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LATERALIZATION IN INSECTS: THEORETICAL AND EXPERIMENTAL APPROACHES

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3.6.1. Materials and methods

Abstract

Recent studies have revealed a variety of left–right asymmetries among vertebrates and invertebrates. In many species, left- and right-lateralized individuals coexist, but in unequal numbers (‘population-level’ lateralization). It has been argued that brain lateralization increases individual efficiency (e.g. avoiding unnecessary duplication of neural circuitry and reducing interference between functions), thus counteracting the ecological disadvantages of lateral biases in behaviour (making individual behaviour more predictable to other organisms). However, individual efficiency does not require a definite proportion of left- and right-lateralized individuals. Thus, such arguments do not explain population-level lateralization. It has been shown that, in the context of prey–predator interactions, population-level lateralization can arise as an evolutionarily stable strategy when individually asymmetrical organisms must coordinate their behaviour with that of other asymmetrical organisms. I extended the mathematical model showing that populations consisting of left- and right-lateralized individuals in unequal numbers can be evolutionarily stable, based solely on strategic factors arising from the balance between antagonistic (competitive) and synergistic (cooperative) interactions.

I also provided empirical evidence to support the prediction from theoretical models suggesting that population-level lateralization is more likely to have evolved in social than in non-social species. I compared olfactory lateralization in two species of Hymenoptera Apoidea, the honeybee (*Apis mellifera*), a social species, and the mason bee (*Osmia cornuta*), a solitary species. Recall of the olfactory memory 1 h after training to associate an odour with a sugar reward, as revealed by the bee

extending its proboscis when presented with the trained odour (Proboscis Extension Reflex – PER), was better in honeybees trained with their right than with their left antenna. No such asymmetry was observed in mason bees. Similarly, electroantennographic (EAG) responses to a floral volatile compound and to an alarm pheromone component were higher in the right than in the left antenna in honeybees but not in mason bees.

Further experiments were conducted to test the lateralized recall of olfactory memory in honeybees, following conditioning of the PER, at 1 or 6 h after training, using a range of different odours. Results confirmed previous evidence that bees learn to associate a new odour of a positive stimulus mainly in neural circuits accessed via their right antenna, and that, after a period of a few hours, memory consolidation occurred accompanied by antennal asymmetry, with bees now being able to recall the odour mainly when using their left antenna. I showed that this peculiar dynamic of memory traces has severe consequences when odours are already known to the bees (either for a biological reason or as a result of previous experience) and are thus already present in the long-term memory store. Response competition arising from multiple memory traces could be observed, with bees showing unexpected lack of specificity in their longer-term olfactory memories.

The behavioural finding that honeybees are better in learning to associate odours with a sugar reward when they are trained through their right antenna can been partially explained by the stronger responsiveness of the olfactory receptor neurons inside the right antenna, as shown in my experiments by EAG recordings. I checked whether this in turn might be associated due to a difference in the number of the olfactory sensilla present on the right and on the left antennae. I found that the number of olfactory sensilla is higher on the right antenna with respect to the left antenna. Surprisingly, I also observed for the first time that the number of non-olfactory sensilla was significantly higher on the left antenna than on the right antenna in all segments except the apex.

I investigated the generality and phylogenetic origins of the antennal asymmetry found in the honeybee *Apis mellifera* by examining three species of Australian stingless social bees (*Trigona carbonaria*, *Trigona hockingsi* and *Austroplebia australis*). Meliponinae (stingless bees) are much older compared to Apidae, Bombinae and Euglossinae; thus, it is maintained that the honeybees did not evolve from the stingless bees but rather independently from some other (asocial) bee type and that any social features the two lines of evolution now share are the result of convergent rather than divergent evolution. I found that stingless bees (Meliponinae) have the same laterality as honeybees (Apinae). This evidence suggests that lateralization evolved prior to the evolutionary divergence of these groups or that it evolved separately in each line. Furthermore, since honeybees and stingless bees are the only highly social bees, it seems that lateralization at the population level was convergent and evolved in association with social behaviour, supporting the hypotheses of the theoretical models that population level lateralization is more likely to evolve in social rather than in non-social species.

Chapter 1 – Introduction

Brain lateralization (i.e. the different functional specializations of the left and right sides of the brain), once considered to be unique to humans (Corballis, 1989), is now well known to be present in all vertebrate classes (reviewed by Rogers and Andrew, 2002; Vallortigara and Rogers, 2005; MacNeilage et al., 2009). Recently, evidence of lateralization in invertebrates has begun to emerge; suggesting that lateralization of the nervous system may be a feature of simpler brains as well as more complex ones (reviewed by Frasnelli et al., submitted).

Why do humans and many others animal species have asymmetrical brains and show left-right asymmetries in behaviour? There is a consensus that a lateralized brain may confer several advantages at the individual level: for instance, sparing neural tissue by avoiding duplication of functions in the two hemispheres (Levy, 1977); processing information in parallel, reducing interference between functions (Vallortigara et al., 1999; Rogers, 2002; Rogers et al., 2004); avoiding competition in the control of responses, making one hemisphere in charge of control of behaviour (which is particularly important in animals with laterally placed eyes; Andrew, 1991; Vallortigara, 2000; Vallortigara and Rogers, 2005). However behavioural (and brain) left-right asymmetries usually occur at the population level, with most individuals showing similar direction of bias. Individual brain efficiency does not require the alignment of lateralization in the population. Moreover, behavioural lateralization at the population level can also present disadvantages, because it makes individual behaviour more predictable to other organisms. Thus, population-level asymmetries are likely to be due to a selection pressures for a particular side to become specialized in the same direction in more than half of the individuals.

From a biological point of view asymmetries can be ‘fluctuating asymmetries’ or ‘adaptive asymmetries’ (Palmer, 1996) The first are random variations in the development of otherwise symmetrical traits as result of disturbances in ontogeny (accidents that could occur during the development such as wounds, illnesses) and the output is a majority of the individuals that are symmetrical with few either left- or right-biased individuals. More interesting are the ‘adaptive asymmetries’ that are genetically or epigenetically influenced; namely that have been selected for a specific function. This type of asymmetries can have two distributions: ‘antisymmetry’ (half left- and half right-biased individuals, i.e. individual-level asymmetry) and ‘directional asymmetry’ (tendency towards one side, i.e. population-level asymmetry).

There could be several advantages of having a lateralized brain at the individual as mentioned above; but what is the reason for the population-level lateralization? Using concepts from mathematical theory of games, it has recently been argued that the alignment of lateralization at the population level may arise as an ‘‘evolutionarily stable strategy’’ when individually asymmetrical organisms must coordinate their behaviour with that of other asymmetrical organisms (Ghirlanda and Vallortigara, 2004). This hypothesis requires that brain asymmetries manifest themselves in behaviour, and thus may have fitness consequences, costs and benefits, in interactions with other organisms. For instance, vigilance behaviour and escape responses to predators often show lateral biases. This idea has been developed in a game-theoretical model considering interspecific interactions between group-living prey subject to predation (Ghirlanda and Vallortigara, 2004). As described in details in the next chapter, I have put this analysis a step further by considering whether a similar scenario also hold when selection pressures on lateralization arise from purely intraspecific interactions of competition and cooperation.

Mathematical models of the evolution of lateralization (Ghirlanda and Vallortigara, 2004; Ghirlanda et al., 2009; Vallortigara, 2006) suggest that stable polymorphism with an uneven distribution of left- and right-forms can be expected to emerge spontaneously in species in which left-right biases have behavioural consequences during everyday interactions between individuals. Thus, population level asymmetries would be more likely to be observed in social rather than solitary species. Some evidence for this will be presented on Chapter 3.

A variety of left-right asymmetries in general morphology has been observed among invertebrates (reviewed by Palmer, 2009) but in this introduction I will focus on asymmetries in the nervous system and behaviour. Invertebrate asymmetries may be crucial to fully understand the evolution of brain asymmetry and, besides, given the relative simplicity of some of their brains, they could provide excellent model systems to investigate the molecular, genetic and developmental aspects of lateralization.

1.1. Sensory asymmetries

Honeybees (Hymenoptera, Apidae, Apinae, Apini) have provided evidence for lateralization in sensory systems, particularly olfaction. Letzkus et al. (2006) showed that honeybees (*Apis mellifera*) display a clear laterality in learning to associate an odour with a sugar reward. The researchers used the proboscis extension reflex (PER) paradigm (Bitterman et al., 1983), in which honeybees are conditioned to extend their proboscis in anticipation of a food reward when they perceive a particular odour. Letzkus et al. (2006) tested bees in two versions of the PER paradigm. In one version, bees were conditioned to extend their proboscis to a scented drop of sugar water but not to an unscented drop of salt water; in the other version, bees were conditioned to extend their proboscis to one odour (dissolved in a sugar solution - reward) but not to another odour (dissolved in a salt solution - punishment). Each version of the learning task was carried out on three groups of bees. The bees in one group had their left antenna covered with a silicone compound, which prevents detection of odour, those in the second group had their right antenna covered, and those in the third group constituted a control in which both antennae were uncovered. Results revealed that the bees with the right antenna covered learnt less well than the bees with their left antenna covered and bees with both antenna uncovered. In fact, the bees trained with only the right antenna in use performed just as well as the untreated controls. The question that arises is whether the difference between bees with the right antenna in use and bees with the left antenna in use is a sensory difference or a memory recall difference. Considering that honeybees were trained through one antenna and the test recall was carried out with the same trained antenna in use the day after, it is difficult to argue that the asymmetry found is due only to a sensory asymmetry.

Letzkus et al. (2006) tried to answer this question hypothesizing that honeybees perform better when trained with the right antenna because of sensory differences between the right and the left antenna. To test the first hypothesis, they compared the number of the olfactory receptor cells, *sensilla placodea*, in the two antennae. Images of ten right antennae and ten left antennae (seven of these left-right pairs originated from the same individuals) were obtained using scanning electron microscopy and the mean numbers of *sensilla placodea* per flagellum on the two antennae were compared. The number was significantly higher on the right than on the left antenna (mean difference of 10%). This finding should been interpreted with caution, however, since only one type of sensilla was considered, and there are other sensilla, such as *sensilla trichodea* (Dietz and Humphreys, 1971), which play an olfactory role in honeybees. Moreover, in the paper of Letzkus et al. (2006), SEM images did not cover the whole antennal segment surface leaving a hidden, non-characterized area. Finally, only 7 out of 10 left-right pairs of antenna were from the same individuals.

Rogers and Vallortigara (2008) extended the work of Letzkus et al. (2006) by investigating whether lateralization could be found in recall of olfactory memory at various times after the bees had been trained using both antennae (i.e. without forcing them to learn with one or the other antenna) and, moreover, whether such lateralities are manifested as side biases in responsiveness to odours in bees with both antennae in use. After PER training with both antennae in use, using lemon plus sucrose solution as the positive stimulus and vanilla plus saturated saline as the negative stimulus, bees were tested for recall 1-2 and 23-24 hours later and with the left or right antenna coated with the silicone compound. At 1-2 hours after training, bees showed excellent recall when tested using their right antenna, but poor or no recall when tested using their left antenna. By contrast, 23-24 hours after training recall was good when the left antenna was in use but not when the right antenna was in use, demonstrating that long-term memory is accessed mainly via the left antenna. Thus, retrieval of olfactory learning is a time-dependent process and involves lateralized neural circuits.

Rogers and Vallortigara (2008) also checked whether the laterality observed in bees was manifested as side biases to odours presented to the left or right side of the bee without any covering of the antennae, and so in a more natural condition than in the paradigm requiring one antenna to be coated with latex. Bees were trained using both antennae and the recall at several intervals (1, 3, 6 or 23 hours) after training was tested using lateral presentation of the two stimuli and no coating of the antennae. The odour was presented to the left or right side of the bee. Use of an exhaust fan apparently meant that the antenna closer to the droplet received a higher concentration of odour reaching the antenna further from it. There were significantly more correct PER responses to odours presented on the right than on the left side at 1 hour after training. No significant left/right difference occurred at 3 hours after training. At both 6 and 23 hours after training the correct responses were higher on the left side than on the right side. The study by Rogers and Vallortigara (2008) clearly demonstrates that the asymmetry is more complex than a difference in learning ability of the right and left antennae and that the difference in number of olfactory sensilla is unlikely to explain entirely the behavioural laterality.

A shift of recall access from one to the other side of the brain has been noted previously in birds (Cipolla-Neto et al., 1982; Clayton, 1993; Andrew, 1999*)*. This is interesting because it suggests that lateralized events in memory formation may be similar in bees and vertebrate species.

It has been shown (Sandoz and Menzel, 2001) that, after a period of 3 hours delay, bees can respond to a conditioning stimulus on the contralateral side, suggesting that both sides of the brain share information about the learned odour.

Thus, a transfer of the learned information between sides of the brain in honeybees or a generalization between odorants on each side has been hypothesized (Sandoz and Menzel, 2001). Moreover, interestingly, in a bilateral differential conditioning procedure, conditioning performances have been shown to be significantly higher on the left than on the right side. Three hours after retention, as in the other experiments, honeybee transfer information about the odour they had been trained on to the opposite side. It is important to note that this finding is in contrast with the findings of Letzkus et al. (2006) that show that honeybees perform better at responding to odours, and at odour discrimination, when they are trained through the right antenna.

A shift of memory access from the right to the left side of the brain would allow the right antenna to learn about new odours without interference from odour memories in long-term stores. In fact, since bees visit different flowers at different times of the day, as nectar becomes available, the formation of different odour associations during the course of the day would be required, and this is a process that might be aided if recall of earlier odour memories is avoided on the side of the brain undergoing new learning. Kells and Goulson (2001) reported that bumblebees *Bombus spp* (Hymenoptera, Apidae, Apinae, Bombini) show preferred directions of circling as they visit florets arranged in circles around a vertical inflorescence. In three out of four species examined the majority of bumble bees circled in the same direction. Since two species circled anticlockwise and one clockwise, it is unlikely that the asymmetry is a function of the structure of the florets. The biased circling might well have something to do with lateralization of antennal responsiveness to odours or lateralized leaning and memory recall.

Further evidence of sensory lateralization in invertebrates has been obtained recently in fruit flies *Drosophila* (Duistermars et al., 2009). The use of bilateral olfactory cues to track odours in flight has been investigated using a modified flight simulator that enables maneuvers in the yaw axis. Results showed that flies readily steer directly toward a laterally positioned odour plume and do so by orienting saccades directly up an odour gradient. This ability is eliminated when the sensory input to one antenna is occluded, precluding odour detection and thus eliminating bilateral spatial comparison. Flies with a single intact antenna performed frequent yaw deviations but these paired saccades frequently occurred outside of the plume. Moreover, odour presentation resulted in a significant bias in flight heading toward the intact antenna and occluding the left antenna had a stronger effect than occluding the right antenna, revealing that sensory signals from the left antenna contribute disproportionately more to odour tracking than signals from the right antenna. Input to the left antenna has been shown to be sufficient to elicit a significant odour-mediated decrease in saccade frequency and to be able to generate a higher proportion of left turns than the right antenna in response to odour. These results reveal a consistent asymmetry in antenna-mediated flight control, suggesting that asymmetrical cross-modal olfactory and mechanosensory signals facilitate stable odour tracking in complex multisensory environments and likely also increase the efficiency of search behaviour.

Letzkus et al. (2007) used a PER paradigm to investigate visual learning of bees using their left or right eye. Bees were tested with their antennae removed, because there is evidence that conditioning to visual stimuli is easier to obtain in bees without antennae (Hori et al., 2006). Bees were tested with both eyes covered (BEC), both eyes exposed (BEE), their right eye exposed (REE) or their left eye exposed (LEE). The conditioned stimulus (CS) was a large yellow rectangle presented on a computer-controlled display. The bees were conditioned to extend their proboscis in anticipation of a food reward (unconditioned stimulus, US) according to the colour of the stimulus CS. Each experiment consisted of two 10-training sessions; the first one was conducted the morning after the eyes were covered and the second one was conducted the morning after the first 10-training session. The two 10-training sessions were subdivided in four five-trial blocks, during which the CS (yellow rectangle on monitor) was presented for 15 s. BEC bees showed 0% learning performance throughout the entire training. The BEE bees' performance rose steadily, with a mean response rate of 39% in the last five trials. REE bees also showed an increase in learning performance, but the response rate was slightly (but not significantly) lower than that of BEE bees throughout the training. Only, in the last five trials REE bees showed a performance level of 33%. LEE bees reached a mean learning performance of only 13%, which was significantly lower than that of BEE and REE groups. LEE bees were statistically different from BEE and REE bees in trials 6–10 and from BEE bees in trials 11–15. Thus, at least in the last part of the test, bees seem to primarily use the right eye for learning and/or detecting objects (for associating a visual stimulus with a food reward, in this case). Since the second 10-training session was performed the day after the first 10-training session, it is questionable whether the results found are dealing with learning or with memory and, if memory, whether is long- or short-term memory. Comparing these findings with the lateralization found in olfaction, Letzkus et al. (2007) argued that sensory inputs from the right side are used preferentially while foraging or feeding. However, this result cannot be compared directly with the asymmetry found in the olfactory response because of several reasons. First, the right antenna has been shown to be involved in short-term memory for odours, i.e. in the learning process of new odours, while the long-term memory is recalled via the left antenna. Thus, as concerns olfaction, it is not possible to generalize saying that sensory inputs from the right side are used preferentially while foraging. Moreover, not all the odours, even if most of them, are connected with the feeding process: they can have a different biological meaning as, for instance, alarm pheromones. For the eye asymmetry, it could be possible that the right eye is involved in feeding while the left eye is involved in predators detection, as found in some vertebrates (Vallortigara and Rogers, 2005), but further studies are required to test this hypothesis. Furthermore it seems important to test antennal and eye preferences for different odours and visual stimuli in naïve honeybees, i.e. without any training.

Asymmetries in eye use have been studied also in cephalopods. Byrne et al. (2002; 2004) measured preferential eye use in octopus *(Octopus vulgaris)* by recording the time animals spent watching a stimulus presented to them outside their tanks while holding on to the front glass of the tank. The stimulus used was a life-sized plastic crab model (resembling the prey crabs fed to the octopuses) mounted on a transparent Plexiglas stick, which was presented at five equidistant positions along the length of the tank and was held at each position for 3 min according to a random sequence, so that the time at each of the five positions was the same to avoid directional bias. Individuals displayed lateral preferences for either the left or right eye (Byrne et al., 2002) but the asymmetry in octopuses followed an anti-symmetrical distribution, with no systematic bias towards left or right at the population level. It is interesting to note that *Octopus vulgaris* is a solitary species (Hanlon and Messenger, 1998) and therefore an antisymmetrical distribution of preferential eye use would be not unexpected according to the theoretical reasoning outlined above.

1.2. Motor asymmetries

Ants (*Formicidae*) and spiders (*Araneae*) have been shown to possess behavioral left-right asymmetries (Heuts et al., 2003). A significant majority of spiders in the field with mainly left-leg lesions was observed and also their weak leg lesions caused by catching them were significantly biased to the left. In ants, appendage severance was significantly more frequent on the left than right side.

The greater vulnerability of left legs compared to right legs in spiders in general is indicated by the evidences of 305 left-leg versus 254 right-leg lesions (obtained from 18 spider families - Heuts and Lambrechts, 1999). It has been shown that separate spider families and species are also significantly left-biased and never significantly right-biased (Heuts and Lambrechts, 1999). The left-side vulnerability may be due to anatomical factors (joint strength and leg length) and/or a complex of various behavioural factors such as a propensity to move the legs of one side when alarmed, or left/right attack biases in spider predators as documented in some vertebrates species (Vallortigara and Rogers, 2005). There are a few direct observations of spider leg use when interacting with spider prey. Ades and Ramires (2002) demonstrated that the spitting spider *Scytodes globula* (Scytodidae) uses its legs in an asymmetric way during prey handling. This species catches its prey by rapidly ejecting a mixture of glue and venom from the chelicerae and, on the direct contact during the handling of the prey, it uses legs I and/or II for longer than the other legs. Looking at the missing legs in spiders collected in the field it was found that in 162 field-collected spiders, 36 had one leg missing; among these 36 the frequency of spiders with a leg I or II missing was significantly higher than that of spiders with a leg III or IV missing. There was a significant difference between those missing the left I leg and those missing the right I (15 missing the left I leg and 4 missing the right I leg) but, even through more spiders lacked left leg II than right leg II, there was no significant bias. Ades and Ramires (2002) also looked at touches during predatory encounters of individual *S. globula* with prey individuals from 3 species of spiders, *Loxosceles intermedia, L. gaucho*, and *L. laeta* and they observed that touching movements with the left anterior legs were significantly more frequent than with the right anterior legs. Spiders, however, were tested only once, and thus repeated testing of the same individuals is required to confirm the constancy of use of the preferred leg. Additionally, it would be interesting to investigate the use of the legs in handling non-spider prey and in other behavioural contexts in order to assess the generality of leg preference. The reason and possible adaptive benefits of a preferential use of both left leg I and II could be explained checking whether both left legs I and II explore by touching after shorter latency than both the right legs or whether there is a higher density of tactile (chemo) receptors in them. In other words, this side bias might reflect a sensory lateralization.

Evidence of lateral biases in ants has been reported too (Heuts et al., 2003). Twelve ant species *Lasius niger* kept mainly to the right on their foraging “streets”, whereas there was only one species which kept to the left. On streets in trees, 49 *Lasius niger* colonies kept to the right versus 26 to the left. In this ant species a significant majority of couples in the laboratory had the left side of their bodies exposed to their partners when resting. This identical left body side exposure when resting and foraging in streets also correctly predicted that lone foraging *L. niger* would significantly more often turn to the right than to the left (the ratio was 14 to 2). The existence of a specific behavioural lateralization (sharp left turns) in *L. niger* and four other ant species when running on unknown ground in an “alarm” situation (24 left *vs.* 4 right turns) makes sense because these sharp left turns increase the chance of remaining on the spot when a potential danger must be countered, whereas the blunt turns to the right during foraging are beneficial to a fast and efficient transport of food, especially so in dense “traffic”.

Bradshaw and Rogers (1993) noted right-claw anatomical and behavioural dominance in a crab species, suggesting stronger right than left appendage muscles like that in ants (an assumption based on their sharp and fast left turns). A more recent study (Backwell et al., 2007) conducted on male fiddler crab (*Uca vocans vomeris*) investigated the consequences of being left-clawed in this species composed of predominantly right-clawed individuals (less than 1.4% of males are left-clawed). Left-clawed males usually fight opposite-clawed opponents. Thus, the prediction, in line with the theoretical models on the evolution of population-level asymmetries (Ghirlanda and Vallortigara, 2004; Ghirlanda et al., 2009) and with the evidence in humans of the advantage conferred by being left-handed in interactive sports like tennis or boxing (Raymond et al., 1996; Brooks et al., 2004) is that left-clawed males would be better fighters due to their relatively greater experience in fighting opposite-clawed opponents. However the expectation did not match with the results that showed that a left-clawed male retains a burrow for a period that is significantly shorter than a size-matched right-clawed male. Moreover, when experimentally displaced from their burrow, even if no difference was found in the tactics used by left- and right-clawed individuals to obtain a new burrow, right-clawed males were significantly more likely than left-clawed males to initiate fights with resident males. In the same way, right-clawed residents engaged in significantly more fights than left-clawed residents. This occurred as a consequence of the previous observation, indeed that a significantly greater proportion of left-clawed males retreat into their burrow observing and avoiding fights. However, when a fight occurs, no significant difference in the proportion won by left- and right-clawed residents has been observed. Eventually left-clawed males appear to be less likely to fight and when they fight they are less likely to win than right-clawed males. An explanation for that may be that that the left-clawedness is causally related to their reduced ability in fights, i.e. it is associated with developmental pathway that affects general performance. Lastly, left-clawed males might be generally inclined to avoid fights, as they are more likely to escalate an encounter with a larger opponent, which they are unlikely to defeat. The underlined strategy will be that it might be easier to assess an opponent's strength when it is of the same clawedness (Hyatt and Salmon, 1978).

With regard to vertebrates, Bradshaw and Rogers (1993) mention a significant bias to left-side lesions in whitefish attacked by lampreys, as in spiders and ants. A possible explanation could be that right-side muscles are stronger than left-side muscles in animals. It is interesting to point to similarities between general trends in vertebrates and the lateralization in spiders and ants. It could be argued that this could be simply chance, but in the case we would expect a Gaussian distribution of side use preference with half population showing a right-side use preference and half population showing a left-side use preference and not an alignment of the direction at the population level. Fish approach a predator more closely for inspection if it is on their left side (Bisazza et al., 1999); the spiders studied by Ades and Ramires (2002) and one of spider species studied by Heuts et al. (2003) similarly inspected a spider opponent by touching it with a left leg. Several fish species and toads tend to expose their left side to conspecifics because they inspect conspecifics with their left eye (Vallortigara et al., 1998; Bisazza et al., 1999); ants exposed their left side to nest-mates when resting and when keeping to the right on their foraging trails. Like various birds and fish that forage for food, the lone foraging *Lasius niger* ants also showed a significant right-turning bias (that might facilitate their keeping to the right when not alone on foraging trails).

Behavioural asymmetries (left-right, anterior-posterior) in arm use have been observed in octopuses, *Octopus vulgaris* (Byrne et al., 2006a). A preferential use of posterior arms for walking has been reported for this species (Mather, 1998) and, in their study, Byrne et al. (2006a) tested the limbs use of 8 animals, 7 of these had been involved in the eye preference study described above (see Byrne et al., 2004). A T-maze containing a food reward was placed in the tank so that the octopus could explore and retrieve the food from the T-maze by inserting only one arm at a time. Within each trial, the researchers scored (1) the contact arm used to first touch the T-maze, (2) the arm used in the first choice (i.e. that used to make a choice into the left or the right) and, the arm used in the second choice (used to search for additional food). The results showed a contact arm preference for frontal arms, while for the first and second choice arm 5 octopuses used frontal arms and 3 used first the frontal arms to touch and, then, the posterior ones to reach into the T-maze. Only 4 octopuses showed a significant preference at the first contact (2 left and 2 right); 2 right-eyed animals preferred the anterior right arm most (conventionally indicated as R1); 2 left-eyed preferred L2 arm. Thus, arm preference in octopuses is present at the individual level. However, in comparison with lateral bias in eye use (Byrne et al., 2004), in which 92% of the population showed a preference for a specific eye, lateral bias in arm use was not as strong (all seven octopuses used their favoured arm in 38.3 ± 5.7% of all cases). Five subjects were tested for both lateral eye and arm use preferences: four of them exhibited a preference for the arms on the same side of the body as the preferred eye. Byrne et al. (2004) argued that lateralized eye use might be based on a task division between the two hemispheres of the octopus brain for different behaviour. The experimental setup in the former study triggered a behavioural exploratory response to a prey-like object. In the latter study, the food item was visible to the octopuses through a transparent T-maze; thus the arm choice could have been influenced by the eye preference. This gives rise to the question of how strongly eye and arm choice influence each other. Byrne et al. (2006b) considered the visual-motor connections between eye and arm. They hypothesized that motor asymmetry may influence the choice of eye use or that lateralized eye use may influence arm preference. To investigate this plastic objects were positioned on three different levels in a water column and the octopuses were observed during approach, contact initiation and exploration of objects. None of the seven subjects showed a left/right bias for approaching the objects, independently of the position of the objects in the water column. A strong association was found between the direction of approach and the first arm used to touch the objects. The most commonly used arm was the one in a direct line between the eye used in looking at the object, and the object. In 99% of all cases, if the object was to the anterior of or above the octopus, the octopus used an anterior arm, if the object was to the posterior of or below the octopus, a posterior arm was used and, if the object was to the right (or left) of the octopus, a right (or left) arm was used. The results of this study indicate that octopuses most commonly use an arm to initiate contact with an object that is in a direct line between the eye used to look at the object and the object. Anatomically this is a logical solution, because it would be more complicated for an octopus to use an arm that is on the other side of its body to grab an object during monocular visual exploration. However, the researchers did not investigate whether the same eye and arm coordination occured in a more demanding task.

Behavioural asymmetries in mating behaviour have been reported in some invertebrate species. In a study conducted on the earwig *Labidura riparia* (Insecta: Dermaptera) by Kamimura (2006) and, in particular, on the males of this earwig species with two penises, nearly 90% of field-collected and laboratory-reared males hold their intromittent organs in the ‘right-ready’ state when not mating as well as when mating. However, not all earwig taxa have two penises, warranting more study of the earwig species. Looking at the phylogenetic relations in this species, Kamimura (2006) suggested an intriguing evolutionary scenario. Male earwigs evolved from a primitive state with both penises held in the ‘not-ready’ orientation when not mating, first through a stage where they always held one penis (either the right or left at random) in the ‘ready’ orientation. Males that still possessed two morphologically indistinguishable penises, but which preferentially held the right in the ‘ready’ orientation represented the next evolutionary step. Finally, the less-preferred (left) penis disappeared altogether, leaving only traces of a closed, non-functional ejaculatory duct. Thus, a purely behavioural asymmetry might have facilitated the evolution of a fully-blown morphological asymmetry.

This is a fine example of a phenotype-precedes-genotype mode of evolution because the right-ready and left-ready penis variants, which are equally common in evolutionary intermediates, and therefore probably not heritable (Palmer, 2004), clearly existed before the genetically captured right-ready phenotype seen in *L. riparia.*

A similar behavioural asymmetry in mating behaviour, due to an anatomical asymmetry dependent by a maternal effect gene, has been observed in the pond snails *Lymnaea stagnalis* (Asami et al., 2008; Davison et al., 2009). The pond snail *Lymnaea stagnalis* is a self-fertile hermaphrodite; in any single mating an individual takes the male role or the female role. Chirality in snails is determined by the single locus of maternal effect (Boycott and Diver, 1923; Sturtevant, 1923). This means that the phenotype of an individual is dependent upon the genotype of their mother. Asami et al. (2008) used crossing experiments to demonstrate that the primary asymmetry of *L. stagnalis* is determined by the maternal genotype at a single nuclear locus where the dextral allele is dominant to the sinistral allele. Dextral is dominant in *Lymnaea* (by convention, D = dextral allele; S = sinistral allele). The dextral and sinistral stocks are genetically DD or SS, respectively. Mating virgin sinistral and dextral, offspring that are either genetically dextral (genotype = DS) but with a shell coil that is either sinistral (sinistral mother) or dextral (dextral mother) can be produced (F1 generation). By allowing the sinistral F1 mother to self-fertilise, offspring were produced with a dextral coil, but which are genetically DD, DS or SS (F2 generation). Dextral SS individuals were identified by virtue of them producing sinistral young. Davison et al. (2009) investigated the occurrence and the inheritance of a potential laterality trait in the pond snail and tried to understand if it is associated with both body chirality and nervous system asymmetry. They found that all dextral “male” snails circled in a counter-clockwise manner, no matter if they were paired with another dextral or a sinistral snail. Similarly, all the sinistral snails, both those paired with dextral and those paired with sinistral, circled in a clockwise manner.

Chirality in mating behaviour is matched by an asymmetry in the brain. *L. stagnalis* has a ring of nine ganglia that form a Central Nervous System (CNS) around the oesophagus, with two more distant buccal ganglia on the buccal mass. In all dextral individuals, the right parietal ganglion was fused with the visceral ganglion, so that the left visceral ganglion was unpaired. By contrast, in all sinistral individuals, the reverse was observed; the left parietal ganglion was formed by fusion with a visceral ganglion. The central nervous system in sinistral pond snails, therefore, has an asymmetry that is reversed compared to that of dextral snails. As the coil of the shell is determined by the maternal chirality genotype and the asymmetry of the behaviour is in accordance with this, it is likely that the same genetic locus or, a closely linked gene determines the behaviour. These findings suggest that the lateralized behaviour of the snails is established early in development and is a direct consequence of the asymmetry of the body. Interestingly enough, in their study Davison et al. (2009) discovered a dextral individual with a typical “sinistral” brain. Although they were, unfortunately, not able to determine the asymmetry in the mating behaviour of this individual, the result is in line with studies in other animals, including humans, where it has been found that a variable proportion of individuals have a brain asymmetry that is not in the direction that might be expected based on their behaviour (McManus, 2002).

Evidence of a population level lateralized behaviour has been found in the giant water bugs, *Belostoma flumineum Say* (Heteroptera: Belostomatidae; Kight et al., 2008). The giant water bugs are large aquatic insects, predators of other aquatic invertebrates and small fishes. Bugs were trained to swim left or right in a T-maze and a significant preference to turn left, even when not reinforced, was observed. To control for environmental cues that might bias the turning direction of water bugs in the maze, the authors ran two separate experiments on independent groups of 20 water bugs. Both experiments were identical with the exception that, after the first group of 20 water bugs was tested, the maze apparatus was rotated 180° in the laboratory room, thus reversing the polarity of all directional environmental cues such as lighting or electromagnetic fields. Again the same left turn tendency was observed. Hence, the explanation of the presence of this bias could be the existence of asymmetries in the nervous system or asymmetric exoskeletal morphology (i.e. leg length) that could cause biased swimming behaviour.

An asymmetry in T-maze behaviour has been reported also in the cuttlefish (*Sepia officinalis*) trained to learn how to enter a dark and sandy compartment at the end of one arm of the maze (Alves et al., 2007). The study revealed that 11 out of the 15 cuttlefish displayed a pervasive side-turning preference. Interestingly, the side bias could be reliably detected in a single probe trial. It is unclear at present whether the asymmetry is motor or sensorial (preferential eye use) in origin. This study, and previous ones (Karson, 2003; Karson et al., 2003), used a sample size too small to establish whether lateralization was at the individual or population level. More recent work by Alves et al. (2009) in a large sample (N=107) has, however, confirmed the existence of a population-level bias; moreover, to find out whether or not visual perception plays a role in determining the direction of turning, cuttlefish were either tested inside the empty apparatus or with attractive visual stimuli (sand and shadow) on both sides of the T-maze apparatus. Alves et al. (2009) found a progressive postembryonic development of a bias to escape leftwards from three to 45 post-hatching days using either an empty apparatus or one in which sand and shadow were provided. In vertebrates, age-dependent biases depend on functional, neurochemical or morphological asymmetries between the left and right sides of the brain (Regolin and Vallortigara, 1996). In cuttlefish, changes in side-turning preference along development could be linked to an asymmetrical postembryonic maturation of the brain. The optic lobes are paired structures of the central nervous system in cuttlefish (Nixon and Young, 2003). It is within the outer cortex of these optic lobes that the major processing of visual information is believed to occur (Williamson and Chrachri, 2004). Axons run from the outer cortex towards a central medulla (Young, 1974) considered to be a visual-motor region. Electrical stimulation of the medulla evokes responses from many effectors of the body (Boycott, 1961; Sanders and Young, 1940), and induces ipsilateral rotation (Chichery and Chanelet, 1976). Alves et al. (2009) recently reported a correlation between side-turning bias and the size of the left and right optic lobes (OL) of cuttlefish. Authors reported a strong correlation at 30 post-hatching days between the laterality index (LI) and the ratio (volume of the Right Optic Lobe) / (volume of the Left Optic Lobe), with the ratio decreasing when the LI is increasing (Alves et al., 2009). Cuttlefish with a right turning preference possess a bigger left OL, and cuttlefish with a left turning preference possess a bigger right OL.

1.3. Asymmetries in the nervous system

In the fruitflies *Drosophila melanogaster* a previously unknown structure has been described, near the fan-shaped body, which connects the right and the left hemisphere (Heisenberg, 1994), i.e., an asymmetrical round body (AB) with a diameter of about 10 µm. In a sample of 2,550 wild-type flies, 92.4% of individuals were found to show the AB in the right hemisphere and the natural exceptions to this asymmetry constituted only 7.6% in the population (Pascual et al., 2004). Wild-type flies presenting symmetric structures were trained to associate an odour with an electric shock: a single training cycle for short term memory testing and five individual training sessions (15-min rest intervals) for long term memory testing. Pascual et al. (2004) observed no evidence of four-day long-term memory in wild-type flies with a symmetrical structure, although their short-term memory was intact. Thus, brain asymmetry is not required to establish short-term memory but it is important in the formation or retrieval of long-term memory in *Drosophila*. It is interesting to compare this finding with the evidence of lateralized memory recall in honeybees (Letzkus et al., 2006; Rogers and Vallortigara, 2008). The right antenna has been shown to be important in the learning of new odours (Letzkus et al., 2006; Rogers and Vallortigara, 2008) and in the recall of the short-term memory for such odours (Rogers and Vallortigara, 2008). On the other hand, the left antenna is important in the recall of long-term memory for odours (Rogers and Vallortigara, 2008). It has not been checked yet whether honeybee's brain has an asymmetrical structure located in one side of the brain as fruitfly does. However, studies comparing the possible difference in volume between the glomeruli in the right and left antennal lobes are carried out (Haase et al., 2010) to investigate the possible asymmetry from an anatomical point of view. Thus, it is difficult to generalize these findings saying that lateralization is important for memory formation in both insect species. In the fruitfly brain, an asymmetrical neural structure is coincident with the ability to form long-term memories (Pascual et al., 2004). The study in fruitflies documented the existence of a single, asymmetrically positioned brain structure that appears to be important in the formation of long-term memory and that enhances long-term memory recall. Anyway, that study does not demonstrate any functional asymmetry in behaviour. For honeybees, it appears that the time-dependent recall of odour memories is lateralized with the transition from shorter-term recall via the right antenna to long-term memory recall via the left antenna taking place at about 3 hours after training (Rogers and Vallortigara, 2008). Although similar neural structures might be involved in memory formation on the left and right sides, it seems that antennal inputs may access different neural circuits on the left and right sides.

Further studies on memory storage for odours have been recently conducted on the terrestrial slug *Limax* (Matsuo et al., 2010). For this species olfaction is the most important sensory modality and this slug is capable of acquiring odour-aversion memories, i.e. when presented with the odour of food in combination with an aversive odour, it avoids that food. It has been demonstrated in bilateral PC ablation experiments that the procerebrum (PC), a secondary olfactory centre of terrestrial molluscs, is necessary for this type of learning (Kasai et al., 2006), and that the PC is the memory storage site. On the other hand, it has been hypothesized that only the unilateral PC is used for odour-aversion learning. It has been shown (Matsuo et al., 2010) that, when the PC is surgically ablated only unilaterally before or after conditioning, approximately half of the slugs are unable to for an odour-aversion association, while the other half keep an intact memory performance. Furthermore, no difference in the memory performance between the right and the left PC-ablated slugs has been found. These findings support the hypothesis that only the PC on one side, randomly determined, is used for the storage of odour-aversion memory in *Limax*. Thus, the terrestrial slug does not show a lateralization at the population level for the storage memory side as both fruitflies (Pascual et al., 2004) and honeybees do (Rogers and Vallortigara, 2008).

Interestingly, no transfer of the unilaterally stored memory from one PC to the other, after up to 7 days post-conditioning, has been observed. The study by Matsuo et al. (2010) shows clearly that either the left or right PC is randomly used for olfactory learning, and that the side of use is determined at the level of the olfactory ascending pathway to the PC. Evidence is different than that found in honeybees (Sandoz and Menzel, 2001; Rogers and Vallortigara, 2008) showing that unilaterally acquired memory for odours is transferred to the other side of the brain, specifically from the right to the left side (Rogers and Vallortigara, 2008) with a time scale of 5-6 hours. Even if no transfer has been observed in the terrestrial slugs, it may be that odour-aversion memory in this species is gradually transferred on a longer time scale.

Another case of asymmetry in the nervous system is that of the snail *Helix aspersa* (Chase, 1986). In this species, morphological and physiological data revealed a right-sided bias in the mesocerebrum, which has a major role in the control of sexual behaviour. Thus, the right lobe has 23% more neurons than the left lobe, and the neurons are 24% larger. It has been observed that excitatory synaptic inputs derive predominately from neurons on the right side (Chase, 1986). Furthermore, the axons of right-side mesocerebral neurons and even those of left-side neurons travel mostly in right-side connective nerves. In the study (Chase, 1986) direct evidence for a role of the mesocerebrum in controlling sexual behaviour was provided through experiments with electrical stimulation. Thus, the asymmetry in the mesocerebrum seems consistent with the asymmetry observed in the sexual behaviour, which is executed almost entirely on the animal's right side.

The nematode *Caenorhabditis elegans* offers a unique opportunity to address how symmetrical neuronal assemblies deviate to create functional lateralization.

Hobert et al. (2002) provided a detailed cellular and molecular perspective on left-right (L–R) asymmetry in the nervous system of *C. elegans*. In this species, 2/3 of the neurons (198 out of a total of 302) are present as bilaterally symmetrical pairs of neurons. These neuron pairs (or neuroblasts) that are initially bilaterally symmetrical - by postmorphogenetic position, morphology and lineage - choose at some stage to execute a L–R asymmetrical programme of further differentiation, in terms of migratory pattern, axonal paths or gene expression patterns.

A case of L-R asymmetry deriving from previously symmetrical cells is the antisymmetry in odoursensory neurons. The AWC neuron class consists of two neurons, AWCL and AWCR, which are bilaterally symmetrical according to several distinct criteria (cell position, axon morphology, outgrowth and placement, dendritic morphology and pattern of synaptic connectivity) and are functionally symmetrical in that they express similar sets of genes and are both required for chemotaxis to specific odorants. Surprisingly, Hobert et al. (2002) found out that these two neurons show an asymmetrical pattern of expression of a putative G-proteincoupled olfactory receptor, encoded by the *str-2* gene. The expression of *str-2* is turned on in one of the two bilateral homologues during late embryogenesis and the induction of this gene occurs stochastically in either the left or the right cell. This is a case of individual asymmetry in which the sidedness is random in a population and, thus, is classified as ‘antisymmetry’. The antisymmetry of calcium-dependent odorant receptor expression in a bilaterally symmetrical *C. elegans* odour sensory neuron class is the best understood case of antisymmetry in the nervous system, in terms of functional relevance and genetic mechanisms.

In contrast, the taste receptor neurons in *C. elegans* are an example of ‘directional asymmetry’, i.e. biased forms of asymmetry. The ASEL/ASER neurons are the main taste receptors of *C. elegans*. ASEL and ASER are bilaterally symmetrical with regard to cell position, axon morphology, outgrowth and placement, dendritic morphology and qualitative aspects of synaptic connectivity patterns. However, three putative sensory receptors of the guanylyl cyclase class, *gcy-5*, *gcy-6* and *gcy-7*, are asymmetrically expressed in ASEL (*gcy-6*, *gcy-7*) and ASER (*gcy-5*), two to left and one to the right. This asymmetry of gene expression correlates with a significant functional asymmetry of the two neurons - laser-ablation studies revealed that each of the individual neurons is responsible for sensing a distinct class of water-soluble chemicals.

A genome wide analysis of all *gcy* genes has been undertaken (Ortiz et al., 2006) to examine the extent of lateralization of *gcy* gene expression patterns in the ASE neurons. The existence of a total of 27 *gcy* genes encoding receptor-type guanylyl cyclases and of 7 *gcy* genes encoding soluble guanylyl cyclases in the complete genome sequence of *C. elegans* has been reported. The receptor-type guanylyl cyclases has been found to be highly biased but not exclusively restricted to the nervous system: 41% (11/27) of all receptor-type guanylyl cyclases are expressed in the ASE gustatory neurons and one-third of all *gcy* genes (9/27) are expressed in a lateral, left/right asymmetric manner in the ASE neurons. A comparative study in the related nematode *C. briggsae* has been conducted (Ortiz et al., 2006) showing that the complement of *gcy* genes in *C. briggsae* differs from *C. elegans* in chromosomal localization, number of *gcy* genes, and expression patterns. Specifically, differences in *gcy* expression patterns in the ASE neurons of *C. briggsae* arise from a difference in cis-regulatory elements and trans-acting factors that control ASE laterality. Results (Ortiz et al., 2006) indicate the existence of a surprising multitude of putative chemoreceptors in the gustatory ASE neurons and suggest the existence of a substantial degree of laterality in gustatory signaling mechanisms in nematodes.

An additional asymmetry has been demonstrated in ASE chemosensory neurons (ASEL and ASER) of *C. elegans* (Suzuki et al., 2008). Optical recordings of calcium concentration in ASE neurons in intact animals showed that ASEL is a ON-cell, stimulated by increases in NaCl concentration, whereas ASER is an OFF-cell, stimulated by decreases in NaCl concentration, with both ASE neurons reporting changes n concentration rather than absolute levels. Moreover, the ON-OFF asymmetry is the result of intrinsic differences between ASE neurons and it extends to the level of behavioural output. Specifically, ASEL lengthens bouts of forward locomotion (runs) whereas ASER promotes direction changes (turns). The asymmetry of ASE neurons at the sensory level (ON-cell versus OFF-cell) and the behavioural level (runs versus turns) are precisely those of a simple yet novel neuronal motif for computing the time derivate of chemosensory information. This means that the net effect of ASE activation is a behavioural signal that approximates the time derivate of salt concentration. It is likely to think that the functional asymmetries of ASE neurons have emerged as a means of computing a quantity that is essential to chemotaxis in this organism. ASE asymmetry is established and maintained by a gene regulator network, whose complexity could be justified by the critical role of chemotaxis in the search of food and habitat.

Very recently, Ortiz et al. (2009) investigated the extent of functional lateralization of the ASE neurons and genes responsible for the left/right asymmetric activity of ASEL and ASER. They showed that a substantial number of salt ions are sensed in a left/right asymmetric manner and that lateralized responses to salt allow the worm to discriminate between distinct salt ions. Examining the chemotaxis behaviour of animals harbouring mutations in eight different receptor-type, transmembrane guanylyl cyclases (encoded by *gcy* genes), which are expressed in either ASEL (*gcy-6*, *gcy-7*, *gcy-14*), ASER (*gcy-1*, *gcy-4*, *gcy-5*, *gcy-22*), or ASEL and ASER (*gcy-19*), the authors identified the molecules involved in sensing salt ions and/or transmitting such sensory information. Disruption of a particular ASER-expressed *gcy* gene, *gcy-22*, results in a broad chemotaxis defect to nearly all salts sensed by ASER, as well as to a left/right asymmetrically sensed amino acid. In contrast, disruption of other *gcy* genes resulted in highly salt ion-specific chemosensory defects.

New insights into the evolution of body plans and left-right specification in Bilateria have been recently provided (Grande and Patel, 2009). The signalling molecule Nodal, a member of the transforming growth factor-β superfamily is used by the molecular pathway that leads to left-right asymmetry in vertebrates and in other dueterotomes, but no nodal orthologue has been reported in the two main clades of Bilateria: Ecdysozoa (including flies and nematodes) and Lophotrochozoa (including snails and annelids) before. Grande and Patel (2009) reported the first evidence for a nodal orthologue in a non-dueterostome group, indicating that the involvement of the Nodal pathway in left-right asymmetry might have been an ancestral feature of the Bilateria. Furthermore, this study suggests that nodal was present in the common ancestor of bilaterians and it too may have been expressed asymmetrically.

Very recently the cellular and molecular mechanisms that lead to neuronal asymmetries in the nematode *C. elegans* has been investigated and compared to the mechanisms involved in asymmetrical neural development in zebrafish *D. rerio* (Taylor et al., 2010). The specification of the left and right AWC neurons of the worm olfactory system and the asymmetry in the fish epithalamus has been analyzed. It has been shown that both these species use iterative cell-cell communication, i.e. reciprocal interactions rather than a simple linear pathway, to establish left-right neuronal identity and reinforce this left-right asymmetry but with different outcomes and molecular details. The functional differences in morphologically identical neurons in the olfactory system of *C. elegans* are the result of gap-junctional communication and calcium influxes; whereas the neuroanatomical left-right differences in the epithalamus of *D. rerio* are the result of morphogenic changes regulated by secreted signalling molecules. Although it is remarkable that the two species considered share some common arguments - the interaction of neurons across the midline during formation of the asymmetrical nervous system and the inherently stochastic nature of some developmental pathway - results need to be interpreted with caution since the evolutionary gap between the 302 neurons of the worm and the estimated 78,000 neurons of the larval fish (Hill et al., 2003) is considerable. However, even if the striking differences in the genetic and cellular pathways underline the improbability that nematode and zebrafish lateralization arose from the same ancestral event, because of the advantages conferred by asymmetrical neural systems it is likely to assume that the left-right differences in the two species have evolved convergently.

Very recently Kharchenko et al. (2010) show that learning involves synchronous and asymmetric serotonin-dependent mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) activation in identified neurons of the food-aversion network in the mollusc *Helix lucorum*. The signal transduction pathway MAPK/ERK plays an important role in the regulation of gene expression during memory formation both in vertebrates and invertebrates. In the mollusc *Helix lucorum*, serotonin induces activation of MAPK/ERK in the central nervous system (CNS) upon food aversion learning. Such learning depends on a neuronal network in which specialized neurons play distinct roles. Kharchenko et al. (2010) used serotonin application to the CNS in order to mimic learning and analysed the MAPK/ERK activation in single neurons of the food-aversion network, focusing both on command neurons, which mediate withdrawal behaviour and process information pertaining to the unconditioned stimulus, and on neurons of the procerebrum, the mollusc's olfactory centre, which process information from the conditioned stimulus. Results showed that after food aversion learning phos-pho-ERK levels increased significantly in RPa(2/3) command neurons of the right parietal ganglia and in the procerebrum. In contrast, no activation of MAPK/ERK was detected in similar conditions in the corresponding neurons of the left parietal ganglia LPa(2/3). This finding that learning involves synchronous and asymmetric serotonin-dependent MAPK/ERK activation may correspond to a lateralization of memory processes in the mollusc brain. It may be argued that as in nematodes (Hobert et al., 2002) in *Helix* the left and right neurons of avoidance behaviour can be asymmetric in the expression of some receptors connected to intracellular MAPK/ERK cascade and thus the asymmetry is associated with the ability of molluscs to discriminate in a tiny way odour or taste information. One more possible explanation of why should the memory trace be asymmetrical in these animals might be related to the developmental processes that build up gastropods, specifically gastropods are characterized by a visceral mass that has undergone a 180 rotation during development. A result of such torsion is the fact that snails develop an asymmetrical nature with the majority of growth occurring on the left or right side.

Chapter 2 – Theoretical approach

2.1. Introduction

In the previous chapter it has been shown how lateralization may confer several advantages at the individual level. One intriguing aspect of lateralization, however, cannot be explained by arguing that lateralized brains are more efficient. The direction of lateralization, in fact, is usually aligned at the population level, with 60–90% of individuals showing the same direction of bias (depending on species and behaviour considered, see Previc, 1991 and Vallortigara and Rogers, 2005). Individual efficiency does not require an alignment of lateralization at the population level, and does not explain why a minority of individuals lateralized in the other direction almost always exists (e.g. left-handedness in humans). One could argue that population-level lateralization is a mere by-product of genetic expression, but it has been proved that selection for the strength of lateralization does not necessarily favour one direction of lateralization over the other (e.g. Collins, 1985).

Two explanations (not mutually exclusive) have been proposed for the evolution of population-level asymmetries. Some genetic models of human handedness (McManus, 1999; Annett, 2002) posit one or more ‘directional’ (D) alleles that cause right-handedness, and one or more ‘chance’ (C) alleles that cause left- or right-handedness at random. A population with a majority of right-handers and a minority of left-handers can be maintained, in these models, if DC genotypes have higher fitness than CC and DD genotypes (heterozygotic advantage), for instance, if intermediate levels of brain asymmetry are superior to both extreme asymmetry and symmetry (Corballis, 2006). Suggested disadvantages of CC and

DD homozygotes include impairments in spatial, verbal and other cognitive abilities (Annett, 2002; Barnett and Corballis, 2002; McManus, 2002).

The second suggested explanation is that the population structure of lateralization may reflect, not a balance between symmetry and asymmetry, but an evolutionarily stable strategy that can arise when individually asymmetrical organisms must coordinate their behaviour with that of other asymmetrical organisms (Vallortigara and Rogers, 2005). This hypothesis recognizes that brain asymmetries manifest themselves in behaviour, and thus may have fitness consequences in interactions with other organisms. For instance, vigilance behaviour and escape responses elicited by predators often show lateral biases (Lippolis et al., 2002, 2005; Vallortigara and Rogers, 2005). This idea has been studied in a game-theoretical model considering group-living prey subjected to predation (Ghirlanda and Vallortigara, 2004; Vallortigara, 2006). Assuming first that lateralization influences the direction of escape from predators, two contrasting selection pressures on lateralization have been considered. On one hand, individuals in large groups have a lesser risk of being targeted by predators (the so-called ‘dilution’ of predation risk, Foster and Treherne, 1981). This favours individuals who tend to escape in the same direction as the majority, thus promoting the same direction of lateralization across the whole population. On the other hand, given that predators may learn to anticipate prey escape strategies, individuals who escape in a different direction from the majority may surprise predators and survive predation attempts more often. This tends to favour populations in which left- and right-lateralized individuals are equally common.

This model shows that population-level lateralization can emerge provided that none of the two selection pressures is much stronger than the other. According to this view, the evolution of brain lateralization would have occurred in two steps: first, individuals became lateralized because of advantages from increased brain efficiency (e.g. Rogers et al., 2004); and second, individually lateralized organisms aligned the direction of their asymmetries when they started to interact to each other in ways that made their asymmetry relevant to each other's behaviour (e.g. in fishes shoaling, Vallortigara and Bisazza, 2002). Here, I investigate whether a similar scenario could hold when selection pressures on lateralization arise purely from intraspecific interactions of competition and cooperation, rather than interspecific prey-predator interactions.

2.2. Model

I study the influence on lateralization of purely intraspecific interactions using a similar modelling strategy as that of Ghirlanda and Vallortigara (2004). I assume that individuals engage in both antagonistic (competitive) and synergistic (cooperative) interactions. An individual's pay-off depends on its success in interactions, which is a function of how common its lateralization is in the population. Synergistic activities tend to favour individuals with the same lateralization (they can, for instance, have an easier time coordinating physical activities, use efficiently the same tools, etc.). Antagonistic activities, on the other hand, tend to favour individuals different from the majority. The reason is similar to the one mentioned above for predation: minority-type individuals will be able to surprise opponents, adopting behaviours to which opponents are less accustomed. For example, it has been argued that human left-handers may hold an advantage in fighting, or in more recent times in certain sporting activities, but only so long as they remain in the minority (Raymond et al., 1996). Thus, if only synergistic interactions were present, the population would be composed entirely of individuals with the same lateralization. If only antagonistic interactions were present, the population would be composed of left- and right-lateralized individuals in proportion of one-half. I study whether, when both kinds of interactions exist, it is possible to maintain a population in which left- and right-lateralized individuals coexist in a proportion different from one-half, and how such a situation is influenced by model parameters.

Let x be the proportion of left-lateralized individuals in the population. The fitness f(x) of such an individual may be written as the sum of a term accounting for antagonistic interactions (a) and one-term accounting for synergistic interactions (s):

$$f(x) = a(x) + cs(x) \qquad (1)$$

where the parameter c weights the relative importance of the two kinds of interactions. I use the following forms for a(x) and s(x)

$$a(x) = \exp(-k_a x) \qquad (2)$$

$$s(x) = 1 - \exp(-k_s x) \qquad (3)$$

where k_a and k_s are positive parameters. In keeping with the assumptions done, a(x) decreases with the proportion of individuals with the same lateralization, while s(x) increases (Figure 1).

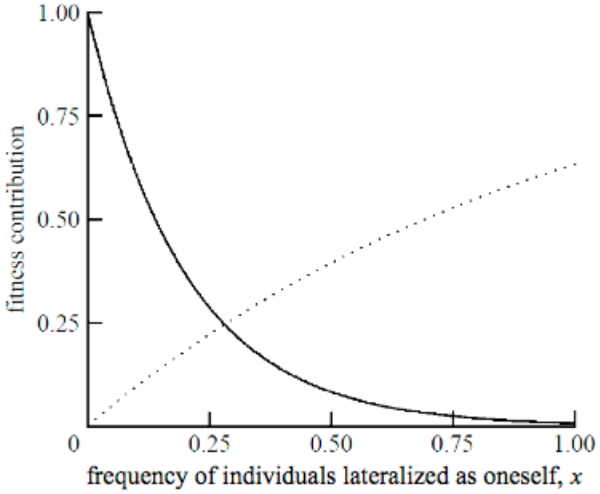


Figure 1. Graph of the functions a(x) and s(x) (equations (2) and (3)), which enter fitness (equation (1)). Parameter values: k_a=5, k_s=1. Since k_a>k_s=1, the fitness contribution of antagonistic interactions a(x) decays more quickly than the fitness contribution of synergistic interactions s(x) increases. Solid curve, antagonistic interactions, a(x); dotted curve, synergistic interactions, s(x).

I have chosen an exponential shape because it is often a good approximation to actual learning curves, that is, how performance on a given task increases as a function of increasing experience with that task (Mackintosh, 1974; Pearce, 1997). In Figure 1, parameter values are chosen so that k_a>k_s (in particular k_a=5, k_s=1). This means that a(x) decreases more rapidly than s(x) increases; i.e. the fitness contribution of antagonistic interactions varies more quickly with strategy frequency than the fitness contribution of synergistic interactions.

The fraction of right-lateralized individuals in the population is 1-x. Since any intrinsic advantage of being left- or right- lateralized is assumed, the fitness of these individuals is

$$f(1-x) = a(1-x) + cs(1-x) \qquad (4)$$

Evolutionary equilibria, x^*, are derived by equating the fitness of left- and right-lateralized individuals:

$$f(x^*) = f(1-x^*) \tag{5}$$

The evolutionary stability of an equilibrium x^* is assessed by asking what happens if the proportion of left-handers deviates slightly from x^*. If the equilibrium is stable, natural selection tends to restore the equilibrium proportion x^*. Thus, an increase in the proportion of left-lateralized individuals from x^* to x^*+E should result in a situation in which their fitness falls below that of right-lateralized individuals. Formally:

$$f(x^*+E) < f(1-x^*-E) \tag{6}$$

Likewise, a decrease in left-lateralized individuals should result in these individuals having a higher fitness:

$$f(x^*-E) > f(1-x^*+E) \tag{7}$$

In the appendix it is shown that these conditions are equivalent to the following condition on the derivative $f'(x)$ of $f(x)$:

$$f'(x^*) + f'(1-x^*) < 0 \tag{8}$$

Combining this condition with the equilibrium condition (6) it is possible to look for evolutionary equilibria and assess their stability. It is also needed to check whether populations composed entirely of left- or right-lateralized individuals are stable, corresponding to the conditions, respectively:

$$f(0) > f(1) \tag{9}$$

$$f(1) > f(0) \tag{10}$$

I have performed this analysis by a mixture of analytical and numerical methods, as detailed in appendix A.

2.3. Results

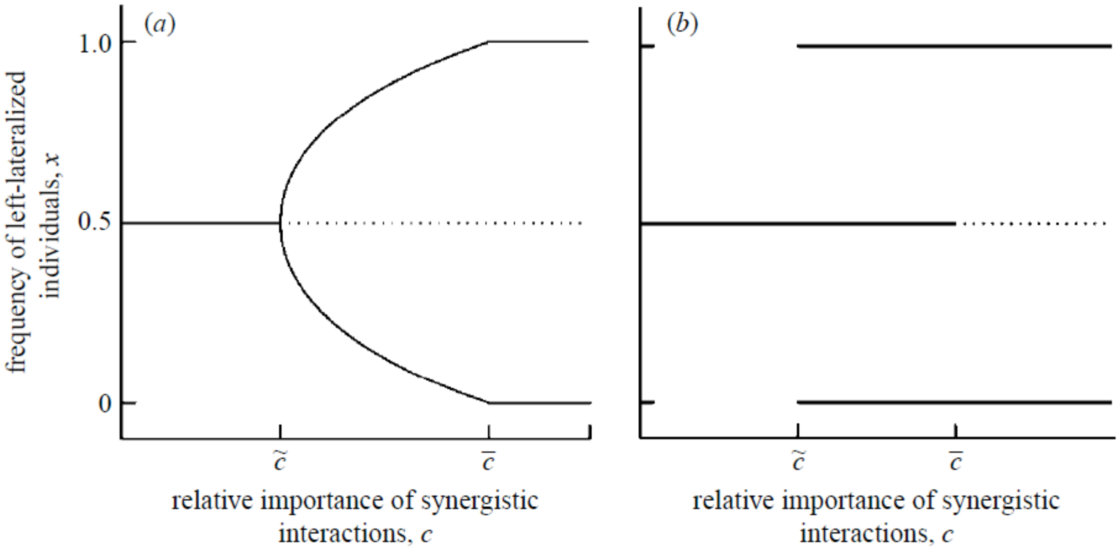


Figure 2. Equilibrium frequency of left-lateralized individuals x as a function of the relative importance of antagonistic and synergist interactions (parameter c in equation (1)). Solid lines represent stable equilibria; dotted lines unstable equilibria.

(a) The case in which frequency dependence is stronger for antagonistic than synergistic interactions ($k_a>k_s$ in equations (3) and (4)). For $c<\tilde{c}$ $\frac{a}{b}$, $x^*=½$ is the only stable solution; for $c>\bar{c}$, $x^*=½$ is unstable while $x^*=0$ and $x^*=1$ (populations with only right- or left-lateralized individuals) are both stable. Between $\tilde{c}$ and $\bar{c}$, two values of x different from one-half are stable equilibria, corresponding to populations in which left- and right-lateralized individuals coexist but are not equally common.

(b) The converse case ($k_s>k_a$). For $c<\tilde{c}$, $x^*=½$ is stable, for $c>\bar{c}$, $x^*=0$ and $x^*=1$ are stable, for intermediate c values $x^*=½$, $x^*=0$ and $x^*=1$ are all stable, meaning that coexistence between left- and right-handers is not possible.

I investigated the model varying the relative importance, c, of synergistic and antagonistic interactions. The nature of the equilibria depends on the relationship between the parameters k_a and k_s. If $k_a>k_s$ the situation is similar to that found by Ghirlanda and Vallortigara (2004) in interspecific prey-predator interactions (Figure 2). There exists a value of c, $\tilde{c}$, below which $x^*=½$ is the only stable solution. In such a situation, synergistic interactions are too weak to cause a departure from the strategic equilibrium favoured by antagonistic interactions. Similarly, there exists a value of c, $\bar{c}$, above which $x^*=½$ is unstable while $x^*=0$ and $x^*=1$ are both stable, corresponding to populations with only left- or right-lateralized individuals. Here synergistic interactions dominate, determining completely the population structure of lateralization.

Finally, there is a range of c-values in between $\tilde{c}$ and $\bar{c}$ where two values of x different from one-half are stable equilibria, corresponding to populations in which left- and right-lateralized individuals coexist but are not equally common. This is the situation we observe in humans and many other vertebrates. The range of c-values in which this situation occurs expands as k_a increases with respect to k_s.

If $k_a<k_s$, for small c, $x^*=½$ is stable and that for large c, $x^*=0$ and $x^*=1$ are stable, but the situation for intermediate c-values is different. Coexistence between left- and right-handers is not possible; rather $x^*=½$, $x^*=0$ and $x^*=1$ are all stable. In this case, synergistic interactions are strong enough to stabilize a monomorphic population, but not strong enough to destabilize a population with 50 per cent right- and left-handers. In summary, existence of lateralized populations requires in our model that $k_a>k_s$, meaning that the graph of a(x) must be steeper than the graph of s(x) (figure 1; see below for interpretations).

2.4. Discussion

The model demonstrates that populations consisting of left- and right-type individuals in unequal numbers can be evolutionary stable based solely on strategic factors arising from intraspecific interactions. The model makes several testable predictions. An important prediction is that the frequency of the minority type depends on the balance between the fitness contributions of antagonistic versus synergistic interactions. When antagonistic interactions are more important for individuals' fitness, the minority type is expected to be more common. Likewise, when synergistic interactions are more important, the minority type is expected to be less common. To evaluate this prediction, it is necessary to obtain data from populations that differ in the balance between antagonistic and synergistic interactions but are otherwise as similar as possible. One possibility is to compare different human groups. Faurie and Raymond (2005) provide data in agreement with the model, showing that the frequency of left-handers in eight traditional societies is strongly correlated with the rate of homicides, ranging approximately between 5 and 25 per cent as the adult homicide rate ranges between 0.01 to more than 1 per 1000 individuals per year. Another source of evidence may be comparative studies of related species. For instance, it is currently debated whether lateralization is more pronounced in humans than in non-human primates. This seems unlikely for cerebral lateralization in general (Vallortigara et al., 1999), but it could hold for certain forms of behavioural lateralization, such as handedness (Rogers, 2007; and see Andrew et al., 2000 for the general issue of lateralization of non-bilateral effectors). Wild chimpanzees show population-level handedness for tool use (Lonsdorf and Hopkins, 2005), but apparently not so strongly as humans do (Annett, 2006). The fact that synergistic interactions are more important in humans (e.g. Jensen et al., 2007) may explain why we are more strongly lateralized at the population level.

Another prediction concerns the fact that the model allows for population-level lateralization only when the condition $k_a>k_s$ is met (see §3 and appendix A). That is, when the frequency of majority- and minority-type individuals varies, the fitness contribution of antagonistic interactions should vary more quickly than the fitness contribution of synergistic interactions. From the point of view of selective pressures, this prediction means that minority-type individuals should lose their advantage in antagonistic interactions very quickly as they become more common, more quickly than they gain an advantage in synergistic interactions. From a behavioural point of view, this corresponds to the fact that individuals should learn quickly how to contrast minority-type individuals in antagonistic interactions, while they should learn more slowly how to cooperate with them in synergistic interactions. This prediction can be put to empirical test (perhaps in experimental populations in the laboratory), but presently, there is not any direct evidence in favour or against it.

In conclusion, I have extended previous results on interspecific interactions to intraspecific interactions, reinforcing the view that strategic factors may have been a powerful force in the evolution of lateralization. A purely strategic model has been considered for simplicity, but future research should also consider how strategic factors interact with other potential determinants of lateralization, such as neurophysiological constraints, the genetic mechanisms of lateralization and, especially in humans, traditions and culture (Laland et al. 1995).

APPENDIX A

A.1 Stability condition (equation (8))

A first order Taylor expansion of equation (6) yields:

$$f(x^*) + f'(x^*)E + O(E^2) < f(1-x^*) - f'(1-x^*)E + O(E^2) \qquad \text{(A 1)}$$

Dropping terms of higher order in E and using the equilibrium condition $f(x^*)=f(1-x^*)$ (equation (5)), equation (8) is obtained. A similar argument shows that equation (7) is also equivalent to equation (8). Given that $f(x)=a(x)+cs(x)$, equation (8) can be written as

$$a'(x^*) + cs'(x^*) + a'(1-x^*) + cs'(1-x^*) < 0 \qquad \text{(A 2)}$$

A.2 Stability of the equilibrium x*=½; (non-lateralized population)

The value x*=½ is always a solution of equation (5), hence it is always an equilibrium. Equation (A2), evaluated for x*=½, implies that this equilibrium is stable if c is smaller than

$$\tilde{c} = \frac{k_a e^{-(k_a/2)}}{k_s e^{-(k_s/2)}} \qquad \text{(A 3)}$$

A.3 Stability of x*=0 and x*=1 (completely lateralized populations)

Considering the situation where the population is composed entirely of left- or right-lateralized individuals, these situations are stable if equations (9) and (10) hold, respectively. Using expressions (1) and (2) both expressions hold if c is larger than

$$\bar{c} = \frac{1-e^{-k_a}}{1-e^{-k_s}} \qquad \text{(A 4)}$$

A.4 Existence of partially lateralized populations

It has been shown that a non-lateralized population (x*=½) is stable if c<$\tilde{c}$ and fully lateralized populations are stable only if c>$\bar{c}$. Thus, a lateralized population with 0<x*<½ or ½<x*<1 can be stable only if $\bar{c}$>$\tilde{c}$. Using expressions (A 3) and (A 4), the latter is equivalent to

$$\frac{1-e^{-k_a}}{1-e^{-k_s}} \qquad \text{(A 5)}$$

or

$$\frac{\sinh\left(k_a/2\right)}{k_a/2} > \frac{\sinh\left(k_s/2\right)}{k_s/2} \qquad \text{(A 6)}$$

which in turn is equivalent to

$$k_a > k_s \qquad \text{(A 7)}$$

because sinh(k)/k is monotonically increasing for k>0. When condition (A 7) holds, the interval of values between $\tilde{c}$ and $\bar{c}$ can be explored numerically to calculate the equilibrium value x*. This calculation was performed, for instance, to build Figure 2a. Two methods were used to guard against numerical instability. The first method used the fsolve function of the OCTAVE software (v. 2. 9. 9), designed to solve nonlinear equations. The second method looks for a solution by iterating the map

$$x' = -\ln\left[e^{-k_a(1-x)} + c\left(1-e^{-k_s(1-x)}\right) - c\left(1-e^{-k_s x}\right)\right]\Big/k_a \qquad \text{(A 8)}$$

obtained from equation (A 2) using equations (1) and (2). The fixed point theorem (Granas and Dugundji, 2003) guarantees that a solution for this recursion exists. The two methods typically produced the same answer, but for some parameter values one or the other method would not converge to a solution. I then used the value obtained by the other method.

Chapter 3 – Experimental approach

3.1. Introduction

Evidence of functional lateralization in invertebrates has begun to appear recently (see Chapter 1), suggesting that lateralization of the nervous system may be a common feature of brains with completely different organizing principles and evolutionary histories. As described in details in Chapter 1, honeybees readily learn to associate odours with reward (Proboscis Extension Reflex, PER; Bitterman et al., 1983) and they learn well when they use the right antenna but poorly when they use the left antenna (Letzkus et al., 2006). Moreover, at 1h after training, using both antennae, recall (of short-term memory) is possible only when the bee uses its right antenna but at 6 and 24h after training the (long-term) memory can be recalled only when the left antenna is in use (Rogers and Vallortigara, 2008).

The current knowledge of the genetics and evolution of brain asymmetry is imperfect (Corballis, 2009; McManus, 2002). However, as described in the previous chapter, a prominent hypothesis has been put forward, based on mathematical games theory modelling (Ghirlanda and Vallortigara, 2004; Ghirlanda et al., 2009), that selective pressures associated with social life might have been crucial in the evolution of lateralization (Vallortigara and Rogers, 2005). The crucial problem addressed by the hypothesis is the fact that, within a species, left- and right-type individuals are often in a proportion different from 50:50 (e.g., hand use in humans), i.e. population-level lateralization. The basic idea can be conveyed straightforwardly: behavioural lateralization as we know it in humans and other animals may have evolved under basically “social” selection pressures, i.e. when individually asymmetrical organisms must coordinate their behaviour with that of other asymmetrical organisms.

Thus, models based on Evolutionary Stable Strategy (ESS) predict that non-social species would be more likely to be lateralized at the individual rather than population level. However, this is difficult to prove empirically because vertebrates are usually quite social species and even current living solitary species certainly had social progenitors (e.g., Orangutans). On the other hand, invertebrates may provide a simple way of testing this hypothesis, because among Hymenoptera strictly related species have developed either into forms of sophisticated eusociality or maintained solitary behaviour. Thus, the first part of the experimental work described in this thesis will focus on the comparison of the lateralization through behavioural and electrophysiological studies in a social (*Apis mellifera*) and a non-social (*Osmia cornuta*) species of bee (Section 3.3; Anfora et al., 2010).

Rogers and Vallortigara (2008) speculated that a shift of memory access from the right to the left side of the brain might allow the right antenna to learn about new odours without interference from odour memories in long-term stores. Since bees visit different flowers at different times of the day, as nectar becomes available, the formation of different odour associations during the course of the day would be required, and this is a process that might be aided if recall of earlier odour memories is avoided on the side of the brain undergoing new learning.

Note, however, that the evidence of a lateral shift in the olfactory memory in honeybees was obtained using two unfamiliar odours (lemon and vanilla). I was interested in checking the generality of this finding using a variety of familiar and unfamiliar odours (Section 3.4, Frasnelli et al., 2010a).

The different sensitivity between the right and the left antenna has been justified by observing that the mean number of *Sensilla placodea*, which are the main type of olfactory receptor structures in honeybee (McIndoo, 1914; von Frisch, 1921; Lacher and Schneider; 1963; Kaissling and Renner; 1968), is significantly higher on the right than on the left antenna (Letzkus et al., 2006). However, this finding should be interpreted with caution since only one type of olfactory sensillum was considered, and other types of sensilla, such as *Sensilla trichodea* and *Sensilla basiconica*, may play an additional olfactory role, together with gustatory and mechanoreceptive functions, in bees (Snodgrass, 1935; Slifer and Sekhon, 1961; von Frisch, 1967; Dietz and Humphreys, 1971; Whitehead and Larser, 1976; Gupta, 1986; Ågren and Hallberg, 1996). Moreover, in the paper of Letzkus et al. (2006), SEM images did not cover the whole antennal segment surface leaving a hidden, non-characterized area. Finally, only 7 out of 10 left-right pairs of antenna were from the same individuals.

In the study described in details in Section 3.5. (Frasnelli et al., 2010b), I first duplicated the behavioural result using forager Italian honeybees (*A. mellifera ligustica Spin.*) and then I checked for anatomical differences in the number of sensilla between the right and the left antenna by using a larger sample of honeybees compared to the number of bees used by Letzkus et al. (2006). Morphological data were collected from bees harvested in 2 different hives (one in North Italy and another one in South Italy). Both antennae of each bee were imaged from four different views and all different types of sensilla were considered.

As last step I investigated the generality and possible phylogenetic origins of the antennal asymmetry in Apidae by testing primitive (stingless) social bees (Meliponinae), as described in details in Section 3.6. (Frasnelli et al., submitted). The study was performed in the field in Valla beach (NSW, Australia) on three species of Australian native, stingless bees (*Trigona carbonaria*, *Trigona hockingsi* and *Austroplebeia australis*). The bees were conditioned to associate an odour with a sugar reward, using the PER paradigm and the lateralization in the recall at 1 and 5 hours was investigated. Stingless bees are believed to be phylogenetically older than honeybees and thus they can provide important information on the evolution of lateralization in Apidae.

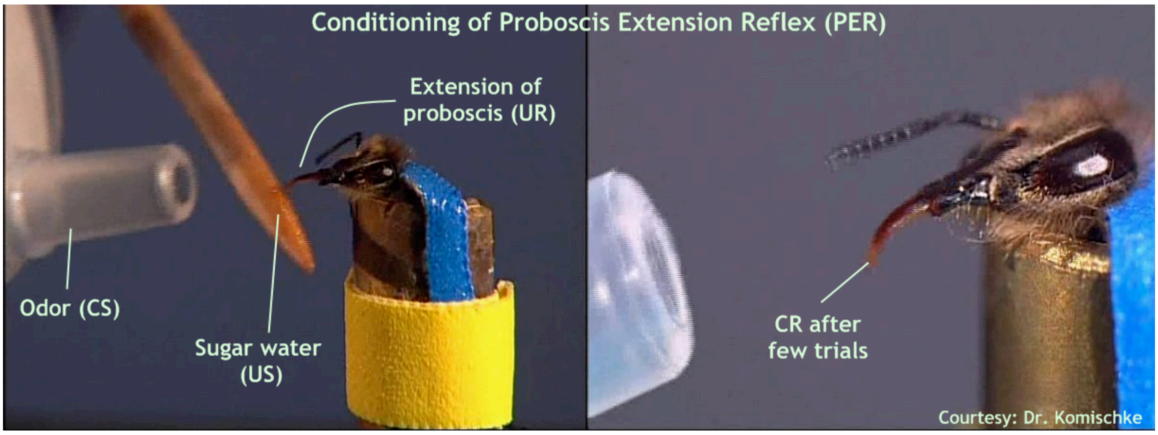
3.2. Materials and methods

In the studies presented in this thesis I investigated antennal asymmetries in honeybees and other related insect species. To do this I performed behavioural experiments using the Proboscis Extension Reflex (PER) paradigm (explained in details in the Section 3.2.1.), electrophysiological measurements with the Electroantennography (EAG) technique (explained in details in the Section 3.2.2.) and morphological investigations using the Scanning Electron Microscopy (SEM) instrumentation (explained in details in the Section 3.2.3.)

3.2.1. Proboscis Extension Reflex (PER) paradigm

Associative learning is a fundamental property of nervous systems governed by rules applicable both across species and across modalities. In classical conditioning (Pavlov, 1927), animals learn that an originally neutral stimulus (conditioned stimulus, CS) can act as a predictor for a biologically significant stimulus (unconditioned stimulus, US). In operant conditioning (Skinner, 1938), animals learn that a given response (operant action) is followed by the occurrence or suppression of a given reinforcement.

The honeybee, *Apis mellifera*, constitutes a traditional model for the study of learning and memory at the behavioural, cellular, and molecular level (Hammer and Menzel, 1995; Menzel and Giurfa, 2001). In a natural context, honeybees learn and memorize the local cues that characterize places of interest such as the hive and flowers in the surroundings. In the laboratory, the bees' learning abilities are mainly studied using a well-established paradigm: the olfactory conditioning of the proboscis extension reflex (PER; Bitterman et al., 1983). When the main chemosensory organs of a hungry bee, the antennae, are touched with sucrose solution, the insect reflexively extends its proboscis (PER) to reach out toward the sucrose and lick it.



Courtesy: Dr. Komischke

a) b)

Figure 3. Conditioning of Proboscis Extension Reflex (PER) paradigm in an immobilized and harnessed honeybee.

a) Training phase: the presentation of the odour (CS) and the sugar reward (US) elicits the extension of the proboscis (UR) and the bee learns the association between the CS and the US;

b) Testing phase: after few trails in the training phase the bee extends its proboscis when presented with the trained odour.

Bees immobilized in individual harnesses learn to associate an odour (conditioned stimulus or CS) with a reward of sucrose solution (unconditioned stimulus or US) delivered to their antennae and to the proboscis. Thus, they learn to extend their proboscis to the mere presentation of the odor (as shown in Figure 3). This effect is clearly associative and involves classical and not operant conditioning (Bitterman et al., 1983) as shown by the omission procedure, in which the occurrence of the conditioned response (the extension of the proboscis) prevents occurrence of the US (Bitterman et al., 1983). Bees trained in this way learn to respond to the odour despite the omission training, thus showing that the association learned is classical and not operant.

3.2.2. Electroantennography (EAG)

The electroantennography (EAG) is a technique that I used to investigate the olfactory response of insects species to odorants. Before explaining how this system operates, I would like to first describe, briefly, how the system of odour perception in insects works.

Olfactory perception starts at the level of the antennae where olfactory receptor neurons are located within specialized hairs called sensilla (Figure 4).

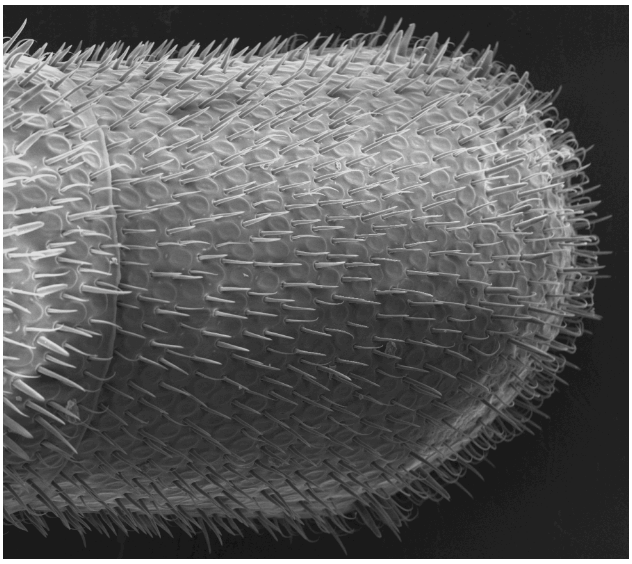
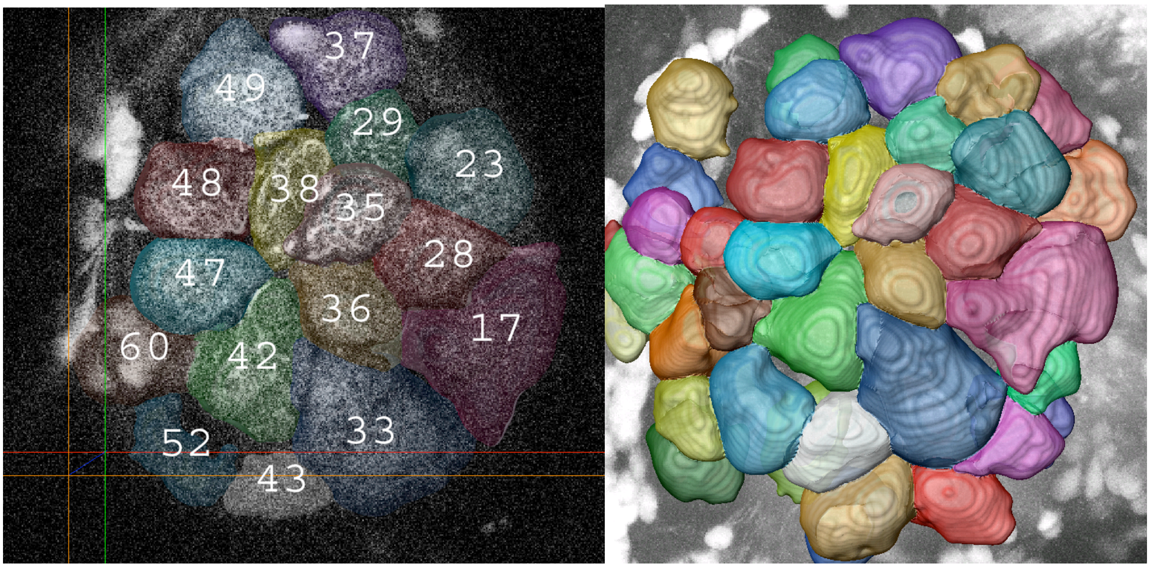


Figure 4. Image acquired with the Scanning Electron Microscope (SEM) of the apex of a honeybee forager's antenna. Note the sensilla covering the whole surface of the antenna.

Sensory neurons endowed with molecular olfactory receptors convey information on odorants to the antennal lobe. The antennal lobe of insects is the functional analogue of the olfactory bulb in mammals, and the first centre where information from the olfactory receptor neurons is processed. This paired structure is a good example of 'dedicated neuropile', i.e. brain regions devoted to the processing of specific sensory information (vision, olfaction, etc.), as it is the primary olfactory centre of the bee brain. Antennal lobes are constituted by globular structures called glomeruli (Figure 5). Glomeruli are synaptic interaction sites between olfactory receptors, local inhibitory interneurons connecting glomeruli laterally and projection neurons conveying processed olfactory information to higher order centers such as the lateral horn or the mushroom bodies. The latter are 'higher-order integration centers' as they receive input from visual and mechanosensory pathways apart from the olfactory pathway (Figure 6).

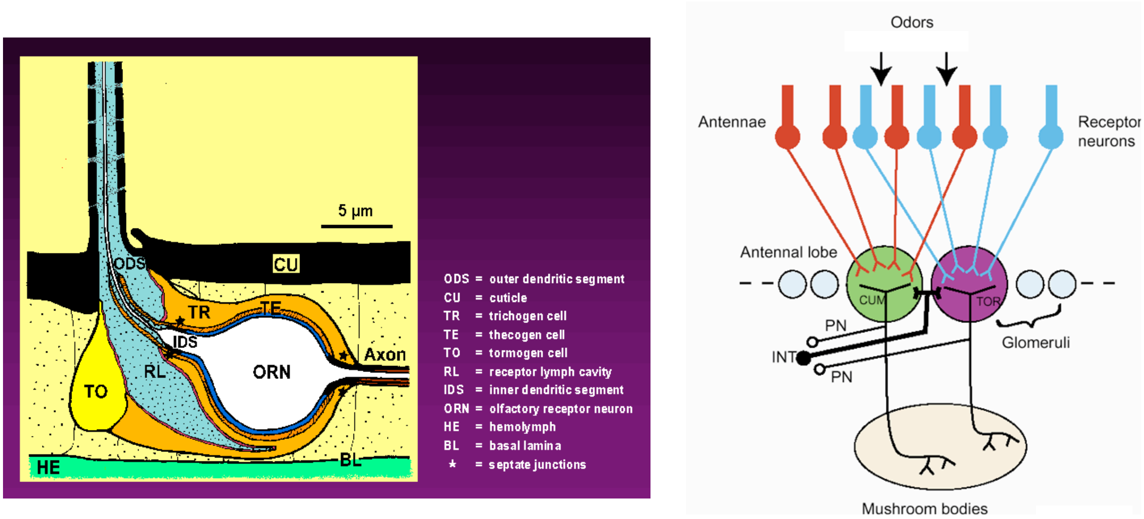


a) b)

Figure 5. The antennal lobe (AL) of a honeybee is composed by 160 glomeruli.

a) Image of the right antennal lobe of a honeybee acquired with a 2-photon microscope. The glomeruli are colored with different colours and enumerated;

b) 3D reconstruction of the antennal lobe starting from a series of images acquired with a 2-photon microscope.



a) b)

Figure 6. Olfactory system in honeybees.

a) The odour stimuli are captured by the olfactory receptor neurons through the pores on the antennal surface;

b) The olfactory receptor neurons (ORNs) on the antennae detect the odours and project to the glomeruli of the antennal lobes, where the incoming information is processed by local interneurons (INT) and then relayed by projection neurons (PN) to the mushroom bodies.

In particular, in the honeybee, on each antenna there are 60,000 ORNs (Esslen and Kaissling, 1976), which project to 160 glomeruli of the antennal lobe (Galizia et al., 1999). Here, the incoming information is processed by about 4000 local interneurons (Witthöft, 1967), and then relayed to higher order brain centres, the mushroom bodies, by 800 projection neurons (Hammer, 1997).

The EAG (Schneider, 1957) is a technique to study the olfactory response of insects to odorants and shows the sensitivity of the insect olfactory system to a specific substance. It measures electrical signals over a section of the antenna, so it cannot tell us which receptors are binding. The antenna is mounted on two glass capillary electrodes filled up with electrophysiological solution and then is stimulated with puffing different odours (Figure 7). The electric response measured is the sum of the depolarization potentials (mV) of the antennal olfactory receptor neurons.

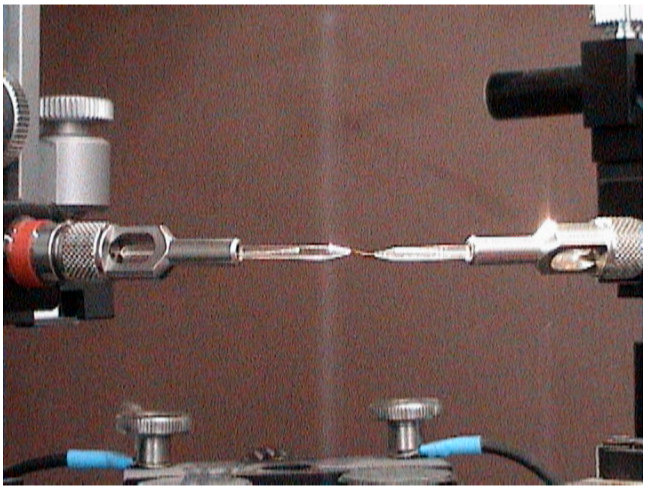


Figure 7. Electroantennography (EAG).

3.2.3. Scanning Electron Microscopy (SEM)

Figure 8. Scanning Electron Microscope (SEM) FEI XL 30 ESEM FEG in the Department of Medicine Laboratory, APSS, Trento.

The scanning electron microscope (SEM) is a type of electron microscope that images the sample surface by scanning it with a high-energy beam of electrons in a raster scan pattern. The electrons interact with the atoms that make up the sample producing signals that contain information about the sample's surface topography, composition and other properties such as electrical conductivity. The signals result from interactions of the electron beam with atoms at or near the surface of the sample. The SEM can produce very high-resolution images of a sample surface, revealing details about less than 1 to 5 nm in size. Due to the very narrow electron beam, SEM micrographs have a large depth of field yielding a characteristic three-dimensional appearance useful for understanding the surface structure of a sample.

In a typical SEM, an electron beam is thermionically emitted from an electron gun fitted with a tungsten filament cathode. Tungsten is normally used in thermionic electron guns because it has the highest melting point and lowest vapor pressure of all metals, thereby allowing it to be heated for electron emission, and because of its low cost. In the SEM FEI XL 30 ESEM FEG I used for this study (Figure 8), the source is a field emission gun (FEG) of the cold-cathode type using tungsten single crystal emitters.

The electron beam, with an energy ranging from 0.5 keV to 40 keV, is focused by one or two condenser lenses to a spot about 0.4 nm to 5 nm in diameter. The beam passes through pairs of scanning coils or pairs of deflector plates in the electron column, typically in the final lens, which deflect the beam in the x and y axes so that it scans in a raster fashion over a rectangular area of the sample surface. In the sample chamber of the SEM there is a stage, where the samples are positioned, that can be tilted in the case of the instrument used in this study until 75 degrees, allowing the sample observation from different view avoiding the chamber access (Figure 9).

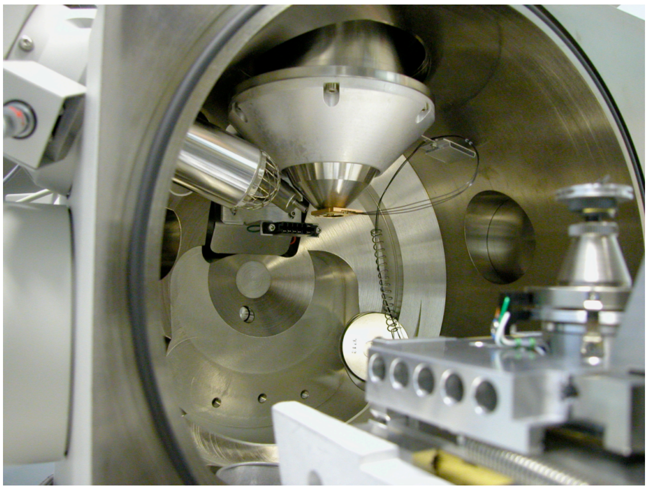


Figure 9. Opened sample chamber of the SEM in the Department of Medicine Laboratory, APSS, Trento.

For SEM, a specimen is normally required to be completely dry, since the specimen chamber is at high vacuum. The effect of the high vacuum on a biological sample not properly prepared corresponds in a loss of the fine structures of cells and tissues, determined by a mechanical damage caused by the violent evaporation of the water contained in the tissues.

In this study the samples are the antennae of honeybees and other insect species that are already quite dried. Thus, the samples were mounted on a specimen stub using an electrically conductive double-sided adhesive tape, and sputter coated with gold before examination in the microscope. Gold has a high atomic number and sputter coating with gold produces high topographic contrast and resolution. However, the coating has a thickness of a few nanometers, and can obscure the underlying fine detail of the specimen at very high magnification. Thus, we used a proper thickness of gold coating (see Section 3.5.1. for further details).

3.3. Experiment 1: Behavioural and electrophysiological lateralization in a social (*Apis mellifera*) but not in a non-social (*Osmia cornuta*) species of bee.

I tested behavioural and electrophysiological lateralization of olfactory responses in two species of the superfamily Apoidea, the social honeybee, *Apis mellifera L.* (Fam. Apidae), and the solitary mason bee, *Osmia cornuta* (Latreille) (Fam. Megachilidae). The common name mason bees derives from their habit of making compartments of thick mud walls in their nests, made in hollow reeds or holes in wood built by wood boring insects. Unlike honeybees, mason bees are solitary: every female is fertile and makes its own separate nest; they do not produce honey or wax and there are no worker bees in these species. Males leave their maternal nest before the females, and compete for mating waiting for the females at the nest entrance. Mating occurs immediately after females leave the original nest, then the females spend two-three days in feeding on flowers before starting the nesting activity (Nepi et al., 2005). Females visit flowers to gather pollen and nectar, and it takes many trips to complete a pollen/nectar provision mass. Once a provision mass is complete, the bee backs into the hole and lays an egg on top of the mass.

3.3.1. Materials and methods

3.3.1.1. Subjects

Forager Italian honeybees, *A. mellifera ligustica* Spin., were collected next to three different outdoor hives during summer 2008 at San Michele all’Adige (Trento, Italy). Female adult mason bees were obtained from over-wintering cocoons collected at Crevalcore (Bologna, Italy) during spring 2009.

3.3.1.2. Behaviour

Bees (*A. mellifera* and *O. cornuta*) were cooled in 750ml containers until immobilised and then secured in holders (Bitterman et al., 1983). They were assigned randomly to groups for the occlusion of one antenna and, 1 hour later, all bees were trained in the same way. For both species, the experiment was carried out on three groups. The bees in one group (N=6 honeybees; N=6 mason bees) had their left antenna coated with a silicone compound (Silagum-Mono, DMG, Germany); those in the second group ((N=6 honeybees; N=6 mason bees) had their right antenna coated, while both the antennae of the bees belonging to the third group were left uncoated (N=6 honeybees; N=5 mason bees).

One hour after the antennae had been coated, each bee in its holder was placed in front of an exhaust fan and trained using β-citronellol (Fluka, Buchs, Switzerland; purity>95%) plus 1M sucrose solution as the positive stimulus (10ul of β-citronellol dissolved in 3 ml of the sucrose solution). The negative stimulus was a saturated salt (NaCl) solution. Three trials spaced 6 min apart were given. On the first trial a droplet of the β-citronellol sugar solution at the end of a 23 gauge needle was held over the bee’s antennae at 1 cm from the antennae and after 5 s the antennae were touched, which led to PER. The bee was then allowed to ingest the drop of β-citronellol sugar solution. The procedure was repeated with the saline solution (trial 2), which did not trigger PER but avoidance by moving the antennae away from the droplet. On trial 3 the procedure of trial 1 was repeated and usually PER occurred without the need to touch the antennae.

Retention was tested 1 hour later by presenting β-citronellol or saturated salt solution in distilled water and holding the droplet 1 cm from the antennae while moving it slightly but being sure not to touch the antennae. The solutions were presented for 5 s alternatively. Each bee was tested in a total of 10 such paired trials, presented in random order and separated by an interval of approximately 60 s. We recorded every time the bee extended the proboscis. The percentages of the correct response (number of proboscis extensions to the β-citronellol over the number of total extensions to both β-citronellol and salt solution) were calculated. Before analysis, the percentage values were arcsin transformed (see Sokal and Rohlf, 1995), and after checking for normality and homogeneity of variances, data were analyzed by analysis of variance with antenna in use and species as between-subject factors.

3.3.1.3. Electroantennography (EAG)

An EAG technique (see Schmidt et al., 2007) involving a standard EAG apparatus (Syntech, Hilversum, The Nederlands) was used. EAG absolute responses (mV) were recorded from honeybee foragers (N=16) and from mason bee females (N=21). The antenna was cut at the level of the scape, which was then inserted into the glass reference electrode filled with Kaissling saline solution. The recording electrode was brought into contact with the last segment of the flagellum from which the distal tip had been cut. For each individual, responses from both the right and the left antenna were obtained and the antenna tested first was chosen at random. The bees were maintained alive between the two recordings.

Five hexane solutions of either isoamyl acetate (Sigma Aldrich, Milano, Italy; >99.7% purity), component of the honeybee alarm pheromone (Boch et al., 1962), or

(-)-linalool (Sigma Aldrich, >98.5% purity), a common floral odour, were prepared, at concentrations ranging from 10^{-2} to 10^{2} µg/µl. Aliquots of the test solutions (25 µl each) were adsorbed on pieces of filter paper (1 cm^2), which were inserted into individual Pasteur pipettes and applied in ascending order at 60 s intervals, after allowing the solvent to evaporate. Stimuli were added to the airstream by injecting vapour from an odour pipette. The dorsal region of the antenna, which has the highest number of placodea olfactory sensilla, was exposed perpendicularly to the airflow direction. A stimulus controller (CS-55, Syntech) was used to steer the procedure. Before and after presenting each series of stimuli, antennae were stimulated with a pipette loaded with 25 µl of hexane and with an empty pipette as controls. Fresh cartridges were prepared before each bee was tested. Data were analyzed by analysis of variance with antenna, scent and dose as within-subject factors.

3.3.2. Results

The results of the behavioural tests are shown in Figure 10. The analysis of variance revealed a significant main effect of the antenna in use ($F_{2,29}=8.69$, $p<0.001$) and a significant antenna x species interaction ($F_{2,29}=11.94$, $p<0.001$). There was a significant asymmetry in honeybees ($F_{2,15}=68.26$, $p<0.001$), with very poor recall in bees with only their left antenna in use, whereas no asymmetry was apparent in the mason bees ($F_{2,14}=0.15$, $p=0.86$).

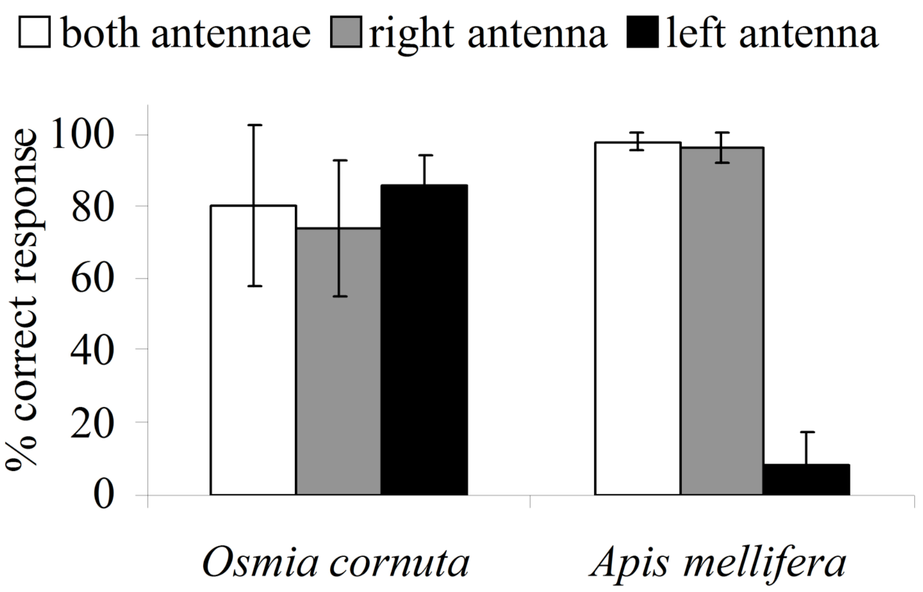


Figure 10. Mean percent ± SEM correct responses in recall of olfactory memory 1 hour after training to associate odours with sugar rewards in honeybees and mason bees tested with both antennae or with only one antenna in use.

The results for electroantennography revealed a similar pattern (Figure 11). In honeybees, the EAG responses elicited by both tested compounds on the right antenna were significantly higher than those on the left one ($F_{1,15}=5.12$, $p<0.050$), whereas there were no significant differences in mason bees. ($F_{1,19}=0.64$, $p=0.80$). Interestingly, though not lateralized at the population level, 15 out of 21 ($\chi^2=3.05$, $p<0.05$) individual mason bees showed significantly stronger responses (estimated by one tailed binomial test, $p<0.05$) either with the right (7 individuals) or the left (8 individuals) antenna.

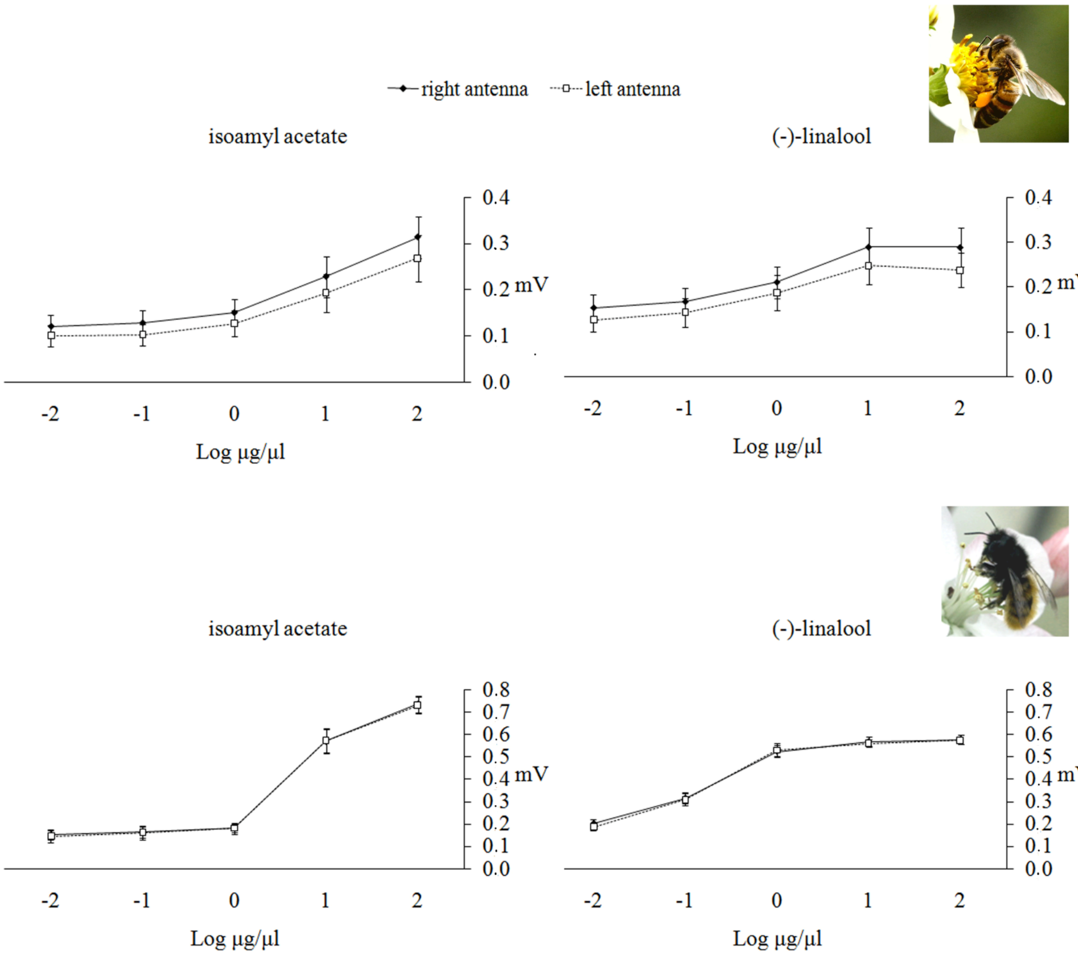


Figure 11. Mean EAG ± SEM responses (mV) of right and left antenna of *Apis mellifera* foragers (upper graphs) and *Osmia cornuta* females (lower graphs) to isoamyl acetate and (-)-linalool at 5 different doses. The dotted lines with empty squares show responses of the left antenna, whereas unbroken lines with black squares of the right antenna. X-axes indicate dose (Log10 µg/µl), y-axes indicate EAG response (mV).

As expected, both species showed a significant increase in their EAG responses with increasing doses of isoamyl acetate and (-)-linalool (*A. mellifera*: $F_{4,60}=43.48$, $p<0.001$) (*O. cornuta*: $F_{4,76}=407.11$, $p<0.001$).

The analysis also revealed significant main effects of the type of odour for *O. cornuta* ($F_{1,19}=49.94$, $p<0.001$) but not for *A. mellifera* ($F_{1,15}=3.10$, $p=0.10$). Significant interactions emerged only between type of odour and dose (*A. mellifera*:

$F_{4,60}=5.86$, $p<0.001$) (*O. cornuta*: $F_{4,76}=77.41$, $p<0.001$). No responses were obtained in the control tests.

Results thus showed stronger PER recall and EAG responsiveness in the right than in the left antenna in the honeybees but not in the mason bees.

3.3.3. Discussion

Results duplicate and extend the findings of Letzkus et al. (2006) and Rogers and Vallortigara (2008) showing a striking right antenna advantage in recall of olfactory memory using PER in honeybees. EAG results suggest that the asymmetry may arise, in part, from different responsiveness of olfactory receptor neurons (see also Letzkus, 2007).

In contrast, mason bees, which are related phylogenetically to honeybees, showed no evidence of being asymmetric at the population level, either in behaviour or in EAG responsiveness. Of course, differences between just two species should be interpreted with caution. Nonetheless the present results confirm that left-right asymmetries at the population level are apparent in some but not all invertebrate species, and not even in species that are strictly related phylogenetically. (Note also that the neuroanatomical characteristics of the mushroom bodies associated with learning during foraging seem to be present in both honeybees and solitary bee species, though with different development paths – Withers et al., 2007).

Mathematical models of the evolution of lateralization suggest that stable polymorphism with an uneven distribution of left- and right- forms can be expected to emerge spontaneously in species in which left-right biases have behavioural consequences during everyday interactions between individuals (Vallortigara, 2006).

The fact that mason bees show evidence of individual-level lateralization in EAG responses (the between-subject design used in the PER study prevented such an analysis for behavioural data) but not population-level (directional) lateralization fits nicely with mathematical modelling (see Ghirlanda and Vallortigara, 2004).

The behavioural traits studied here (olfactory learning and electroantennographic responsivity) are not obviously social in nature (although it is not possible to exclude that the original drive for antennal asymmetries could be related to social interaction during e.g. trophallaxis). It is conceivable, however, that some forms of asymmetries that are unlikely to have been directly selected as evolutionarily stable strategies (ESSs, see Chapter 1 and 2) in social contexts could have evolved as population-level biases as by-product of other biases that in fact evolved as ESSs. It is likely that when an individual-level asymmetry is stabilized as a directional (population-level) asymmetry, other asymmetries that in principle would not require any alignment at the population level because irrelevant to any social interaction would organize themselves as directional as well simply because a directional organization in the two sides of the brain already exists.

Little is known about side biases in invertebrates observed in their natural behaviour in the wild. Interestingly, however, Kells and Goulson (2001) have reported that primitively eusocial bumble bees, *Bombus spp.,* show preferred directions of circling as they visit florets arranged in circles around a vertical inflorescence. Since two species circled anticlockwise and one clockwise, it is unlikely that the asymmetry is a function of the structure of the florets. Comparative research with several species of Hymenoptera both in the field and in the laboratory may thus provide important insights on the evolution of left-right asymmetries in behaviour and in the nervous system.

3.4. Experiment 2: Response competition associated with right–left antennal asymmetries of new and old olfactory memory traces in honeybees.

This part of the research has been performed at the Centre for Neuroscience and Animal Behaviour at the University of New England (Armidale, NSW, Australia). The study is divided into three experiments that I will enumerate as A, B and C. In Experiment A, I investigated whether the right to left antenna shift occurs for recall of memories of a range of odours, using both simple (cineol and eugenol) and compound (lemon, rose and vanilla) odours. I found an unexpected loss of memory specificity in recall of long-term memory by bees trained with lemon (+) and rose (-). I hypothesized that the phenomenon could be due to the familiarity of rose odour, assuming that the bees tested had visited roses before capture or that they had been exposed to this odour in the hive. Hence, the training with rose as the negative stimulus might have involved reversal learning of an odour previously established in memory as a positive stimulus.

In Experiment B I used the major component of the alarm pheromone, isoamyl acetate (IAA), to check whether familiarity (as established biologically rather than by learning during the course of ontogenesis) might affect lateralized memory consolidation. Since IAA stimulates alarm and attack I considered it to be a negative stimulus. I observed a suppression of the response from both the right and the left side of the stimuli presentation at 1 hour after training, while at 6 hours bees responded to both odours from both sides.

In Experiment B I tried a direct test of the effects of reversal learning and familiarity. I trained a group of honeybees with lemon(+)/vanilla (-). After 16 hours bees were re-trained under reverse contingencies, lemon(-)/vanilla(+), and tested at either 1 hour or 6 hours after the reverse training session.

3.4.1. Materials and methods

3.4.1.1. Subjects

Experiments were performed in Armidale (NSW; Australia). Feral honeybees, which in Australia are a mix of European subspecies, were captured when they were foraging on banksia (*Banksia marginata*) or Hakea (*Hakea decurrens*) flowers at about 0.900h and cooled in 700ml containers in the refrigerator (5^0C) until immobilised. Then they were harnessed in metal holders, using the method of Bitterman et al. (1983), as modified by Rogers and Vallortigara (2008), by adding a piece of paper to cover the thorax of the bee so that the bee was not damaged by the adhesive tape and could be released after the test.

Three experiments on seven groups of 20 bees each (total of 140 bees) were performed. Each group was trained using different odour pairs (Table 1). The concentrations of lemon and rose were 10µl of the scent essence (Queen Fine Food Pty Ltd) dissolved in 3ml of 1M sucrose solution or saturated saline solution. Two concentrations of isoamyl acetate (IAA) were used: (1) high concentration (concentrated), 10µl of 98% IAA, Aldrich Chemical Company, in 3ml of 1M sucrose and (2) low concentration (dilute), dilution of the high concentration 1:150 (IAA odour still easily detected by humans). The pure odours used were cineol (99% Alrich Chemical Company) 1µl in 5ml 1M sucrose and eugenol (99% Alrich Chemical Company) 1µl in saturated saline.

EXPERIMENT NUMBER	GROUP NUMBER	POSITIVE STIMULUS (+) FOR THE RECALL TEST	NEGATIVE STIMULUS (-) FOR THE RECALL TEST
A	1	LEMON	VANILLA
	2	CINEOL	EUGENOL
	3	LEMON	ROSE
B	4	CONCENTRATED IAA	ROSE
	5	DILUTE IAA	ROSE
	6	DILUTE IAA	VANILLA
C	7	VANILLA	LEMON

Table 1. – A schematic summary of the experiments. Three experiments were performed on a total of seven groups of 20 bees each (total of 140 bees). Each group was trained using different odour pairs as positive (+) and negative (-) stimuli. Bees in group 7 were trained twice (the second time in a reverse way). The positive (+) and negative (-) stimuli in the table refer to positive (+) and negative (-) stimuli used in the recall test. IAA = isoamyl acetate.

3.4.1.2. Experimental procedures

One hour after the bees were placed in the holders training commenced. The harnessed bee was placed in front of an exhaust fan and then trained using different odours as positive and negative stimuli (Table 1). The positive stimulus was the odour plus 1M sucrose solution, the negative stimulus was the other odour plus saturated saline. The bee was trained with both antennae in use. Positive and negative stimuli were presented as a drop of the solution at the end of a 23-gauge needle held over both of the bee’s antennae at 1 cm from the antennae. Three trials were given at 6-min. intervals. In the first trial, after holding the droplet of odour in sucrose solution over the antennae for 5 seconds the antennae were touched with the droplet to trigger the proboscis extension response (PER). The bee ingested the drop and immediately after was presented with the negative stimulus. Once again the droplet was held over the antennae for 5 sec. and then both antennae were touched with the solution. After 6 min, a second trial was performed and the bee usually responded to the positive stimulus with a PER without the need to touch the antennae with the droplet. After another 6 minutes bees were given the third and last trial and then placed in a forced-draught incubator at 25^0C.

Bees trained with each odour pair were tested either at 1 hour or at 6 hours after the completion of training; i.e. 10 bees tested per group at each time after training. During the interval between training and testing, the bees were kept in an incubator at 26° C and 60% humidity. We used the method of Rogers and Vallortigara (2008) to test for laterality of responding. It involved presenting both odours dissolved in distilled water at the same concentrations as used in training. Droplets of these solutions were held on the left or right side of the bee, out of reach of the antennae or proboscis at approximately 1 cm from the bee. Each odour was followed immediately by the other odour and 10 presentations of each pair of odours were made on each side of the bee. Side of presentation was random. The odours were presented for 5 sec. and whether or not PER occurred was noted. Referring to group 1 in Table 1 (lemon as positive stimulus and vanilla as the negative stimulus), the responses were scored as follows: A, extension of the proboscis to lemon and no extension to vanilla; B, extension of the proboscis to both lemon and vanilla; C, extension of the proboscis to vanilla but not lemon; D, no extension of the proboscis to lemon or to vanilla.

A seventh group of 20 bees was captured, harnessed and trained with lemon in sucrose and vanilla in saline at approximately 15.00h. Following training these bees were placed in the incubator overnight to allow time for memory consolidation (Menzel, 1999; Gerber and Menzel, 2000). After 16 hours they were trained again with the reverse condition, lemon in saline and vanilla in sucrose. They were then returned to the incubator until testing at either 1 hour or 6 hours after the reverse training session.

3.4.1.3. Statistical analyses

For each group of bees and for each side (right and left) the mean number of A responses and the mean number of B responses with the respective standard errors (SE) were calculated. Results are shown in Figure 12. C and D responses were too rare to warrant inclusion in the analysis.

Data were analysed by repeated-measurement analysis of variance (Anova) for each group of bees with ‘antenna’ as the within-subjects factor. Comparison between the different groups was performed with ‘odour’ as between-subjects factor.

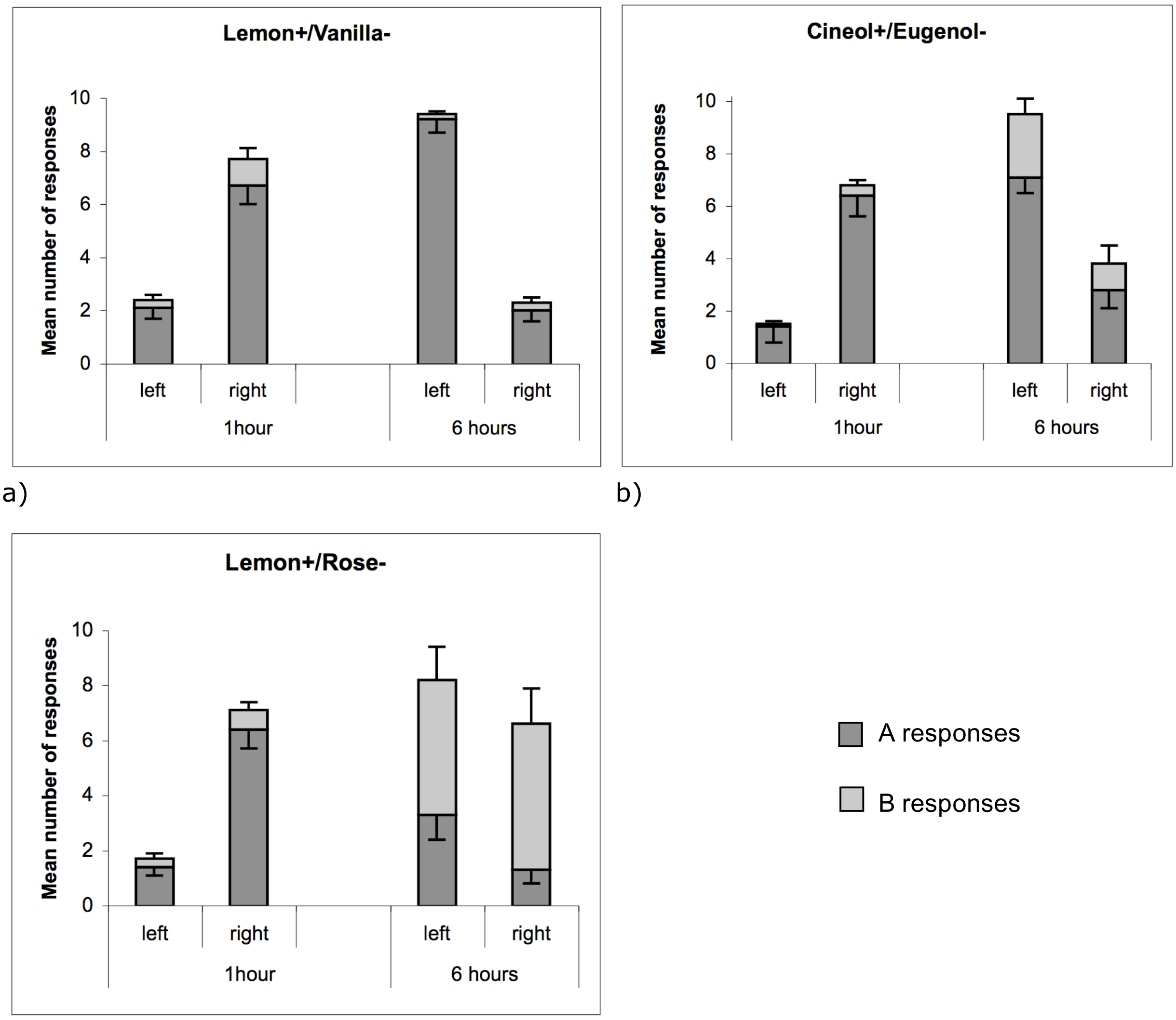
3.4.2. Results

3.4.2.1. Experiment A: Does the right to left antenna shift occur for recall of memories of a range of odours?

Results of recall of lemon (+) and vanilla (-) replicated those found by Rogers and Vallortigara (2008), showing a significant difference in A responses between the right and the left antenna at both 1 hour and 6 hours after training (Figure 12a). On recall at 1 hour the bees responded significantly better when tested on the right side than when tested on the left side (F(1,9)=103.500; p=0.0001); whereas, on recall at 6 hours, bees performed significantly better when tested on the left side than when they were tested on the right one (F(1,9)=342.250; p=0.0001). The same pattern was found using cineol and eugenol (see Figure 12b): there was a significant difference in the number of A responses when the bees were tested on the right side versus the left side at 1 hour (F(1,9)=51.136; p=0.0001) and 6 hours (F(1,9)=27.689; p=0.001). At 1 hour recall was possible testing the bees on the right side, whereas at 6 hours it was possible testing them on the left side. B responses were low in number in all of the cases.

Bees trained with lemon as the positive stimulus and rose as the negative stimulus (Figure 12c) showed a similar pattern of responses when they were tested for recall: at 1 hour the number of A responses was significantly higher when they were tested using the right antenna than when using the left antenna (F(1,9)=48.913; p=0.0001). At 6 hours this difference was reversed: bees responded more when tested on the left side than when tested on the right side (F(1,9)=6.429; p=0.032), but there were a high number of B responses on both sides (F(1,9)=0.419; p=0.534).

This loss of memory specificity in recall of long-term memory by bees trained with lemon (+) and rose (-) was unexpected. I thought that it could be due to the fact that the experiments were conducted in Armidale, NSW, Australia, at an altitude of almost 1000 meters, where no vanilla or lemon plants grow and nor do any plants related to these, whereas as roses are quite common and were in bloom at the time of conducting the experiments. Thus, the different result found for the bees trained with lemon and rose could be related to the familiarity of rose odour, assuming that the bees tested had visited roses before capture or that they had been exposed to this odour in the hive. Hence, the training with rose as the negative stimulus might have involved reversal learning of an odour previously established in memory as a positive stimulus.



a)

b)

c)

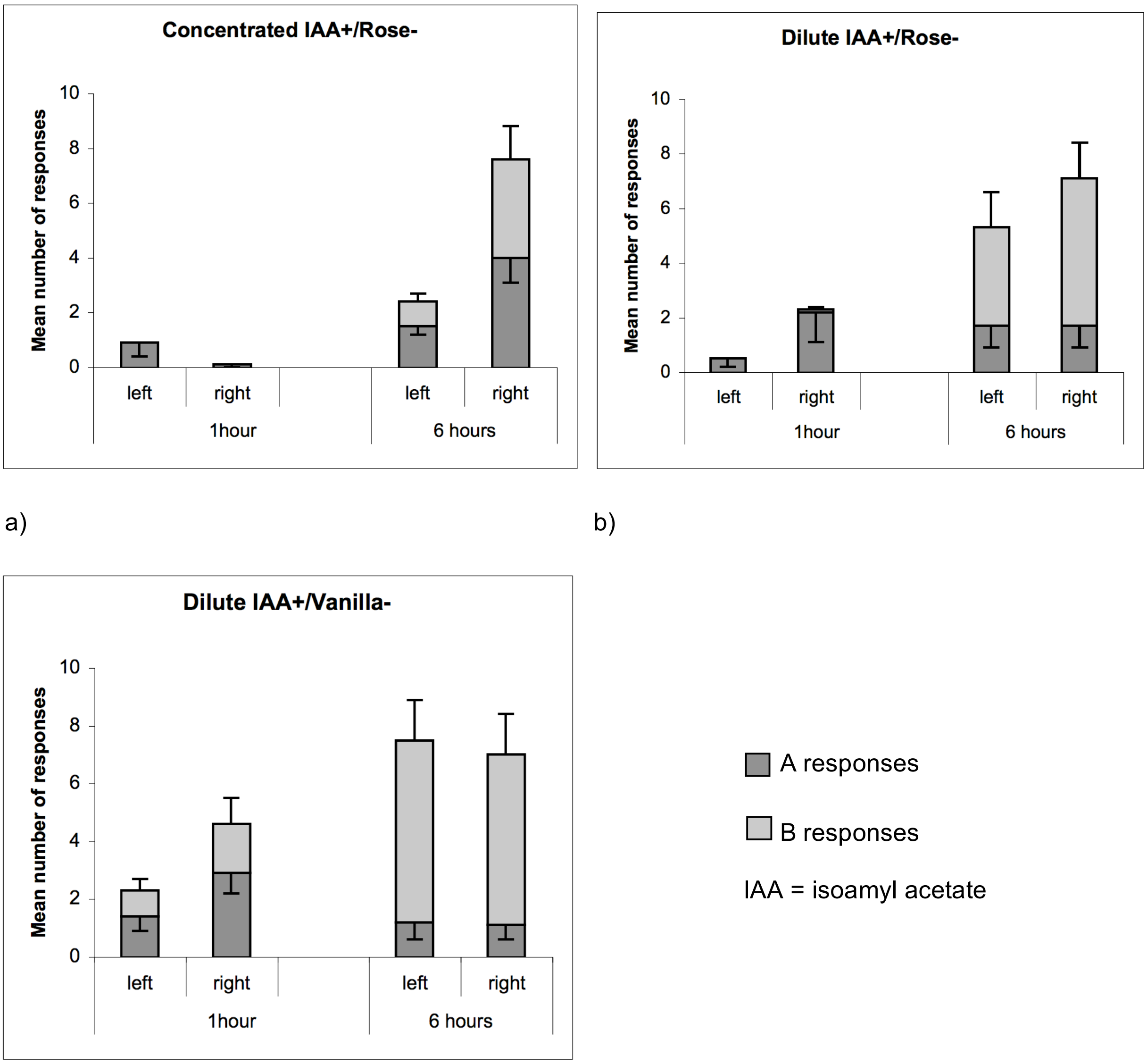
Figure 12. Experiment A. Recall of memory at 1 and 6 hours after training with a) lemon (+)/vanilla (-); b) cineol (+)/eugenol (-); c) lemon (+)/rose (-). All bees were trained using both antennae and tested for recall by presenting the odours on the bee's left side (left) or right side (right). The mean numbers (standard errors) of type A responses (proboscis extension response, PER, to S+ and not S-) are plotted as dark grey bars, the mean numbers (standard errors) of type B responses (PER to both odours) are plotted as light grey bars.

3.4.2.2. Experiment B: Does the familiarity of odours affect memory formation?

To investigate whether reversal learning leads to non-specific long-term memory I chose a biological odour which elicits alarm responses and may be an established negative stimulus. Isoamyl acetate (IAA) is an organic compound that is released by a honeybee's stinging apparatus, where it serves as a pheromone beacon to attract other bees and provoke them to sting (Boch et al., 1962). The idea was to train bees using IAA as a positive stimulus and probably, therefore, in a reversal learning paradigm.

The IAA was used at two different concentrations: concentrated IAA and dilute IAA (see Materials and Methods). Honeybees trained with IAA as positive odour and rose as negative odour gave few responses of any kind when tested for recall at 1 hour and this was the case for both concentrations of IAA used and irrespective of the antenna in use. No significant difference was found between the right and left antenna at one hour in the bees trained with concentrated IAA (Figure 13a) (F (1,9)=1.946; p=0.196) or in the bees trained with dilute IAA (Figure 13b) (F (1,9)=3.819; p=0.082) although the latter showed a trend in the expected direction of higher responsiveness on the right side. At 6 hours after training all of the bees trained with IAA and rose were more responsive than at 1 hour, but they responded to both odours (responses type B). Bees trained with concentrated IAA as the positive stimulus and rose as the negative stimulus (Figure 13a) and tested 6 hours later showed a slight but significant difference in the A responses between presentation on the right and on the left sides (F(1,9)=5.488; p=0.044), the right antenna responding more than the left. The number of B responses was also significantly higher for the right antenna than for the left antenna (F(1,9)=8.191; p=0.019). Bees trained with dilute IAA and rose (Figure 13b) did not show any difference in A responses between the right and the left antenna at 6 hours (F(1,9)=0.0001) but a significant difference in B responses (F(1,9)=12.356; p=0.007); they were higher on the right than the left. Additional statistical analysis (t-tests, two-tailed) revealed no significant differences between the performance of bees trained with concentrated IAA and rose (Figure 13a) and bees trained with dilute IAA and rose (Figure 13b) (left side at 1 hour: F(1,18)=0.431; left side at 6 hours: F(1,18)=0.052; right side at 1 hour: F(1,18)=3.466; right side at 6 hours: F(1,18)=3.550). Thus, the concentration of IAA does not affect the result.

I checked whether this pattern might be obtained only when IAA is used as a positive stimulus in combination with rose as a negative stimulus. Thus, I replicated the experiment by training bees with the dilute IAA as positive odour and vanilla as the negative odour (Figure 13c). Bees trained with diluted IAA as the positive stimulus and vanilla as the negative stimulus (Figure 13c) performed similarly to those trained with diluted IAA as the positive stimulus and rose as the negative stimulus (Figure 13b). One hour after training, there was a slight but significant difference in the number of A responses between the right and the left side - the bees responded better when tested on the right side than on the left side (F(1,9)=5.260; p=0.048) - but no significant difference in the number of B responses. On recall at 6 hours, there was no significant difference between the right and the left antenna either in A responses (F(1,9)=0.018; p=0.897) or in B responses (F(1,9)=0.247; p=0.631). There was only a slightly significant difference between bees in Figure 13b and bees in Figure 13c in the number of B responses for the left antenna at 1 hour (F(1,18)=4.893; p=0.040).



a)

b)

c)

Figure 13. Experiment B. Recall of memory at 1 and 6 hours after training with a) concentrated IAA (+)/vanilla (-); b) cineol (+)/eugenol (-); c) lemon (+)/rose (-). All bees were trained using both antennae and tested for recall by presenting the odours on the bee's left side (left) or right side (right). The mean numbers (standard errors) of type A responses (proboscis extension response, PER, to S+ and not S-) are plotted as dark grey bars, the mean numbers (standard errors) of type B responses (PER to both odours) are plotted as light grey bars.

3.4.2.3. Experiment C: Direct test of the effects of reversal learning

In Experiment C I tried a direct test of the effects of reversal learning and familiarity by training bees twice, the second time in a reverse way. Bees were first trained with lemon as the positive stimulus and vanilla as the negative stimulus, so that in the left side of the bee's brain a long-term memory for these odours would be formed. Sixteen hours later, bees were trained with reversed contingencies (vanilla+/lemon-) and the recall 1 hour and 6 hours after re-training was tested. Bees showed a pattern of results similar to that of the bees trained with dilute IAA and vanilla (Figure 14). On recall at 1 hour, the responsiveness was very low and there was no evidence of a significant difference between the right and the left sides either in A responses (F(1,9)=1.976; p=0.193) or in B responses (F(1,9)=1.000; p=0.343). At 6 hours responding was high but there was no significant difference between the sides for A responses (F(1,9)=0.474; p=0.509) or B responses (F(1,9)=2.139; p=0.178).

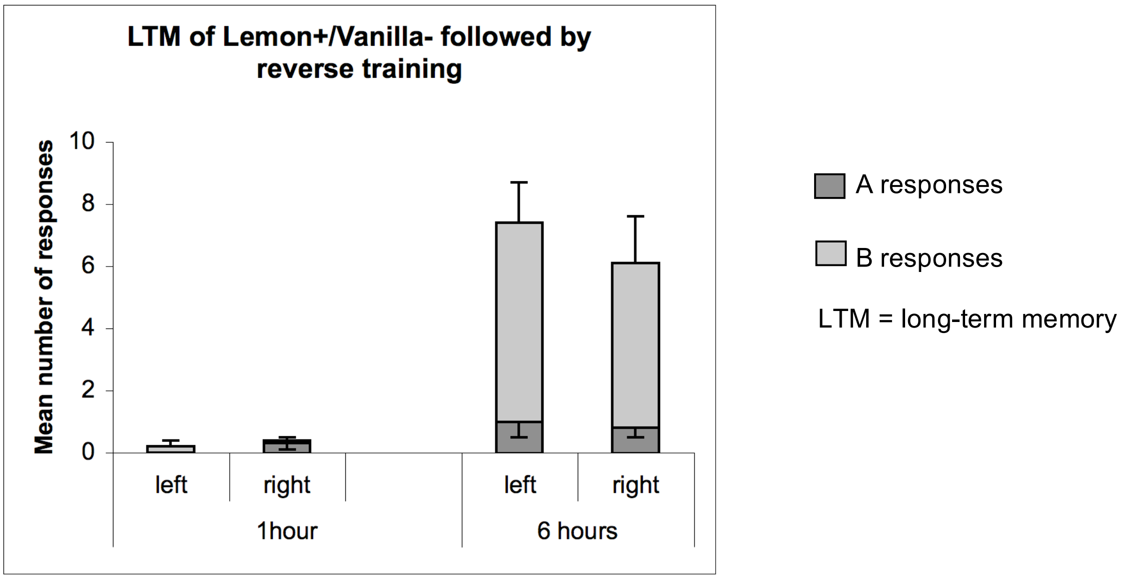


Figure 14. Experiment C. Recall of memory at 1 and 6 hours after training. All bees were trained using both antennae and tested for recall by presenting the odours on the bee's left side (left) or right side (right). The mean numbers (standard errors) of type A responses (proboscis extension response, PER, to S+ and not S-) are plotted as dark grey bars, the mean numbers (standard errors) of B responses (PER to both odours) are plotted as light grey bars.

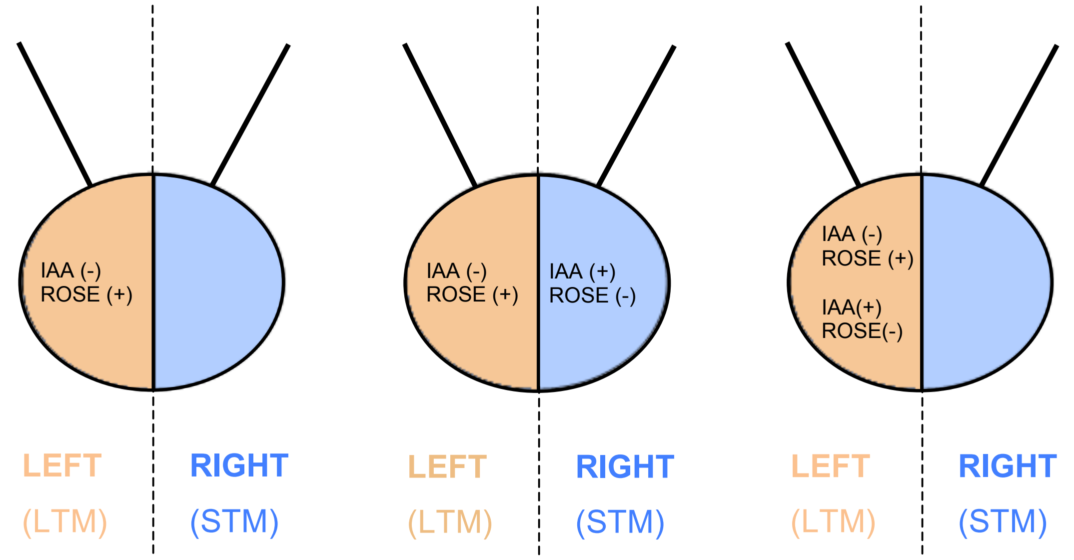
3.4.3. Discussion

The findings of Experiment A replicate those of Rogers and Vallortigara (2008). Recall of memory of the single odours (cineol and eugenol) showed the same pattern of lateral shift of olfactory memory recall as they had found for lemon (+) and vanilla (-). At 1 hour after training the bees responded to the positive stimulus (cineol) mainly when it was presented on their right side (i.e. using their right antenna), whereas at 6 hours after training they did so when it was on presented on their left side (i.e. using their left antenna). At both times of recall they discriminated this from the negative stimulus (eugenol), to which they did not respond with a PER.

Lemon (+)/rose (-) gave the same responses at 1 hour as did lemon (+)/vanilla (-) and cineol (+)/eugenol (-) but, in contrast, the memory was unspecific at 6 hours, even though A responses were still biased to the left side. I argued that this pattern of results could be due to familiarity with the rose odour, implying that reversal learning (negative stimulus) of an already established positive stimulus (rose) had occurred during training. If the bees had already formed a memory of rose as a positive stimulus, they may have failed to establish a long-term memory of rose as a negative stimulus.

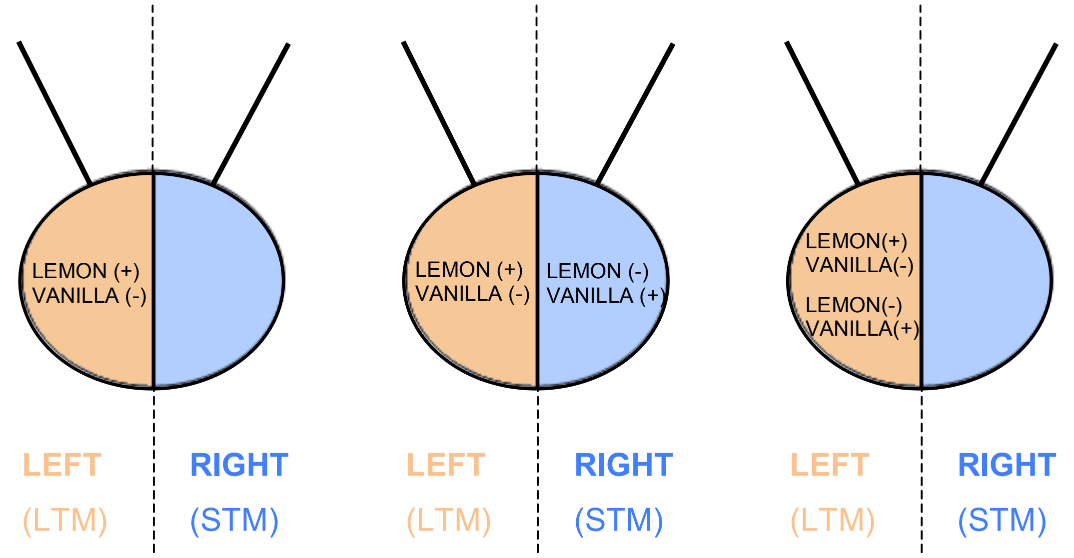
In Experiment B, IAA was used because we reasoned that it is probably a biologically established negative stimulus. Therefore, to investigate whether reversal learning leads to non-specific long-term memory recall we used IAA as the positive stimulus during training. For both concentrations of IAA used, at 1 hour after training on rose (+)/IAA (-) a suppression of the response was observed with no significant difference between the right and left antenna. At 6 hours after training the bees responded to both odours (type B responses), slightly more when the stimuli were presented to the right side than to the left side. I also used vanilla as the negative stimulus in combination with diluted IAA as the positive stimulus to check whether the pattern obtained was unique to IAA (+)/rose (-). A similar result was obtained. There was only a slightly significant difference between the two groups and that was in the number of B responses for the left antenna at 1 hour, which was higher for the bees trained with dilute IAA (+) and vanilla (-) than it was for the same concentration of IAA (+) and rose (-).

A simple model could account for these results. Research on retroactive interference suggests that honeybees seem to hold on to memories, i.e. new memories do not wipe out old ones (Cheng and Wignall, 2006). Three broad, but not mutually exclusive, retroactive interference effects have been put forward: 1) *memory loss*, where a second training causes the memory for the first task to be irretrievably eliminated; 2) *retroactive inhibition*, where the second task causes inhibition that actively suppresses the memory formed in the first task; 3) *response competition*, where multiple memories are intact, but the animal is uncertain as to which memory to exhibit in performance (see Cheng and Wignall, 2006). Let us consider the olfactory pathway and the brain of the bees as divided in two equal parts (right and left sides), with the right side mainly associated with short term memory and the left side with long-term memory (this is likely to be an oversimplification but here - see Figure 15 - simply serves the purpose of taking into account time-dependent shift of memory recall; see Rogers and Vallortigara, 2008). Familiar odours would be already present in the left side of the bee's brain (in the long-term memory store) and, in particular, memory of IAA would be encoded for biological reasons as a negative stimulus, whereas memory of rose may be already present as a positive stimulus because of previous learning.



Before training After training, at recall at 1 hour At recall at 6 hours

a)



After the 1st training After the 2nd training, at recall at 1 hour At recall at 6 hours

b)

IAA = isoamyl acetate; STM = short-term memory; LTM = long-term memory

Figure 15. A schematic representation of the model. a) represents what happens in the brain of bees trained with IAA(+)/rose(-). Before training, both IAA and rose were already present in the long-term memory (left side of the brain) as respectively negative (-) and positive (+) stimuli. After training with IAA(+)/rose(-), on recall at 1 hour, a short-term memory was available from stores \ in the right side of the brain with IAA(+)/rose(-) and bees showed reduced responsiveness, as a result of retroactive inhibition. On recall at 6 hours (after the memory shift from the right to the left side of the brain) IAA and rose would be present in the long-term memory as both negative (-) and positive (+), producing response competition due to multiple memories and making the bees respond to both odours. b) the same for bees in Exp. 3, trained twice, once in a reverse way. After the first training (lemon+/vanilla-) a long-term memory was allowed to form in the left side of the bee's brain, with lemon as positive and vanilla as negative (both these odours were unfamiliar to bees before training). The day after, bees were trained a second time with reversed contingencies (vanilla+/lemon-). On recall at 1 hour a short-term memory with vanilla(+) and lemon(-) was available on the right side. Six hours after reversal training, after the shift from right to left had taken place, in the left side memories of both odours would be present as both positive and negative stimuli, making bees respond to both odours.

When lemon and rose are used during training as, respectively, positive and negative stimuli, a short-term memory would be formed on the right side of the brain. One hour after training, recall would be possible from the right side (right antenna) and the bee would be able to discriminate between the two odours and respond only to the positive one (lemon). At 6 hours, when the shift from the right to the left side would be completed, in the left side of the bee's brain the rose odour will be present as both positive (as a result of long-term memory acquired before) and negative (as a result of the new long-term memory acquired after the training) thus producing response competition due to multiple memories (Cheng and Wignall, 2006) and, since the bee can no longer recognize whether rose is a positive or a negative odour, it responds to both the odours.

A similar explanation would work for Experiment B: the IAA would be already present in the long-term memory (left side of the brain) as a negative stimulus (see Figure 15a). Thus, at 6 hours (after the memory shift) IAA would be present in long-term memory as both negative and positive, producing response competition due to multiple memories and making the bee respond to both odours. Bees trained with IAA also showed low responsiveness after 1 hour, however. The suppression of responding at 1 hour could be interpreted as an example of retroactive inhibition (Cheng and Wignall, 2006), likely caused by the difficulty of associating a biologically known negative odour with a reward.

In order to check this hypothesis, in Experiment C I tried to simulate experimentally a retroactive interference effect, by training bees twice and, on the second occasion, in a reverse way. Three or more hours after the first training (lemon+/vanilla-) a long-term memory would form in the left side of the bee’s brain (see Figure 15b), with lemon as positive and vanilla as negative (both these odours were unfamiliar to bees before training). On the following day, these bees were trained with reversed contingencies (vanilla+/lemon-) and recall 1 hour and 6 hours after reversal training was tested. As expected (see above), 1 hour after the reversal training the bees showed reduced responsiveness, as a result of retroactive inhibition. At 6 hours, after the shift from right to left had taken place, bees responded to both odours, replicating the situation of response competition between the two memories (due to the fact that in the left side both odours would be present as memories of both positive and negative stimuli).

The model is based on the idea that memory shifts from the right to left side of the brain. Another possible explanation for these results could be that the information is encoded over different time frames in each side of the brain, rather than being transferred from one side to the other, and hence there would be asymmetry in the effectiveness of recall. As far as I can see, the result would be the same as predicted by our model.

Overall, the results of this series of experiments confirm the surprising finding (see Rogers and Vallortigara, 2008) that bees learn to associate a new odour of a positive stimulus mainly in neural circuits accessed via their right antenna, and that, after a period of a few hours, memory consolidation occurs accompanied by antennal asymmetry, with bees now being able to recall the odour mainly when using their left antenna. Moreover, this particular dynamic of memory traces has severe consequences when odours are already known to the bees (either for a biological reason or as a result of previous experience) and are thus already present in the long-term memory store. As a result, response competition arising from multiple memory traces can be observed, with bees showing unexpected lack of specificity in their longer-term olfactory memories.

3.5. Experiment 3: Morpho-functional asymmetry of the olfactory receptors of the honeybee (*Apis mellifera*).

In this study I first duplicated the PER behavioural result using forager Italian honeybees (*Apis mellifera ligustica* Spin.) and than I checked for anatomical differences in the number of sensilla between the right and the left antenna by using a larger sample of honeybees compared to the number of bees used by Letzkus et al. (2006). Morphological data were collected from bees harvested in 2 different hives (one in North Italy and another one in South Italy). Both antennae of each bee were imaged from four different views and all different types of sensilla were considered.

3.5.1. Materials and methods

3.5.1.1. Subjects

For the behavioural experiments, forager Italian honeybees were collected next to three different outdoor hives during summer 2009 at San Michele all’Adige (Trento, Italy). For the scanning electron microscopy, honeybees were collected during summer 2008 from two different outdoor hives: 1) the hive in San Michele all’Adige (Trento, Italy) and 2) a hive in Reggio Calabria (South of Italy). They were preserved in a freezer at -20°C and used as specimen for the scanning electron microscopy in autumn 2008.

3.5.1.2. Types of sensilla

On the basis of the literature and according to my own preliminary observations, I distinguished the following sensillum types (Figures 16 and 17).

Sensilla placodea or oval pore plate organs (McIndoo, 1914; von Frisch, 1921; Lacher and Schneider, 1963; Kaissling and Renner, 1968) (Figures 16a and 17a).

Sensilla trichodea, inserted into a surface depression, can be divided into thick trichodea (type a) (Figures 16a-16c and 17a), straight, shorter and larger, and thin trichodea (type b) (Figures 16a and 17a), longer and slender, tapering to the tip (Dietz and Humphreys, 1971; Al Ghamdi, 2006; Zakaria and Allam, 2007). The entire wall of the sensilla trichodea (type a) possesses pores.

Sensilla basiconica, inserted into a slightly depressed round socket, are distinguished into thick basiconica (Figures 16a-16b and 17a), and tapered basiconica (Figures 17a-17c) (Yokohari et al., 1982). The tip surface of the former shows porous structures.

Sensilla coeloconica, referred also to as ampullacea, which are involved in perception of temperature, carbon dioxide and humidity (Figure 16a and 17a) (Dietz and Humphreys, 1971; Yokohari et al., 1982; Al Ghamdi, 2006; Zakaria and Allam, 2007).

Sensilla campaniformia or coelocapitular, considered as hygro- and thermoreceptors and/or mechanoreceptors (Figure 16a) (Kuwabara and Takeda, 1956; Dietz and Humphreys, 1971; Yokohari et al., 1982; Al Ghamdi, 2006; Zakaria and Allam, 2007).

Sensilla chaetica, are stout bristles with their base inserted into a socket and are sensitive to mechanical or gustative stimuli (Figure 17a-17b) (Whitehead and Larsen, 1976; Amornsak et al., 2000). They house an apical pore.

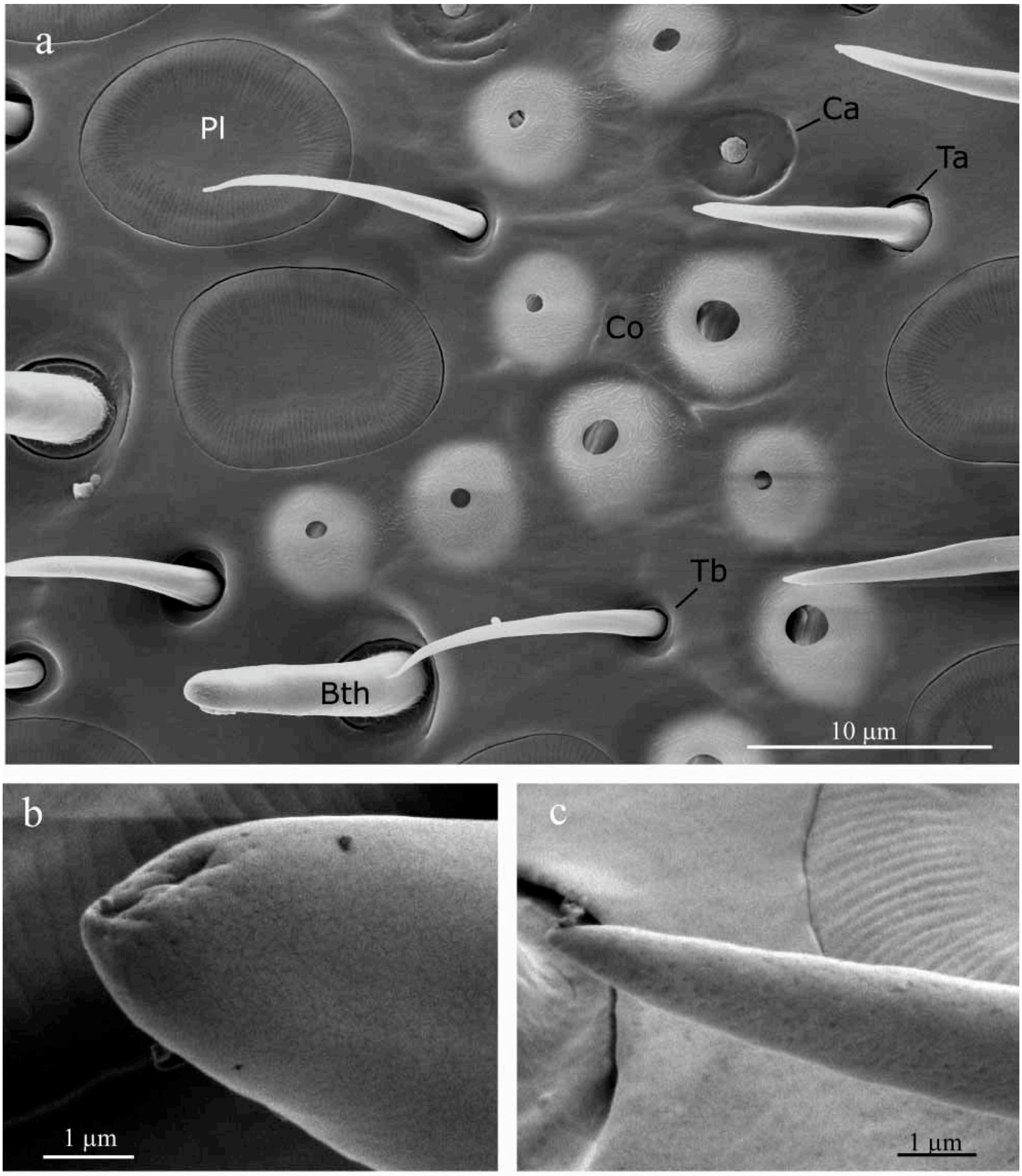


Figure 16. Scanning electron micrographs of the antenna of *Apis mellifera* forager: (a) dorsal view of a medial segment of the flagellum; (b) detail of a sensillum basiconicum thick; (c) detail of a sensillum trichodeum type a. Pl, sensillum placodeum; Ta, sensillum trichodeum type a; Tb, sensillum trichodeum type b; Bth, sensillum basiconicum thick; Co, sensillum coeloconicum; Ca, sensillum campaniformium.

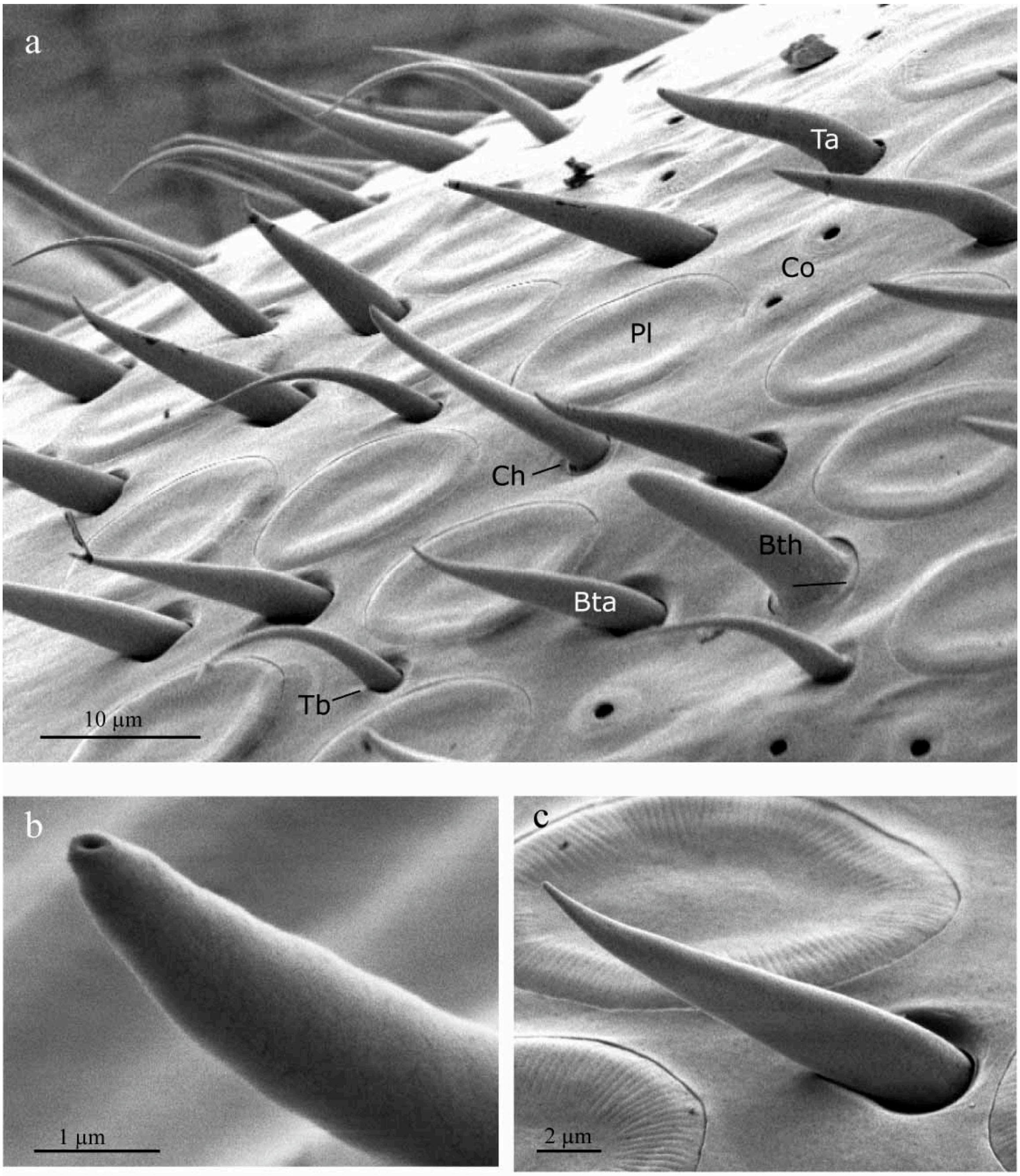


Figure 17. Scanning electron micrographs of the antenna of *Apis mellifera* forager: (a) dorsal view of a medial segment of the flagellum; (b) detail of a sensillum chaeticum; (c) detail of a sensillum basiconicum tapered. Pl, sensillum placodeum; Ta, sensillum trichodeum type a; Tb, sensillum trichodeum type b; Bth, sensillum basiconicum thick; Bta, sensillum basiconicum tapered; Ch, sensillum chaeticum; Co, sensillum coeloconicum.

3.5.1.3. Behaviour

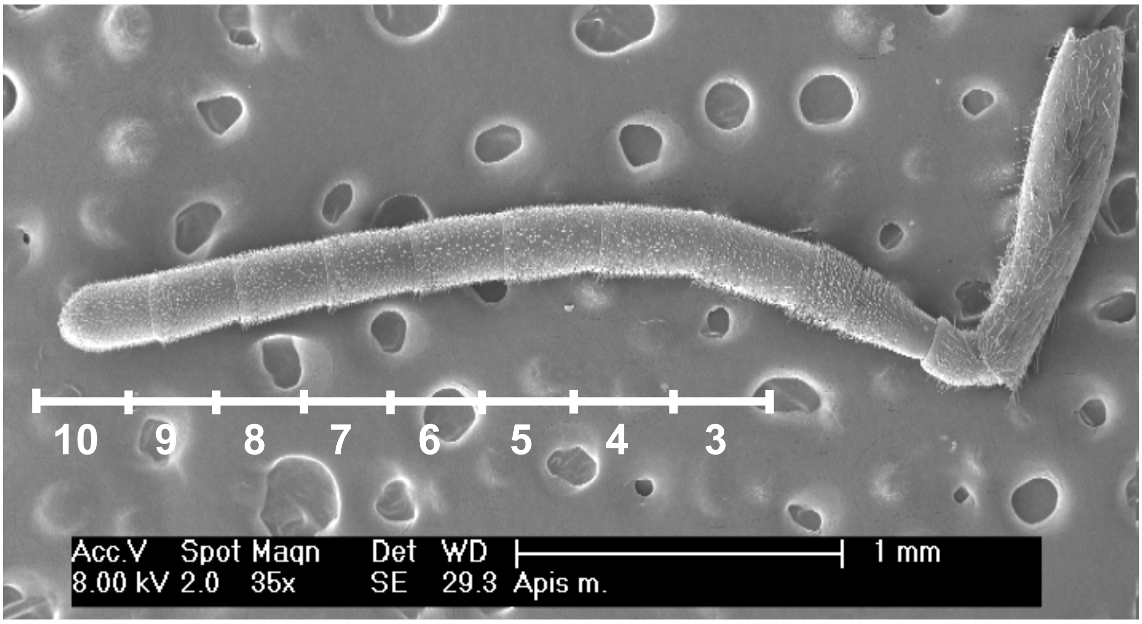
Honeybees were cooled in 750ml containers until immobilised and then harnessed in metal holders using the method of Bitterman et al. (1983), as modified by Rogers and Vallortigara (2008), by adding a piece of paper to cover the thorax of the bee so that the bee was not damaged by the adhesive tape and could be released after the test. They were assigned randomly to groups for the occlusion of one antenna and, 1 hour later, all bees were trained in the same way. The experiment was carried out on three groups. The bees in one group (N=9) had their left antenna coated with a silicone compound (Silagum-Mono, DMG, Germany); those in the second group (N=9) had their right antenna coated, while both the antennae of the bees belonging to the third group were left uncoated (N=9).

One hour after the antennae had been coated, training commenced. Each bee in its holder was placed in front of an exhaust fan and trained using (-)-linalool (Sigma Aldrich, >98.5% purity), a common floral odour, plus 1M sucrose solution as the positive stimulus (10ul of (-)-linalool dissolved in 3 ml of the sucrose solution). The negative stimulus was a saturated salt (NaCl) solution. Three trials spaced 6 min apart were given. On the first trial a droplet of the (-)-linalool solution at the end of a 23 gauge needle was held over the bee’s antennae at 1 cm from the antennae and after 5 s the antennae were touched, which led to PER. The bee was then allowed to ingest the drop of (-)-linalool sugar solution. The procedure was repeated with the saline solution (trial 2), which did not trigger PER but avoidance by moving the antennae away from the droplet. On trial 3 the procedure of trial 1 was repeated and usually PER occurred without the need to touch the antennae.

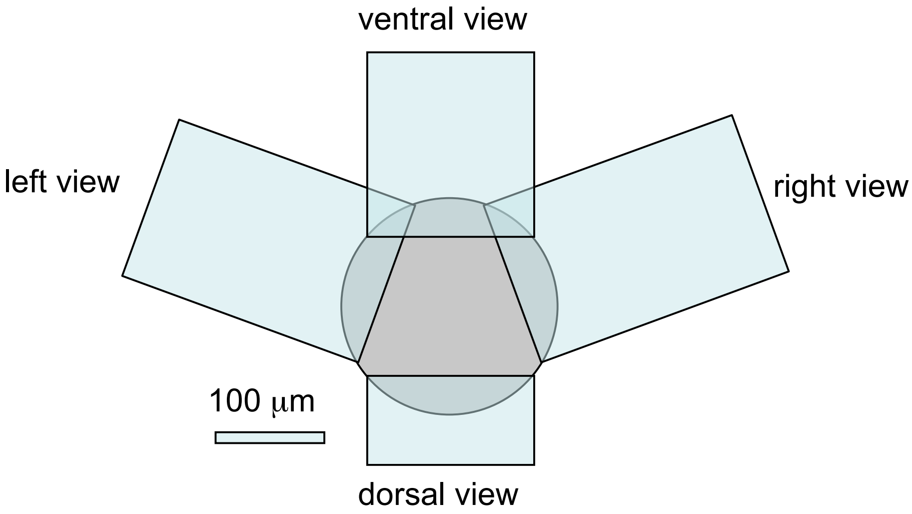
Recall was tested in three groups of bees one hour after the training ended. Both (-)-linalool and saturated salt solutions were presented as dissolved in distilled water at the same concentration as used in training. Droplets of these solutions were held over the bee, out of reach of the antennae or proboscis at approximately 1 cm from the bee. The odour presentation was followed immediately by the saline solution and 10 presentations of each pair of solutions were made separated by an interval of approximately 60 s. The solutions were presented for 5 sec. and whether or not PER occurred was noted. The responses were scored as follows: A, extension of the proboscis to (-)-linalool and no extension to saturated salt solution; B, extension of the proboscis to both (-)-linalool and saturated salt solution; C, extension of the proboscis to saturated salt solution but not (-)-linalool; D, no extension of the proboscis to (-)-linalool or to saturated salt solution.

3.5.1.4. Scanning Electron Microscopy (SEM)

The bees (N=18) collected during summer 2008 were preserved in a freezer for about 2 months. After defrosting at room temperature for 4 hr, left and right antennae were cut at the base of the pedicel with a razor blade. The basal segments of each antennae pair were attached to a circular stub by double-sided conductive tape (TAAB Laboratories Equipment Ltd. Aldermaston, UK). All samples were gold-coated for guaranteeing electrical conductivity during imaging with a XL 30, Field Emission Environmental Scanning Electron Microscope. (FEI-Philips, Eindhoven, Netherlands). Each antenna was imaged from four different viewpoints (Figure 18a): ventral view (holder at 0°), right view (sample tilted at -75°, imaging of the right antenna side), left view (sample tilted at +75°, imaging of the left antenna side), and dorsal view (after removing antenna from stub and replacing upside). The four views allowed imaging almost completely the antenna surface as sketched in Figure 18b. Because there are no olfactory receptors on the first two segments of the honeybee flagellum, only segments 3rd to 10th were scanned. Each segment from 3rd to 9th was scanned longitudinally at a magnification of 600 times. A magnification of 800 times was used for the 10th smaller segment (apex). Four images per segment were collected according to the four different viewpoints: ventral, right, left, and dorsal.



a)



b)

Figure 18. Morphological characterization of *Apis mellifera* antenna.

a) Low-magnification SEM image of the ventral view of *Apis mellifera* left antenna. Segments from 3rd to 10th were imaged for subsequent count of sensilla.

b) Schematic representation of the four views used for imaging each segment (antenna diameter is about 200 µm); the SEM field of view at a magnification of 600 times is 205 µm in width and 154 µm in height.

Fourteen out of 18 subjects were selected for SEM imaging, excluding damaged antennae during sample preparation or rotation.

Sensilla placodea, sensilla trichodea type a and b, sensilla basiconica thick and tapered, sensilla coeloconica, sensilla campaniformia and sensilla chaetica were identified according to their specific morphological characteristics as previously described (Figures 16 and 17). Each type of sensillum was then tagged and counted on all acquired images by using image analysis software (UTHSCSA ImageTool Version 3.0). Data were clustered according to the four viewpoints, eight antennal segments, two antennae, and eight sensilla types.

3.5.1.5. Statistical analyses

For the behavioural data, the mean number of A responses with the respective standard error of the mean (SE) were calculated for each bee of each group (right antenna, left antenna, both antennae in use). B, C and D responses were too rare to warrant inclusion in the analysis. Data were analysed by analysis of variance (Anova) with antenna in use as between-subjects factor.

For the images acquired by SEM, we counted all the sensilla on all the images. The mean number of olfactory sensilla (sensilla placodea, sensilla trichodea a and b, sensilla basiconica thick and tapered) with the respective standard error (SE) were calculated and plotted for each segment number. Data were analysed by analysis of variance (Anova) with antenna, segment, type of sensilla as within-subjects factors. Further analyses were done considering the putative olfactory sensilla and the sensilla involved in other functions (sensilla coeloconica, sensilla campaniformia and sensilla chaetica).

3.5.2. Results

Results of behavioural tests are shown in Figure 19. The analysis of variance revealed a significant main effect of the antenna in use ($F_{2,24}=5.31$, $p<0.05$). Post hoc comparison using the Tukey HSD test revealed a significant difference between bees using the right antenna and bees using the left antenna ($p<0.05$) and between bees using the left antenna and bees using both antennae ($p<0.001$), whereas there were no significant differences between bees using the right antenna and bees using both antennae ($p=0.55$).

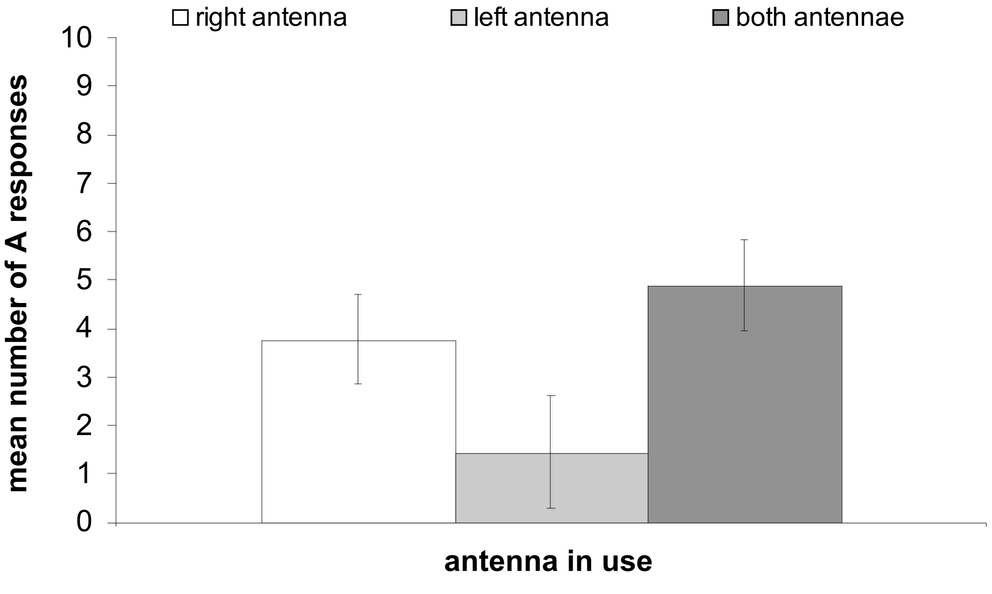
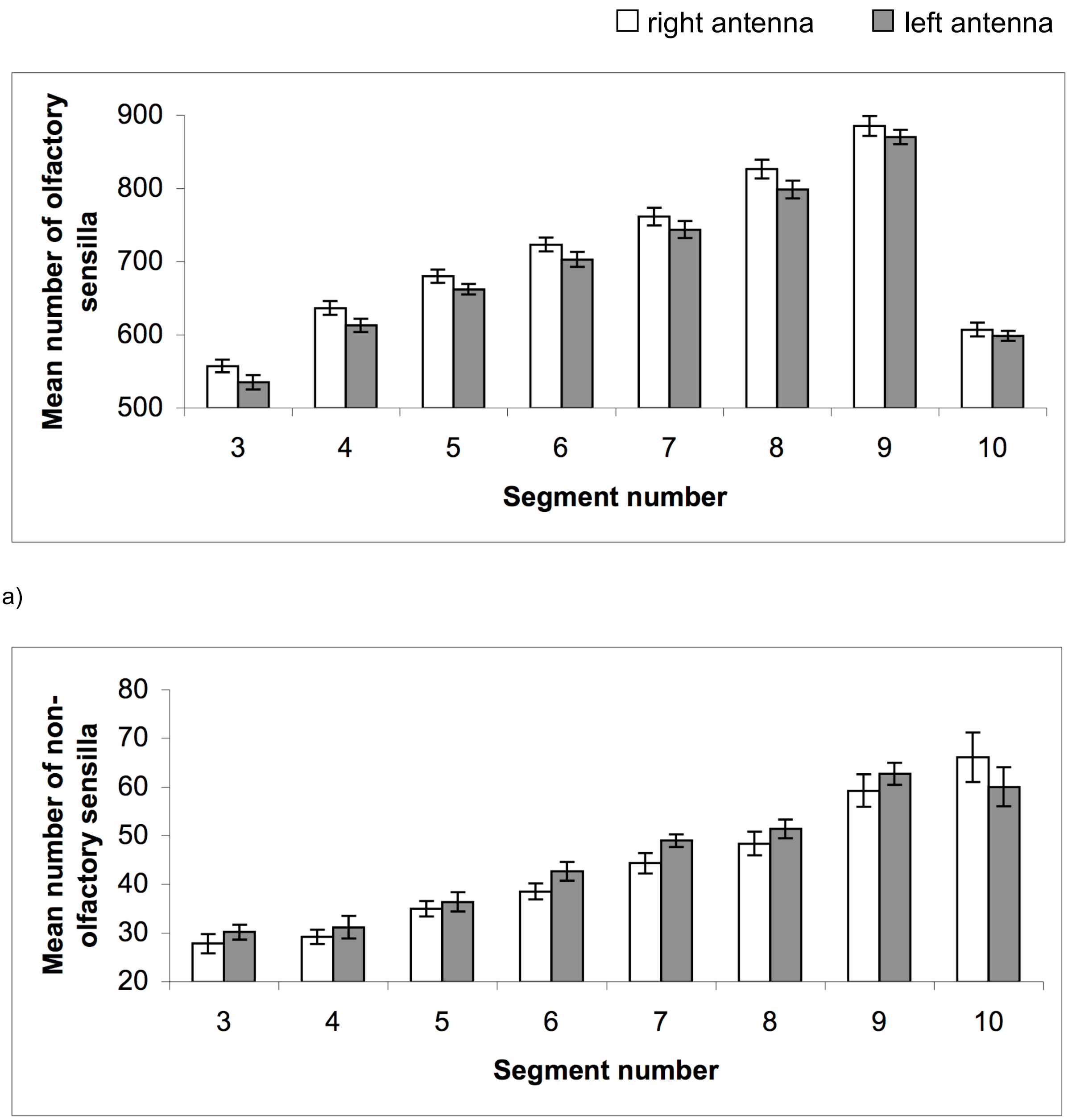


Figure 19. Behavioural asymmetry during recall in honeybees *Apis mellifera,* after trained on the proboscis extension reflex (PER). The mean number of A responses (extension of the proboscis to (-)-linalool and no extension to saturated salt solution), plotted with standard error bars, in recall tested at 1 hour after training in honeybees tested with both antennae or with only one antenna in use: white bars for honeybees tested with the left antenna coated and the right antenna in use (right antenna); light grey for honeybees tested with the right antenna coated and the left antenna in use (left antenna); dark grey for honeybee tested with both antennae in use (both antennae).

Results from SEM are summarized in Figure 20. The Anova with antenna, segment and type of sensilla as within-subjects factors revealed significant main effects of segment ($F_{7,91}$=837.21, p<0.0001) and sensilla type ($F_{7,91}$=1623.47, p<0.0001), and significant antenna x sensilla type ($F_{7,91}$=3.07, p<0.01) and segment x sensilla type ($F_{49,637}$=180.45, p<0.0001) interactions. Sensilla that are likely to be involved in olfaction (sensilla placodea, sensilla trichodea a and b, sensilla basiconica thick and tapered) showed to be higher in number on the right than on the left antenna for all segments ($F_{1,13}$=5.56, p<0.05, Figure 20a). For sensilla involved in non-olfactory functions (sensilla coeloconica, sensilla campaniformia and sensilla chaetica) there was evidence of asymmetry in only the segments 3-9 but in the opposite direction: there were in fact more non-olfactory sensilla on the left than on the right antenna (excluding the 10th segment, $F_{1,13}$=6.07, p<0.05, see Figure 20b).



a)

b)

Figure 20. The mean number of a) olfactory sensilla and b) non-olfactory sensilla for the right antenna (white bars) and for the left antenna (dark grey bars) *of Apis mellifera* with the respective standard error (SE) in function of the segment number.

3.5.3. Discussion

Results of the behavioural test confirm the finding of Letzkus et al. (2006) that the honeybees are lateralized in responding to learned odours and in particular that after training on the proboscis extension reflex (PER) paradigm, bees respond to odours better when they are trained through their right antenna. Bees trained with the left antenna coated performed similarly to the control group (bees with both antennae left uncoated) while those trained with the right antenna coated performed worse. I checked whether this difference is due to a difference in the number of sensilla which house the receptor cells involved in the olfactory responses. I extended the work done by Letzkus et al. (2006) by considering a larger sample and both antennae of each bee. Moreover I imaged the whole antenna surface from 4 different views. All the sensilla present on each image were counted distinctly for a total of eight sensilla types: five of which with possible olfactory function (sensilla placodea, sensilla trichodea type a and b, sensilla basiconica thick and tapered) and the other three sensilla types involved in others, not olfactory, functions (sensilla coeloconica, sensilla campaniformia and sensilla chaetica). I found that the number of olfactory sensilla was significantly higher on the right antenna than on the left antenna. Surprisingly, I observed for the first time that the number of non-olfactory sensilla was significantly higher on the left antenna than on the right antenna in all segments except the apex. The functional significance of this opposing morphological asymmetry is at present unclear but behavioural asymmetries in other sensory domains appear to be worth testing.

It is worth of noting that in a previous study on an another eusocial Apidae species, *Apis florae* Fabr., the olfactory sensilla were shown to be slightly more abundant on the left antenna (Gupta, 1986). Thus, comparative work on different species is needed in order to understand the generality and the significance of antennal morphological asymmetries in Apidae.

It should also be stressed that morphological differences between the honeybee's antennae in the number of olfactory sensilla could not account entirely for the asymmetries observed in behaviour. Firstly, because both anatomical and EAG asymmetries (Anfora et al., 2010) seem to be quite modest in magnitude to account for the behavioural bias, and secondly, and most important, because asymmetries in recall seem to change in direction with memory consolidation. Rogers and Vallortigara (2008; see also Frasnelli et al., 2010a) investigated whether lateralization could be found in recall of olfactory memory at various times (1, 6 and 24 hours) after the bees had been trained using both antennae and they showed that the left-right asymmetry in olfactory memory depends on time. At 1 hour after training, bees showed excellent recall when tested using their right antenna, but poor or no recall when tested using their left antenna. By contrast, 6 and 24 hours after training recall was good when the left antenna was in use but not when the right antenna was in use, demonstrating that long-term memory is accessed mainly via the left antenna. Thus, a lateral shift had occurred from the right to the left antenna, showing that the consolidation or retrieval of olfactory learning is a time-dependent process and involves lateralized neural circuits. It seems likely that the asymmetry in peripheral receptors is part of a cascade of asymmetrical mechanisms associated with detection, storage and retrieval of olfactory events (see for similar mechanisms in vertebrates Rogers and Andrew, 2002). It will be therefore important to look at the morphological and physiological processes going on in the honeybee left and right olfactory brain centers (i.e. antennal lobes and mushroom bodies) following olfactory learning at different times during memory consolidation.

3.6. Experiment 4: Right-left antennal asymmetry of odour memory recall in three species of Australian stingless bees

In this research I was interested in investigating the generality and phylogenetic origins of the antennal asymmetry found in the honeybee Apis mellifera (Letzkus et al., 2006; Rogers and Vallortigara, 2008; Anfora et al., 2010; Frasnelli et al., 2010a) and therefore studied whether it could be observed in stingless, social bees. Australian stingless bees (Hymenoptera, Apidae, Meliponinae, Genera *Trigona* and *Austroplebeia*) are among the most primitive social bees (Dollin, 1996) and stingless bees are more primitive than honeybees (the oldest known bee fossil, a *Trigona prisca* worker from late Cretaceous, preserved in amber in New Jersey, USA, is 74-94 million years old, and is very similar to the extant species of *Trigona spp.* from the neotropics; Michener and Grimaldi, 1988), suggesting that Meliponinae (stingless bees) is an ancient group. The other taxa (Apinae, Bombinae and Euglossinae) seem to be more recent: the oldest known fossil of a honeybee (*Apis*) is 22-25 million years old (Michener, 1990).

a)

b)

c) d)

Figure 21. – Australian native stingless bees: a) *Trigona carbonaria*, b) *Trigona hockingsi*, c) *Austroplebia australis* and d) the barrier made by *A. australis* over the entrance to the hive.

Studies carried out on Brazilian stingless bees (Mc Cabe et al., 2007; Mc Cabe and Farina, 2009; Mc Cabe and Farina, 2010) showed possible differences in odour-learning abilities compared to honeybees, *Apis mellifera* (Mc Cabe et al., 2007). Gustatory responsiveness was found to be similar in the three species studied but honeybees showed the best ability to discriminate, *M. quadrifasciata* an intermediate, and *S. aff. depilis* bees showed no discrimination. The presence of long-term memory has been documented in *M. quadrifasciata* bees (Mc Cabe and Farina, 2009).

In this study *Trigona carbonaria*, *Trigona hockingsi* and *Austroplebia australis* stingless bees were tested*. Trigona carbonaria* (4 mm; Figure 21a) is found on the east coast of Australia from North Queensland to southern NSW (Dollin, 1996). *Trigona hockingsi* (4.5 mm; Figure 21b) is found in the northern and eastern parts of Queensland and in the Northern Territory. *Austroplebia australis* (4 mm; Figure 21c) is distributed throughout northern NSW and in Queensland.

Stingless bees were trained using lemon and vanilla as odours and tested their recall by presenting the stimuli on the right or left side of the bee at 1 hour and at 5 hours after training (see Rogers and Vallortigara, 2008). As in previous studies, the proboscis extension reflex (PER) paradigm was used (Bitterman et al., 1983, and Letzkus et al., 2006), in which bees are conditioned to extend their proboscis in anticipation of a food reward (unconditioned stimulus) when they perceive a particular odour (conditioned stimulus). The procedure of testing and the set up were, however, modified to accommodate testing of the very small stingless bees.

3.6.1. Materials and methods

3.6.1.1. Subjects

The bees used were all from established hives in boxes. Worker bees were captured in tubes (length 100 mm x diameter 20 mm) as they exited their hive and transported to the laboratory nearby. Experiments were conducted during summer (February and early March, 2010) in Valla, northern NSW, Australia, which is within the natural range of *T. carbonaria* and just outside of the range of *A. australis.*

Due to their small size (4.0 to 4.5 mm) these bees could not be handled in the same way as honeybees. Also, due to their susceptibility to low temperatures, they were not subjected to cooling before being placed in the training holders for testing. Instead, I was able to take advantage of their consistent and obvious negative geotaxis, which meant that they would climb upward into a tube for testing. The tube was made by cutting off the end of a pipette cap of 10 µl to make a hole of diameter at the tip so small that the bee could protrude only its head and not escape. The size of the hole had to be adjusted to each species to prevent the bee escaping. Once the bee had adopted this position in the tube it would remain there provided that the tube remained upright, as shown in Figure 22, and could be trained by presenting odours and reward (sugar) or punishment (saline).

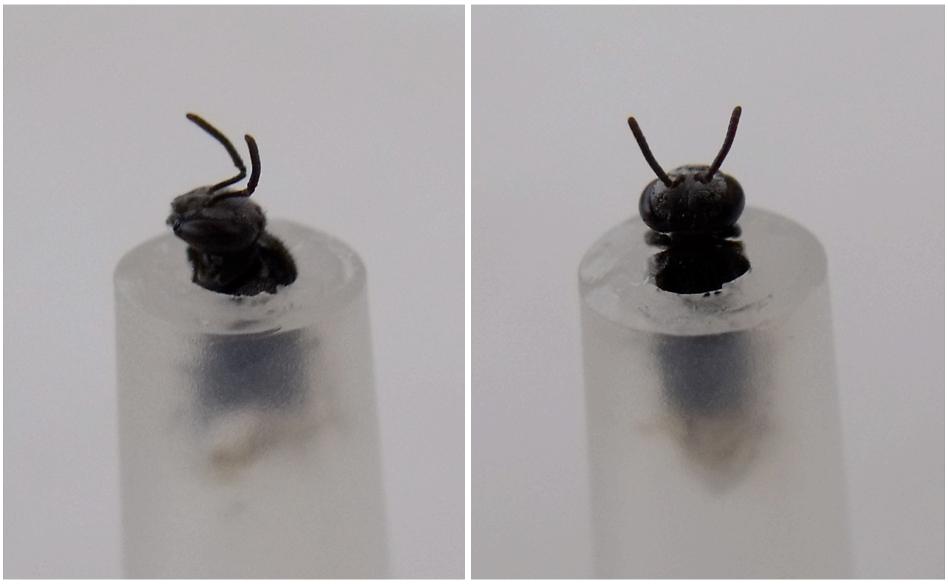


Figure 22. A bee positioned in the tube for training and testing.

Six groups of bees (total of 67 bees) were tested. Three groups (one of each species) were tested for short-term memory (N=12 *T. carbonaria*, N=13 *T. hockingsi* and N=10 *A. australis*) and another three groups (one of each species) were tested for long-term memory (N=8 *T. carbonaria*, N=11 *T. hockingsi* and N=13 *A. australis*).

Each group was trained using lemon odour as the positive stimulus and vanilla odour as the negative stimulus. The concentrations of lemon and vanilla were 10 µl of the scent essence (Queen Fine Food Pty Ltd) dissolved in 3 ml of 1M sucrose solution or saturated saline solution. In order to check whether the shift in lateralized recall was due to the elapse of time per se or to changes in motivation associated with increasing hunger, I performed a control experiment in which I fed the bees with sugar 1 hour before the recall test at 5 hours using a group (N=7) of *T. hockingsi* bees.

3.6.1.2. Experimental procedures

One hour after the bees had been restrained in the testing tubes training commenced. The bees were trained with both antennae in use using lemon plus 1M sucrose solution as the positive stimulus and vanilla plus saturated saline as the negative stimulus. Positive and negative stimuli were presented (one after the other one) using cotton buds soaked in one or the other solution and holding the bud over both of the bee's antennae at 1 cm from the antennae. Three trials were given at 6-min intervals. In the first trial, after holding the bud with the odour in sucrose solution over the antennae for 5 sec, the antennae were touched with the bud to trigger the proboscis extension response (PER). The bee was allowed to ingest the sugar solution from the bud for 5 sec. Then the negative stimulus was presented, again with touching of the antennae, which led to withdrawal. After 6 minutes, a second trial was performed and the bee usually responded to the positive stimulus with a PER without the need to touch the antennae with the bud. After another 6 minutes bees were given the third and last trial and then held for either 1 hour (in the holder) or 5 hours (released into a 125 ml tube until 1 hour before testing) at room temperature (varying from 28° C to 32° C).

Bees trained were tested either at 1 hour or at 5 hours after the completion of training. I used the method of Rogers and Vallortigara (2008) to test for laterality of responding. It involved presenting the odours dissolved in distilled water at the same concentrations as used during training. A cotton buds with either of these solutions was held on the left or right side of the bee, out of reach of the antennae or proboscis at approximately 1 cm from the bee. Each odour was presented 10 times on each side of the bee in completely random order for a total of 40 randomized presentations (10 lemon on the right side, 10 lemon on the left side, 10 vanilla on the right side and 10 vanilla on the left side). The odours were presented for 5 sec and whether or not PER occurred was recorded. A full PER involved complete extension of the proboscis, which included extension of the two outer sheaths of the proboscis and the tongue. Partial extension of the proboscis, which involved extension of the sheaths but not full extension of the tongue, was also recorded. I refer to the latter as ½ PER.

In the experiment controlling for motivation to feed (above), bees were trained in the same way but they were fed with 1M sucrose solution without any odour presented on a cotton bud at 1 hour before testing recall at 5 hours after training.

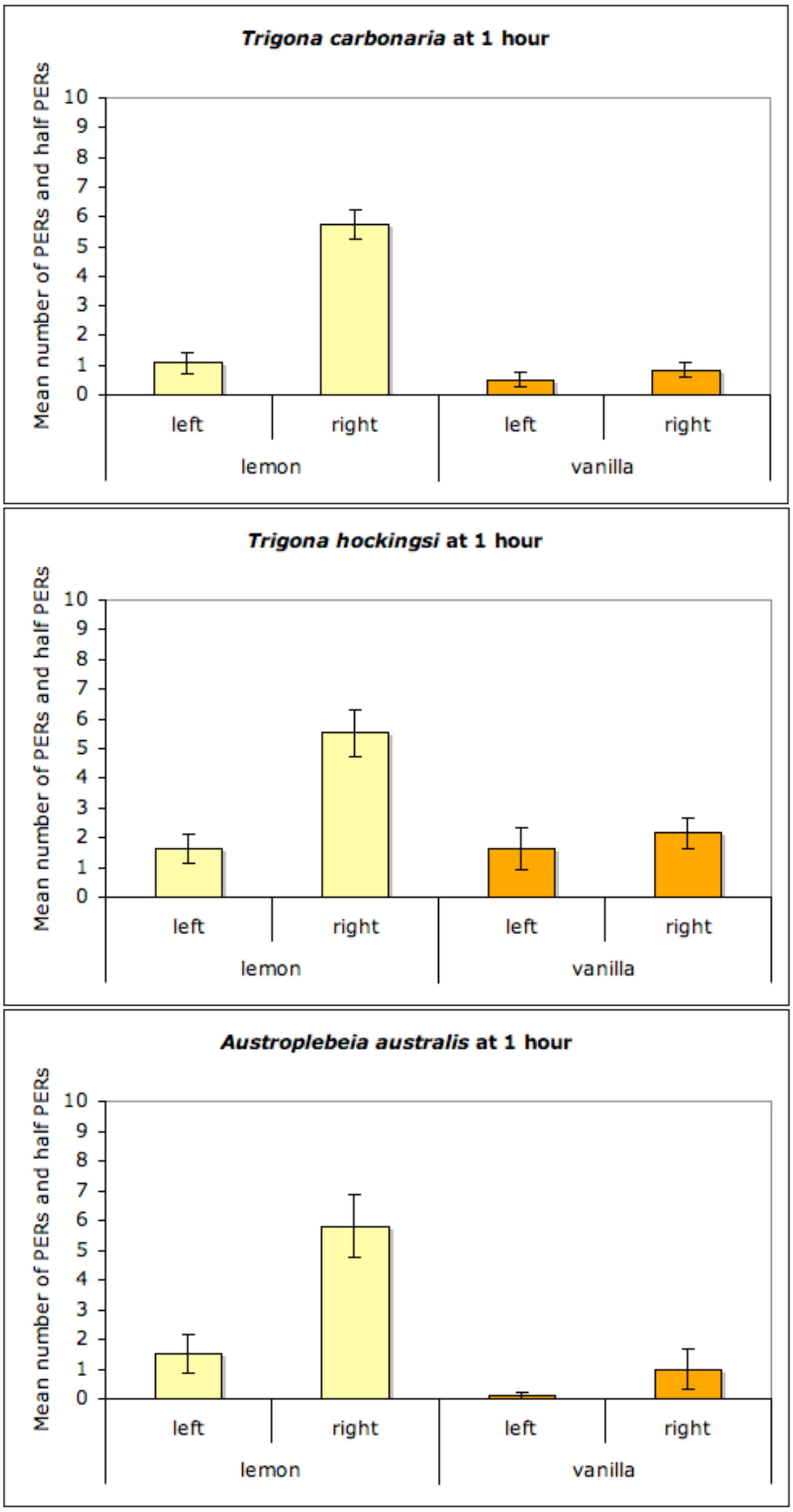
3.6.1.3. Statistical analyses

Before analysis data were log-transformed to account for heterogeneity of variances. For each group of bees and for each side (right and left) the mean number of responses to lemon and the mean number of responses to vanilla with the respective standard errors (SEM) were calculated. Both the full proboscis extensions (full PER) and extension of only part of the proboscis (half PER, ½ PER) were considered. Because of the differences in the numbers of full PER recorded between the three species, data are plotted considering both full PER and ½ PER (Figure 23) and considering full PER only (Figure 24).

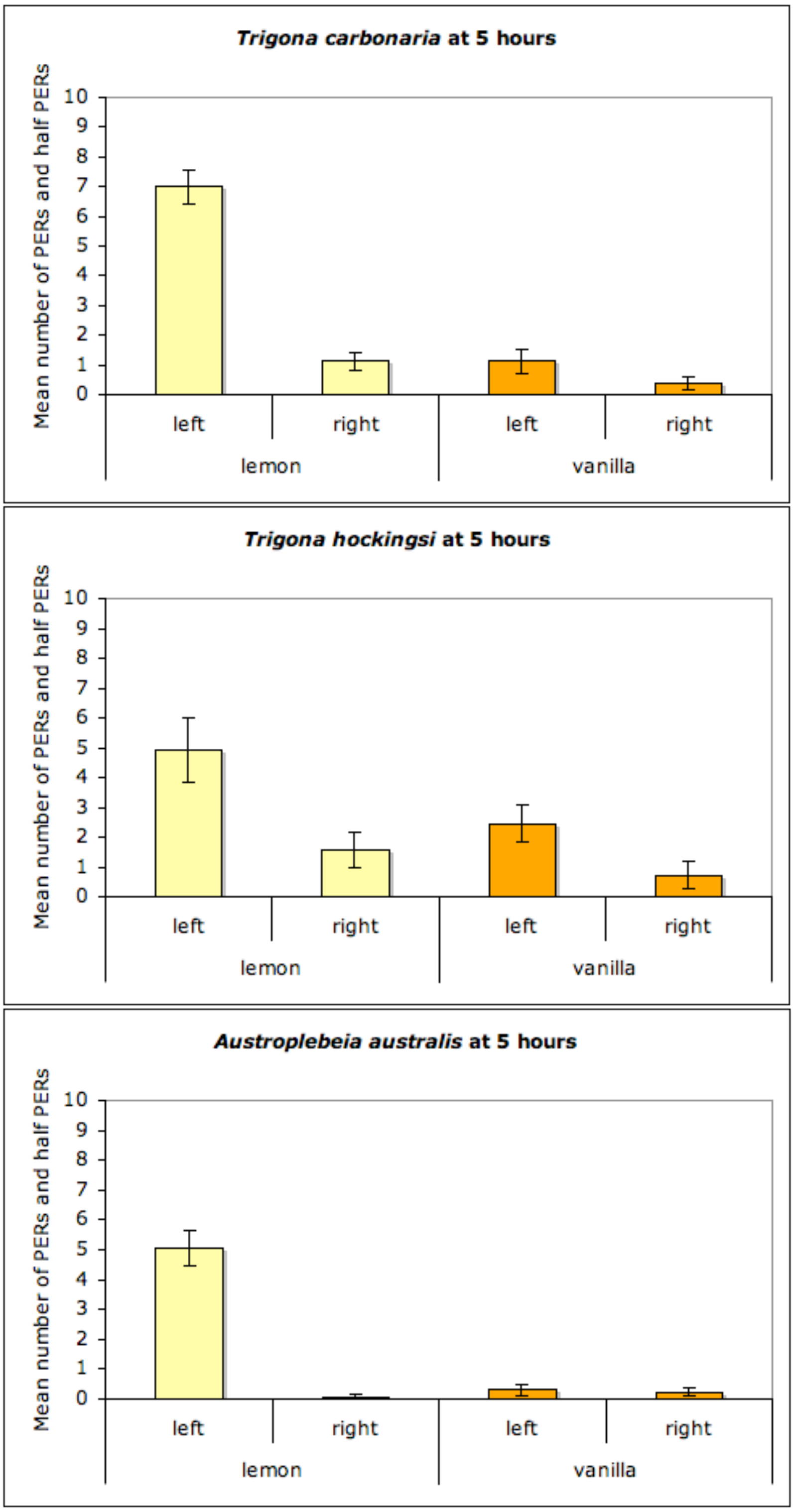
Data were analysed by analysis of variance (Anova) with 'antenna' and 'odour' as within-subjects factors, and 'species' as between-subjects factor. Data from the control experiment conducted on the *T. hockingsi* bees fed with sugar one hour before the recall test at 5 hours were compared with those of the *T. hockingsi* bees tested for recall at 5 hours in the main experiment using 'experiment' as between-subjects factor.

3.6.2. Results

Considering both full PER and ½ PER responses, the bees responded differently to lemon (+) and vanilla (-) when using the right and the left antenna at both 1 hour and 5 hours after training in all the three species tested (Figure 23).


Trigona carbonaria at 1 hour
Mean number of PERs and half PERs
0
1
2
3
4
5
6
7
8
9
10
left
right
left
right
lemon
vanilla
Trigona hockingsi at 1 hour
Mean number of PERs and half PERs
0
1
2
3
4
5
6
7
8
9
10
left
right
left
right
lemon
vanilla
Austroplebeia australis at 1 hour
Mean number of PERs and half PERs
0
1
2
3
4
5
6
7
8
9
10
left
right
left
right
lemon
vanilla

a)



b)

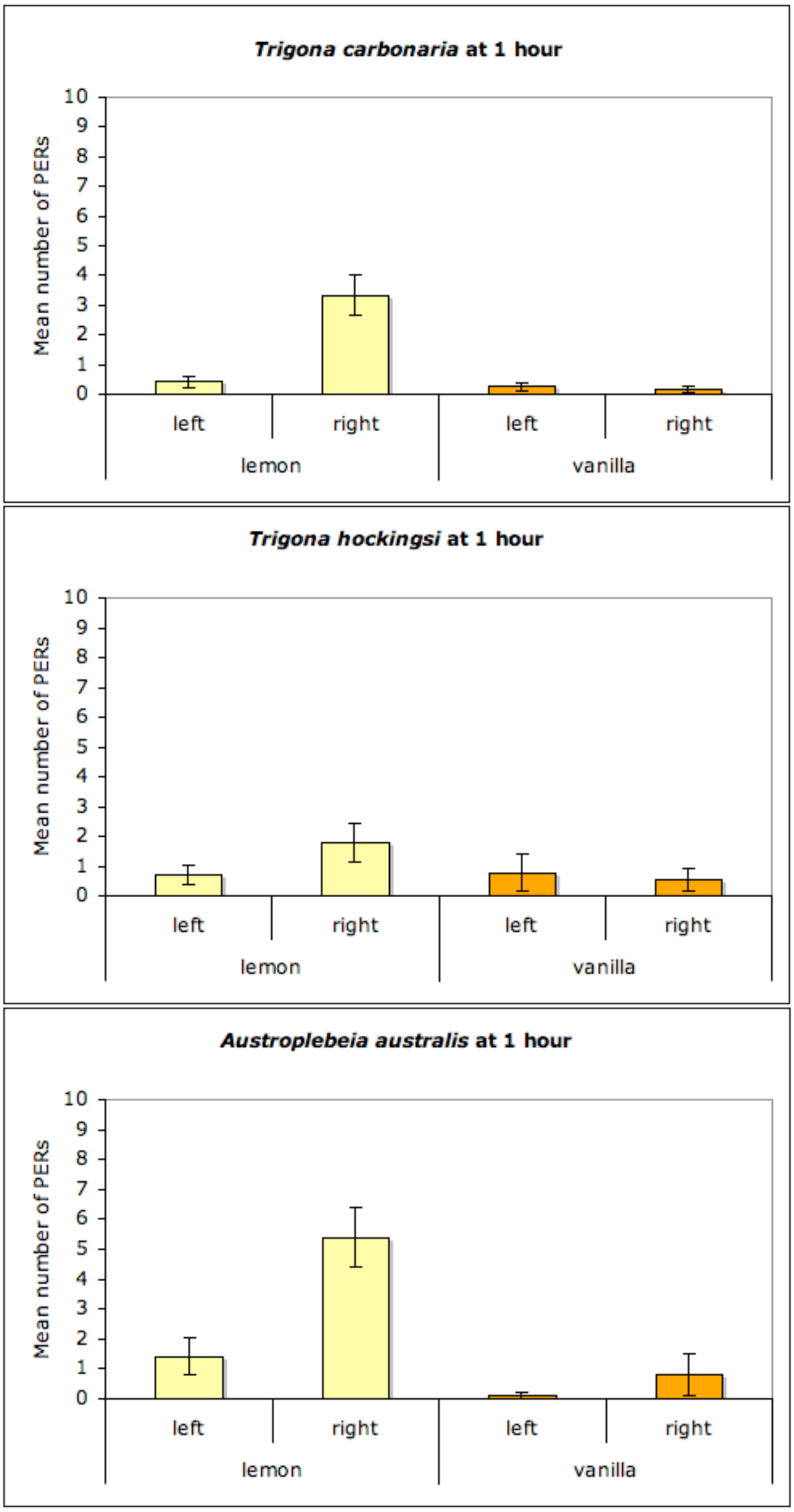
Figure 23. Recall of memory of the three Australian native stingless bees (*Trigona carbonaria, Trigona hockingsi* and *Austroplebia australis*) at a) 1 hour and b) 5 hours after training with lemon (+)/vanilla (-). All bees were trained using both antennae and tested for recall by presenting the odours on the bee's left side (left) or right side (right). The mean number (with standard error) of full

Proboscis Extension Reflex (PER) and half PERs responses (see text) to both odours (yellow for lemon and orange for vanilla) are plotted for the right and the left side of presentation.

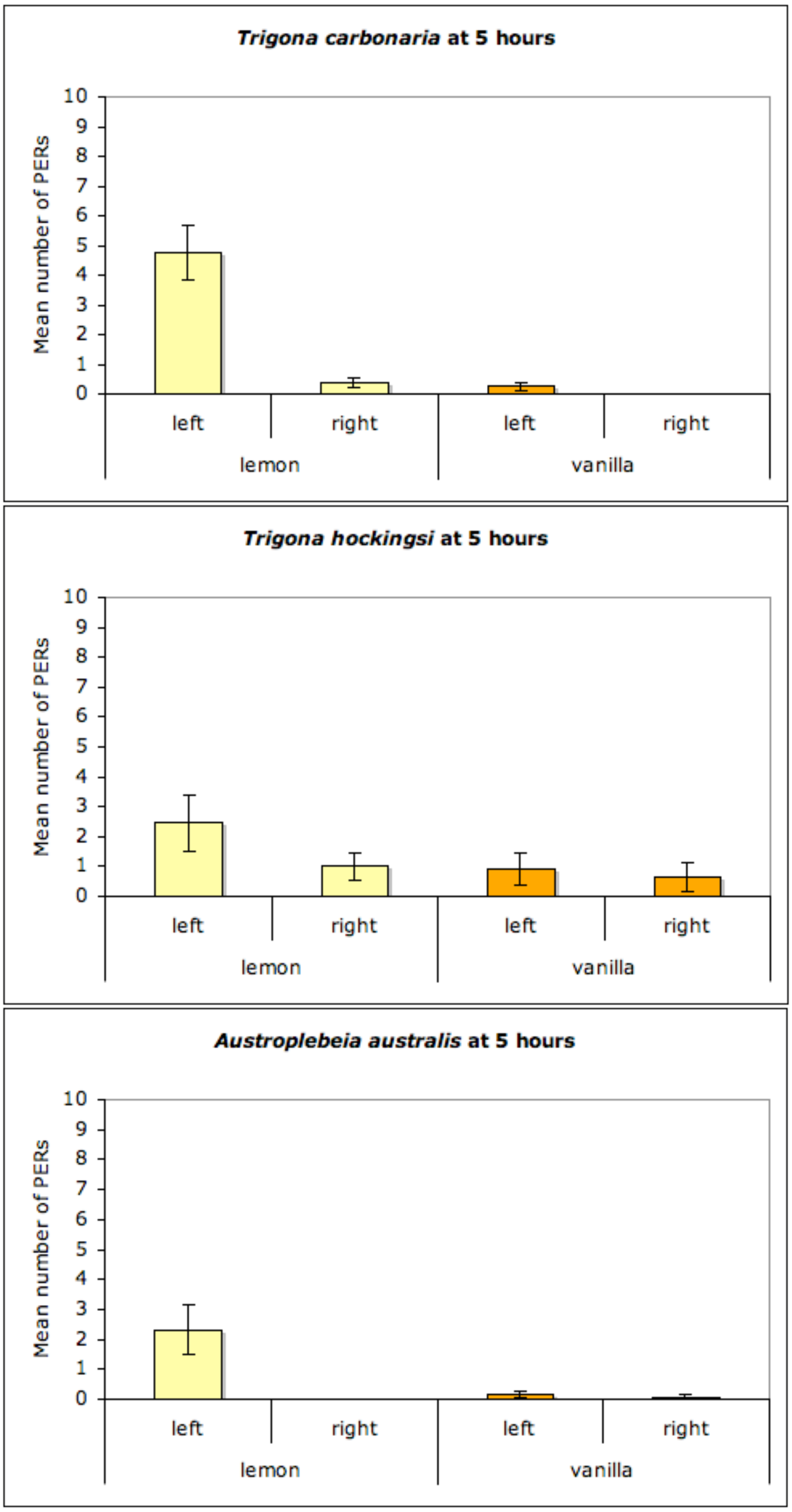
The analysis of variance showed that on recall at 1 hour stingless bees responded significantly better when the lemon odour was presented on their right side than they did when the same odour was presented on their left side (F(1,32)=69.375; p<0.0001; Figure 23a). There was a significant main effect of odour (F(1,32)=57.074; p<0.0001), due to lack of response to the vanilla odour, and a significant odour x antenna interaction (F(1,32)=27.657; p<0.0001), whereas no inter-species significant differences were apparent (F(2,32)=2.237; p=0.123). The Anova did not reveal any other significant effects (odour x species (F(2,32)=2.559; p=0.093), antenna x species (F(2,32)=0.051; p=0.950), odour x antenna x species (F(2,32)=0.444; p=0.645)). The percentage discrimination on the right side was similar in the three species (F(2,32)=0.882; p=0.424).

On recall at 5 hours, on the other hand, the three species of Australian stingless bees performed significantly better when the lemon odour was presented on their left side than when it was presented on their right side (F(1,29)=51.349; p<0.0001; Figure 23b). There was a significant main effect of odour (F(2,29)=79.785; p<0.0001), due to non-response to the vanilla odour, and a significant main effect of species (F(2,29)=10.254; p<0.0001), due to lower responsiveness in *A. australis* compared to the other two species. Significant interactions were found between odour x antenna (F(1,29)=35.200; p<0.0001) and odour x antenna x species interaction (F(2,29)=8.653; p=0.001). The latter was due to the percentage discrimination on the left side differing between the three species (F(2,29)=10.056; p<0.0001): *T. carbonaria* and *A. australis* discriminated between lemon and vanilla (80% and 100% respectively) but *T. hockingsi* discriminated less well between the two odours (65%). No odour x species (F(2,29)=4.001; p=0.029) nor antenna x species (F(2,29)=0.022; p=0.979) interactions were observed.

Considering only the number of full PER, a significant difference in the number of responses to lemon between the right and the left antenna at both 1 hour and 5 hours after training was observed. The same results were found as above for PER and ½ PER, although slightly less strongly (Figure 24).


Trigona carbonaria at 1 hour
Mean number of PERs
0
1
2
3
4
5
6
7
8
9
10
left
right
left
right
lemon
vanilla
Trigona hockingsi at 1 hour
Mean number of PERs
0
1
2
3
4
5
6
7
8
9
10
left
right
left
right
lemon
vanilla
Austroplebeia australis at 1 hour
Mean number of PERs
0
1
2
3
4
5
6
7
8
9
10
left
right
left
right
lemon
vanilla

a)



b)

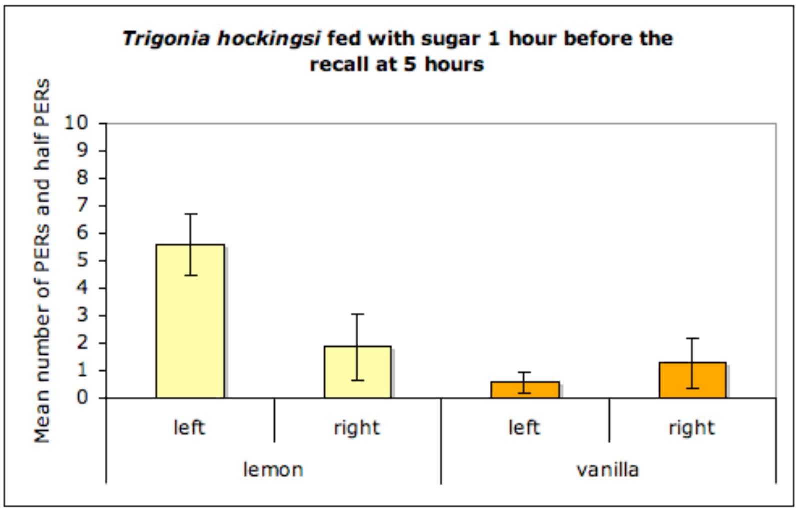
Figure 24. Recall of memory of the three Australian native stingless bees (*Trigona carbonaria*, *Trigona hockingsi* and *Austroplebia australis*) at a) 1 hour and b) 5 hours after training with lemon (+)/vanilla (-). All bees were trained using both antennae and tested for recall by presenting the odours on the bee's left side (left) or right side (right). The mean number (with standard error) of full

Proboscis Extension Reflex (PER) responses to both odours (yellow for lemon and orange for vanilla) are plotted for the right and the left side of presentation.

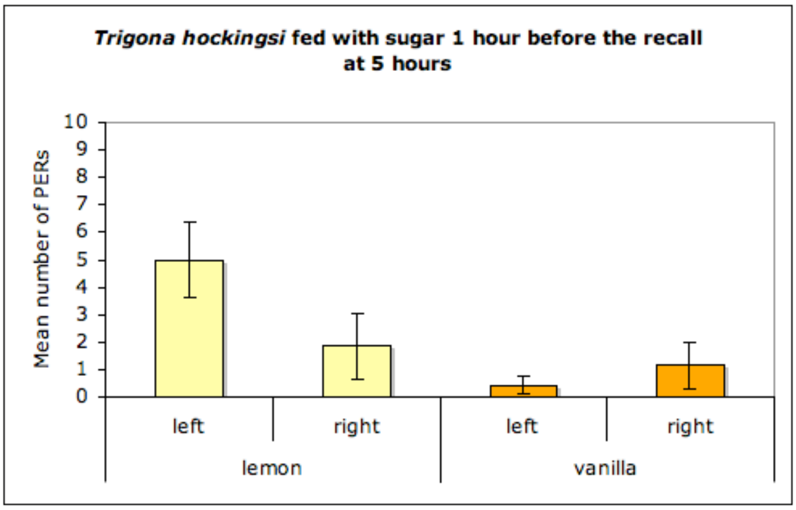
The analysis of variance revealed that on recall at 1 hour the stingless bees responded significantly better when tested on their right than on their left side (F(1,32)=32.114; p<0.0001; Figure 24a). There was a significant main effect of odour (F(1,32)=42.924; p<0.0001), and significant odour x antenna interaction (F(1,32)=39.634; p<0.0001). No main effect of species was observed (F(2,32)=1.410; p=0.259) but there was a significant odour x species interaction (F(2,32)=4.057; p=0.027) due to *A. australis* giving more full PER responses to lemon than did the other two species. In fact, *T. hockingsi* gave very few full PER responses to lemon. The Anova did not reveal any other significant interactions (antenna x species: F(2,32)=3.127; p=0.057; odour x antenna x species: F(2,32)=2.563; p=0.093). The percentage discrimination on the right side did not differ significantly between the three species (F(2,32)=2.505; p=0.098).

On recall at 5 hours the three stingless species of bees performed significantly better when tested on the left than on the right side (F(1,29)=21.343; p<0.0001); Figure 24b). There was a significant main effect of odour (F(1,29)=46.860; p<0.0001), a significant odour x antenna interaction (F(1,29)=35.047; p<0.0001), a significant odour x species interaction (F(2,29)=4.637; p=0.018) and a significant odour x antenna x species (F(2,29)=3.876; p=0.032) interaction; no significant main effect of species (F(2,29)=1.740; p=0.193) or antenna x species interactions (F(2,29)=1.862; p=0.173) were observed. The three-way interaction was caused by greater responsiveness by *T. carbonaria* to lemon odour presented on the left than was the case for the other two species. The percentage discrimination on the left side differed slightly significantly among the three Australian stingless species of bees (F(2,29)=3.582; p=0.041): *T. hockingsi* was less able to discriminate lemon from vanilla than were the other two species.

The results of the control experiment conducted on *T. hockingsi* bees fed with sugar 1 hour before the recall test at 5 hours after training are shown in Figure 25.



a)



b)

Figure 25. Recall of memory at 5 hours after training with lemon (+)/vanilla (-) of *Trigona hockingsi* bees fed with sugar one hour before the recall test. All bees were trained using both antennae and tested for recall by presenting the odours on the bee's left side (left) or right side (right). The mean number (with standard error) of a) full Proboscis Extension Reflex (PER) and half PERs responses; b) full Proboscis Extension Reflex (PER) only are plotted for the right and the left side of presentation.

Considering both full PER and ½ PER responses (Figure 25a), there was a significant main effect of odour (F(1,6)=49.892; p<0.0001), but not of antenna (F(1,6)=4.679; p=0.074 n.s.), and a significant odour x antenna interaction (F(1,6)=23.543; p=0.003). There was a significant main effect of the antenna (F(1,6)=10.419; p=0.018) on presentations of lemon odour but not vanilla odour (F(1,6)=2.323; p=0.178). The bees responded to lemon, but not vanilla, on their left side but not on their right side.

Comparing the performance at the recall at 5 hours of the *T. hockingsi* bees of the main experiment with that of the *T. hockingsi* bees of the control experiment, the Anova with 'experiment' as a between-subjects factor revealed no significant main effect of the experiment (F(1,16)=0.257; p=0.619), a significant main effect of odour (F(1,16)=28.549; p<0.0001) and antenna (F(1,16)=10.208; p=0.006), a significant odour x antenna interaction (F(1,16)=12.335; p=0.003) and odour x antenna x experiment (F(1,16)=8.006; p=0.012) interaction. The percentage discrimination on the left side differed significantly between the two groups of *T. hockingsi* bees (F(1,16)=8.624; p=0.010): the bees fed with sugar (without lemon or vanilla odour) 1 hour before the recall test discriminated better than the *T. hockingsi* bees not fed with sugar. No significant odour x experiment (F(1,16)=0.637; p=0.437) or antenna x experiment interactions (F(1,16)=0.637; p=0.437) were apparent.

When only full PER were considered (Figure 25b), the Anova revealed a significant main effect of odour (F(1,6)=21.779; p=0.003), but not of antenna (F(1,6)=2.252; p=0.184 n.s.). There was a significant odour x antenna interaction (F(1,6)=11.696; p=0.014) and a trend for a significant antennal effect was observed for lemon (F(1,6)=5.497; p=0.057) but not for vanilla (F(1,6)=2.400; p=0.172). Considering only the full PER responses, we compared the performance on recall at

5 hours of the *T. hockingsi* bees of the main experiment with that of the *T. hockingsi* bees of this control experiment. The Anova with 'experiment' as between-subjects factor revealed no significant main effect of the experiment (F(1,16)=0.824; p=0.377), a significant odour (F(1,16)=22.733; p<0.0001) and a significant odour x antenna interaction (F(1,16)=12.772; p=0.003). This was due more full PER to the lemon odour on the left following feeding with sugar 1 hour prior to testing than in the bees not fed during the interval between training and testing. In fact, comparison of Figures 24b and 25b shows that, following feeding with sugar, the performance of *T. hockingsi* is similar to that of *T. carbonaria* not fed sugar. No significant main effect of antenna (F(1,16)=2.871; p=0.110), odour x experiment interaction (F(1,16)= 3.441; p=0.082), antenna x experiment interaction (F(1,16)=0.109; p=0.745) or odour x antenna x experiment interaction (F(1,16)=4.410; p=0.052) was revealed.

As for the PER and ½ PER responses the performance at the recall at 5 hours of the two groups of *T. hockingsi* bees was compared. In this case the percentage discrimination on the left side was similar between the two groups of *T. hockingsi* bees (F(1,16)=2.650; p=0.123).

3.6.3. Discussion

Australian stingless bees showed lateralized behaviour in recall of olfactory memory. At 1 hour after training the bees demonstrated that they had learned the association of lemon with the sugar reward and responded to lemon mainly when using their right antenna. In contrast, recall at 5 hours after training was better when the odour was presented to the left than to the right side of the bees. This result is the same as that reported previously for honeybees, *Apis mellifera* (Rogers and

Vallortigara, 2008; Frasnelli et al., 2010a). Hence, these findings add to the growing number of invertebrate species showing population-level lateralization and confirm that neural circuits accessed by the right antenna are able to recall short-term memory of odours, whereas circuits accessed by the left antenna recall long-term memories of odours.

During recall at 1 hour, all the three species respond mainly with PER to lemon on the right. If PER plus ½ PER are compared to PER only responses, species differences are apparent. *T. hockingsi* bees performed as many ½ PER responses as the other species, showing that they remembered the odour, but they performed fewer full PER responses compared to the other two species. It seems that, despite their ability to recall the learnt memory, they were less motivated to feed. In fact, *A. australis* was the species most likely to give full PER to lemon odour at 1 hour after training. Note that *T. hockingsi* has the most tropical distribution and suggest that it might more responsive to higher concentrations of odour since odours are usually more pungent in tropical regions. *T. hockingsi* bees also discriminated between the lemon and vanilla odours somewhat less well than the other two species, which could indicated that they required more training to learn the discrimination than did the other two species. This can now be tested. In fact, differences in learning ability have been observed in some species of Brazilian stingless bees (Mc Cabe et al., 2007) and may be due to differences in foraging and recruiting strategies.

At 5 hours after training the data for all the three species responded to lemon only on the left side, showing that the recall of long-term olfactory memory takes place via neural pathways connected to the left antenna. Also, on recall at 5 hours *T. hockingsi* bees were less responsive than *T. carbonaria* bees and so were *A. australis* bees. As in recall at 1 hour, at 5 hours *T. hockingsi* bees discriminated lemon form vanilla less well than did *T. carbonaria* and *A. australis* bees: the percentage discrimination between the odours on the left side at 5 hours for *T. hockingsi* was significantly less than that of the other two species. Interestingly, this lower responsiveness and reduced ability to discriminate between the odours in *T. hockingsi* disappeared if the bees were fed odourless sugar 1 hour prior to testing recall at 5 hours after training. It seems, therefore, that the performance of *T. hockingsi* deteriorates due to lack of sugar. Since it is a tropical species, it may depend on more frequent intact of sugar.

T. hockingsi bees fed with sugar 1 hour before recall at 5 hours performed PER only to presentations of lemon odour on the left side. Hence, the shift from right antennal recall and 1 hour to left antennal recall at 5 hours is not due the difference in the duration of food deprivation but, instead, it is a difference resulting from short- versus long-term memory.

As stated at the beginning of this Section 3.6., the geographic distribution and fossil record support the hypothesis of greater antiquity for Meliponinae (stingless bees) compared to Apidae, Bombinae and Euglossinae, and that Meliponinae presents a considerably remote relationship or possibly no direct relationship to the other Apidae (Camargo and Pedro, 1992). Thus, it is generally believed that the honeybees did not evolve from the stingless bees but rather independently from some other (asocial) bee type and that any social features the two lines of evolution now share are the result of convergent rather than divergent evolution. Thus, the findings that stingless bees (Meliponinae) have the same laterality as honeybees (Apinae), suggest that lateralization evolved prior to the evolutionary divergence of these groups or that it evolved separately in each line (i.e. was convergent and evolved in association with social behaviour). Honeybees and stingless bees are the only highly social bees, with large perennial colonies, morphologically distinct worker and queen castes, and an intricate division of labour and recruitment to food sources (Roubik, 1989). Some recent theoretical models (Vallortigara and Rogers, 2005; Ghirlanda and Vallortigara, 2004; Ghirlanda et al., 2009) have argued that social behaviour may have been instrumental in forcing the evolution of individual-level asymmetries towards their alignment at the population-level. Empirical data in vertebrates (Bisazza et al., 2000) and invertebrates (Anfora et al., 2010) seem to support this hypothesis. Thus, it is not unlikely that the basic similarity found here between honeybees and the three Australian stingless bees species in terms of population-level lateralization is linked with the social features shared by the two taxa.

Chapter 4 - Discussion

In Chapter 2 (Ghirlanda et al., 2009), the evolution of lateralization has been investigated from a theoretical point of view, extending the previous results on interspecific interactions (Ghirlanda and Vallortigara, 2004). Considering purely strategic factors arising from intraspecific interactions of cooperation and competition, the model demonstrates that populations consisting of left- and right-type individuals in unequal numbers can be evolutionary stable.

To verify the hypothesis predicted by models based on evolutionary stable strategy (Ghirlanda and Vallortigara, 2004; Ghirlanda et al., 2009) that population level lateralization is more likely to evolve in social species rather than in solitary species, I approach the issue of the evolution of (population level) lateralization from an experimental point of view. In Experiment 1 (Section 3.3. – Anfora et al., 2010) the lateralization in two species of Hymenoptera, the honeybees, which are a social species and the mason bees, which are a solitary species was investigated through behavioural studies (PER) and electrophysiological measurements (EAG) and compared. Results showed a striking right antenna advantage in recall of olfactory memory using PER in honeybees, confirming and extending the findings of Letzkus et al. (2006) and Rogers and Vallortigara (2008). EAG results demonstrated different responsiveness of olfactory receptor neurons on the right antenna compared to those on the left antenna, suggesting that the asymmetry found in the behavioural tests may arise, in part, from an electrophysiological difference. In contrast, mason bees, a solitary species related phylogenetically to honeybees, showed no evidence of being asymmetric at the population level, either in behaviour or in EAG responsiveness, but interestingly they were found to be lateralized at the individual level in the responsiveness of the olfactory receptors neurons either on the right antenna or on the left antenna. These results confirm that left-right asymmetries at the population level are apparent in some but not all invertebrate species, and not even in species that are strictly related phylogenetically. Moreover these differences between the two species, even if should be interpreted with caution, fit in well with mathematical models of the evolution of lateralization. The fact that mason bees (solitary species) show evidence of individual-level lateralization in EAG responses but not population-level (directional) lateralization as honeybees (social species) do fits nicely with mathematical modelling (Ghirlanda and Vallortigara, 2004; Ghirlanda et al., 2009). As already discussed (Section 3.3.3.) note that olfactory learning and electroantennographic responsivity are not obviously social in nature although we cannot exclude that the original drive for antennal asymmetries could be related to social interaction during for example trophallaxis, that is the transfer of food or other fluids among members of a community. On the other hand, it is likely that when an individual-level asymmetry is stabilized as a directional population-level asymmetry, other asymmetries that in principle would not require any alignment at the population level because irrelevant to any social interaction would organize themselves as directional as well simply because a directional organization in the two sides of the brain already exists.

In Experiment 2 (Section 3.4. – Frasnelli et al., 2010a) the generality of the findings of Rogers and Vallortigara (2008) was tested. Recall of memory of the single odours (cineol and eugenol) showed the same pattern of lateral shift of olfactory memory recall as they had found for lemon (+) and vanilla (-). At 1 hour after training the bees discriminated the positive stimulus (cineol) from the negative stimulus (eugenol) and responded to the first one mainly when it was presented on their right side (i.e. using their right antenna), whereas at 6 hours after training they did so when it was on presented on their left side (i.e. using their left antenna). However, when trained with either a familiar appetitive odour (rose) as a negative stimulus, or with a naturally aversive odour (isoamyl acetate, IAA) as a positive stimulus, bees showed suppression of the response from both the right and the left side at 1 hour after training (likely due to retroactive inhibition) and at 6 hours responded to both odours on both sides. I argued that at 6 hours, when access to memory has completed the shift from the right to the left side, memory of these familiar odours in the left side of the brain would be present as both positive (rose)/negative (IAA) (as a result of long-term memory either biologically encoded or acquired well before testing) and negative (rose)/positive (IAA) (as a result of the long-term memory of training) stimuli, thus producing response competition. As a direct test of this hypothesis, bees were first trained with unfamiliar lemon(+)/vanilla(-) and then (16 hr later) re-trained with vanilla(+)/lemon(-); as predicted, 6 hours after re-training bees responded to both odours on both the left and right side.

In Experiment 3 (Section 3.5. – Frasnelli et al., 2010b) I verify once more that honeybees are better in responding to learned odours when trained through their right antenna rather than through their left antenna and the possible morphological difference in the number of sensilla between the right and the left antenna has been investigated extended the study of Letzkus et al. (2006). Results of the behavioural test confirm again the previous finding (Letzkus et al., 2006) that after training on the proboscis extension reflex (PER) paradigm, honeybees respond to odours better when they are trained through their right antenna. To check whether this difference is due to a difference in the number of sensilla which house the receptor cells involved in the olfactory responses, I considered a larger sample of bees compared to that used by Letzkus et al. (2006) and both antennae of each bee were surfaced from 4 different views. Furthermore, all the sensilla present on each image were counted distinctly for a total of eight sensilla types: five of which with possible olfactory function (sensilla placodea, sensilla trichodea type a and b, sensilla basiconica thick and tapered) and the other three sensilla types involved in others, not olfactory, functions (sensilla coeloconica, sensilla campaniformia and sensilla chaetica). Results showed that the number of olfactory sensilla was significantly higher on the right antenna than on the left antenna. Surprisingly, I observed for the first time that the number of non-olfactory sensilla was significantly higher on the left antenna than on the right antenna in all segments except the apex. It should also be stressed that the morphological differences between the honeybee's antennae in the number of olfactory sensilla found (Frasnelli et al., 2010b) and the EAG asymmetries (Anfora et al., 2010) seem to be quite modest in magnitude and thus cannot account entirely for the asymmetries observed in behaviour. Moreover, note that asymmetries in recall have been found to change in direction with memory consolidation (Rogers and Vallortigara, 2008; Frasnelli et al., 2010a), i.e. the left-right asymmetry in olfactory memory depends on time. Thus, probably the asymmetry in peripheral receptors observed in honeybees is part of other asymmetrical mechanisms of the olfactory pathway.

Experiment 4 (Section 3.6. – Frasnelli et al., submitted) focused on the generality and the evolutionary origins of the asymmetry in the olfactory responses found in honeybees by looking at primitive social bees, in particular at 3 species of Australian stingless bees: *Trigona carbonaria, Trigona hockingsi* and *Austroplebeia australis.* All the three Australian stingless bees species were able to associate lemon with a sugar reward and, more interesting, they showed lateralized behaviour in recall of olfactory memory as honeybees *Apis mellifera* (Rogers and Vallortigara, 2008; Frasnelli et al., 2010a). At 1 hour after training they responded to lemon mainly when using their right antenna; at 5 hours after training they responded to lemon mainly when the odour was presented to their left than to their right side. Results showed some interspecific differences. Both in recall at 1 hour and at 5 hours *T. hockingsi* bees discriminated lemon form vanilla less well than did *T. carbonaria* and *A. australis* bees. Note that interestingly, this lower responsiveness and reduced ability to discriminate between the odours in *T. hockingsi* disappeared if the bees were fed odourless sugar 1 hour prior to testing recall at 5 hours after training. It seems, therefore, that the performance of *T. hockingsi* deteriorates due to lack of sugar. Since it is a tropical species, it may depend on more frequent intact of sugar. In fact, the group of control of *T. hockingsi* bees fed with sugar 1 hour before recall at 5 hours responded only to lemon odour when presented on the left side. Thus, this demonstrates not only that the reduced ability to discriminate between the odours in *T. hockingsi* disappeared if the bees were fed with sugar, but that the shift from right antennal recall and 1 hour to left antennal recall at 5 hours is not due the difference in the duration of food deprivation but, instead, it is a difference resulting from short- versus long-term memory.

Chapter 5 - Conclusions

Summing up, it seems that invertebrates not only share the attribute of lateralization with many vertebrates, but may also show some similarities in its appearance. This raises the question of whether lateralization is determined by homologous genes in insects and vertebrates (Vallortigara and Rogers, 2005) or whether there has been an analogous evolutionary development of lateralized function in the two taxa. I think it is most likely that an analogous evolutionary development happened, but presently I am not aware of evidence in favour or against this hypothesis.

In this thesis the generality and phylogenetic origins of the antennal asymmetry found in the honeybee *Apis mellifera* (Letzkus et al., 2006; Rogers and Vallortigara, 2008; Anfora et al., 2010; Frasnelli et al., 2010a) was investigated and observed in three species of Australian stingless social bees (*Trigona carbonaria*, *Trigona hockingsi* and *Austroplebia australis*). The hypothesis of greater antiquity for Meliponinae (stingless bees) compared to Apidae, Bombinae and Euglossinae, and of the considerably remote (or possibly no direct) relationship of Meliponinae to the other Apidae is strongly supported by the geographic distribution and fossil record (Camargo and Pedro, 1992). Thus, it is likely to suppose that the honeybees did not evolve from the stingless bees but rather independently from some other (asocial) bee type and that any social features the two lines of evolution now share are the result of convergent rather than divergent evolution. Therefore, the findings of this thesis that stingless bees (Meliponinae) have the same laterality as honeybees (Apinae), suggest that lateralization evolved prior to the evolutionary divergence of these groups or that it evolved separately in each line. In other words, it seems that lateralization at the population level was convergent and evolved in association with social behaviour. It is important to stress that honeybees and stingless bees are the only highly social bees, with large perennial colonies, morphologically distinct worker and queen castes, and an intricate division of labour and recruitment to food sources (Roubik, 1989). Interestingly, the compared study conducted on mason bees, a solitary species belonging to the same superfamily (i.e. Apoidea) of the honeybees showed that mason bees are not lateralized at the population level neither in the behavioural test nor in the electroantennography measurements (Anfora et al., 2010).

The results of these studies (Anfora et al., 2010; Frasnelli et al., submitted) point out the connection between the sociality and the population level asymmetries. Indeed the empirical data fit in well with some recent theoretical models (Vallortigara and Rogers, 2005; Ghirlanda and Vallortigara, 2004; Ghirlanda et al., 2009) supporting the hypothesis that social behaviour may have been instrumental in forcing the evolution of individual-level asymmetries towards their alignment at the population-level. Thus, it is not unlikely that the basic similarity found here between honeybees and the three Australian stingless bees species in terms of population-level lateralization is linked with the social features shared by the two taxa.

As concerns the theoretical approach I have extended previous results on interspecific interactions (Ghirlanda and Vallortigara, 2004) to intraspecific interactions (Ghirlanda et al., 2009), reinforcing the view that strategic factors may have been a powerful force in the evolution of lateralization. Since a purely strategic model has been considered for simplicity, the major aim of my future research will be to consider how strategic factors interact with other potential determinants of lateralization, in particular genetic mechanisms of lateralization. Specifically, I plan to integrate our game-theoretical approach with more traditional genetic models based on research on human handedness. Theories of handedness based on the presumed existence of a single gene locus, assume that one allele predisposes to right-handedness and the other does not. The two alleles are assumed to be held in balance by heterozygotic advantage in fitness, ensuring that the proportion of left-handers cannot exceed 50%. Variation is possible governed by the relative fitness of the two homozygotic genotypes. Following McManus, there are two alleles: D specifying dextrality and C specifying change direction of handedness at the individual level. Thus there are three phenotypes: the homozygote DD, that produces right-handedness in all offspring; the homozygote CC that results in fluctuating asymmetry 50% of offspring being right handed and 50% left handed and the heterozygote CD, for which the effect of the alleles is additive producing 25% left-handers.

I plan to look in the future at the influence of synergistic and antagonistic interaction in populations composed by individuals of these three different genotypes: CC, CD and DD. The frequency of the alleles would influence the fitness of the right-type and left-type individuals and I want to investigate mathematically the sort of scenario that will arise. Sex differences in the ratio between left-handers and right-handers in humans have been documented in 63 studies founding a 27.4% higher incidence of left-handers in males than females. In 18 studies the parental and offspring handedness in relation to the sex of parents and offspring had been investigated and the so-called 'maternal effect' had been reported: left-handed mothers have more left-handed offspring than do left-handed fathers. Thus, I plan to include sex as a variable in my model. The idea would be to investigate the effects of competitive and cooperative interactions on lateralization in a population composed by females and males, assuming that competitive and cooperative behaviour may occur with different probabilities in the two sexes.

As for the empirical part, the results of this series of experiments conducted on honeybees confirm the surprising finding (see Rogers and Vallortigara, 2008) that bees learn to associate a new odour of a positive stimulus mainly in neural circuits accessed via their right antenna, and that, after a period of a few hours, memory consolidation occurs accompanied by antennal asymmetry, with bees now being able to recall the odour mainly when using their left antenna. Moreover, I showed here (Frasnelli et al., 2010a) that this particular dynamic of memory traces has severe consequences when odours are already known to the bees (either for a biological reason or as a result of previous experience) and are thus already present in the long-term memory store. As a result, response competition arising from multiple memory traces can be observed, with bees showing unexpected lack of specificity in their longer-term olfactory memories.

The behavioural finding that honeybees are better in learning to associate odours with a sugar reward when they are trained through their right antenna may be partially explained by the stronger responsiveness of the olfactory receptor neurons inside the right antenna as revealed by the electroantennography measurements (Anfora et al., 2010). This in turn may be somewhat due to the higher number of the olfactory sensilla present on the right antenna compared to the left one (Frasnelli et al., 2010b). Surprisingly, I observed for the first time that the number of non-olfactory sensilla was significantly higher on the left antenna than on the right antenna in all segments except the apex. The functional significance of this opposing morphological asymmetry is at present unclear but behavioural asymmetries in other sensory domains appear to be worth testing. It is worth of noting that in a previous study on an another eusocial Apidae species, *Apis florae* Fabr., the olfactory sensilla were shown to be slightly more abundant on the left antenna (Gupta, 1986). Thus, comparative works on different species are needed in order to understand the generality and the significance of antennal morphological asymmetries in Apidae.

The fact that the right antenna has more olfactory sensilla and a stronger electroantennographic signal may explain the reason because it is involved in odour learning. Since the behavioral asymmetry in the recall of olfactory memory shift from the right to the left olfactory pathway, it is likely to suppose that probably the asymmetry in peripheral receptors observed is part of other asymmetrical mechanisms associated with detection, storage and retrieval of olfactory events. It will be therefore important to look at the morphological and physiological processes going on in the honeybee left and right olfactory brain centers (i.e. antennal lobes and mushroom bodies) following olfactory learning at different times during memory consolidation.

Moreover, it will be very remarkable to perform comparative research with other species of Hymenoptera showing different degrees of sociality, both in the field and in the laboratory that may provide important insights on the evolution of left-right asymmetries in behaviour and in the nervous system. For example, behavioural studies, electroantennographic measurements and scanning electron microscopy in a primitively eusocial bumble bees, *Bombus spp.* could provide more information on the link between the evolution of population level asymmetries and the degree of sociality. Furthermore, it will be very interesting to study species of insects that could change their degree of sociality on the basis of ecological factors, like for example the locust *Schistocerca gregaria*.

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