Olfactory representation in the honey bee antennal lobe: Investigations on a filter's functions and dysfunctions.

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XXVIII Cycle

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Abstract.

The honeybee, *Apis mellifera,* is an established model for the study of olfactory processing, olfactory learning and memory, and the related plasticity. The primary centre for olfactory processing in the bee brain, the antennal lobe, has a very important function in odour coding and odour discrimination. Nevertheless, both its structure and its function are plastic. In this thesis, I analysed the structural antennal lobe plasticity related to associative learning, and that related to a non-associative experience, *i.e.* prolonged odour exposure, in the adult honeybee. Subsequently, I analysed the functional modification taking place in the latter case, within the output units of the antennal lobe, showing that parallel structural and functional changes occur. In the last part of the thesis, I focused on the effects of a common neonicotinoid pesticide, imidacloprid, on antennal lobe function and the discrimination abilities of honeybees. I demonstrated that both are strongly impaired in the acute treatment of the brain with such substance.

Chapter 1 - Introduction.

1.1 Why studying olfactory coding and plasticity in the honeybee, Apis mellifera.

A major problem in neuroscience is that of understanding how sensory information collected in the environment is mapped into functional schemes of activity within the brain. The issue can be addressed under the perspective of different sensory modalities and different animal species, leading to quite different answers. However, even if the variation in forms across animals is huge, some solutions are widely shared. Some building schemes and modules of function can be found almost identical in vertebrates and invertebrates, which are located far apart in the evolutionary tree. This is the case of the first processing centres for olfactory information incoming in the brain. In vertebrates, they are known as olfactory bulbs; in insects, as antennal lobes. These two types of olfactory processing centres - with apparently independent evolutionary origins - not only share the same function, but also the same modular organization and main principles of information encoding (Wilson 2008). Hence, studying odour coding and plasticity in the honeybee antennal lobe is relevant to a more general understanding of olfaction. Moreover, principles of plasticity as those displayed within the honeybee antennal lobe recur also elsewhere across animal brains (*i.e.* in different brain areas and with regards to different sensory modalities; Ghose 2004). In conclusion, if we were interested in how brains represent the sensory environment the animals are embedded in, and how this representation is changed by meaningful experiences, such as learning, we would better choose to work with an animal showing robust and easily testable performances of learning. Then, we would preferentially focus on a brain area able to perform complex computations, though within a simple morpho-functional framework. The honeybee antennal lobe meets all these desiderata, not to mention that its size is perfectly fitting the axial penetration capacity of a two-photon microscope, rendering the *in vivo* and *ex vivo* analysis of the area quite convenient in that imaging setting.

1.2 Why is olfaction so important to the honeybee?

Honeybee communities are based on a subdivision of work which is partially genetically determined, and partially reflects the progression through the life phases. First of all, specialization depends on the individual sex, which is already set at egg laying: from fertilized eggs, female honeybees will develop, and drones (males) from the unfertilized. Further specialization is defined throughout rearing. The queen is a female honeybee raised by the workers through a sustained royal jelly diet during the larval stage. All other females develop into sterile workers, handling different tasks at different ages. At the beginning of their lives, they work within the hives, first as nurses, taking care of the larvae, then building combs, and lately storing food supplies and guarding the hive entrance. The last part of the worker life is dedicated to foraging: then, it is when the worker bee first leaves the hive, initially for short orientation flights, and subsequently to find and collect food and resources (nectar, pollen, water) for the whole colony (Tautz 2008). All bees emit a number of pheromones, which are important in keeping the colony cohesive, and which should be readily recognized by the olfactory system of conspecifics. Pheromones, as for their definition, trigger innate behavioural responses: therefore, their neural representation should be robust and remain stable over the course of life of an animal (Slessor et al. 2005; Lamprecht et al. 2008; Sandoz 2007). More interesting for us, however, is the case of those odorants, which become important at certain moments in life, and should, hence, be learned. Their salience and value might change considerably based on the context. The classical example is that of odorants which are associated with nectar and pollen reward during foraging. The smell of a certain flowering might promptly acquire a rewarding value following experience, and motivate reorientation and foraging flights (Chaffiol 2005).This association can also be acquired in hive (Arenas et al. 2007; Farina et al. 2007). In addition, important odours in the honeybee's everyday life are encountered in form of mixtures, requiring further contextualization. Simple odorant molecules might in fact participate in mixtures of hugely differing values, from alarm pheromone to floral blends. It is indeed the overall odorant cue representation, which acquires meaning in the bee behaviour, rather than the simple components

(Chandra and Smith 1998; Deisig et al. 2010). Therefore, representation and meaning should both be quite flexible (Sandoz 2011).

1.3 Bee learning odours.

At the beginning of the XXth century, the Austrian zoologist Karl von Frisch was the first to investigate sensory perception in the honeybee, proving the sensibility of those insects to colours and odours. The combination of these two kinds of stimuli - together with the communication strategy based on waggle dancing – is indeed able to guide the animals in their search for food. Von Frisch could also demonstrate the capability of bees to remember an odour which has been presented in association with food, and the later preference that they show for sources of food marked with the same scent. However, he is mostly famous for his studies on bee communication about rewarding sites through waggle dance, for which he won the Nobel prize for physiology in 1973 (see Fig. 1; von Frisch 1966).

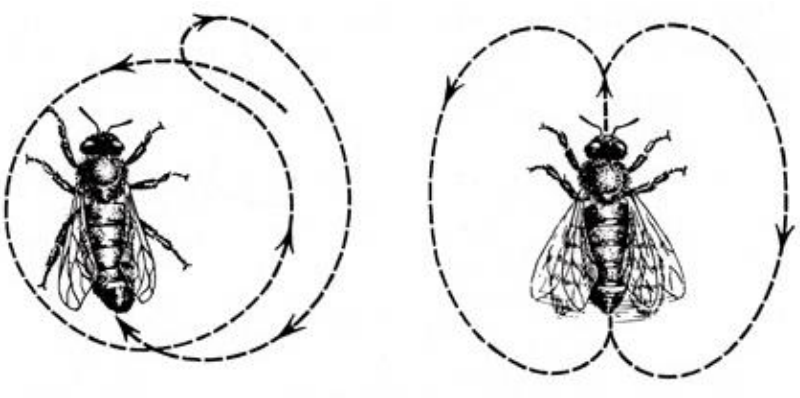

Figure 1 – On the left: The round dance is performed to signal nearby food. On the right: The waggle dance indicates precisely the direction and distance of food which is more distant than ~100 m around the hive. Adapted from (von Frisch 1957).

In 1961, Kimishi Takeda introduced the protocol for olfactory (classical) conditioning of the proboscis extension response (PER; Takeda 1961). In this protocol, which was then perfected over the following 20 years (Bitterman et al. 1983), harnessed bees are trained to associate a specific odour (conditioned stimulus, CS) with a sucrose reward (unconditioned stimulus, US). The association is produced through repeatedly presenting the odorant to the bee antennae, followed in close temporal sequence by the presentation

of sucrose to the antennae and mouthparts (Fig. 2). Contact of the sucrose solution with the antennae elicits the extension of the bee's proboscis, a naturally occurring reflex. The subsequent contact with the mouthparts allows the bee to feed on the solution, so that the reinforcing value of the sucrose on the memory of the association may be exerted. After a few presentations, the bee is able to remember and anticipate the reward, so that olfactory stimulation alone (CS) is now capable of eliciting the reflex. PER will then be measurable even before the stimulation of the antennae with the sucrose solution (US) has happened, indicating that learning has occurred. Memory of the association is maintained for several days after the procedure (Giurfa and Sandoz 2012). For a comprehensive review on the topic see Matsumoto et al. 2012.

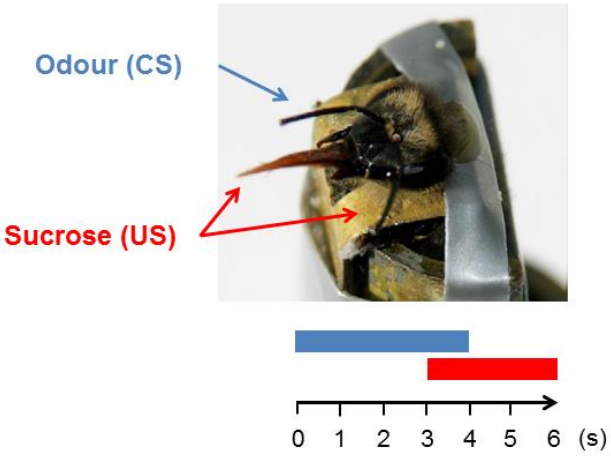

Figure 2 – Scheme of proboscis extension response (PER) conditioning. In each trial, the bee is presented with an odour (4 s), followed in close temporal association by a sucrose solution (3 s, with 1 s overlap). The sucrose solution should come in contact first with the antennae, to stimulate the reflex, and then with the proboscis, allowing the bee to drink it. Adapted and modified from (Matsumoto et al. 2012).

1.4 Environmental threats to the bee olfactory learning and olfaction.

As a consequence of honeybee colonies survival issues and reports of colony collapse disorder (Farooqui 2013) in the last decades, studies on olfactory learning upon exposure to various chemicals (encountered by the bees in

their environment) have seen a revival. A major problem, nowadays, is that of assessing the sub-lethal effects of neonicotinoid pesticide, which have been shown, among other effects, to disrupt olfactory learning and memory (Farooqui 2013). A comprehensive review on such topic will be presented in 3.2. Threats to olfaction *per se* will be discussed based on the data.

1.5 The honeybee olfactory pathway.

Olfactory learning and the neural and molecular bases of odour memory in the honeybee have been extensively investigated. In the following sections, I will discuss the anatomical bases of olfactory processing and learning, with an emphasis on the antennal lobe.

1.5.1 General overview.

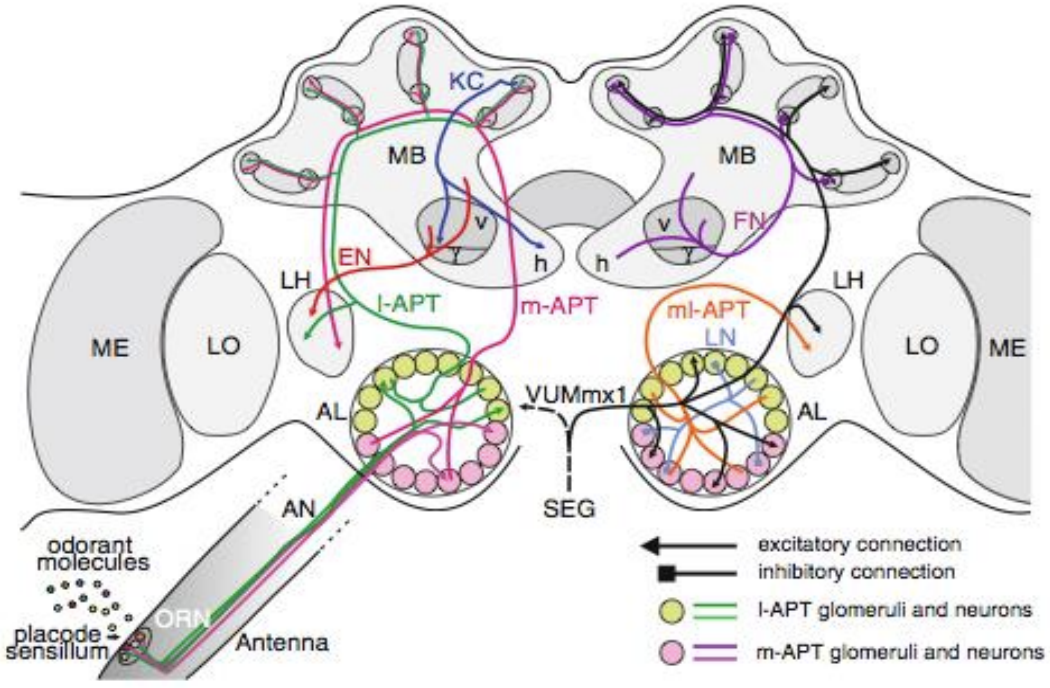

Figure 3 – Scheme of the olfactory pathways within the bee brain. In the left part of the picture, the major excitatory pathways are shown. Inhibitory and modulatory pathways are depicted on the right. The olfactory receptor neurons, ORNs, located in the antennae, transmit information relative to the odorants to the antennal lobe, AL. There, ORNs synapse within the glomeruli with local interneurons and projection neurons (PNs). The latter cell type project the information to higher brain areas through different antenno-protocerebral tracts (APTs). In particular, excitatory uniglomerular PNs (uPNs) send their axons to the mushroom bodies (MBs) and to the lateral horn (LH) through both the medial and lateral-APT (m-APT

and l-APT), which project to the two areas in inverse order. In the MBs, uPNs synapse on Kenyon cells (KCs), whose cell bodies form the calyces of the MB, while their axons form the peduncle. Inhibitory feedback neurons (FNs) project from the peduncles back to the calyces. Excitatory extrinsic neurons (ENs) constitute the main output of the MBs and mainly project to the LH. Finally, the VUMmx1 neuron – whose cell body is located in the subesophageal ganglion (SEG), where it receives gustatory inputs – brings the information relative to appetitive rewards to the AL, MB, and LH. Adapted from (Sandoz 2012).

The olfactory pathway within the bee brain is summarized in Figure 3. Odour processing starts at the periphery, *i.e.* in the honeybee antenna, where ~60,000 olfactory receptor neurons (ORNs), expressing specific olfactory receptors, interact with different odorant molecules. ORNs are hosted inside cuticular structures of various conformations called *sensilli*. Sensilli are filled with lymph, allowing the interaction between dissolved odorants and the ORN dendrites (Galizia and Lledo 2013).

Every receptor type interacts with various molecular odorants, binding them with different affinities. The information about the odorants is then transmitted to the second order neurons of the olfactory pathway, *i.e.* the projection neurons (PNs). In particular, ORNs' axons, reaching the AL through the olfactory nerve, synapse onto the dendrites of ~800 PNs - whose cell bodies are located at the periphery of the AL - forming the AL neuropile's units called glomeruli. It is believed that, as for the fruit fly (Gao et al. 2000), ORNs expressing the same olfactory receptor will converge bilaterally onto single, defined, glomeruli (mainly based on the evidence that number of presumptive functional olfactory receptor genes, Ors, ≈163, is equal to the number of AL glomeruli; Robertson and Wanner 2006).

The stimulation of the antennae with a particular molecular odorant causes a specific and reproducible spatio-temporal pattern of activation within the glomeruli (Sachse et al. 1999). This is the consequence not only of the already-mentioned sparse interaction of odorants with odorant receptors at the periphery, but also of the computations introduced on the odour-evoked activity by the antennal lobe local network, *i.e.* the local neurons (LNs). LNs (~4,000 per AL) are mainly inhibitory, GABAergic, neurons (Galizia 2014). However, also other neurotransmitters have been reported to act in this area

(see Gauthier and Grünewald 2012), and histamine has been suggested to be the neurotransmitter used by a subpopulation of inhibitory LNs (Sachse et al. 2006). See more on the AL cytoarchitecture and cytochemistry in paragraph.1.4.2.

The PNs are the output units of the AL. Their axons leave the AL through different antennal lobe-protocerebral tracts, APTs (the multiglomerular PNs, mPNs, through the mediolateral-APT, and the uniglomerular PNS, uPNs, through the median- and lateral APTs; Kirschner et al. 2006), and reach higher associative areas such as the mushroom bodies (MBs) and lateral protocerebrum (see Fig.3).

In the MBs, PNs project onto ~180,000 Kenyon cells (KCs), whose dendrites constitute the bilateral cup-like structures of the calyces (medial and lateral). Various regions of the calyces are specialized for receiving inputs from different sensory modalities. Olfactory information is mapped onto the lip region and inner half of the basal ring. Kenyon cell axons project to more frontal areas forming the mushroom body peduncles. Subsequently, each axon bifurcates, with branches reaching the (vertical) α-lobe and the (medial) β-lobe, respectively. Multisensory integration is thought to occur both in the calyx neuropile and in the two lobes. Further tracing of a purely olfactory pathway is, hence, no longer possible from here on (Heisenberg 1998).

In summary, while ALs would be the pre-processing sites of odour information - carrying out operations such as increasing signal-to-noise ratio, magnifying weak signals, implementing gain control, threshold-control, and selective lateral processing (with consequences on finer discrimination, possibly dependent on learning and/or contextual cues; for a review see Galizia 2014) -, MBs would be the sites responsible for odour identification, *i.e.* the place where complex patterns of activity inherited from PNs acquire uniqueness and might be linked to other sensory cues by memories.

For what concerns the lateral protocerebrum (LP), the third main brain area involved in olfactory processing, it has been suggested as an odour evaluation and pre-motor site (Galizia 2014). However, there might be ways

through which evaluation would be started already the AL level, as it has been demonstrated in *Drosophila* (Knaden et al. 2012).

Interestingly, these three brain areas also represent sites of convergence of information relative to the both the conditioned and unconditioned stimuli with regards to appetitive olfactory conditioning. Indeed, the olfactory information processed at these locations might be evaluated based on an octopaminergic signal delivered from a neuron with cell body located in the suboesophageal ganglion (SOG), called VUMmx1 (ventral unpaired median cell of maxillary neuromere 1), and innervating the three areas. The activity of VUMmx1 is directly correlated to the presence of a reinforcing stimulus and is sufficient in eliciting the association CS-US, causing PER (Hammer 1993). Therefore, in all the three areas, phaenomena of Hebbian learning relative to the conditioning would be expected.

1.5.2 The antennal lobe.

1.5.2.1 Structure and morphology.

As shown in the previous sections the antennal lobe (AL) represents the first centre for processing of olfactory information in the bee brain. It is the equivalent of the vertebrate olfactory bulb (OB), and like the OB it shows a modular organization with morpho-functional subunits called glomeruli. In fact, the honeybee antennal lobe has a remarkably regular structure. The organization in glomeruli is evident already from a first view under the microscope (at a 10 or 20x magnification). The glomeruli have stereotypical shapes and positions across different individuals. Therefore, they can be unambiguously identified based on standard atlas (Galizia et al. 1999; see Fig. 4). In the worker (and queen) honeybee, esteems of glomerular number oscillate between 156 and 166 (Galizia et al. 1999a; Arnold et al. 1985), in comparison to the ~52 of the fruit fly *Drosophila melanogaster* (Grabe et al. 2016) and ~430 of the worker ant *Camponotus japonicas* (Mizunami et al. 2010), to make some examples. The number of glomeruli is lower (~107) in drones, and they show different morphology (Kropf et al. 2014; Arnold et al.

1985). However, in this thesis we will speak more generally of the “honeybee”, meaning the “worker honeybee”, on which most of the literature focuses.

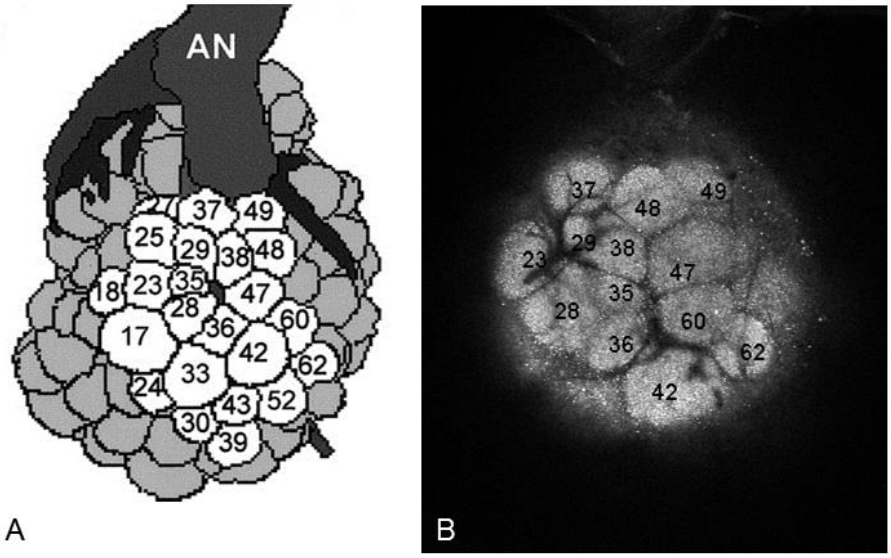

Figure 4 – The antennal lobe structure. A) 3D graphical reconstruction of the AL superior view, with glomeruli of the T1 tract identified. The antennal nerve (AN) is shown in dark grey. Adapted and modified from (Sachse and Galizia 2002). B) α-synapsin immunostaining of the AL and identification of a subset of T1 glomeruli.

As mentioned before, on each AL ~60,000 ORNs project onto ~800 PNs. The number of presumptive functional Ors, ≈163 (Robertson and Wanner 2006), is similar to that of glomeruli, leading to the hypothesis that the separate convergence of the various OR-expressing-ORNs on the glomeruli, demonstrated *e.g.* in the mouse (Vassar et al. 1994) and in the fruit fly (Gao et al. 2000), would be a rule also in the honeybee. However, this principle was never demonstrated in this species.

ORN axons bundle in the antennal nerve. The antennal nerve (AN) is composed of six tracts, T1 to T6, of which four (T1-T4) innervate different subdivisions of the AL, while T5 and T6 bypass the AL to innervate the antenno-mechanosensory motor centre (also termed “dorsal lobe”), the posterior protocerebral lobe, and the subesophageal ganglion (Rybak 2012). The T1-T4 segregation corresponds to distinct groups of PNs that leave the AL following different tracts (Abel et al. 2001).

In most AL glomeruli (at least those belonging to the T1-T3 tracts), the ORN axons terminate in the so-called cortex (exterior part) of glomeruli, without innervating the core (see Fig. 5). On the contrary, uniglomerular PN (uPN) dendrites branch throughout the whole volume of the glomeruli (Sinakevitch et al. 2013; Fonta et al. 1993; Fig. 8B). Therefore, direct synapses between the two neuronal types, if present, should be located in the glomerular cortex.

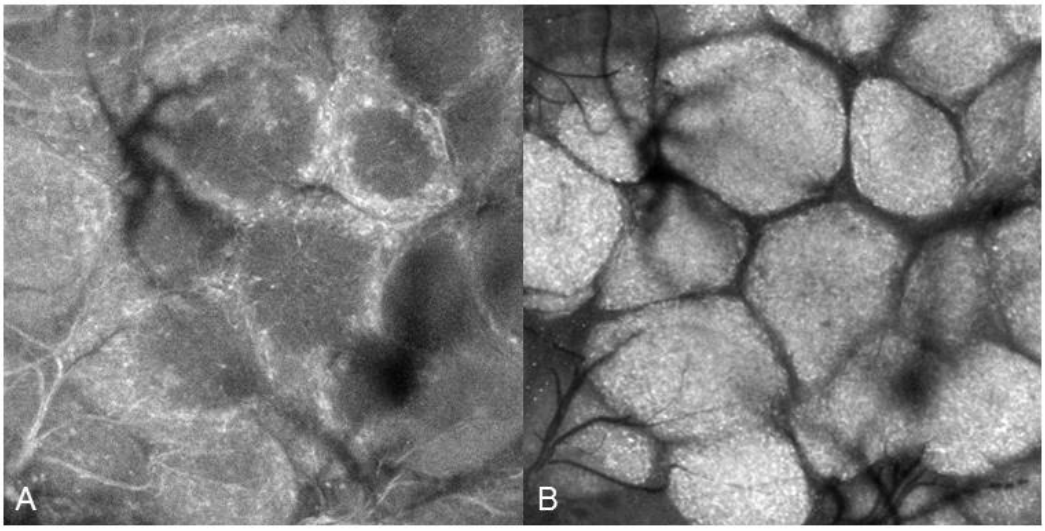

Figure 5 – Double staining ORNs-synapsin, 60x magnification. A) Olfactory receptor neurons (ORNs) have been stained with fixable Alexa Fluor 488. Their axon terminals innervate the core portion of the AL glomeruli (bright ring at the periphery of the glomeruli). B) α-synapsin immunostaining. Synapses are densely present over the whole glomerular volume. Square view dimensions: 176 µm x 176 µm.

Each glomerulus receives innervation from ~5-6 uniglomerular PNs, uPNs, which innervate single glomeruli (Rybak 2012). uPN axons leave the AL through the m-APT and l-APT. Multiglomerular PNs (mPNs), on the contrary, innervate numerous glomeruli (predominantly in T1 and T3 subdivisions; Menzel and Rybak 2010). Their neurites, differently from those of uPNs, branch over the core only (Fonta et al. 1993). mPNs project to the LP giving rise to the mlAPT. mPNs connecting both ALs and brain sides have also been described (Abel et al. 2001). All PNs – both uPNs and mPNs - have their cell bodies grouped at the periphery of the AL. The outer boundary of the AL, as that of each glomerulus, is also lined by glial cells (Galizia 2008).

Local interneurons (LNs) – with cell bodies also located at the periphery of the AL - are present in the honeybee AL in the number of ~4,000 (the highest ever counted among insects; Witthöft 1967). Their name implies that their neurites are not leaving the AL: indeed, they serve as connection *within* the AL glomeruli. Different morphologies have been reported. The so-called homo-LNs innervate most - if not all - glomeruli homogeneously. On the other hand, hetero-LNs have a glomerulus that they innervate predominantly, and other less densely innervated glomeruli (Flanagan and Mercer 1989; Sun et al. 1993). Hetero-LNs are the most numerous in honeybees (Galizia 2008).

Extrinsic neurons other than ORNs are also innervating the AL. One example is the already-mentioned VUMmx1 neuron, signalling the presence of a sucrose reinforcement (Hammer 1993). Other neurons with similar morphology and still unknown functions have been reported (Schröter et al. 2007). Finally, various feedback neurons might also participate in the network, *e.g.* a feedback neuron named ALF-1 has been described, which connect the MBs with the AL (Kirschner et al. 2006). Its function is still unknown.

The exact pattern of connection among all these neuronal types remains elusive in the honeybee. However, more details are available regarding the cockroach and the fruit fly AL (Galizia 2008). In those species, almost all possible types of connections among the main AL cell types (*i.e.* ORNs, PNs, and LNs) have been reported. This allows tracing models of the expected connectivity within the honeybee AL (see Fig.6).

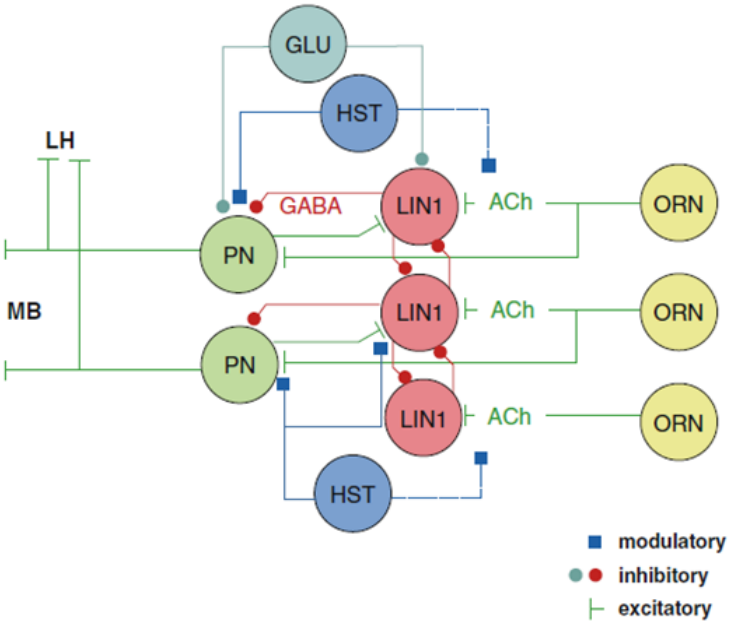

Figure 6 – Scheme of putative connectivity among the different cell types present in the honeybee AL. ORNs send cholinergic excitatory inputs to the local interneurons (LIN1) and PNs. PNs send further excitatory cholinergic input to higher brain areas, i.e. MB and LH. LIN1s form inhibitory, GABAergic, synapses among them and with the PNs. Further histaminergic (HST) and glutamatergic (GLU) signalling have also been demonstrated in the bee AL. They might depend on other types of local interneurons. HST cells are thought to synapse on both ORNs and PNs, while GLU cells are probably active just on the latter neuronal type. Adapted from (Grünewald 2012).

1.5.2.2 Neurochemistry.

Of the main cell types innervating the AL, the ORNs are thought to be cholinergic. First, the antennal nerve shows strong activity of the acetylcholine (ACh) degrading enzyme acetylcholine esterase (AChE). Secondly, binding sites for α-bungarotoxin have been visualized in the AL, and ACh–induced current were described in the AL-dissociated cells (Gauthier and Grünewald 2012).

A portion of the PNs are supposed to be cholinergic as well. The m-APT has indeed been stained for AChE. Accordingly, the postsynaptic Kenyon cells (KCs) express ACh nicotinic receptors (nAChRs). On the other hand, l-APT neurons have been stained for taurine-like immunoreactivity. Finally, at least a subpopulation of ml-PNs are immunoreactive to an antibody against GABA (Gauthier and Grünewald 2012; Sinakevitch et al. 2013).

For what concerns the LNs, most of them are inhibitory, GABAergic neurons. However, a picrotoxin-insensitive inhibitory network has been described as well (Sachse and Galizia 2002). Histamine is probably a second inhibitory neurotransmitter of the local network (with ~260 positive neurons counted; Dacks et al. 2010; Sachse et al. 2006; Bornhauser and Meyer 1997). It has proposed to be the neurotransmitter of at least a subpopulation of hetero-LNs, similarly to what has been reported for the bumble bee, *Bombus impatients* (Dacks et al. 2010). Glutamate might also be a candidate, as it was observed to cause Cl^- currents in AL dissociated cells (Barbara et al. 2005). Cholinergic excitatory LNs have currently been described only in the fruit fly, *Drosophila melanogaster* (Shang et al. 2007). The presence of homologous in the honeybee is not to be excluded.

Octopamine positive neurons, such as VUMmx1, innervate the AL, and both their excitation and the release of octopamine have been shown to necessary and sufficient to elicit associative learning (Hammer 1993; Hammer and Menzel 1998; Farooqui et al. 2003).

Finally, dopamine, serotonine, and tyramine positive fibers have all been visualized in the AL. Their function is still to be elucidated, but there is evidence that dopamine is involved in aversive learning (Gauthier and Grünewald 2012).

1.5.2.3 Function.

From the 90s, the honeybee AL function was intensely investigated through *in vivo* imaging technique (see below) and electrophysiological (see *e.g.* Sun et al. 1993) methods. The first attempts to image the odour-evoked activity with voltage sensitive dyes (VSDs) and calcium sensitive dyes were often carried out on dissociated heads (Galizia et al. 1997, 2000; Joerges et al. 1997; Lieke 1993). Subsequently, the preparation was perfected in order to use intact animals (Galizia et al. 1998).

First results showed that the AL glomeruli were behaving as functionally coherent units, responding to odours with evident depolarization and intracellular calcium increases, respectively (Galizia et al. 1997). Also, these

first studies showed that each odour was evoking a different pattern of glomerular activation (Galizia et al. 1997), and that patterns activated in the two ALs were perfectly symmetrical in each individual (Galizia et al. 1998). The consistency of activated patterns among individuals was also demonstrated (Galizia et al. 1999b). First attempts to correlate odour codes to molecular structures of the odorants were carried out (Sachse et al. 1999; Fig.7).

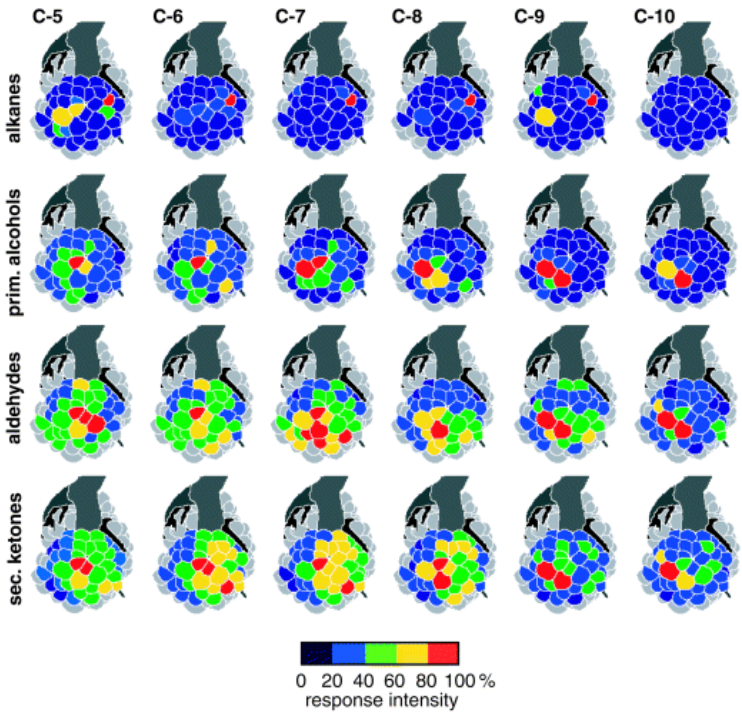

Figure 7 – Schemes of activation patterns obtained through calcium green bath staining. The glomeruli which display calcium increase at the odour presentation are shown with different colours based on their response intensity. There is some regularity in odour response patterns relative to stimuli either sharing the same functional group or, particularly, the same chain length. However, there is not any defined glomerulus responding specifically to one of those features. Adapted from (Sachse et al. 1999).

However, VSD signals had proved to be less robust to noise, and – soon - calcium imaging techniques became the preferred tool for *in vivo* imaging. Moreover, bath applied cell-permeable dyes (such as calcium green 1- and 2-AM) were in 2002 substituted with injectable cell-impermeable calcium-sensitive dyes (such as fura 2-dextran), with which specific populations of neurons could be stained. From recording of the mix population signal (ORNs, PNs, LNs) characterizing the first method, scientists moved to recording

specific PN calcium signal, following retrograde staining (see Fig. 8). The two odour codes proved to be correlated, but not identical (as probably the calcium green signal was dominated by ORNs). The PN odour code, despite slight variations, was also conserved among individuals (Sachse and Galizia 2002). Reading the output of the AL – in terms of PNs activity – proved very useful to better understand the overall activity of the AL network. Indeed, one of the first applications of the new imaging strategy was elucidating the role of GABAergic inhibition in the AL. By applying GABA and picrotoxin, while at the same time visualizing the PN odour response, Sachse and colleagues demonstrate the existence of a second, non-GABAergic, inhibitory network in the AL (Sachse and Galizia 2002).

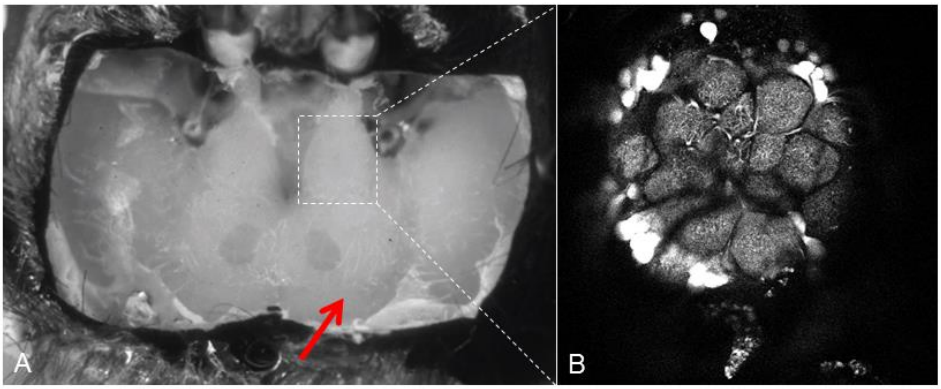

Figure 8 – A) Injection site for uPNs staining. The red arrow indicates the site of injection of cell impermeable calcium sensitive dyes for projection neuron calcium imaging. The area is crossed by both the l-APT and the m-APT. Adapted and modified from (Galizia and Vetter 2004). B) During the following hours, the fluorescent dye has been translocated to the PN cell bodies (intensely stained areas at the AL boundaries), and to the PN dendrites, making the glomeruli fluorescent (fura 2-dextran staining, square view dimensions: 406 µm x 406 µm).

Subsequently, by combining the two methods of staining, important outlooks on how the AL local network performs contrast-enhancement at high stimulus concentrations (with increasing ORN input) were achieved (Sachse and Galizia 2003). In summary, the study by Sachse and Galizia showed that, by increasing the AL input, the overall inhibition also increases, so that relative output activity of the AL PNs allows encoding odour identity over up to four log-unit concentrations. Another relevant result obtained through AL imaging was the visualization of the response to the major queen pheromone

component in the drone macroglomerulus MG2 (Sandoz 2006). Other pheromone components, however, are processed in ordinary glomeruli and other three drone macroglomeruli are still “orphans” (*i.e.* their specialization is unknown). Finally, an important study confirmed that similarity between patterns of AL activity in response to different odours is directly related to the perceived similarity of those odorants, and with the degree of generalization in behavioural tasks (Guerrieri et al. 2005). The result constitutes a starting point to build hypothesis on odour discrimination based on calcium activity recorded in the glomeruli.

Calcium imaging recordings from PNs and, notably, hetero-LNs, through iontophoretical injection of calcium sensitive dye paralleled by intracellular recording, were also achieved (Galizia and Kimmerle 2004). This latter study suggested that the main innervated glomerulus of a hetero-LN is probably pre-synaptic to it, and further confirmed that calcium transients recorded in PNs reflect their spiking activity. Other studies also addressed the local network through intracellular recordings (see *e.g.* Sun et al. 1993).

Lately, two-photon imaging has been implemented for imaging odour-evoked activity in the bee AL (Haase et al. 2010; Franke 2009). One of its first results, thanks to the higher spatiotemporal resolution that it provides compared to classical fluorescence microscopy (see Fig. 9), was showing the coherence of the PNs innervating one glomerulus (Franke 2009). This approach, with its enhanced penetration depth, would also promise a deeper access to the AL and gathering of new information regarding traditionally neglected glomeruli. An important point to consider, in fact, is that the AL is constituted of subsystems, such as the subdivision in glomeruli whose uPNs innervate the l-APT and those contributing to the m-APT. Remarkably, most calcium imaging study, both in bath application and with retrograde PN staining, particularly before the advent of two-photon imaging, were conducted on T1 glomeruli (*i.e.* glomeruli innervated from T1 tract of the antennal nerve), which are the most optically accessible from above. So, most on the information currently available about odour coding in PNs is based on this subpopulation of uPNs (forming the l-APT system).

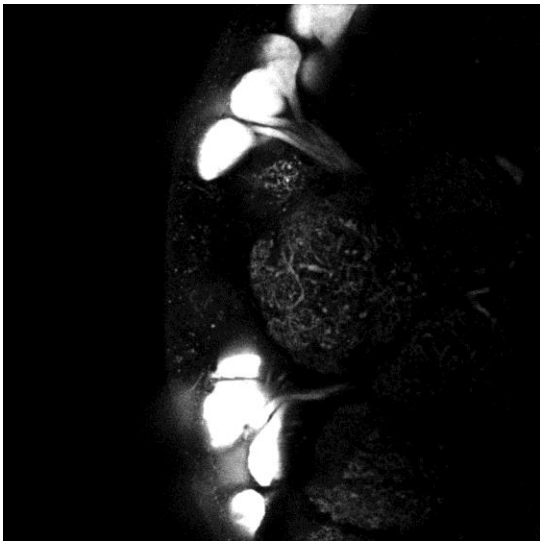

Figure 9 – Two-photon microscopy allow visualizing the individual PN cell bodies with high resolution. Fura 2-dextran staining of uPNs, fluorescent (fura 2-dextran staining; 40x magnification; square view dimensions: 264 µm x 264 µm).

A first study addressing the odour-evoked activity of T3 glomeruli (whose PNs converge, together with those of the T2 and T4 portions, to the m-APT system), imaged through a gold mirror, reported no qualitative differences in odour response between T1 and T3 glomeruli (Galizia et al. 2012). Other studies addressed the question both through extracellular recordings (Brill et al. 2013, 2015, 2014) and optical imaging (Carcaud et al. 2012, 2015b), and find that widely overlapping odour information is encoded in the two tracts. Only slight differences in stimulus specificity and temporal latencies (Brill et al. 2013), and in within-tract synchronicity (Brill et al. 2015) were reported. Moreover, different pheromones were shown to be represented exclusively in one or the other of the two tracts (Carcaud et al. 2015b). Finally, a recent study showed impaired associative learning under selective lesion of the m-APT (Carcaud et al. 2015a). However, a clear picture of how and why parallel processing occurs is still missing (Rössler and Brill 2013).

Another point that would need further elucidation is the temporal component of the PN calcium response. Odour identity is not only coded in the quality of their responses (which glomeruli are responding) but also in the specific temporal courses of those responses (*i.e.* the same glomerulus responds with different shapes of calcium transients to the stimulation with different

odorants, with phasic, tonic, and phasic-tonic profiles observed; Fig. 10). Where and how this temporal code is then read is still a question without answer.

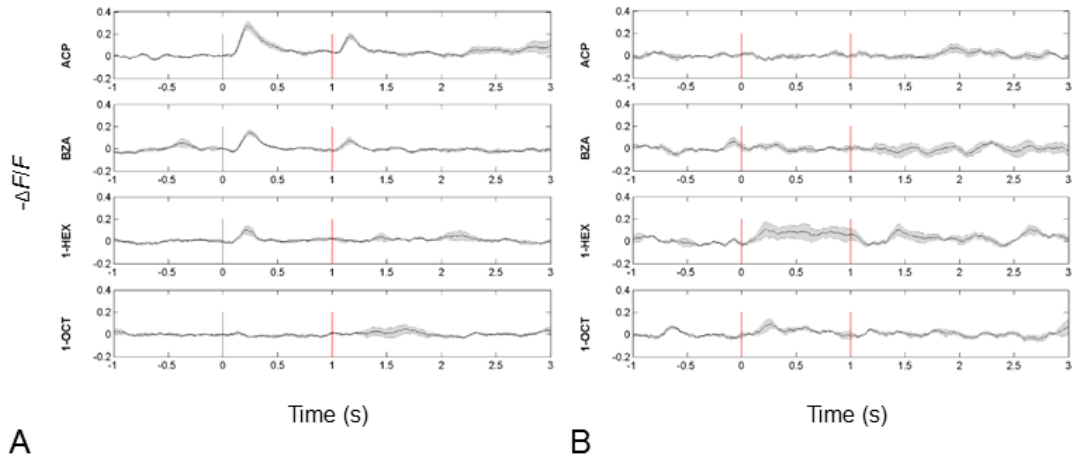

Figure 10 – Temporal features of the glomerular odour responses. A) Odour-evoked PN responses in a glomerulus identified as T1-48 to acetophenone (ACP), benzaldehyde (BZA), 1-hexanol (1-HEX) and 1-octanol (1-OCT). The glomerulus shows calcium transients in response to ACP, BZA, and 1-HEX. The response to ACP and BZA has phasic properties with a second intracellular calcium peak at the end of the stimulus. On the contrary, the response to 1-HEX is composed of a single peak at the onset of the stimulus. B) Another glomerulus recorded in the same bee and identified as T1-28 displays a tonic odour-evoked response to 1-HEX. Odour onset and offset are indicated by vertical red lines. The response profiles are shown as mean (of three stimulus repetitions) ± SEM.

To conclude, it is worth mentioning that information passed from the AL to downstream areas includes a further dimension consisting in the rhythmicity and synchronicity of spiking of PNs (recordable in terms of oscillations of the local field potential). Stimulus-evoked oscillatory synchronization (frequencies ≈20-30 Hz) has been described in various insects, *e.g.* the locust *Schistocerca americana* (Laurent and Davidowitz 1994), where it has been demonstrated to rely onto the GABAergic local network (MacLeod and Laurent 1996). The same modulation is present in the honeybee AL. There, disruption of GABAergic signaling through picrotoxin application has been shown to disrupt fine odour discrimination (Laurent et al. 1997). As synchronization has been shown in the locust to increase by repetitive stimulation with the same odour (Stopfer and Laurent 1999), a possible

mechanism has been put forward by Galan and colleagues (Galán et al. 2006). They showed in honeybee that glomeruli, which are co-activated in the odour response, become attractors of spontaneous activity in the post-stimulus phase - and are subsequently more probably re-activated together in the following ~1-2 min. I will not go more in detail about AL local field synchronization, as this is beyond the scopes of this thesis. As a final remark, a new bandwidth of power spectra modulation in the AL (1.5-5 Hz) related to odour stimulation has been identified in our lab recently, suing two-photon calcium imaging. It is both odour and glomerulus specific. The source of this modulation and its significance for olfactory coding are still unknown (Paoli et al. 2016b).

Chapter 2 – Experience shapes the filter.

2.1 Summary.

In this Chapter, I will first introduce the topic of olfactory learning and memory in the honeybee, and then review evidences of neural plasticity related to these process. The brain areas showing plasticity effects include the antennal lobes (ALs), as well as other upstream and downstream areas. However, I will mainly focus on the ALs. I will discuss evidences of both structural and functional plasticity, with concerns to both associative and non-associative learning. Subsequently, in 2.4, I will present and discuss the results of an experiment aimed at assessing structural plasticity within the AL glomeruli following associative learning. In 2.5 and 2.6, non-associative odour experience will be addressed, assessing, respectively, structural and functional modification in the AL following prolonged odour exposure. Conclusions are presented in 2.7.

2.2 Introduction.

2.2.1 General overview.

Adaptation is not only a matter of evolutionary timescales. Under a certain point of view, it is a matter of everyday life. Animals have evolved a number of mechanisms specifically directed to maintain their brains as flexible as possible, in order to continuously adapt their behaviours to the ever-changing environment. This form of adaptation is better known as “learning”, and the mechanisms that support it are included in the extensive definition of “plasticity”.

The idea that the modifications induced by experience would be engraved into the neural circuits, in terms of changes in the functional properties and/or in the connectivity of the networks, is nowadays widely accepted. Some invertebrate species have become thoroughly studied models for investigations in the field. Due to the relative simplicity of their brain anatomies, often found in combination with a variety of easily observable

learning behaviours, they allow researchers to study the topic under various perspectives at the same time (Glanzman 1995; Menzel 2012; Giurfa 2013).

As anticipated in Chapter 1, the bee is one of those models. Behavioural performances of learning and memory – particularly in the olfactory domain - are indeed well established and easily reproducible (see section 1.3). Moreover, good knowledge of the brain areas responsible for olfactory processing allows building hypotheses on the physiological and structural modifications involved.

The typical situation in which a bee has to learn a huge amount of information relative to different sensory modalities, as anticipated in Chapter 1, is during foraging flights, and particularly when encountering novel sources of food. The bee has then to record, among others, the olfactory cues that characterize the specific flowering. Such olfactory memory has been widely studied, and it is known to be acquired through classical (Pavlovian) associative learning. The odour, acting as conditioned stimulus (CS), is associated through repeated temporally contingent presentations to the sucrose reward, or unconditioned stimulus (US), triggering an observable reflex: the proboscis extension response (PER; Giurfa and Sandoz 2012). As mentioned in Chapter 1, PER conditioning can be reproduced in laboratory setting.

The sites of convergence between the reinforcement (US) and the olfactory (CS) pathways become of particular relevance when searching for traces of plasticity linked to an associative memory. In the honeybee, good knowledge exists regarding the anatomical substrates of both US and CS. I already described the brain areas involved in olfactory processing, and the role of the octopaminergic VUMmx1 neuron in signalling the presence of the reward (Hammer 1993). This neuron, like other VUM neurons (Schröter et al. 2007), is innervating bilaterally the three brain sites responsible for olfactory information processing: the ALs, the MBs, and the LHs. The plasticity associated with this form of learning is, hence, to be searched there.

2.2.2 Vocabulary of learning.

I will here briefly define a few of the most commonly used terms in the field of honeybee associative learning:

- “Acquisition” is the time-consuming process of storing the information about the association in a stable form.

- “Retrieval” is the later access to this stored information, guiding appropriate behavioural responses. The effectiveness of the acquisition is, however, only evident through retrieval itself, so the two processes are hard to disentangle (but they were confirmed to be two separate processes in 1988, when Michelsen demonstrated that dopamine interfere with retrieval without affecting storage; Michelsen 1988).

-“Memory” is the persistence of the consolidated information over time. Again, this is only accessible to the observer through retrieval.

-“Extinction” is the weakening of the memory relative to the association, following acquisition, through subsequent presentations of the CS, which are not paired to the US anymore.

2.2.3 Phases of memory.

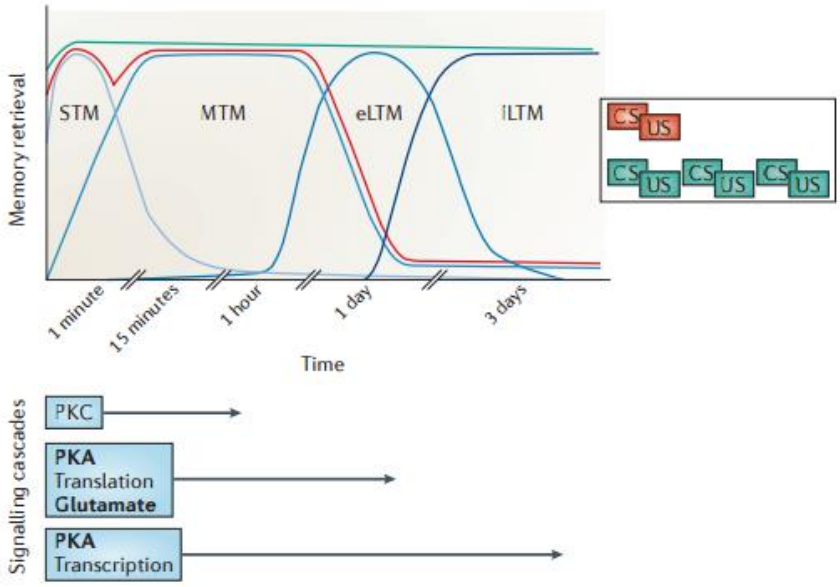

Figure 11 – Scheme of the different phases of memory (blue) developing at different times and transforming one into the other after one (red) or more (green) associations of CS and US have been experienced. The different phases are dependent on different signalling cascades. Adapted from (Menzel 2012).

Different phases of olfactory memory have been described based on their sensitivity to procedures inducing retrograde amnesia, and the molecular pathways involved (see Fig. 11). Briefly, one or more associative-learning trials induce short-term memories (STM, respectively early and late) in the seconds and minutes timescale. STM is initially dominated by unspecific appetitive arousal and sensitization of the PER response. The extent of consolidation and later retrieval of this memory is directly dependent on number of trial repetitions (Giurfa and Sandoz 2012). STM is transformed in medium-term memory (MTM), which spans over the course of several hours and is less sensitive to extinction, through activation of PKC/PKM in the antennal lobe. STM, in the case of multiple trials, is also accompanied by a strong activation of both cAMP/PKA pathway and of NO synthase (NOS), later required for the transition to long-term memory (LTM). In this way, formation of MTM and LTM can occur parallel and independently (Menzel 2012, 2001).

In LTM two phases might be identified, respectively called early (1-2 days after conditioning) and late (>3 days after conditioning) long-term memory, eLTM and lLTM. The second form might be elicited specifically by spaced PER conditioning, while the massed version of the paradigm predominantly leads to eLTM (Matsumoto et al. 2012). LTM is in any case dependent on protein synthesis, but while eLTM is translation-dependent, lLTM is also transcription dependent (Eisenhardt 2014).

Antennal lobes and mushroom bodies are both involved in those memory phases. Converging evidences were achieved through studies on retrograde amnesia induced by cooling and studies on CS-US pairing (obtained via topic octopamine applications). PER acquisition requires forward CS-US pairing at the AL level (showing that STM builds up in the ALs). However, forward pairing at both locations (MB and AL) is able to produce significant performances 20 min later, showing that MTM is build up through transfer of information between the two (Hammer and Menzel 1998). LTM is also started by the ALs, as a single cAMP uncaging event at this level is able to elicit it (Müller 2000), and later involves the MBs. Similarly, cooling of the AL 1 min after conditioning induced amnesia, but 5-7 min after, cooling of the MBs is needed. No retrograde amnesia was observed after cooling the LH (Menzel et al. 1974; Erber et al. 1980).

2.2.4 Plasticity in the mushroom bodies.

It is widely accepted that the MBs in insects are important sites of multi-sensory integration and storage of complex memories (Menzel 2014). There are evidences of MB structural plasticity linked to age and olfactory experience, and particularly to foraging (Durst et al. 1994; Withers et al. 1993; Farris et al. 2001; Krofczik et al. 2008; Maleszka et al. 2009). Briefly, age produces a slow growth of MB calyx volumes (Durst et al. 1994), which is then the basis for further growth of sensory specific areas (lip, collar) at the start of foraging (Withers et al. 1993; Durst et al. 1994), accompanied by an increased complexity in Kenyon cells (KCs) branching (Farris et al. 2001). At the same time, foraging experience causes a decrease in density of microglomerular complexes, *i.e.* the complexes formed by the synapse

between PN boutons and KC dendritic spines in the calyces, while boutons (pre-synaptic sites) increase their size (Krofczik et al. 2008). For what concerns studies on learning in laboratory setting, conditioning of 7-day-old bees causes an increase in the density of microglomeruli in the lip (Hourcade et al. 2010). Functional changes in the Kenyon cells following learning have also been reported, such as an increase in odour responses strength following associative learning, and a decrease following non associative learning (Szyszka et al. 2008). At the MB output, odour-specific functional plasticity is evident as well, as highlighted studies on various MB extrinsic and feedback neurons (Okada et al. 2007; Haehnel and Menzel 2012; Strube-Bloss et al. 2011; Mauelshagen 1993; Grünewald 1999; Menzel and Manz 2005). However, an exhaustive review on these results is beyond the scopes of this thesis. After a short notice on peripheral plasticity, in next paragraphs I will rather focus on evidences of plasticity in the AL.

2.2.5 Plasticity of receptor neurons.

A few works describe plastic processes taking place at the very periphery of the system, in the antennae. Change in the electroantennogram following differential learning were described (de Jong and Pham-Delègue 1991), that in some cases were concentration specific (Bhagavan and Smith 1997). Another study found a decrease in OR expression in the worker bee antennae following conditioning, specifically concerning the OR that is the most responsive to the conditioned odour. Unpaired presentation of the odorant and sucrose were not able to elicit any change (Claudianos et al. 2014). The authors suggested that this might be an effect aimed at balancing the increased odour acuity gained, via conditioning, in other olfactory areas (ALs, MBs).

2.2.6 Plasticity in the antennal lobes.

2.2.6.1 Structural plasticity: effects of age, sensory experience and associative conditioning.

The antennal lobe glomeruli are neuropile aggregates formed by the synapses among the three cell types present in the AL (ORNs, PNs, LNs).

They undergo volume changes during growth. In worker bees, an increase in volume in specific glomeruli is observed at the onset of foraging (Winnington et al. 1996; Sigg et al. 1997). The described volume changes can be accompanied or not by a corresponding increase in synapse number (Brown et al. 2002). Some of the volume changes that follow foraging onset (as observed *e.g.* in the induction of precocious foraging) can also be triggered, independently, by age (Brown et al. 2004).

Notably, associative learning can also affect glomerular volume. Bees exposed to a scented (either 1-hexanol or 1-nonanol) sucrose solution between day 5 and 8, besides showing increased rate of PER response to the odour at day 17, display odour-specific volumetric changes (Fig. 12; Arenas et al. 2012). It was suggested that the glomeruli involved were not the most responsive to each odour, but the ones that were changing their functional response after the procedure the most.

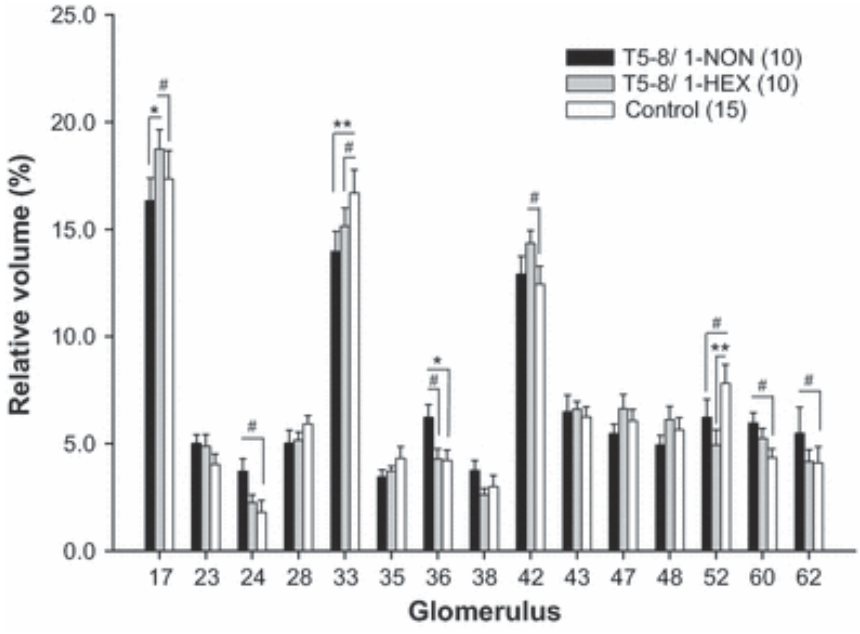

Figure 12 – Volumetric changes (volume is measured as relative contribution to the overall measured volumes) in a subset of T1 glomeruli following early (5-8 d) experience with a scented sucrose solution. The experience is resulting in increase of PER to the odour presentation at day 17, meaning that CS-US association was elicited. Volumetric changes were directly correlated to changes in functional response, so that glomeruli newly recruited to the odour response were the ones in which volumes showed the greater increases. In black, animals which experienced 1-nonanol (1-NON) are shown; in grey, animals which

experienced 1-hexanol (1-HEX). In white, control animals are depicted. Bars represent mean ± SEM. **: $p < 0.01$, *: $p < 0.05$, #: $p < 0.10$. Adapted from (Arenas et al. 2012).

Odour conditioning with 1-hexanol and 1-nonanol in adult bees was demonstrated in one study to affect glomerular volumes as well. The identity of the affected glomeruli could not be easily deduced by their involvement in the odour response. Rather, the authors found a weak negative correlation between inhibition received by the other measured glomeruli and volume change (Fig. 13; Hourcade et al. 2009).

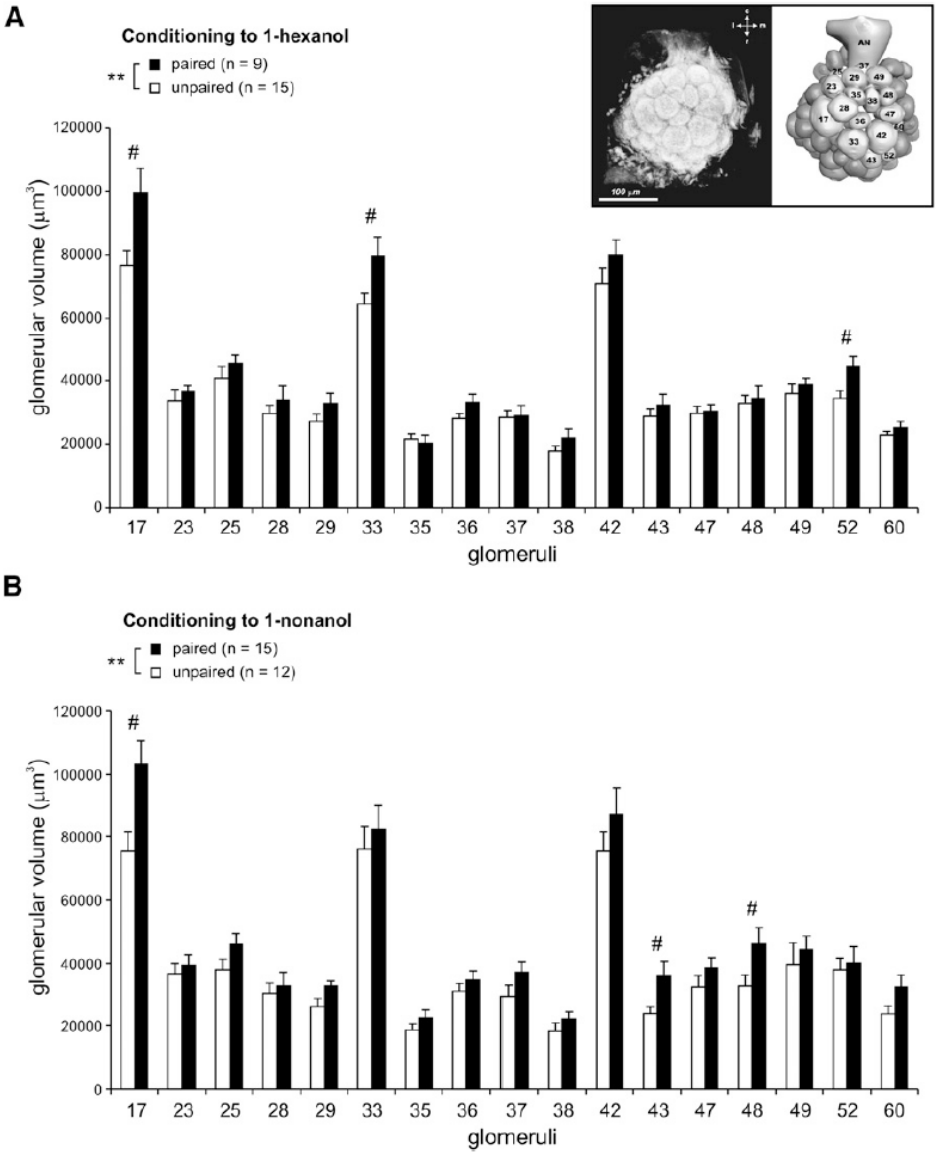

Figure 13 - Volumetric changes in a subset of T1 glomeruli following associative conditioning to 1-hexanol (above) and to 1-nonanol (below). In black, the paired, and in white the unpaired

(pseudo-conditioned) group. Bars represent mean ± SEM. #: $p < 0.05$.Adapted from (Hourcade et al. 2009).

A first attempt to replicate the data on 1-hexanol conditioning in our lab produced, however, completely different results (Fig. 14; Rigosi 2013). The conditioning procedure with both 1-hexanol and (-)-linalool produced no significant changes in glomerular volume. Anyhow, a trend for a decrease of glomeruli T1-17 and T1-33 in 1-hexanol conditioning was observed. Right/left measures were compared as well, but no lateralization of the effects was observed (Rigosi 2013).

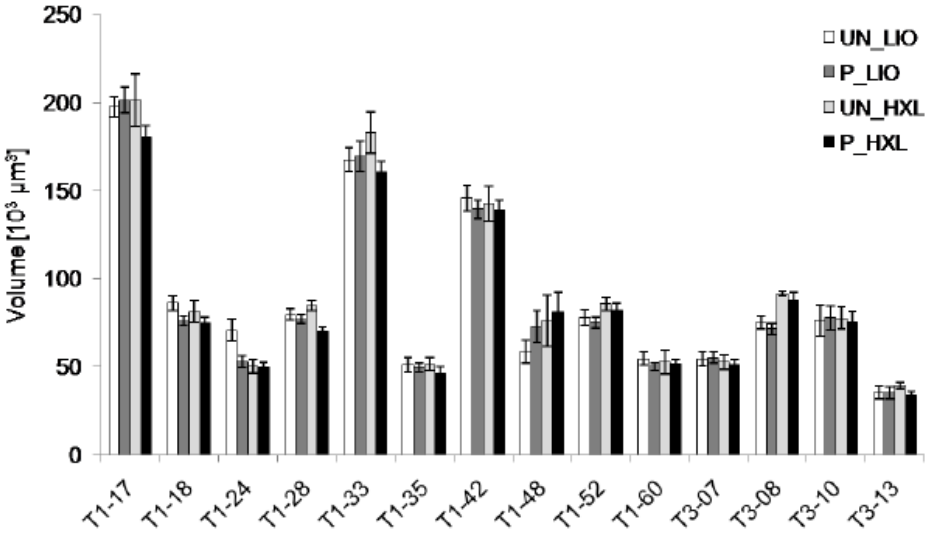

Figure 14 – Glomerular volumes following associative conditioning to 1-hexanol (HXL) and to linalool (LIO). 1-hexanol paired (P_HXL) and unpaired (UN_HXL) groups are depicted in in black and light grey, respectively, while linalool paired (P_LIO) and unpaired (UN_LIO) animals are coloured in grey and white, respectively. No significant changes were found. Bars represent mean ± SEM. Adapted from (Rigosi 2013)

The results of these two experiments are difficult to reconcile. The procedure of conditioning was performed in the same way in both studies, and brains were collected in both cases at 72 h after training. It has to be noted that, while both studies used fixed tissue, the type of staining differs: neutral red was used in one case (Hourcade et al. 2009), and α-synapsin/phalloidin staining and in the other (Rigosi 2013).The number of bees used (6 : 13 unpaired : paired) was slightly lower in the second study in comparison to that

used by Hourcade and colleagues (15 : 9 unpaired : paired). Season might be another bias factor. In the case of Rigosi, the bees were collected between starting of spring and early summer. The information is not available in case of Hourcade and colleagues.

2.2.6.2 Associative learning causes functional plasticity.

Learning-related changes in the AL functionality have been investigated various times. However, the results of these studies are sometimes discordant and difficult to compare, due to the use of different staining and recording methods, or quite different behavioural paradigms (see below).

Moreover, the topic is somehow controversial, because of the role of "olfactory decoder" that the AL seems to play. Indeed, the function of this brain structure is to provide a first interpretation of the multiplicity of olfactory cues encountered in the environment, so that higher brain areas can lately assign them different values and meanings (Menzel 2014; Galizia 2014). It is difficult to imagine that modifications in the activity of the ALs would be adaptive. However, the problem is now by most scientists regarded as outdated, as it is more and more accepted that memory traces should be engraved in the brain in a quite distribute way, at all levels of processing of sensory information. Moreover, the inconsistency depicted above is just an illusory one. It is possible to think of a qualitatively invariant representation of an odour, which nevertheless is susceptible of being modified by experience and learning in its relative discriminability against other odours, *i.e.* in its "acuity". This is exactly what happens in the AL.

By using calcium imaging in bath-staining and a differential conditioning procedure, Faber and colleagues showed enhanced calcium activity at the presentation of the odour that during the training was associated with a reward (CS+), and a slight increase for a novel (control) odour. The odour that during the differential conditioning had no reinforcement (CS-) showed unchanged calcium activity. The patterns of activation triggered by the two stimuli (CS+ and CS-) became more de-correlated after training. The after-

training measurements were collected at maximum 30 min after training (Faber et al. 1999).

A later study using the same staining technique but a different behavioural paradigm, taking advantage of the within-animal control available in the side-specific conditioning (*i.e.* each animal was conditioned A+B- on one antenna and A-B+ on the other antenna), found slightly different results. The authors measured changes 24 h after training, by just comparing sides within individuals. Notably, as was previously demonstrated, odour representations in ALs are symmetrical in control bees (Galizia et al. 1998). As a result of an enhanced recruitment of initially silent glomeruli to the rewarded odour response, an increased Euclidean distance between the representations of each of the training odours in the two ALs in conditioned versus control animals was observed. Interestingly, in the same task, no further de-correlation between the rewarded and unrewarded odour was detected within one side (Sandoz et al. 2003).

On the contrary, using absolute conditioning and the same staining technique, another study found no change in the representation of the trained odour at 3 days after conditioning (Hourcade et al. 2009). It might be tempting to speculate that functional changes in the glomerular activity are apparent only when the animal has to distinguish more effectively a salient odour *versus* an irrelevant one, but other discrepancies in the methods of these three studies suggest caution with conclusions. In the latter, the training condition was compared to a control, which was a completely different group of individuals (while in the other two studies, within-individual comparisons were performed). This choice complicated the results, although it was an obliged one, given the experimental procedure. The animals were indeed tested for effect of olfactory LTM at 3 days (instead of minutes, or hours) after conditioning. So, it was impossible to record from the same animals before and after training. Of course, the different timescale represents another not negligible difference.

Finally, another study addressed the question from a developmental perspective, and showed that odour representation were different between the

ALs of animals that were experiencing early (5-8 d) olfactory learning or were left untreated (Arenas et al. 2009).

However, from bath staining application of calcium-sensitive dyes, only rather unspecific conclusions can be drawn. Even if a plastic change in the activity can be highlighted, it is difficult to identify the neuronal population responsible for it. Therefore, further studies focussed on plasticity at the output units of the AL. Functional analyses on uniglomerular PN (or uPN subpopulations) performed through injection of cell-impermeable calcium sensitive dyes led as well to controversial results. A first study using retrograde Fura-2 l-APT-uPN staining, found no differences in the signals before and after both absolute and differential conditioning (Peele et al. 2006). However, the changes were investigated up to only ~20 min after training. In a study published some years later, an increase in Euclidean distances between CS+ and CS- representations was on the contrary found 24 hours after training with a differential paradigm (using binary mixtures AB in different A and B proportions as CS+ and CS-, see Fig. 15; Fernandez et al. 2009).

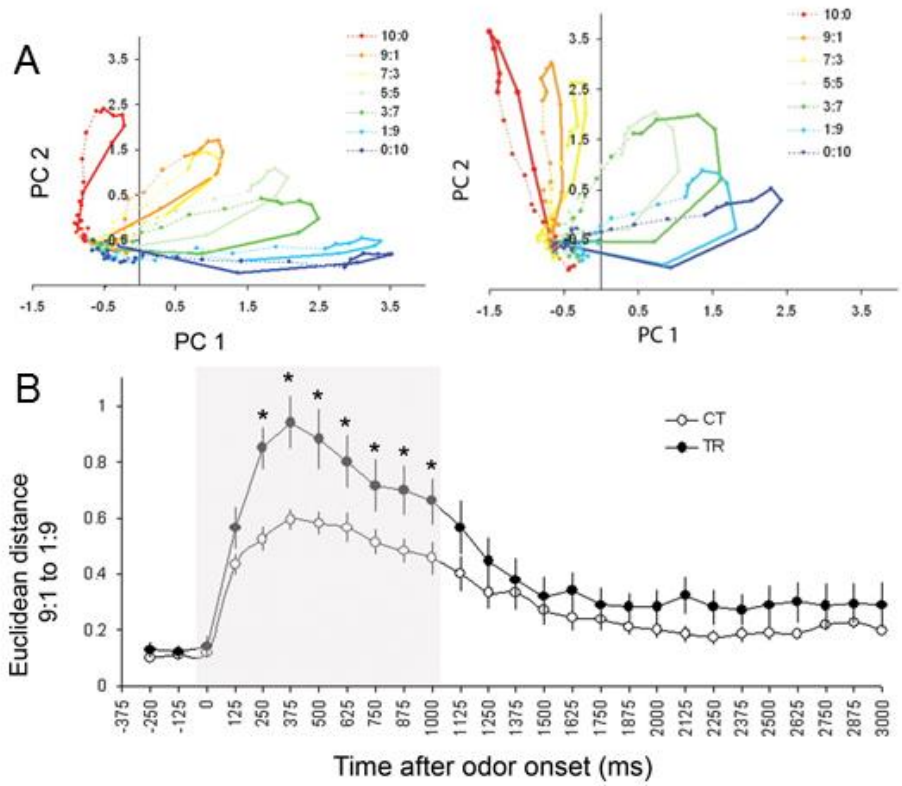

Figure 15 – Representations of different binary mixtures AB (proportions from 10:0 to 0:10), where A: 1-hexanol and B: 2-octanone. A) *Left:* The representation of different proportion-binary mixtures is graded in control animals. Trajectories of representation in time are shown here in the first two principal components obtained by reducing the glomerular response space. *Right:* In animals differentially conditioned to 9:1 (CS+) against 1:9 (CS-), the representation of 9:1 moved away from 0:10, 1:9 (CS-), 3:7 and 5:5 and became more similar to 10:0, meaning that conditioning causes a "peak shift", which allows the more salient components of a mixture to dominate the representation. B) Relative Euclidean distances between ratios 9:1 and 1:9 in control and trained bees (stimulus pulse from 0 to 1000 ms). Asterisks indicate significant differences ($p < 0.05$). Adapted and modified from (Fernandez et al. 2009).

Concerning the first study, there is the possibility that the injection itself was the cause of poor learning, at least on the stained side (also, retrieval was absent or impaired, so real rates of learning are hard to judge in that case; Peele et al. 2006). A later study, in fact, found that learning and retrieval are possible after PN injection, but at a lower rate compared to untreated animals (Rath et al. 2011). Anyhow, injection sites were slightly different in the two cases.

The same later study conclusively demonstrated that differential conditioning would lead to a general decorrelation CS+/CS- (measured 2-5 hours after training). The authors went further, tracing back the change to the activity of specific glomeruli (and PN subsets): those responding selectively to the CS+ would increase their activity following training, while those responding only to the CS- would be left unchanged. The glomeruli responding to none of the two odours would also increase their response strength, but those responding to both – therefore adding ambiguity to the overall activity pattern - would decrease it (Rath et al. 2011).

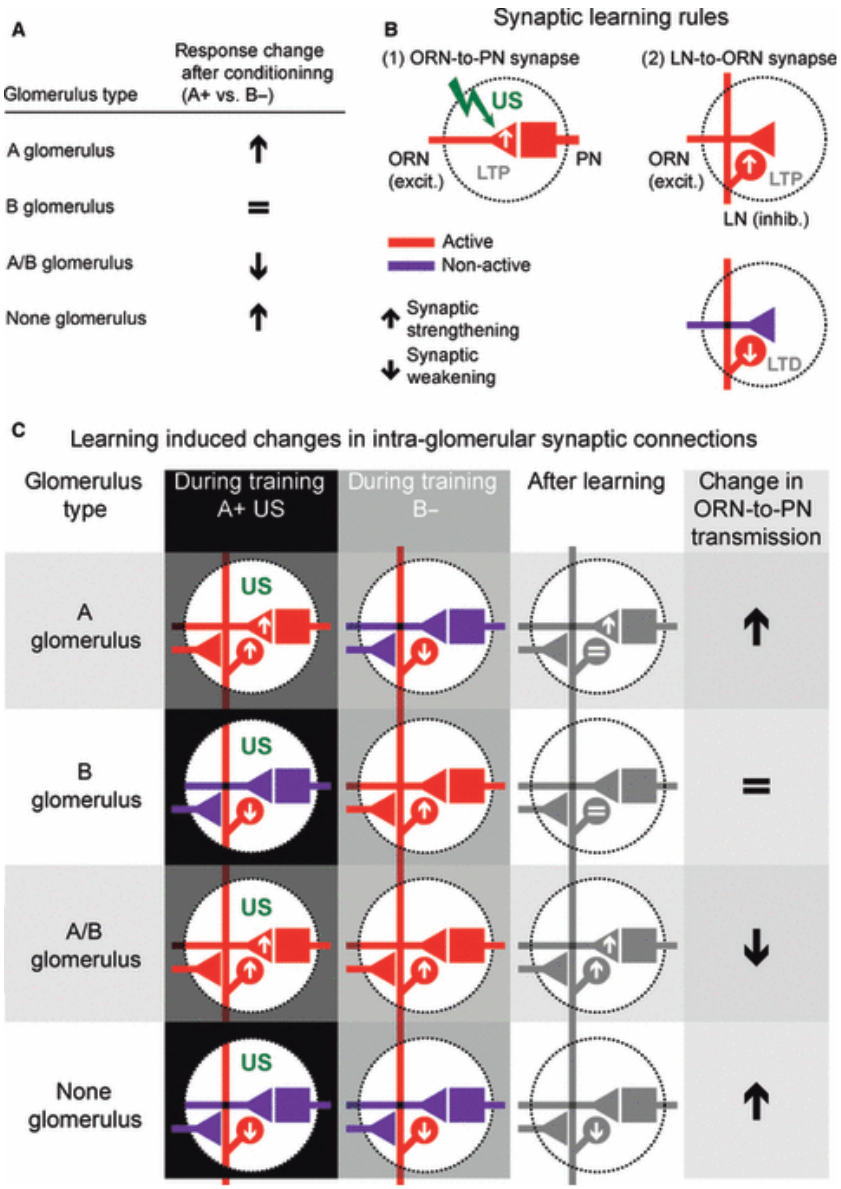

Figure 16 – Scheme of modifications induced in the AL synapses by learning. A) Activity of glomeruli with different profile of responses to odours A and B are modified in different

directions by differential A+B- conditioning. B) LTP (upward arrow) and LTD (downward arrow) are thought to occur at different synapses following CS-US association, depending on which cell types are active (red) or inactive (purple). C) The resultant of these processes of LTP/LTD would be a change in ORN-to-PN transmission which is able to sharpen the two odour representations, and further differentiate them. Adapted from (Rath et al. 2011).

The model that is taking shape from the latest results (see Fig. 16) is very interesting, and totally in accordance with the hypothesis that modifying the "decoder" output (*i.e.* the PN activity) would be efficient in terms of improved acuity, and discrimination. To conclude, data from extracellular recordings of the PNs also seem to confirm that the spiking activity of these cells would be changed by conditioning in a complex way (Denker et al. 2010). It is still uncertain whether this would happen at fixed (in terms of glomeruli, or PNs) locations, consistent across different individuals.

Further investigation are also needed to clarify the many questions still open in the field, and to better understand how plasticity at this level might coordinate with plasticity at more peripheral and more central sites (Sandoz 2013).

2.2.6.3 Non-associative learning related plasticity.

As described in the previous chapter, associative conditioning has been extensively investigated for its potential to modify representations of the training odours in the AL.

To summarize these results, it seems that the AL acts as a filter, increasing the relative gain of the rewarded odour pattern with respect to other confounding stimuli. More generally, in complex paradigms, most "important" patterns (with either positive or negative anticipatory value with respect to a reward) are advantaged, as demonstrated in the experiments with graded binary mixtures conducted by Fernandez and colleagues. There, after differential training, unbalanced mixtures were perceived more similarly to the most efficient anticipatory cue of the mixture (the prevailing one) with comparison to controls (Fernandez et al. 2009). The resulting patterns, simplified and modified in terms of "utility" for the shaping of an appropriate

behavioural response, are then forwarded to higher brain areas, where the odour's valence is evaluated and motor response is triggered (Galizia 2014).

Locatelli and colleagues in 2013 reported, for the first time, functional modifications in the AL following non-associative learning. An odour that is repeatedly presented to the bee olfactory system, associated with neither positive nor negative reinforcement, loses relevance to the animal. PNs modify their activity accordingly. Indeed, in the above-mentioned study, a clear effect on binary mixture representations in the AL was observed: when presented with a binary mixture formed by the pre-exposed odour and a novel odour, pre-exposed animals perceived it as more similar to the novel odour alone. The responsible for this functional change were suggested to be the LNs-PNs synapses, based on modeling. The shift in representation was also confirmed in behaviour, by showing occurrence of asymmetric overshadowing in a memory test with the two pure odorants, after the animals were conditioned with the binary mixture of the two (Locatelli et al. 2013). In a nutshell, the AL filter would reduce the relative gain on the response pattern relative to the pre-exposure odour, with respect to other odours, transforming it in a background odour (Fig. 17).

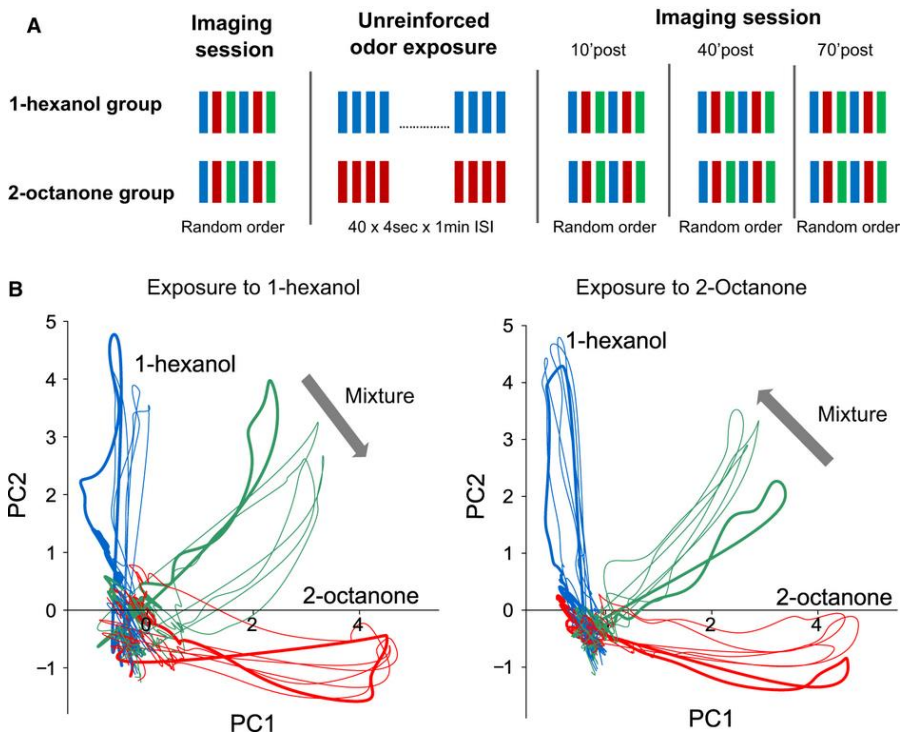

Figure 17 – Effects of unreinforced odour exposure. Odour of exposure: either 1-hexanol or 2-octanone. A) Paradigm of unreinforced odour exposure. B) Effects of unreinforced odour exposure on binary mixture representation (here shown in principal components): the mixture becomes more similar to the novel odour with respect to the odour of pre-exposure. Adapted from (Locatelli et al. 2013).

PNs' plasticity following both associative and non-associative learning was put in a common frame by later work, which assessed the appearance of the contrary effect following associative learning. In fact, conditioned animals perceived a binary mixture composed of the conditioned odour and a novel odour as more similar to the conditioned odour. By modeling the AL network, the authors of this study assessed that the most probable responsible for both type of plasticity were also in this case the inhibitory synapses formed by LNs on PNs (Chen et al. 2015). Therefore, even if the mechanisms and the underlying chemistry might be very different - while the former mechanism is probably due to octopamine-based modulation of LN activity (Sinakevitch et al. 2013; Chen et al. 2015), the latter would be dependent on recurrent inhibition on PNs via LNs (Sudhakaran et al. 2012) - the consequences of associative and non-associative learning on PNs are similar and opposite in sign.

Empirical confirmation of the role of the inhibitory local network in the functional modification related to non-associative learning came from studies in the fruit fly. There, functional plasticity related to long-term odour exposure, *i.e.* a reduction of PN responses to the pre-exposure odorants, leading to behavioural habituation (Sachse et al. 2007; Das et al. 2011), is paralleled by structural plasticity at defined location in the AL. In particular, glomeruli responding specifically to the exposed cue appear to increase their size following exposure procedure (Sachse et al. 2007; Das et al. 2011). This effect was demonstrated to be GABAergic interneuron dependent, *i.e.* GABAergic interneurons increase their branching and synaptic connections, and therefore their inhibitory action, onto specific PNs, reducing odour response of the latter (Sachse et al. 2007; Das et al. 2011). Both functional and structural effects are *rutabaga* dependent (Das et al. 2011). The odour specificity of the effect is based on recurrent feedback from PNs (see Fig. 18), requiring $GABA_A$Rs and NMDARs in PNs, as well as VGLUT and cAMP signaling in the inhibitory local interneurons (LN1 type; Sudhakaran et al. 2012).

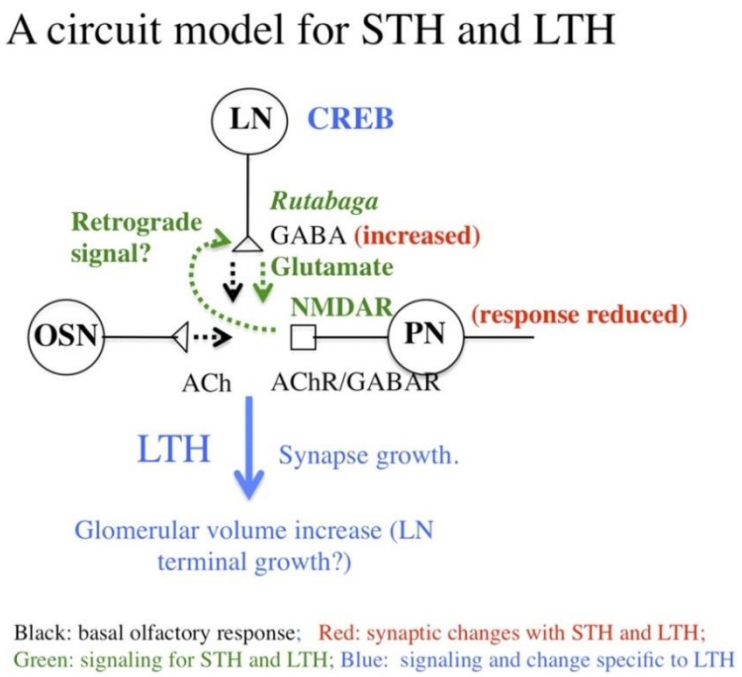

Figure 18 – A circuit model for short-term habituation (STH) and long-term habituation (LTH). The connections among the different cell types in the glomeruli and the neurotransmitters involved in their reciprocal communication are shown. Signals and consequences of STH and

LTH are depicted in green and blue, respectively. Their common effects on synaptic efficiency are marked in red. Adapted from (Das et al. 2011).

A single study, however, found decreased glomerular volumes following odour exposure. These plastic changes were ascribed to synaptic loss. The study was performed before having any indications of the odour responses of the various glomeruli, so it is unclear whether the modified glomeruli were the ones responding to the pre-exposure odour or not (Devaud et al. 2001). Notably, in both cases of observed increased and decreased glomerular volumes, olfactory receptor neurons (ORNs) were shown to be morphologically and functionally unchanged by the procedure, excluding a role of peripheral adaptation in the described morpho-functional modifications (Sachse et al. 2007; Devaud et al. 2001).

The behavioural outcomes of non-associative learning and unrewarded odour exposure have been investigated both in the honeybee and in the fruit fly. These effects range from diminished olfactory avoidance of a previously habituated odorant (Sachse et al. 2007; Devaud et al. 2001; Das et al. 2011), to latent inhibition (Lubow 1973), *i.e.* a delay in the acquisition of an associative memory of the same odour following odour exposure. In the honeybee, both repeated (Chandra et al. 2010) or prolonged (Fernández et al. 2009) presentations of an unreinforced odour can produce latent inhibition. This evidence suggests that repeated and prolonged odour exposure might trigger similar mechanisms in the AL, aimed at filtering out irrelevant information. Another effect that has been reported following repeated exposure is the already-mentioned imbalance in overshadowing during conditioning with a binary mixture (Locatelli et al. 2013).

For what concerns structural plasticity, or the neurochemical or molecular pathway involved in non-associative learning, much is still to uncover in the honeybee. It is reasonable to speculate that results obtained in the fruit fly would apply also to the bee, but slight differences in the cytoarchitecture of their ALs suggest caution with generalization.

2.3 Aim of the experiments.

The aim of the experiments presented hereafter was to answer the questions of how much plastic the antennal lobe - given its well-known function of filter of olfactory information responsible of odour pre-processing and odour discrimination - might be, and through which mechanisms. On the one hand, we expect that the antennal lobe odour representations are stable enough so that a specific odour percept maintains its identity throughout the bee life. On the other hand, as previous studies have shown, the filter might be subtly reshaped by learning, so that relative representations of odours with different salience and value are shifted the one with respect to the other. This is surely adaptive in order to immediately extract the most relevant information while navigating the environment. But how much plastic the system is? Are there structural changes happening that might cause the functional reshaping? How are the different glomeruli involved? We tried to answer all those questions. In 2.4 I will present the results of an experiment aiming to duplicate the results of Hourcade and colleagues (Hourcade et al. 2009), showing volumetric changes in the AL glomeruli following appetitive conditioning. I will also present a proof-of-principle approach to relate volumetric data with analysis of synapse density in 2-photon microscope. Even if we were not able to replicate those results (Hourcade et al. 2009), the experiment led us to a more basic question, *i.e.* if unrewarded odour exposure, which in the above-mentioned study served as a control condition, might also be a source of structural plasticity. The repeated presentation of the odour stimulus, disassociated from any reinforcement, might in fact cause non-associative learning, which in the fruit fly has been shown to be the cause of structural reshaping. Therefore, in the experiments that will be discussed in 2.5 and 2.6, we focused on unrewarded odour exposure. In 2.5, I will describe the effects of odour exposure on the AL morphology, showing for the first time that this treatment is able to affect glomerular volumes in adult bees. In 2.6, using a calcium imaging approach, I will try to relate the described structural changes to changes in the glomerular odour responses and overall odour codes. Data presented in 2.6 were collected with the help of Ben Timberlake, and are partially discussed in his Master's thesis, that I co-supervised (Timberlake

2015). Data and text in 2.5 and 2.6 were adapted with minor modifications from Methods, Results and Discussion of an under-review paper (Andrione et al.).

2.4 Associative learning-related plasticity in the honeybee antennal lobe.

2.4.1 Methods.

2.4.1.1 Animals.

Forager honeybees were captured at the entrance of the hive between October and November 2013 in Mattarello (TN), using a transparent pyramid. The bees were kept for 30' in a shady plastic container where they were free to fly. Then, they were chilled to immobility and individually harnessed in metal chunks (Fig. 19) leaving only their head and mouthparts free to move (Bitterman et al. 1983). They were fed with a small drop of a 50% w/w solution, and then they rested for at least 3 h in the dark. Following conditioning, the bees were fed twice per day with sucrose solution, up to satiation.

Figure 19 – Bee harnessed in a metal tube for conditioning.

2.4.1.2 Conditioning and memory test.

The bees underwent a protocol of appetitive olfactory conditioning which is able to set a late long term memory of the association (Matsumoto et al. 2012). Briefly, the protocol consists of five 4 s-presentations of the odour (1-hexanol; Sigma-Aldrich, >99% purity) coupled with sucrose reward (50% w/w

solution, 3 s, with 1 s overlap with the odour), 10 min spaced one from the other. The odour presentations were preceded by ~26 seconds of habituation to the air flow and followed by other ~20 seconds of rest in the air flow. The protocol also included a group of pseudo-conditioned animals, which were subject to five presentations of the odour (1-hexanol) and five presentations of the reward, randomly ordered and separated by 5 minute intervals (Hourcade et al. 2009). Conditioned animals were therefore placed five additional times in the setup without any stimulation, to equalize experience with the experimental context. We also included a group of naïve bees, which were captured at the same moment and kept in the same way of the other two groups. The naïve animals were exposed ten times to the air flow and the set-up, similarly to bees in the other groups, and presented five times with the reward, but had no experience with the odour. Animals of all groups not able to produce PER (tested with sucrose stimulation of the antennae) were discarded before the conditioning/pseudo-conditioning procedure. Acquisition was assessed in terms of observable PER at the odour presentations (1= presence of PER, 0= absence of PER). After training, bees were fed to satiation and kept in the dark. The bees were tested for their long-term memory, LTM, regarding the association three days (72 h) after conditioning. Ability to perform PER following sucrose stimulation was assessed again, and animals which did not show the reflex were discarded. A novel odour (1-nonanol; Sigma-Aldrich, >98% purity) was presented along with 1-hexanol (both: 4 s, with an inter-stimulus interval of 10 min) during the test.

2.4.1.3 Odour stimuli.

The odours were presented in form of a 4 µL drop of the pure substance poured on a filter paper inserted in a glass pipette. The air space was delivered to the animal's antennae via a constant air flow developing through a system of plastic tubes and the glass pipette. The animals were positioned with their antennae at around 1 cm, facing the end of the tubes.

2.4.1.4 Selection of animals based on their response.

In the “conditioned” group, only bees showing at least three out of five “1” scores in the acquisition phase, or two consecutive “1” in the final trials, were considered for further testing (LTM test). Conditioned animals were subsequently assigned to the group of “learners” or “non-learners” based on the specific memory (response to 1-hexanol, but not to 1-nonanol) on the third day. Only “learners” were considered for further analyses (see 2.4.1.4 and following). Pseudo-conditioned and naïve animals responding with PER to one or both odours during the LTM test were discarded. This choice was intended to simplify the variation across animals in the different groups.

2.4.1.5 Brain dissection and immunohistochemistry.

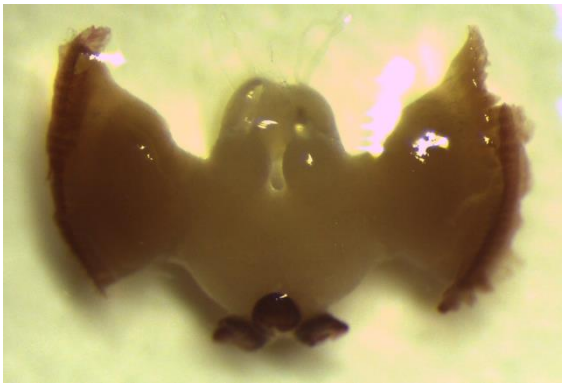

Figure 20 – Fixated and dissected bee brain, before transparentization.

The heads were collected after the memory test and partially dissected in Ringer’s solution (130 mM NaCl, 6 mM KCl, 4 mM $MgCl_2$, 5 mM CaCl2, 160 mM sucrose, 25 mM glucose, 10 mM HEPES, pH 6.7, 500 mOsm/L; Galizia and Vetter 2004). They were plunged in ice-cooled 4% PFA solution, where they were kept O/N at 4°C. After completing the dissection in PBS (Fig. 20), the brains were rinsed three times in 1% Triton X-100 PBS and pre-incubated in 5% normal goat serum for 1h at RT, and rinsed again. Then, they were incubated for 2 days at 4°C with 3% α-synapsin mouse monoclonal antibody (SYNORF1, clone 3C11; DSHB). After rinsing again, brains were incubated O/N at 4°C in 3% anti-mouse Alexa Fluor 546 (Invitrogen) antibody. Afterwards, the rinsed brains were incubated and kept for three weeks in an

optical clearing solution (30% glycerol 4 M urea), in order to enhance their transparency.

2.4.1.6 Optical set-up.

Images were acquired via two-photon microscopy (Ultima IV, Prairie Technologies-Bruker), equipped with an ultra-short pulsed laser (Mai Tai Deep See HP, Spectra-Physics) as excitation source. Excitation wavelength was tuned to 800 nm. Fluorescence was separated by a dichroic beam-splitter (Chroma Technology Corp), filtered by a 70 nm bandwidth filter centreed at 525 nm, and detected by Photomultiplier tubes (Hamamatsu Photonics). Excitation and signal collection were performed in epi-fluorescence configuration via a water-immersion objective (20x, NA=1, Olympus). Liquid of immersion was the same optical clearing solution described above.

2.4.1.7 Image acquisition and processing.

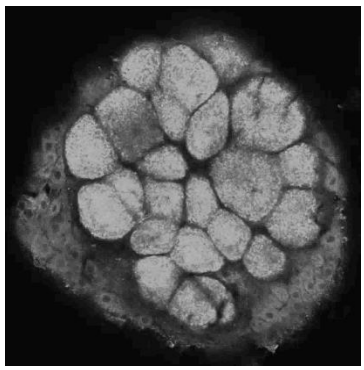

Figure 21 – α-synapsin-immunostained AL visualized at the two-photon microscope after transparentization.

The tissues were imaged under two-photon microscope and stacks of the ALs were acquired through optical sectioning (20x, 512x512 px resolution, *z*-axis step size: 3 µm; Fig. 21). Both ALs were acquired in each animal. The ALs were 3D-reconstructed and the glomeruli identified, where possible, through comparison with the standard atlas (Galizia et al. 1999a). With the software Amira (FEI), it was possible to segment and calculate putative volumes of the reconstructed glomeruli (based on a semi-automatized thresholding method). During measuring, I was blind for the group that each AL belonged and whether it was right or left (via prior horizontal flipping of *z*-stacks of the right

ALs). Each analysed glomerulus was measured three times on different days by myself, and measures were subsequently averaged. For further analyses, only those animals in which each right and the left glomerulus could be unambiguously identified and reconstructed were considered.

2.4.1.8 Statistical analyses.

Acquisition was tested through Cochran's Q test and compared between groups via Mann-Whitney U test. Proportion of PER during the LTM test were compared between groups with Fisher's exact tests, and within groups with McNemar's tests. Volumes were analysed via three-way repeated measure ANOVA with side and glomerulus as within-subject factors and treatment as between-subject factor. Subsequently, averaged volumes were compared through a two-way repeated measure ANOVA with glomerulus as within-subject factors and treatment as between-subject factor. The values of L in the different groups and different glomeruli were tested against a normal distribution (Z-test) with mean 0.5 and standard deviation equal to the deviation within each sample. All analyses were conducted in MATLAB (Mathworks).

2.4.2 Results.

2.4.2.1 Absolute conditioning.

Learning rates during conditioning are shown in Figure 22. Bees of the conditioning group (n=57) showed increasing rate of PER to the rewarded odour (1-hexanol), while pseudo-conditioned animals (n=40) do not increase their PER to the odour presentations over the course of five trials. Acquisition was tested through two Cochran's Q tests (Conditioning: Q_4=78, $p<10^{-15}$, Pseudo Conditioning: Q_4=6.4, p=0.17). The rate of learning differed between conditioned and pseudo-conditioned bees (Mann Whitney U test, Z_1=5.5, $p<10^{-7}$).

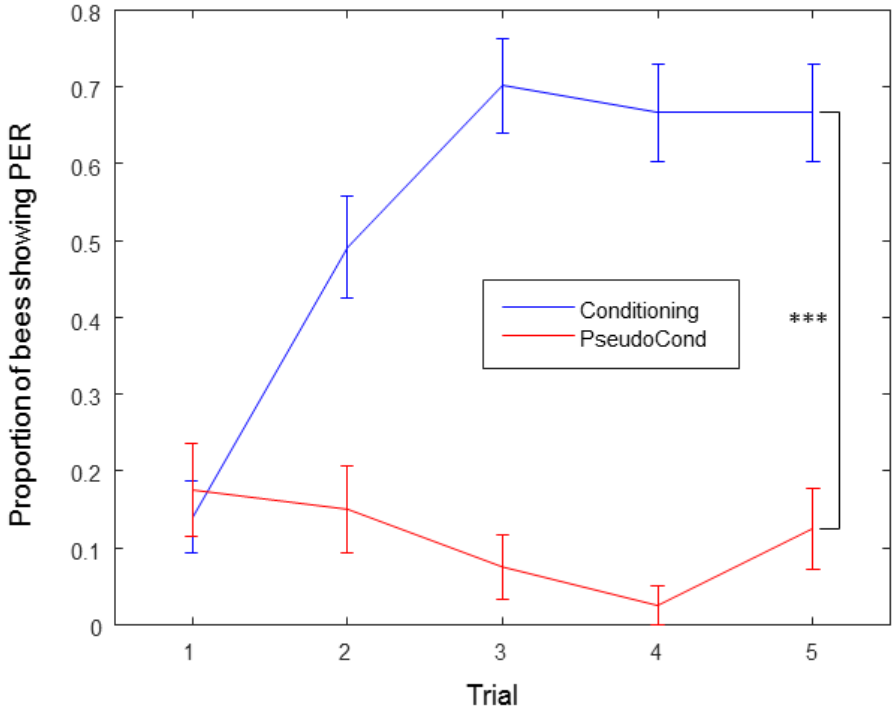

Figure 22 - Proportion of PER responses were computed for the two treatments (conditioning: n=57, pseudo-conditioning: n=40), for each of the five trials of the conditioning paradigm (conditioned stimulus, CS = 1-hexanol). Data are shown as mean ± SEM. ***: $p<0.001$.

2.4.2.2 Memory test.

At 72 memory of the association was tested in conditioned, pseudo-conditioned and naïve bees. Naïve bees showed no responses to either the conditioned odour (1-hexanol) or the novel odour (1-nonanol). The proportion of bees responding to each of the two stimuli was different between conditioned and pseudo-conditioned bees, with conditioned bees showing a higher proportion of PER to both odours (tested through two Fisher's exact tests: Conditioned odour: $p<10^{-3}$; Novel odour: $p=0.0068$). The proportion of responses to both stimuli was, on the contrary, not significantly different between pseudo-conditioned and naïve bees, as expected. So, there was a clear effect of conditioning on LTM (Fig. 23).

Generalization of the memory was quite high. Many bees, in fact, responded to both odours (notably, no bees responded only to the novel odour). The proportions of conditioned bees responding, respectively, to the conditioned

odour (CS) and to both the conditioned and the novel odours (Nod) were not significantly different (McNemar's test: $\chi 2=0.41$, $p=0.52$; see Fig. 23).

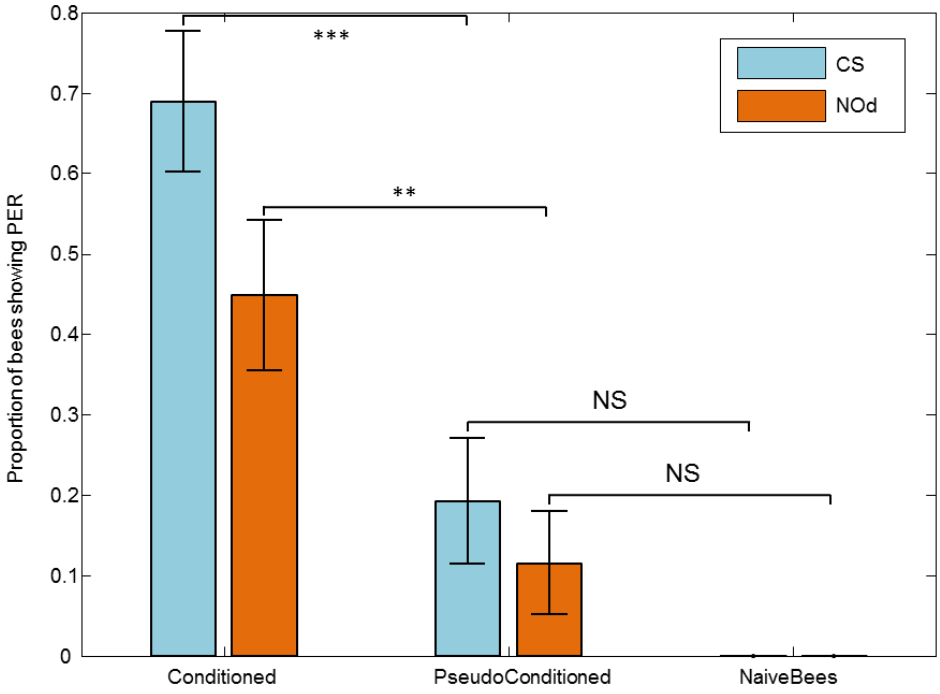

Figure 23 - Proportion of bees within each group (conditioned: n=29; pseudo-conditioned: n=26; naïve bees: n=20) showing long-term memory of the association at 72 hours. PER is measured in response to presentation of 1-hexanol (CS) and a novel odour (Nod), 1-nonanol. Some bees will respond to both stimuli (shown as "NOd"). However, no bees responded only to the novel odour. Data are shown as mean ± SEM. ***: $p<0.001$, **: $p<0.01$.

2.4.2.3 Brain dissection and glomeruli reconstruction.

For the morphological analyses, subsets of bees belonging to the three groups were considered. I chose, respectively:

- Conditioned bees: those bees displaying specific memory at the LTM test, *i.e.* non-generalizers.
- Pesudo-conditioned bees: a subset of those bees showing no response to either the CS or the novel odorant.
- Naïve bees: a subset of those bees showing no response to either the CS or the novel odorant.

Their brain were dissected, and stained in order to reveal synapsin localization, as described in Methods (2.4.1.5). After optical clearing was

achieved, they were imaged under the two-photon microscope. Then, they were visually analysed to bilaterally identify glomeruli of the T1 tract, through comparison with the standard atlas. Those brains, in which glomeruli could be unambiguously identified on both sides, were 3D-reconstructed in Amira (see Fig. 24).

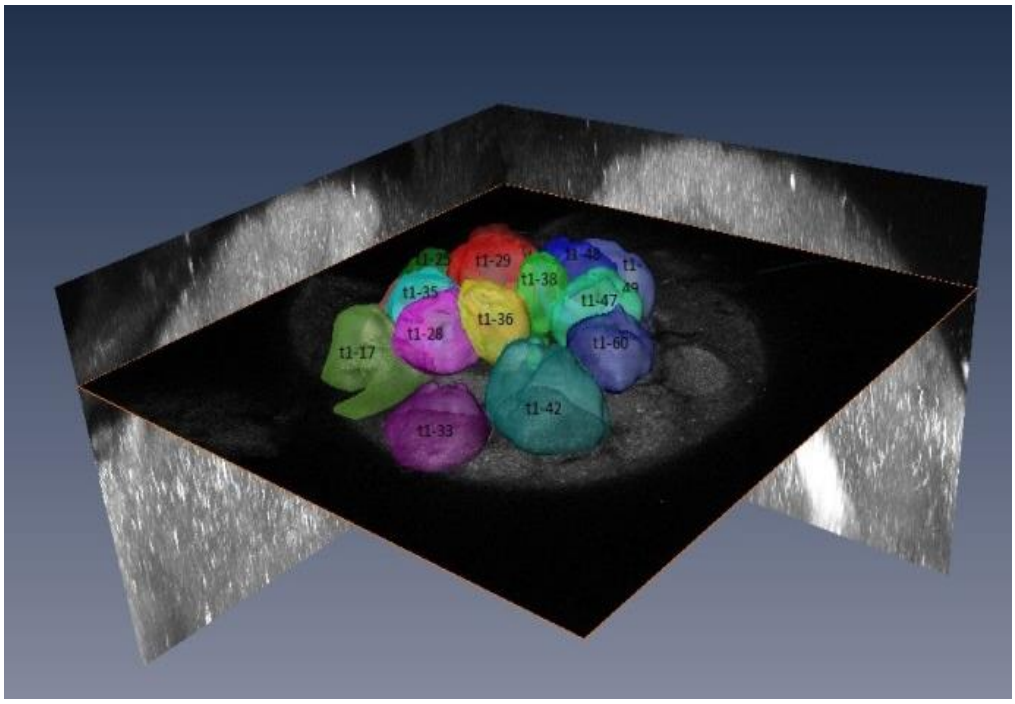

Figure 24 – 3D-reconstructed glomeruli plotted on top of an AL immuno-labelled coronal optical section. On the two sides: medial and posterior projections.

2.4.2.4 Volumetric measurements.

Four glomeruli for which volumetric changes were described before (T1-17, T1-33, T1-52, T1-42 (Hourcade et al. 2009; Arenas et al. 2012; Rigosi 2013) and three for which they were never described (T1-47 and T1-36, T1-28) - one responding strongly (T1-28), one responding slightly (T1-36) and one not responding (T1-47) to 1-hexanol - were bilaterally measured and compared across groups (Fig. 25). A three-way repeated measure ANOVA with side and glomerulus as within-subject factors and treatment as between-subject factor showed neither effect of treatment, nor interaction of treatment with the other two factors. Glomeruli, however, were significantly affecting volumes, as expected. Surprisingly, side was a significant factor too (three-way repeated measures ANOVA: Treatment: $F_{1,\ 12}$=0.32, p=0.60; Glomerulus $F_{1,\ 12}$=76,

$p<10^{-5}$; Side: $F_{1,\ 12}=46$, $p<10^{-4}$; Treatment × Glomerulus interaction: $F_{1,\ 12}=0.13$, $p=0.73$, Treatment × Side: $F_{1,\ 12}=0.35$, $p=0.56$, Treatment × Glomerulus × Side: $F_{1,\ 12}=0.14$, $p=0.71$).

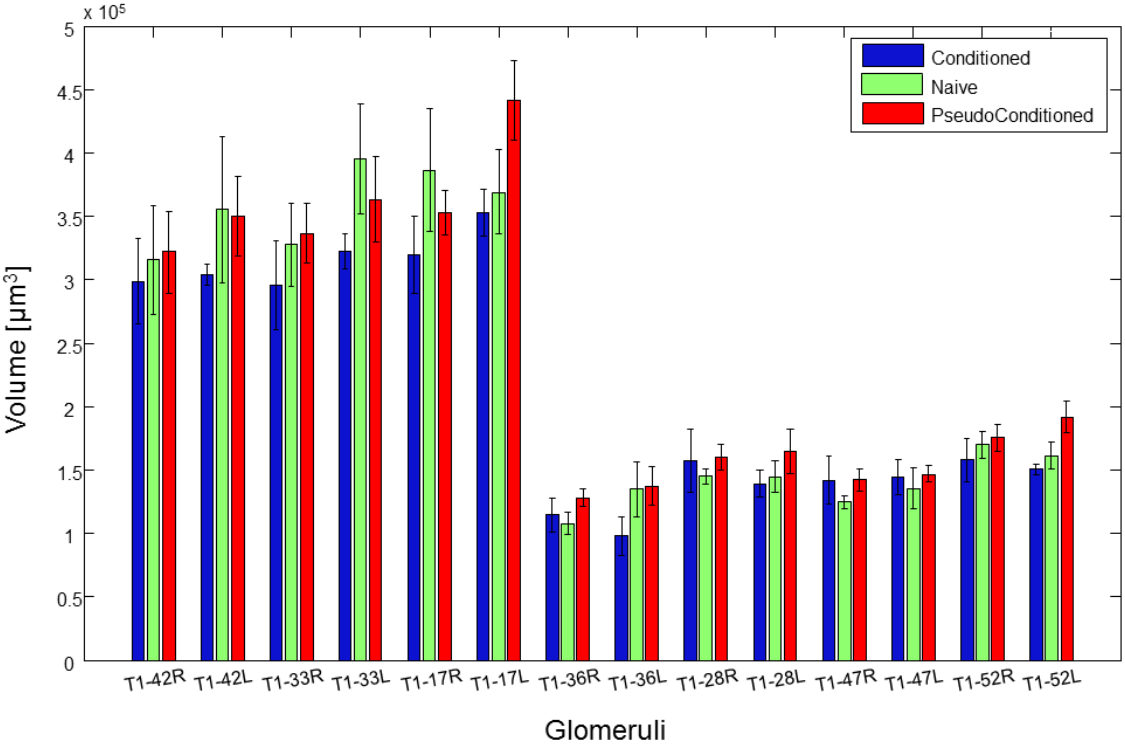

Figure 25 - Comparison of volumes (µm^3) among different glomeruli (T1-42, 33, 17, 36, 28, 47, 52), the two brain sides (R: right, L: left), and the three groups (group colours: blue: Conditioned (n=3), red: Pseudo-conditioned (n=6), green: Naïve (n=5)). Data are shown as mean ± SEM.

By averaging right and left sides, and performing a two-way repeated measure ANOVA with glomerulus as within-subject factors and treatment as between-subject factor, treatment and treatment × glomerulus were still non-significantly affecting volumes (two-way repeated measures ANOVA: Treatment: $F_{1,\ 12}=0.32$, $p=0.58$; Glomerulus $F_{1,\ 12}=76$, $p<10^{-5}$; Treatment × Glomerulus interaction: $F_{1,\ 12}=0.13$, $p=0.73$), as expected from results of the previous analysis. Averaged volumes are shown in Figure 26.

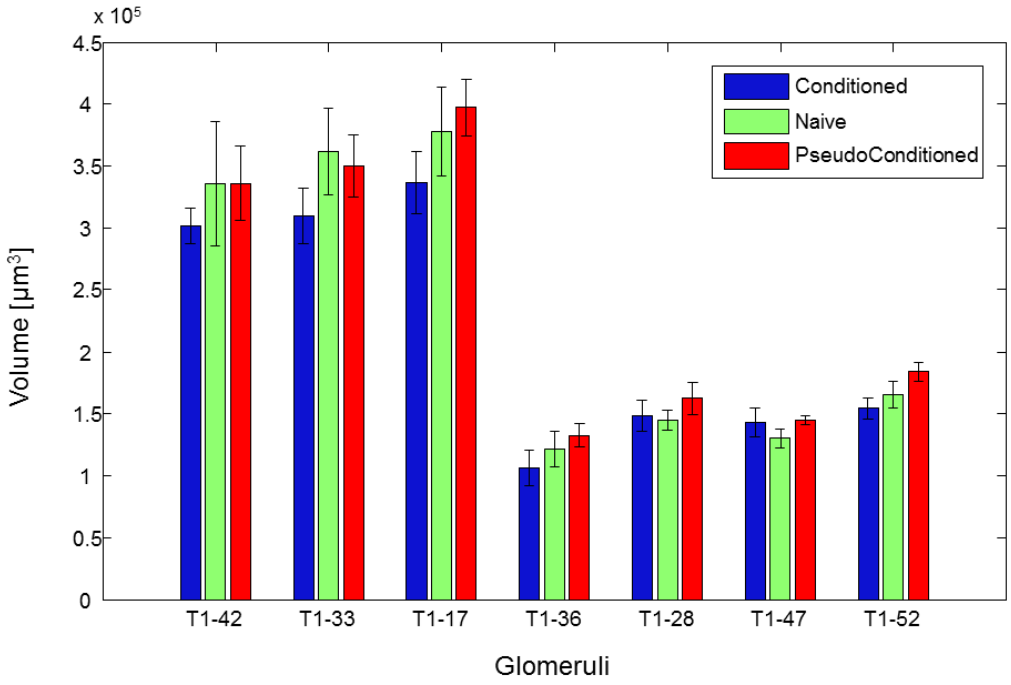

Figure 26 - Comparison of average right/left volumes (μm^3) among different glomeruli (T1-42, 33, 17, 36, 28, 47, 52), in the three groups (group colours: blue: Conditioned (n=3), red: Pseudo-conditioned (n=6), green: Naïve (n=5)). Data are shown as mean ± SEM.

2.4.2.5 Lateralization index.

Considering the analyses described before, there was no effects of treatment or treatment × side on glomerular volumes. However, we still wondered if treatment might affect lateralization. This question arose from two issues: 1) optical clearing might have influenced volumes differently in individual bees (further discussed in 2.4.3), so that effects of the treatments might have been missed in the ANOVA, and 2) a trend in lateralization is observable in Glomerulus T1-17 in Pseudo-Conditioned bees (see Fig. 25). This trend might have failed to reach significance due to the same reason in (1).

Therefore, we computed lateralization indexes, L, described as:

$L = VR/(VR+VL)$,

where VR is the right volume and VL the left volume, for all bees and all glomeruli. Average values of L are reported in Fig. 27.

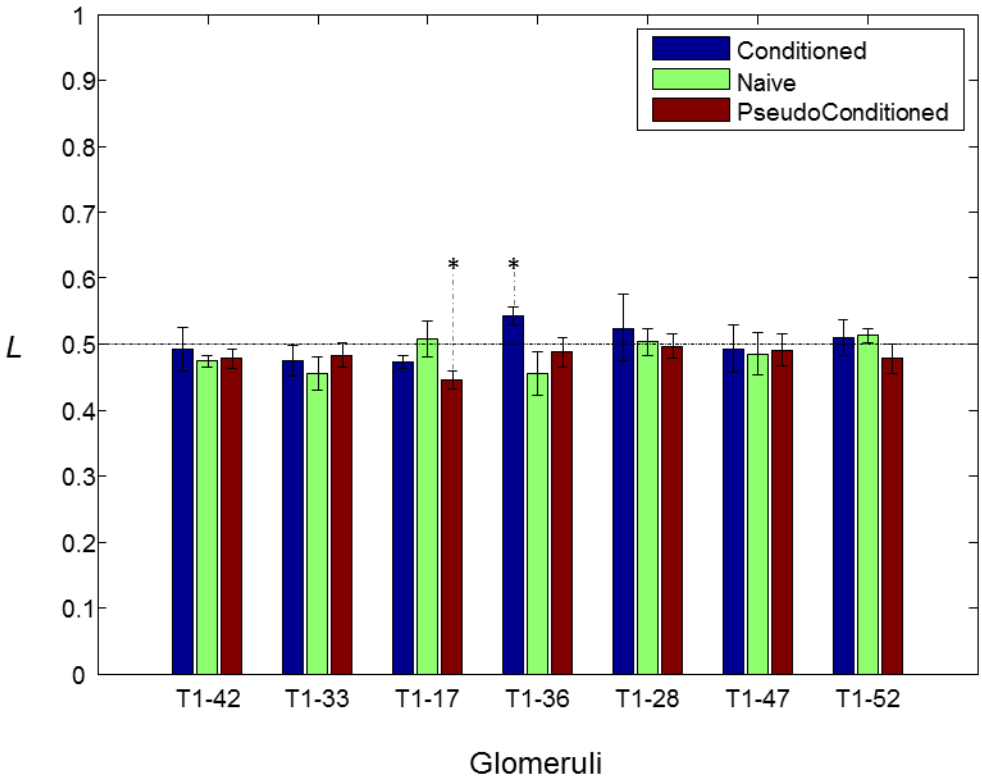

Figure 27 – Lateralization indexes calculated for the different glomeruli (T1-42, 33, 17, 36, 28, 47, 52), in the three groups (group colors: blue: Conditioned (*n*=3), red: Pseudo-conditioned (*n*=6), green: Naïve (*n*=5)). Data are shown as mean ± SEM. *: $p<0.05$.

Glomeruli T1-17 in pseudo-conditioned bees and T1-36 in conditioned bees show significant deviations from a normal distribution of *L* with mean=0.5, *i.e.* they might be lateralized. In particular, left glomerulus T1-17 in 1-hexanol pseudo-conditioned bees and right glomerulus T1-36 in 1-hexanol conditioned bees seem to be larger than the contralateral (*Z*-test: respectively, $p=0.048$ and $p=0.034$).

2.4.2.6 Optical clearing.

The optical clearing procedure, associated to α-synapsin immunostaining, proved successful in revealing details of the overall synapse distribution in the glomeruli (see Fig. 28 and Fig. 29). Since we did not observe any specific volumetric change caused by the treatment (see section 2.4.2.4), we did not use (optical) synapse density as a measure to evaluate whether volumetric change are paralleled by synapse increase. However, we propose it as a proof of principle method to analyse structural plasticity within glomeruli.

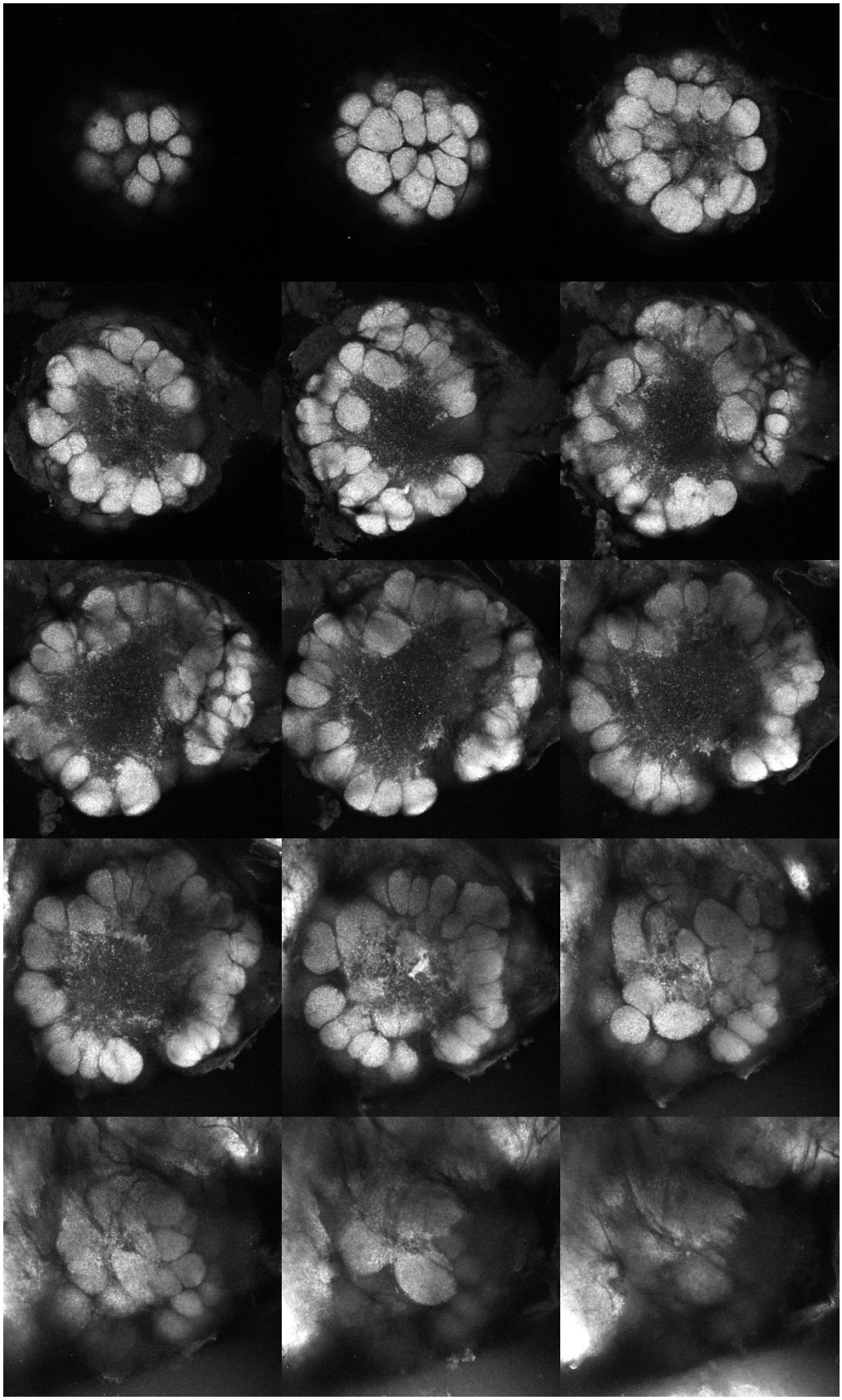

Figure 28 - Optical sections (step size: 30 µm; square view dimensions: 528 µm x 528 µm) of a right AL after treatment with anti-synapsin antibody and optical clearing. The tissue was

imaged in two-photon microscopy under a 20x objective. Glomeruli and the synapse compartment within them are visible throughout the whole AL depth (here, 450 µm).

In association with double-labelling of ORNs (see Fig.5), it might as well provide useful to study synapse density in the glomerular sub- compartments, *i.e.* glomerular core and cortex. The synapse-dense areas are indeed visible in great detail at high magnification (Fig. 29).

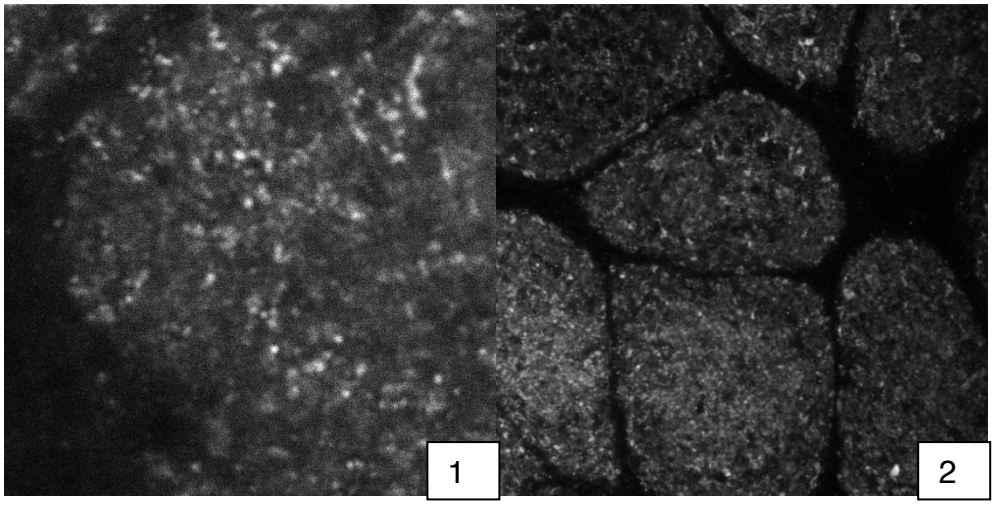

Figure 29 - Optical sections of AL glomeruli at high magnification. In (1) the AL is imaged under 100x objective, with a 3x optical zoom, up to a total magnification factor of 300x (square view dimensions: 35 µm x 35 µm). In (2) the AL is imaged under 100x objective, without further zooming (square view dimensions: 106 µm x 106 µm).

2.4.3 Discussion.

The experiments presented in this part were aimed at assessing associative learning-related plasticity in the honeybee AL. Our results do not support the previously published evidences of plasticity, *i.e.* volumetric increase of T1-17 and T1-33 in 1-hexanol conditioned animals (Hourcade et al. 2009). A previous study conducted in our lab also failed to replicate those results and, on the contrary, showed a trend for a volume decrease in both glomeruli (Rigosi 2013).

In particular, results presented here do not show any significant change in any glomerulus of any group. However, in our case, subtle changes might have been masked by the optical clearing procedure. Indeed, all brains were treated at the same time, but imaged at the two-photon microscope over the

course of several weeks. Although cleared tissue should stabilize after initial shifts in volume (Hama et al. 2015), after a three weeks period we observed that glomerular volumes keep slightly changing over time. The estimated volumetric change over a 1 month-period was different in each recorded brain and glomerulus (data not shown), so that modelling the trajectory of change based on the time of imaging was impossible.

Given the limitation above, we defined a relative confidence interval *r.C.I.* for the change in volume that would be observable in our data, with

$$r.C.I. = 1.96 \ \max\left(\frac{\sigma}{V}\right)/\sqrt{n}$$

where *n* is the average *n* across groups, σ and *V* the standard deviation and mean of each glomerular volume, and 1.96 the factor corresponding to a two-sided test with 95% significance level. We can therefore exclude, since our analysis did not show significant differences across groups, relative volumetric changes larger than *r.C.I.* ≈ 33% of glomerular volume. Largest volume changes reported by Hourcade and colleagues were ~40% of the glomerular volume. We are not able to exclude the occurrence of smaller changes.

For what concerns lateralization, more information could be reliably extracted from the data. Measures of *L* in fact indicate that some lateralized effects do exist. Lateralization index *L* would still constitute a reliable measure, given the artefacts. Indeed, we expect that, within the same brain, symmetric structures - such as the same glomeruli on the two brain side - should be affected by those volume oscillations in the same way (they have very similar volumes, and should be exposed to the external solution in the same way).

Another issue in our data is the low *n.* The choose to reduce variability by selecting only animals with a precise pattern of response in the memory test was a good attempt to make the small changes in volumes, that we are looking for, more evident. Anyhow, odour conditioning produced high number of generalizers at 72 h within the conditioned bees, which should be excluded. Other issues such as mortality (which should always be considered when subjecting the bees to many-days procedures), and the impossibility to

include all imaged brains in the measure, due to damage to the tissues or ambiguity in glomerular identification, either unilateral or bilateral, further reduced the number of subjects.

If repeating the same experiment again, we would hence suggest increasing the initial *n* for the conditioning procedure, and selecting age-controlled foragers grown in an indoor hive, so that initial variability among individuals is kept to a minimum. Indeed, the effort required to harness, condition, nourish and test the bees, and that of dissecting, staining, imaging, reconstructing and measuring (three times in this case) the brains requires long times, and *n* should be optimized to be the minimum useful to make it possible for slight volume changes to be evident in the analyses.

For what concerns the optical clearing procedure, associated to α-synapsin immunostaining and to two-photon microscopy at high magnification, it has proven very successful in visualizing the synaptic compartment of glomeruli with great detail. We believe that, in association with ORNs tracing (see 1.4.2 for a proof-of-principle image of double ORN-synapsin staining), it will also prove very useful to study synapse density in the sub-glomerular compartments. However, we suggest that volumes should be studied *before* the rinsing and the storage of tissues in the clearing solution, to avoid volume oscillation artefacts. Also, imaging and *z*-stack acquisition at low magnification (for volume reconstruction) should be planned to happen to in the shortest time possible for all brains.

Finally, the lateralization effect we observed within individuals (leftwards imbalance of glomerulus T1-17 in pseudo-conditioned bees and rightwards imbalance of T1-36 in conditioned animals) is very interesting and suggest two important evidences: 1) AL plasticity changes might happen within a specific brain side rather than bilaterally, and 2) changes might emerge in the pseudo-conditioned group as well as in the conditioned.

For what concerns (1), it is unclear whether Hourcade and colleagues measured glomeruli on one side or both sides of the brain, and if they averaged among bilateral measures (Hourcade et al. 2009). If they measured

always on one side of the brain, their results might reflect changes specific to that side. However, in a previous experiment in our lab, volumetric changes following conditioning were evaluated bilaterally and no lateralization emerged (Rigosi 2013). We do not know how to explain this discrepancy; anyhow, a low *n* might be the major issue also in this case (*e.g.* for 1-hexanol pseudo-conditioned bees, *n*=6 in both our and Rigosi's data).

For what concerns (2), may the result be confirmed by higher *n*, it is very interesting to notice that pseudo-conditioning represents an experience *per se*. Changes previously highlighted in conditioned animals by Hourcade and colleagues do not equal to saying that - in their data - conditioned animals did diverge from control, naïve, bees. It is as well possible that changes highlighted in that study did result from a divergence of pseudo-conditioned bees from control, naïve, bees (Hourcade et al. 2009). In fact, naïve bees were not considered in that study.

We claim here that in any future analyses of plasticity related to associative learning two controls should be included: pseudo-conditioning, and naïve animals. Of course, comparing volumes across three groups, instead of two, would require even higher *n*.

For this reason, we think that, first of all, effects of odour exposure (unrewarded, or anyhow not related to associative learning) on the adult honeybee AL should be evaluated. This problem has never, to our knowledge, been addressed before. This consideration led us to the experiments that I will describe in next sections.

2.5 Odour exposure-related plasticity in the honeybee antennal lobe (structural).

2.5.1 Methods.

2.5.1.1 Animals.

Forager honeybees were collected with a transparent plexiglass pyramid at the entrance of the hive in spring and summer 2015, in Rovereto (TN).

2.5.1.2 Odour pre-exposure.

The bees were caged in groups of ~30-40 individuals in insect tents (BugDorm-2120). The exposure procedure started at 4 pm and lasted 72 h. Odorants used were 1-hexanol (HEX) in one tent and 1-nonanol (NNL) in a second enclosure (all odours: 1:50 dilution in mineral oil up to a total volume of 100 µl). In a third tent, a control group was exposed to mineral oil only (all compounds from Sigma-Aldrich).

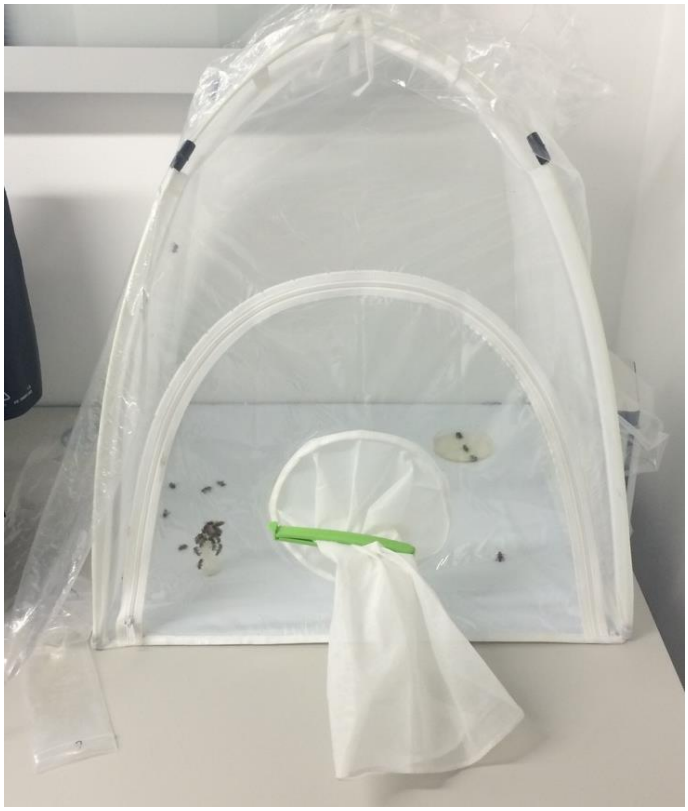

Figure 30 – Bees within each group were caged in tents, where a food source (50% w/w sucrose; on the left in the picture), water, and odour exposure (in the picture, the two Petri

dishes on the right), were provided, according to a daily schedule. The tents were loosely covered in order to avoid excessive odour leakage.

All odour samples were suffused onto a piece of filter paper, enclosed in a plastic Petri dish with a perforated lid, and set inside each tent. Bees had access to a feeder in the form of a glass Boardman bottle containing 50% w/w sucrose solution seeping up through cylindrical dental filters. The feeder was spatially separated from the odour source. An additional Petri dish carrying pure water was added at a third location to provide drinking water and to keep humidity at a constant level (see Fig. 30). The tents were kept in a single room at a distance of ~ 3 m and were loosely covered with transparent plastic wrap in order to avoid excessive odour leakage. On the first day, bees were first provided with food and water. The odour dispenser was added after one hour. Each following day, food and odours were removed independently for certain periods, according to a daily schedule shown in Figure 31, and renewed afterwards. The temperature and humidity of the room were monitored and kept respectively between 22°-26 °C and 45-55%. Lights were turned on in the morning and off in the evening.

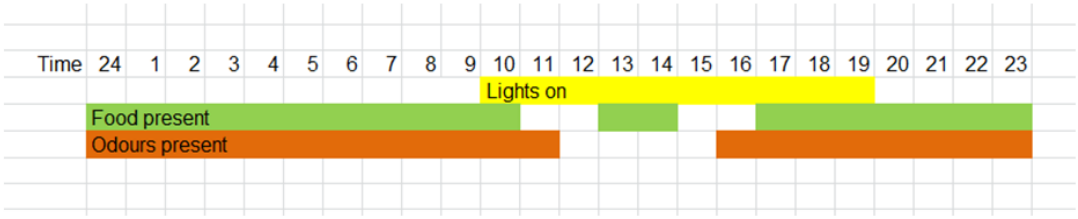

Figure 31 - Daily schedule of odour/food/light exposure. On the first day, bees were first provided with food and water. The odour dispenser was added after one hour. Each following day, food and odours were removed independently for certain periods, according to the schedule, and renewed afterwards.

2.5.1.3 Brain dissociation and immunohistochemistry.

After 72 h of odour exposure, a subset of bees was sacrificed, and brains were dissociated and processed for immunohistochemistry. Briefly, bees were decapitated and heads were fixed in a 4% paraformaldehyde solution (4° C O/N). Brains were later carefully dissociated in PBS. Subsequent washing and immunostaining procedures were all conducted in a 0.5% Triton X-100 PBS

solution. After blocking of non-specific sites through incubation in a 5% normal goat serum solution (1 h RT), the brains were incubated (48 h, 4° C, 4% in Triton-PBS) with anti-synapsin antibodies (DSHB Hybridoma Product 3C11 (anti SYNORF1)). The binding sites were then revealed with an Alexa Fluor 546 secondary fluorescent antibody (Alexa Fluor 546 donkey anti-mouse IgG (H+L), Invitrogen Molecular Probes; incubation 48 h, 4° C, 2% in Triton-PBS).

2.5.1.4 Optical setup.

Same as in previous section, see Optical set-up 2.4.1.6.

2.5.1.5 Acquisition and 3D reconstruction.

Z-stacks of the right and left ALs (3µm step size along the antero-posterior axis) were evaluated in ImageJ (Schneider et al. 2012a), and glomeruli were identified. In cases where both the right and the left sides were perfectly intact and unambiguous labelling of the glomeruli could be achieved, the images were processed in Amira (FEI) for 3D reconstruction and volumetric measurements of the glomeruli of interest. The whole procedure was conducted blindly with respect to both group and side, necessitating prior horizontal flipping of *z*-stacks of the right AL.

2.5.1.6 Statistical analyses.

Volumes were analysed via a mixed ANOVA with side and glomerulus as within-subject factors and treatment as between-subject factor. Each glomerulus was then analysed via a repeated-measure ANOVA with side as within-subject factor and treatment as between-subject factor. Independent sample *t*-test comparisons were performed within each glomerulus, using a right-left averaged measure of each glomerulus in each bee. All analyses were conducted in MATLAB (Mathworks).

2.5.2 Results.

2.5.2.1 Odour experience.

Bees were divided into three groups and pre-exposed over 3 days to one of two floral odorous compounds, 1-hexanol (HEX) and 1-nonanol (NNL), or to

mineral oil as a control (CTR). The exposure schedule was specifically intended not to create anticipation relationship between odorant and sucrose (see 2.5.1.2).

2.5.2.2 Effects of odour exposure on glomerular volumes.

Following a fluorescent immunolabelling procedure of the synaptic compartments, brain morphology was imaged and analysed for a subset of animals from each treatment group. The ALs' glomeruli were identified, segmented, and 3D-reconstructed. A subset of glomeruli was bilaterally measured in each individual. The chosen glomeruli were selected for responding strongly either to HEX (glomeruli T1-38 and T1-28), to NNL (glomeruli T1-17 and T1-33) or to neither (glomeruli T1-47 and T1-42; Sachse and Galizia 2002).

Measured glomerular volumes are reported in Figure 32. A mixed ANOVA was performed, with right/left side and glomerulus number as within-subject factors and treatment as between-subject factor. Treatment, glomerulus, and their interaction significantly affected volumes (Treatment: $F_{2,40}$=7.8, p=0.0014; Glomerulus: $F_{5,200}$=450, $p<10^{-106}$: Treatment*Glomerulus: $F_{10,200}$=3.0, p=0.0017). Brain side did not show any significant main effect or interaction with the other factors (Side: $F_{1,40}$=0.060, p=0.81; Treatment*Side: $F_{2,40}$=1.4, p=0.26; Glomerulus*Side: $F_{5,200=}$0.29, p=0.92; Treatment*Glomerulus*Side: $F_{10,200}$=0.80, p=0.63), meaning that the volumetric changes we observed were not lateralized (Rigosi et al. 2011; Haase et al. 2011; Frasnelli et al. 2014). Accordingly, for further independent sample *t*-test comparisons, corresponding right and left glomeruli were averaged in each bee.

Non-associative odour experience led to a decrease in volume of specific glomeruli. In particular, glomeruli T1-33 and T1-17 were both reduced in cases of NNL pre-exposure. A trend was also observed for a reduction in T1-28 volume in HEX pre-exposed bees.

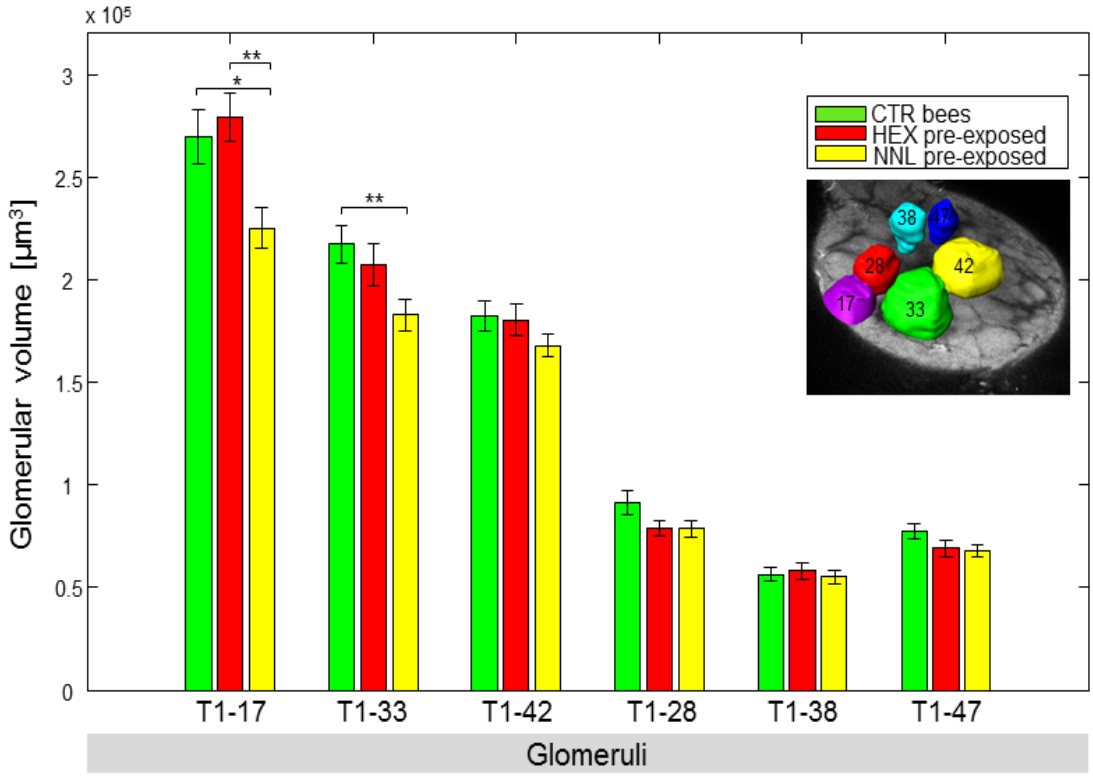

Figure 32 - The average volumes of the six glomeruli in the three treatment groups (CTR bees, HEX pre-exposed, and NNL pre-exposed bees). An example of segmented and 3D-reconstructed glomeruli T1-17, 33, 42, 28, 38, 47 from an immunolabelled left AL image stack is shown in the inset. Volume data were obtained by left-right averaging (see Methods). Bars represent mean ± SEM (n_{CTR}=13, n_{HEX}=15, n_{NNL}=15). Groups with significantly different means are indicated (*: p<0.05, **: p<0.01).

2.5.2.3 Lateralization index.

Even if side was not a significant factor in the ANOVA, we decided to compute lateralization indexes *L* across bees, to highlight possible subtle effects of lateralization. In fact, as we observed in the previous experiment, *L* might reach significance independently of results in the ANOVA.

However, *L* in the six measured glomeruli did not differ among the three groups of bees (Fig. 33). Therefore, we can exclude any structural lateralization in the considered glomeruli following HEX and NNL exposure.

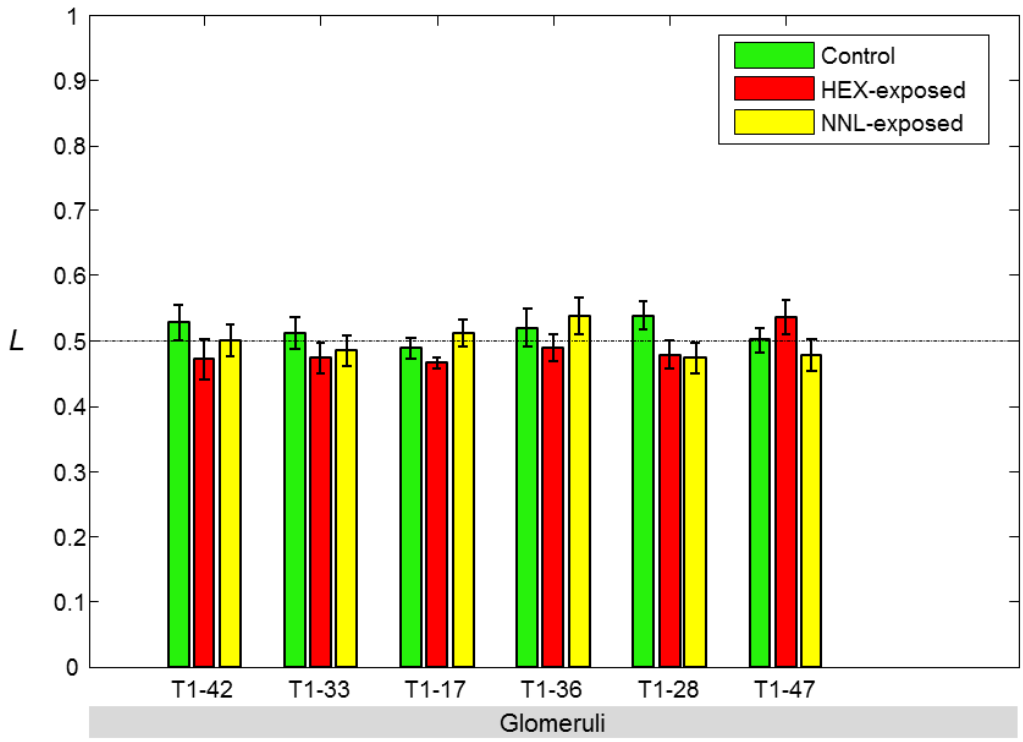

Figure 33 - Lateralization indexes calculated for the different glomeruli in the three groups (CTR bees, HEX pre-exposed, and NNL pre-exposed bees). Bars represent mean ± SEM (n_{CTR}=13, n_{HEX}=15, n_{NNL}=15).

2.5.3 Discussion.

We used a paradigm of prolonged pre-exposure of honeybees to single-odour compounds, similar to those that have been shown to produce measurable morphological and functional changes in *Drosophila melanogaster* (Sachse et al. 2007; Das et al. 2011; Devaud et al. 2001). In the above-mentioned study, this kind of non-associative odour experience has been shown to cause non-associative learning *e.g.* a reduction of avoidance response to a habituated odorant (Sachse et al. 2007).

The literature in *Drosophila*, suggests that odour-specific volumetric changes in the glomeruli would happen only during a certain time window following eclosion (Devaud et al. 2003). However, we observed structural plasticity in adult forager bees, well past any such window. The high flexibility that foraging activity requires, regarding individuation of food sources in space and

time, may account for the preservation of plasticity in the honeybee olfactory structures.

Volumetric changes in the AL glomeruli have been reported in the adult bee alongside associative learning (Hourcade et al. 2009). Here we report, for the first time, volumetric changes accompanying non-associative odour experience. Our volumetric measurements on reconstructed glomeruli show significant changes in at least two of the six sampled glomeruli (T1-33 and T1-17) for at least one of the two pre-exposure odours (NNL). Volumetric changes were not lateralized.

It is very interesting that the two affected glomeruli are both highly responsive to the pre-exposure odour. To better understand the mechanisms behind this specificity, we also analysed the AL of those animals from a functional point of view (see following section).

Notably, we did not find any structural changes in the measured glomeruli following HEX pre-exposure. We expected such treatment to cause similar effects to 1-hexanol pseudo-conditioning (see previous section), *i.e.* T1-17 lateralization. Repeated and prolonged odour exposure might indeed share mechanisms through which they are established in the olfactory pathway, as they both have been shown to cause latent inhibition of PER in adult bees (Chandra et al. 2010; Fernández et al. 2009). However, it might as well be that the two manipulations cause similar behavioral consequences through different mechanisms. Another possibility is that effects on pseudo-conditioned bees in the previous experiment (on associative learning, see 2.4) were inflated by small *n*. Also, we cannot exclude that plastic changes in the AL following HEX pre-exposure might happen at sites different from those that we have considered.

To conclude, for the first time we demonstrated that simple odour exposure is able to cause plastic changes in the honeybee AL. Plasticity in the adult brain, therefore, is able to produce similar volumetric changes in different individuals, irrespective of foraging experience before the experiment. It is

anyhow likely that effects would be even stronger under experience- and age-controlled conditions.

2.6 Odour exposure-related plasticity in the honeybee antennal lobe (functional).

2.6.1 Methods.

2.6.1.1 Animals.

Same as in previous section, see Animals, 2.5.1.1.

2.6.1.2 Odour pre-exposure.

Same as in in previous section, see Odour pre-exposure, 2.5.1.2.

2.6.1.3 Functional imaging protocol.

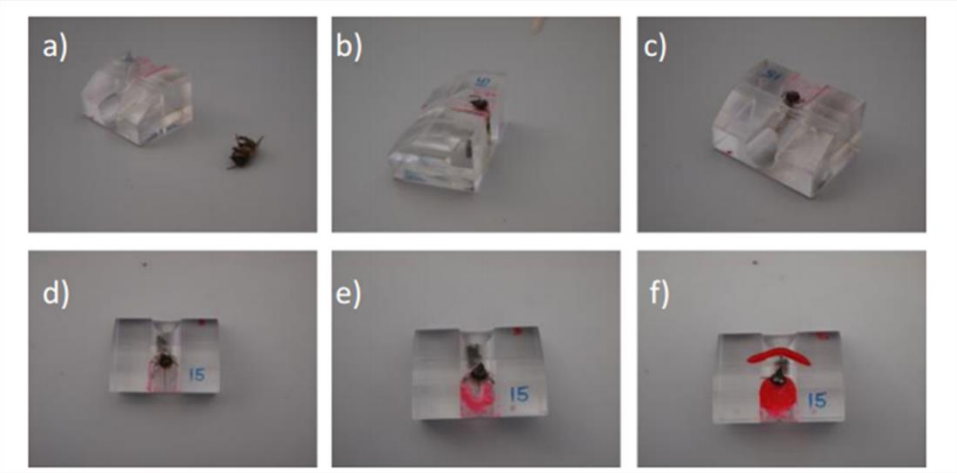

Figure 34 – Staging of the bee for PN injection and functional imaging: the neck is inserted in a fissure within a custom-designed imaging stage. The head is then fixed with a sealed plastic foil covering the fissure and soft dental wax, to avoid movements. Adapted from (Paoli et al. 2017).

A subset of bees was prepared for *in vivo* calcium imaging. Briefly, after chilling to immobility, the bees were mounted onto imaging stages (see Fig. 34). After opening a cuticle window on top of the head, on one side of the brain, dextran-conjugated fura 2 (Life Technologies) was injected into the medial and lateral-antennoprotocerebral tracts (APTs; Paoli et al. 2016b; see

Fig. 8) via a pulled borosilicate glass needle. The next day, fluorescence changes in the ALs were recorded while stimulating the animals with 1-hexanol (HEX), 1-nonanol (NNL), and a binary 50:50 mixture of the two (MIX). Calcium-dependent fluorescence changes were acquired through repeated scanning of a 1D trace crossing all glomeruli of interest within one focal plane (Fig. 35).

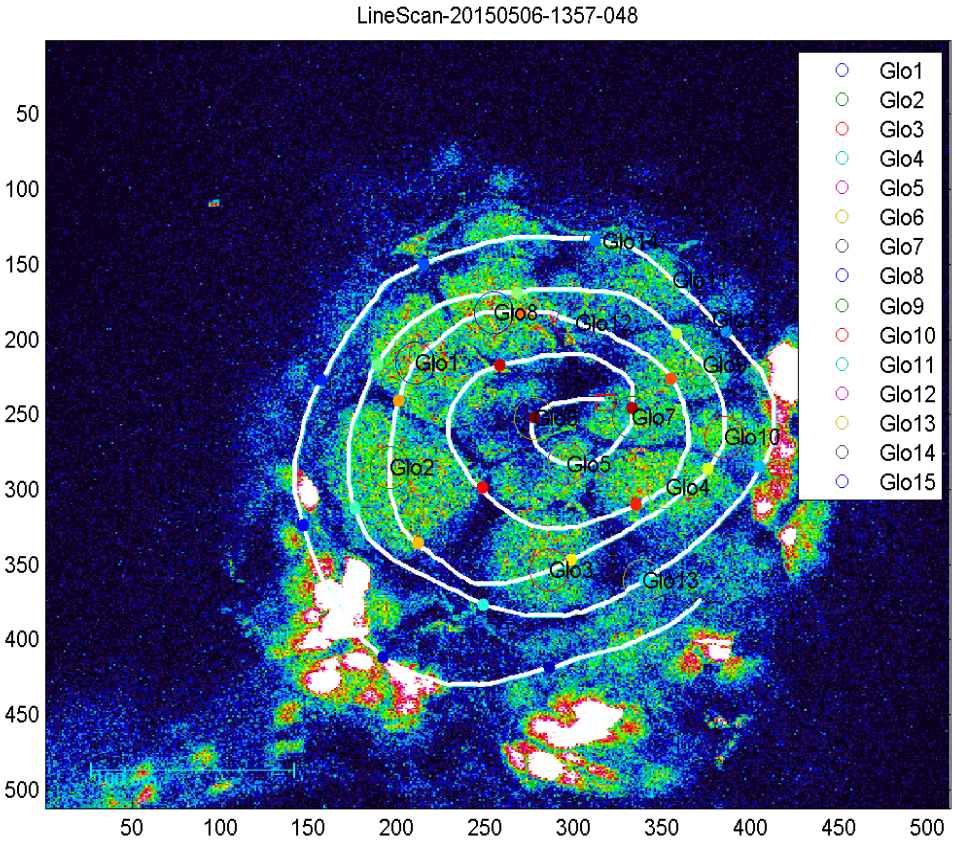

Figure 35 – Example of AL scanning path. The optical path (white) crossing the glomeruli of interest is scanned repeatedly by directing the laser beam through galvanometric mirrors. The fluorescence is then collected, and the intensity over time of the scanline pixels recorded. The fluorescence intensity is here represented in pseudo-colours. Intervals of the scanline are later assigned to different glomeruli (Glo) based on manual identification of the glomerular boundaries.

The raw data obtained are of the type shown in Figure 36. For further details see Paoli *et al*, 2016 (Paoli et al. 2017).

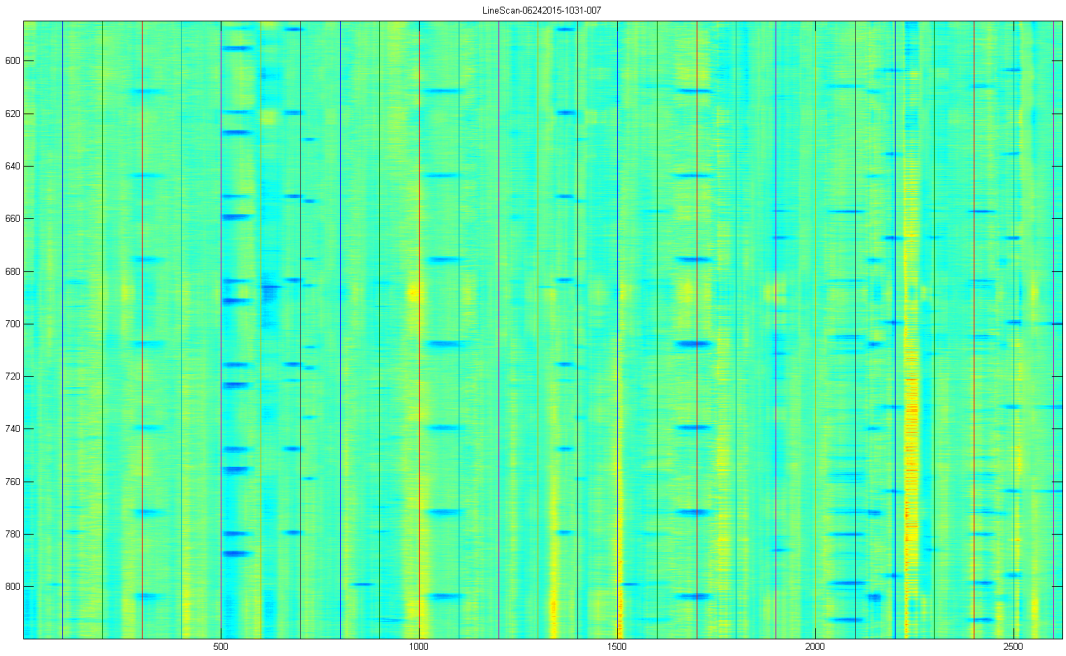

Figure 36 – Fluorescence over time (raw data). In this example of raw data, the fluorescence intensity (in pseudo-colours) of >2500 pixels composing a scanline (*x*- axis) is plotted over time (> 800 s, *y*- axis). Repetitive stimulations with odours produce repetitive fluorescence changes (dark horizontal lines) at location corresponding to active functional units.

2.6.1.4 Odour stimuli.

Odour stimuli were delivered to the bee antennae through a constant air flow via a custom-made stimulus generator (Fig. 37), controlled via a LabVIEW interface and a National Instruments board (Paoli et al. 2016a).

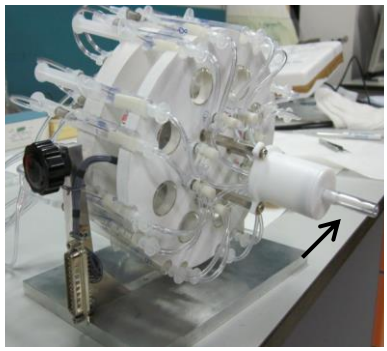

Figure 37 – The so-called “odour-revolver”, delivering up to 8 different olfactory stimuli to the bee through its “nose” (indicated by the black arrow).

The odour stimuli consisted of a head space of glass vials containing HEX diluted 1:500 or NNL diluted 1:250 (due to its lower volatility), both in mineral

oil. The binary mixture was created by coherent summation of both individual channels. Each pure odour application had duration of 1 s and was repeated 6 times. The initial delay was 2 s for the novel odour and 8 s for pre-exposed odour. By using stimulus periods of 4 s for the novel odour and 2 s for the pre-exposed odour, a stimulus pattern is created which presents the novel odour, the pre-exposed odour and the binary mixture in a pseudo-random manner (see Fig. 38). CTR bees were randomly given the exposure protocol of either HEX or NNL pre-exposed bees.

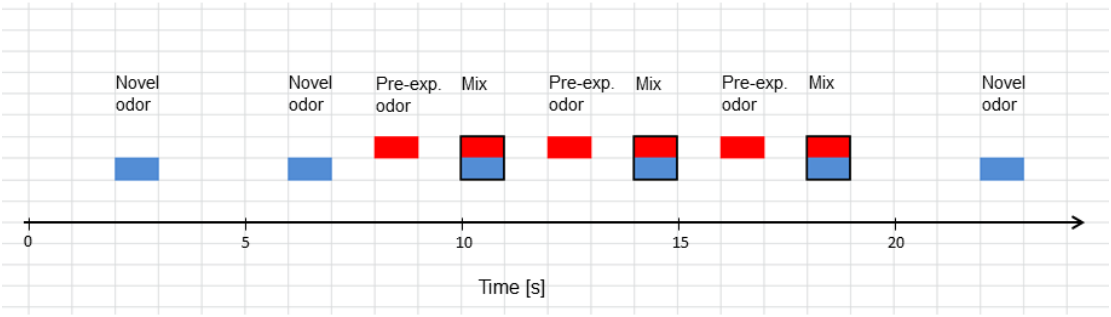

Figure 38 – Paradigm of odour stimulation.

2.6.1.5 Optical imaging setup.

Same as in previous section, see Optical set-up, 2.5.1.4.

2.6.1.6 Functional data analyses.

Calcium imaging data (fluorescence intensity along the scanline as a function of time) were post-processed using MATLAB (Mathworks). After de-noising via spatial and temporal averaging, responding regions were assigned to single glomeruli in a semi-automatic algorithm. The temporal signal was then split into single stimulus windows, from 0.5 s pre-stimulus to 1 s post-stimulus, for each glomerulus. The relative activation was obtained by normalizing the fluorescence intensity to the pre-stimulus baseline: $-\Delta F/F$. Only glomeruli responding with an average stimulus-evoked activity (200-500 ms after stimulus onset) significantly (1.96 σ) deviating from the average baseline activity were considered for quantitative analysis of the odour response pattern. The average $-\Delta F/F$ (200-400 ms after stimulus onset) was used as a measure of maximum response (Fernandez et al. 2009). To analyse the

separation of the odour response maps in glomerular coding space, the Euclidean distance ED between two odours *x* and *y* was calculated:

$$ED_{x,y} = \sqrt{\sum_{i=1}^{n}(x_i - y_i)^2},$$

where x_i and y_i are the maximum responses of a single glomerulus *i*, which are summed over all *n* considered glomeruli. Normalized distances between pure odours and mixture were obtained by dividing their EDs by the ED between the pure compounds NNL and HEX within each group. The reliability of the EDs calculated on these large-dimension samples was assessed via bootstrap resampling with replacement (*N*=1000 repetitions). The sampling distribution of differences between two EDs was then tested by a *z*-test against a standard normal distribution (after confirming its normality). A similar procedure was used to compare the ratios of the EDs. The ratio distributions were obtained by bootstrapping (*N*=1000 repetitions) and each sampling distribution of differences between ratios was tested by a *z*-test against a standard normal distribution (after confirming its normality).

To visualize the response dynamics, the odour coding space dimension was reduced via a principal component analysis (PCA) of the joint coding space of all three groups. The transformation matrix was obtained based on the average activity 200-400 ms after stimulus onset. The first two principal components explain, respectively, 76.75% and 23.25% of the overall variation.

Single glomerular responses of T1-33 and T1-17 to the odour stimuli were compared between treatment groups via one-way ANOVA and independent sample *t*-tests. All pre-processing steps and analyses were conducted in MATLAB (MathWorks).

2.6.2 Results.

2.6.2.1 Odour experience.

Same as in in previous section, see Odour experience, 2.5.2.1.

2.6.2.2 Odour pre-exposure effects on the odour code.

A subset of animals was prepared for PN calcium imaging and imaged on the following day, as described in 2.6.1.3. Stimulus-evoked activity was evaluated within each animal in response to the single odours HEX and NNL and a binary 50:50 mixture (MIX).

The change in fluorescence over time was analysed for several glomeruli (by assigning specific pixels of the scanning path to specific glomeruli). The glomeruli were recorded in limited number within a single focal plane in each bee, so as to avoid odour overexposure by excessive repetition of the same paradigm of stimulation. The plane was chosen to contain the landmark glomeruli T1-17, T1-33, T1-42. Due to deviations among these focal planes, glomerular subsets were not fully overlapping across different bees. Therefore, instead of averaging over all animals (n_{CTR}=5, n_{HEX}=5, n_{NNL}=4), glomeruli of all bees of the same treatment were pooled together in order to construct overall odour representations in each group (n_{CTR}=56, n_{HEX}=62, n_{NNL}=44).

The dimensionality of this coding space was then reduced by principal component analysis (PCA). The odour-response dynamics (1 s during stimulus and 1 s post-stimulus) are shown in the first two principal components (Fig. 39). The PCA shows a relative difference in odour representation among the three pre-exposed groups. In the CTR group, the binary mixture MIX activation vector is centred between the two pure components HEX and NNL, while in the odour pre-exposed groups, MIX shifts away from the familiar compound and toward the novel odour.

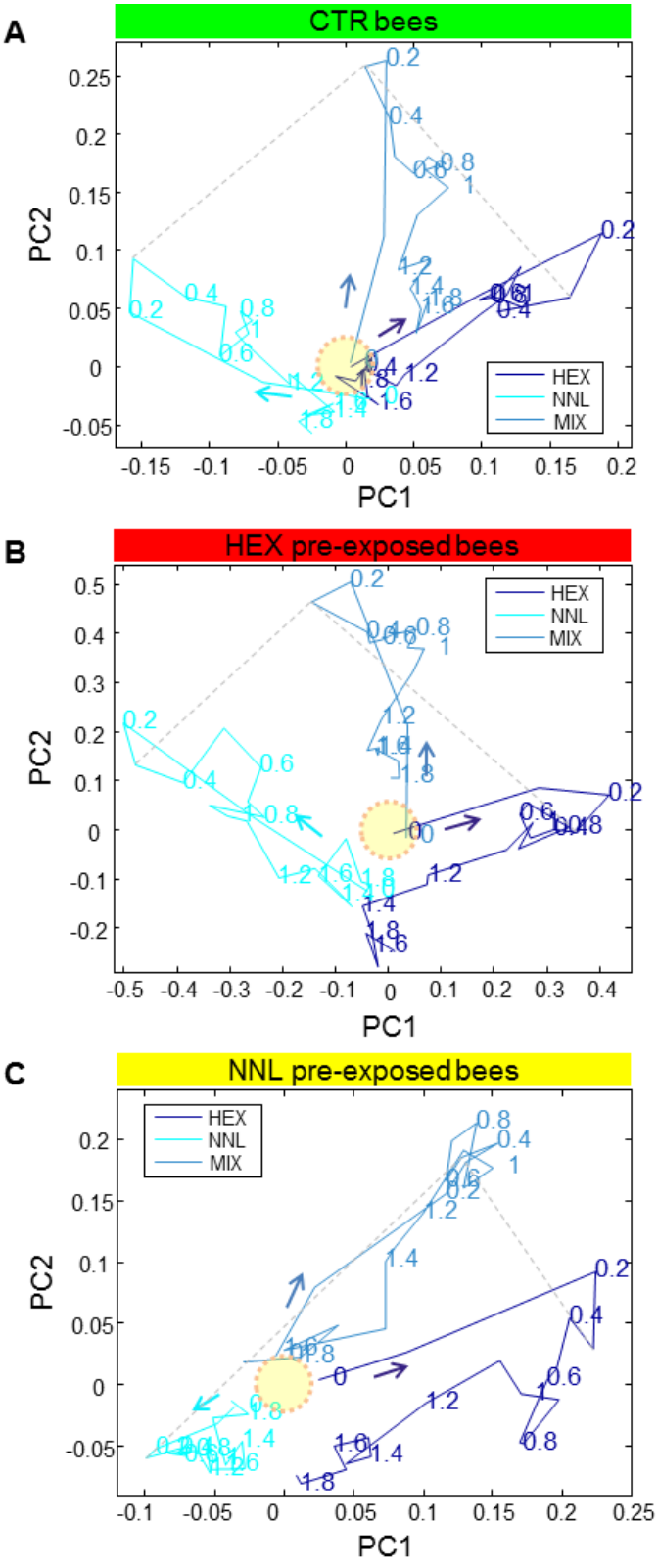

Figure 39 - Activation dynamics during the presentation of three odour stimuli in the principal component coordinate system (1 s stimulus and 1 s post-stimulus) for the 3 groups: (A) Control (CTR) bees, (B) Hexanol (HEX) pre-exposed bees and (C) Nonanol (NNL) pre-exposed bees. Dynamics account for activity of all measured glomeruli in a given treatment

group. HEX activation vector is shown in blue, NNL in cyan, and their binary mixture (MIX) in light blue. The origin, marked by a yellow circle, corresponds to baseline activation. Arrows show the temporal axis; numbers indicate time after stimulus onset in seconds. Dashed grey lines connect the 300 ms time-points, which are the centres of the maximal odour separation period.

This effect can be quantified by the Euclidean distance (ED) between odour pairs in the individual groups. The EDs were calculated based on the average activity of the recorded glomeruli in the interval 200 to 400 ms after stimulus onset. We normalized the EDs within each group to the ED between the pure compounds (Fig. 40). In the CTR group, in which animals were exposed to mineral oil only, single odours are again equidistant from the 50:50 mixture, while in the odour-pre-exposed groups, the ED between mixture and novel odour is reduced. However, this difference was significantly different from zero only in the case of NNL pre-exposed bees (*z*-test of sampling distribution of differences, p=0.016).

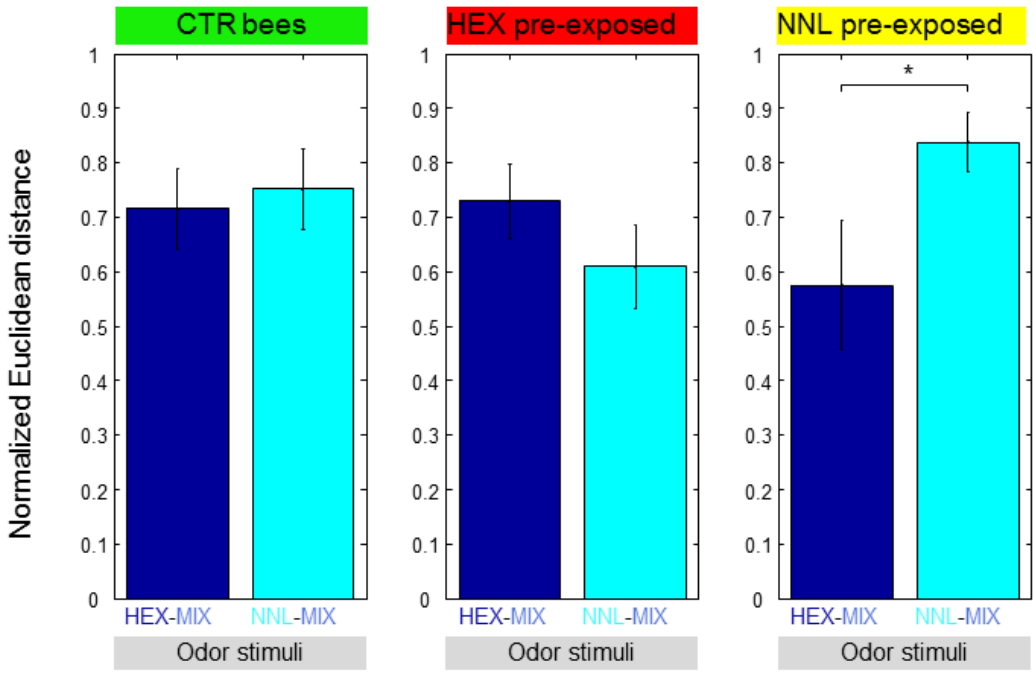

Figure 40 - Euclidean distances between representations of MIX and pure compounds within each group, normalized to the ED between the two pure compounds. Error bars represent standard deviations obtained via bootstrap resampling with replacement of glomeruli (N=1000). The difference was significant in the NNL pre-exposed group (*z*-test, *: p=0.019).

To compare this relative shift among groups, we computed ratios between the HEX-MIX and the NNL-MIX distances within each group (Fig. 41). This ratio was approximately 1 in the case of CTR bees, while it increased for HEX pre-exposed bees and decreased for NNL pre-exposed bees. The difference between the two groups appeared to be significant (*z*-test of sampling distribution of differences, p=0.041).

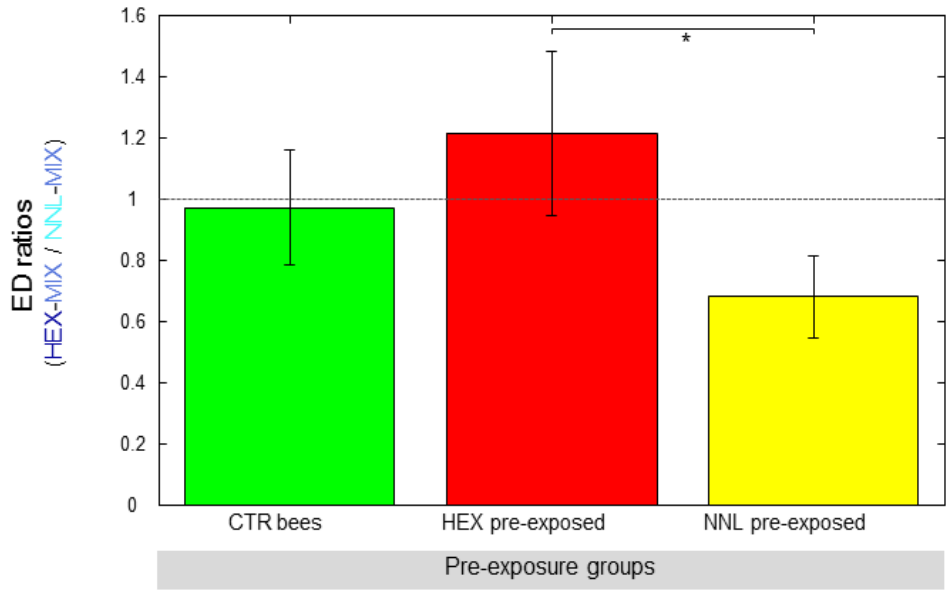

Figure 41 - Ratios of normalized EDs within each group. Error bars represent standard deviations obtained by bootstrapping. The shift between the HEX and the NNL pre-exposed groups was significant (*z*-test, *: p=0.041).

2.6.2.3 Odour pre-exposure effects on the individual odour responses.

Based on the observation that glomeruli T1-33 and T1-17 were most (structurally) changed by odour pre-exposure (2.5.2.2), their single odour-evoked response profiles were compared among groups (Fig. 42).

T1-17 response profiles to the three stimuli HEX, NNL, and MIX are shown in Figure 42A; those of T1-33, in Figure 42B. Odour responses are clearly changed following olfactory pre-exposure. In the CTR group (green bars), neither glomerulus responded to HEX (left columns), but both were strongly excited by NNL (center) and the MIX (right). Glomeruli in the HEX pre-exposure group (red bars) shifted the response to HEX towards inhibition (left columns), although not significantly. Meanwhile, the responses to NNL

(middle) and to the MIX (right) increased in comparison to those of CTR bees (significantly in the case of glomerulus T1-33). Responses of the NNL pre-exposed group (yellow bars) shifted in the opposite direction, with a decreased response to NNL (middle) and to the MIX (right) in both glomeruli. Also, responses to HEX were shifted to a slight excitation in T1-33.

The two pre-exposure groups gave rise to significantly different response to both NNL and MIX in the case of glomerulus T1-33 and to MIX in the case of glomerulus T1-17.

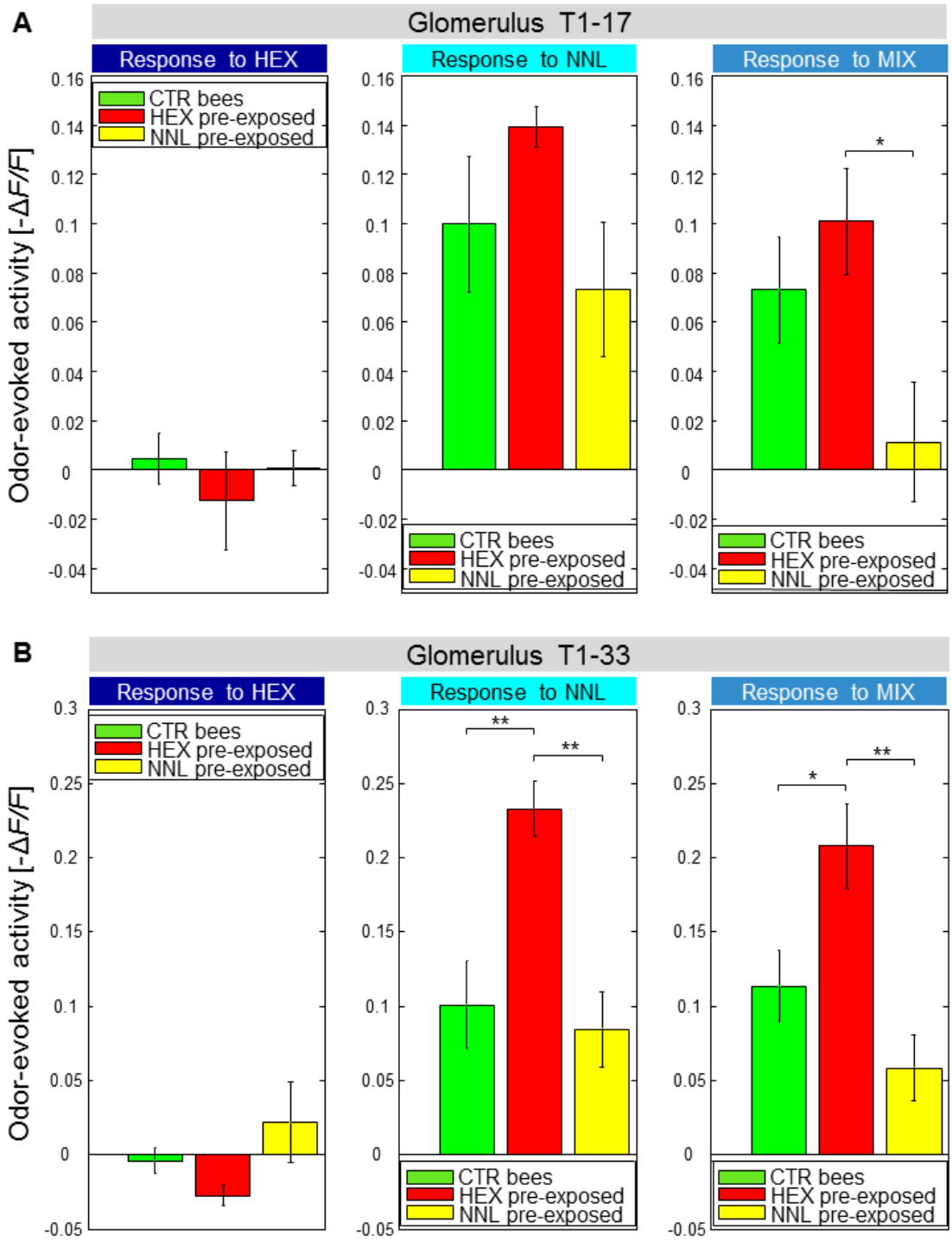

Figure 42 - Odour-evoked activity of glomeruli T1-17 (A) and T1-33 (B) in the three treatment groups, averaged over single bees. Bars represent mean ± SEM (n_{CTR}=5, n_{HEX}=5, n_{NNL}=4). The left histograms show the responses to 1-hexanol of all three treatment groups; the middle histograms, to 1-nonanol; and the right histograms, to the 50:50 mixture. The treatment effect was found to be significant via one-way ANOVA, and group means were confronted via two-sample *t*- tests (*: p<0.05, **: p<0.01).

2.6.2.4 Effects on individual odour responses do not depend on the stimulation paradigm.

Given the different order and inter-stimulus intervals of the two stimuli in the two pre-exposure groups (see *Methods, Odour stimuli, 2.6.1.4*), a question can be raised, regarding the possibility that the stimulation paradigm, rather than the pre-exposure procedure, influences the functional responses recorded in the two groups. Hence, we compared odour-evoked responses in example individual bees (1 NNL pre-exposed bee, 1 HEX pre-exposed bee, and 2 CTR bees which received one or the other stimulation paradigm) in glomeruli T1-17 and T1-33. The profiles of response for the glomerulus T1-17 are shown in Figure 43. The profiles of response for the glomerulus T1-33 are shown in Figure 44.

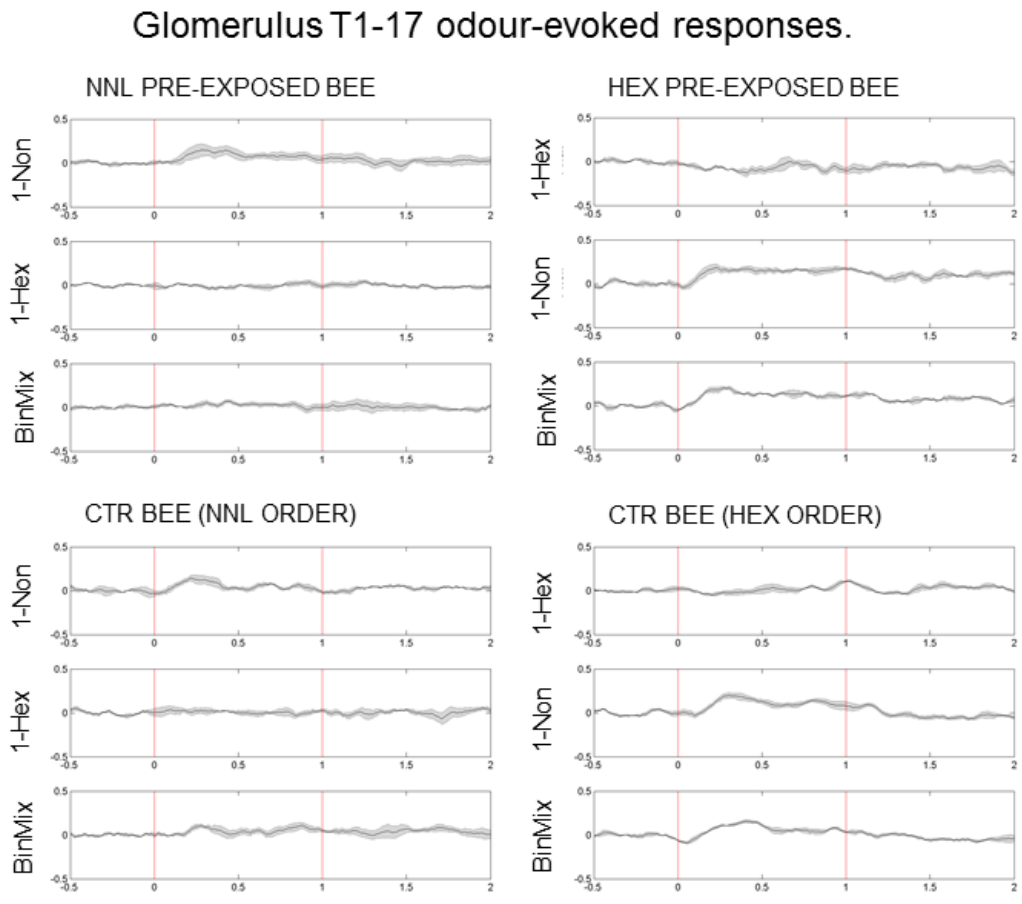

Figure 43 – Odour-evoked responses in bees recorded under different stimulation paradigms in glomerulus T1-17. Irrespective of the stimulation paradigm, the odour-evoked responses in the example control bees are similar to those of HEX-pre-exposed animals, while NNL pre-

exposed animals show different responses. In particular, in CTR and HEX pre-exposed bees, the profile response to1-hexanol (1-Hex) is found almost unchanged in the response to the binary mixture. In NNL pre-exposed bees, response to binary mixture is flat, as it is the one to 1-hexanol. Notice that the three stimuli are in different order in the right and left column. The plots represent three odour repetition averaged ± SEM (shadow). Red vertical bars represent odour onset and offset.

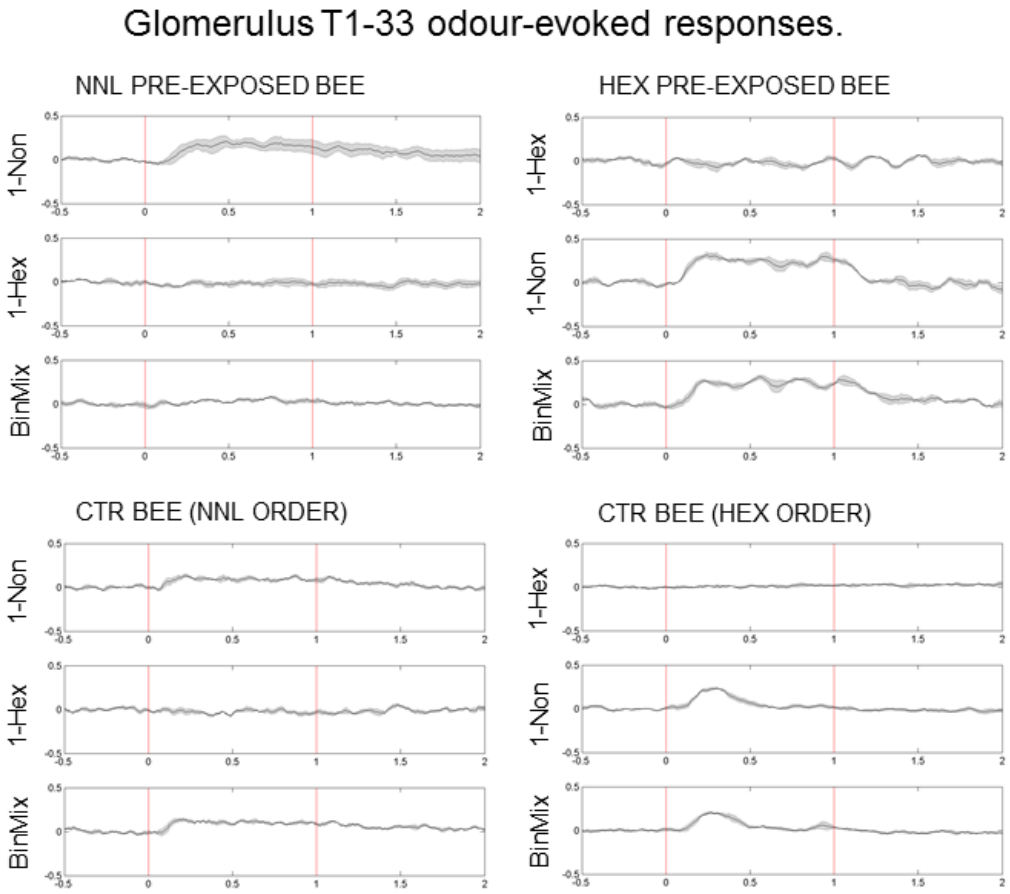

Figure 44 - Odour-evoked responses in bees recorded under different stimulation paradigms in glomerulus T1-33. Irrespective of the stimulation paradigm, the odour-evoked responses in the example control bees are similar to those of HEX-pre-exposed animals, while NNL pre-exposed animals show different responses. In particular, in CTR and HEX pre-exposed bees, the profile response to1-hexanol (1-Hex) is found almost unchanged in the response to the binary mixture. In NNL pre-exposed bees, response to binary mixture is flat, as it is the one to 1-hexanol. Notice that the three stimuli are in different order in the right and left column. The plots represent three odour repetition averaged ± SEM (shadow). Red vertical bars represent odour onset and offset.

The responses diverge between NNL pre-exposed and HEX pre-exposed animals. Response in control animals are similar to those of HEX pre-exposed

bees irrespective of the stimulation paradigms they were subjected. This allows us to exclude major effects of the stimulation paradigm on the recorded functional patterns.

2.6.3 Discussion.

We used a paradigm of prolonged pre-exposure of honeybees to single-odour compounds, similar to exposures that have been shown to produce measurable morphological, behavioral (habituation), and functional changes in *Drosophila melanogaster* (Sachse et al. 2007; Das et al. 2011; Devaud et al. 2001).

In the honeybee, repeated short stimulation with a single odorant has been demonstrated to induce a change in the relative representation of the binary mixture composed of this odour and a novel compound, in a saliency-based tuning of the AL filter (Locatelli et al. 2013). Although our bees were treated instead with a continuous, long-term pre-exposure, functional imaging revealed the same effect, *i.e.* a suppression of the exposed odour representation in the odour response code of the mixture. This confirms the results of Locatelli *et al.* (Locatelli et al. 2013) and shows that non-associative plasticity occurs in the honeybee not only during repeated exposure, but also during prolonged exposure, as applied in our experiment. In fact, the latent inhibition of the proboscis extension response conditioning, a well-known consequence of unreinforced odour exposure in the honeybee, has been shown to appear in cases of both repeated (Chandra et al. 2010) and prolonged (Fernández et al. 2009) odour exposure.

A part of overall functional changes in the odour representation, we observed specific changes in the odour response of individual glomeruli, which were previously identified as being the most affected (structurally) by the pre-exposure experience. In particular, we found significant differences in how glomeruli T1-17 and T1-33 respond to the mixture in the two pre-exposure groups (the response to the MIX stimulus was significantly different in HEX pre-exposed versus NNL pre-exposed bees in both T1-17 and T1-33). The

changes suggest that the overall effects of odour exposure on binary mixture representation (Locatelli et al. 2013) are distributed across different glomeruli.

Moreover, the pre-exposure procedure seems to have reduced (or shifted towards inhibition) the response to the odour of pre-exposure in both glomeruli. A slightly inhibitory HEX response, absent in CTR bees, manifests in HEX pre-exposed bees in both T1-17 and T1-33. NNL excitatory responses in both glomeruli were also slightly decreased in NNL pre-exposed animals in comparison to CTR. However, differently from those in MIX responses, those modifications were not significant.

It is difficult to hypothesize which mechanism might have produced the shifts in odour response depicted in 2.6.2.3. To summarize the modifications mentioned above, we could say that if T1-17 and T1-33 outputs (PNs) are usually tuned to signal NNL, the tuning becomes even sharper in the case of HEX pre-exposure, but less specific (see the slightly excitatory response to HEX in T1-33) in the case of NNL pre-exposure.

Considering the functional data together with the volumetric data (2.5.2), a modification at the level of olfactory receptor neurons (ORNs) or at the level of LNs can be hypothesized. A reduction of connections from ORNs to the two glomeruli might indeed cause a loss of tuning in the case of NNL pre-exposed bees, and the reduction in volumes. However, this hypothesis would be at odds with findings in *Drosophila*, where number, morphology, and physiology of ORNs were not affected by odour pre-exposure (Sachse et al. 2007; Devaud et al. 2001).

Alternatively, a decrease in volume at specific locations can be explained by a decrease in branching and synapses from inhibitory LNs onto PNs. This would be in accordance with results in *Drosophila*, where an enhancement of GABAergic transmission from LNs to active PNs (recurrent inhibition) followed odour pre-exposure, and caused volume increase in specific glomeruli (Sachse et al. 2007; Das et al. 2011; Sudhakaran et al. 2012). Another study in *Drosophila*, on the contrary, found decreases in volumes of glomeruli following odour exposure, but without a clear understanding of their

involvement in the odour response (Devaud et al. 2001). We found the same effect in NNL pre-exposed bees, in glomeruli T1-17 and T1-33, which are strongly activated by that odour (Sachse and Galizia 2002). However, if a loss of inhibition underlies the volume loss in T1-17 and T1-33 in NNL-exposed bees, our functional results suggest that it favors the transmission of HEX, rather than NNL information.

Responses to HEX and NNL in the honeybee are in fact far more distributed across glomeruli than those to CO_2 in the fruit fly (one responsive glomerulus) (Sachse and Galizia 2002; Sachse et al. 2007). It might well be that a HEX stimulus (in CTR bees) is able to reach glomeruli T1-17 and T1-33, but is usually blocked rather than passed on, to the advantage of the NNL contribution. This would be in accordance with a model in which functional inhibition, rather than stochastic or morphological, shapes the responses of PNs to similar odours (Linster et al. 2005). From this perspective, inputs of ORNs activated by HEX and projecting to different glomeruli would indirectly prevent PNs projecting to T1-33 and T1-17 from responding to the same odour, thus sharpening the response to NNL, as suggested elsewhere (Sachse and Galizia 2002). This default block, or “occlusion”, would be partially lost in our NNL pre-exposed bees, causing the MIX to be represented more similarly to HEX, accompanied by volume loss in the glomeruli in question.

To conclude, we demonstrated that prolonged odour pre-exposure causes functional plasticity, shifting the relative representation of a binary mixture of the pre-exposure odour and a novel odour towards that novel compound. The odour of pre-exposure, losing functional relevance, is partially suppressed in the mixture representation that is projected to higher brain centres. We identified single glomeruli causing these changes by measuring absolute differences in the responses to the two odours and their mixture depending on the odour of pre-exposure. We observed for the first time accompanying structural plasticity in these glomeruli, manifested as a decrease in volume. We suggest as an underlying mechanism the loss of inhibition (or “occlusion”) of the novel compound with respect to the pre-exposed odour.

2.7 Conclusions.

The results presented in this chapter add to previously reported evidences of AL plasticity in the adult honeybee. We were unable to replicate results on structural plasticity following associative learning (Hourcade et al. 2009). However, for the first time, we reported on prolonged odour exposure-related structural plasticity (2.5). This type of treatment represents a non-associative form of experience (with no specific spatio-temporal association between odour and reward), and we demonstrated that, like repeated odour exposure (Locatelli et al. 2013), it is sufficient to cause functional shifts in a binary mixture representation. Those shifts favour the novel odour components over the pre-exposed components, meaning that, also in the case of prolonged odour exposure, the pre-exposed odorant becomes irrelevant to the bee. However, we did not test effect of such manipulation on the behaviour. We might expect the bees to become habituated to the odour and to reduce avoidance or appetitive responses to it, as shown for bees and fruit fly in similar settings (Sachse et al. 2007; Das et al. 2011; Locatelli et al. 2013; Chandra et al. 2010; Fernández et al. 2009). From this perspective, we would describe the plastic changes we have observed non-associative learning-related. However, we do not have a specific measure of learning to do so, and, therefore, we might just hypothesize that some form of learning has occurred.

The functional modification of PNs that we observed are in accordance with previously reported results (Fernandez et al. 2009; Rath et al. 2011; Locatelli et al. 2013; Chen et al. 2015). The above-mentioned studies, taken together, show the ability of PNs to “tune” very quickly to most important olfactory “messages”: being those either the conditioned odour, following conditioning, or all other odours, following unrewarded odour exposure. In other words: what is most relevant, or salient, is made more separate from the rest (in terms of representation), and is more easily transmitted to higher brain area, when encountered within a complex background (Locatelli et al. 2013; Chen et al. 2015; Linster and Smith 1997).

For this reason, we think that that of “filter” is a proper metaphor to describe the AL function, and its output units in particular. As many studies have highlighted, the most probable site for both associative and non-associative learning-related plasticity to happen, is the LN-PN synapse (Chen et al. 2015; Locatelli et al. 2013). For non-associative learning, this was also empirically demonstrated in the fruit fly (Sachse et al. 2007; Das et al. 2011; Sudhakaran et al. 2012). It is very interesting that both types of plasticity have been proposed to act on the same site, as it means that this is the most efficient way to transiently modify odour representation. By letting the ORN-PN synapse unmodified, stable stimulus encoding would be, on the other hand, ensured.

So, when the saliency of an odour A is modified through one of either associative or non-associative manipulation, the percept of A is left unchanged: A is still encoded as “A”. But, since the animal is immersed in a complex olfactory environment, A is perceived in competition with, and along with other odorants. Learning happens in such a way, so that the previous experience influences the *probability* of A of affecting behaviour: enhancing it in case of associative learning, and decreasing it in the case of non-associative learning. This all happens at the LN-PN stage, but of course parallel plastic changes in higher brain areas are not be excluded (and were, in fact, proved in the case of associative learning, see *e.g.* Hourcade et al. 2010). These plasticity mechanisms might also be seen as a distributed form of *attention*, requiring no further top-down intervention to be hypothesised in order to be effective.

In our work, we have traced the functional changes back to the activity of single glomeruli. We demonstrated that the tuning of glomeruli, which were structurally changed by odour exposure, is modified (2.6.2.3). If LNs are responsible of both structural and functional effects, the question arises of which type of LNs would be involved. An interesting hypothesis would be that hetero-LNs are the responsible of such odour-specific plasticity in the honeybee. Indeed, two characteristics of the honeybee AL may perhaps be related, and dependent the one on the other: the huge plastic capacity

retained throughout adulthood (differently from what happen in the fruit fly; Devaud et al. 2003), and the huge presence of hetero-LNs, creating asymmetrical inhibition (Fonta et al. 1993)

This hypothesis would require further studies to be tested. A related question is which neurotransmitter, or neuromodulator, would be responsible of the modification occurring in non-associative learning in the AL. For what concerns associative learning, the role of octopamine is well established. This neurotransmitter would be released by the VUMmx1 on several brain areas, including the AL, and octopaminergic receptors have been reported to co-localize with GABA in LNs (Sinakevitch et al. 2013). The temporal coincidence of octopamine and ACh (released by the activated PNs) signals onto LNs would cause modifications of specific LNs-PNs synapses (Chen et al. 2015). However, the neurochemistry of non-associative learning is still unknown.

Chapter 3 - Neonicotinoids cause dysfunctions of the filter.

3.1 Summary.

In this Chapter, I will introduce the topics of neonicotinoid pesticides, their action on the insect brain, and their sub-lethal effects in the honeybee. The focus will be, in particular, on those behavioral effects which are related to olfaction. Based on an extensive literature reporting impaired olfactory learning and memory following exposure to such substances, we wondered whether neonicotinoid might affect odour discrimination in the antennal lobe. We addressed this question through both a behavioural (3.4) and a functional (3.5) approach. Conclusions are presented in 3.7.

3.2 Introduction.

3.2.1 General overview.

Neonicotinoids pesticides were introduced in the market in the 1990s and their application immediately spread worldwide, partly due to the fact that the previous available insecticides (organophosphate and carbamate derivatives) had already induced the evolution of many forms of resistance, and partly because of their desirable biological properties, such as wide spectrum, low application rate and quick uptake (Tomizawa and Casida 2004). Imidacloprid, the first launched neonicotinoid, observed the largest sales of any insecticides worldwide. For what concerns crop protection, neonicotinoids serve different ways of application, from seed coating to foliar spray. Moreover, they are commonly used in domestic animals health care against parasites. Hence, their presence in the environment is massive (Thany 2010).

Concern has been raised regarding the possible link between neonicotinoid use and the observed decline in populations of domestic and wild pollinators. Neonicotinoids applied via seed coating are indeed translocated in the whole plant, including nectar and pollen, and plant exudates. Foliar spraying, on the other hand, harms pollinators in even more direct ways – by contact, and by

causing the highest level of pollen and nectar contamination. Moreover, when these substances are sprayed, they can spread as dust in the air under windy conditions and accumulate in ground and surface water, with possibility of re-entering the feeding cycle of insects at many points (Goulson 2013). Several studies have therefore addressed the issue, particularly over the last 15 years. Evidences have accumulated that neonicotinoids at sublethal doses harm pollinators in many ways, both individually and at the colony level. However, whether these studies are fully representative of environmental occurring exposure and how their results should affect policies, are matter of debate (Godfray et al. 2014, 2015).

Regarding the honeybee, *Apis mellifera*, many noxious effects of both individual and colony exposure have been described. Neonicotinoids have also been proposed as a co-factor responsible for cases of sudden disappear of honeybee colonies, *i.e.* the so-called “colony collapse disorder” (for a review on the argument see Farooqui, 2013). For all these reasons, the European Union (EU) decided to impose a partial restriction on their use in December 2013. The consequences on bee health are currently being evaluated.

In the next subchapters I would first introduce the mechanisms of acute neonicotinoid action, and then briefly review the behavioural effects of sub-lethal neonicotinoid exposure in the honeybee, with a particular focus on olfaction-related behaviours.

3.2.2 Neonicotinoid action on the nicotinic receptor.

Commercially available neonicotinoids include: acetamiprid, clothianidin, dinotefuran, imidacloprid, nitenpyram, thiacloprid and thiamethoxam. The first to be released was imidacloprid in 1990. It was based on a structural and substituent optimization of nithiazine, a compound obtained in the 1970s in the search for new insecticidal molecules. Nithiazine, a molecule based on nicotine structure, proved very effective but very photoinstable. Imidacloprid overcame this limitation, and could be commercialized. The structures of commercially available neonicotinoids are visible in Figure 45.

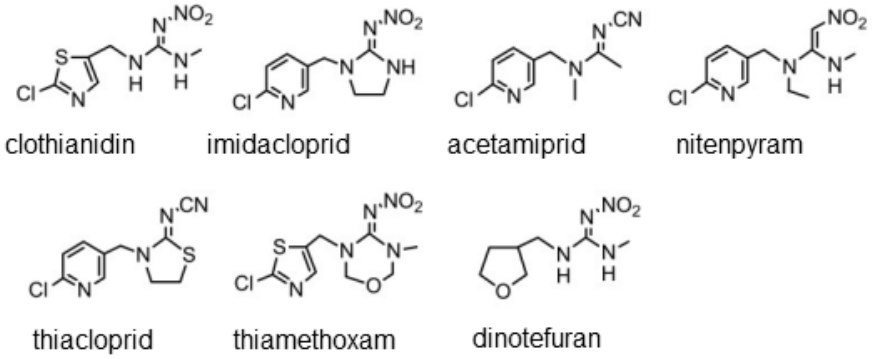

Figure 46 – Commercially available neonicotinoids.

Most neonicotinoids show a pyridine-like ring in their structure, similarly to nicotine. So-called chloropyridylmethyl compounds include: imidacloprid, thiacloprid, nitenpyram, and acetamiprid. The last two have acyclic structures attached to the chloropyridylmethyl, differently from the imidazoline ring of imidacloprid. Thiacloprid also has a cyclic structure. Clothianidin and thiamethoxam – of successive production – are defined as chlorothyazolylmethyl compounds. Dinotefuran has a different structure from all other neonicotinoids listed above, not involving any pyridine-like ring, as it was designed starting from acetylcholine structure, rather than nicotine (Thany 2010).

The mode of action of neonicotinoids mimics that of nicotine, *i.e.* they bind to the acetylcholine (ACh) nicotinic receptor, nAChR, with efficacy spanning between that of partial agonists (*e.g.* imidacloprid) and that of super-agonists (*e.g.* clothianidin; Brown et al. 2006; Matsuda et al. 2001). Electrophysiological studies in the cockroach showed that imidacloprid is able to activate both alpha-bungarotoxin(α-Bgt)-sensitive and α-Bgt -insensitive nAChRs, as well as a “mixed” nicotinic and muscarinic cholinergic receptors (Buckingham et al. 1997). Nicotinic receptors are composed of five subunits arranged around the central ion channel (a cation channel permeable to K^+, Na^+, and Ca^{2+}). The efficacy of specific neonicotinoid compounds is not only dependent on their molecular structure but can also be modulated by the subunit composition of the nAChRs they interact with. This property is

species-specific and cell type-specific (Tricoire-Leignel and Thany 2010; Jones and Sattelle 2010; Thany et al. 2007).

Binding sites for imidacloprid have been highlighted in a honeybee head preparation. They show some degree of competition for nicotinic binding sites with α-Bgt and other classical nicotinic ligands (Nauen et al. 2001). Imidacloprid, as many other partial agonists, is able to reduce the response of the cell to the full agonist, *i.e.* acetylcholine (Déglise et al. 2002).

Eleven nicotinic subunits - the largest family so far described in insects - have been identified in the honeybee genome (Jones et al. 2006). It is not totally clear how these subunits would combine in receptors expressed at different brain areas, and at different developmental stages (Dupuis et al. 2011, 2012). Neonicotinoid effects may well show dependency on these parameters. However, studies on dissociated cells – *e.g.* Kenyon cells (KCs) and antennal lobe (AL) preparations – allowed examining the effects of neonicotinoids at specific locations. I will here focus on imidacloprid (Fig. 47) effects.

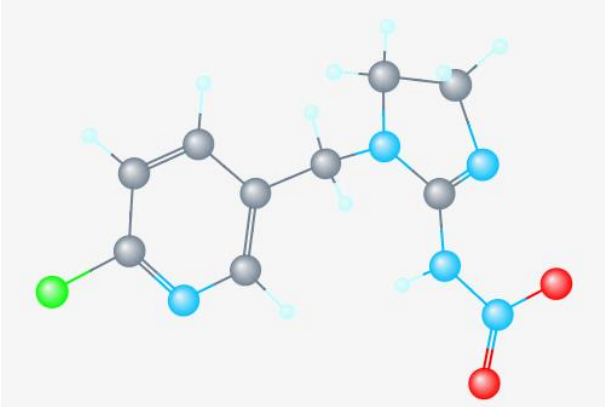

Figure 48 – Structure of the neonicotinoid imidacloprid, with C atoms coloured in grey, O in red, N in blue, and Cl in green. H atoms are indicated in transparent cyano. Double bonds are shown. From: https://pubchem.ncbi.nlm.nih.gov/.

Imidacloprid (as well as its olefin and 5-OH-imidacloprid metabolites; for an in-depth analysis of metabolism and the distribution of imidacloprid metabolites in the bee body see Suchail et al. 2004a, 2004b, 2001) is able to induce currents in those AL dissociated cells which are at the same time responsive to acetylcholine –however, the response to imidacloprid is usually smaller. It has been suggested that at least two different populations of nAChRs exist in

the adult honeybee AL, differing in their agonistic response to imidacloprid, but not to acetylcholine (Nauen et al. 2001). This finding was not confirmed in a further pupal (Barbara et al. 2005) and adult AL preparation (Barbara et al. 2008). The discrepancies could have risen due to the fact that in these preparations it was not possible to discriminate the different neuronal types. Therefore, it may be that the respective authors were recording from different cell types.

In pupal Kenyon cell preparations, similar results were obtained, *i.e.* imidacloprid acts there also as a partial agonist of the nicotinic receptor. A partial antagonistic interference of imidacloprid with GABAergic transmission was also described (Déglise et al. 2002). A confirm of this latter result came from studies on heterologous expression of insect GABA receptors containing the Rdl (resistant to dieldrin) subunit (Taylor-Wells et al. 2015).

The first study to describe effects of imidacloprid on integrated KCs (by recording from an acutely isolated adult honeybee brain) demonstrated that both imidacloprid and its major metabolite imidacloprid-olefin are able to induce sustained depolarization in those cells. In particular, these compounds induce a tonic inward current, with a variable degree of desensitization (Palmer et al. 2013). As a result, and as was suggested before (Déglise et al. 2002), imidacloprid reduces KC responses to ACh (Palmer et al. 2013).

The only – to our knowledge – calcium imaging study testing the action of imidacloprid on the intracellular calcium was a study performed on *Drosophila melanogaster*'s dissociated larval cholinergic neurons. In that study, it was demonstrated that both ACh and imidacloprid are able to induce transient increases in the intra-cellular calcium concentration (usually lower for imidacloprid in comparison to acetylcholine). In both cases, such responses are blocked by most nicotinic toxins. However, only in the case of imidacloprid, the calcium increase was sustained by voltage-gated calcium channels (Jepson et al. 2006). It is anyhow not clear whether such composite calcium dynamics might as well occur in the adult honeybee.

3.2.3 Behavioural effects of sublethal imidacloprid exposure in the honeybee, *Apis mellifera*.

The effects of sublethal neonicotinoid exposure on the bee physiology are numeorus – both the honeybee and the bumblebee have intensively been investigated in this regard (Blacquière et al. 2012). However, for the purpose of this thesis, I will mainly focus on behavioural effects in the honeybee *Apis mellifera*.

The presence of nAChRs in the insect central nervous system is huge and diffuse, as acetylcholine is its main excitatory neurotransmitter (Kreissl and Bicker 1989). Not surprisingly, neonicotinoids – even at very low doses – can hence interfere with a number of behaviours. The interference ranges from more basic effects regarding the motility (Charreton et al. 2015; Williamson et al. 2014; Medrzycki and Montanari 2003), which is impaired by exposure, to complex effects regarding navigation, communication, and learning and memory (for a review see Farooqui, 2013). In the following paragraphs, imidacloprid exposure will be mainly considered.

One of the first reported imidacloprid effects was a modification of dance frequencies (Kirchner 1999). Subsequently, effects on habituation of the proboscis extension response (PER), again caused by imidacloprid, were described (Guez et al. 2001; Lambin et al. 2001). A few years later, imidacloprid (acute treatment with 12 ng/bee) was reported to impair memory formation in a paradigm of olfactory classical conditioning, possibly through targeting of KCs (Decourtye et al. 2004a). The memory impairment was highlighted also in semi-field conditions (Decourtye et al. 2004b). The bee sensitivity to the substance, and the occurrence of such effects, were suggested to be modulated by the season (Decourtye et al. 2003).

Imidacloprid was also shown to enhance sucrose response thresholds in a PER assay (already at 0.21 ng/bee), and confirmed to reduce waggle dancing (Eiri and Nieh 2012). Further studies on associative learning assessed the effects of acute and chronic field-relevant doses of imidacloprid (*i.e.* 1.28 ng/bee; Williamson et al. 2013; Williamson and Wright 2013). In the acute

treatment, imidacloprid was shown to have little or no effect on learning, a part of reducing aspecific responses in a long-term memory test. In combination with coumaphos, however, it was demonstrated to enhance learning (Williamson et al. 2013). The opposite result was obtained with a 4-day treatment with the same dose: imidacloprid impaired learning, memory and discrimination (Williamson and Wright 2013). This discrepancy highlights the importance of assessing acute and chronic effects separate. In another study, feeding the larvae with imidacloprid for 4 days resulted in learning problems at 15 days after eclosion, showing that the effects of this substance accumulate in the long-term (Yang et al. 2012). The same treatment was also shown to decrease microglomerular density in the mushroom body calices (Peng and Yang 2016).

At the colony level, imidacloprid was shown to reduce egg-laying and motility in queens, and to reduce foraging and hygienic behaviours in workers (Wu-Smart and Spivak 2016; Schneider et al. 2012b; Yang et al. 2008). In particular, regarding foraging efficiency, neonicotinoids were demonstrated to impair homing flights following bee dislocation, which require access to older landscape memories acquired during orientation flights (Fischer et al. 2014). All of these effects are not surprising given the established role of cholinergic transmission in memory formation (Gauthier et al. 2006; Gauthier and Grünewald 2012; Louis et al. 2012).

Finally, and very importantly, it was also assessed that honeybee and bumblebee do not avoid food contaminated with neonicotinoids. On the contrary, they show a marked preference for it. This effect would also be explained by the action of nicotinic agonists on the circuits responsible for memory formation, rather than by a gustatory sensitivity to the neonicotinoid compounds (Kessler et al. 2015).

3.3 Aim of the experiments.

The influence of imidacloprid on the mushroom bodies' Kenyon cells, and on processes of olfactory memory formation, clearly emerges from the previously discussed literature (*e.g.* Déglise et al. 2002; Decourtye et al. 2004a; Palmer

et al. 2013; Williamson and Wright 2013). However, nicotinic receptors expression has been described also in the AL (*e.g.* Kreissl and Bicker 1989; Dupuis et al. 2011) and imidacloprid has been demonstrated to induce currents in AL dissociated cells (Barbara et al. 2008, 2005; Nauen et al. 2001). Impairments of the AL functionality may, hence, be hypothesised, which would result in compromised odour processing and odour discrimination. In the following sections, I will describe two experiments aimed at analysing these possible effects. First, we evaluated discrimination ability in a differential conditioning task (4.4). I will briefly discuss methods and results of this experiment, as an introduction to the second experiment. The data presented in 4.4 were collected by Martina Puppi during her Master's thesis with my co-supervision, and are presented extensively in her thesis (Puppi 2015). However, they were re-analysed for the purpose of this thesis and some new analysis was added. Secondly, we analysed AL functionality *in vivo*, through a calcium imaging experiment, in order to assess odour processing and odour discrimination ability following imidacloprid exposure more unambiguously (4.5). Data and text in 4.5 were adapted with minor modifications from Methods, Results and Discussion of an under-review paper (Andrione et al.).

3.4 Differential conditioning upon imidacloprid exposure.

3.4.1 Methods.

3.4.1.1 Animal preparation and experimental schedule.

Data were collected over a 4-weeks period in May and June 2015 in Rovereto (TN). Groups of forager honeybees (*Apis mellifera ligustica*) were captured at the entrance of the beehive via a Plexiglass pyramid, and transferred in a 40x40 cm bug dorm with access to food. Individual bees were then cooled to immobility by placing them on ice for ≈5 min, and subsequently harnessed in a hollow metal stage with a piece of paper covering the thorax, fixed with adhesive tape. Only the head of the bee was emerging from the tube. Harnessed bees were kept in a quiet room with temperature oscillating between 21 and 23 °C, and constant humidity. Each bee was randomly

assigned to either one of the two experimental conditions, the control group and the pesticide-treated group. The experimental schedule was designed as follow: on Day 1, bees were captured from the hive and mounted on the metal supports; Day 2 was a rest day; on Day 3, bees were conditioned to either one of two odours (see 3.4.1.5 *Conditioning and memory tests* below); on Day 4, early long term memory was tested; Day 5 was a rest day; on Day 6, long term memory was tested. The number of survived bees was recorded at the starting of every experimental day.

3.4.1.2 Pesticides and diet regime.

Imidacloprid (<99% purity, Sigma-Aldrich, St Louis, MO, USA) was first dissolved in a small amount of acetone and then in tap water (through successive dilutions) to a concentration of 10 nM/L. The same passages (without imidacloprid) were followed to prepare a control water solution to feed the bees of the control group. Tubes containing the solutions were wrapped in aluminium foil to prevent photodamage and kept in the freezer. Before the experiment, we added saccarose to the imidacloprid/control solutions, in order to obtained 50% sucrose solutions to feed the bees with (Matsumoto et al. 2012). The final concentration of imidacloprid obtained after this passage was 5.8 nM. Bees of each group were fed up to satiation (*i.e.* until they stopped responding to food with extension of the proboscis) two times before the start of the conditioning experiment: once in Day 1(immediately after being harnessed), and once (morning and afternoon) in Day 2. Bees of pesticide-treated and control group were fed with imidacloprid sucrose solution and sucrose control solution, respectively. From after the conditioning experiment (Day 3) until the last day of the experimental regime (Day 6), bees of both groups were fed to satiation with sucrose control solution (without imidacloprid). The amount of food ingested by each bee was recorded on every experimental Day.

3.4.1.3 Experimental set-up.

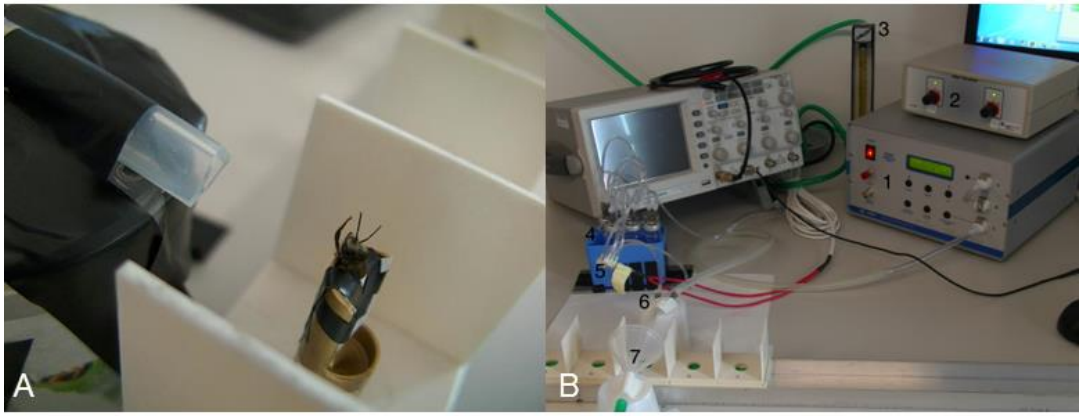

Figure 49 – The new set-up for conditioning. A) Bees were placed sequentially in front of the tube carrying odour stimuli thanks to a custom-built sliding bar. Timing was provided by acoustic signals hearable to the experimenter via headphones. B) The rest of the set-up was composed by: 1) stimulus controller (generating the carrier flow); 2) odour revolver (controlling the valves); 3) flowmeter; 4) glass vials containing odours and mineral oil; 5) valves; 6) nose; 7) air exhaustion system.

Odours (1-hexanol and 1-nonanol) were delivered to the bee antennae through a custom-made olfactometer where a constant air stream is split into two channels, each composed of only two alternate paths: an odour chamber and a blank chamber. Single channels are switched by electronic valves controlled by a USB-6008 multifunction board (National Instruments) and programmed via a LabView-based user interface (Paoli et al. 2017). The exit flow (total: 50 mL/sec) of the olfactometer was introduced through syringes onto a plastic tube encompassing a carrier air flow (about 600 mL/sec), in order to reach proper odorous stimulation for conditioning, and to avoid excessive odour accumulation at the end of the tube. Bees were placed about 2 cm in front of the opening of the plastic tube (from here onwards: the “nose”), in a custom-built sliding hard plastic bar. The bar could be pushed longitudinally into a metal runner, so that it was possible to swiftly place one bee after the other in front of the nose. The LabView program provided different acoustic signals (hearable by the experimenter, wearing headphones) at stimulus onset and time of reward, as well as indicating when to switch subject. The set-up was completed by a continuous air exhaustion

system placed 2 cm behind the bee stage, in order to remove residual odours (see Fig. 50).

3.4.1.4 Odour stimuli.

Odour stimuli were 1-hexanol and 1-nonanol (Sigma- Aldrich, Saint Louis, MO) diluted in mineral oil. 1-hexanol was used in concentration of 1:100 and

1-nonanol was used in concentration 40:100. The difference in concentration was due to the different vapour pressures of the two molecules. This value at room temperature is about 40 times as low for 1-nonanol than for 1-hexanol (calculated from https://www.nist.gov/).

3.4.1.5 Conditioning and memory tests.

The conditioning experiment was carried out for groups of about 16/20 bees at a time, half belonging to the control group and half to the pesticide-treated group. Half of the bees were conditioned to 1-hexanol and half to 1-nonanol (with the other odour being the unrewarded one), with random assignment. Each conditioning experiment consisted in 8 trials, 4 of which were rewarded and 4 unrewarded. The sequence of rewarded and unrewarded trials was randomized. In each trial, the bee was placed for about 15 seconds in front of the nose for familiarization, than exposed to the odorous stimulus for 4 seconds, finally kept in front of the nose for about 11 more seconds (in order to avoid association between the positioning in front of the nose and the reward). After that period, the bar was pushed, so to position the next bee in front of the nose. In this way, each bee spent 30 seconds in front of the nose and inter-trial periods within each bee had a fixed duration of 10 minutes (*i.e.* every 10 minutes a bee was presented with either the rewarded or the unrewarded stimulus; 10 minutes-spaced trials have been showed to yield the highest learning rates, see Matsumoto et al. 2012). During a rewarded trial, 3 seconds after odour onset, the bee antennae were quickly touched with a cotton bud previously soaked in a 50% sucrose solution. Then, the bee was allowed to lick for ≈2 seconds the sucrose solution from the cotton bud. In an unrewarded trial, the bee was simply exposed to the odour. Proboscis extension response PER, *i.e.* spontaneous extension of the proboscis beyond

the virtual line between the mandibles during the first 3 seconds of odour stimulus, was recorded as a yes/no response. If a bee did not raise its proboscis even after antenna stimulation during the first trial, it was discarded and excluded from later analyses. On rare occasions, a bee would raise its proboscis as soon as it was placed in front of the air stream; in such cases, since it was not possible to discern whether the PER response had occurred because the bee had sensed odour traces from previous trials, or rather had been conditioned to the airstream, the response was counted as 0. The experimenter was blind with respect to the subjects' group (control or treated).

Early long term memory (eLTM) and long term memory (LTM) were tested 24 and 72 hours after conditioning, respectively (*i.e.* in Day 4 and Day 6). First, the bee was presented with 4 seconds pulse of the rewarded odour, and a few minutes later it then trial it was presented with 4 seconds pulse of the unrewarded odour. The PER response was recorded. Again, the experimenter was blind with respect to the subjects' group (control or treated).

3.4.1.6 Statistical analyses.

Statistical analyses were conducted in MATLAB (MathWorks). Data on mortality were compared across sessions through independent sample *t*-test, after assessing normality of residuals (Kolmogorov-Smirnov test) and homoscedasticity (Levene's test). Data on food intake were also compared through independent sample *t*-test. Acquisition of the conditioned PER was assessed through Cochran's Q tests. Mann Whitney U tests were used to compare learning performances regarding the rewarded and the unrewarded odour in the two groups. McNemar's tests were used to assess specificity of the odour memory within each group. Fisher's exact tests were used to compare proportions of bees responding to the rewarded and the unrewarded odour in the two memory tests between the two groups. Proportions of True Learners, generalizers, Wrong Learners and Non Responders (see Results for definitions) were also compared through Fisher's exact tests. All analyses were conducted in MATLAB (MathWorks).

3.4.2 Results.

3.4.2.1 Mortality and food ingestion.

Bees were fed with either control or imidacloprid-containing sucrose solution two times throughout the two days prior to the conditioning. Our treatment could hence be defined as semi-chronic. Each treated bee ingested on average 0.05 ng of imidacloprid over the two days.

Bees fed with the different sucrose solution did not show different rates of mortality over the 6 days (independent sample *t*-test, t_{14}=-0.62, p=0.55). Briefly, the number of dead bees in each experimental group in each session was evaluated at Day 6 of each session. The mean percentages ± SEM across sessions (n=8) are shown in Figure 48.

This result further confirms that we were using sublethal doses.

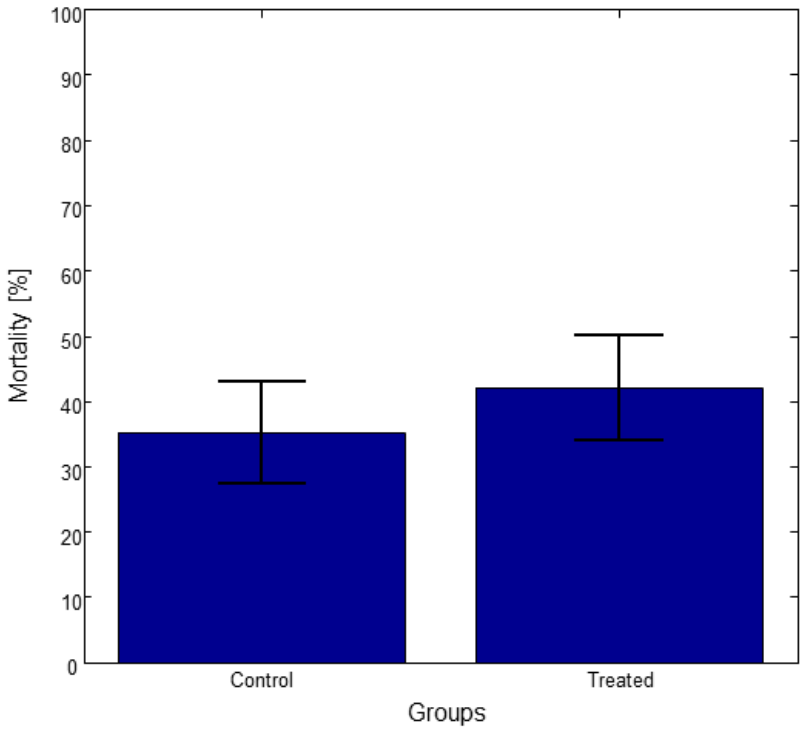

Figure 48 – Percentages of dead animals in each 6 d-session (n=8) are shown for the two groups. The rate of mortality is not significantly different between treated and control bees.

Since for each bee we recorded the amount of food ingested throughout the treatment, we could make comparison on this parameter, too. Over the two

days prior to the conditioning, bees did not differ in the average quantity of sucrose solution they accepted (control bees: 35.7 µL, treated bees: 37 µL; independent sample *t*-test, t_{121}=-0.3859, p=0.70).

3.4.2.2 Conditioning.

The proportion of bees that failed to produce PER at sucrose solution, and were hence discarded from following analyses, was not different between groups (data not shown; Puppi 2015).

Each bee was differentially conditioned to either 1-hexanol or 1-nonanol, while the other odour provided unrewarded exposure. Conditioning to the two odours produced equal rates of learning (data not shown; Puppi 2015).

In Figure 49 and in the following analyses, the data about the two odour combinations (1-hexanol^{+}/1-nonanol^{-} and 1-nonanol $^{+}$/1-hexanol $^{-}$) are pooled together. Bees of both treatment groups showed increasing rate of PER to the rewarded odour, while they did not increase their PER to unrewarded odour presentations over the course of four trials (tested through four Cochran's Q tests, Control-rewarded: Q_3=78, $p<10^{-15}$, Treated-rewarded: Q_3=53, $p<10^{-10}$, Control-unrewarded: Q_3=1.3, p=0.72, Treated-unrewarded: Q_3=0.67, p p=0.90).

The rate of learning of the rewarded odour differed between control (n=56) and pesticide-treated bees (n=56; Mann Whitney U test, Z_1=2.2, p= 0.032; Fig. 49). This was not true for the unrewarded odour (Mann Whitney U test, Z_1=0.94, p=0.35).

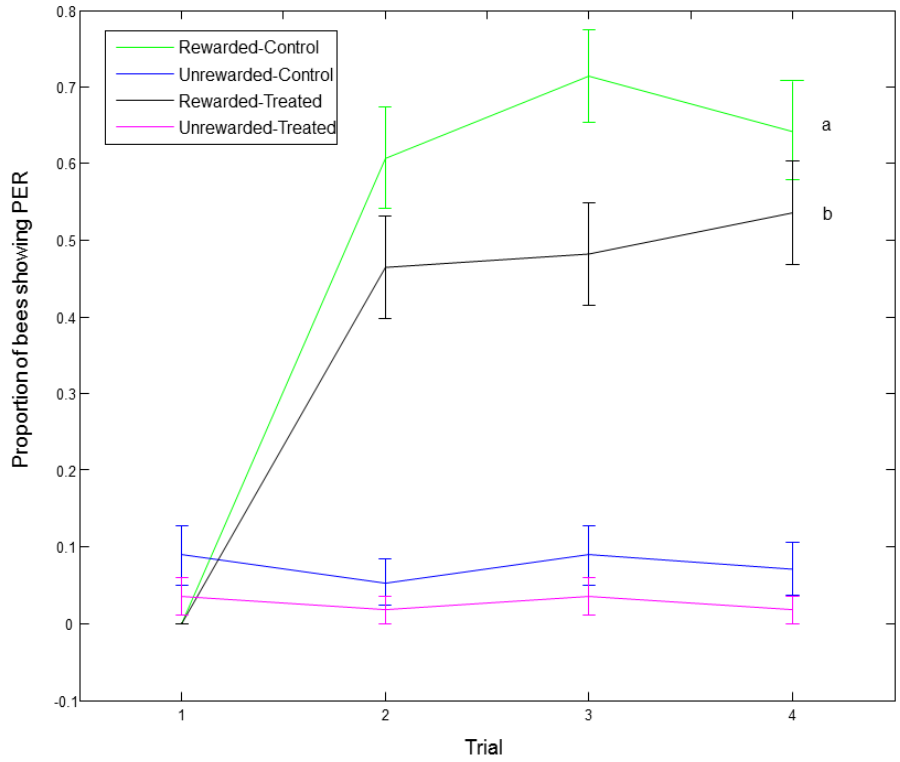

Figure 49 – Proportion of bees showing PER in response to the CS+ and CS- in the two groups (respectively, control bee responses to CS+: green, control bee responses to CS-: blue, treated bee responses to CS+: black, treated bee responses to CS-: magenta). The overall rate of response to the CS+ is different between treated (*n*=56) and control (*n*=56) animals (b, a). This is not true for the CS-. Error bars represent SEM. Notice that CS- curves do not start from zero because, even if the sequence of 8 stimuli was randomized, the CS- was never presented as first odour to any bee.

3.4.2.3 Memory tests.

The bees were tested for their early long-term memory (eLTM) and for long-term memory (LTM), respectively at 24 and 72 hours after conditioning. Each bee received a pulse with the previously rewarded odour and one with the previously unrewarded odour. The results are shown in Figure 50.

At 24 h there was a specific memory of the association in both the control bees and the treated bees. On the other hand, at 72 h the specificity of the odour memory was evident in the control but not in the treated bees (tested through four McNemar's tests: 24h-Control: $\chi 2=10$, $p=0.0013$, 24h-Treated: $\chi 2=3.7$, $p=0.054$, 72h-Control: $\chi 2=7.6$, $p=0.006$, 72h-Treated: $\chi 2=0.31$, $p=0.58$).

However, the proportion of bees responding to the two stimuli at the two time points are not significantly different between the two groups (tested through four Fisher's exact tests: eLTM-rewarded: p=0.50, eLTM-unrewarded: p=0.95, LTM-rewarded: p=0.70, LTM-unrewarded, p=0.99).

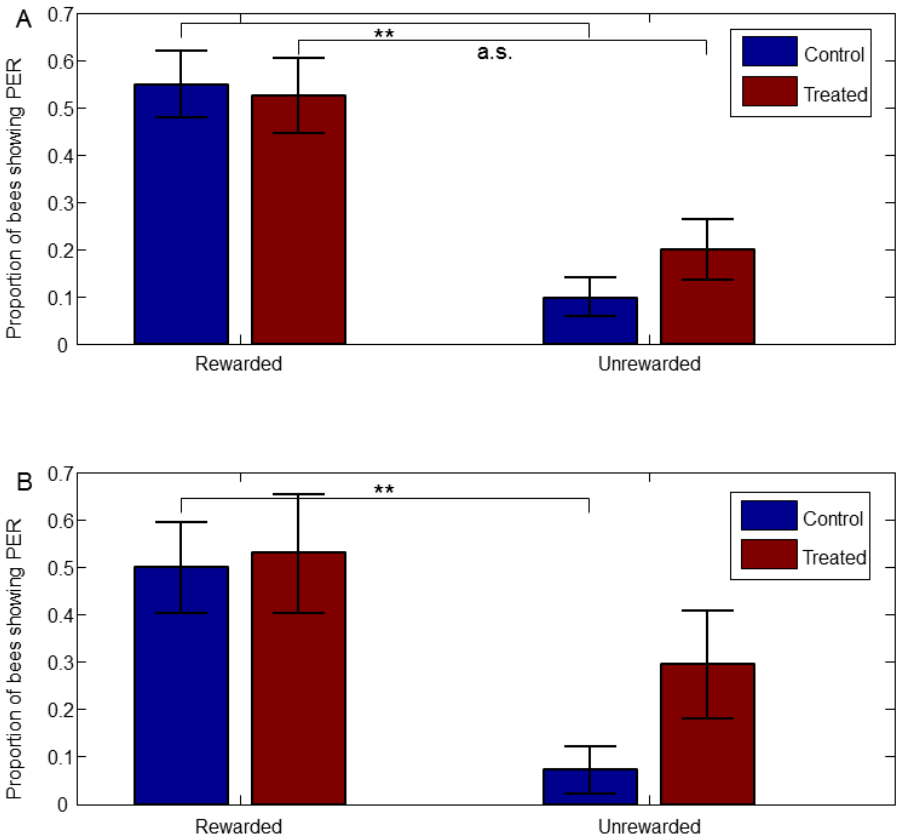

Figure 51 – Proportion of bees responding with PER to the long-term memory test. A) eLTM test (24 h). The specificity of the memory is evident in both control (n=51) and treated (n=40) bees. **: p<0.01; a.s., almost significant: p=0.054. B) LTM test (72 h). The specificity of the memory is evident in control (n=28) but not in treated (n=17) bees. **: p<0.01).

As each Fisher's exact test took into consideration the response to one stimulus at a time, we further categorized bees into four groups, based on the combined response to the two odour stimuli. We defined bees responding to the both the rewarded and the unrewarded odour as Generalizers, bees responding to the rewarded odour only as True Learners, bees responding to the unrewarded odour only as Wrong Learners, bees non responding to neither of both as Non-Responders.

We compared those proportions across groups in the two memory tests (Fig. 51). The groups were not significantly different with respect to these categories (tested through Fisher's exact tests).

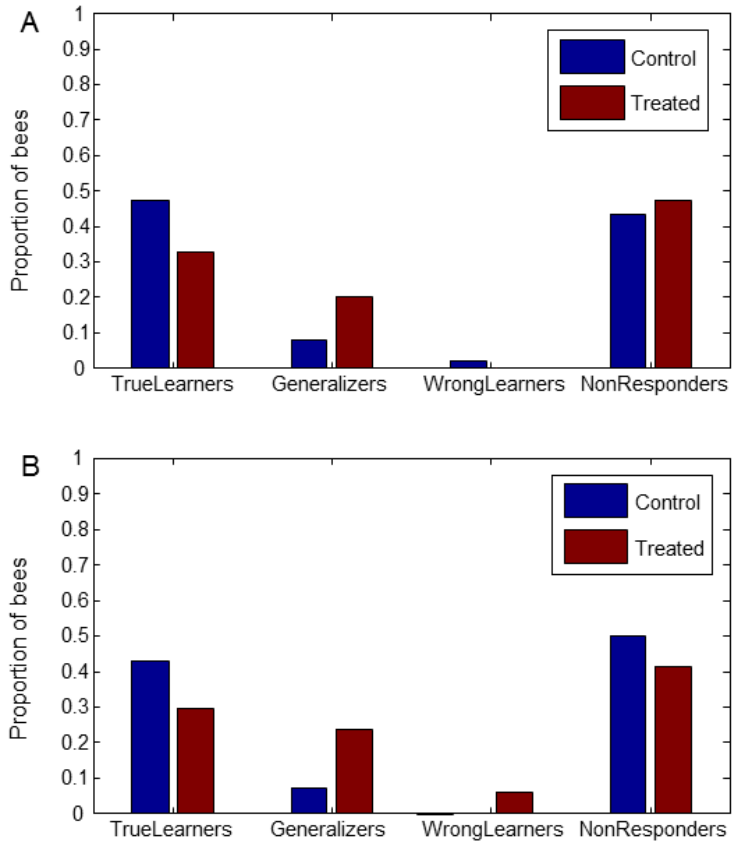

Figure 52 – Proportions of True Learners, generalizers, Wrong Learners, and Non-Responders are not different between control and treated bees in neither the eLTM nor LTM tests (*n*=51, 40, 28, 17).

3.4.2.4 Dose-performance relationship.

As the effects of pesticide treatment were mainly evident on the acquisition phase, we wondered whether we may establish a correlation between amount of imidacloprid ingested and performance during conditioning trials. We therefore assigned a rank *Pf* to the performance of each bee based on the number of specific PERs during conditioning trials 2 to 4:

$$Pf = 3 + (\mathrm{RW2} + \mathrm{RW3} + \mathrm{RW4} - \mathrm{UR2} - \mathrm{UR3} - \mathrm{UR4}),$$

where RW2, 3, 4 are the PER scores in trials 2, 3, 4, with presentation of the rewarded odour, while UR2, 3, 4 are the PER scores in trials 2, 3, 4, with

presentation of the unrewarded odour. *Pf* scores of the treated bees are shown in Figure 52. Correlation of *Pf* with amount of imidacloprid ingested was not significant (R=0.24, p=0.072).

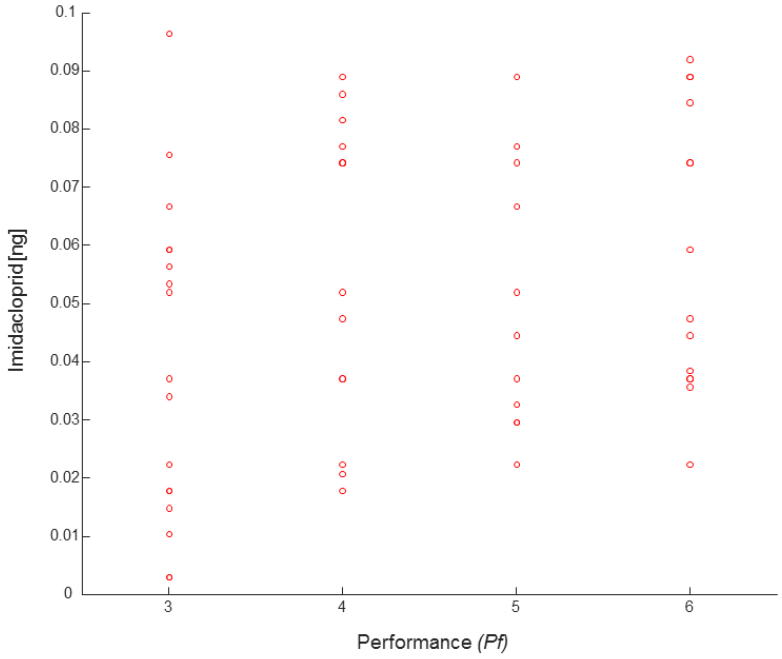

Figure 53 – Scores of *Pf* in individual treated bees (n=56) show no significant correlation to the amount of imidacloprid ingested.

3.4.3 Discussion.

A semi-chronic treatment with the neonicotinoid imidacloprid was shown to affect olfactory learning and discrimination. In particular, we found that acquisition of PER to the rewarded odour - during a differential olfactory conditioning task - was reduced. However, in the same task, responses to the unrewarded odour did not differ from those of controls.

Moreover, in the LTM test (at 72 h) discrimination was impaired, as responses to the unrewarded odour did not differ from those to the rewarded odour in treated animals. However, the proportion of bees generalizing their memory, despite being slightly higher in the treated group, was not significantly different between groups.

Our results confirmed previous findings of reduced acquisition of olfactory memory following both acute (Decourtye et al. 2004a) and chronic (Williamson and Wright 2013) treatment with imidacloprid. Our administration method was not acute, but quite too short to be considered as chronic, so we defined it semi-chronic. The doses we used (≈0.05 ng/bee) were lower than what used in both studies mentioned above, and can easily occur in-field (Williamson and Wright 2013).

For the first time to our knowledge, we analysed neonicotinoid action on learning though a paradigm of olfactory differential conditioning. The reduction in responses to the rewarded odour may also be caused by discrimination problems, but it is most probably due to retrieval impairment. Indeed, if bees were unable to discriminate between the two odours at this stage, we would expect PER rate to the unrewarded odour to be increased as well. However, this is not the case.

On the other hand, discrimination impairments emerged during the memory test, in particular at 72 h after learning. Others have already described discrimination problems during memory tests (see *e.g.* Williamson and Wright 2013). Since we were presenting the bees with the same two odorants used in the conditioning, differences between the two phases in how distant the two odours were - with respect to the perceptive space of the bees - should be excluded. We do not know how this temporal discrepancy - with discrimination problems being absent in the conditioning phase, but present 72 h later – might arise. It is probably not a difference between imidacloprid action and the action of metabolites produced within the bee, because our administration method was not acute, so we expect imidaclorpid to be already metabolized when we condition the animals (Suchail et al. 2004b).

The problem of how bees process and discriminate odours following neonicotinoid treatment is probably more directly and unambiguously addressed through *in vivo* imaging of the bee AL. In the following chapter we explained how we exploited this approach.

However, we would like to stress that a possible replicate of differential conditioning upon neonicotinoid chronic treatment should probably be designed so that bees have to compare between odours that are as much perceptually similar as possible, at doses equal or higher than the one we applied.

3.5 Odour processing upon imidacloprid exposure.

3.5.1 Methods.

3.5.1.1 Animal preparation.

Forager honeybees were collected with a transparent plexiglass in 2015, in Rovereto (TN). The procedure of PN injection is adapted from Galizia and Vetter (2004). Forager honeybees were collected at the entrance of the beehive in a Plexiglass pyramid and fixed on a mounting stage after immobilisation at 4°C. PNs were backfilled with the calcium indicator fura-2 in its dextran-conjugated form (ThermoFisher Scientific). A small volume of the crystallized dye was manually injected via a custom-made glass capillary at the intersection of the lateral- and medial-antenno-protocerebral tracts between medial and lateral calices of the MBs (for an image of the injection site see Fig. 8). To avoid lateral biases, left and right ALs were prepared alternately (Rigosi et al. 2011, 2015). After injection, animals were fed on a 50/50 w/w sucrose solution and kept in the dark until imaging on the following day, allowing the dye to diffuse retrogradely into the AL.

3.5.1.2 Data acquisition.

Imaging was performed via a two-photon fluorescence microscope (Ultima IV, Bruker) combined with an ultra-short pulsed laser (Mai Tai Deep See HP, Spectra-Physics-Newport), tuned to 800 nm for fura-2 excitation (Haase et al. 2010). The beam was focussed by a water-immersion objective (20x, NA 1.0, Olympus). The fluorescence was collected in epi-configuration, selected by a dichroic mirror, and filtered with a band-pass filter centred at 525 nm with 70 nm bandwidth (Chroma Technology Corp). Finally, it was detected by photomultiplier tube (Hamamatsu Photonics). An optimal signal-to-noise ratio

was achieved with a laser power ≈10 mW, without any sign of photobleaching.The AL was repeatedly scanned by a set of galvanometric mirrors along a spiral line of interest crossing all glomeruli within a selected focal plane. The frame rate was ≈30 Hz. Changes in the intracellular calcium concentration manifested themselves as temporal variations of the fura-2 fluorescence intensity.

3.5.1.3 Odour stimulation.

Odour stimulation was performed through a custom-made olfactometer where a constant air stream is split into eight channels, each composed of two alternate paths: an odour chamber (1:500 dilutions in mineral oil) and a blank chamber (mineral oil). Single channels are switched by electronic valves controlled by a PCIe-6321 multifunction board (National Instruments) and programmed via a LabView-based user interface (Paoli et al. 2017). Acetophenone, benzaldehyde, 1-hexanol, 1-octanol (all Sigma-Aldrich) were applied sequentially as pulsed stimuli. Each odour pulse (duration: 1 s, inter-stimulus interval: 7 s) was repeated 25 times. Because the 4 odours were alternated, this produced an interval of 32 s between subsequent stimulations with the same odour.

3.5.1.4 Imidacloprid administration.

During the imaging sessions, the bee brain was continuously perfused via input and output capillaries, embedded laterally in the imaging mount (Fig. 54).

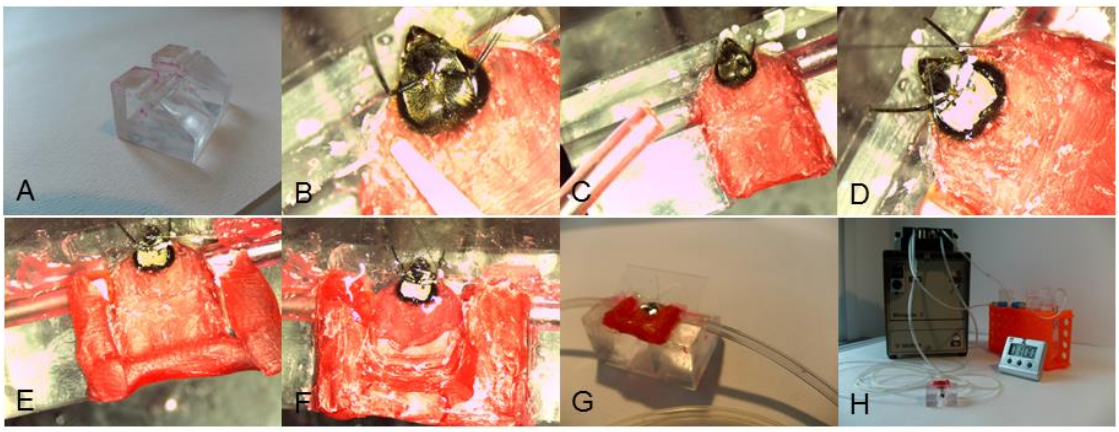

Figure 55 – Dynamic imidacloprid application: preparation procedure. A) A modified version of the bee imaging stage was designed, where B) bees are staged, following chilling, and C) capillaries are embedded in two lateral fissures of the stage. D) The head capsule is opened for PN injection. E) A barrier of soft dental wax is manually shaped to avoid liquid leakage from the back, in addition to the plastic foil protecting the antennae on the front. F) The imaging chamber is sealed with Kwick-Sil (WPI), without covering the brain. G) The stage is ready for imaging and H) perfusion, by connecting the capillaries with a peristaltic pump, which can be manually activated. Perfusion liquid can be switched by manually operating a valve positioned on the entrance capillary.

Via a peristaltic pump, Ringer's solution (130 mM NaCl, 6 mM KCl, 4 mM $MgCl_2$, 5 mM CaCl2, 160 mM sucrose, 25 mM glucose, 10 mM HEPES (all Sigma Aldrich), pH 6.7, 500 mOsm/L; Galizia and Vetter 2004), was injected at a flow rate of ≈ 1 ml/min. During the treatment phase perfusion was switched for 60 s to Ringer's with 10 µM imidacloprid (Sigma Aldrich) added to it.

3.5.1.5 Data analyses.

Images acquired via the microscope software Prairie View were de-noised and processed using custom-written MATLAB (MathWorks) codes. Glomerular response signals were extracted by manual identification of single glomeruli centres and averaged over 5-pixel intervals (1 µm/pixel) around those. The mean pre-stimulus activity *F* (averaged over 1s) was subtracted and served as a normalization factor. The time series of this relative activity - $\Delta F/F$ were the basis for further analysis. The mean odour-response activity in each of the recorded glomeruli was obtained by averaging over a time window

between 200 and 400 ms after stimulus onset. This period shows the maximal separation of PN odour response patterns in the bees (Fig.54).

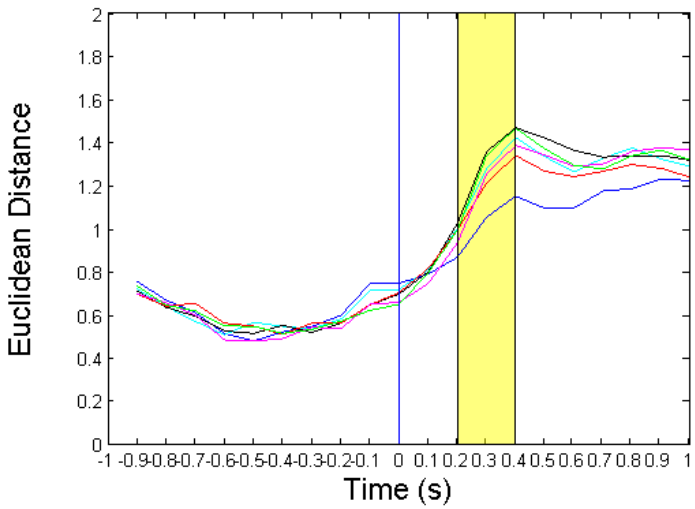

Figure 54 - EDs between odour pairs show a maximum at 0.4 s after odour onset. The six curves represent the 6 odour pairs, obtained from the 4 odours used in the experiment described in Chapter 3.5. Here, distances over time were calculated by pooling all glomeruli of all bees in all groups (first 3 odour repetitions).

Glomeruli were identified as responsive if their average activity over the whole stimulus period deviated significantly from the background activity ($p<0.05$: $|\Delta F|>F+2\sigma$), and classified as either “excitatory” or “inhibitory” based on their sign. Response delays were calculated for significant responses, as the time from stimulus onset to time when half of the maximum activation was reached. To quantify odour separation in the *n*-dimensional glomerular space (*n* is the number of glomeruli recorded in each bee), Euclidean distances were calculated. For a definition of Euclidean distances, see 2.6.1.6 *Functional data analyses*.To visualize the dynamics of the treatment effects on the odour response, dimensionality of that coding space was reduced by a principal component analysis (PCA). To be able to compare all bees within common coordinates, a transformation projected the whole coding space spanned by all glomerular responses (n=172 (86 glomeruli pertaining to treated bees and 86 to control bees) × 4 odours) to principal components (PCs). The first 3 PCs explained all variance between the 172 × 4 glomerular responses averaged over the 4 pre-treatment stimuli (PC1: 47%, PC2: 36%, PC3: 17%). In the PC space, responses to each odour were averaged over the bees within the

single groups (*n*=5), producing a time sequence of 25 points corresponding to the 25 subsequent stimuli.

3.5.1.6 Statistical analyses.

- Figure 56: the first 24 repetitions of 1-octanol stimulation in a treated bee were averaged in groups of 4 to obtain the six subsequent windows shown in this figure. One-way ANOVA (after assessing normality of residuals and homoscedasticity of the samples) was performed to compare values of peak response (the maximum after stimulus onset) and the integral of the response (area under the curve of response from stimulus onset to return to baseline) in these six "groups". Via Dunnett's *post-hoc* test, all later time windows were tested against the first.
- Figure 58: the number of responding glomeruli and the average intensity of both excitatory and inhibitory responses were averaged across bees within each group (control bees: *n*=5, and treated bees: *n*=5). After assessing approximate normality of residuals and homoscedasticity of the samples (via Kolmogorov-Smirnov test and Levene's test, respectively), in each case a two-way repeated measures ANOVA was performed (with time as within-subject factor and group as between-subject factor). Where ANOVA showed significant effects, pairs of values were further compared through paired (within one group) and unpaired (between groups) sample *t*-tests.
- Figure 60: glomeruli that showed significant odour-evoked responses both before treatment and after EOT – either at 1 min after EOT or 8 min after EOT – were used for comparisons. Differences in onset delays were extracted from pairs of significant responses and compared via two-way ANOVA, with time and group as two between-subject factors. Two-way ANOVA was chosen because we wanted to test a possible effect of interaction of these two factors. However, since the analysis showed no significant effect of time × group, we further

compared the two groups via a Kruskal-Wallis test, more suitable in this case, as residuals deviated from normality.

- Figure 63: EDs were calculated relative to the six odour pairs and the three time windows in each bee. ED changes were normalized with respect to the pre-treatment values: $(ED_{after}-ED_{before})/ED_{before}$, applied to both time points 1 min and 8 min after end of treatment (EOT). We then averaged across odour pairs (n=6) and bees per group (n=5). Results were compared via a two-way repeated measures ANOVA (after assessing normality of residuals and homoscedasticity of the samples), with time as repeated measure and group as between-subject factor.

3.5.2 Results.

3.5.2.1 Static imidacloprid application.

Staining PNs with fura 2-dextran allowed *in vivo* functional imaging of the AL glomerular responses upon stimulation with different odorants (1-hexanol, 1-octanol, acetophenone, and benzaldehyde).

Application of an imidacloprid solution proved quite challenging, as, in order to precisely compare glomerular odour responses before and after treatment, the same area should be focused on throughout the procedure. Manual bath-application of the solution - after removal of the whole volume of haemolymph covering the brain surface – proved to be problematic from this point of view.

However, when the same glomerulus was compared before and after application of an imidacloprid solution of concentration as little as 1 µM, we often observed the odour response disappearing, without being restored in the course of following ≈30 min. Figure 55 show an example of such imidacloprid effects.

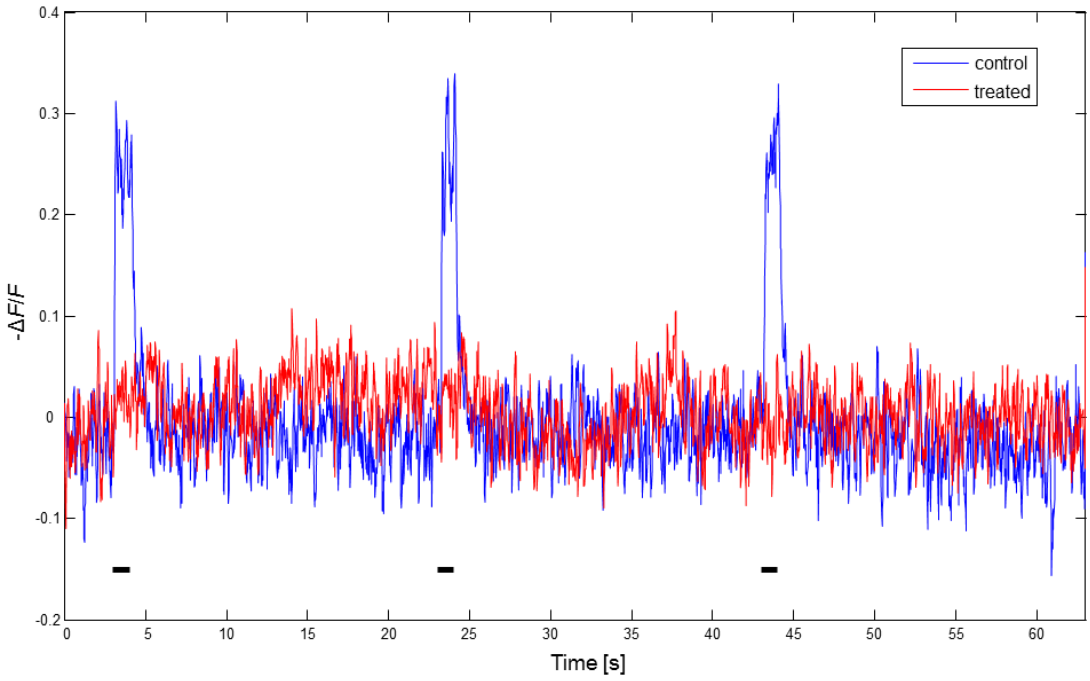

Figure 55 - Odour-evoked activity (odour pulses are represented by the black horizontal bars) in a single glomerulus T1-37 before (blue) and after (red) the application of a 1 µM imidacloprid solution. Odour of stimulation is benzaldehyde. Imidacloprid solution is applied in a time-unrestricted manner, in substitution of the whole volume of haemolymph covering the brain surface. In this setting, there is no movement of the buffer solution or washing phase. 2-3 min after the application of a concentration of the drug as low as 1 µM, the odour-evoked activity is lost. In the perfusion set-up, used in later experiments, effects comparable to those that are shown here set in only at a concentration 10 times higher.

3.5.2.2 Dynamic imidacloprid application.

For the further analyses, in order to establish a more reliable approach, and to increase both temporal precision and spatial accuracy, we modified our imaging stage as shown in Methods (Fig. 53). Briefly, experiments were designed to allow continuous imaging of odour-stimulated activity from the same AL area (a single focal plane allowed access to 14 to 21 glomeruli depending on the bee) before, during, and after treatment of the brain with an imidacloprid solution. This was achieved via controlled perfusion of the brain with a buffer solution, to which substances could be added during well-defined periods, without creating interruptions or movement artefacts in the images.

The administration of a 10 µM solution of imidacloprid for 1 min, followed by washing with physiological solution, resulted in significant reduction in the

mean and peak odour-evoked calcium responses within single glomeruli (see Fig. 56). Indeed, we noticed that, with respect to static bath-application, in the perfusion approach we had to reach ten-fold concentration in order to observe similar effects on the odour response. Recovery was visible after a few minutes (Fig. 56E, F).

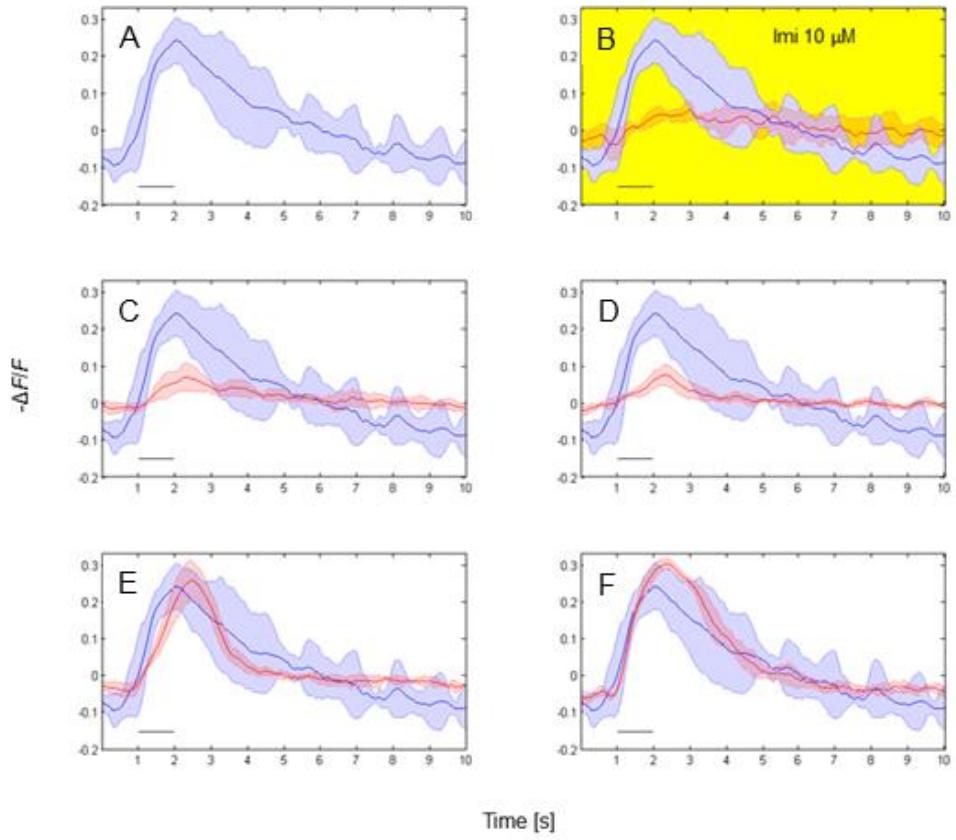

Figure 56 - Odour-evoked activity in a single glomerulus, identified as GloT1-17, in a treated bee. Boxes (A-F) represent subsequent time windows. In each, the change in fluorescence over time (blue in A, red in B-F) is averaged across four subsequent repetitions of the odour stimulus (1-octanol; horizontal black line). In (B-D) both peak response (one-way ANOVA, $F_{5, 18}$=34, p<10-7, followed by Dunnett's post hoc test) and integral response (one-way ANOVA, $F_{5, 18}$=16, p<10-5, followed by Dunnett's post hoc test) are reduced with respect to (A). Shadows represent standard deviations. The profile of response in (A) is reported (in blue) in all the subsequent windows for comparison. In yellow: the time window (B) corresponding to imidacloprid application. Before and after: physiological (Ringer's solution) washing.

3.5.2.3 Variability of effects (across glomeruli and bees).

The onset of the effects on brain activity was slightly different among bees. This might be due to variations in the kinetics of perfusion and diffusion of the

substance into the tissue. Around 1 min after the end of imidacloprid application, however, the effect on the odour-evoked responses was visible in all bees. A few minutes after imidacloprid administration, on the other hand, the response amplitude in some of the glomeruli had recovered to initial values, though calcium transient dynamics could still be different. In one case, not all odour responses followed the same trend, and the recovery was odour-specific (Fig. 57, Glomerulus T1-37).

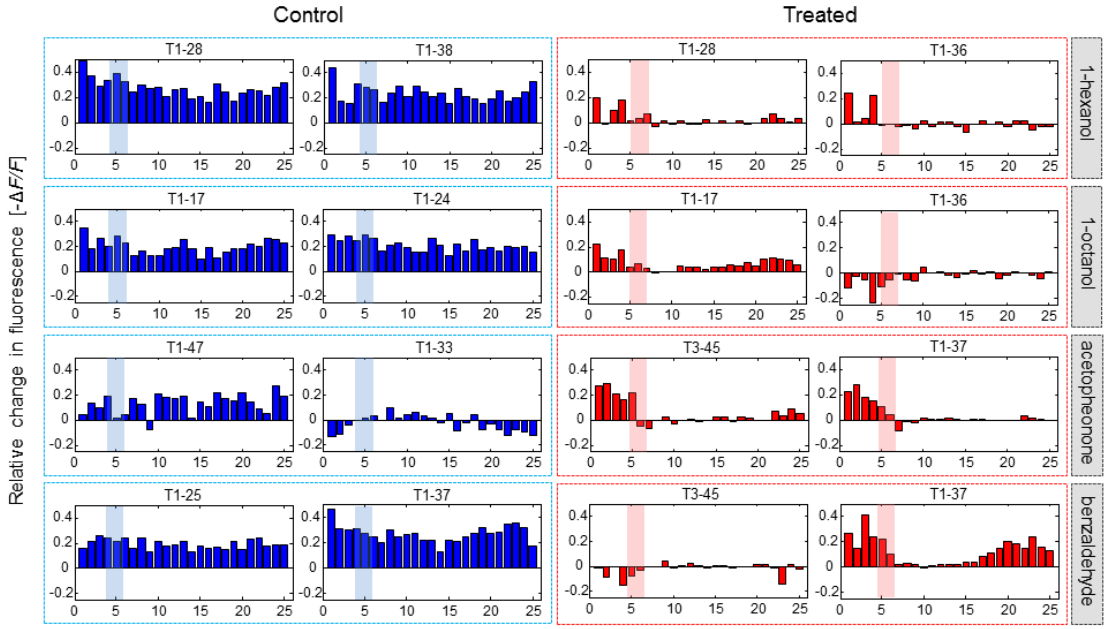

Figure 57 - Example of two representative glomeruli of the odour response pattern relative to each odour in a control bee (left; blue) and a treated bee (right; red). Odour-evoked responses (-Δ*F*/*F*) are averaged over the 200-400 ms interval after stimulus onset, for each of the 25 stimuli repetitions (shown on the *x*-axis). Odours are reported on the right. Shadows: administration of Ringer's solution (blue) or imidacloprid (red).

Due to the temporal variability mentioned above, and in order to compare effects across animals, we focused on three time windows, each encompassing three stimulus repetitions (≈ 1.5 min): before treatment, 1 min after the end of treatment (EOT), 8 min after EOT.

First, we evaluated the distributions of "excitatory" and "inhibitory" odour-evoked responses (see Methods) in the two groups relative to the three moments (Fig. 58A). Shrinkage of the excitatory response distribution in treated animals following treatment (red, 1 min after EOT) suggested that

these responses diminish in both intensity and number compared with those of control bees. We tested this hypothesis by comparing the number of excitatory/inhibitory responses and their average intensity in bees of each group (Fig. 58B).

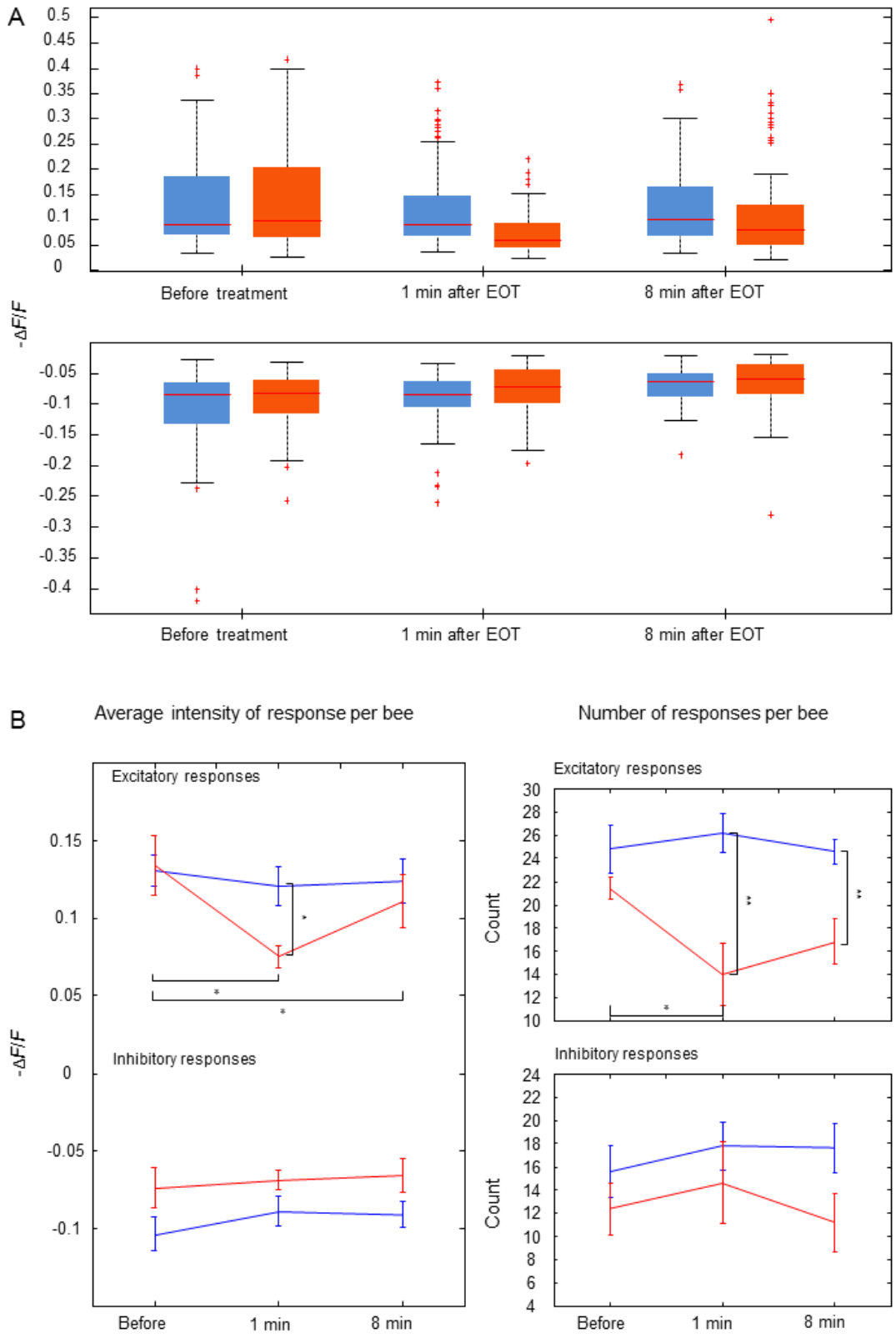

Figure 58 - Odour-evoked glomerular responses were gathered and automatically classified in excitatory, inhibitory, or no response. This was done for responses before treatment (Before), 1 min after EOT (1 min) and 8 min after EOT (8 min). The intensity (average -Δ*F*/*F* between 200 and 400 ms after odour onset) distributions of excitatory (top) and inhibitory

responses (bottom) are shown in (A), with data from control animals (n=5) reported in blue and those from treated animals (n=5) in red. Boxplots indicate median, quartiles, and outliers of the distributions. In (B), the average intensity (-ΔF/F; left) and the number (right) of both excitatory and inhibitory responses across bees are shown (control bees, n=5, in blue, and treated bees, n=5, in red). Error bars represent SEM. Average intensity of excitatory responses varied following treatment (two-way repeated measures ANOVA showed a significant effect of time $F_{2, 16}$=9.4, p=0.0020 and group × time interaction, $F_{2, 16}$= 4.7 p=0.025). Number of excitatory responses were also reduced by the treatment (two-way repeated measures ANOVA showed a significant effect of group × time interaction, $F_{2, 16}$= 3.9, p=0.042, and group: $F_{1, 8}$=17, p=0.003). Pairs of measurements were further compared via paired and unpaired t-tests, respectively (*: p<0.05, **: p<0.01).

Indeed, we found that both number and average intensity of excitatory responses were reduced in treated bees following treatment. The two-way repeated measures ANOVA on response number revealed significant effects of group: $F_{1, 8}$=17, p=0.003 and group × time interaction: $F_{2, 16}$= 3.9, p=0.042, while time was not significant: $F_{2, 16}$=2.0, p=0.16. The two-way repeated measures ANOVA on average intensity showed a significant effect of time: $F_{2, 16}$=9.4, p=0.0020 and group × time interaction: $F_{2, 16}$= 4.7 p=0.025, but not group: $F_{1, 8}$=1.1, p=0.33. These effects did not occur in inhibitory responses (two-way repeated measures ANOVA on number: time: $F_{2, 16}$=0.86, p=0.44, group × time: $F_{2, 16}$=0.53, p=0.60, group: $F_{1, 8}$=2.2, p=0.18; two-way repeated measures ANOVA on intensity: time: $F_{2, 16}$=1.5398, p=0.24; group × time: $F_{2, 16}$=0.3, p=0.74, group: $F_{1, 8}$=4.5, p=0.07).

3.5.2.4 Effects on onset delay.

When analysed more in detail, the odour-specific effects on the above-mentioned glomerulus T1-37 (Fig. 57) unveiled further delay effects (Fig. 59).

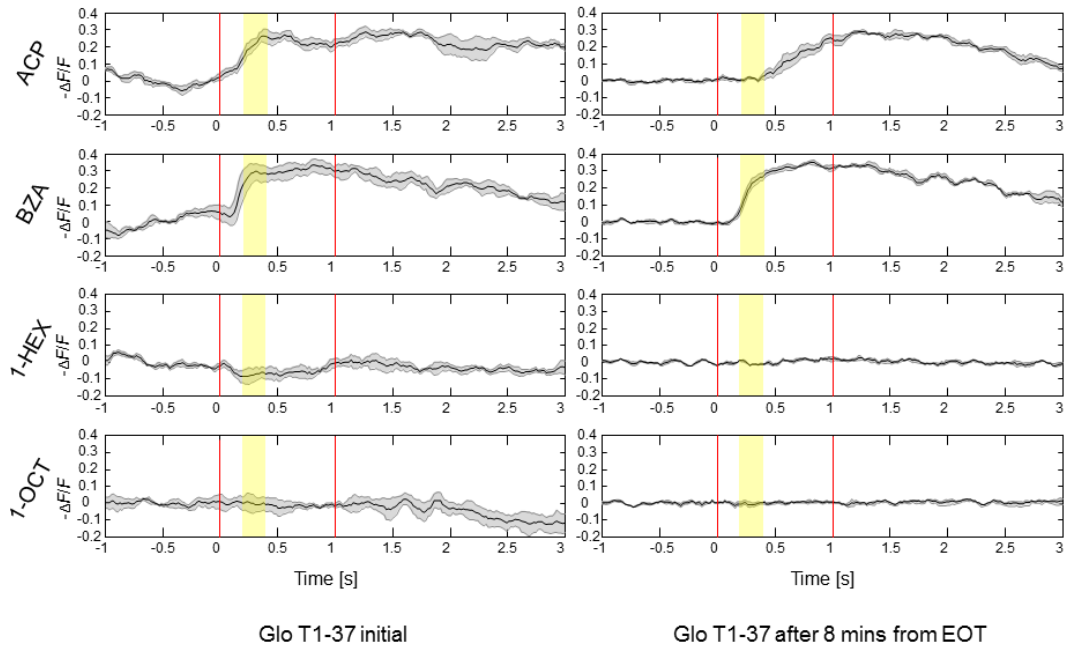

Figure 59 - Odour-evoked activity and odour-specific recovery in a single glomerulus, identified as GloT1-37, in a treated bee, from 1 s prior to stimulus onset to 2 s after stimulus offset. The glomerular activity is shown in response to the four different odours used in the stimulation paradigm (ACP=acetophenone, BZA=benzaldehyde, HEX= 1-hexanol, OCT= 1-octanol). Each window represents the average -Δ*F/F* calculated over the course of three subsequent repetitions before (left column) and 8 min after the EOT (right column). While the recovery of the response to BZA is complete, the recovered response to ACP is delayed (compared to the beginning), such that it will not show up in the peak activity calculated at 200-400 ms (yellow area) after stimulus onset (see Fig. 57 for comparison). Red vertical bars: stimulus onset and offset. Grey shadows represent SEM.

Therefore, parallel to the amplitude analysis, we computed average delays in onsets of odour-evoked responses in the three time windows. Odour-evoked responses from treated bees show a significant increase in onset delay at both time points after the treatment (Fig. 60). This is probably contributing to the incomplete recovery that emerges from the analysis of average responses (calculated between 200 and 400 ms after stimulus onset).

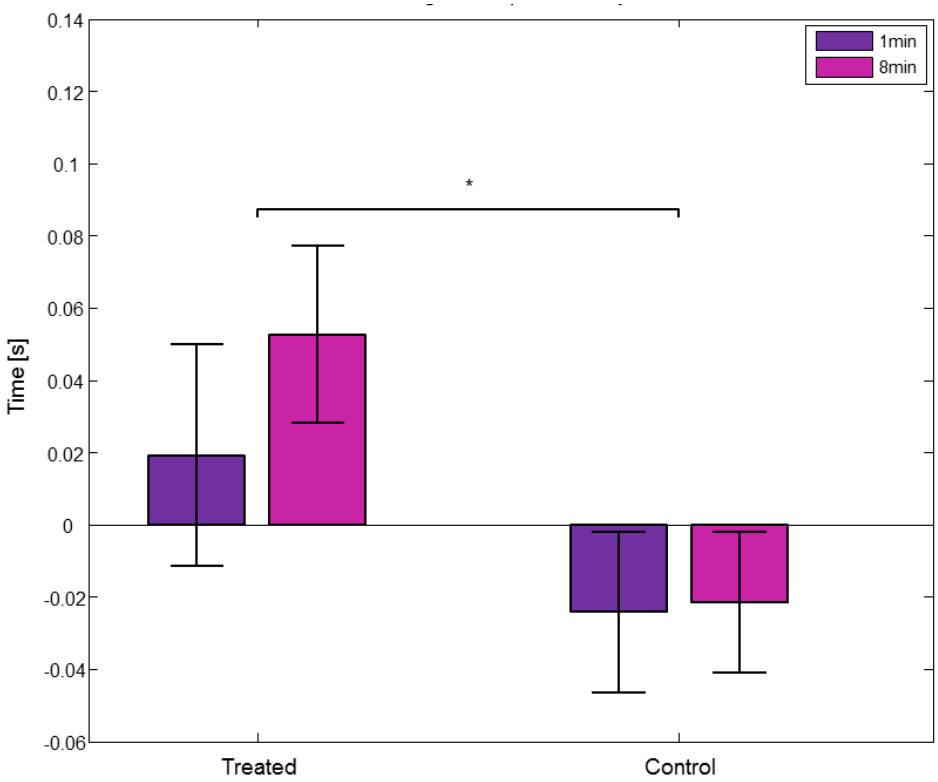

Figure 56 - Change in delay of the response onset $delay_{after}$-$delay_{before}$ at 1 min (in violet) and 8 min (in fuchsia) after EOT. Only active glomeruli were considered (*i.e.* those in which the mean -Δ*F/F* during stimulus pulse deviated from the mean -Δ*F/F* calculated during 1 s before stimulus by at least 2 standard deviations; n=76, 88, 138, 135 odour responses). Error bars represent SEM. The two groups show significantly different delays in response onset (Kruskal-Wallis, H_1=6.2, p=0.013).

3.5.2.5 Effects on Euclidean distances.

The results on the individual odour-evoked responses led to the question of how the odour codes and odour discrimination would be affected by the treatment.

To answer this question, as a measure of distinguishability, Euclidean distances (EDs) between pairs of odour-response patterns were computed, and the dynamics of this measure over the course of the 25 stimulations around imidacloprid administration were studied. ED dynamics relative to an example control bee and a treated bee are shown in Figure 61.

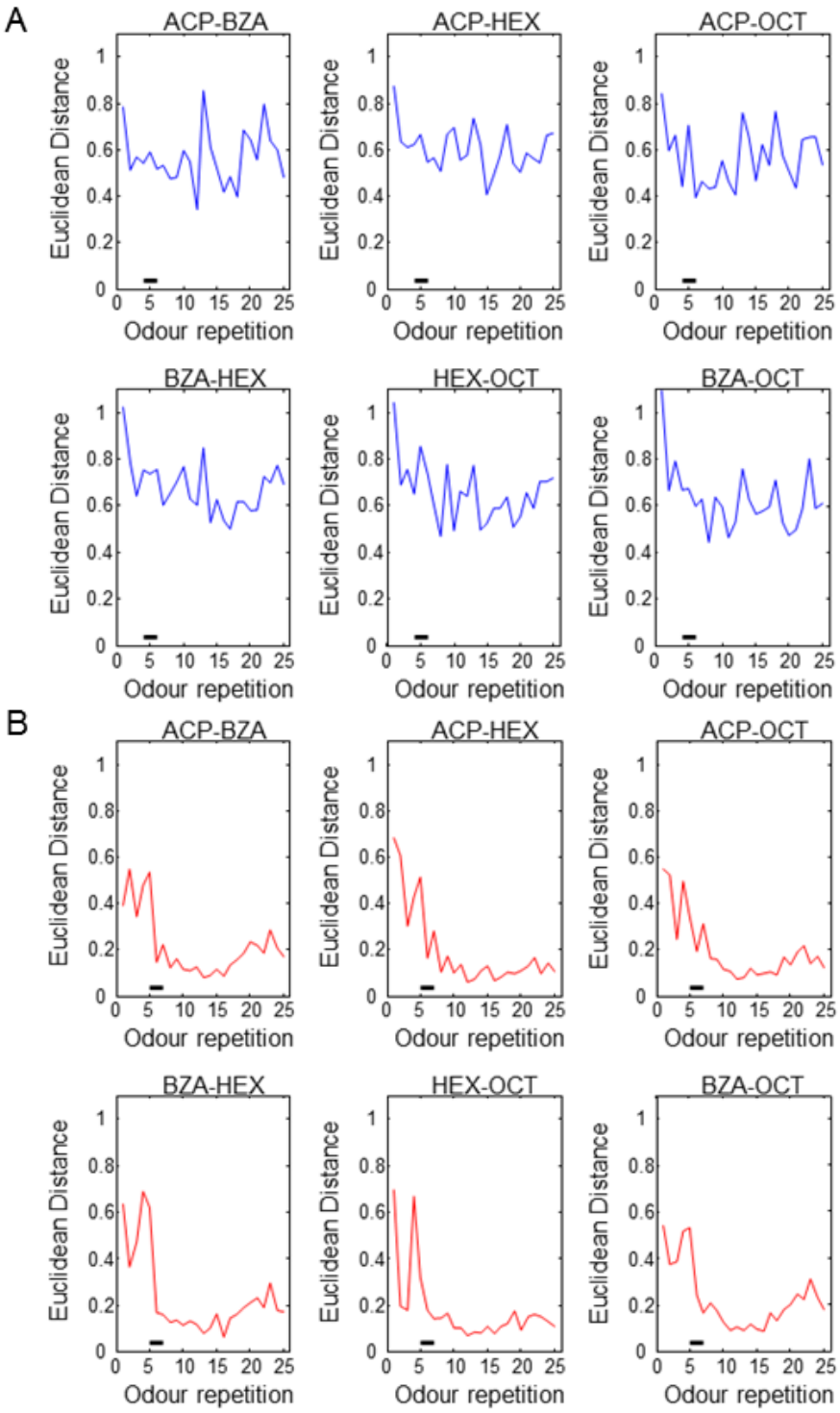

Figure 57 - Euclidean distances between pairs of odours (ACP= acetophenone, BZA= benzaldehyde, HEX= 1-hexanol, OCT= 1-octanol) across 25 stimulations within one control (A) and one treated (B) bee. The EDs are strongly reduced in the treated bee by the treatment (black horizontal line) with a slight tendency for recovery in the later repetitions.

Again, to compare changes in EDs across bees, we focused on the three time windows described above (before treatment, 1 min after EOT, 8 min after EOT). In each bee, we extracted EDs relative to each period, and then we averaged across groups (Fig. 62).

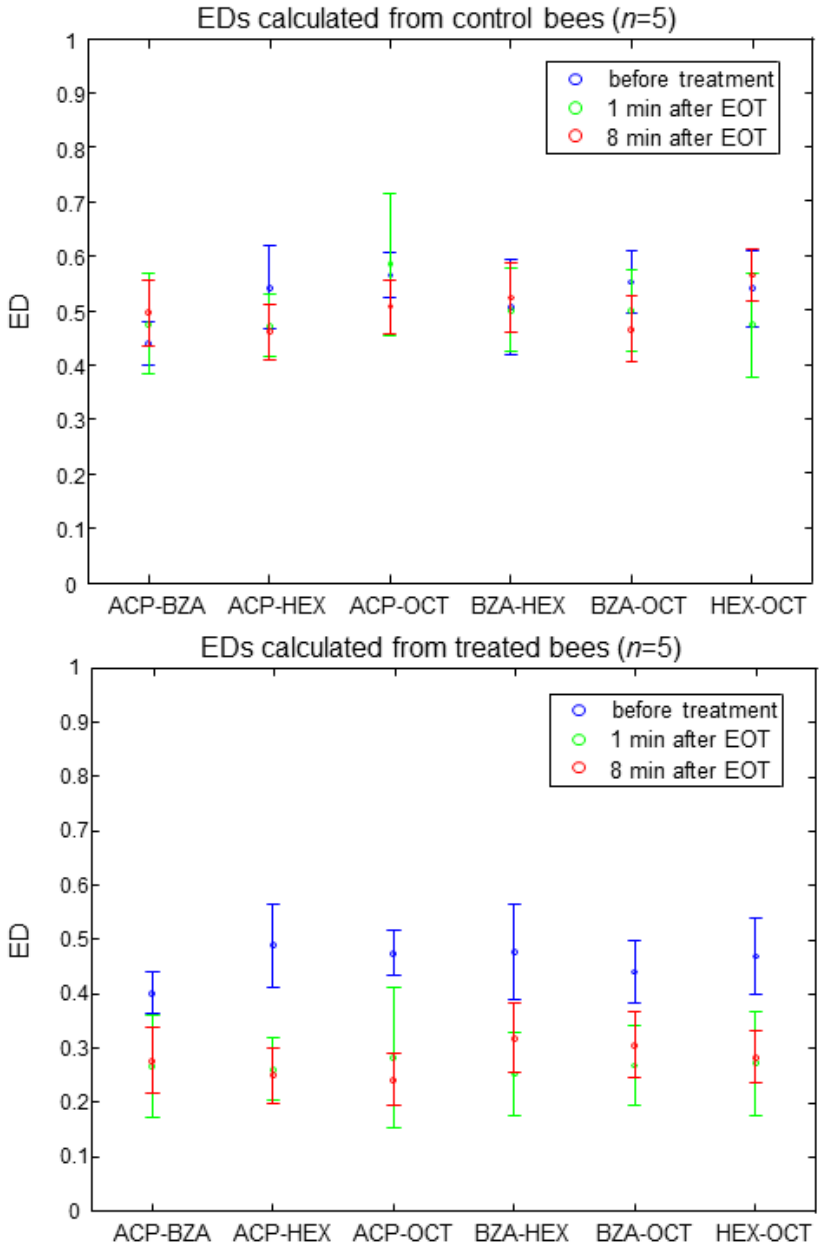

Figure 58 - Average Euclidean distances between pairs of odours calculated before the treatment (blue), at 1 min (green), and 8 min (red) after EOT, in controls (above) and treated bees (below). Error bars show SEM (*n*=5 bees per group).

Then, we computed a measure of normalized change in ED: $(ED_{after}-ED_{before})/ED_{before}$, for both time points after EOT. Normalized changes in ED

were averaged across odour pairs, and compared across bees of both groups (Fig. 63).

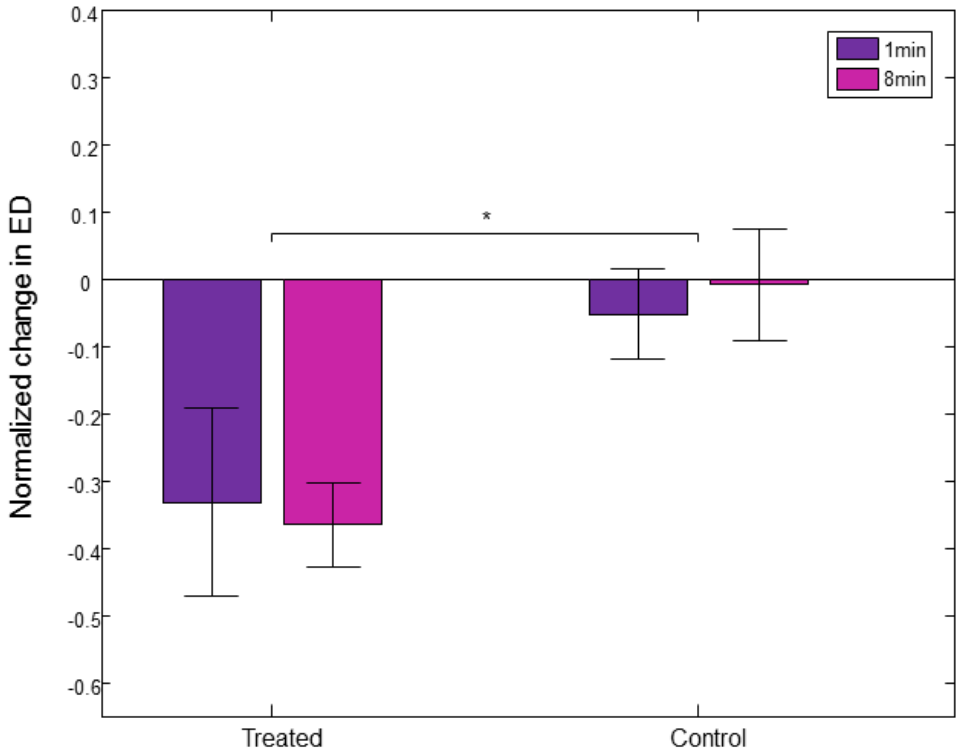

Figure 59 - Normalized changes in Euclidean Distances over time: (EDafter-EDbefore)/EDbefore in treated and control bees. There is no significant effect of time on ED measurements, nor of time × group interaction. However, EDs are significantly reduced in the treated group at both time points after EOT (1min: 1 min after EOT, shown in violet, and 8min: 8 min after EOT, shown in fuchsia) with respect to the control group (n=5 bees per group; two-way repeated measures ANOVA, group: $F_{1, 8}$= 8.3, p=0.020). Error bars represent SEM.

The treated group showed a significant reduction in the diversity of the odour codes following treatment, with respect to the control, at both time points after treatment (two-way repeated measures ANOVA revealed a significant effect of group: $F_{1,8}$= 8.3 , p=0.020, but not of time: $F_{1, 8}$=0.28, p=0.61, or group × time interaction: $F_{1, 8}$= 0.29, p=0.61).

3.5.2.6 Principal components analysis.

To visualize the alteration of the response codes relative to the 4 odour stimuli during and after the treatment, we reduced the dimensionality of the coding space by a principal component analysis (PCA). Transforming the n-dimensional space of all recorded glomeruli in all bees (n=172) we find that

the first three principal components (PCs) explain all variance across mean activities. Subsequently, we averaged mean activities in PCs across bees within each group (*n*=5). In this way we obtain for each odour a temporal sequence of the mean group response to the 25 repeated stimuli. The evolution of the mean responses to all odours for treatment and control group is shown in the three-dimensional PC space (Fig. 64).

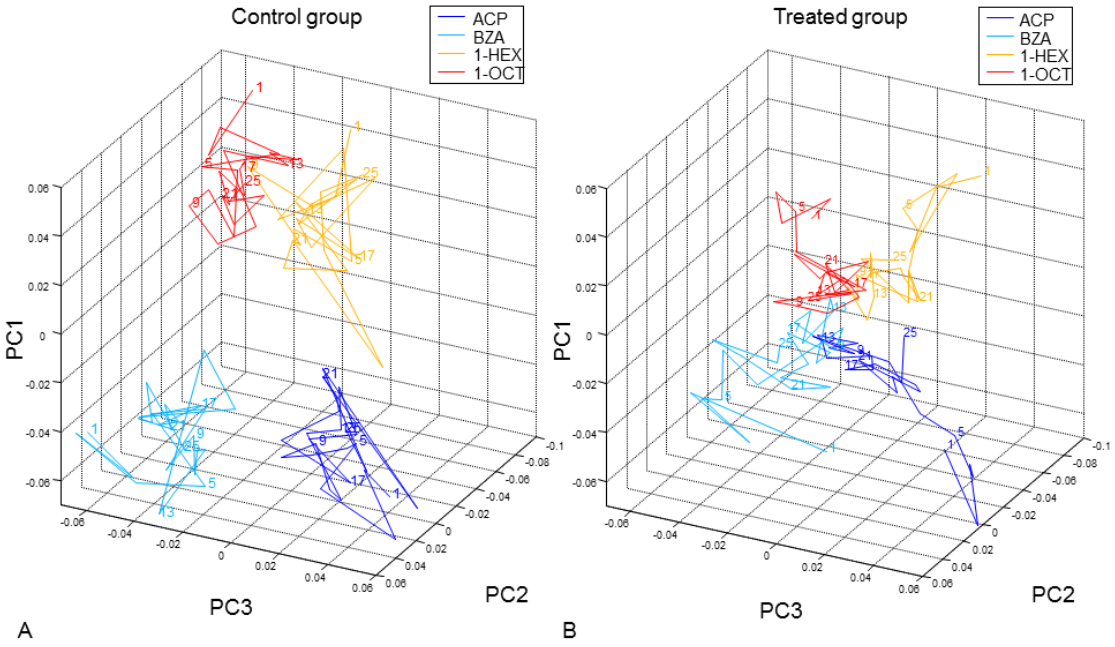

Figure 64 - Average odour representation in time (*n*=5 bees per group; ACP: acetophenone, shown in blue, BZA: benzaldehyde, in cyan, 1-HEX: 1-hexanol, in yellow, and 1-OCT: 1-octanol, in red) during 25 stimulus repetitions (repetitions are marked by numbers along the trajectories) in control (A) and treated (B) bees. Imidacloprid was administered on average between trials 4.6 and 6.6 to the treatment group (B), while, in the same window, the control group (A) was administered with Ringer's solution from a second vial. Odour responses are shown in principal components (PCs) in order to reduce the coding space dimensionality. PCs and axes are identical for (A) and (B), allowing comparison of odour code separation.

The odour representations within the control group (Fig. 64A) were confined to a given area of the coding space, remaining stable over the course of 25 stimulation cycles. On the contrary, in the treated group (Fig. 64B) the representations of the 4 odours, which were well separated at the beginning, collapsed after treatment (administered on average between trials 4.6 and 6.6; see marker 5 and following, Fig. 64B) onto the origin of the coding space. A trend was noticeable in the final stimulation cycles (see *e.g.* marker 25, Fig.

64B) of some of the odours to separate again within the glomerular coding space. However, the overall distances between different odorants were still reduced compared to the initial condition.

3.5.3 Discussion.

Effects of imidacloprid on the dissociated antennal lobe cells have been previously described (Barbara et al. 2008; Nauen et al. 2001; Barbara et al. 2005). However, in those studies, it was not possible to determine the identity of the recorded cells, and it was therefore impossible to make any hypotheses on how this substance would affect the AL network when tested *in vivo.*

We have shown here that the odour-specific calcium transients evoked in the AL PNs are greatly reduced by imidacloprid application. This reduction is probably due to inactivation of PNs. Indeed, it has been demonstrated that, at least in the case of Kenyon cells, imidacloprid induces a tonic inward current, making the cells unable to respond to acetylcholine (Palmer et al. 2013). We assume the same happens in PNs, which would explain the vanishing of all odour-induced calcium response soon after treatment. The partial reversibility of the observed effect can be explained by a progressive detachment of imidacloprid molecules from their binding sites, supported by the sustained washing with the physiological solution. Additionally, a delayed recovery of the intracellular calcium concentration, initially raised by imidacloprid (Jepson et al. 2006), might contribute.

However, the observed reversibility was not complete, as it did not encompass all glomeruli. Moreover, in glomeruli in which the response amplitude was restored, sometimes the shape of the odour-evoked calcium transient was different compared to that of pre-treatment, as we showed in the case of response onsets, which were often delayed. All this prevented the odour code from being fully regained. In fact, the Euclidean distances – measures of distinguishability between odour pairs – remained below initial values during the complete post-treatment period. So, even if the recovery on a single-glomerulus scale seemed to have saturated within ≈10 min after the EOT, some important part of the odour code was probably never regained. It

has been previously suggested that different nAChRs might be expressed at different locations in AL (Barbara et al. 2008; Dupuis et al. 2011), and a possible explanation for our results is that these receptors show different sensitivity to imidacloprid. This is a point that will need to be further elucidated.

It seems reasonable to argue that PNs are not the only cell type responsible for the effects we observed. This is suggested *e.g.* by the response pattern of glomerulus T1-37 (Fig. 57 and Fig. 59). Within the same glomerulus, two different odour-evoked responses were differently affected by the treatment, implying that the information pertaining to those two odorants is conveyed to these PNs via two distinct cellular pathways, *i.e.* as direct input from ORNs and via the LNs. Therefore, we hypothesise that this latter cell type (most probably also expressing nAChRs) might not be able to restore its normal activity after the treatment.

Thus, the effect could be not only glomerulus-specific, but rather nAChR-type-specific (in terms of subunit composition; Dupuis et al. 2011) and cell-type-specific. Neuronal actors other than PNs and inhibitory LNs might also be involved. Excitatory cholinergic LNs have been described in the fruit fly, *Drosophila melanogaster* (Huang et al. 2010; Olsen et al. 2007; Shang et al. 2007), and there may be homologs in the honeybee. Moreover, the equivalent of a putative feedback neuron, ALF-1 (Kirschner et al. 2006), transmitting information from the mushroom bodies back to the ipsilateral antennal lobes, has been demonstrated in the fruit fly to be a cholinergic neuron (Hu et al. 2010). Finally, cholinergic feedback onto the ORNs might also contribute to glomerulus-specific effects.

Another non-cholinergic effect that may contribute to imidacloprid's interference with odour coding is its partial blocking of GABA-induced currents, observed for the first time in Kenyon cells (Déglise et al. 2002). All of these contributions might accumulate and give rise to the complex effects that we observed.

Our results add to evidences collected by others in conditioning studies showing that imidacloprid and other neonicotinoids interfere with olfactory learning (Williamson and Wright 2013; Williamson et al. 2013; Wright et al. 2015; Yang et al. 2012). We demonstrated here that malfunctioning of the olfactory pathway under neonicotinoid exposure starts as soon as at the AL level, where odour coding is disrupted. We suggest that imidacloprid impairs olfactory learning in several ways by acting at different locations in the bee brain. Along the odour processing pathway, odour discrimination is the first function to be impaired.

Finally, we would like to discuss an apparent contradiction between our results and those of Williamson and colleagues (Williamson et al. 2013), who in a classical conditioning study reported enhanced odour discrimination following acute treatment with imidacloprid (1.28 ng/bee). However, at higher doses (Decourtye et al. 2004b) or under chronic treatment (Williamson and Wright 2013), imidacloprid impairs acquisition, memory, and discrimination. So, effects on the olfactory pathway are strictly dependent on doses. Our treatment is also acute, but instead of oral administration, the pesticide is bath-applied to the brain. Concentrations reaching the synapses are hard to compare but most likely higher in our case.

To conclude, we demonstrated for the first time an effect of a neonicotinoid pesticide, imidacloprid, on the AL functionality in the honeybee, *Apis mellifera*. The experimental result – that imidacloprid disrupts odour coding within the AL, reducing the EDs between odour pairs –, allows the inference of a decreased odour distinction capacity (Guerrieri et al. 2005). Diminished odour discrimination is likely to contribute to the previously reported impairment of olfactory learning and memory, since a specific and robust stimulus-coding pattern is necessary to form and recall odour-specific memory.

3.6 Conclusions.

The results presented in this chapter constitute new evidences of sub-lethal effects of neonicotinoids in the honeybee, *Apis mellifera*. Odour discrimination and AL functionality were here investigated, for the first time, from this point of

view. Behavioural experiments on olfactory learning, *e.g.* the one on differential learning described in 3.4, can give some indications of odour discrimination problems. However, olfactory conditioning *per se* would hardly give conclusive evidences regarding pure discrimination. Memory and retrieval are always an issue in this paradigm, even during the acquisition phase. A more clear result of impaired discrimination during differential learning would be an undifferentiated rate of PER to the rewarded and unrewarded odour in treated bees. In fact, PER was reduced in this group in response to the rewarded odour. However, it was not increased in response to the unrewarded one. This result means that treated bees are still able to differentiate between the two stimuli, and possibly only their memory, or retrieval, is impaired. A critical point here might be that we used two stimuli, which are too easy to differentiate. We suggest that further experiments on differential learning upon neonicotinoid treatment should be conducted with two odorants, which are much closer in the perceptive space. Anyhow, it is also possible that discrimination problems just set up at very high doses of imidacloprid, or possibly would require longer time to emerge (as we observed discrimination impairments in the long term memory test).

In vivo visualization of the odour coding in calcium imaging is, in this perspective, a very powerful tool, as it allows assessing the AL functionality in a very direct way. A measure of discrimination can be extracted by the overall odour response, without confounds of other cognitive capabilities which might also be impaired. We exploited this approach to answer the question of the effects of imidacloprid on odour discrimination. From our results, it emerges very clearly that imidacloprid is able to disrupt the odour code and, therefore, discrimination. The direct application of the drug on the brain was a necessary tool in order to study the effects of neonicotinoids on the AL network in the most direct way possible. The question, in fact, was never addressed before. Study on dissociated cells showed the existence imidacloprid-evoked currents, but without being able to identify the neuronal type involved (see *e.g.* Barbara et al. 2008). So, the effects on the circuit could not be predicted. It is not even yet clear whether all the neuronal types express nAChRs and in which fashion they are connected the ones with the others.

The results I described in this thesis point to two separate effects on at least two cell populations within the AL. This is suggested by cases, such as the one highlighted in Figure 57 and Figure 59, where one glomerulus (T1-37) shows odour specificity in the recovery of its odour response. If the information regarding the two odorants (acetophenone and benzaldehyde) reach the T1-37 uPNs through a single route (*i.e.* the same synapse pool), then we would not observe a different profile of recovery for the two signals. The odour-evoked responses should be - in that case - both fully recovered, or both delayed. However, this is not the case.

So, we can hypothesize that imidacloprid acts on different cell types, or at least on different types of synapses. We do not know if the effects might be more transient in some cases, or rather the odour-specific differences we observed in odour recovery might be due to an uncomplete washing. We are currently analysing the functional data in 3.5 in terms of correlation among spontaneous activity in the different glomeruli, in order to establish if AL connectivity might be changed following the treatment. This would be a clearer indication of LN dysfunction.

The next important step would be that to evaluate the occurrence of such effects on the odour code following treatment in a more natural setting, *i.e.* by feeding the bees with neonicotinoids, either in acute or in semi-chronic paradigm. This step is necessary to establish whether the effects that we have observed do contribute to the threats to bee survival caused by those pesticides. It might well be that different brain areas might be susceptible to different concentrations of neonicotinoids, and so different effects might appear at different doses. Therefore, precise evaluation of the thresholds for settling of specific effects is of high value for environmental and economic considerations on neonicotinoid use.

Chapter 4 – Final remarks.

4.1 Summary of conclusions.

- We could not replicate results on structural plasticity following odour conditioning reported by Hourcade and colleagues (Hourcade et al. 2009). However, failure to do so might have resulted from artefacts linked to the optical clearing of the tissues, or from a too low *n*.
- Structural lateralization in glomerulus T1-17 could be observed in pseudo-conditioned bees. This points out to the need for a naïve group along with a pseudo-conditioning group in such conditioning studies.
- We described a proof-of-principle approach to analyse structural changes within glomeruli in terms of synapse density (quantifiable in optically cleared α-synapsin-immunolabelled brains, visualized at the two-photon microscope).
- For the first time, we report on volumetric changes in the AL glomeruli following prolonged odour exposure. Two glomeruli (T1-17 and T1-33) are reduced in size in 1-nonanol pre-exposed bees.
- Using the same manipulation, we analysed functional changes in the AL (through *in vivo* calcium imaging analyses), observing shifts in binary mixture representation, similarly to those described by Locatelli and colleagues (Locatelli et al. 2013).
- We also observed changes in odour responses within individual glomeruli. Particularly, glomeruli whose size appeared changed in the morphological analysis also showed a differentiated pattern of odour response depending on the odour of pre-exposure.
- We observed reduced rates of differential learning in imidacloprid semi-chronically treated bees. Such reduction might be caused by both memory/retrieval problems and discrimination problems.
- Discrimination impairments are anyhow evident in the long term memory of the association, as previously reported (Williamson and Wright 2013).

- When functionally analysed (through *in vivo* calcium imaging and acute application of imidacloprid), the AL resulted to be heavily affected by the neonicotinoid. In particular:
    I. Odour responses within individual glomeruli are transiently switched off.
    II. Following washing, some odour responses are regained.
    III. However, some will never recover.
    IV. Some will be changed in shape. In some cases, latencies of calcium response onsets are increased.
    V. The modification in the glomerular responses causes huge changes in the overall odour code, and the Euclidean distances between odorants are permanently reduced.
    VI. Thus, odour discriminability is impaired by the treatment.

4.2 Future directions.

- The analysis on associative-learning related plasticity should be conducted on larger sample of bees, including both a “pseudo-conditioning” and a “naïve” control groups, possibly in age-and-experience-controlled setting. The brains might be α-synapsin and ORNs double-stained. The volumes should be estimated before optical clearing of the tissue. Optical clearing might add a deeper layer of analysis in case volumetric changes were to be highlighted (and ORN tracing would in that case provide useful to study synapse density in the different glomerular sub-compartments).
- Experiments with neurotransmitter and neuromodulator blockers (such as GABA and histamine blockers) might highlight asymmetrical local computation across the glomeruli. One test would be applying such blockers in a restricted manner on either T1-17 or T1-33 in control bees, and observe whether the odour responses in those glomeruli (in particular to 1-hexanol) are changed.
- A different approach to address the same issue would be that of specifically ablating glomeruli which are strongly responding to 1-

hexanol (*e.g.* either T1-28 or T1-38), and observe whether response to the same odour might be enhanced in T1-17/T1-33.

- Differential learning in imidacloprid treated bees should be assessed upon slightly larger doses (in the experiment reported in 3.4, we applied a dose that is in the low-range of the environmentally occurring exposure), and by using highly similar odours, in order to make the task more difficult.
- Latencies in PER response during a differential conditioning task should be compared between treated and untreated bees, by video-recording the procedure. The question would be here that of reaction times - and specifically if those might be increased by imidacloprid treatment (as we observed that the peak of calcium response is delayed in many glomeruli: would that be a sign that a proper odour encoding/ discrimination occur later in treated bees?).
- Connectivity among glomeruli recorded in 3.5 should be evaluated before and after the treatment. These data might possibly result in a clearer indication of the cell types involved in the functional impairments. We are currently analyzing the data in this regard.
- Euclidean distances among a panel of quite similar odours should be assessed via calcium imaging in control and imidacloprid treated bees. In this case, bees should be treated orally, in order to test the effects of neonicotinoids on the AL functionality in a more natural setting.

4.3 Contributions.

All experiments were designed by me. All analyses were performed by me, with the help of my advisor Dr. Albrecht Haase. MATLAB scripts for data processing and statistical analyses were written by me. Data were collected by me, with two exceptions: 1. The behavioural data on differential conditioning upon imidacloprid exposure were collected by Martina Puppi, under my co-supervision (analyses on this part, as for what shown in this thesis, were also performed by me). 2. The behavioral paradigm for unrewarded odour exposure was set up with the help of Ben Timberlake, and

the calcium imaging data on the same part were collected by the two of us together.

4.4 Acknowledgements.

I would like to thank all the people that made this thesis work possible and who helped me throughout the years of my PhD.

First of all I would like to thank my advisor, Dr. Albrecht Haase, for the freedom he left me in designing the experiments and choosing the questions to address, providing at the same time constant advise on approaches, analyses, and, in particular, help on all kind of technical problems with the two-photon microscope.

Then, I would like to thank my co-advisor, Prof. Giorgio Vallortigara, for the invaluable insight he provided throughout the years, helping to direction this work.

I would also like to thank Marco Paoli, for the many technical advises he provided, and the help, and Elisa Rigosi, for teaching me bases of how to condition the bees at the beginning of my PhD.

I am very grateful to Martina Puppi and Benjamin Timberlake for their rigorous work, which directly contributed to this thesis.

Then, to Quentin Badouin: for helping me figuring out how the bee perfusion-imaging stage should be designed; and to Seth Levine and David Pascucci: for teaching me rudiments of MATLAB at the beginning of my PhD.

For advises they gave at various stages of my work, I am grateful to: Prof. Randolf Menzel (FU Berlin), Prof. Giovanni C. Galizia, Dr. Paul Szyszka, and Nicholas Kirkerud (University of Konstanz), Dr. Irina Sinakevitch (Arizona State University), Prof. Martin Giurfa (CNRS, Toulouse) and Prof. Jean-Christophe Sandoz (CNRS, Paris).

Finally, thanks to all the people working in the in the ACN Lab in Rovereto for the nice and funny moments, that made work enjoyable.

Funding was provided by Autonomous Province of Trento (Project IBRAIM), Autonomous Province of Bolzano (project B26J16000310003), and Fondazione Cassa di Risparmio di Trento e Rovereto.

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