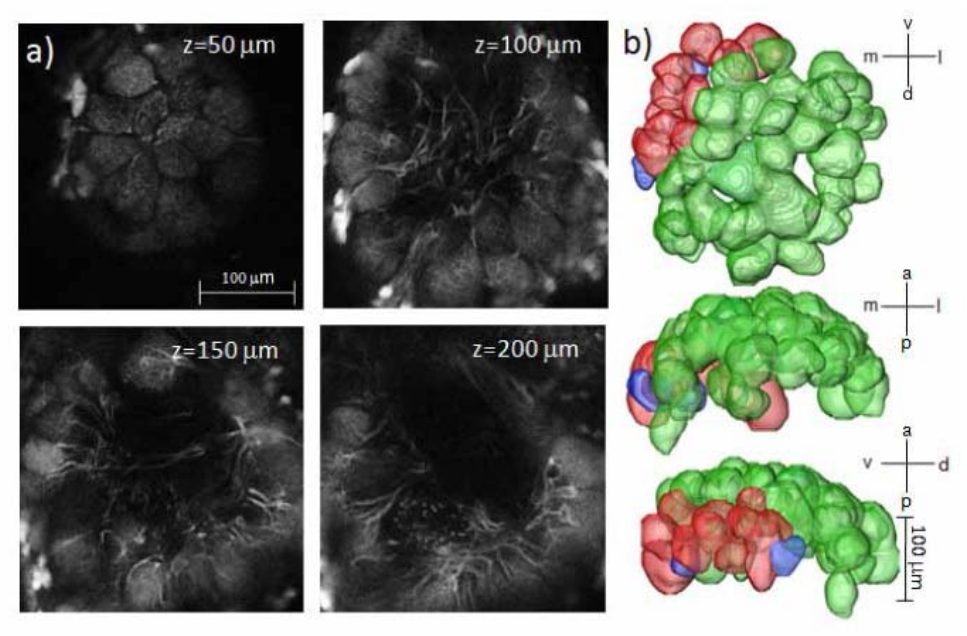

Fig 4.1.5 a) Image stack examples down to 200μm penetration depth into the right AL. b) Reconstructed glomerular volume images in all projections, glomeruli colored in green are from the T1 tract projecting into the l-ACT, the red glomeruli are from the T3 tract projecting into the m-ACT and the blue one form lm-ACT.

In Figure 4.1.6 we show an example of these data. Figure 4.1.6a represents the glomerular response map to a 1-Hexanol stimulus at a depth of 150μm. Apart from the standard post-processing methods this map has been averaged over 3 consecutive recordings with one minute recovery intervals in between. This allows to distinguish random fluctuations from specific signal changes which becomes more crucial at that depth. In Figure 4.1.6b the corresponding focal plane together with the laser scan trace are visualized. The points where activity has been detected are marked by dots. Comparing these points with our morphological reconstruction we could attribute all signals to specific glomeruli which are labeled according to (Galizia *et al*., 1999b). The green colored points represent signals from the T1 tract. In some of the cases the glomeruli are located in an upper layer above the imaging plane and the signal comes only from the few projection neurons connected to them. In these cases the absolute fluorescence signal is very faint, but never the less a relative drop of up to 20% can be detected also here. The data points colored in blue and red mark glomeruli from the T2 and T3 tract, respectively. These glomeruli are located in the focal plane leading to a strong absolute fluorescence signal. For the first time we were able to optically record activity also from these glomeruli deep within the antennal lobe without the needs of specific apparatus to add or a changing in the orientation of the specimen. Figure 4.1.6c

shows examples of single response curves for the T2 and T3 glomeruli. Strong activity signal and high temporal resolution are also here giving hope to a much needed systematical extension of the odor response maps to this glomerular classes.

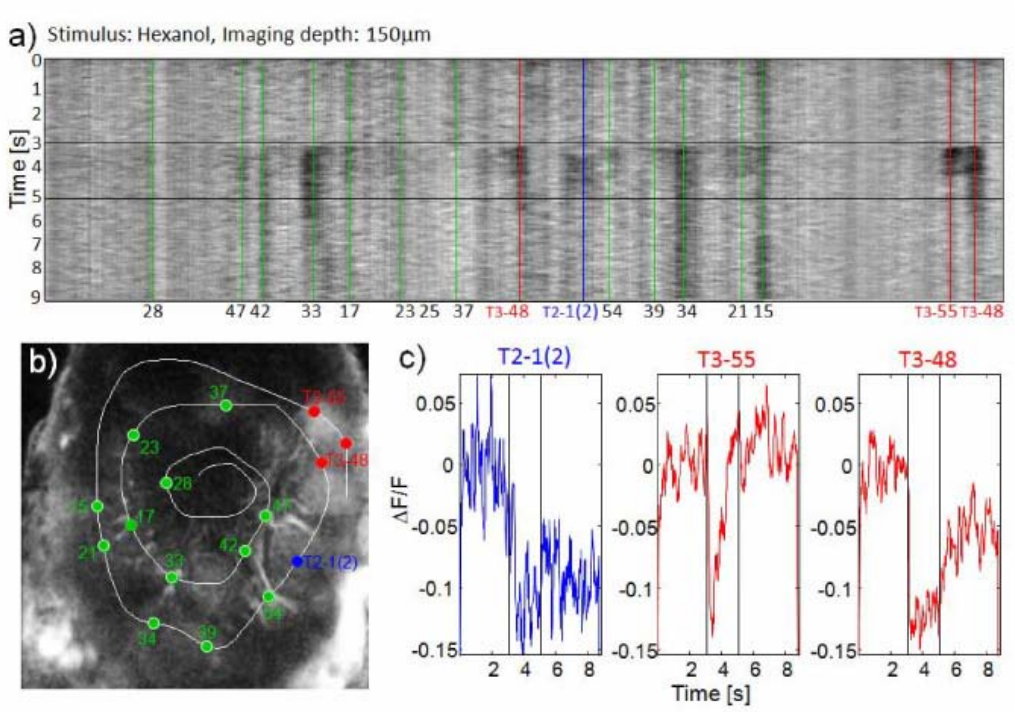

Fig. 4.1.6 a) Functional image map of responses to a 1-Hexanol stimulus recorded at 150μm depth from the left AL. b) The imaged focal plane together with the laser scan line. The responding centers are marked by points and labeled by the corresponding glomerular number. Green points correspond to T1, blue ones to T2, and red ones to T3 glomeruli. c) Single time traces of the responses from glomeruli of the T2 and T3 tract.

Discussion

The two-photon microscopy functional data we have presented in this work suggest that our imaging platform offers the capability to extend the specific AL odor response maps that have been measured in the past for many different odor components and in most of the T1 glomeruli (Lieke *et al*., 1993). Two-photon imaging has been already exploited for analyzing *in-vivo* evoked activity in the insect ALs such as in *Drosophila melanogaster* (Wang *et al*., 2003) and in *Schistocerca Americana,* up to single neuron level (Moreaux and Laurent, 2007). More recently, the advantage of two-photon microscopy even in non-genetically manipulated context has been revealed in ants (Ruchty *et al*., 2010; Brandstaetter *et al*., 2011). Exploiting the spatial resolution of two-photon microscopy, Ruchty *et al* were able to either functionally record thermal

information and morphologically indientified involved glomeruli in the ant's ALs. Two-photon imaging in fact allows to obtain high resolution images right after recording neural activity, overcoming the problems of identification of less visible glomeruli.

Nevertheless, in honeybees, so far these maps contained only the static parameters response strength and consistency range, which might now be supplemented by adding temporal features as the response latency or oscillatory components. The intrinsic axial resolution and the extended imaging depth of two-photon microscopy has allowed us to resolve profound functional data. Odor response maps could therefore be completed by measuring the more profound classes of T2,T3, and T4 glomeruli. This new possibility is of special interest because these glomerular classes are projecting into the m-ACT axonal tract and have been hypothesized to show fundamental differences regarding their odor coding properties (Müller *et al*., 2002; Yamagata *et al*., 2009), as well as their memory related plasticity after odor conditioning (Peele *et al*., 2006). The morphological division of the olfactory pathway into the two axonal tracts (Kirschner *et al*., 2006) seems to be accompanied by a complete functional division into two parallel processing branches. Recently, novel preparations (Galizia *et al*., 2012; Carcaud *et al*., 2012) suggested a quite redundant activity in both tracts with different roles in the separability of odours on the basis of functional groups in the m-ACT rather than carbon length (Carcaud *et al*., 2012). Nevertheless, with these new methods morphological identification of single glomeruli was not possible suggesting that two-photon microscopy is still worthwhile for identifying high-resolved structures and activities coming from still concealed areas of the AL.

In addition we have obtained a 4-fold increase in the functional-related fluorescence change with respect to similar experiments using wide-field imaging. Another promising feature of a two-photon microscopy approach is the possibility to investigate sub-glomerular structures down to single neurons (Franke, 2009). This becomes even more crucial if imaging is extended to higher order brain structures such as the mushroom body, where a meta-structure comparable to the AL's glomeruli is absent (Faber and Menzel, 2001). Finally, aside the resolution's improvements, the intrinsic two-photon limited photo-damage has offered extended imaging sessions up to 5 hours. This should allow in the future for *in-vivo* real time studies of the antennal lobe plasticity after odor conditioning (Hourcade *et al*., 2009; Rath *et al*., 2011) also in deeper glomerular classes like T3 glomeruli whose role in AL memory traces is still unknown.

Finally, considering the limited knowledge about physiological correlates of lateralised behavior (see chapter 1.2.1), it might be a challenging and intriguing question to address. In honeybees, in fact, the only evidence at the central level about lateralisation has been recently revealed (see chapter 1.2.2); Biswas and colleagues in fact demonstrated a difference between sides in the distribution of neuroligin-1, a protein involved in learning and memory when honeybees were only left or right antennae amputated (Biswas *et al*., 2010). Therefore a more specific analysis between right and left ALs is necessary and two-photon *in-vivo* imaging seems to be promising. A first comparison of *in-vivo* odor-evoked activity between sides is in fact needed, both to unravel fine temporal discrepancies and to have accessibility to those glomeruli that have been never imaged with such a high-resolution.

4.2 Asymmetrical odor coding in the honeybee Antennal Lobe

Introduction

Functional asymmetries of the nervous system are widespread among vertebrate and invertebrate species (see Chapter 1). Lateralization of visual processing, for example, is a common trait among vertebrates (for a review see Rogers and Andrew 2002; Rogers *et al*., 2013). Besides anatomical differences between the bilateral visual tracts which connect peripheral and central areas, there are only a few examples which show lateralization at neurophysiological level (Folta *et al*., 2004; Peirce and Kendrick, 2002; Town *et al*, 2011). Folta and colleagues (2004) showed for the first time a left right physiological difference in visual information processing: in pigeons, neurons in the right nucleous rotundus responds with shorter latency to visual stimuli, and this might account for faster visuomotor responses of the left eye (due to partially crossed of the visual fiber).

Investigations on invertebrate animal models, on the other hand, have started recently to deeply explain in few cases cellular and molecular mechanisms underneath functional lateralization (see Chapter 1.2). In *C. elegans,* for instance*,* two bilateral symmetrical head neurons with respect to position and neural connections, are differently involved in taste perception, with the left (ASEL) specialized for being active perceiving increased Na^+ concentration and the right (ASER), responding to decreasing Cl^- concentration (Suzuki *et al*., 2008). This asymmetrical encoding is necessary for the animal to perform appropriate context-dependent behaviors during chemotaxis (Suzuki *et al*., 2008). The left side activated leads to forward behaviours while the right one triggers searches in other directions. The lateralized coding of the environment may in some cases come out from anatomically symmetrical bilateral circuits on a large scale (reviewed in Concha *et al*., 2012). In rats' hippocampus, for example, the symmetrical CA3-CA1 synapses express different amounts of glutamate receptors between sides (Shinohara *et al*., 2008), with the left side showing significant higher levels. This difference has demonstrated to promote a stronger long-term potentiation on the left CA1 axons when CA3 cells were optogenetically stimulated (Kohl *et al*., 2011). In *Drosophila melanogaster*, a left-right asymmetry in encoding different odours has been revealed (Rodrigues, 1988). Differently from other insects, *D. melanogaster*'s primary

olfactory centres, the antennal lobes, receive inputs bilaterally from both the antennal nerves (Couto *et al*., 2005). This symmetry is broken when odours with different biological meanings are presented to the animals. When attractive odours are puffed unilaterally, in fact, they are spatially encoded restrictively in the ipsi-lateral Antennal Lobe (AL); while repulsive odorants induce activation in both ALs (Rodrigues, 1988).

Despite the needs of identifying how left-right asymmetries take place in the nervous system, the understanding of lateralized neural circuits remains mostly unsolved and unaddressed (see Chapter 1.2). The honeybee has become an interesting target for investigating olfactory asymmetries at the behavioural level and physiological level (see Chapter 1.2.2). Briefly, a left-right difference in short-term olfactory memory recall has been demonstrated, favoring the right side (Rogers and Vallortigara 2008, Frasnelli *et al*., 2010a; Anfora *et al*., 2010). Moreover, there is a disproportionate distribution in number of *sensilla*, the olfactory structures on the antenna that house odour receptor neurons, with the right antenna showing more *sensilla* in each segment (Letzkus *et al*., 2006; Frasnelli *et al*., 2010a). Finally, an asymmetry in the responses of the antennal nerves has been demonstrated when two biological relevant odours were puffed, again with the right antennal nerve being more sensitive (Anfora *et al*., 2010).

To date, there are no studies that address the neural correlates of these differences inside the honeybee brain. Along the olfactory pathway, antennal lobes represent the first olfactory brain area for coding odour information (see Chapter 1.3). Odour induced activity from the antennal nerve is integrated with the local interneurons` activity, then encoded in projection neurons` (PNs) activity and forwarded to higher brain areas (see Galizia and Szyszka 2008 and see Chapter 1.3). PNs get synaptic inputs in sphere-like substructures, called glomeruli. There is good evidence that PNs` activity indeed encodes odour identity as the perceived odour similarity (measured behaviorally as generalisation) corresponds to the similarity between odour evoked glomerular response patterns (Guerrieri *et al*., 2005; Szyszka *et al*., 2011). Therefore, PNs are an excellent candidate for searching any encoding of asymmetry, moreover, they can be selectively stained and imaged *in-vivo* (see Chapter 4.1). Here, we compare left-right PNs' activity when stimulating with single odorants and a binary mixture. To our knowledge, it is the first comparison of odour coding in a single neuron population between sides, conducted in the same conditions over an extended sample. We found a left-right asymmetry in the distances between odour representations and in

mixture interactions. These differences are neither due to a significant lateralization of activity strength between the sides nor to the number of activated glomeruli.

Methods

Measures came from a dataset of Rath *et al*., 2011 and unpublished data from the same experiment. The recordings were performed on either the left or the right AL. In total, 66 bees were imaged; 33 left ALs and 33 right ALs.

Animals

Experiments were performed with foragers of the honeybee, *Apis mellifera*. Bees were taken from hives kept in containers with a stable temperature (16°C at night and 25°C during the day), light/dark cycle (12/12 h) and humidity (about 75%).

Staining and Preparation

Each experiment took 2 days. On the first day in the morning bees were caught from feeders located 1 to 2 meters in front of the hives. Shortly after they were paralized by cooling on ice and harnessed in Plexiglas stages with help of dental wax (Deiberit 502, Dr. Böhme und Schöps Dental GmbH, Goslar, Germany). The mandibles were pushed open and fixed to the stages with hard wax. A rectangular opening was cut into the head capsule, glands and tracheae covering the mushroom body calyces were removed, the PN of both ALs were stained by injecting a glass needle covered with the calcium sensitive dye Fura-2 dextran (Invitrogen, Molecular Probes, Eugene, OR, USA) into the axon tract between the calyces of the mushroom bodies, and the head was closed again. After this operation, bees were fed with a 1.25 M sucrose solution until satiation, and were kept in a moist box until the next day (14 to 16 hours) to let the dye travel to the PN dendrites in the AL.

One day later bees were imaged. Before imaging, the antennae were fixed at the scapus-pedicellus joint so they pointed frontwards. Then the head capsule was opened again and the tracheae above the ALs were removed. To reduce movements, the esophagus and the surrounding muscles were lifted through a small window in the clypeus. The window was sealed with two component silicon (Kwik-Sil, World Precision Instruments, Inc, Sarasota, USA). A plastic cover slide was used to shield the antennae and Kwik-Sil and soft dental wax (Utility Wax, Carmel Group Inc., Canada) was used to

seal the chamber. A piece of foam (1 cm x 1 cm x 3 cm) was pushed against the bees body to reduce movements. The brain was covered with a thin layer of Kwik-Sil in order to stabilize it. Within 10 to 30 minutes after the preparation bees were put under the microscope and heated to around 28 °C with an infra red lamp. Finally, the AL that showed better staining and spontaneous activity was brought into focus.

Odour stimulation

2-octanol, 1-hexanol and 2-nonanol were used for odour stimulation (all from Sigma Aldrich, Deisenhofen, Germany). The pure odorants were diluted to 1:100 in mineral oil (Sigma Aldrich). Odorants were prepared freshly every four weeks. 200 µl of each odour were loaded onto a cellulose stripe (Sugi, REF 31003, Kettenbach GmbH & Co. KG, Eschenburg, Germany) located in a 3 ml syringe (Norm-Ject, Henke-Sass, Wolf GmbH, Tuttlingen, Germany). Syringes were prepared freshly every day. Odour stimuli were delivered as 4 s pulses with a custom built computer controlled olfactometer (Szyszka *et al*., 2011). Each channel of the olfactometer consisted of two syringes, an empty one for equalizing air flow and one containing the odourant. The olfactometer had 6 channels. The air stream through each channel was 300 ml/min each controlled by a flow meter (Analyt-MTC GmbH, Müllheim, Germany). Odors were injected into a continuous carrier air stream (1200 ml/min) in a glass tube (1 cm in diameter), which was directed to the bee sitting 1 cm in front it. A magnetic two way valve controlled the odor pulses by diverting air from the empty syringe to the odorant syringe. The six channels added up to 1800 ml/min, and total air stream was 3000 ml/min. In addition to the single odors the bees were stimulated with a binary mixture of 1-hexanol and 2-octanol by opening the valves of the two channels simultaneously. Continuous air suction behind the bee cleared residual odor.

Imaging

PN in the AL were imaged with a water dip objective (20x, NA 0.95, Olympus, Tokyo, Japan). The imaging system consisted of a fluorescence microscope (BX-50WI, Olympus), a light source (Polychrome IV, Till Photonics, Gräfelfing, Germany) and a CCD camera (Imago QE, Till Photonics). 8x8 pixels of the camera were binned on-chip resulting in a resolution of 172 x 130 pixels. Each recording lasted 29 s and consisted of 232 double frames recorded with 340 and 380 nm excitation light at a frame rate of 8 Hz. Excitation and emission light were separated with a 420 nm dichroic mirror and a

490-530 nm emission filter. Bees were stimulated with octanol and hexanol, the mixture of octanol and hexanol, nonanol and the solvent mineral oil as blank control. Odors were presented in a pseudo randomized order with an inter-trial interval of 2 min. Odor stimulation was controlled by the acquisition software of the imaging system (Till Vision, Till Photonics).

Data analysis

Imaging data were analyzed using custom-written programs in IDL (RSI, Boulder, USA). Movies were movement corrected by aligning frames within and between measurements. Glomeruli were segmented with help of an unsharp masked image of the raw fluorescence and a correlation image where the correlation of the signal traces between neighboring pixels was calculated. The ratio of the images acquired at 340 and 380 nm excitation wavelength was calculated and the average background fluorescence was subtracted from every frame of a measurement to get the ΔF340/380. The background fluorescence was calculated as mean of 66 frames (frame 4 - 69) before stimulation. No filtering was used for quantitative analysis.

Response strength was quantified as the mean signal during 1 s (frames 72 - 79 after stimulus onset). To calculate the global response over the whole AL, for each bee, the response of each glomerulus was calculated and averaged over all glomeruli. The distance between odor response patterns was quantified as follows: for each bee, the response of each glomerulus was calculated and the odor response pattern was represented as vector of these values. Distance between two odor responses was quantified by calculating the Euclidean distance between the two vectors as follows:

$$d_{ij} = \sqrt{\sum_{k=1}^{p} \left(X_{ik} - X_{jk}\right)^2}$$

With *i* and *j* indicating odours; *p* the glomeruli; *Xik* the activity of the *k* glomerulus when stimulated with odour *i*.

Odour distances were analyzed separately using RM ANOVA with distances as a within factor and side as a between factor.

Spontaneous activity was calculated as mean standard deviation (SD) before stimulus onset (frames 1-70). For each bee, the averaged SD was calculated and left-right spontaneous activities were compared with an independent *t-test* (two tails).

Glomeruli whose activity during the first second of odour response showed a signal ≥6*SD before stimulus were considered as active. We compared numbers of glomeruli at the level of the AL (averaged per bee) using a RM ANOVA with odours as within factor and side as a between factor.

For evaluating differences in mixture interactions between sides we calculated the stronger odour for each glomerulus. Stronger odour (SO) is the strongest response to one of the two component of the mixture (i.e. is the least responses to mixture if no interaction would occur with the mixture) and it's a measure used for investigating mixture interactions (see for an example Deisig *et al*., 2010). We took all the glomeruli which showed a positive signal during odour activiation (frames 72-103). For each glomerulus we compared SO and mixture (paired t-test, two-tails) in each antennal lobe. An interaction index (*i*=mixture-SO) was calculated and right and left indices were compared using an independent t-test (two tails). An index of distances between the components of the mixture in each antennal lobe (i=SO-Weaker odour) was calculated and Pearson correlation analyses were performed between the two indices in each antennal lobe. Finally, we selected those glomeruli that showed SO ≥ 3*Weaker odours and we compared within each glomerulus in the two antennal lobes SO with mixture responses (paired *t-test*, two-tails) as well as the interaction indices between left and right antennal lobes (independent *t-test*, two tails).

Quantitative and Statistical Analysis were performed using MATLAB (Mathworks R2010b) and SPSS (PASW Statistic 18).

Results

Background activity, odour response strength and number of activated glomeruli are equal between sides

We first compared the background activity between left and right ALs, measured as standard deviation before stimulus onset. The background activity was 0.63(±0.05) in the left and 0.66 (±0.041) in the right AL and there was no significant differences between sides (independent *t-test* $t(64)= -0.485$; $p=0.629$).

We then compared the odour response strength (average signal during the first second of odor reseponse) between the left and right AL and again, no difference were apparent (Fig. 4.2.1). The ANOVA revealed an odour effect ($F(3,192)=25.57$, $p<0.001$)

but neither side effect (side F(1,64)=2.59; *p*=0.112) nor interactions (odour*side F(3,19)=1.390; *p*=0.25) were found.

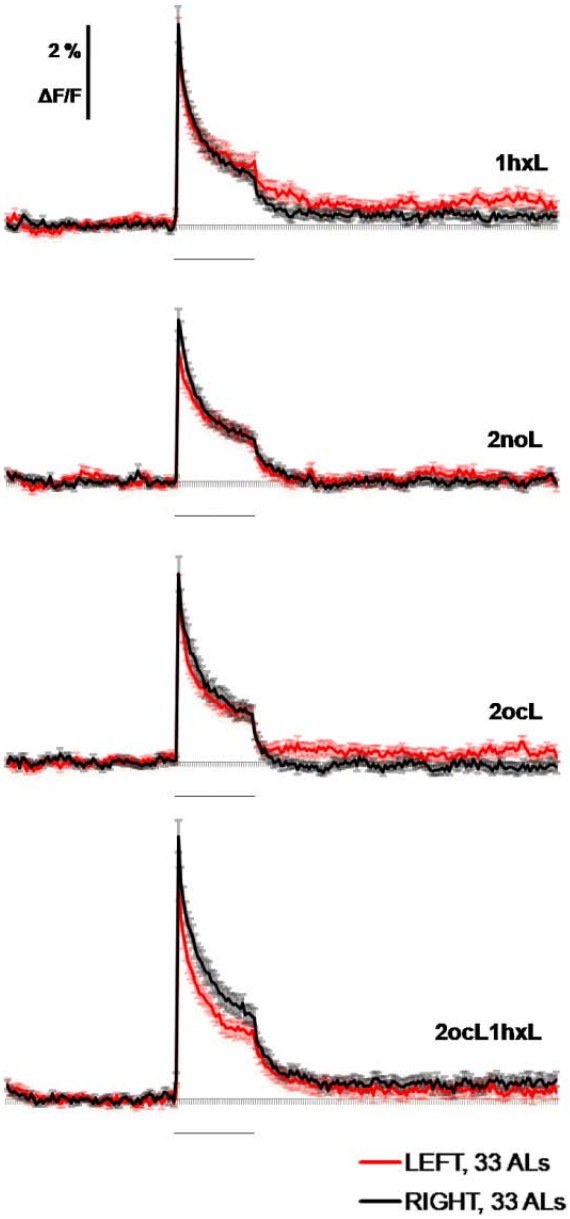

Fig.4.2.1 The response does not differ between sides. Time-course of global PNs responses to odours along the whole recordings (29s) (average over all glomeruli; mean±SEM); 1hxL: 1-hexanol; 2noL: 2-nonanol; 2ocL: 2-octanol; 2ocL1hxL: 1:1 2-octanol:1-hexanol mixture; *x* axis: time (s); *y* axis: PNs activity (ΔF/F %) Gray bars indicate the 4 s stimulus pulse along the whole recording time (29s).

Odours activated between 20 and 50 % of the visible glomeruli (Fig. 4.2.2). The percentage of activated glomeruli differed between odours (F(3,192)=23.436, *p*<0.001) but not between sides(F(1,64)=0.143, *p*=0.706) and there was no interaction between side and type of odorant (odour*side F(3,192)=0.706, *p*=0.529) .

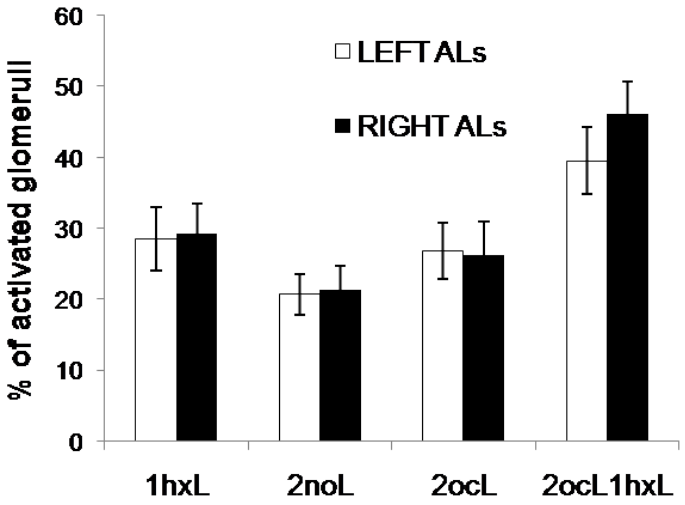

Fig.4.2.2 The number of odour-activated glomeruli is equal between sides. Percentage of activated glomeruli for different odour stimuli. Each glomerulus was considered active when activity during the first second of odour response showed a signal ≥6*SD before stimulus onset. For each bee the percentage of activated glomeruli was calculated as mean +SEM for left and right ALs (*N*=33 per side).

Distances between odour representations are higher in the right antennal lobe

We next asked whether there is a qualitative difference in odour representations between the left and right ALs and calculated the Euclidean distances between glomerular response patterns of different odours. The distances between odour evoked glomerular response patterns correlate well with the perceived dissimilarity between them (Guerrieri *et al*., 2005). Thus, distances between odour evoked glomerular response patterns contain behavioral relevant odour information, and left-right differences in inter-odour distances would indicate differences in the discriminatory power of the odour code. The analysis of variance revealed a general side effect with the right AL showing higher distances compared to the left ALs ($F(1,64)=5.209$, $p=0.026$ fig.4.2.3). There was also a general difference among odour distances ($F(5,320)=17.670$, $p<0.001$) but no effect due to interaction between odour distance and side ($F(5,320)=0.654$, $p=0.588$).

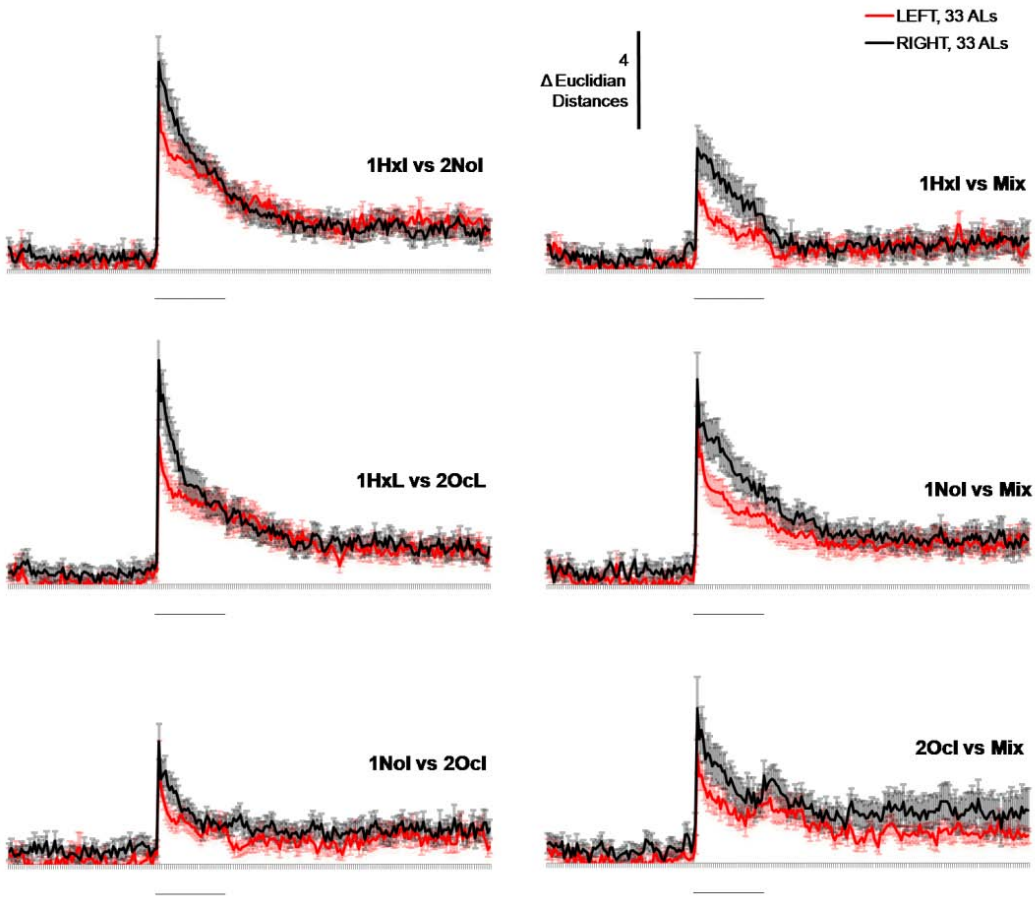

Fig. 4.2.3 Distances between odor response patterns are greater in the right AL. Time course of Euclidian Distances for each odour couple (average over all bees; mean±SEM). For each odour-pair left (red line) and right (black line) AL distances are represented. 1hxL: 1-hexanol; 2noL: 2-nonanol; 2ocL: 2-octanol; mix: 1:1 2-octanol:1-hexanol mixture; *x* axis: time (s); *y* axis: ΔEucidian distances.
Bar indicates the 4s stimulus pulse along the whole recording time (29 s).

Mixture interaction differs between sides

We noted that the global AL response strength to the mixture was larger in the right than in the left AL (Figure 4.2.1, $p<0.026$), although there was no significant left-right difference in response strength when the other odors were taken into account. We asked whether the left-right difference reflects a difference in the processing of mixture and compared mixture interactions between the left and right ALs. Each glomerulus` mixture response was compared with its response to the stronger component (Figure 4.2.4). In the right AL the response strength for the mixture was stronger than the response strength for the stronger component ($t(337)=-5.675$; $p<0.001$). This additive mixture processing corresponds to the stronger mixture response observable in the right

AL (Fig. 4.2.1). In the left AL the response strength for mixture was not different from the response strength for the stronger component ($t(321)=1.289$; $p=0.198$). The lack of inhibitory mixture interaction was surprising given its abundance in insect PNs (Galizia *et al*., 2000; Deisig *et al*., 2006, 2010; Joerges *et al*., 1997; Najar-Rodriguez *et al*., 2010; Silbering and Galizia, 2007; Silbering *et al*., 2008; Stierle *et al*., 2013). We wondered whether the absence of inhibitory mixture interactions might be due to the fact that 1-hexanol and 2-octanol activate largely overlapping sets of glomeruli (Sachse *et al*., 1999; Galizia and Menzel 2001; Deisig *et al*., 2006; 2010): Inhibitory mixture interactions in PNs are mainly mediated by lateral inhibition and the effect of lateral inhibition might be masked when the components of a mixture activate the glomerulus equally strong. In contrary, the effect of lateral inhibition might become more prominent with increasing difference between the components.

To reveal whether differences in component response strength influences mixture interaction, we separately analyzed those glomeruli where the SO was stronger than the weaker component (e.g. 3x stronger as in Weaker Odour (WO)). In the left AL there was a significant mixture suppression while there was no mixture interaction in the right AL (left: $t(79)=2.214$; $p=0.030$; right $t(92)=-0.837$; $p=0.405$) (Fig.4.2.4 Middle Graph).

Moreover, we calculated the Pearson's correlation between the mixture interaction index and the distance between components. While in the right antennal lobe there was no significant correlation ($\rho=-0.086$; $p=0.115$) the left antennal lobe showed a significant negative correlation ($\rho=-0.442$; $p<0.01$). Thus, only in the left antennal lobe, mixture suppression increases with increasing difference between the components (Fig. 4.2.4, Lower graph).

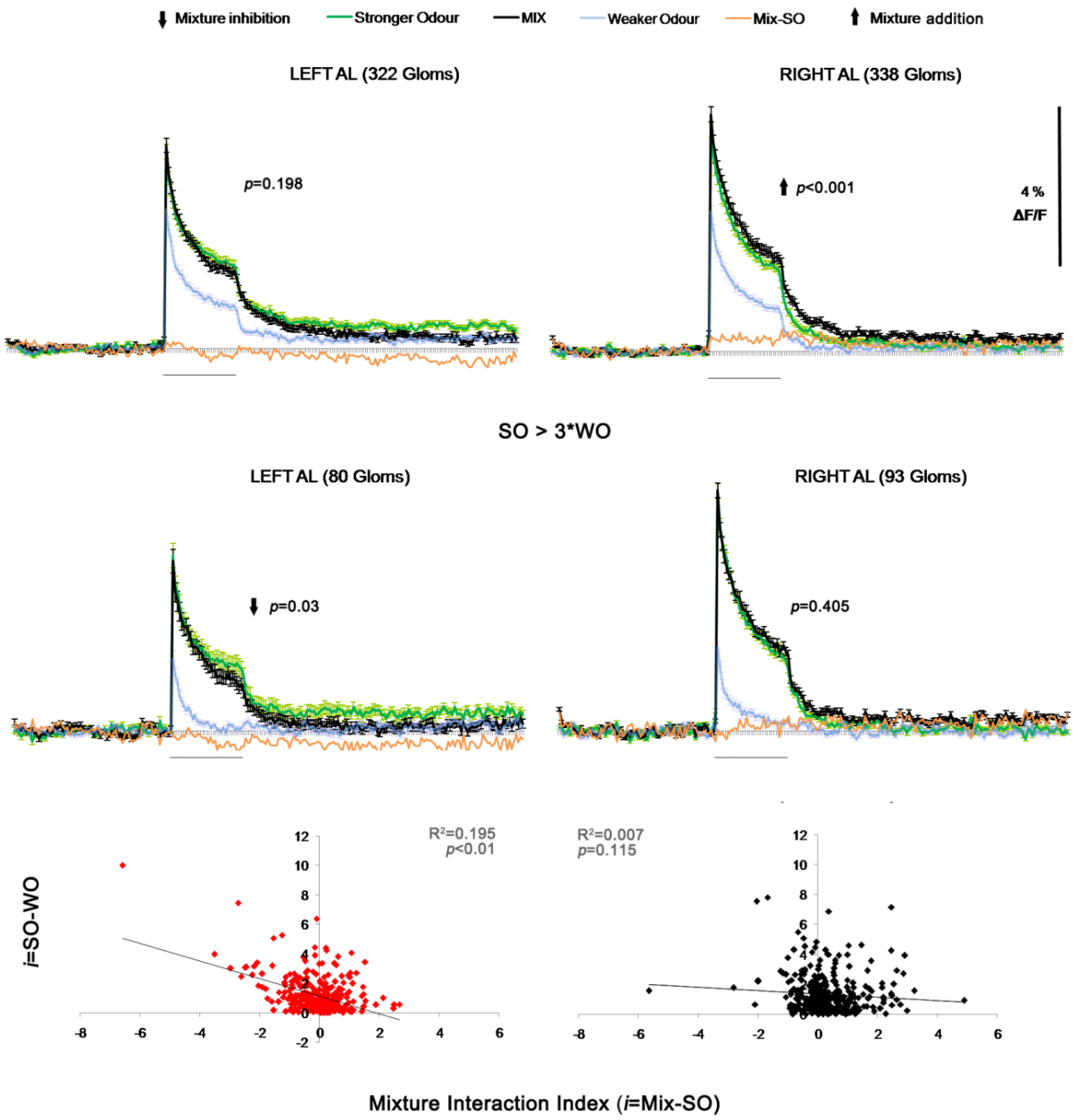

Fig. 4.2.4 Mixture processing differs between left and right AL. Upper Graph: Time course of the global PNs activity in all glomeruli that showed positive responses to both components, in the left and right antennal lobes. **Middle Graph:** Time course of the global PNs activity in the left and right antennal lobes Averaged activities are shown (mean±SEM) only for those glomeruli where SO ≥ 3*WO. For both Upper and Middle Graphs averaged activities are shown (mean±SEM). Black lines correspond to the mean mixture activity; green and cyan to the Stronger Odour and Weaker Component respectively; orange line is represents the difference between Mix-Stronger value. left panel: left antennal lobe, 322 Glomeruli; right panel: right antennal lobe, 338 Glomeruli. *x* axis: time (s); *y* axis: PNs activity (ΔF/F %). **Lower Graph:** Similarities among interaction index (*negative values indicate mixture suppression; positive values indicate additive mixture processing*) and index of distances between odour component in the left AL (red dots: 322 glomeruli) and in the right AL(black dots: 338 glomeruli).Only in the Left Antennal Lobe a correlation between two Indices was apparent. SO: Stronger Odour; Mix: Mixture; WO: Weaker Odour

We next asked whether this left-sided dependence between mixture suppression and component-difference at glomerular level is also visible at antennal lobe level, i.e. when averaging the glomerular response for each antennal lobe. In both groups of glomeruli (all glomeruli and glomeruli with 3 times larger response to the stronger component that to the weaker component). Averaging the glomeruli within each side, we still found mixture interaction differences between right and left AL, both considering all glomeruli (Fig.4.2.5; right AL additive mix interaction t(64):-2.429; *p*=0.018;no effect in the left AL t(32)=0.810 p=0.424) and the subset of glolmeruli with high difference (3*) between components' responses (a statistical trend between sides : *t*(56):-1.820; *p*=0.074 and a statistical mixture suppression only on the left AL *t*(29)=2,203; *p*=0.036; Fig. 4.2.5).

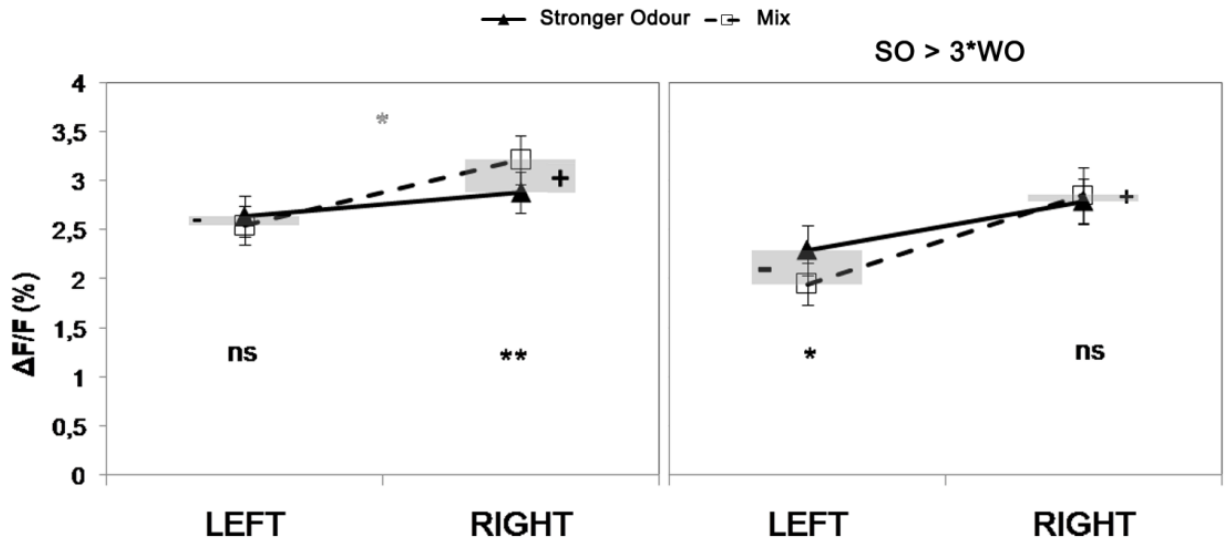

Fig. 4.2.5 Left and right AL differ in sign and degree of mixture interaction Averaged Stronger Odour (black triangles)) and Mixture (Mix, open squares) responses in each antennal lobe ±SEM (*N*=33 per side) calculated in those glomeruli that showed positive responses to both mixture components (left panel) and or those glomeruli which showed the Stronger Odour (SO) ≥ 3* Weaker odour (WO). Gray rectangles indicate the value of the averaged interaction index (calculated as the difference between mixture responses and SO), signs stands for direction of interaction (+: additive interaction; - : suppression). Gray asterisks indicate differences between sides in the interaction indices. Black asterisks and 'ns' represent the statistic within each side (* p<0.5; ***: p<0.001, 'ns': not significant).

Discussion

Using *in-vivo* calcium imaging of selectively stained PNs, we found qualitative difference in odour representations and mixture processing between left and right antennal lobes in honeybees, *Apis mellifera*. The same population of projection neurons, were stained and imaged either on the left or in the right hemisphere of the bee brains. The odour coding at the level of the whole AL was analyzed in the two sides when 3 single odorants were presented alone or as a binary 1 : 1 mixture of two of them.

These results contradict conclusions from previous studies which investigated whether the honeybee ALs signal was symmetrical between sides (Galizia *et al*., 1998; Sandoz *et al*., 2003). A first analysis comparing left-right AL activity showed the signal was indeed symmetrical between sides (Galizia *et al*., 1998). However, the investigation was conducted comparing correlation between sides through pixel-based activity, leading to a gross-scale analysis. A second study comparing activity patterns between sides within each individual failed to find any difference both in odour-evoked activity and in the Euclidian distances (Sandoz *et al*., 2003). However, both the latter studies imaged ALs using bath staining technique, for which it's not clear what is the contribution of what class of cells during signal acquisition, though receptors neurons are considered to be the most abundant contributors (Galizia and Vetter, 2005). Therefore, the so far supported bilateral symmetry might represent symmetrical neural coding other than PNs signals, and the left-right difference which we found might be due to left-right difference in processing within the AL network rather than left-right difference in ORN activity.

Higher discriminatory power of odor representations in the right AL

Along the olfactory processing, the AL leads to increase separability between odorants in the PNs response maps, compared to the input coding (Deisig *et al*., 2010; Bhandawat *et al*., 2007; Wilson *et al*., 2004) This separability of odour representations has been demonstrated to be fundamental for odour perception and discrimination at the behavioural level in honeybees: honeybees capability to discriminate between two odours (by testing their odour generalization after classical odour-sugar conditioning) corresponds to the distance between glomerular response pattern in the AL odour

(Guerrieri *et al*., 2005, Szyszka *et al*., 2011). Thus, the higher distance between odour representations which we found in the right AL might provide the bee with higher discriminatory power that could be exploited for odour discrimination tasks. On the other hand, the lower inter-odour distance in the left Al might be exploited for odour generalization tasks. Functional compartmentalization of different tasks in the left-right sides has reported both in vertebrates and invertebrate species (see for a Review Rogers *et al*., 2013; Frasnelli *et al*., 2012a) optimizing neural capacity. Specializing one side might be a further advantage when processing two tasks at the same time (see for instance Rogers *et al*., 2004; Piddington and Rogers, 2012. Kanwal *et al*., 2012; see for a review Rogers *et al*., 2013).

Asymmetry in mixture processing

We found a left-right difference in mixture processing: Inhibitory mixture interactions occurred mainly in the left antennal lobe, while additive mixture processing occurred mainly in the right antennal lobe. Moreover, in the left antennal lobe inhibitory mixture interactions increased with increasing difference between the components response strength, while in the right antennal lobe there was no correlation between component response difference and the sign of mixture interaction.

At the level of the antennal lobe blends processing may be either analytic (elemental), enhancing the information about the individual components or synthetic (configural), rising as a new information (Lei and Vickers, 2008). A specialization between the two antennal lobes may be postulated for coding mixture information, the right antennal lobe being specialized for analytic processing (segregating mixture into components identity) and the left AL tuned for synthetic configuration that tagged mixture itself with no information about its components (Wiltrout *et al*., 2003; Linster and Cleland 2004). This hypotheses fits nicely with the differences between antennal lobe we found in the odour distances between mixture and their components, with the right AL showing higher odour distances between mixture compounds compared to the left AL (see fig. 4.2.3). Whether this is the case also at the perceptual level (i.e. with the right side being able to segregate better the components of a mixture) it has to be unraveled, together with the potential role of interglomerular inhibition that has been postulated to affect blend coding (Linster and Cleland 2004). It has to be noted that a recent study on odour evoked activity at the level of the Projection Neurons in the bee AL, revealed a specific increased mixture analytic processing in specific condition of the mixture presentation

(Stierle *et al*., 2013), the same condition triggers better capacity of bees in segregating between mixture and their components at the behavioural level (Szyszka *et al*., 2012).

Function of asymmetric odor processing

The functional distinction in odour coding between sides might enhance the capacity of processing odour information in honeybee. Besides left-right specializations, compartmentalization of odour information is indeed a feature within the first olfactory neuropil (Krofczik 2008; Yamagata *et al*., 2009; Galizia *et al*., 2012; Carcaud *et al*., 2012; Brill *et al*., 2013). In the antennal lobe, in fact, half of the glomeruli are innervated by m-APT PNs and the other half by l-APT. Both types of PNs response to the same odors but differ in their inter-odor distances, odour response strength, mixture interaction and response latencies. It has been suggested that this within-AL compartmentalization has advantages for odor processing allowing parallel coding information. On the other hand, the result we found might sharpen per se the difference between hemispheres triggering to an emergent enhanced discrimination between sides rather than within sides. To evaluate this assumption, a within-individual comparison of PNs activity is needed allowing for a more detailed analysis at the glomerular level. Moreover, behavioral experiments to test odour elemental/configural discrimination in bees with one-antennae are required together with pharmacological experiments that would also examine the neural substrates standing for this asymmetrical coding. Interestingly, picrotoxin, a GABAa receptor antagonist, has been found to increase generalization of similar odours in insects and increase discrimination threshold (Stopfer *et al*., 1997; Mwilaria *et al*., 2008). Equally, an impairment in mixture processing has been demonstrated when GABAa was blocked in the antennal lobe of honeybee (Choudhary *et al*., 2012) with treated bees not able to distinguish at the behavioural level between mixture and its components. Whether odour discrimination is optimized behaviourally in the right antennae and through which mechanisms has to be investigated both behaviourally and neurophysiologically, opening the possibility for new steps towards revealing and understanding the asymmetrical neural coding in a key insect model.

Chapter 5 Left-right antennal asymmetry during honeybees' social interactions

Summary

Recent literature and previous chapters showed a difference between sides in honeybees favouring the right antenna both for detection of odours and their neurophysiological representation at a central level. Although at the behavioural level the right antenna has been shown to be specialized for the recall of specific short-term odour memories, no studies have investigated honeybee olfactory asymmetry under more ecological conditions. This chapter aims to address this question observing whether any directional biases in antennal use are apparent when two bees interact with each other. Interestingly, our results showed the right antenna being significantly more related to context-dependent social interactions.

Introduction

As illustrated in previous chapters, the quest for neural correlates of lateralized behaviours is one of the fascinating still open issues in the study of brain asymmetries (see Concha *et al*. 2012; Chapter 1.2). On the other hand, it's worth noting that a cerebral lateralization might or might not trigger an apparent asymmetrical behaviour (discussed in Rogers *et al*., 2013). Moreover, the majority of studies assessing functional lateralization have been conducted by testing single individuals on specific tasks under laboratory conditions (for a review on vertebrates Vallortigara *et al*., 2011; invertebrates: Frasnelli *et al*., 2012a). Despite the observation of these asymmetrical behaviours in single organisms, the resulting occurrence in a more ecological context is not obvious or present in interactions between individuals. Taking the fruit fly *D.melanogaster* as an example, a clear asymmetrical pattern of antennal activation during odour tracking has been shown, with the left antenna being more activated compared to the right one (Duistermars *et al*., 2009). What this really means from the ecological point of view and how it is evident in everyday behaviour (if at all) is completely unresolved. Among vertebrates, however, there are a few examples in which a more natural context allows the observers to better understand the valence of lateralized behaviours and their evolution. Recent studies on primates might represent a

nice example. Great apes show right-handedness at the population-level in using tools, although this has been debated over the last decades (see Vallortigara *et al*., 2011). Only recently, it has been suggested that there is a common bias of unilateral hand actions and this is strongly dependent on social context (Forrester *et al*., 2011; Forrester *et al*., 2012). Gorillas, as do chimpanzees, show right-handedness (left-hemisphere dominance) for hand actions involving touching of non-animate objects rather than animate target (i.e. strongly context-specific) (Forrester *et al*., 2012). Besides providing a more specific delineation of what is shown to be lateralized, these studies are important also for providing evidence in the understanding of the handedness as an evolutionary inherited tract in different species (Forrester *et al*., 2013).

Visual asymmetries and lateralized social recognition in vertebrates is another striking example that links individual observations together with their social aspects (for a review see Rosa-Salva *et al*., 2012). It's well described that both humans and apes show right hemisphere dominance for face recognition (Rogers *et al*., 2013; Rosa-Salva *et al*., 2012), as well as an asymmetry in the time looking at face stimuli, with the left part of a face stimulus triggering higher fixation rates in the observer (Guo *et al* 2009). Sheep show the same right hemisphere superiority also in electrophysiological recording of specific face cells in the temporal cortex: there is an advantage (only in latency) of the right hemisphere particularly for fine discrimination (Peirce and Kendrick, 2002). In chickens the asymmetrical ability in the left- and right-eye system to discriminate between a familiar social companion and an unknown one has also been shown (Vallortigara and Andrew, 1991). Covering one eye at a time, Vallortigara and Andrew (1991) showed the left-eye choice is comparable to the one expressed by binocular chicks, linking the right-hemisphere (due to almost complete crossing of the optic nerve fibers) to a better discrimination between companion and strangers (see also Vallortigara and Andrew, 1994). Interestingly, the results presenting a hemisphere specialization for social discrimination fit also nicely with an ecological advantage of lateralized chickens in social groups. It has been demonstrated, in fact, that lateralized individuals develop a social hierarchy that is more defined compared to non-lateralized ones (Rogers & Workman, 1989).

In honeybees, a different side specialization for odour detection and for recalling odour memories has been demonstrated (Letzkus *et al*., 2006; Rogers and Vallortigara 2008; Anfora *et al*., 2010, see Chapter 1.2.2). However investigations have to date been conducted on single restrained individuals only. Considering honeybees' eusociality

(Michener, 2000) and their asymmetry in odour processing and memory (see Chapters 1.2 and 4.2) here we wanted to assess whether any difference in antennal use may be revealed in the pattern of interaction between two honeybees.

Methods

Subjects

Honeybee foragers (*Apis mellifera* L.) were collected between 9.00 and 10.00 a.m. at the entrance of two hives placed in Rovereto (Trento, Italy). Honeybees were individually collected in single vials (60mL) and taken to the laboratory where bees of each hive were randomly divided into 3 groups. To be sure that bees underwent the same treatment all of them were anesthetized, cooled (at 4°C) till immobilization for about 10 min. After that, in one group of bees the left antenna was cut at the base of the scape, in a second group the right one was removed and in the third, the control group, both antennae were left intact. Immediately after bees recovered from the anaesthesia, they were fed with 10-20 μl of 50% sugar solution in water (w:w) in order to control for physiological differences in social interactions due to different sates of satiations (see Wright *et al*., 2012). Bees were left in single vials for 2h at 25°C and 60% humidity before test began.

Apparatus

Tests were conducted in a self-costumized arena,modified from the one in Wright *et al* (2012) as follows: Two petri-dishes (9cm diam x 1.5cm depth each, Sigma-Aldrich) were juxtaposed, fixed together with transparent tape on the lids and used upside-down (lids touching the ground). At the point of conjunction, in each petri-dish openings were incorporated (0.7x1.3cm) both in the mobile upper parts and the lower parts which were fixed to each other. When all the openings were juxtaposed, bees were able to move independently around in both of the petri-dishes (see Figure 5.1), while turning the upper parts and their openings away from the junction point, each petri-dish was a closed independent space. 7 symmetrical holes in the upper part of the petri-dishes allowed for air circulation (see Wright *et al*., 2012). The apparatus was put in a bigger arena made of white cardboard (diam 31 cm, high 35 cm) and covered at the top by white fabric to create a homogeneous environment.

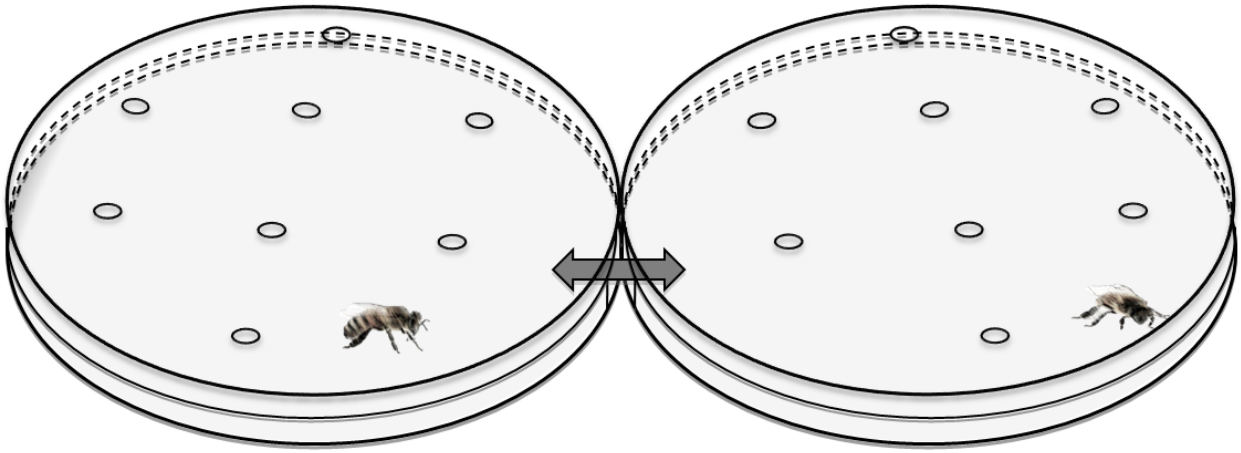

Fig. 5.1 The apparatus used in the test. Two petri-dishes were juxtaposed, upside down and the basal plates fixed to each other. Openings in both the upper and lower parts at the level of the junction point allowed for movements of the bees between the two compartments (indicated by the arrow). At the same time the two dishes could be separated by turning the upper lids away from the junction (see text for details).Small circles indicates holes for airs.

Procedure

Dyads tested (total: *N*=70) contained bees from the same hive (nestmates) or from two different hives (non-nestmates). Within each of these conditions 3 groups of different dyads were tested (6 types of dyads in total): pairs with only their Left Antenna in use (i.e., right antenna removed, LA); with only their Right Antenna in use (left antenna removed, RA) and with Both Antennae intact (control, BA). From 11 to 14 dyads were tested for each of the 6 conditions. At each test, type of dyads tested was randomly chosen, each bee was put in a single petri-dish with upper openings turned away in order to let the bee acclimatize in a single petri for 5 min. After 5 min, the openings of the upper parts of the arena were juxtaposed so that the bees were free to interact for 5 min. Both pre-tests and tests were recorded for each pair with a video-camera (LifeCam Studio for Microsoft 1080p HD) placed at 15cm above the petri-dishes.

Behavioural coding and Statistycal Analysis

Off-line coding of behaviours was done using behavioural scoring already used for honeybees (Richards and Packer 2010; Wright *et al*. 2012). Each pair was considered statistically as a unit as we were interested in differences in amount and kind of social interactions between different pairs rather than between individual of the same dyads. We measured latency to contact, number of Proboscis Extension Reflexes (PER), C-

postures (when the bee bends her abdomen forming a “C”; see Peso and Richards, 2010), and mandibulations (number of times that bees open its mandibulae).

Dyads-behaviours were scored by three independent observers, in particular one was blind in respect to the type of pair. We compared the score of the latter observer in respect to the other two using Sperman’s correlation test, they were strongly correlated (Spearman correlation, all behaviours pooled: r=0.989, p<0.001; for latency: r=0.998, p<0.001; for number of PER: r=0.923, p<0.001; for number of C-responses r=0.915, p<0.001).

We used non-parametric statistics. We checked for independence of measures (Pearson’s correlation test). For each behaviour coded and for each condition, Mann-Whitney U-tests were used (2 tails in every behaviour except for PER, since the direction of that was predicted on the basis of previous literature).

Results

To investigate differences in left/right antennae use during bees interactions, we scored different social behaviours (latency to contact; numbers of PER; number of C-responses; number of mandibulations) in pairs of bees coming either from the same colony or from different colonies. For both these conditions we measured the occurrences of behaviours in three different types of dyads: with LA only, RA only and BA as a control. The measures were all independent as revealed by Pearson’s correlation analysis with a trend for aggressive behaviours (PER-Latency: r=-0.197, p=0.102; Latency-C-responses: r=-0.129, p=0.288; Latency-Mandibulation: r=-0.193, p=0.110; PER-C-responses: r=0.052, p=0.669; PER-Mandibulations: r=-0.017, p=0.889; C-responses-Mandibulations: r=0.213, p=0.077). Results are shown in Figure 5.2 as mean occurrences of behaviours (±SEM) in different types of dyads.

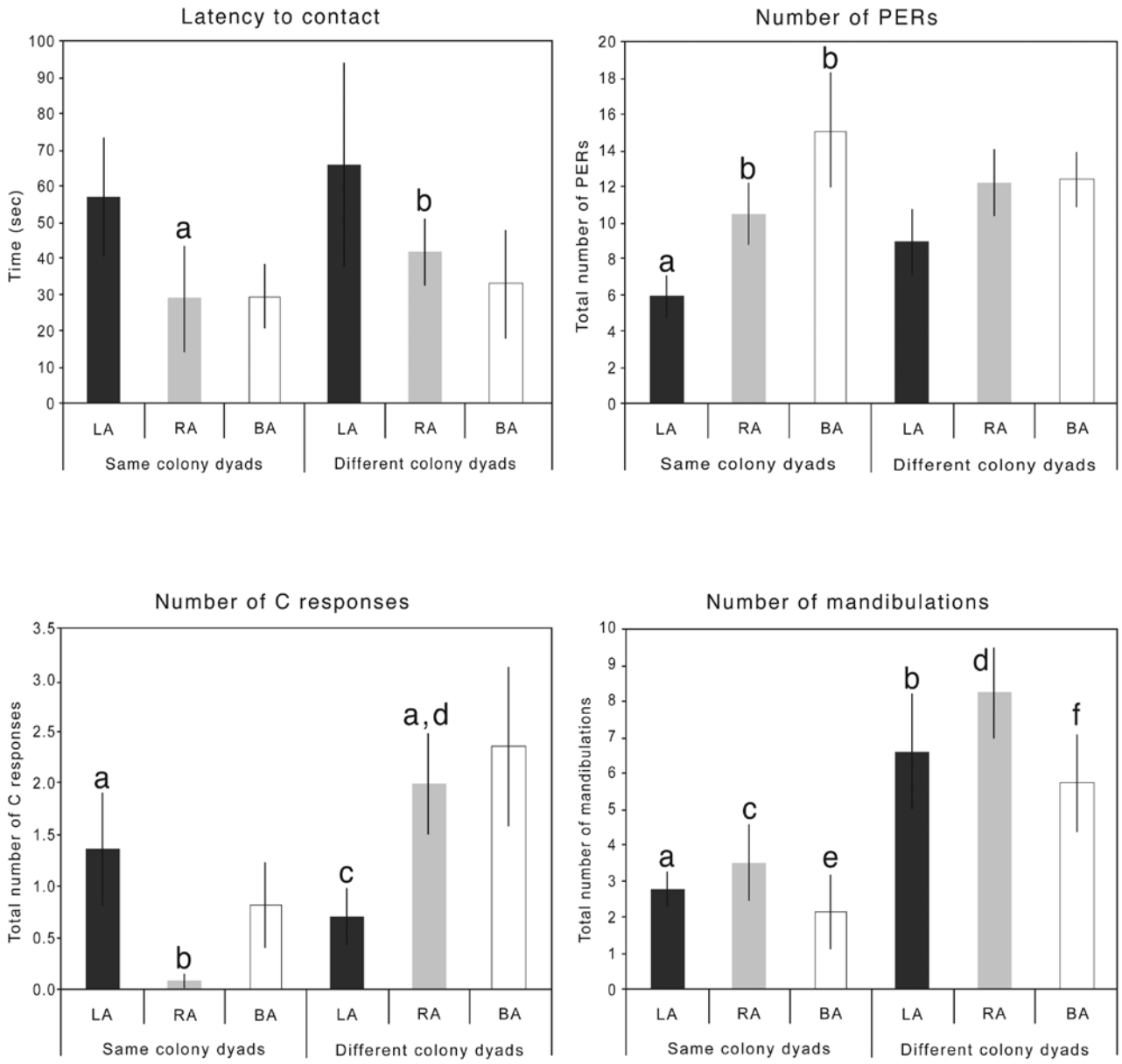

Fig. 5.2 For each behaviour scored, means (±SEM) calculated for the 5 min test are shown: Latency to contact (Upper left), number of PER (Upper right), number of C- responses (Lower left) and mandibulations (Lower right). For each of these measures results of dyads coming from the same colony and from different colonies are presented (left and right part of each graph, respectively). LA: bees with only their left antenna in use (black bars); RA: bees with only their right antenna in use (gray bars); BA: bees with both antennae in use (white bars). Letter 'a' on bars indicates a statistically significant difference to bars marked 'b'; Letter 'c' indicates differences to 'd' and the same was for 'e' and 'f'($p<0.05$).

Comparing nestmates dyads, differences on the basis of antenna in use are apparent. In particular, among pairs coming from the same hive, LA and RA showed a difference in number of PER, C-postures, and a trend for latency. LA, showed a lower number of PER (U=52.5, p=0.036, N=27), a trend for higher latency (U=54, p=0.073, N=27), and

an increased number of C-responses (*U*=43.5, *p*=0.006, *N*=27) compare to RA. While BA did not show differences in PER nor in latency to contact with RA (Latency: *U*=56, *p*=0.396, *N*=24; *U*=53.5, *p*=0.296, *N*=24), and a trend for reduced number of C-responses in RA (*U*=50, *p*=0.079, *N*=24). Numbers of PER were significantly different between BA and LA (*U*=35.5, *p*=0.011, *N*=25).

Comparing non-nestmates dyads, C-responses were different between LA and RA, with LA bees performing fewer C-postures than RA (*U*=28.5, *p*=0.05, *N*=21). In all non-nestmates dyads, mandibulation was higher in respect to nestmates dyads (BA: *U*=27, *p*=0.026, *N*=22; LA: *U*=36.5, *p*=0.047, *N*=24; RA: *U*=15.5, *p*<0.001, *N*=24).

Discussion

Aggressive interactions are significantly elevated in non-nestmate dyads

C-responses were more common in RA dyads of non-colony members than in the RA dyads from the same colony; moreover number of mandibulations increased significantly in all the nestmate dyads compared to non-nestmate ones. Based on comparisons of aggressive behaviour only, we can observe a difference between members and non-members of the same colony and thus infer occurrence of nestmate recognition in the tested conditions (Peso and Richards 2010).

Right antenna for social interactions?

We found for the first time an asymmetry between bees that interact and have only their right antenna intact compared to those that have only their left antenna intact. In particular, when nestmates are observed interacting, RA dyads have comparable latency, number of aggressive interactions and frequency of proboscis extensions as BA. In contrast, RA bees showed higher rates of PER extensions, fewer C-responses, and shorter latencies than LA bees. Surprisingly, when two bees from different colonies interact, LA showed fewer C-responses compared to LA of same-colony dyads, while RA increased aggression when the two observed bees were not same-colony members. Considering the asymmetry in C-responses and the general increased aggression rates towards non-nestmates, we might conclude that the right antenna controls social behaviour appropriate to context. Dyads using their LA, do not adjust their agonistic

behaviour (C-responses) according to the social context (i.e. a decreased aggression towards nestmates compared to non colony members).

PER scores included sampling the scent/taste of another bee and trophallaxis. In bees taste perception is important both during foraging and during social interaction (see for a review De Brito Sanchez 2012). Gustatory perception, allows nestmate recognition because cuticular hydrocarbons are not airborne soluble and gustatory detection might be the specific chemical channel for same-colony members recognition (De brito Sanchez, 2011). An asymmetrical frequency of PER between RA and LA might be read as a different amount of information on cuticle hydrocarbons and therefore be involved in the incongruent aggression responses of LA bees. This might be masked partially by gustatory sampling occurring without any overt behaviour. Gustatory perception in honeybees is, in fact, spread over the antennae, mouthparts and forelegs (Haupt, 2004). On the other hand, PER might express begging, playing behaviour, and trophallaxis, the latter in more than 95% of cases does not involve exchange of food and is, as for other PER, an aspect of non-agonistic social interaction (Korst & Velthuis, 1982). Given that PER score differed between LA and RA, further investigations on the nature of this asymmetry are needed. For example, by inducing a donor/receiver in each pair thus allowing for occurrence of trophallaxis and by assessing the social context dependency.

Although no lateralization has been found in mandibulation behaviours, our results show that at least three most important measures of social interaction, latency to contact, proboscis extension and C-responses, depend on use of the antennae in a lateralized way. The RA is therefore not only specialized for learning about new odours associated with food sources, but also for exchange of odoriferous information between same-colony worker bees and in control of aggressive responses between different-colony worker bees. It is also the use of the RA that motivates bees to approach and contact each other. Although the use of the LA does not cause bees to completely avoid each other, social behaviour performed is not context-appropriate. We can therefore hypothesize that this might be due to an inability by LA to distinguish between hive mates and bees from another hive.

Considering the right antennal lobe being specialized for increasing odour distances (see Chapter 4.2) the functional lateralization shown here fits well with right antennal dominance for discrimination and possibly favoured nestmate recognition.

The difference in the use of antennae shows a directional bias

Results described in this chapter revealed that a behavioural asymmetry in the use of the antennae occur at the population-level (i.e. the majority of the bees showed congruity in the direction of the bias). Therefore, these findings provide new evidence about the link between the evolution of directional asymmetries and social context (see chapter 1.1). On the other hand, considering the honeybee as invertebrate model for cognitive feats (see Menzel 2012; Giurfa 2013) it raises the question whether and how functional asymmetries are related to enhanced cognition at the individual and/or group level. In vertebrates there are some examples of enhanced cognition in lateralized individuals compared to non-lateralized ones (i.e. an advantage in performing a task; McGrew and Marchant, 1999; Gunturkun *et al*., 2000; Rogers *et al*., 2004) but whose generalized value is still strongly debated (see Rogers *et al*., 2013 for a discussion). As a result, the observations reported here open the door to investigation of lateralization or side biases in other forms of social behaviour of honeybees, including communication by dancing, which might also benefit from asymmetry of function.

CHAPTER 6 General Discussion

The results reported in the previous chapters confirmed and extended the evidence for lateralized behaviours in Apoidea. We were able to demonstrate that another species of the superfamily Apoidea, *B. terrestris*, showed a behavioural asymmetry in short-term olfactory memory. Bumblebees, trained with only one antenna to extend their proboscis in association to a sugar reward (PER protocol), showed a different performance in the memory recall depending on the antennae was used. One hour after training, bumblebees with only their right antenna in use showed significantly better performance (measured as number of PERs when the odour was presented without a food reward) compared to bumblebees with only their left antenna in use. This right side dominance occurred at the population level, i.e. the favoured side was consistent in the majority of the individuals thus presenting a direction in the population. Interestingly, the direction of the preference was the same as that showed by *Apis mellifera* in the PER task (Rogers and Vallortigara, 2008; Frasnelli *et al*., 2010a; Anfora *et al*., 2010). Given that in honeybees the behavioural asymmetry was associated with morphological and electrophysiological differences at the level of the antennae, we assessed whether a similar peripheral lateralization was visible in the primitively eusocial bumblebees. For each individual we compared right and left electrophysiological responses of the antennal nerve to biological relevant odours (a floral odour and to an aggregative pheromonal component). Bumblebees showed an individual level asymmetry when the whole antennal nerve depolarization was compared between individuals in the majority of the individuals tested. When we compared the number of olfactory *sensilla* between antennae, we found a difference in the number in only one type of olfactory *sensilla*, namely *sensilla* trichodea A. The asymmetry in olfactory *sensilla* and the lack of asymmetry in EAG responses raises a general question as to where the behavioural bias comes from along the olfactory neural pathway. We tried to investigate this aspect, in the model *A. mellifera*. In this species, a population level asymmetry at the behavioural level has been shown (with a right side favouring short term odour memory and left-side favouring long-term one, Rogers and Vallortigara, 2008). Due to this shift in antenna dominance during odour recall, a simple lateralization in the electrophysiological asymmetries (right bias in naïve individuals; Anfora *et al*., 2010) might not solely account for olfactory asymmetry. Consequently, we decided to investigate whether any side-bias existed at a central

level, in the first olfactory neuropil of the honeybee brain, the antennal lobe. We found that when functional units that formed this neuropil, namely the glomeruli, were compared between sides in naïve individuals, no morphological differences were apparent. We were able to show, that a subset of identified glomerul involved in the coding of odour that triggers lateralization, were volumetrically symmetric between sides. This was shown using a staining technique coupled with two-photon microscopy that allows measuring absolute volumes without extracting the brain. Concurrently, we found that odours which activated mostly the selected glomeruli did not lead to a lateralized memory recall by default. There exists an odour dependency of the lateralization in olfactory memory performance. We hypothesized that floral odours trigger lateralized memories and green-plant volatiles do not. Furthermore, we used a standard immunohistochemical procedure for measuring glomerular volumes in bees that underwent a training for long-term olfactory memory. For this second morphological study we chose a wider number of glomeruli compared to the one conducted in naïve organisms being able to consider also those glomeruli that are normally not accessible using conventional functional microscopy (Galizia *et al*., 1999a; Galizia *et al*., 2012; Galizia and Menzel, 2001). We did not find any side difference in trained bees although glomerular-specific differences after learning were apparent. In particular we noted a shrinkage in glomeruli that were slightly activated by the odours during *in-vivo* recordings. In Chapter 4 we presented a method for *in-vivo* functionally imaging of the bee antennal-lobe that produces highly resolved odour-dependent activity maps both in space and time. We were able to obtain *in-vivo* responses of the projection neurons, the output neurons of the antennal lobe, improving also the imaging depth and resolution. We then wondered whether the odour activation map would differ between sides, to this aim we used data from published work using conventional microscopy technique. Besides a symmetrical glomerular pattern of responses, the right antennal lobe, presented an increased odour separability in the *in-vivo* representation of odorants. When odour distances and mixture interactions were analyzed, the right antennal lobe showed higher distances between odours compared to the left and an opposite mixture interaction between sides was evident. Finally, we investigated for the first time bees with only one antenna in use interacting with each other, both nestmate pairs and non-nestmate dyads. We were able to find antennal-dependent differences in the social behavioural repertoire when two honeybees interacted socially. In particular, left-antenna-only bees showed an impairment in social behaviors (with higher aggression)

compared to bees that have only their right antenna in use, the latter did not differ from control bees (with both antennae in use). The results showed a possible context-dependent impairment in left-antenna-bees that might be linked to a right antenna dominance for better discrimination.

Brain-Behavioural lateralizations are common traits

Along these chapters we showed novel traits among Apoidea that are lateralized, i.e. the short-term olfactory memory in *B. terrestris* and social interactions in *A. mellifera* together with a difference in coding information in the brain of the latter species. These results showed once again how brain-behavioural asymmetries are widespread among the animal kingdom and are not a unique trait of vertebrates. Only in the last decade, invertebrate lateralization started to be investigated proving that asymmetry is a trait present also in simpler (in term of numbers of neurons) brains (Frasnelli *et al*., 2012a).

The evidence of their occurrence is already a relevant a indication about its evolutionary value. It has been stressed and discussed (see Rogers *et al.* 2013) how the multiple appearances of brain asymmetries in such distant taxa demonstrate the high-fitness values of this trait. Benefits of having a lateralized brain, have been shown both in vertebrates and in invertebrates (Pascual *et al*., 2004; Rogers *et al*. 2004; McGrew Marchant, 1999; Sovrano *et al*., 2005 and Bisazza & Dadda, 2005). Advantages are evident in terms of acquisition of function and regarding the optimization of neural pathways. For instance, it was hypothesized that in owls, ear asymmetry evolved at least five times independently to adapt the individual for a localization along the vertical line through an ear comparison (Norberg, 1977). Lateralization may generate a new gain of function as in the latter example (the new vertical component comparison) and/or a more efficiency in specific tasks (*D. melanogaster* (Pascual *et al*., 2004); *C. elegans* (Wes and Bargmann, 2001); chicks (Rogers & Workman, 1989), fish (Sovrano *et al*., 2005), and chimps (McGrew and Marchant, 1999). Again, an advantage might also be obtained in solving problems involving two tasks in parallel (see primates (Paddington and Rogers, 2012); chicks (Rogers *et al*., 2004) and fish (Dadda and Bisazza 2006)).

Considering the behavioural asymmetries we found in bumblebees, it has been hypothesized that having one side (the right one in particular) specialized for short-memory tasks, i.e. for acquisition of new odours, might represent an advantage during foraging (Rogers and Vallortigara, 2008). This hypothesis was firstly put forwards for

honeybees (Rogers and Vallortigara, 2008) where the left antenna was shown to be specialized for long-term memory. Thus, during honeybees interaction tests with a single antenna in use (Chapter 5) we might have expected a left antenna increased performance for nestmate discriminations (due to the supposed left side specialization for odour memories). This is not what we observed, but we rather found a better discrimination in bees with the right antenna only. This might be related to the specialization of the right neuopil for odour separability (Chapter 4.2) a trait that was never reported so far at the behavioural level (with single antenna in use and PER bioassay). These considerations point out two main, still open issues in the study of brain asymmetry: (i) how many and how much different biased circuits are present in the same individual (discussed in Concha *et al*., 2012) and (ii) whether both advantages and occurrence of lateralization at the behavioral level are strictly task-dependent (see Rogers *et al*., 2013). Recently, Dadda and colleagues showed in the fish *G. falcatus* how the advantages associated with being lateralized are strongly dependent on the task that has to be performed (Dadda *et al*., 2009). The authors presented evidence showing how lateralization might hinder when the task required hemisphere rapid communication, for instance. Dadda and colleagues were able to prove that lateralized fish performed poorly compared to non-lateralized ones when they had to generalize a task in a symmetrical way, e.g. in a modified paradigm of the bisection test as well as when they had to choose between two shoal of different biological values with the two eyes (Dadda *et al*., 2009). Thus, also at the individual level, having a bias not necessarily improves by default a performance but the asymmetry found has to be conceived and investigated in a wider context-dependency and not necessarily as evolutionarily driven (see also Vallortigara and Rogers, 2005).

Does alignment stand for sociality?

The brain-behavioural lateralizations we presented here (short-term olfactory memory in bumblebees (Chapter 2), a context dependent interaction in honeybees (Chapter 5) and differences in odour neural coding (Chapter 4.2) showed a similar direction of bias in the majority of the individual tested (i.e. they appeared at the population level). These outcomes tighten the link between social context and population-level biases. In the perennial eusocial honeybee a directional asymmetry in the recall of olfactory memories was already demonstrated, with the right antenna being specialized in short-term olfactory memory (Anfora *et al*., 2010; Rogers and Vallortigara,

2008; Frasnelli *et al*., 2010a). The same bias was revealed here for the first time, in the primitively eusocial bumblebees. It has been shown with a mathematical model how the evolution of population asymmetries might be a stable strategy in social contexts in which asymmetrical individuals have to cooperate among each other (Ghirlanda and Vallortigara, 2004; Ghirlanda *et al*, 2009). Moreover, it was pointed out that in social context the direction of asymmetry should present frequency-dependent polymorphisms on the basis of what has a higher fitness value between synergistic and antagonistic behaviors in the same population (Ghirlanda *et al*., 2009). Thus the olfactory asymmetries at the population level we found might be considered another evidence of the correlation between sociality (i.e. cooperation needed in a group) and alignment of the asymmetry in a population. We used the same protocol used in other laboratories for assessing the same question in a different species (see Frost *et al*., (2012) for eventual caveats in different PER protocols). It has been demonstrated in fact that *A. mellifera* as well the Australian social bees (3 species) showed a population-level lateralization, which is not evident in the solitary bees *Osmia cornuta* (Anfora *et al*., 2010; Frasenelli *et al*., 2012b). For their annual colonies and primitive eusociality, *B. terrestris* is a species in between the perennial eusocial *A. mellifera* and the solitary *O. cornuta* (Goulson 2003; Michener, 2000). It is interesting that the outcome at the level of the behavioral task in bumblebees shows such a lateralization as strong as that found in the perennial eusocial honeybee. Nonetheless, the peripheral detection of odorants seems not to show the same population-level dependency.

A puzzling and interesting question arises about the evolutionary link between peripheral and central asymmetries. Data so far are suggesting the more social is a group, the more lateralized is it at the population-level (both centrally as well as peripherally). It has also to be stressed that whenever a population-level asymmetry was not detected in a social species or a population-level bias was found in a non-social one, it does not necessarily need to be a disprove of the evolutionary stable strategy of population level asymmetry. A false negative is highly conceivable considering for instance the evolution of solitary individual as evolutionary derived from a common social ancestor (Vallortigara and Rogers, 2005; discussed in Frasnelli *et al*., 2012a). Some species among helictinies tropical bees, for example, have been shown to derive from social species (Wcislo and Danforth 1997) being able to interact with other solitary individuals in a context-dependent successful way, at least in *L(D.) figuresi* species (Wcislo, 1997).

Neural correlates of behavioural asymmetries in honeybees

Besides the differences in sensitivities between antennae in honeybees (Anfora *et al*., 2010; Frasnelli *et al*., 2010a) we did not find any volumetric differences in the functional unit that formed the first olfactory neuropil of the honeybee brain. There is evidence of behavioural asymmetries that showed a correlated morphological asymmetry of neural circuitry at a gross-scale (see for instance Koshiba *et al*., 2003; Gunturkun, 1997). Recently Concha (Concha *et al*., 2012) described two different systems by which an asymmetry can take place in the nervous system, the so called type I in which an asymmetry comes up from a bilateral equivalent circuits (Koshiba *et al*., 2003; Gunturkun, 1997; Shinohara *et al*., 2008; Young and Govind, 1983; Schotten *et al*., 2011) and a second type in which a specialization is designed breaking the bilateral structures of the nervous system itself (Concha *et al*., 2000; Pascual *et al*., 2004). *D. melanogaster*, for instance, represents one of the example of the latter type with the presence of an asymmetrical body in the central complex of the brain which correlates with the capacity of the flies' long-term memory to be formed (Pascual *et al*., 2004). The honeybee brain has been described previously as symmetrically shaped (Winnington *et al*., 1996), we were anyway interested both in potential laterality after learning and in exploiting a more precise method for possible detection of asymmetry.

Though no side-bias was revealed in volume, we are anyhow far from saying that no differences exist at all in the bee's glomeruli between sides. The difference in the rat's hippocampi to encode for long term-memory in the left-side, for instance, is due to a different amount of neurotransmitter and size of synapses between sides (Shinohara *et al*., 2008; Kawakami *et al*., 2003). Moreover, though a symmetrical functional activity between the antennal lobes has been so far described (Galizia 1998; Sandoz 2003) we found a difference in encoding the olfactory information in this neuropil. There are few examples that are able to show an asymmetrical bilateral processing in the nervous system when a symmetrical stimulus is presented to the subject. Among these, studies conducted on sheep and in bats are of particular interest (Peirce *et al*., 2002; Kanwal, 2012). They both showed a difference in latency between sides through electrophysiological recordings in single neurons, in particular in bats, a difference in response to specific classes of sounds between sides has been shown (Kanwal, 2012).

In honeybees for the first time we demonstrated a lateral-bias not at the level of single recordings but in the three-dimensional odour evoked maps and their neural

representations. It is interesting to speculate about possible scenarios. Is it a gain of function and specialization of one side or rather a new parallel code (of the two lobes) when incoming information has to be compared? We might suggest that asymmetrical specialization but without complete loss of function of the other side might be the most suitable scenario for increasing fitness, in particular when lateralization is established to be made of symmetrical equivalent structures (see Concha *et al*., 2012).

Future Outlook

The appearance and evolution of an asymmetrical aligned trait in a population is certainly an intriguing open-issue in the field of brain lateralization. Further data to evaluate the model by Ghirlanda and colleagues (Ghirlanda *et al*., 2009) are necessary, in particular focusing on closed species of superfamily Apoidea. It would be worthwhile to investigate the correlation of frequency-dependent left/right polymorphisms in a population and compare them among different social structures. It is fascinating how the alignment in a direction occurs in other systems, having as a result optimization and more coordination among subjects. Changing scale, a molecule's conformation within a complex structure seems to follow nicely the same theoretical principles as cited above. When a molecule has to arrange, two possible scenarios come up, namely the left or right chiral forms, two different versions that are mirror images of each other. It has been demonstrated that (i) even with a symmetric proportion of both enantyomeric forms in a crystal a final "pure" crystal form with just one of the two enantiomers will turn out necessarily (Viedma, 2005) and also (ii) that chiral asymmetry and its alignment among different molecules can emerge from symmetrical conditions (Edlund *et al*., 2012). We are dealing with a different process but the common optimization of the system (either in term of low energy conformation in a molecule or the coordination in a superorganism) it is in both cases consistent with the congruency of a direction (either left/right, it is not relevant which one of the two). Of course much is different, but certainly the issue poses fascinating input to the research in "the progressive enantiomeric amplification of a certain handedness" (from Viedma, 2005) or rather the evolution of a progressive alignment towards a certain handedness

. In particular, behavourial asymmetries in perennial eusocial bees inside the colony environment (where the need for optimization of cooperation is maximized) should be deeply investigated. Ants, for instance showed a population level asymmetry in foraging trips coordination, with the majority of the studied species keeping right in

their “streets” (Heuts *et al*., 2003). It might be interesting to study honeybees interactions between scouters and foragers for instance at the entrance of the hive where nestmate discrimination should be overt. Contacts between organisms inside the nest (for instance body-contact side preference) would be worth investigating. Moreover the evaluation of asymmetries at different developmental scales would be worthwhile in honeybee, this species, in fact, is a well-known model presenting age polyethism (Kolmes, 1986).

With respect to the neural mechanisms underneath behavioural asymmetries, further studies are needed for both disentangling odour memory biases and odour discrimination differences between sides in honeybee. At the level of the antennal lobe, it might be worthwhile testing for instance whether a difference in the amount of the neurotransmitters either excitatory or inhibitory, would exist (in particular due to a difference in odour mixture interactions between sides presented in Chapter 4.2). In addition, two-photon microscopy would allow a higher detectability of any possible difference between sides, for instance in the temporal domain at the level of glomeruli or even single cells. Temporal differences in the electrophysiological responses of equivalent bilateral neurons have been shown in sheep with the left side specialized for face recognition (Peirce *et al*., 2002). Two-photon would exploit the benefit of 3D imaging with a high temporal resolution in specific areas of the bee brain, overcoming electrophysiological limitations (Potter, 2000). On the other hand, so far no evidence for an asymmetry of learning has been demonstrated in the bee brain. Besides a study showing a different expression of a protein *–neuroligin I-* related to synaptic transmission in bees with left or right antenna amputated (Biswas *et al*., 2010), no literature is available on this topic. Mushroom bodies and lateral horns would be the natural loci to look for any asymmetries both for early or late expression genes after learning and in the odor-evoked activity in naive and trained bees. In particular, it would be interesting to face this question at the level of the lateral horn, a site that is both involved as the ultimate site for odour-driven behavior as well as for innate odour dependent behaviour (Hansson and Christensen 1999; Heimbeck *et al*, 2009). Further evidence of a bias after learning has been put forward showing both a difference between right and left expression of epigenetic modifications after odour learning (Shvestov *et al*., unpublished data) and different expression between sides has been revealed in the sugar reward pathway (McNaill *et al*., unpublished observations). Though there are not confirmed published data yet, this anyhow enhances the need for

future investigations, giving promising outlook to what might explain side asymmetries in olfactory memories.

Another fundamental question regarding differences in olfactory perception that has been described in honeybee (Anfora *et al*., 2010; Letzkus *et al*., 2006) is related to chemotaxis. Many invertebrates, and bees as well use the information coming from the two antennae for cue-orienting navigation, at least near a source (Martin, 1965). It has been demonstrated that flies, for example, require both the antennae for odour-tracking with the left antenna being more sensitive in steering towards the odour source (Duistermars *et al*., 2009). A bee with one antenna only is able (though with less accuracy) to correctly orientate towards an odour cue (Martin, 1965), it might be worthwhile to see whether any difference in the ability of cue-oriented pathfinding showed any side-bias. Although counter-intuitive, a sensory asymmetry might be useful in odour navigation; models of sensory-motor vehicles that have to orient towards a gradient cue, showed in fact embedded asymmetries in the sensor-motor system that enhance their capacity (Holland and Melhuish, 1996). On the other hand, it was recently shown that a little asymmetry in coding external asymmetrical information can control completely different behaviours (Gaudry *et al*., 2012), this might open the door to the unresolved but relevant meaning of brain-behavioural lateralizations.

Appendix A

Here we report *in-vivo* imaging data from our lab showing antennal lobe activation of specific glomeruli when (-)-linalool was presented to the bee's antennae. For information about *in vivo* imaging of the antennal lobe see Chapter 4.1. In Figure 3.2A.1 a 2D image of the right antennal lobe is reported (depth: 30 µm), glomeruli, the spherical functional units that form the insect antennal lobe, are visible in the whole neuropil.

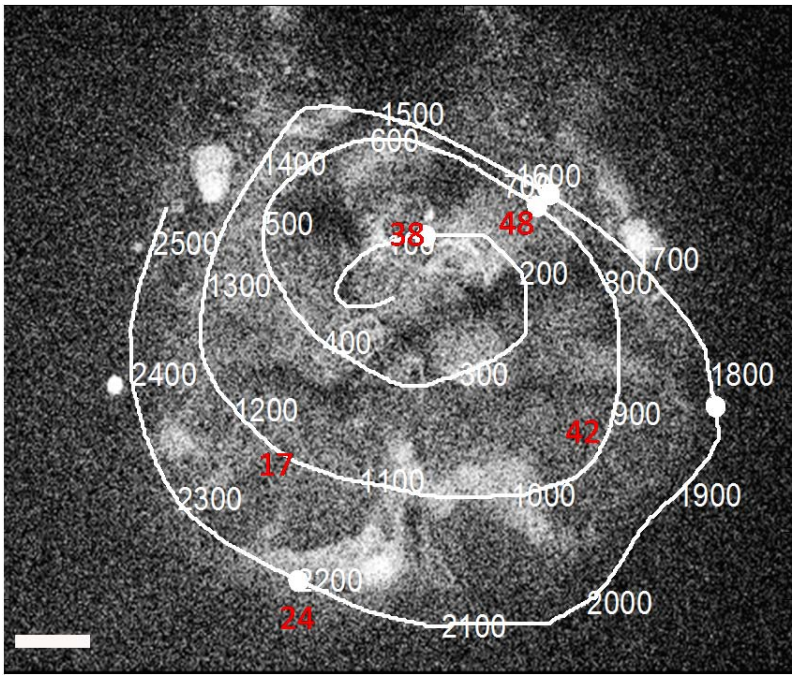

Fig 3.2A.1 Image of a right antennal lobe at 30μm depth: The line indicates the laser scanning trace, the dots label the measurement's reference positions corresponding to the vertical lines in Figure 3.2A.2. White numbers indicate space position along the line. Red numbers indicate glomeruli of interest. White bar: 45 μm .

When puffing (-)-linalool to the bee antenna the activation map (Figure 3.2A.2) reported an activation of glomeruli T1-38 and T1-48 (imaged as dark areas along the time lines for specific dimensions corresponding to antennal lobe glomeruli); a slight activation of glomerulus T1-24 is reported. See discussion of Chapter 3.2 for functional meaning of these neurophysiological data.

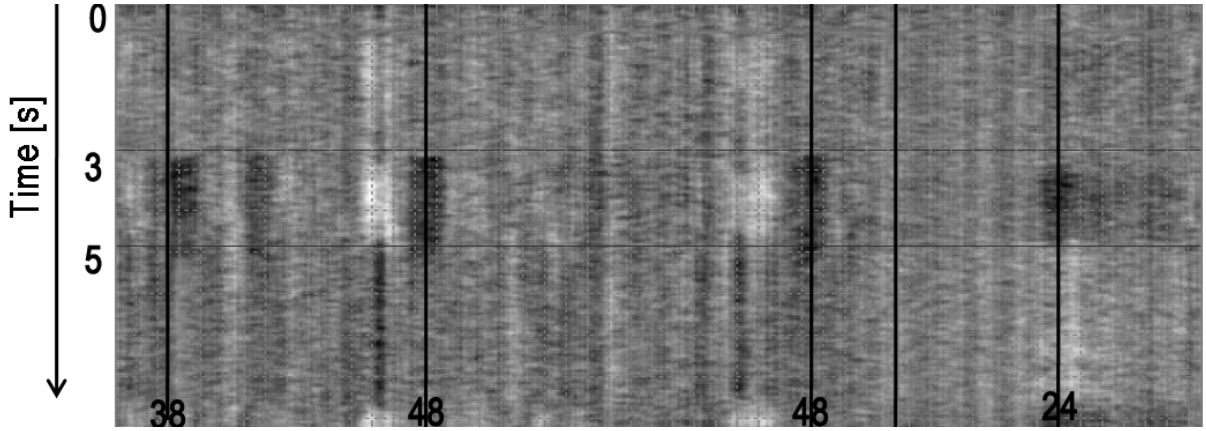

Fig 3.2A.2 Calcium response map for (-)-linalool recorded along the scanning trace in Figure 3.2A.1. The stimulus period is enclosed by the horizontal lines, responding glomeruli centers are marked by vertical lines, numbers label the identified glomeruli.

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