# Experience-dependent plasticity in brain structure and olfactory learning capacities in honey bees (*Apis mellifera*).

Amélie Cabirol

BSc, MSc

*This thesis is submitted for the degree of Doctor of Philosophy at*

*Macquarie University*

Department of Biological Sciences

&

*Université Toulouse 3 Paul Sabatier*

Centre de Recherches sur la Cognition Animale

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# Table of contents

# Summary

Learning capacities, and the structure of the brain centres supporting them, vary greatly between individuals, partly due to different life experiences. In honey bees, experience-dependent plasticity has been reported in multisensory brain centres involved in learning and memory: the mushroom bodies (MBs). The consequences of such plasticity on learning performances are still unknown. The aim of my thesis was to examine the relationships between experience, learning capacities and MB organization in honey bees. The age-related division of labour in honey bees gave me the opportunity to study experience-dependent plasticity both in young bees working inside the hive, and in older bees foraging outdoors.

I first observed that bees exposed to a sensory-impoverished environment for the first days of adulthood had a higher number of synaptic boutons in the MBs, and a reduced performance in a MB-dependent learning task; reversal learning. This suggests the occurrence of experience-dependent synaptic pruning in the natural environment, which improves learning capacities.

I observed similar effects of environmental enrichment when the bees started foraging. Foraging onset was accompanied by a decrease in the number of synaptic boutons in the MBs, as well as by an improvement in reversal learning performance. Prolonged foraging activity, however, had the opposite effects, especially when a stress applied to the colony induced bees to forage earlier. Therefore, I highlighted a negative relationship between the number of synaptic boutons in the MBs and performance in reversal learning.

I then confirmed the negative impact of foraging activity on learning capacities using a different MB-dependent task; positive patterning. I revealed the involvement of the cholinergic signalling pathway in this experience-dependent cognitive decline.

This thesis presents the first integrated analyses of experience-dependent plasticity in both brain structure and cognitive capacities in honey bees. It helps to understand the mechanisms linking synaptic connectivity to learning performances, and will encourage further studies on the role of environmental stressors in the reported cognitive decline in foragers.

# Declaration

I certify that this thesis entitled « Experience-dependent plasticity in brain structure and olfactory learning capacities in honey bees (*Apis mellifera*) » has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree to any other university or institution other than Macquarie University and Université Toulouse 3 Paul Sabatier (as per the cotutelle agreement).

I also certify that the thesis is an original piece of research and it has been written by me. Contributions of co-authors have been appropriately acknowledged, and so has any assistance that I have received in my research.

I certify that all information sources and literature used are indicated in the thesis.

As this research was on invertebrates, the research presented was not subject to approval by the Macquarie University Ethics Review Committee.

Amélie Cabirol (29/06/17)

# Preface

The chapters of this thesis constitute an ensemble of papers ready for submission, submitted, accepted or published in peer-reviewed journals. Therefore, some repetitions were unavoidable, but I still consider this format to be the more efficient to highlight my work.

**Publications included in this thesis**

Cabirol A., Brooks R., Groh C., Barron AB., Devaud JM. (accepted). Experience during early adulthood shapes the learning capacities and the number of synaptic boutons in the mushroom bodies of honey bees (*Apis mellifera*). *Learning & Memory*

- Included as Chapter 2.

    CA and DJM designed the experiment – CA conducted the experiment – CA and BR analysed the data – CA, GC, BAB and DJM wrote the paper.

Cabirol A., Cope AJ., Devaud JM., Barron AB. (submitted). Evidence for a relationship between brain structure and cognitive and foraging performance in honey bees. *Proceedings of the National Academy of Sciences*

- Included as Chapter 3.

    CA, DJM and BAB designed the experiment – CA conducted the experiment – CA and BAB analysed the data – CAJ developed the model – CA, CAJ, DJM and BAB wrote the paper.

Klein S.*, Cabirol A.*, Barron AB., Devaud JM., Lihoreau M. (2017). Why bees are so vulnerable to environmental stressors. *Trends in Ecology and Evolution* 32, 268-278

[*Co-first authors]

- Included as Chapter 5.

    CA, BAB and DJM reviewed the behavioural and brain literature – KS, BAB and LM reviewed the behavioural and ecological literature – KS and CA wrote the first version of the review which was then corrected and improved by all authors.

**Conference presentations during candidature**

Cabirol A., Barron AB., Devaud JM. (2016) Experience during adult brain maturation is required for the development of complex cognitive capacities in honey bees. *Poster*

*presentation delivered at the International Society for Neuroethology congress in Montevideo, Uruguay*.

Cabirol A., Devaud JM., Barron AB. (2017) Experience-dependent variability in learning capacities and related brain plasticity in honey bees. *Oral presentation delivered at the International Heisenberg & Menzel meeting in Aspet, France*.

Cabirol A., Devaud JM., Barron AB. (2017) Experience-dependent plasticity in brain structure and learning capacities in foraging honey bees. *Oral presentation delivered at the French Club of Invertebrates Neurobiology in Dijon, France*.

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Un grand merci à mes parents, mon frère, ma sœur et toute ma famille, pour tout le soutien, l'éducation et l'amour que vous m'avez apporté! Je remercie également Hervé et Véronique pour leur soutien, même lorsque j'ai dû m'éloigner de leur fils le temps de deux hivers pour cette thèse.

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Last but not least, I thank Anthony. A few words are not enough to explain how important your continuous support and love were during these three years. Thank you for understanding my passion for science, thank you for everything!

# Abbreviations

Ach: Acetylcholine

AL: Antennal lobe

EN: Extrinsic neuron

GABA: γ-aminobutyric acid

GNG: Gnatal ganglion

IN: Input neuron

IS: Inversion score

KC: Kenyon cells

LH: Lateral horn

MB: Mushroom body

NGS: Normal goat serum

OL: Optic lobe

PBS : Phosphate buffer saline

PCT : Protocerebral tract

PER: Proboscis extension response

PN: Projection neuron

RFID: Radio frequency identification

SRS: Sucrose response score

Tx : Triton X-100

WRS: Water response score

# Chapter 1.

General introduction

## Experience-dependent plasticity in learning capacities and brain structure

Individuals' learning abilities can be greatly influenced by their previous experiences. Positive experiences, such as environmental enrichment, and negative experiences, such as stressful events, have opposite effects on cognitive capacities (Wright and Conrad 2008). Frequently, enriching the environment of laboratory-reared animals, with sensory-motor and social stimulation, increases animals' learning and/or memory performance in a range of different learning tasks (*crickets*: Mallory et al. 2016; *cuttlefishes*: Dickel et al. 2000; *fishes*: Salvanes et al. 2013; *rodents*: Simpson and Kelly 2011; Leger et al. 2015). Stressful experiences, on the contrary, frequently compromise these capacities (*fishes*: Gaikwad et al. 2011; *rodents*: Cazakoff et al. 2010; Hurtubise and Howland 2016).

The impact of such experiences on brain structures involved in learning processes are thought to play a major role in the observed inter-individual variability in cognitive capacities (Kanai and Rees 2011; Kolb and Gibb 2013). Different specific experiences are often associated with synaptic turnover in the brain, with some synapses being formed and others pruned as a result of information storage (Caroni et al. 2012). In mice this synaptic turnover is enhanced by environmental enrichment, resulting in improved learning and memory (Bednarek and Caroni 2011). Enriched environments are also associated with increased brain size, dendritic arborisation and neurogenesis in rodents and humans (van Praag et al. 2000; Clemenson et al. 2015). The effects of stressful experiences on brain structure are more varied since they can induce both dendritic retraction and branching, depending on the brain region and the gender of individuals (Leuner and Shors 2013).

Whether and how the volume and synaptic connectivity of specific brain regions determine learning performance remains, however, poorly understood (Kanai and Rees 2011). Through this thesis, I have shown that honey bees (*Apis mellifera*) can be a relevant model system with which to study experience-dependent brain plasticity and cognitive variability. The neural bases of learning and memory in this insect have been intensively investigated (Giurfa 2007; Menzel 2012), and neuroimaging techniques have been developed to access synaptic connectivity and ultrastructure in the bee brain (Groh et al. 2012). Honey bees naturally have a rich behavioural repertoire and show excellent learning and memory capacities (Robinson 1992b; Giurfa 2015). Experience-dependent plasticity of brain regions related to such capacities has been documented in this species (Withers et al. 1993a; Farris et al. 2001; Krofczik et al. 2008). Last but not least, understanding the impact of environmental stressors on experience-dependent brain and behavioural plasticity in honey bees is critical in a context of current declines in pollinator populations.

## Honey bees experience a naturally complex and stressful environment

Honey bees live in a naturally complex social environment. Honey bee society is structured by a division of labour, with groups of specialised individuals performing different tasks inside or outside the colony (Robinson 1992b). A honey bee's specific experience and behavioural development is influenced by two factors: (1) division of labour is age-related, each worker progresses through different roles during its life in a predictable sequence, and (2) this pattern of division of labour is socially modulated according to the needs of the colony (e.g. amount of brood, food availability in the field, predation) (Robinson 1992b; Huang et al. 1996; Pankiw 2004).

After emerging from a pupal cell as an adult, honey bees typically perform duties inside the hive such as cell cleaning and then brood caring (nursing) (Seeley and Kolmes 1991). In the hive, they encounter an environment rich in meaningful olfactory stimulation whether it be pheromonal signals from conspecifics (e.g. brood and queen pheromones) (Slessor et al. 2005) or odorants from the food gathered by foragers (nectar, pollen) (Grüter et al. 2006). Later in life, honey bees switch to outside duties (Seeley and Kolmes 1991). They first perform short-distance orientation flights around the hive to learn the surrounding landmarks and thereby be able to locate their hive in the environment (Capaldi et al. 2000; Degen et al. 2016). They then start to forage on flowers. Thus, during the transition from indoor to outdoor activities, bees are considered to experience an enrichment of their environment as they encounter new visual and spatial information (landscape, flower cues) and new olfactory stimuli (floral aromas), together with intense physical activity (Winston 1987).

In parallel to this environmental enrichment, honey bees also have to deal with different stressors present in the foraging environment (Potts et al. 2010b; Vanbergen et al. 2013). These include natural stressors, such as predators and weather conditions (Tan et al. 2013), but also human-induced stressors such as pesticides and habitat fragmentation (Morimoto et al. 2011; Goulson et al. 2015). Importantly, old bees exhibit physiological senescence and are less resistant to stress (Remolina et al. 2007; Williams et al. 2008). As mentioned above, changes in the colony needs and demography influence the age at which bees switch from one task to the other (Robinson 1992b; Huang et al. 1996). In case of a loss of foragers, for instance, young bees start to forage earlier. Such precocious foraging, which can be induced by a wide range of environmental stressors (Goblirsch et al. 2013; Perry et al. 2015), affects bees' spatial memory and survival, and is thought to place a colony at risk of failure (Perry et al. 2015; Ushitani et al. 2016). It is therefore important to assess how the richness of a natural environment and the stressors it contains interact to affect the brain and learning capacities of honey bees.

## Honey bees as a model to study the neural bases of olfactory learning

The honey bee has been established as a model system for studying learning, memory and cognition (Giurfa and Menzel 2001). As central-place foragers, honey bees must navigate between the different food sources and the colony, and optimise routes between those locations (Buatois and Lihoreau 2016). In addition, they are able to discriminate between different flowers as not all of them provide nectar or pollen. Bees tend to show floral constancy: they forage on the same floral species as long as it provides profitable food (Grant 1950). As the environment is constantly changing in terms of resource location and availability, however, honey bees must adapt their foraging behaviour and therefore demonstrate flexibility in learning processes (Menzel 1999).

The ability to learn and discriminate between olfactory cues can be easily studied in the laboratory using the olfactory conditioning of the Proboscis Extension Response (PER): an appetitive Pavlovian conditioning established by Takeda more than 50 years ago (Takeda 1961). The PER is triggered by touching the bee's antennae with sucrose solution (unconditioned stimulus) **(Figure 1.1A)**. In olfactory conditioning of the PER, bees are sequentially presented with a neutral odour (conditioned stimulus) and a sucrose reward with a temporal overlap between the two stimulations. They are allowed to sip the sucrose briefly when they extend the proboscis. Once the odour is learned as positively reinforced, subsequent presentation of the odour alone is sufficient to trigger the PER.

Ambiguities can be introduced into the conditioning protocol, therefore increasing the task complexity. In a reversal learning paradigm, for instance, there is a temporal ambiguity between two learning phases in which bees must learn opposite rules (Bitterman et al. 1983) **(Figure 1.1B)**. In the first phase, an odour A is reinforced with sucrose, but not an odour B (A+B-), and in the second phase, bees must reverse their responses as the sucrose reinforcement becomes associated with the odour B and not with the odour A anymore (A-B+). In another task, positive patterning, the ambiguity comes from the stimuli themselves (Deisig et al. 2001) **(Figure 1.1C)**. Bees must respond to a mixture of two odours reinforced with sucrose (AB+), but not to the non-reinforced components of the mixture when presented alone (A-/B-).

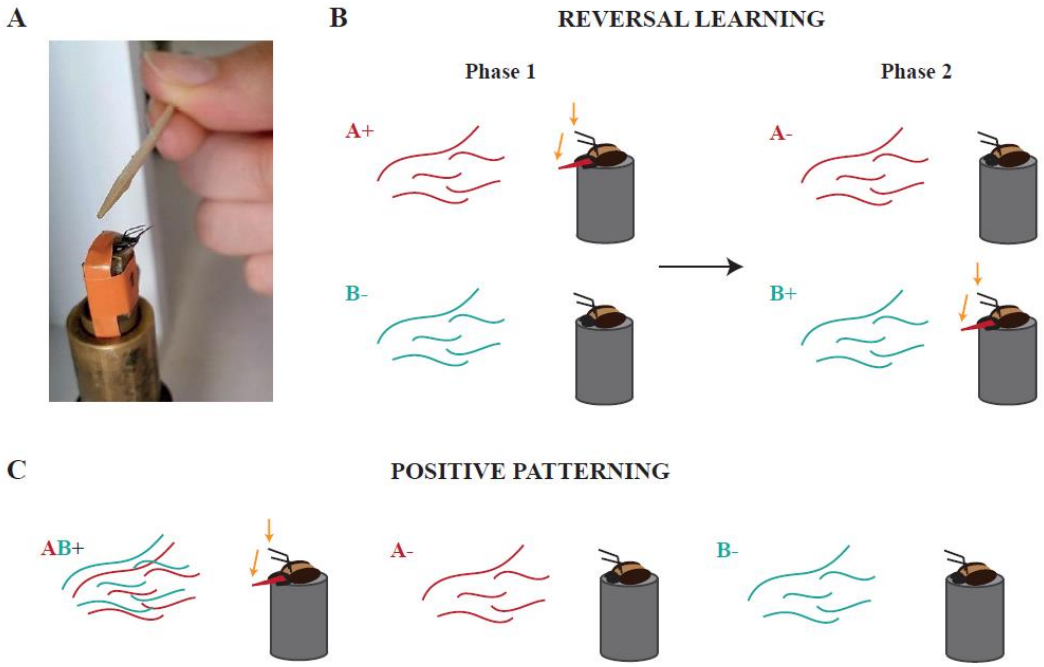


**Figure 1.1. Ambiguous learning tasks based on the olfactory conditioning of the Proboscis Extension Response (PER). A:** Photograph of a bee extending the proboscis in the conditioning set-up in response to a stimulation of its antennae with a toothpick soaked in sucrose solution (photo courtesy of Amelie Noël). **B:** Schematic representation of the reversal learning paradigm. In phase 1, the bee is presented either with an odour A reinforced with sucrose, or with a non-reinforced odour B (A+B-). In phase 2, the odour B is reinforced, but not odour A anymore (A-B+). **C:** Schematic representation of the positive patterning paradigm. The bee is presented either with a mixture of two odours A and B reinforced with sucrose (AB+), or with the individual components of the mixture presented alone without any reinforcement (A-/B-). The orange arrows represent the application of the sucrose reinforcement on the antennae first, and then on the proboscis to allow the bee to drink it.

These learning protocols, applied to restrained individuals, are compatible with various interventions to manipulate the brain **(Figure 1.2A)**, and have thereby established the honey bee as a model system for study of the neural bases of learning and memory (Giurfa and Sandoz 2012a). In the honey bee brain **(Figure 1.2B)**, olfactory information is first processed in primary sensory centres, the antennal lobes (ALs), and then transferred by projection neurons (~800 PNs) to higher-order brain centres, the mushroom bodies (MBs) and the lateral horns (LHs) (Rössler and Brill 2013). The MBs are multisensory integration centres receiving olfactory and visual information in different subregions of their neuropil (Gronenberg 2001): the lip receives olfactory information from the ALs, the collar receives visual information from the optic lobes (OLs), while the basal ring receives both olfactory and visual inputs **(Figure 1.2C)**. Within the MB neuropil, PNs connect the dendrites of multiple MB neurons, the Kenyon cells (KCs), thus forming synaptic boutons (also called microglomeruli) (Groh et al. 2012) **(Figure 1.2D)**. The

MBs also get gustatory information, about a sucrose stimulation of the proboscis for instance, from the gnathal ganglion (GNG) (Mobbs 1982). In the GNG, the VUMmx1 neuron responds to sucrose stimulation, and its activation can replace the sucrose reinforcement during olfactory conditioning of the PER (Hammer 1993). VUMmx1 connects the olfactory processing pathway at the level of the ALs, LHs and MBs (Hammer 1993; Schröter et al. 2007). It is therefore thought to mediate information about the sucrose reinforcement in olfactory learning. Although functional MBs are dispensable for learning the association between odours and sucrose reward, they are required for more complex olfactory learning tasks such as reversal learning and positive patterning (Devaud et al. 2007, 2015). *The aim of this thesis was to establish the relationship between experience, MB structure and MB-dependent learning capacities at different stages of the honey bee life, taking advantage of the natural richness of its environment.*

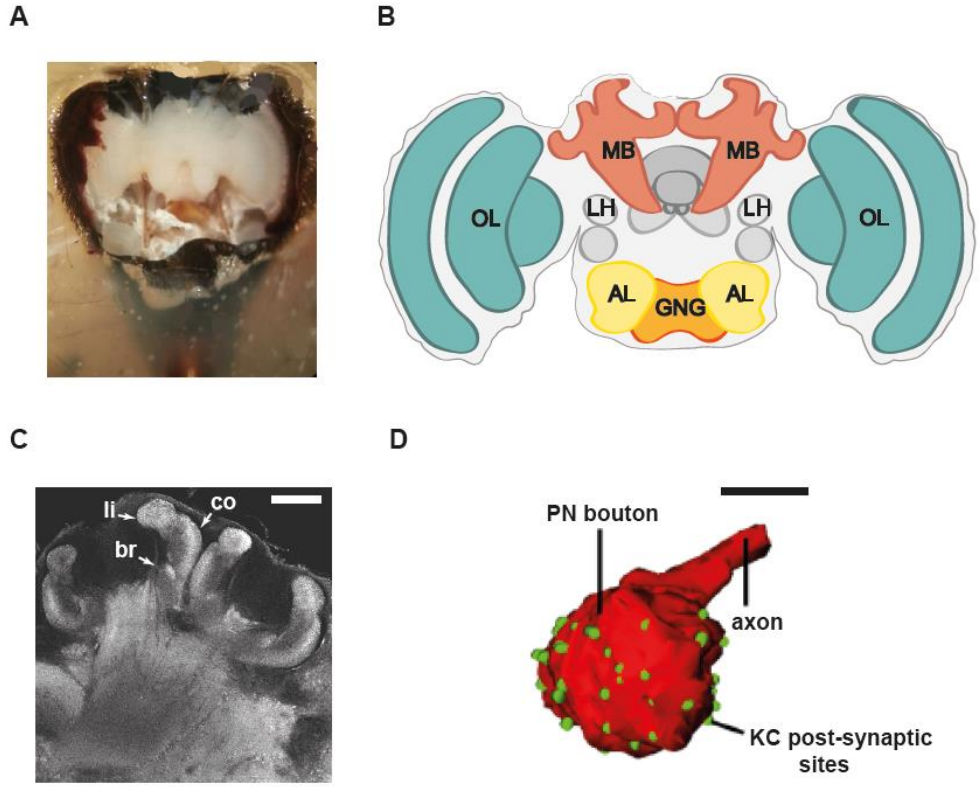


**Figure 1.2. Description of the Honey bee brain. A:** Frontal view of the brain in the head capsule. **B:** Schematic frontal view of the brain highlighting the mushroom bodies (MBs), the lateral horns (LHs), the optic lobes (OLs), the antennal lobes (ALs), and the gnathal ganglion (GNG). Adapted from Klein et al. (2017). **C:** Frontal confocal section of the left MB immunolabeled for synapsin (pre-synaptic protein) (scale bar = 100µm). White arrows indicate the lip (li), collar (co) and basal ring (br). **D:** Serial-section electron microscopy reconstruction of a projection neuron (PN) bouton (red) in the MB neuropil, connecting multiple dendrites of the Kenyon cells (KCs; green) (scale bar = 1µm). Adapted from Groh et al. (2012).

## How do learning capacities vary with experience in honey bees?

How specific experiences acquired by honey bees change their learning capacities is unclear. In the field, individuals' foraging efficiency, measured as the quantity of food they collect, temporarily increases after 4 days of foraging (Dukas and Visscher 1994; Dukas 2008). These authors suggested that this improvement reflects a progressive learning by foragers of how to locate, identify and handle flowers. Among the studies comparing associative learning performances between nurses and foragers, some have indeed reported improved performance in foragers (Ray and Ferneyhough 1999; Scheiner et al. 2017). Others, by contrast, observed a cognitive decline in foragers, based on assessments of learning performance using PER conditioning protocols (Ben-Shahar et al. 2000; Behrends et al. 2007; Scheiner and Amdam 2009; Münch et al. 2013). This discrepancy may be due to differences in the age and experience of the tested bees. Indeed, if, as suggested by Dukas (2008), there is a transient improvement in learning performance after a few days of foraging, the foraging experience of bees sampled for the learning task in laboratory conditions might have a critical impact on the resulting comparison of performance with nurses. Also, it is risky to attribute differences in appetitive learning performance between nurses and foragers to differences in cognitive capacities because the sensitivity to sucrose, which is known to affect appetitive learning (Scheiner et al. 2001, 2003), is significantly higher in foragers than nurses (Scheiner et al. 2017). However, the comparison of performance of young and old foragers in different learning tasks also suggests that foraging activity induces a cognitive decline in honey bees (Behrends et al. 2007; Münch et al. 2010).

As mentioned earlier, foraging occurs in a very rich environment, but at the same time, it is a more stressful activity. This paradox may underlie the current discrepancies in the literature regarding the consequences of foraging on learning capacities. *My hypothesis was based on the variation in foraging performance described by Dukas (2008): rich experiences such as the transition from in-hive duties to foraging, might improve learning capacities, but, on the contrary, stressors met in the foraging environment subsequently decrease learning performance. This natural variation in learning performance might be accompanied by structural changes in brain centres involved in learning processes.*

## How does mushroom body structure vary with experience in honey bees?

Behavioural flexibility of honey bees corresponds with brain plasticity, in particular in the MBs. During the first days of adulthood, the volume of the MB neuropil increases whether the bees

are reared in the natural in-hive environment or isolated in a sensory-impoverished environment (Withers et al. 1993a; Fahrbach et al. 1998). This volumetric change was suggested to reflect a programmed (experience-independent) maturation process, observed in absence of sensory or social stimulations, which would prepare the brain for the foraging task. Consistently, precocious foragers exhibit an accelerated increase in MB volume, so that they have similar MB volume values as normal-age foragers at foraging onset (Withers et al. 1993a). An increase in MB volume was also reported later in the honey bee life, during the second week of foraging (Farris et al. 2001; Ismail et al. 2006). This appears to be due mainly to dendritic arborisation, and seems to be the result of foraging experience (Withers et al. 1993a; Farris et al. 2001).

The number of synaptic boutons in both the lip and collar is lower in old foragers compared to young in-hive workers (Groh et al. 2012; Muenz et al. 2015). This suggests the occurrence of synaptic pruning that might result from increased sensory stimulation at foraging onset, as suggested by a decreased number of synaptic boutons in the collar of bees exposed to light (Scholl et al. 2014) or in the lip of leaf-cutting ants exposed to a rich olfactory environment (Falibene et al. 2015).

One of the neural pathways that has been suggested to be involved in experience-dependent MB plasticity is the cholinergic pathway. Acetylcholine (Ach) is the main excitatory neurotransmitter in the honey bee brain and mediates part of the olfactory information transferred from the ALs to the MBs (Kreissl and Bicker 1989; Oleskevich 1999). A chronic stimulation of the muscarinic receptors for Ach mimics the increase in MB volume and dendritic branching observed in foragers (Ismail et al. 2006; Dobrin et al. 2011). Consistently, activity of acetylcholinesterase (the enzyme degrading Ach) is decreased in the brain of foragers compared to nurses, suggesting greater cholinergic transmission in foragers (Shapira et al. 2001). The cholinergic pathway is a target of many pesticides present in the foraging environment (Casida and Durkin 2013). Therefore, it is important to assess the involvement of Ach in the experience-dependent cognitive changes, and to identify the link between MB structure and learning capacities.

## Thesis overview

Through my thesis, I have highlighted the relevance of the honey bee as a model to study the neural bases of experience-dependent variability in cognitive capacities. In addition to describing the effects of experience on learning performance and on the structure of the brain regions supporting them, I investigated the link between brain structure and learning

performances. In a context of pollinator decline, I also emphasized the impact of environmental stressors on the structure and function of the bee brain.

In the second chapter, I assessed whether the programmed maturation of the MBs described during the first days of adulthood was sufficient for the development of reversal learning capacities, and if, as in mammals, experience of an enriched environment (here the hive) improves learning performance.

In the third chapter, I precisely quantified individual bees' amounts of foraging experience to test the consequences of foraging on the plasticity of the MBs and the learning capacities they support, using a reversal learning task. I could therefore bridge the gap between structural and cognitive plasticity.

In the fourth chapter, I used a positive patterning task to study the effect of foraging experience on a different MB-dependent learning paradigm. I stimulated chronically the muscarinic receptors to Ach to highlight the role of the cholinergic pathway in the foraging-induced cognitive changes.

Finally, the fifth chapter provides a review of the literature (co-first author with Simon Klein) to emphasize the importance of studying the impact of environmental stressors on the brain and cognitive processes, as they are relevant to honey bee performance, longevity and colony survival.

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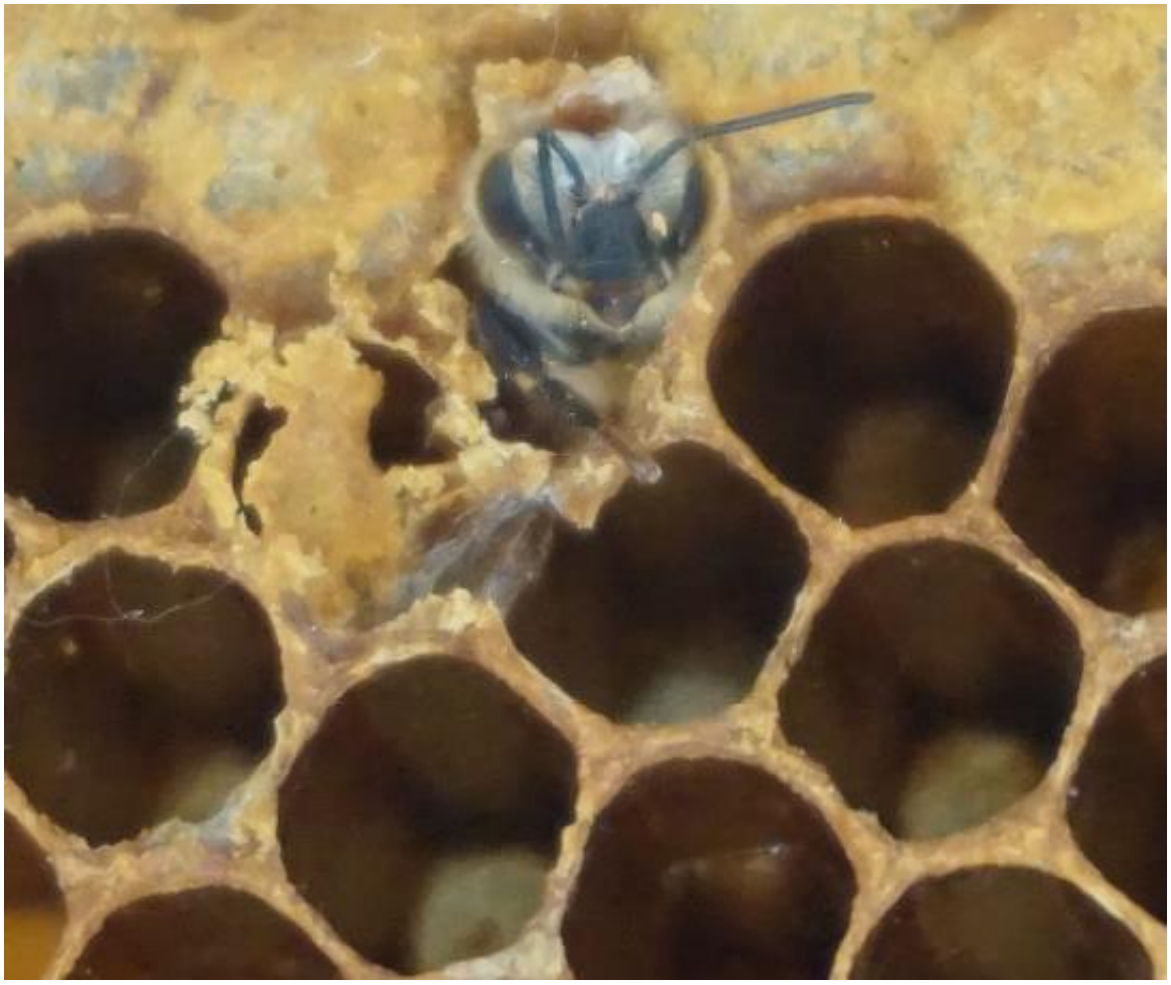
# Chapter 2.

Experience during early adulthood shapes the learning capacities and the number of synaptic boutons in the mushroom bodies of honey bees (*Apis mellifera*).



Cabirol A., Brooks R., Groh C., Barron AB., Devaud JM. (accepted). Experience during early adulthood shapes the learning capacities and the number of synaptic boutons in the mushroom bodies of honey bees (*Apis mellifera*). *Learning & Memory*

Cabirol, A., Brooks, R., Groh, C., Barron, A. B., & Devaud, J.-M. (2017). Experience during early adulthood shapes the learning capacities and the number of synaptic boutons in the mushroom bodies of honey bees (Apis mellifera). *Learning and Memory*, 24, p. 557-562.

DOI: 10.1101/lm.045492.117

## Supplemental figure

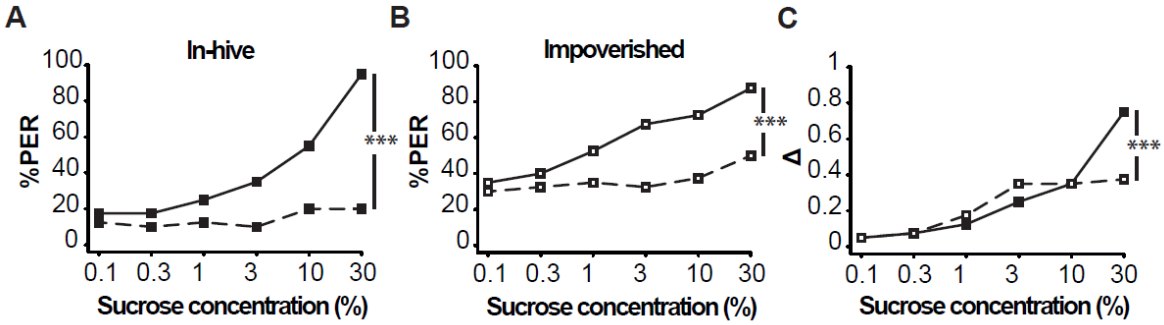



**Figure 2.S1.** Sucrose responsiveness and sensitization of bees from the *in-hive* [N= 40, **(A)**] or *impoverished* [N= 40, **(B)**] environment. Sensitivity to sucrose and to water was measured 2 hours before conditioning, according to a standard protocol (Scheiner et al. 2004): the antennae of each bee were stimulated with increasing concentrations of sucrose (0.1%, 0.3%, 1%, 3%, 10%, 30% w/w) alternating with water presentations. The percentage of PER elicited by increasing concentrations of sucrose (solid line) or by successive presentation of water (dashed line) is represented in **(A)** and **(B)**. To assess sensitivity to sucrose independently of sensitization, six Δ values were calculated for each bee as the difference between the responses to sucrose and to water and averaged for the bees reared in the *in-hive* (solid line) or *impoverished* (dashed line) environment **(C)**. The between-groups difference in Δ found for the 30% sucrose solution is likely due to the diet of the bees, that could not be controlled in the *in-hive* environment. However, the sum of the six Δ values were not correlated with the learning scores calculated for each bee as the sum of its responses to the 5 presentations of each odour during the reversal learning experiment (Spearman rank correlation; A+: rho = 0.09, p = 0.4354; B-: rho = 0.15, p = 0.1990; A-: rho = 0.05, p = 0.6756; B+: rho = 0.09, p = 0.4489).

*** $p<0.005$ (Tukey HSD on the last presentation, following a RM-ANOVA)

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# Chapter 3.

## Evidence for a relationship between brain structure and cognitive and foraging performance in honey bees

Photo courtesy of Simon Klein

Cabirol A., Cope AJ., Devaud JM., Barron AB. (submitted). Evidence for a relationship between brain structure and cognitive and foraging performance in honey bees. *Proceedings of the National Academy of Sciences*

## Abstract

Brain structure and function both vary with experience, but the link between them is unclear. Here, we investigated whether experience-dependent variability in learning performance can be explained by structural plasticity of the brain in foraging honey bees. In the honey bee brain, the mushroom bodies (MB) are required to solve ambiguous olfactory learning tasks such as reversal learning. Using radio frequency identification technology, we assessed the effects of foraging onset and duration, as well as the age when first foraging, on performance in reversal learning and on synaptic connectivity in the MB. We showed that learning performance improved at foraging onset, but declined with greater foraging experience. Analyses of brain structure in the same bees showed that the number of synaptic boutons in the MB decreased when bees started foraging, and then increased with greater foraging experience. In addition, we showed that if bees start foraging before the normal age, as a result of a stress applied to the colony, the decline in learning performance with foraging experience was more apparent. In a model of the MB, reversal learning performance could be altered by changing synaptic bouton number at the MB input and thereby changing the sparseness of coding of sensory information across the MB neuron population. We propose, therefore, that synaptic bouton number in the MB could directly alter cognitive performance. Our study provides the first insight of the mechanistic relationships between learning capacities and MB structure in honey bees, and how these are changed by foraging experience.

## Introduction

A core proposition of behavioural neuroscience is that variation in learning performance should be related to variation in neural systems (Kolb and Gibb 2013; Leger et al. 2014). However, rather few studies have demonstrated this relationship (Kanai and Rees 2011), especially for naturally-occurring ethological examples. To address this, here we examined how the structure of a multisensory-integration region of the honey bee brain (the mushroom bodies, MB) changes with accumulated flight and foraging experience, and how these changes correlate with performance in a cognitive task which is dependent on the MB (Devaud et al. 2007).

Given the richness of their natural environment and of their behavioural repertoire (Robinson 1992b), as well as their outstanding learning capacities for a relatively simple brain (Giurfa 2013), honey bees are an important invertebrate model system for behavioural neuroscience and neuroethology. Since the development of a Pavlovian olfactory conditioning for bees by Takeda in 1961 (Takeda 1961), the neural bases of learning and memory have been intensively investigated and well described (Giurfa and Sandoz 2012a). This work has highlighted the role of the MB (Menzel 2014). The MB are not needed for simple associative learning, but are required for cognitive tasks in which the solution is ambiguous, such as reversal learning (Devaud et al. 2007, 2015). In reversal learning, bees learn, in a first phase, to respond to a rewarded odour A and not to a non-rewarded odour B (A+B-). In a second phase, they learn the reverse contingencies (A-B+). The resolution of this task requires a flexibility in learned behaviour, and relies on functional MB (Devaud et al. 2015), but even so not all bees are able to solve this more complex task.

Both MB structure and learning capacities demonstrate experience-dependent plasticity in foraging honey bees. Foraging relies on cognitively demanding skills such as navigating between food sources and the hive, and identifying flowers providing nectar or pollen, in an ever-changing environment (Giurfa and Menzel 2001; Klein et al. 2017). Due to an age-related division of labour, this task is carried out by older individuals in a colony, who have previously worked on various tasks inside the hive (comb building, nursing, and guarding) (Robinson 1987, 1992a). The onset of foraging is preceded by a series of orientation flights in which bees learn the hive location (Robinson 1992a; Capaldi et al. 2000).

These behavioural changes are accompanied by plastic changes in brain structure. Foragers have larger MB than nurses, and the MB continue to increase in size with accumulated foraging experience. This is related to enhanced dendritic arborisation (Farris et al. 2001; Ismail et al. 2006; Dobrin et al. 2011; Muenz et al. 2015) in the input subregions of the MB, the lips and collars of the calyx, which receive olfactory and visual inputs respectively (Durst et al. 1994; Muenz et al. 2015). In both subregions, axon terminals of input neurons connect to the dendrites

of intrinsic MB neurons, thus forming synaptic boutons (also called microglomeruli). Despite the growth in volume, foragers have fewer synaptic boutons in the lip and collar regions than younger nurse workers, suggesting a synaptic pruning in foragers (Muenz et al. 2015). Synaptic pruning in the collars might actually occur during the orientation flights (Stieb et al. 2010; Scholl et al. 2014), and it is unknown whether it continues with further foraging experience. The functional consequence of this MB structural plasticity has been much speculated on (Withers et al. 1993b; Farris et al. 2001; Shapira et al. 2001), but remains unclear.

Here, we investigated the relationships between foraging experience, MB structure and learning performance in a MB-dependent task; reversal learning. Our results reveal that the onset of foraging increased cognitive performance and reduced the number of synaptic boutons in the MB neuropil. The opposite changes were observed with increasing foraging experience. Consistently, fewer synaptic boutons in the MB neuropil was related to improved performance in reversal learning. In a computational model of the MB, sparsening the connectivity between afferent olfactory neurons and MB neurons improved performance in reversal learning. Hence, we propose experience-dependent pruning of synaptic boutons as a possible mechanism for enhanced cognitive performance in bees. In addition, stress-induced precocious foraging affected the relationship between learning capacities and foraging experience, which raises issues for foraging performance in stressed colonies.

## Materials and methods

Experiments were carried out during the summer of 2016 at Macquarie University (Sydney, Australia). Approximately 1,500 newly emerged adult honey bees (*Apis mellifera*) were obtained from three different colonies, including a colony headed by a single inseminated queen for the brain immunohistochemical analyses. Newly emerged bees were collected from frames of emerging brood placed in a dark incubator (33 °C) for 24 h.

### RFID system

Approximately 1,500 newly emerged bees were equipped with a radio frequency identification (RFID) tag (INVENGO) (Chang et al. 2015; Perry et al. 2015; Søvik et al. 2015a) glued to their dorsal thorax with super glue, and marked with a dot of paint on the tag to identify their birth date. Bees tagged in this way were introduced in the host hive equipped with an RFID antenna (INVENGO) at the entrance which could detect each bee going outside or inside, thanks to the unique 12-byte hexadecimal identifier of each RFID tag. The data, collected into a .csv file, contained the date and time each bee was recorded as well as its RFID identifier, which enabled the reconstruction of each bees' flight history. Trips of < 30s were removed from the data as they were considered to include misreads from the hardware. The hive was displaced once

before introducing tagged bees to remove a portion of the foraging force and therefore incite young bees to begin foraging early (precocious foragers) (Huang et al. 1996).

**Reversal learning**

When tagged bees were between 22 and 26 days-old, 94 of them were randomly collected from the hive entrance in the afternoon of the day before the reversal learning experiment. Collected bees were immobilized on ice and harnessed in metal tubes allowing movements of the antennae and mouthparts only. They were then fed 15µL of sucrose solution (50% w/w) and kept in darkness, at room temperature, for one night. The reversal learning task started on the following morning. Only bees that demonstrated proboscis extension response (PER) when touching the bee's antennae with a toothpick soaked in sucrose solution (50% w/w) were used.

Reversal learning is a Pavlovian conditioning of the PER that includes a temporal ambiguity between two learning phases. In the first phase, bees were trained to associate an odour A with sucrose reinforcement but not an odour B (A+ vs. B-). In the second phase, one hour later, bees had to learn the reversed rule (A- vs B+). Each phase consisted of 5 presentations of each odour (5 trials) in a pseudo-random order, with an inter-trial interval of 8min (Devaud et al. 2007; Boitard et al. 2015). The odours used for conditioning were 1-nonanol and heptanal (Sigma-Aldrich). Their use as odour A or B alternated between testing days. During each learning trial of 40s, the bee was placed in front of the odour delivery system for 15s before the presentation of the odour (familiarisation time). The odour was then presented for 4s, the last second of which overlapped with sucrose presentation which lasted for 3 more seconds allowing the bee to drink the sucrose solution. The presence or absence of PER during the odour presentation was noted as 1 or 0 respectively. Inversion Scores (IS) were then calculated for each bee as the difference between its responses to B+ and A-, for each of the last two trials of the second phase of learning. These trials were used to define learners (IS = 1) and non-learners (IS = -1 or 0). Inversion scores were not calculated before trial 4 as the proportion of learners in trials 2 and 3 was not sufficient to allow statistic comparisons. Also, the performance of an individual in trial 4 reflects its learning speed.

**Immunostaining procedure**

Of the conditioned bees, 18 were sampled arbitrarily to analyse the structure of their MB. Synapsin immunostaining of whole-mount brains was performed following the procedure of Groh et al (Groh et al. 2012). Briefly, brains were dissected and fixed in paraformaldehyde (4% in Phosphate Buffer Saline – PBS - 0.01M) overnight at 4°C. Brains were then rinsed with PBS, permeabilized in PBS-Triton X-100 (Tx) (2% and 0.2% successively), blocked with 2% normal goat serum (NGS) in 0.2% PBS-Tx for one hour and incubated with the α-synapsin primary antibody (SYNORF1; DSHB; 1:10 in 0.2% PBS-Tx - 2% NGS) for 4 days at 4°C. Brains were

rinsed again in PBS and incubated with the secondary antibody (Alexa Fluor 488–conjugated goat anti-mouse; Fisher Scientific; 1:250 in 1% NGS-PBS) for 3 days at 4°C. After rinsing in PBS, brains were dehydrated in an ascending ethanol series. Whole brains were cleared and mounted in methyl salicylate for imaging.

**Image acquisition and analyses**

Images of the whole-mount brains were acquired using a laser scanning confocal microscope (LEICA SP5). For volume measurements of the lip and dense collar regions of the MB, stacks were imaged through the entire right medial calyx with a 5µm interval between the optical sections (10x/0.4 objective, digital zoom 3). To quantify synaptic boutons in the same calyx, optical sections were taken at 0.5µm interval over a depth of 10µm (63x/1.4 objective, digital zoom 2).

Images were processed using the 3D reconstruction software AMIRA 3.0 (FEI Visualization Sciences Group, Düsseldorf, Germany). To measure the lip and dense collar volumes, the boundaries of each region were traced manually and reconstructed by interpolation by the software. The number of synapsin-positive profiles within cubic sampling volumes (1000µm$^3$) located within the lip and dense collar (4 and 3 sampling volumes respectively) were counted. The density of synaptic boutons was averaged over the sampling volumes from each individual. The absolute number of synaptic boutons per lip and dense collar was obtained by extrapolating the mean density to the measured volume of the brain region.

**Computational model description**

A computational model of MB function was developed inspired by an abstraction of the MB circuit proposed by Bazhenov et al (2013) to model simple learning tasks. The main structure of the model consists of an associative network with three neural network layers. Adapting terminology and features from the insect brain, we labelled these: input neurons (IN), a large middle layer of MB intrinsic neurons called Kenyon cells (KC), and a small output population of MB extrinsic neurons (EN).

To provide inputs from the odorants A and B the IN neurons were divided into two subsets of 16 neurons, one for each odorant. The input values when the odorant is presented were chosen randomly in the range {0.9, 1.1} and fixed for the duration of the experiment.

The connections between the IN and KC were formed by a fixed matrix, where a connection between the ith IN and the jth KC is denoted cij. The probability of an IN and KC neuron being connected determines how sparse or dense the connectivity is; the lower this value the sparser the connectivity, and the higher the denser. For a probability of one all neurons are connected, and a probability of zero leads to no connections. For this model we used two values for the

probability: sparse (0.12) or dense (0.16), mathematically described by pIN->KC = {0.12, 0.16}. These values are slightly higher than those used by Bazhenov et al (36) to compensate for the sparsening effect of inhibition and therefore maintain the number of active KC for the sparse case. All connections have a fixed strength of one.

Each model KC neuron sums its inputs, subtracts a threshold value b, and outputs the final value if it is greater than zero using the Heaviside function θ. The value of b is chosen to ensure only KC with two or more active inputs produce an output, and therefore is set to a value of 1.2.

The connections from the KC to the EN are plastic and changed as the model was rewarded and learned, and every KC neuron is connected to every EN neuron. The connection strength between the jth KC and the kth EN (denoted wjk) can take a value between zero and one. Learning takes place in all synaptic weights according to the equation:

$$\Delta w = \alpha(R - R_b) \times presynaptic \text{ with probability } p = 0.1$$

where α=0.13 is the learning rate of the weights, R=1 if reward is given, and zero in all other cases, Rb=0.62 is a reward baseline. These values were chosen so that the synapse learned at approximately half the rate that it forgets. With these values acquisition rate matched that found in real bees. The term presynaptic is 1 if the presynaptic neuron is active and 0 elsewhere. It should be noted that reward was given on proboscis extension only.

The extrinsic neurons, EN, form two distinct sub-populations dedicated to triggering proboscis extension (which we shall term Extend) and retraction (termed Retract). The model proboscis is extended if the total output of the Extend sub-population is greater than the total output of the Retract sub-population, as long as the total activity of both sub-populations together is greater than 0.1 (i.e. once a suitable threshold for the decision has been reached).

Finally, we considered the GABAergic inhibitory protocerebral tract (PCT) neurons in the model. The output of the lth neuron in this population is described by the variable sl. This inhibition increases the sparseness of active KC neurons by suppressing weakly active neurons below the threshold for activity, leading to fewer KC neurons being active for the same stimulus with PCT inhibition as without (37, 38). As these neurons are fed by all of the KC a high value of 150 for bs (the threshold for output) was used. A global weighting wPCT = 0.4 was used to set the level of inhibition to replicate the performance of the experimental control bees.

Mathematically the model is formulated as follows where xi is the output of the ith IN neuron, yj is the output of the jth KC neuron, zk is the output of the kth EN neuron and sl is the output of the lth PCT neuron. The constant values are as described above.

$$y_j = \theta(\sum_{i=0}^{N_{IN}} c_{ij}x_i - b - w_{PCT}\sum_{l=0}^{N_{PCT}} s_l)$$

$$z_k = \theta\left(\sum_{j=0}^{N_{KC}} w_{jk}y_j\right)$$

$$s_l = \theta(\sum_{j=0}^{N_{KC}} y_j - b_s)$$

The numbers of neurons in each population were as follows: NIN=32 is the number of IN; NKC=5000 is the number of KC. There are 6 PCT neurons, and 4 EN in each of the Extend and Retract subsets.

Using the model, we examined performance of virtual bees in the reversal learning task. The experimental protocol for the model was identical to that used with real bees. We presented three conditions for the virtual bees: sparse connectivity between the IN neurons and the KC neurons (probability of connection is 0.12); dense connectivity between the IN neurons and the KC neurons (probability of connection is 0.16); and finally, with the inhibitory PCT neurons silenced (wPCT = 0.0). For each condition we used a 'models as animals' approach. Different random seeds for generating the EN to KC connectivity were used to create a set of 50 virtual bees, and each bee was tested individually.

**Statistical analyses**

R 3.2.3 was used for data analyses and graphic representations (R development core team 2015). In reversal learning, the responses to the odours were analyzed using a repeated-measurement ANOVA as the data met the criteria to apply an ANOVA to a dichotomous dependent variable (Lunney, 1970). Response levels to the two odours at the last learning trial were compared, within a group, using a Tukey honest significant difference (HSD) *post hoc* analysis. Inversion scores and neuroanatomical differences between groups were compared using a Mann-Whitney U test.

## Results

RFID data provided the cumulative time spent outside the hive and the age when first foraging for each bee. Bees were assumed to have begun foraging when they had accumulated > 30 min time outside the hive. Bees with < 30 and > 0 minutes of time outside the hive were considered as performing orientation flights ('orientating bees') (Capaldi et al. 2000; Perry et al. 2015). Bees that began foraging when less than 14 days old as adult were defined as precocious

foragers (Perry et al. 2015). Therefore, we were able to compare reversal learning performance of bees with different foraging durations (based on cumulative time foraging), in the whole sample and also in precocious and normal-age foragers independently.

**Reversal learning performance declines with foraging experience**

We first investigated the effect of foraging duration on performance in reversal learning **(Figure 3.1)**. For this, our sample was divided into four groups of increasing foraging durations, defined by the 1st quartile (113.8min), the median (381.3min) and the 3rd quartile (653.5min) of the distribution of foraging durations recorded in our sample of 83 bees (maximum foraging duration is 2751.33min). Foraging duration clearly affected performance in the second phase of reversal learning, but not the ability to solve the simple discriminative task of the first phase. Indeed, the responses to the rewarded odour (A+) and non-rewarded odour (B-) did not differ between the 4 foraging-experience groups in the first learning phase (Repeated-measure ANOVA; *Group* effect: $F = 0.58$, $p = 0.63$). They all responded gradually more to A+ than to B- (*Trial x Odorant* interaction: $F = 79.42$, $p < 0.0001$), until reaching significant discrimination in the last trial (Tukey HSD *post hoc* analysis; $p < 0.0001$ in all groups). In the second phase, however, although all groups changed their response patterns (*Trial x Odorant* interaction: $F = 107.10$, $p < 0.0001$), only bees in the first quartile of foraging durations responded more to B+ than to A- by the last trial ($p < 0.0001$). None of the other groups reversed the previously learned contingency by the end of the second learning phase ($p > 0.40$ in all three groups). We conclude that greater than 113.8 minutes of foraging activity, reduced performance in a reversal learning task.

This value was subsequently used as a threshold between 'short' and 'long' foraging durations in the following analyses.

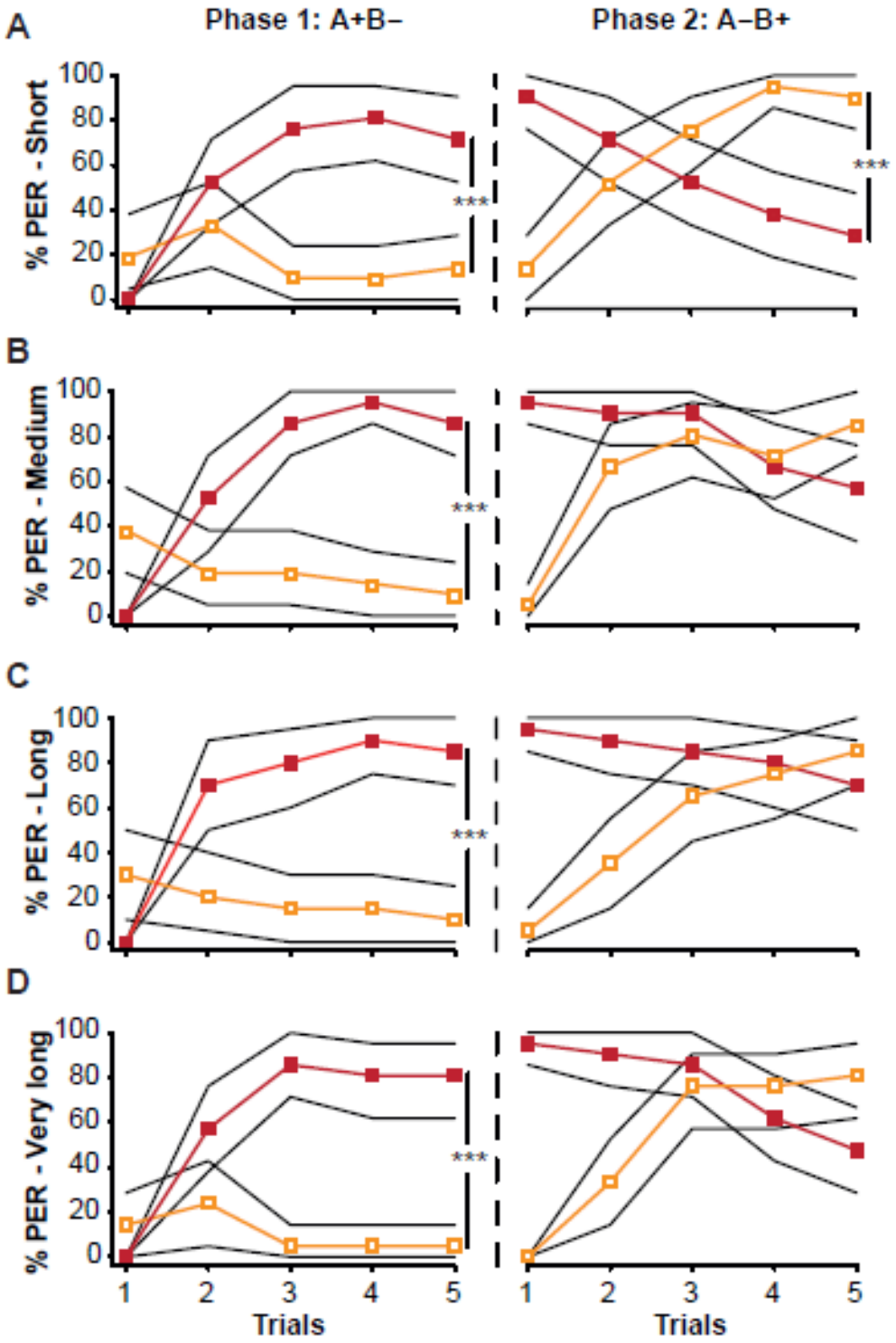


**Figure 3.1. Change in reversal learning performance with amount of foraging experience.** Percentage of individuals displaying PER in response to odours A (*red line*) and B (*orange line*) is shown, during phases 1 (A+B-) and 2 (A-B+) of the reversal learning task. Results are presented for bees with a short [n = 21, **(A)**], medium [n = 21, **(B)**], long (n = 20, **(C)**] or very long [n = 21, **(D)**] foraging duration. These groups were defined using the 1st quartile (113.8min), the median (381.3min) and the 3rd quartile (653.5min) of the total amount of time foraging of the whole sample. The bootstrapped 95% confidence intervals are indicated by the solid dark lines.

*** $p < 0.0001$, Tukey HSD *post hoc* analysis.

**Precocious foragers are more affected by the decline in reversal learning performance**

The decline of reversal learning performance with foraging experience was most apparent in precocious foragers **(Figure 3.2)**. Indeed, precocious foragers with a long foraging duration had lower inversion scores (IS) compared to those with a short foraging duration in the last two trials of the second phase (Mann-Whitney U-test: *Trial 4*: U = 310.5, p < 0.001; *Trial 5*: U = 286, p < 0.01). This was not the case in normal-age foragers (*Trial 4:* U= 165; p = 0.0781; *Trial 5*: U = 146, p = 0.4163).

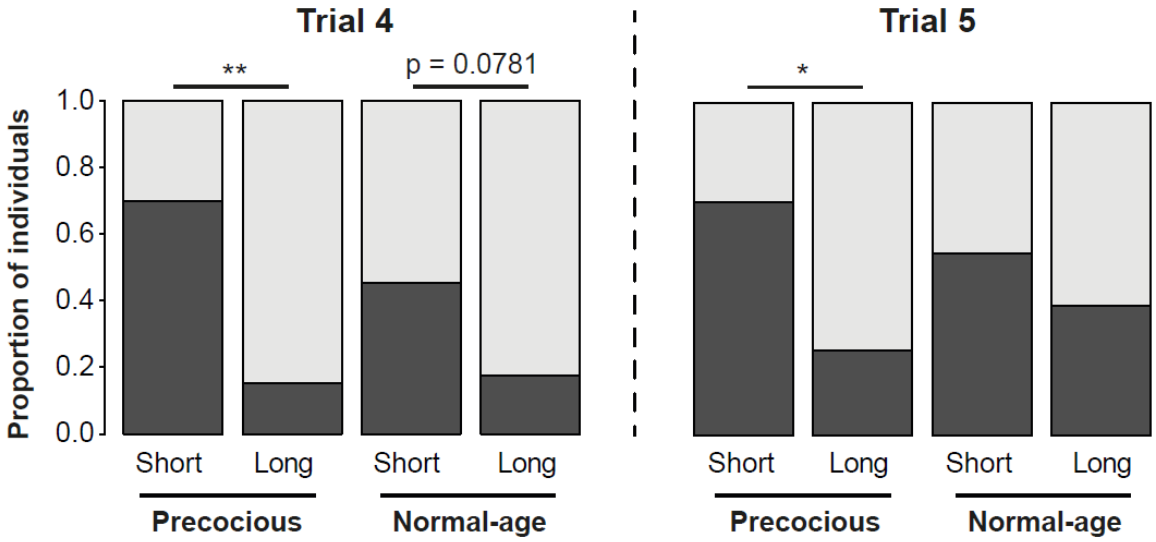


**Figure 3.2. Reversal learning performance of precocious and normal-age foragers with short or long foraging durations.** The proportions of non-learners (*NL*: *light grey*) and learners (*L*: *dark grey*) in the last two trials of the reversal phase (trial 4 and 5 of phase 2) are displayed. For each trial, bees were defined as non-learners or learners according to the value of their individual inversion score (see Methods; NL: IS= -1 or 0; L: IS=1). The IS were compared between precocious and normal-aged foragers, with either short or long foraging durations corresponding respectively to durations within or outside the 1st quartile of the whole sample (113.8min). [*Precocious:* short: n = 10, long: n = 39; *Normal-age:* short: n = 11, long: n = 23] * p < 0.01; ** p < 0.005, Mann-Whitney U-test.

Foraging behaviour also differed between the two groups **(Figure 3.S1)**. Precocious forager exhibited a shorter foraging duration per foraging day (defined as foraging intensity) compared to normal-age foragers (U = 1113, p < 0.01) **(Figure 3.S1A)**. Overall, foraging intensity was related to performances in the 4th trial of the second phase, as non-learners had a higher foraging intensity than learners (U = 888, p < 0.05) **(Figure 3.S1B)**. Yet, although normal-age foragers exhibited a higher foraging intensity than precocious foragers, their IS did not differ (*Trial 4*: U = 889.5, p = 0.5370; *Trial 5*: U = 940, p = 0.2546) **(Figure 3.S1C)**. It confirms that normal-

age foragers are more resistant than precocious foragers to the foraging-related decline in reversal learning capacities.

**Beginning foraging is associated with an improvement in reversal learning abilities**

The effect of foraging onset on reversal learning was assessed by comparing the performance of orientating bees (total amount of time outside < 30min) with that of foraging bees with short and long foraging duration **(Figure 3.3)**. The IS differed markedly among the three groups in the last two trials of the reversal phase (Kruskall-Wallis H-test; Trial 4: $p < 0.001$; Trial 5: $p < 0.05$). Beginning foraging was associated with an increase in the acquisition rate, as bees with short foraging duration had a higher IS in the 4$^{th}$ trial than orientating bees (Trial 4: $U = 61.5$, $p < 0.05$; Trial 5: $U = 149$, $p = 0.1349$). As previously demonstrated, reduced learning performance was observed in foragers with long foraging durations compared to those with short foraging durations in both trials (Trial 4: $U = 949.5$, $p < 0.001$; Trial 5: $U = 866.5$, $p < 0.01$).

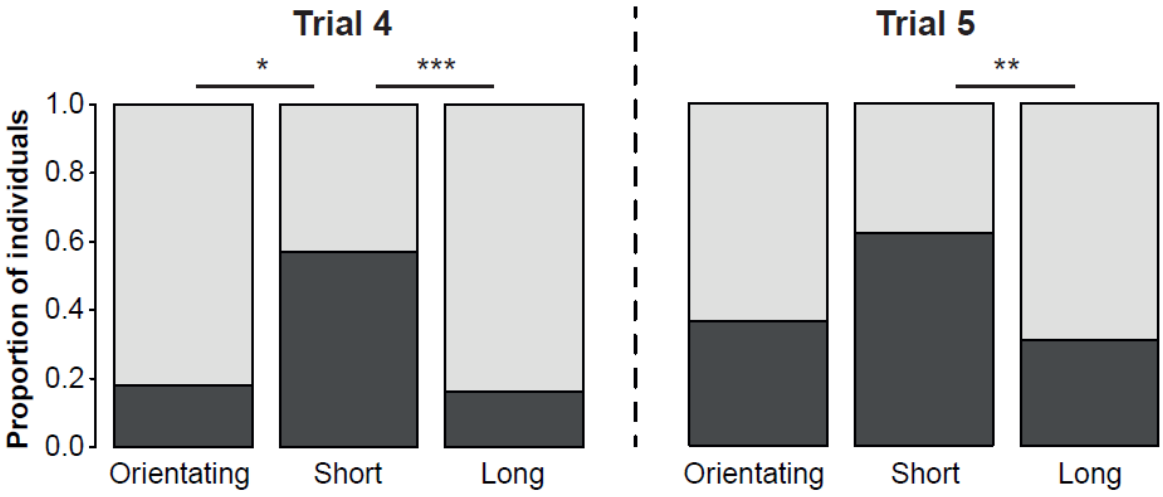


**Figure 3.3. Reversal learning performance of orientating bees and foragers with short or long foraging durations.** The proportions of non-learners (*NL*: *light grey*) and learners (*L*: *dark grey*) in the last two trials of the reversal phase (trial 4 and 5 of phase 2) are displayed. For each trial, bees were defined as learners or non-learners according to the value of their individual inversion score (see Methods; NL: IS= -1 or 0; L: IS=1). The IS are compared between orientating bees and foragers, with either short or long foraging durations corresponding respectively to durations within or outside the 1$^{st}$ quartile of the whole sample (113.8min). [*Orientating*: n = 11; *Foragers-Short*: n = 21; *Foragers-Long*: n = 62] * $p < 0.05$; ** $p < 0.01$; *** $p < 0.0005$, Mann-Whitney U-Test.

**Structure of the MB varies with foraging onset and experience**

MB structure was compared between orientating bees and foragers **(Figure 3.4A)**. The volumes of the lip and dense collar did not differ significantly between orientating bees and foragers (Mann-Whitney U-test: *lip*: U = 18, p = 0.1734; *collar*: U = 16, p = 0.1172) **(Figure 3.4B)**, and neither did the density of synaptic boutons (*lip:* U = 5, p = 0.0595; *dense collar:* U = 15.5, p = 0.6375) **(Figure 3.4C)**. The extrapolated total number of synaptic boutons in the lip and dense collar was lower in foragers than orientating bees (*lip*: U = 3, p < 0.05; *collar*: U = 3, p < 0.05) **(Figure 3.4D)**. Therefore, the transition from orientation flights to foraging was accompanied by a decrease in synaptic bouton number in both regions.

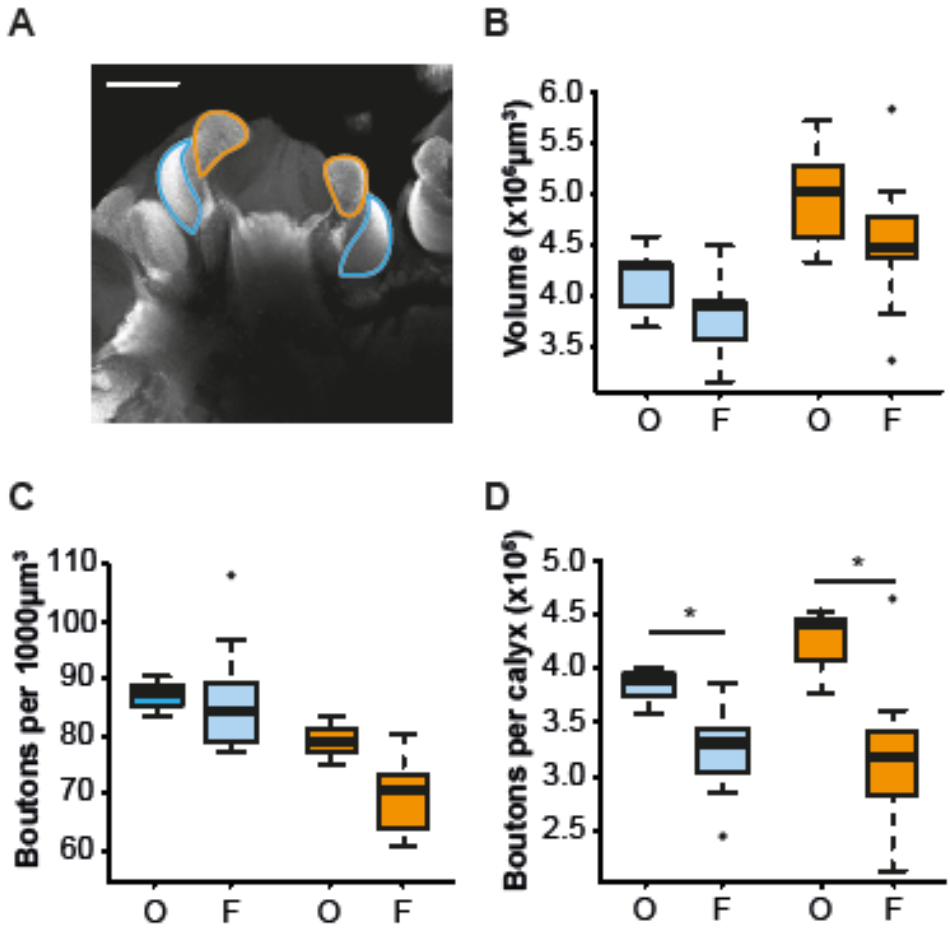


**Figure 3.4. Mushroom body structure of orientating bees and foragers**. **(A)** Frontal confocal image of the right median MB labelled for synapsin (scale bar = 100µm). Borders of the lip (*orange*) and dense collar (*blue*) are highlighted. Boxplots showing the characteristics of the dense collar (*dCo; blue*) and lip (*Li; orange*) of a sample of orientating bees (*O*, n = 5) and foragers (*F*, n = 13): **(B)** neuropil volume, **(C)** density of synaptic boutons, **(D)** number of synaptic boutons per neuropil. * p < 0.05, Mann-Whitney U-Test.

Mushroom bodies structure subsequently varied with foraging intensity (foraging duration/foraging day) **(Figure 3.S2)**. Foraging intensity was positively correlated with the volume of the lip and dense collar (Spearman's rank correlation; *lip*: $R^2$= 0.6648, $p < 0.05$; *collar*: $R^2$= 0.7857, $p < 0.005$), and with the total number of synaptic boutons in both regions (*lip*: $R^2$= 0.6099, $p < 0.05$; *collar*: $R^2$= 0.5220, $p = 0.0706$). Intense foraging was therefore associated with a larger MB neuropil containing a higher number of synaptic boutons.

**A low number of synaptic boutons in the MB neuropil promotes success in reversal learning**

Finally, we compared the MB structure of bees that successfully reversed their learning in the last two trials of the reversal phase (learners) and bees that did not (non-learners) **(Figure 3.5)**. Reversal learning performance was not associated with volume differences in either neuropil **(Figure 3.5A)** (Trial 4: *lip*: U = 49, p = 0.2496; *collar*: U = 53, p = 0.1246; Trial 5: *lip*: U = 40, p = 1; *collar*: U = 44, p = 0.7618). However, synaptic boutons in both regions were less dense in learners than in non-learners in the 4$^{th}$ trial, but not the 5$^{th}$ **(Figure 3.5B)** (Trial 4: *lip*: U = 56.5, $p < 0.005$; *collar*: U = 50, $p < 0.05$; Trial 5: *lip*: U = 49, p = 0.0829; *collar*: U = 48.5, p = 0.0927). The total number of synaptic boutons in the lip and dense collar was lower in learners than in non-learners, when considering the 4$^{th}$ trial only **(Figure 3.5C)** (*Trial 4*: lip: U = 53, $p < 0.05$, collar: U = 48, p = 0.0559; *Trial5:* lip: U = 42, p = 0.3282, collar: U = 43, p = 0.2786). These results suggest that a fast acquisition by the 4$^{th}$ trial of the second phase of reversal learning was associated with fewer synaptic boutons in the MB neuropil.

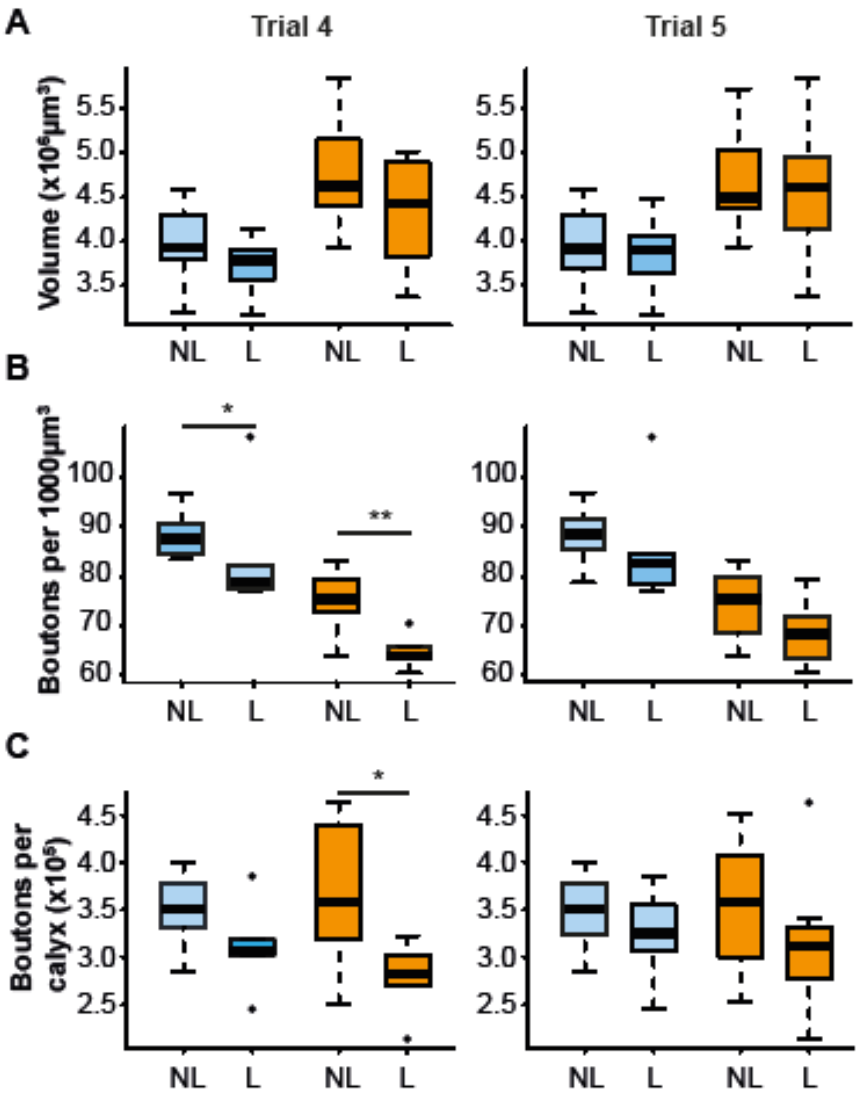


**Figure 3.5. Mushroom body structure and reversal learning performance.** Boxplots showing the characteristics of the dense collar (*dCo; blue*) and lip (*Li; orange*) of non-learners (*NL*, IS = -1 or 0) and learners (*L*, IS = 1) for each of the last two trials of the reversal phase: **(A)** neuropil volume, **(B)** density of synaptic boutons, **(C)** number of synaptic boutons per neuropil. [*Trial 4*: n = 12 NL and 6 L; *Trial 5*: n = 10 NL and 8 L] * $p < 0.05$, ** $p < 0.005$, Mann-Whitney U-Test.

Using a modelling approach, we asked whether changing the connectivity between input neurons and MB neurons could impact on reversal learning performances **(Figure 3.6)**. Simulations showed that decreasing sparseness by increasing the number of input connections onto MB neurons, thus mimicking a high number of synaptic boutons, impaired reversal despite efficient learning in the first phase. The same effect was obtained by removing inhibitory feedback from the GABAergic PCT neurons onto MB neurons, thus demonstrating in the model the results of a previous pharmacological study (Devaud et al. 2015).

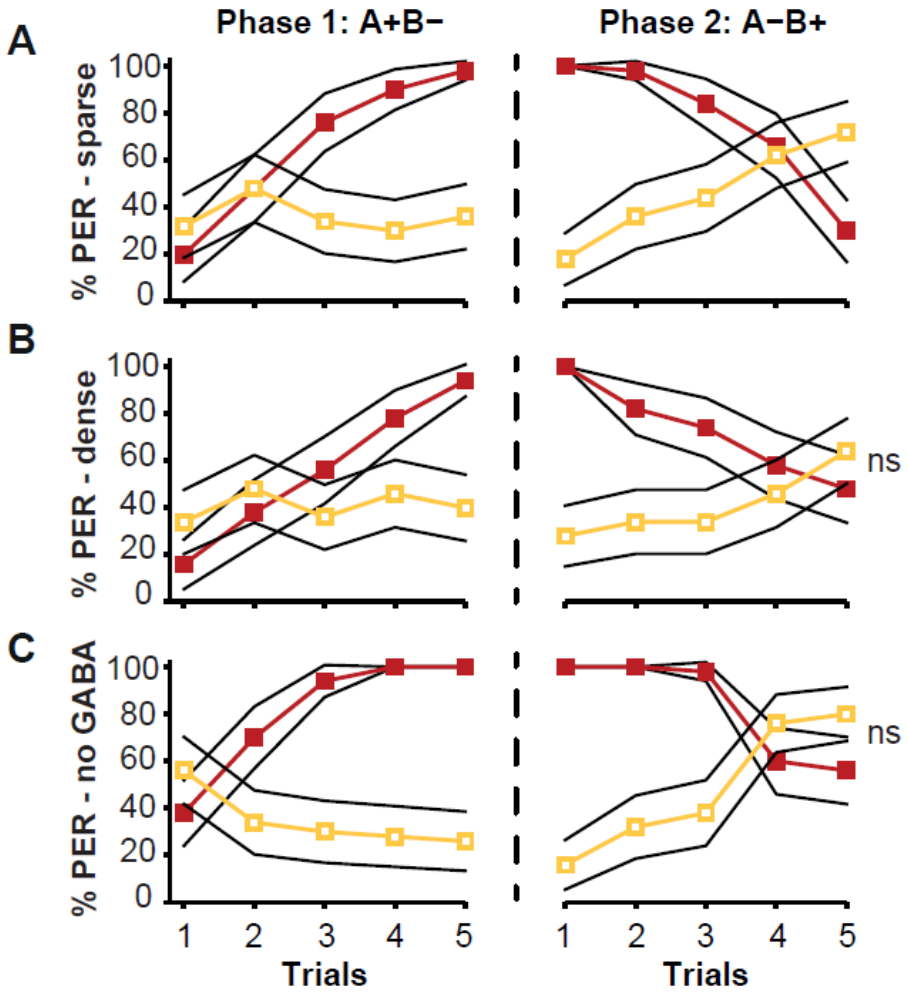


**Figure 3.6. Modelled consequences of changing connectivity in the mushroom bodies on reversal learning performance.** Modelled percentage of individuals displaying PER in response to odours A (*solid red line*) and B (*dashed red line*) during the reversal learning paradigm. Three different models were run simulating a normally sparse **(A)** or dense **(B)** distribution of excitatory connections onto MB neurons (KCs), and **(C)** suppressed inhibitory input from the GABAergic PCT. 50 agents (virtual bees) were modelled for each model configuration. The 95% confidence intervals are represented by the solid dark lines.

## Discussion

This study reports a functional relationship between experience-dependent plasticity in honey bee MB structure and variation in cognitive capacity. We show that a reduced number of synaptic boutons in the MB neuropil after the orientation flights period is associated with improved performance in reversal learning. As bees accumulate more time foraging, however, synaptic bouton number increases while learning performance decreases.

**Cognitive improvement and reduction in synaptic bouton number occur at the onset of foraging**

The transition from orientation flights to foraging was accompanied by an improvement in reversal learning performance (Figure 3.3), as well as a decrease in synaptic bouton number in both the lip and dense collar of the MB (Figure 3.4). Such synaptic pruning might be a consequence of the drastic change in environment and activity concomitant with orientation flights and the onset of foraging. Indeed, synaptic pruning has been reported previously in the dense collar of bees and ants following exposure to light (Stieb et al. 2010, 2012; Scholl et al. 2014). Also, exposure to a rich olfactory environment was demonstrated to reduce synaptic bouton number in the lip of leaf-cutting ants (Falibene et al. 2015). The decrease in synaptic bouton number at foraging onset may represent a self-organised optimisation of MB connectivity for the encoding of different stimuli in the newly explored environment outside the hive.

**Decline in reversal learning abilities and increase in synaptic boutons number with additional foraging experience**

The improvement in reversal learning performance after foraging onset is transient, since long foraging durations and intense foraging activity were associated with poor performance (Figure 3.1). This is consistent with previous observations showing that foraging activity reduces performance in various learning tasks (Behrends et al. 2007; Scheiner and Amdam 2009; Münch et al. 2010, 2013). An alternative hypothesis could be that the decrease in reversal learning performance with foraging experience was due to a lack of sleep in intense foragers. Although our RFID data provided no information about the time allocated to sleeping for each individual, sleep was indeed shown to be critical for learning and memory in honey bees and fruit flies (Hussaini et al. 2009; Donlea et al. 2011; Beyaert et al. 2012).

Because of the precise measures of foraging experience provided by our RFID data we can report a biphasic response of MB plasticity to foraging with an initial pruning of synaptic boutons at foraging onset followed by an increase in synaptic bouton number with more and more intense foraging (Figure 3.1 & 3.S2). Our data are consistent with previous reports of experience dependent plasticity in bees (Farris et al. 2001; Ismail et al. 2006; Muenz et al. 2015), but illustrate more sophistication than has been previously recognised.

What might cause the increased synaptic bouton number in experienced forgers? Previous studies have shown that the increase in MB volume and dendritic arborisation observed in foragers can be triggered by a chronic stimulation of the muscarinic receptors to acetylcholine (Ismail et al. 2006; Dobrin et al. 2011). In addition, excitatory cholinergic neurotransmission,

which is involved in olfactory processing, is higher in the MB of foragers than in nurses (Shapira et al. 2001). Greater activation of cholinergic pathways in the brain might explain why bees that foraged intensely have a high number of synaptic boutons in our experiment (Gogolla et al. 2007).

**Precocious foraging accentuates the foraging-related decline in reversal learning performances**

The decrease in reversal learning performance with foraging experience was more apparent in precocious foragers than in normal-age foragers (Figure 3.2). Precocious foraging can result from a stress applied to the colony, such as depleting a part of the foraging force (Huang et al. 1996; Amdam 2011). Precocious foragers perform less well than normal-age foragers in a range of foraging related metrics (Chang et al. 2015; Perry et al. 2015; Ushitani et al. 2016) and here we show an impact of precocious foraging on olfactory learning performance. This apparent susceptibility of precocious foragers to foraging-related cognitive decline is reminiscent of examples from the mammal literature suggesting that a same experience acquired at various ages can have a different impact on brain and behaviour (Kolb and Gibb 2013). Importantly, this might also contribute to understanding why precocious foragers perform so poorly as foragers in the field (Perry et al. 2015).

**MB structure and reversal learning**

For the first time in honey bees, our results highlight a relationship between brain structure and learning capacities. We have shown that a high number of synaptic boutons in the MB lip was associated with a reduced performance in reversal learning, but not in a simple discrimination task (Figure 3.5). A reason for this relationship was suggested by our modelling study (Figure 3.6). In our model, sparseness of KC activation by sensory input was critical to solve the reversal learning task. An inference from our models is therefore that the higher number of synaptic boutons at the input region of the MB seen in experienced foragers could decrease sparseness of sensory representation and reduce learning performance. This hypothesis from our model is consistent with earlier studies in bees and fruit flies showing that the sparseness of KC responses to odorants in the lip is necessary to discriminate similar odours (Lin et al. 2014), and that GABAergic input to the MB (presumably from feedback PCT/A3 neurons), which is known to maintain sparse coding of olfactory representation in the lip (Froese et al. 2014), is also required to solve a reversal learning task (Wu et al. 2012; Boitard et al. 2015). Our model theoretically links directly sparse coding and reversal learning performance for the first time.

In this study we observed that the number of synaptic boutons increased in both the lip (olfactory input region to the MB) and the dense collar (visual input region) with foraging experience (Figure 3.S2). We have documented an experience-dependent decline in an olfactory based cognitive task. We propose that bees with an experience-dependent increase in synaptic boutons number in the dense collar might show reduced performance in a complex visual learning task also. In fruit flies disrupting GABAergic input to their MB disrupts visual reversal learning (Ren et al. 2012), and hence it is possible that visual learning in bees could also be affected by a change in MB connectivity to visual inputs. More research is needed to explore how experience-dependent changes in synaptic connectivity in the MB affect other cognitive capacities and other sensory modalities.

**Conclusion**

To conclude, for the first time in honey bees, we propose a causal link between experience-dependent variation in brain structure and experience-dependent changes in cognitive performance. The onset of foraging was accompanied by a reduction in the number of synaptic boutons at the input region of the MB and an improvement in reversal learning performance. With additional accumulated foraging experience, synaptic bouton number increased and reversal learning performance declined. We propose that these changes in performance can be explained if reversal learning is dependent on a sparse representation of sensory information in the MB neuronal population. We also noted a greater experience-dependent cognitive decline in precocious than normal-aged foragers indicating that stress accelerates this process.

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## Supplementary figures

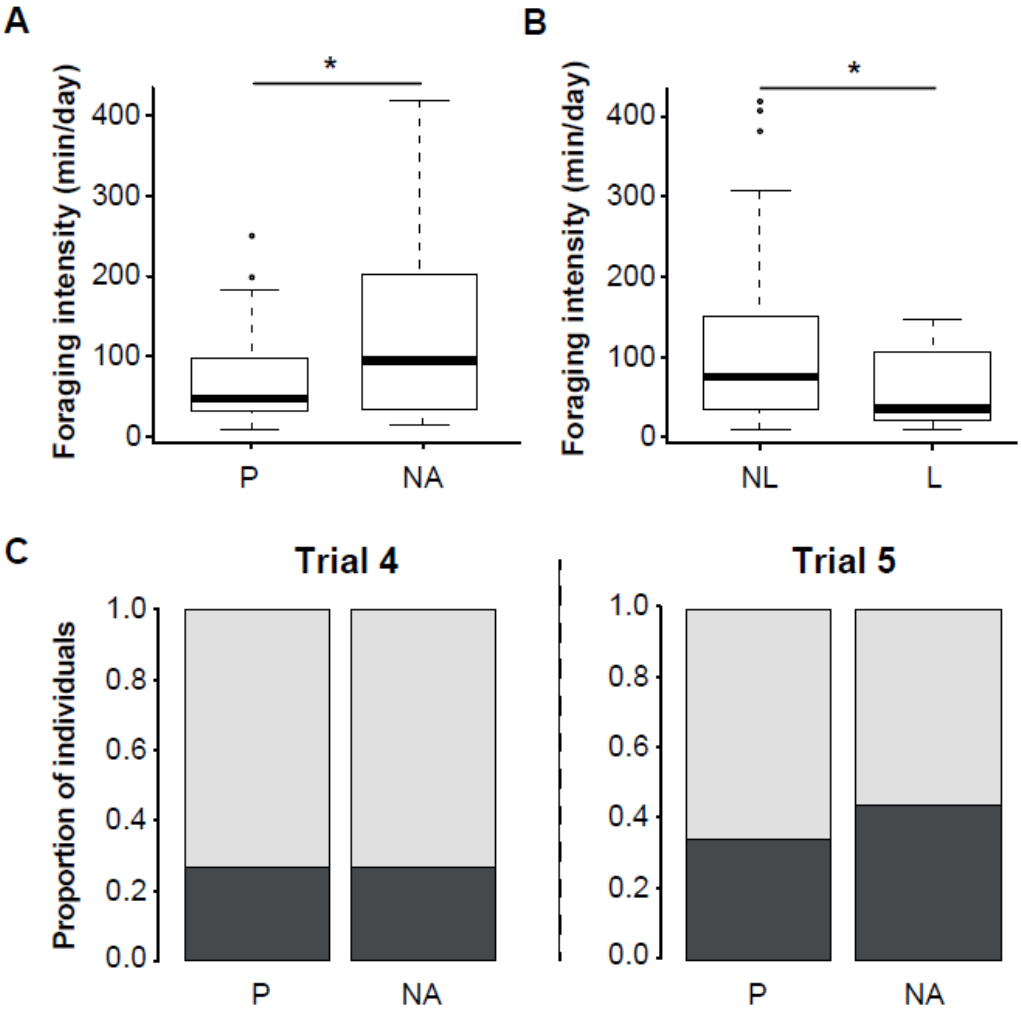


**Figure 3.S1. Foraging behaviour and reversal learning performance of precocious and normal-age foragers.** Foraging intensity (foraging duration/foraging day) of precocious (*P*) and normal-age (*NA*) foragers **(A)**, and of non-learners (*NL*) and learners (*L*) in the 4$^{th}$ trial of the reversal phase **(B)** are shown. The inversion scores (IS) in the last two trials are compared between precocious and normal-age foragers, and the proportion of non-learners (*light grey*) and learners (*dark grey*) is indicated for each group **(C)**. [P: n = 49; NA: n = 34; NL: n = 61; L: n = 22] * $p < 0.05$, Mann-Whitney U-Test.

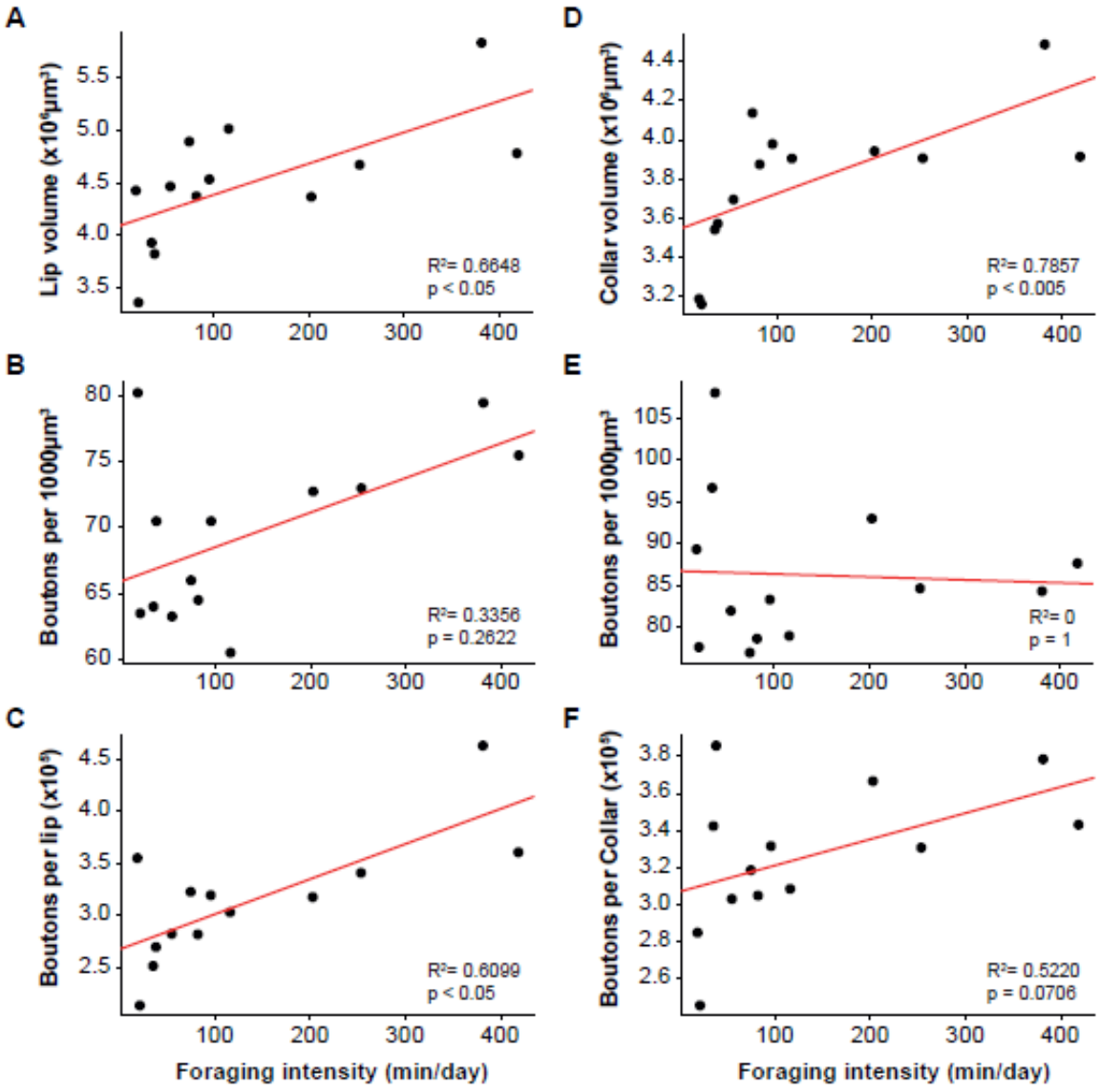


**Figure 3.S2. Correlations between foraging intensity and structural characteristics of the mushroom bodies.** Individual values (n = 13) for the parameters of the lip **(A, B, C)** and dense collar **(D, E, F)** are plotted against foraging intensity: neuropilar volume **(A, D)**, density of synaptic boutons **(B, E),** total number of synaptic boutons **(C, F)**. Spearman rank correlations.

Some outliers are observed that seem to be mostly bees with a small foraging intensity. This could be due to the fact that they represent both bees that foraged at a low intensity over many days, and bees that have just started foraging.

# Chapter 4.

Cholinergic signalling involvement in the decreased learning performances of foraging honey bees

Cabirol A., Devaud JM., Barron AB. (unpublished data).

## Abstract

Cholinergic signalling, in particular via the muscarinic receptors, is involved in olfactory learning and memory in many species, and is thought to mediate the plasticity of the mushroom bodies (MB) associated with foraging experience in honey bees. Here we assessed the performances of bees with different amounts of foraging experience, chronically treated or not with a muscarinic agonist, pilocarpine, in a positive patterning task. In this Pavlovian conditioning task bees learn to associate a mixture of two odorants with a sucrose reinforcement, while the odorants presented separately are not reinforced (AB+ vs. A-/B-). Learning this task requires functional MB. Performance in the task was impaired in bees that had been foraging for 12 days. Foragers with one week of experience, maintained in cages in an incubator for an additional 5 days and fed with pilocarpine exhibited a reduced performance compared to control bees fed with sucrose. Neither foraging activity, nor the pilocarpine treatment, affected the sucrose responsiveness of bees. Therefore, muscarinic signalling seems to be partly involved in the foraging-induced decline in learning capacities in honey bees.

## Introduction

Honey bees exhibit impressive learning and memory capacities in order to discriminate and locate the different food sources in the environment (Giurfa and Menzel 2001). These capacities are thought to be critical for the efficiency of foraging and therefore for colony survival (Klein et al. 2017). Foraging activity has, however, been suggested to decrease performance in some associative learning tasks (Ben-Shahar et al. 2000; Behrends et al. 2007; Münch et al. 2010, 2013). In a context of pollinator losses, it is important to unravel the neurobiological mechanisms responsible for the changes in foragers' learning capacities. Here, we investigated the role of the cholinergic neurotransmitter pathway, which is targeted by some pesticides (Casida and Durkin 2013), in the cognitive decline observed in foraging honey bees.

Acetylcholine (Ach) is the main excitatory neurotransmitter in the honey bee brain (Kreissl and Bicker 1989), and plays crucial roles in learning (Gauthier 2010). The catalytic activity and expression of acetylcholinesterase (the enzyme degrading Ach at synapses) is lower in the brains of foragers compared to in-hive workers (Shapira et al. 2001). This is particularly apparent in the mushroom bodies (MBs), which are involved in learning and memory processes (Menzel 2001; Devaud et al. 2007, 2015) and which receive olfactory information via cholinergic input from the antennal lobes (Oleskevich 1999). As in vertebrates, Ach binds two receptor subtypes: the ionotropic nicotinic receptors and the metabotropic muscarinic receptors (Zhi-Yong Huang and Knowles 1990). An increase in the volume of the MBs, as well as in dendritic arborisation therein, has been reported during the second week of foraging, and can be reproduced by stimulating chronically the muscarinic receptors with the agonist, pilocarpine (Ismail et al. 2006; Dobrin et al. 2011). This implies that the anatomical changes observed in the MBs of foragers are due to elevated cholinergic signalling in their MBs (Shapira et al. 2001). Yet, the consequences of a chronic pilocarpine treatment on learning capacities are still unknown.

In the previous chapter, we have shown that foraging onset is associated with an improvement in reversal learning abilities, but that this is followed by a decline as foraging goes on. Here we chose a different MB-dependent learning task, positive patterning, to explore whether the decline affects other MB-dependent capacities as well (Devaud et al. 2007, 2015). The olfactory positive patterning paradigm is based on the conditioning of the proboscis extension response (PER) (Takeda 1961). To solve this task, bees must extend their proboscis in response to a mixture of two odorants that is reinforced with sucrose solution (AB+) but not to the non-reinforced olfactory components of the mixture when presented separately (A-/B-). A pharmacological blockade of MBs function prevents bees from correctly solving this task

(Devaud et al. 2015). Because sensitivity to sucrose is known to influence olfactory learning performance in MB-independent learning tasks (Scheiner et al. 2001, 2003), we investigated the effects of foraging experience and pilocarpine treatment on sucrose responsiveness, as well as the relationship between sucrose responsiveness and performance in positive patterning. Our results show a negative impact of foraging and pilocarpine treatment on performance in positive patterning, but no effect of treatments on sucrose responsiveness, suggesting that the cholinergic pathway modulates learning *per se* and is involved in the cognitive decline observed in foragers.

## Materials and methods

Experiments were conducted during spring and summer 2016, 2017. Honey bees (*Apis mellifera*) came from different colonies maintained on the campus of the University Paul Sabatier in Toulouse, France.

### Experimental groups

Approximately 3,000 newly emerged bees (less than 24 h old as adults) were collected from brood combs placed in an incubator (34°C, 60% humidity). They were painted on the thorax with a specific colour identifying their birth date, and added to the host colony. The host colony was displaced to induce a precocious foraging of the focal bees (young bees started foraging when they were 5-6 days old) and therefore increase the probability of their survival for a second week of foraging. Seven days later, the hive entrance was observed daily for 5 hours, and any focal bee seen foraging was marked on the thorax with a new colour each day. These new foragers were marked over 5 days, but the foragers marked on the first day were excluded from the experiment since their first day of foraging was unknown. After one week of foraging, bees were either left foraging for five additional days (experienced foragers), or placed in small cages (4x8x5 $cm^3$; 10 bees per cage) in an incubator (28°C, 60% humidity) to receive their pharmacological treatment for 5 days. The treatment consisted in feeding the bees with 50% (w/w) sucrose solution containing the muscarinic agonist pilocarpine ($10^{-6}$ M) (Ismail et al. 2006; Dobrin et al. 2011), while a control group was fed with sucrose (50% w/w). The performance of experienced foragers, pilocarpine treated bees and control bees was assessed in a positive patterning task after which their brains were dissected for immunohistochemistry.

### Animal preparation

On the day before the conditioning experiment, 10 experienced foragers were collected at the hive entrance and placed in cages with 50% sucrose solution in the same incubator as the caged

bees. One hour later, bees from all experimental groups were immobilized on ice and harnessed individually in metal tubes allowing movements of the antennae and proboscis only. Hungry bees received 5µL of sucrose solution in order to improve survival during the night. Bees were placed back in the incubator for the night.

**Sucrose responsiveness**

On the morning of the conditioning experiment, the sucrose responsiveness of bees was tested by individually touching their antennae with a toothpick soaked in increasing concentrations of sucrose (0.1%, 0.3%, 1%, 3%, 10%, and 30%). The presence or absence of proboscis extension response (PER) was recorded as 1 or 0 respectively. A sucrose response score (SRS) was calculated as the sum of the PER to the 6 concentrations of sucrose. Sucrose presentations were interspaced by presentation of water as a measure of sensitization, and the water response score (WRS) corresponded to the sum of the PER to the 6 presentations of water. Bees were allowed to recover in darkness for one hour at room temperature before the positive patterning experiment.

**Positive patterning**

We used the same procedure as in Devaud et al. (2015). Bees received 10 presentations of the odour mixture reinforced with sucrose (AB+) and 5 unreinforced presentations of each odorant of the mixture separately (A-/B-) in a pseudo-randomized order. Each learning trial lasted 40s. Bees were placed in the conditioning setup, in front of an odourless airflow for 15s for familiarisation with the context. The odour was then presented for 4s, followed by the sucrose presentation (in case of a reinforced trial) for 3s, with a 1s overlap. Bees were kept in the setup in front of the odourless airflow until the end of the trial. The inter-trial interval was 8min. The odorants used were pure nonanal and 2-nonanone (Sigma-Aldrich, Lyon, France). They were applied on different pieces of filter paper (4µL), introduced into two different syringes of the conditioning setup. During reinforced trials, the airflow was sent through the two syringes containing the odours (A and B), so that the bees receive the mixture. During unreinforced trials, the airflow was sent through one syringe containing the odour (A or B) and an empty one, so that the bees were always presented with the same amount of A or B, either alone or within the mixture. A learning score was calculated as the difference between the sum of responses to AB+ and the sum of responses to A-/B-.

**Statistics**

R 3.2.3 was used for data analyses and graphic representations (R development core team 2015). Because the response levels to A and B were overall equivalent in positive patterning, the results were pooled and the responses presented as AB+ vs. A−/B-. The responses to the

odours were analysed using a repeated-measurement ANOVA as the data met the criteria to apply an ANOVA to a dichotomous dependent variable (Lunney, 1970). Response levels to the two odours in a specific learning trial were compared, within a group, using a Tukey honest significant difference (HSD) *post hoc* analysis. The same statistics were used to analyse the responses to sucrose and water. A between-group comparison of learning scores was performed using a Kruskall-Wallis test followed by Mann Whitney U-tests.

## Results

We observed a negative impact of foraging experience and the pilocarpine treatment on performance in the positive patterning task **(Figure 4.1)**. Indeed, although bees from all three groups (experienced foragers, pilocarpine treated and control bees) progressively changed their responses to the reinforced odour mixture AB+ and to its unreinforced components A-/B- (RM-ANOVA; *Odour x trial interaction:* $F = 22.36$, $p < 0.0001$), experienced foragers were not able to solve the positive patterning task by the end of the 5 trials (Tukey HSD *post-hoc* analysis; $p = 0.2301$) **(Figure 4.1A)**. By contrast, both control bees **(Figures 4.1B)** and pilocarpine treated bees **(Figure 4.1C)** responded more to the mixture AB+ than to its components A-/B- in the last trial (control: $p < 0.0001$; pilocarpine: $p < 0.001$). Control bees learned faster as they solved this task successfully by the 4$^{th}$ trial (Tukey HSD *post-hoc* analysis; $p < 0.001$), which was not the case for pilocarpine treated bees ($p = 0.1956$). This was particularly apparent when comparing the learning scores between the three groups (Kruskall-Wallis test; $p < 0.05$). Indeed, control bees had significantly higher scores compared to both pilocarpine-treated bees (Mann Whitney U-test; $U= 852.5$; $p < 0.05$) and experienced foragers ($U= 569$; $p < 0.05$). The latter groups did not differ significantly in their learning scores ($U= 402.5$; $p = 0.345$). Therefore, the pilocarpine treatment decreased bees' ability to solve the positive patterning task, which was also impaired in experienced foragers.

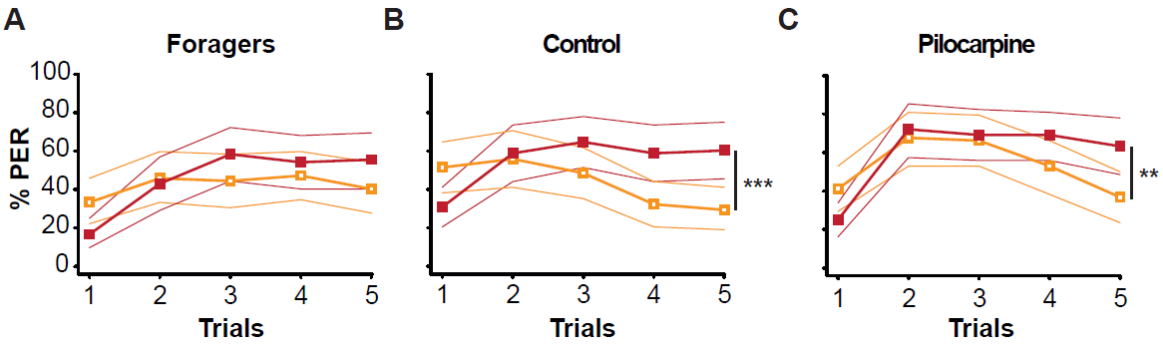

**Figure 4.1. Positive patterning performances of experienced foragers, control bees and pilocarpine treated bees.** The percentage of PER elicited by the odour mixture AB+ (thick red lines) and its independent components A-/B- (thick orange lines) is represented for foragers [N = 36; **(A)**], control bees [N = 34; **(B)**] and pilocarpine treated bees [N = 34; **(C)**]. The bootstrapped 95% confidence intervals are represented by the fine lines of the respective colour.
*** p < 0.0001; ** p < 0.001. Tukey HSD *post hoc* analysis

We then investigated whether sensitivity to sucrose and water was influenced by our treatments **(Figure 4.2)**. Neither foraging experience nor the pilocarpine treatment affected sucrose and water responsiveness (RM-ANOVA; *group effect*: sucrose: F = 0.13, p = 0.88; water: F = 0.22, p = 0.80). Bees from all three groups responded progressively more to the increasing concentrations of sucrose (*presentation effect:* F = 28.30, p < 0.0001) **(Figure 4.2A)**, and progressively less to the repeated presentations of water (*presentation effect:* F = 9.77, p < 0.0001) **(Figure 4.2B)**.

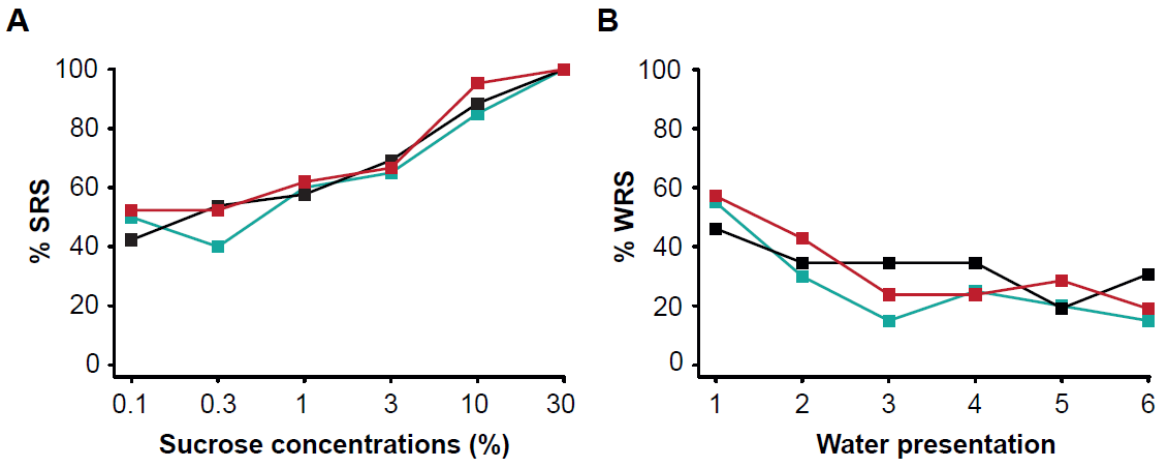


**Figure 4.2**. Sensitivity to sucrose and to water of experienced foragers, control bees and pilocarpine treated bees. The percentage of PER elicited by the 6 different concentrations of sucrose **(A)** or by the 6 presentations of water **(B)** is represented for foragers (N = 26; black), control bees (N = 20; blue), and pilocarpine treated bees (N = 21; red).

Performance in the positive patterning task was not affected by sensitivity to sucrose and water **(Figure 4.3)**. Indeed, there was no significant correlation between either the sucrose response score (SRS), or the water response score (WRS), and the positive patterning learning score (Spearman rank correlation; SRS: rho = -0.1553, p = 0.2096; WRS: rho = -0.0401, p = 0.7471).

Therefore the effects of foraging experience and the pilocarpine treatment on performance in positive patterning could not be attributed to variations in the processing of gustatory information about the sucrose reinforcement.

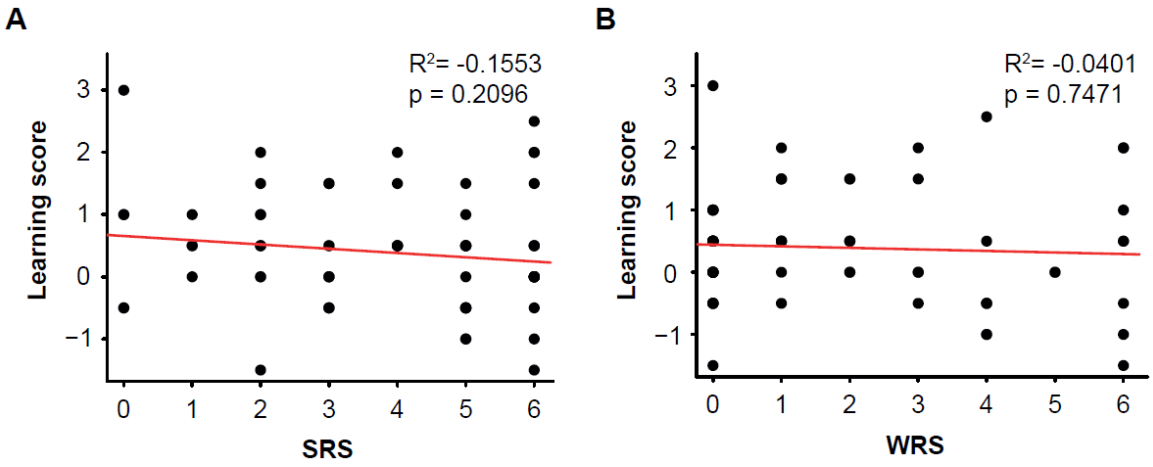


**Figure 4.3. Correlations between sucrose or water sensitivity and learning performance in a positive patterning task.** Individual values (N = 67) for the Sucrose Response Score [SRS; **(A)**], or the Water Response Score [WRS; **(B)**] are plotted against the learning score obtained in the positive patterning task. Spearman rank correlation

## Discussion

Our study reports a drop in performance in a positive patterning task with foraging experience that can be partly reproduced by a chronic stimulation of the muscarinic receptors with pilocarpine. This drop cannot be attributed to variation in sucrose responsiveness, suggesting that cognitive processes are affected.

Unlike control bees, experienced foragers were not able to solve the positive patterning task (Figure 4.1). In this study, control bees foraged for one week and then were caged for 5 days while fed sucrose. The difference between experienced foragers (which foraged for 12 days) and the control group could possibly be interpreted as a cognitive improvement in control bees since we do not know what would be the performance of 1-week foragers (before the caging period). However, given that other learning capacities are affected by foraging activity (Behrends et al. 2007; Scheiner and Amdam 2009; Münch et al. 2010), and that there is certainly no evidence that caging would improve cognition, it is likely that 1-week foragers perform similarly to control bees and that the 5 additional days of foraging has had a negative impact on positive patterning performance. This is consistent with the observation that the volume of

the MBs is not different between 1-week foragers and control bees (1-week foragers caged for 7 days and fed with sucrose), but is increased in 2-weeks foragers (Ismail et al. 2006). Given that control bees solved the positive patterning task, although they were kept outside of the hive, the impaired performance of foragers might not be due to the reduced time spent inside the hive but to foraging itself. The mechanisms by which foraging experience decreases learning capacities still need to be uncovered.

Our observation of a reduced performance in the positive patterning task, in bees treated with pilocarpine suggests that cholinergic signalling, via the muscarinic receptors, is one of the processes involved in the cognitive decline seen in foragers. A chronic exposure to organophosphate pesticides, which inhibit acetylcholinesterase, has been shown to reduce olfactory learning performance (Williamson and Wright 2013). This effect could be mediated by either the nicotinic receptors, the muscarinic receptors or both. Here we explicitly targeted muscarinic receptors with pilocarpine because this treatment could reproduce anatomical changes associated with sustained foraging in the MBs, contrary to the activation of nicotinic receptors (Ismail et al. 2006). In a different study from the same group, a chronic treatment with the same dose of pilocarpine improved odour-based nestmate recognition in new-born adult bees (Ismail et al. 2008). This suggests a different effect of a muscarinic stimulation on odour discrimination throughout a bee life, especially as brain maturation has been reported during the first days of adulthood (Fahrbach et al. 1998). Our study represents the first demonstration of a negative impact of a chronic stimulation of the muscarinic receptors on learning capacities.

Acute blockade of muscarinic receptors is known to reduce olfactory learning and memory in insects (Gauthier et al. 1994; Lozano and Gauthier 1998; Lozano et al. 2001; Silva et al. 2015), and in mammals (Hasselmo 2006; Mandairon et al. 2006). A decreased concentration of muscarinic receptors in the cellular membrane of neurons after a chronic stimulation of these receptors might explain the altered learning capacities (Siman and Klein 1983). The previously reported decreased activity of acetylcholinesterase in the brain of foragers (Shapira et al. 2001) might therefore be deleterious for learning capacities through the overstimulation of muscarinic receptors. Whether this decreased activity is physiological or induced by the pesticides met in the environment is unknown. If this is physiological, we can speculate that organophosphates may have an additive effect and promote the cognitive decline in foragers (Williamson and Wright 2013). In a previous study, a chronic stimulation of the muscarinic receptors, which might occur when the activity of acetylcholinesterase is low, increased the volume of the MBs and the dendritic arborisation therein, thus reproducing the plasticity associated with foraging experience (Ismail et al. 2006; Dobrin et al. 2011). Although it is tempting to relate it to the

decline in cognitive capacities, our previous study has shown that the MBs volume is not a relevant measure to explain variation in learning performance: but rather the number of synaptic boutons therein is (chapter 2). Since in Chapter 2 we used a different learning task (reversal learning), further experiments are needed to assess the relationship between the number of synaptic boutons in the MBs and the performance in positive patterning, as well as the effect of a chronic treatment with pilocarpine on bouton number.

Finally, the effects of foraging and pilocarpine treatment could not be attributed to changes in gustatory processing or motivation since the SRS was not significantly different between the groups, neither was it related to the positive patterning performance. A higher sucrose responsiveness in foragers compared to nurses has previously been reported (Scheiner et al. 2017). Since we did not observe differences in sucrose responsiveness between control bees and experienced foragers, the change in sucrose responsiveness might occur at the transition from nursing to foraging, and might not be affected by subsequent foraging experience. It is not impossible, however, that the pilocarpine treatment affected olfactory perception since the primary olfactory centres in the insect brain (antennal lobes) receives cholinergic inputs from the olfactory receptor neurons located in the antennae (Hansson and Anton 2000). Further studies should assess the impact of a pilocarpine treatment on the structure of the antennal lobes, which also undergo plasticity with foraging experience (Sigg et al. 1997; Brown et al. 2004). Since sucrose responsiveness did not influence the positive patterning performance, while it does influence MB-independent learning tasks, however, (Scheiner et al. 2001, 2003), the observed impairment in positive patterning might reflect changes in higher brain centres, the MBs. Also, performance in a simple olfactory discriminative task was not affected by foraging experience in our previous chapter, which implies that olfactory processing might not be altered (Chapter 2).

To conclude, we have demonstrated that a chronic stimulation of the muscarinic receptors to Ach is deleterious for olfactory learning capacities, and that this signalling pathway might be one of the mechanisms responsible for the cognitive decline associated with foraging experience in honey bees. Here is another study warning on the impact of pesticides which increase the stimulation of muscarinic receptors by inhibiting the enzyme degrading Ach.

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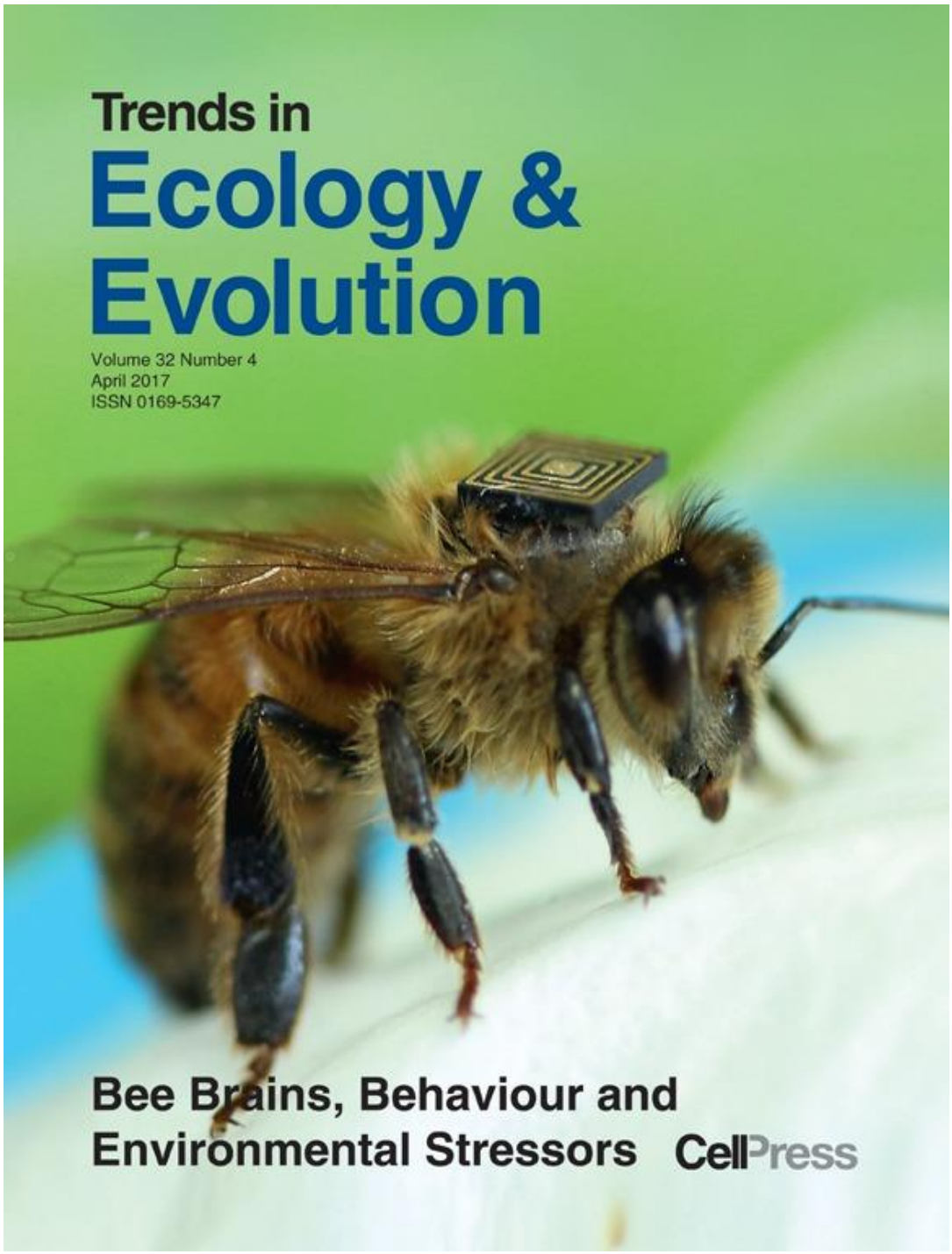
# Chapter 5.

Why bees are so vulnerable to environmental stressors




Klein S*, Cabirol A*, Devaud JM, Barron AB, Lihoreau M (2017) Why bees are so vulnerable to environmental stressors. *Trends in Ecology and Evolution* 32, 268-278

[* These authors contributed equally to the work]

## Abstract

Bee populations are declining in the industrialised world raising concerns for the sustainable pollination of crops. Pesticides, pollutants, parasites, diseases and malnutrition have all been linked to this problem. Here we consider neurobiological, ecological and evolutionary reasons why bees are particularly vulnerable to these environmental stressors. Central-place foraging on flowers demands advanced capacities of learning, memory and navigation. However, even at low intensity levels, many stressors damage the bee brain, disrupting key cognitive functions needed for effective foraging, with dramatic consequences for brood development and colony survival. We discuss how understanding the relationships between the actions of stressors on the nervous system, individual cognitive impairment and colony decline can inform constructive interventions to sustain bee populations.

## Bees are exposed to multiple environmental stressors

Bees are ecologically and economically vital pollinators for both wild and cultivated flowers. Presently many populations are in decline (Potts et al. 2010a; Rundlöf et al. 2015; Ollerton et al. 2015; Woodcock et al. 2016), while demand for pollination dependent crops continues to rise, generating understandible alarm and debate about the possibility of an emerging 'pollination crisis' (Holden 2006). Many causal factors have been identified, including a range of pathogens and parasites (Cornman et al. 2012; Francis et al. 2013), human-induced stressors such as pesticides (van der Sluijs et al. 2013; Henry et al. 2012; Gill et al. 2012) and forms of environmental degradation (Goulson et al. 2015). Very few of these stressors can be considered new, but many have increased in intensity over the last decade in much of the industrialised world. Our objective in this review is to consider why bees are particularly sensitive to these environmental stressors, even at low levels, and why their populations are now declining.

Bees, with the exception of parasitic species, raise their brood in a single defensible nest (Michener 2000). We argue that in these insects, central-place foraging on ephemeral, dispersed and highly variable floral resources places particularly heavy demands on cognitive capacities. Individuals must learn to forage at an energetic profit, locate high quality feeding sites, efficiently handle flowers and navigate back to the nest to provision their brood with the right mix of nectar and pollen. The cognitive capacities underpinning these complex behaviours require optimal development and function of central brain structures, and precisely regulated plasticity of brain circuits necessary for learning, memory and navigation (Giurfa 2013; Menzel 2012). These brain systems are very easily disrupted, and it is especially problematic that many pesticides found in floral resources directly target key neural pathways (Palmer et al. 2013; Peng and Yang 2016). Pathogens and nutritional deficits also compromise cognitive functions (Arien et al. 2015; Iqbal and Mueller 2007). Even quite mild damage to the brain can significantly reduce foraging performance, thus rendering bees especially vulnerable to these environmental stressors. In social species, such as honey bees, bumblebees and stingless bees, efficient division of labour and coordination of tasks across nest mates provide buffering against environmental stressors, since individuals share a fortress-factory stocked with stored resources (Hölldobler and Wilson 2009). However, this buffering capacity has limits, which can be exhausted by frequent stressors. Once this occurs the result is a catastrophic colony decline (Bryden et al. 2013; Perry et al. 2015; Khoury et al. 2011).

Here we develop a neurobiological, ecological and evolutionary thesis to explain why central-place foraging bees are particularly sensitive to environmental stressors. First we describe the complex cognitive challenges bees face when foraging and the neural substrates supporting

these abilities. Next we review evidence that these essential cognitive abilities are impaired by a range of stressors, ultimately threatening brood development, colony function and survival. Finally, we discuss how understanding the mechanisms of action of the different stressors and their consequences on individuals and colonies can help better manage and protect these vital pollinators.

## Central-place foraging on flowers imposes significant cognitive challenges

Bees must gather large volumes of highly dispersed pollen and nectar, and return with it to the nest to feed their brood (Michener 2000). Accordingly, these insects have evolved excellent memory and navigation skills enabling them to exploit complex and variable foraging environments, and more than a century of research has identified the underlying neural circuits (Giurfa 2013; Menzel 2012). Although most studies have focused on a few economically important social species, such as honey bees and bumblebees, solitary bees appear to show similar behaviours (Michener 2000), cognitive capacities (Jin et al. 2015) and overall brain organisation (Farris 2016). In the bee brain **(Figure 5.1)**, visual and olfactory stimuli are first processed by their respective sensory lobes (for detailed reviews see (Dyer et al. 2011; Sandoz 2011)), which then convey information to multisensory integration centres, such as the mushroom bodies (MBs) and the central complex (CX), that are specialised for learning and memory and spatial navigation tasks, as we describe below.

### Learning to recognize flowers

Despite a large variety of available floral species, individual bees tend to forage on the same flower type as long as it provides sufficient nectar or pollen (von Frisch 1966). This floral constancy demonstrates the abilities of bees to learn the association between food rewards and particular floral cues (odour, colour, shape, temperature etc.) (Chittka et al. 1999). In many cases, bees learn more complex associations by generalising specific floral cues to learn conceptual features common to a range of flowers from the same species (Giurfa 2013). The amount of reward offered by flowers can change very rapidly, and bees can update their learned flower preferences accordingly (Dyer et al. 2014; Raine and Chittka 2012). Bees can also use combinations of floral and social cues, including the presence of conspecifics or other bee species on flowers, to locate and learn rewarding flowers (Dawson et al. 2013).

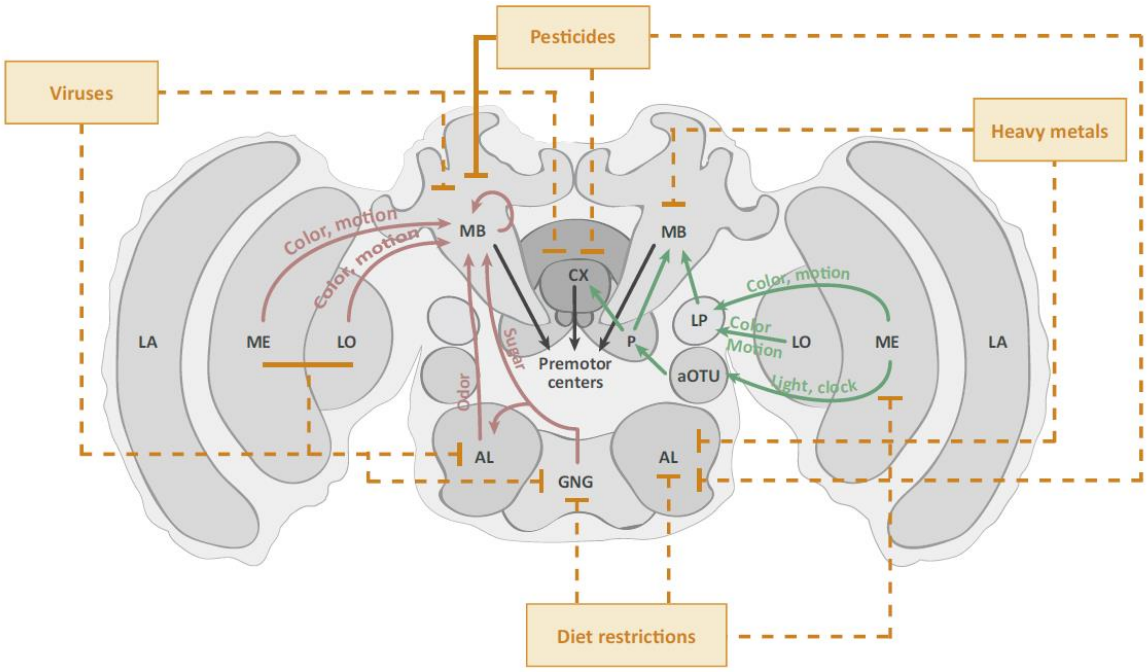



**Figure 5.1. Brain structures supporting the cognitive capacities needed for foraging and how they are impacted by stressors.** Schematic frontal view of a bee brain. Sensory information from the environment is first processed in specialised brain structures. The antennal lobes (AL) process olfactory information. The lamina (LA), medulla (ME) and lobula (LO), as part of the optic lobes, process visual information. The gnathal ganglion (GNG) receives gustatory information, and is sensitive to sugar. Sensory signals are then conveyed to higher-order centers (arrows). The mushroom bodies (MB) are involved in stimulus classification (odour, colour), complex associative learning and memory. They receive information directly from the sensory centers or indirectly through the lateral protocerebrum (LP) and the protocerebrum (P). The central complex (CX) receives processed visual input through the structures of the protocerebrum including the anterior optic tubercle (aOTU) and bulbs. The central complex locates the bee in space using celestial information and visual landmarks and is key for orientation and navigation. Environmental stressors (orange boxes) alter functions of various systems in the brain, and can alter the neural pathways supporting learning (purple arrows) and navigational capacities (green arrows). Dashed orange lines indicate impacts of stressors that have not been directly demonstrated for bees, but can be inferred by behavioural observations or have been observed in other insects.

Many of these mechanisms of learning and memory have been examined in details using experimental approaches **(Box 5.1)**. For instance acquisition of associative memories linking floral cues with food rewards relies on changes in neural activity induced by locally coincident activity in neural networks processing such cues and those signalling food detection (Giurfa and Sandoz 2012a). Plastic changes in connectivity in either the antennal lobes (ALs) or the MBs **(Figure 5.1)** can support associative learning about odorants, and both structures modify

their activity following learning (Sandoz 2011). In particular, the MBs are required for some complex forms of olfactory learning as well as for the formation of olfactory long-term memory (Webb and Wystrach 2016). Although less is known about visual learning, there is visual input from optic lobes (OLs) to the MBs **(Figure 5.1)**, and it is increasingly likely that associative learning of visual features and colours also involves the MBs (Webb and Wystrach 2016). Memorising simple odour-food associations involves excitatory signalling through acetylcholine in the ALs and MBs **(Figure 5.1)** (Giurfa 2013), a neurotransmitter system specifically targeted by many common pesticides, such as neonicotinoids and organophosphate miticides (Palmer et al. 2013).

**Orienting, navigating and learning places**

Bees use multiple different sources of information to orient (Webb and Wystrach 2016). Path integration requires storing information on distances and directions travelled during the outward journey, in order to plot a direct return path to the nest (Collett et al. 2013). Distance is estimated from optic flow (Srinivasan 2000), which is the movement of the image of the environment across the eye during flight. Direction is determined using the position of the bee relative to the sun (el Jundi et al. 2014) and/or the pattern of polarised light in blue sky (Dovey et al. 2013). Bees possess specialised mechanisms to compensate for the apparent movement of the sun (and the polarisation pattern it generates) across the sky during the day (Zeller et al. 2015). Bees are also sensitive to other global sources of navigational information such as fine magnetic field variations, and can learn to relate them to local landmarks so that they can still navigate when celestial cues are blocked by cloud (Wajnberg et al. 2010).

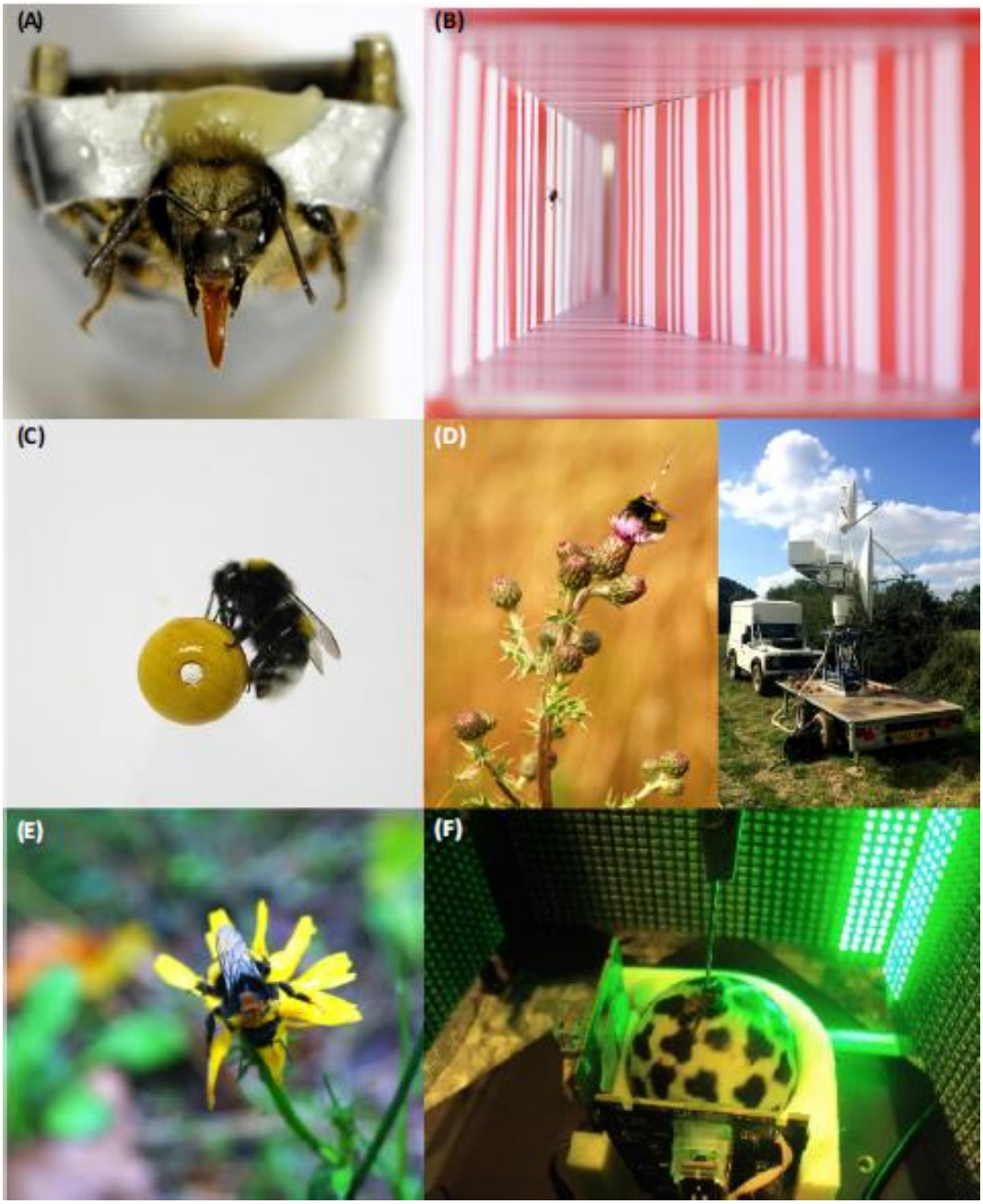
Bees can also learn locations by memorising visual scenes. They use these stored ‘snapshots’ for navigation by positional image-matching (Collett et al. 2013), which compares their current view of the environment with a visual memory of the goal. The degree of matching provides a cue for guidance (Collett and Collett 2002). Bees form snapshot memories of the nest surroundings on their first foraging attempts outside the nest and also of the location of food sources (Philippides et al. 2013). For visual matching, individuals use salient objects (flower patches, trees, buildings), which can be either local cues or panoramic landmarks (Collett et al. 2013). Honey bees can also perform optic flow matching, using the direction of optic flow caused by major landmarks as a navigational cue (Dittmar et al. 2010). Processing information on optic flow and landmarks while flying demands integrating visual and proprioceptive input with a temporal component. Responses to motion stimuli and colour are displayed by neurons connecting the OLs to central areas, the lateral protocerebrum (LP) and the MBs (Paulk et al. 2009) **(Figure 5.1)**, and some of these neurons are involved in visual landmark detection (Mertes et al. 2014).

The functions of the central complex (CX) **(Figure 5.1)** are presently poorly understood, but data from other insect species suggest that it is crucial for navigation (Webb and Wystrach 2016). Besides being a likely substrate for a sky compass (el Jundi et al. 2014), the CX could also support visual short-term (working) memory and spatial memory (Pfeiffer and Homberg 2014). A recent study using a virtual reality assay **(Box 5.1)** in *Drosophila* showed that activity of the ellipsoid body neurons of the CX represented the orientation of the fly relative to visual landmarks (Seelig and Jayaraman 2015). Thus it is increasingly likely that neural activity in the CX contributes to internal representation of position for path integration (Seelig and Jayaraman 2015).

**Box 5.1. Studying the mechanisms of learning and memory in bees**

Experimental work addressing the fine scale neural and behavioural bases of bees' cognitive capacities has relied primarily on Pavlovian conditioning, where an individual is trained to associate an initially neutral stimulus (the conditioned stimulus, CS) with an unconditioned stimulus (US) that elicits an innate response (Giurfa 2013; Menzel 2012). Learning the CS-US association leads the animal to respond to the CS. Historically, the dominant paradigm has been the appetitive conditioning (using a sugar solution as the US) of the proboscis (tongue) extension reflex (PER) using a restrained bee (Figure IA) (Giurfa and Sandoz 2012a) although aversive paradigms also exist (Junca and Sandoz 2015). This method allows study of elemental associations between two prescribed events, and also non-elemental associations (when individuals respond in an adaptive manner to novel stimuli using learned information in a new context). In recent years considerable progress has been made by combining PER conditioning with pharmacological treatments, electrophysiological recordings and brain functional imaging, to unravel mechanisms of learning and memory, especially for olfactory learning (Devaud et al. 2015).

So far, attempts at associative conditioning of visual CS in PER conditioning with restrained bees has yielded low performance levels (Lichtenstein et al. 2015). By contrast, impressive visual learning capacities have been described using free-flight assays, in which bees obtain a sugar reward if they make a correct choice when learning to navigate in a maze (Figure IB) (Srinivasan 2014) or foraging in arrays of artificial flowers (Figure IC) (Dawson et al. 2013). Automated tracking systems, such as harmonic radars (Figure ID) (Lihoreau et al. 2012; Fischer et al. 2014), radio frequency identification (RFID) (Figure IE) (Henry et al. 2012; Perry et al. 2015; Gill et al. 2012), or computer vision (Crall et al. 2015), allow precise quantification of behavioural data in laboratory or semi-field conditions. These approaches have revealed bees' cognitive abilities for learning complex visual features and relational properties between stimuli (Giurfa 2013). New developments in virtual reality assays, in which tethered bees walk on a locomotion compensator (Figure IF) (Paulk et al. 2014) or fly (Taylor et al. 2013) to make foraging decisions in response to stimuli displayed on a screen, hold considerable promises to explore the neural mechanisms of visual learning and navigation.




**Box 5.1. Figure I. Methods for studying bee learning and memory. (A)** Restrained honey bee showing proboscis extension reflex (PER) (C. Fresillon/CNRS). **(B)** Free-flying honey bee in a flight tunnel covered with visual patterns generating optic flow (F. Vrignaud/DGA) (Taylor et al. 2013). **(C)** Bumblebee foraging on an artificial flower (M. Lihoreau). **(D)** *Left:* Bumblebee with a radar transponder in the field (J.L. Woodgate). *Right:* Harmonic radar (J.C. Makinson). **(E)** Bumblebee with a RFID tag in the field (S. Klein). **(F)** Tethered honey bee walking on a locomotion compensator, in a controlled visual environment displayed onto LED panels (G.J. Taylor) (Taylor et al. 2013).

## Learning foraging circuits

Bees can use their spatial memories dynamically to establish and optimise foraging routes. In nature, foragers must sometimes visit hundreds of patchily distributed flowers to collect sufficient nectar and pollen in a single trip (von Frisch 1966), and many species revisit familiar

patches over consecutive hours or days in stable sequences called ‘traplines’ (Janzen 1971). Recordings of bumblebee flight paths using harmonic radar **(Box 5.1)** show that foragers attempt to minimise the overall travel distances between discovered flower patches, a complex optimisation task akin to the Travelling Salesman Problem (Lihoreau et al. 2013). On each new foraging trip, bees try different visitation sequences, ultimately finding (or approximating) the shortest possible path to visit all patches once, starting and ending at the nest (Lihoreau et al. 2010). Route optimisation is an iterative improvement process based on learning and memory of flight vectors between feeding locations, supported by path integration and visual guidance (Reynolds et al. 2013). This process allows for route flexibility and rapid adjustment of trapline geometry in response to changes in spatial distribution of floral resources, for instance when a patch becomes depleted or a more rewarding one is discovered (Lihoreau et al. 2012).

**Foraging performance improves with foraging experience**

On their first foraging attempts, bees make orientation flights to systematically acquire information about the nest location without collecting food (Degen et al. 2015). Foraging performance then improves over the first week of foraging, likely due to learned flower identification and handling, and route optimisation (Gill and Raine 2014; Dukas 2008). Dramatic changes in the structure of the adult brain are seen during this period (Muenz et al. 2015). Foraging activity is reflected by an allometric increase in MB volume (Withers et al. 1993b; Jones et al. 2013). In honey bees this expansion is caused by increased dendritic arborisation of the MB intrinsic neurons receiving visual and olfactory input accompanied by the pruning of microglomeruli (synaptic boutons) (Groh et al. 2012; Muenz et al. 2015), partly due to the activation of cholinergic receptors (Ismail et al. 2006). The selective localisation of these structural changes suggests activity-dependent synaptic plasticity as an underlying mechanism (Muenz et al. 2015). Dendritic growth can provide a substrate for the formation of new synapses to support stable memories (Hourcade et al. 2010). At the same time selective growth and pruning of connections is thought to optimise the performance of brain centres in the rich visual and olfactory environments experienced during foraging (Muenz et al. 2015).

## Stressors affect brain functions, cognition and behaviour

Successful foraging is based on the precise integration of information processed across the major brain networks, as well as dynamic structural modifications of such networks. Therefore even subtle disturbances of neural function could have dramatic consequences on individual cognitive abilities and hence foraging performance. From this perspective it is a major concern

that most of the stressors presently impacting on bees target the brain. The range of stressors has been well reviewed previously (Potts et al. 2010a; Goulson et al. 2015). Here we emphasise how many of these impair cognitive abilities and foraging performance at exposure levels far below those that kill the bee.

**Pesticides and heavy metals**

Many pesticides affect bee cognition. In recent years, neonicotinic insecticides have drawn the most attention (Field et al. 2015). These insecticides disrupt cholinergic transmission, the main excitatory pathway in the insect brain, vital for effective learning and synaptic plasticity (Sandoz 2011; Giurfa 2013). While acute exposure to very small doses of neonicotinoids has been shown to inactivate MB neurons (Palmer et al. 2013), chronic exposure can impair the whole MB development (van Tomé et al. 2012; Peng and Yang 2016). These effects almost certainly explain the dramatic impacts of sublethal doses of neonicotinoids on learning and memory in honey bees (Tan et al. 2015), bumblebees (Stanley et al. 2015), and solitary bees (Jin et al. 2015), which can be linked to deficits in MB plasticity (Peng and Yang 2016). Pesticide exposure also disrupts visuo-spatial memory and navigation (Fischer et al. 2014; Stanley et al. 2016; Henry et al. 2012), most likely through disruption of processing in the corresponding pathways **(Figure 5.1)**, but this has yet to be demonstrated. Alarmingly, bees learn to prefer nectar containing neonicotinoids over non contaminated nectar because of incidental actions of pesticides on the nicotinic receptors involved in reward processing (Kessler et al. 2015).

Fipronil, a widely used insecticide and acaricide, targets neuronal receptors involved in inhibitory transmission by gamma-amminobutyric acid (GABA) and glutamate (Simon-Delso et al. 2015). In honey bees GABA signalling is vital for normal MB function, particularly for complex learning (Devaud et al. 2015; Boitard et al. 2015). Acute fipronil treatment severely reduces olfactory learning and memory performance (Bernadou et al. 2009). Additional indications of neuronal cell death in the MBs following fipronil exposure suggest possible long-term cognitive impairments in honey bees (Roat et al. 2013) and stingless bees (Jacob et al. 2015).

Some pesticides contain manganese, which induces precocious foraging in honey bees (Søvik et al. 2015b). Its effect on sucrose responsiveness suggests that it interferes with signalling pathways important for associative learning, as indicated by the abundant expression of a manganese transporter in MBs and ALs (Søvik et al. 2015b) **(Figure 5.1)**. Selenium, another heavy metal found in crop treatments, has been found to change sucrose responsiveness, olfactory learning and long-term memory (Burden et al. 2016).

**Parasites and viruses**

Human activities have intensified the pathogen pressures on bees through dispersion of pathogens across the world (Cornman et al. 2012). While few parasites or pathogens act directly on the brain, many have a strong impact on the behaviour of bees (Cornman et al. 2012). Part of this can be explained by the activation of the immune system, which might interfere with energy supply or signalling mechanisms. Even an immune response induced by non-pathogenic molecules can reduce olfactory associative learning abilities (Alghamdi et al. 2008; Jaumann et al. 2013).

The microsporodian *Nosema cerana* and the mite *Varroa destructor* are two major parasites of honey bees. Exposure to either of them induces specific but overlapping patterns of altered gene expression in their host's brain (McDonnell et al. 2013). *Varroa* infection alters brain expression of many genes involved in neurotransmitter signalling, including through GABA (McDonnell et al. 2013). These impacts on the brain are thought to induce poor navigation performances by infected bees (Wolf et al. 2016, 2014).

*Varroa* carries many viruses, and a *Varroa* infection of a colony is a complex syndrome of many co-associated pathogens. Part of the effects of varroensis is due to viral infections (McDonnell et al. 2013; Francis et al. 2013). For example, the deformed wing virus (DWV) impacts on olfactory learning, possibly by targeting brain areas of importance for foraging (Iqbal and Mueller 2007). Although there is no known impact of DWV on bee visual learning and navigation, other viruses, such as the Israeli acute paralysis virus (IAPV), affect homing behaviour (Li et al. 2013).

**Malnutrition**

Intensive farming and the expansion of monocultures have imposed strong constraints on the dietary diversity of bees since only rather few food resources are available to them, often in limited flowering seasons (Goulson et al. 2015). Bee nutrition is partitioned between nectar, the main source of carbohydrates, and pollen, which provides proteins, lipids, vitamins and other micro-nutrients (Vaudo et al. 2016). Limited food intake reduces performance in a simple learning task (Jaumann et al. 2013), but having enough food is not necessarily sufficient for optimal cognitive processing. In honey bees, olfactory associative learning is disrupted by qualitative changes in essential lipids (Arien et al. 2015) or amino acids (Simcock et al. 2014). Pollen shortage during development can also lead adults to forage earlier and for a shorter period (Scofield and Mattila 2015), whereas nectar deprivation increases impulsive, suboptimal food choices (Mayack and Naug 2015).

## From reduced foraging performances to colony collapse

Few of the stressors we have considered would kill bees outright at ecological levels. Nonetheless, impairment of the cognitive abilities and food collection performance by low stresses can have extremely severe consequences on bee functions and survival, and critically on their capacity to successfully rear brood and maintain colonies. Hence these stresses can have very significant impacts on populations.

Comparative research on bee declines suggests bees' resilience to stressors depend on their level of sociality (Cresswell et al. 2012; Rundlöf et al. 2015), although this needs to be confirmed by more studies **(Box 5.2)**. In principle, solitary bees are the most vulnerable since reduced foraging efficiency of the female following stress exposure immediately jeopardises the development of her brood. These species lack the profusion of specialised group behaviours observed in social bees (e.g. corpses and diseased brood removal, social fever, collection of antimicrobial and antiviral plant resins) that can mitigate the impact of pathogen stressors on colonies (Cremer et al. 2007).

However, the stress tolerance of social bees is not without limits and stressors, even at low levels, can also have extremely severe consequences on colonies. In the most social species, such as honey bees, foraging is undertaken by middle-aged adults that have completed a period of orientation flights and brain maturation to prepare them for the cognitive demands of foraging (Muenz et al. 2015; Withers et al. 1993b). Stressors not only disrupt foraging performance, but also the process of preparing for foraging. For honey bees, a very common response to many stressors is to begin foraging prematurely (Perry et al. 2015) **(Figure 5.2)**. It has been argued that delaying high-risk tasks to later in life is an effective strategy to extend mean longevity of workers and increase their total contribution to the colony (Woyciechowski and Moroń 2009). But if worker lifespan is reduced, workers react by proportionally compressing their time allocation to each task, and commence foraging early. This is likely an adaptive response to acute stress, since it would temporarily compensate the foraging effort of the colony. However, in conditions of prolonged stress, this response can accelerate colony decline since bees that start foraging precociously complete fewer trips in their lifetime (Ushitani et al. 2015) and live less long (Perry et al. 2015).

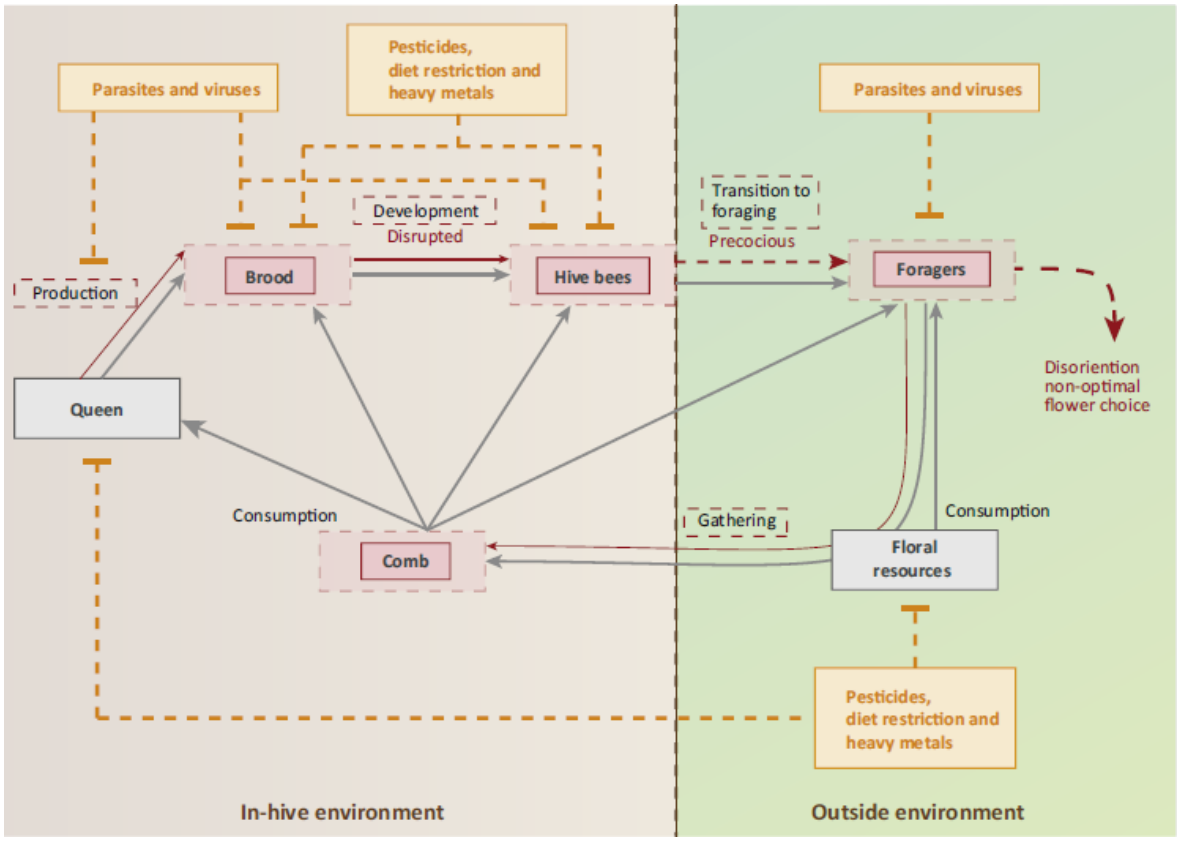



**Figure 5.2. Effects of stressors on honey bee colony dynamics.** In a non-stressed colony (grey arrows), the brood (eggs, larvae and pupae) develops into in-hive bees (e.g. nurses) that begin to forage two weeks later. Foragers gather nectar and pollen from floral resources for storage in the hive (comb). The food stock is consumed by the queen, the larvae, the in-hive bees and the foragers. Individual bees can be exposed to environmental stressors (orange boxes) at different stages, potentially disrupting the whole colony dynamics. Stressors reduce brood production, alter development, induce a precocious foraging onset of in-hive bees and affect the cognitive performances of foragers, leading to disorientation and less efficient food gathering (red arrows). The synergistic action of stressors at different levels of this complex system can lead to dramatic colony collapse. Plain red arrows indicate quantitative changes. Dashed red arrows indicate qualitative changes. Adapted from (Khoury et al. 2011).

Simulation models suggest that continuous stress can create a situation in which the foraging force is dominated by precocious foragers (Becher et al. 2014; Perry et al. 2015), and becomes so inefficient that it can no longer support the colony, at which point the colony population dramatically collapses **(Figure 5.2)**. Stressed bumblebee colonies, although smaller and socially simpler than honey bee colonies, also show highly non-linear responses to environmental stressors (Gill et al. 2012; Bryden et al. 2013). Various impairments of colony function (including foraging, but also thermoregulation, defence and hygienic behaviour) can generate changes in population dynamics via feedback loops affecting rates of hatching and adult death, sometimes leading to colony collapse (Bryden et al. 2013). These complex

dynamics might explain the observed widespread declines of wild and managed bee populations (Rundlöf et al. 2015; Ollerton et al. 2015; Woodcock et al. 2016; Potts et al. 2010a). The known stressors of bees are not new, and many populations have been in a steady decline for decades, but the accelerated declines described recently suggest that we are now reaching the point at which the cumulative stress on colonies is exceeding their capacity to tolerate it (Ollerton et al. 2015; Woodcock et al. 2016; Rundlöf et al. 2015).

## Summary and future prospects

Central-place foraging bees are particularly vulnerable to many current environmental stressors. These insects have evolved refined cognitive abilities to enable them to effectively exploit complex and changing foraging environments to provision their nest. Such capacities demand the optimal function and coordination of major systems in the small bee brain. Many stressors disrupt brain function with the consequence of reduced foraging performance, ultimately compromising brood or whole colonies. These gradual and pervasive effects might explain why eco-toxicological studies, alone, have failed to provide accurate predictions of how stressors can damage bee colonies. We therefore argue that more integrated research that considers actions of the different stressors on bee behaviour, cognition and colony function is urgently needed to understand the declines of these major pollinators and manage their populations **(Box 5.2)**.

**Box 5.2: Outstanding questions: research to sustain bee populations**

1. What are the points of greatest vulnerability in the bee brain? Neurogenomic profiling has started to provide an overview of the gene expression changes occurring in the brain in response to pathogens (McDonnell et al. 2013), but we have yet to understand the signalling pathways involved and the functional relevance of these changes. More integrative work is now needed to identify precisely how stressors damage the brain to reduce foraging performance. This must couple genomic studies with functional analyses of changes in circuit performance and behaviour. If the points of vulnerability in the developing and adult brain can be identified, it would help design neuroprotective treatments to improve the resilience of managed bees.

2. Are all bee species similarly vulnerable to stressors? Bees greatly vary in their social organisation (from solitary to social), feeding ecology and habitats (Michener 2000). While most attention has focused on managed populations of generalist species with a social lifestyle, such as honey bees and

bumblebees, comparative research is now needed to assess the general impact of stressors on the wide diversity of pollinators.

3. How can pesticides and bees be managed to keep populations at a 'safe level' of exposure? A key issue is determining what cocktails and levels of pesticide exposure populations can tolerate. Often there are multiple different pesticides at use in the landscape. We need more information on how these chemicals might accumulate and persist in nests, and how they interact to impact bee physiology and behaviour.

4. How then can the agricultural environment be managed to ensure bees receive adequate nutrition from diverse floral sources? Can we design nutritionally optimised plant assemblages to preserve bee populations? Crops provide huge amounts of foods but these plants that have been selected to optimise production and typically yield poor quality diets to bees (Vaudo et al. 2015). Research is needed to quantify the precise nutrient needs of bees, how they vary across colony developmental stages, species and in the face of specific stressors, and their impact on behaviour and cognition.

5. Can the pollination performance of managed social bees (honey bees and bumblebees) be sustainably improved by manipulating colony composition? Within a colony, social bees show high levels of inter-individual behavioural and cognitive variability. In honey bees a small number of individuals complete a disproportionately high number of foraging trips (Tenczar et al. 2014). Characterising this variability between bees, what causes it, and how it changes under stress conditions is needed to understand the consequences of environmental stressors on the resilience of colonies.

Pesticides provide an informative case in point. Agriculture has become increasingly reliant on the 'next generation' neonicotinoid pesticides because they are so effective at killing pest insects at low doses by directly targeting the insect central nervous system (van der Sluijs et al. 2013). Recent research describing the neural impacts, behavioural impairments and changes in colony dynamics at field contamination levels by pesticides (van der Sluijs et al. 2013; Gill et al. 2012; Henry et al. 2012; Gill and Raine 2014; Stanley et al. 2016) has forced a re-evaluation of the 'safe-level' of pesticide exposure for individual bees and colonies (Field et al. 2015). Using this new knowledge we must now determine how pesticides can be managed in the agricultural landscape in a manner that is compatible with sustaining bee populations. Many other stressors contribute to colony decline (Potts et al. 2010a; Goulson et al. 2015), for which the precise mechanisms of action need to be unravelled **(Box 5.2)**.

As discussed above the stress tolerance of a colony is not without limits, and given the increase in bee declines seen in the last decade it would appear we are very close to exhausting those limits for some key pollinating bee species. But this is far from a hopeless story.

Combining conceptual and methodological advances in neuroscience, ecology and evolutionary biology can bring considerable insights into how specific stressors affect bee behaviour and colony dynamics, and help identify ecological interventions to ameliorate stress on bees. Most of the stressors damaging bee populations are human induced, and can be reduced or eliminated from the environment if there is sufficient will, or economic imperative.

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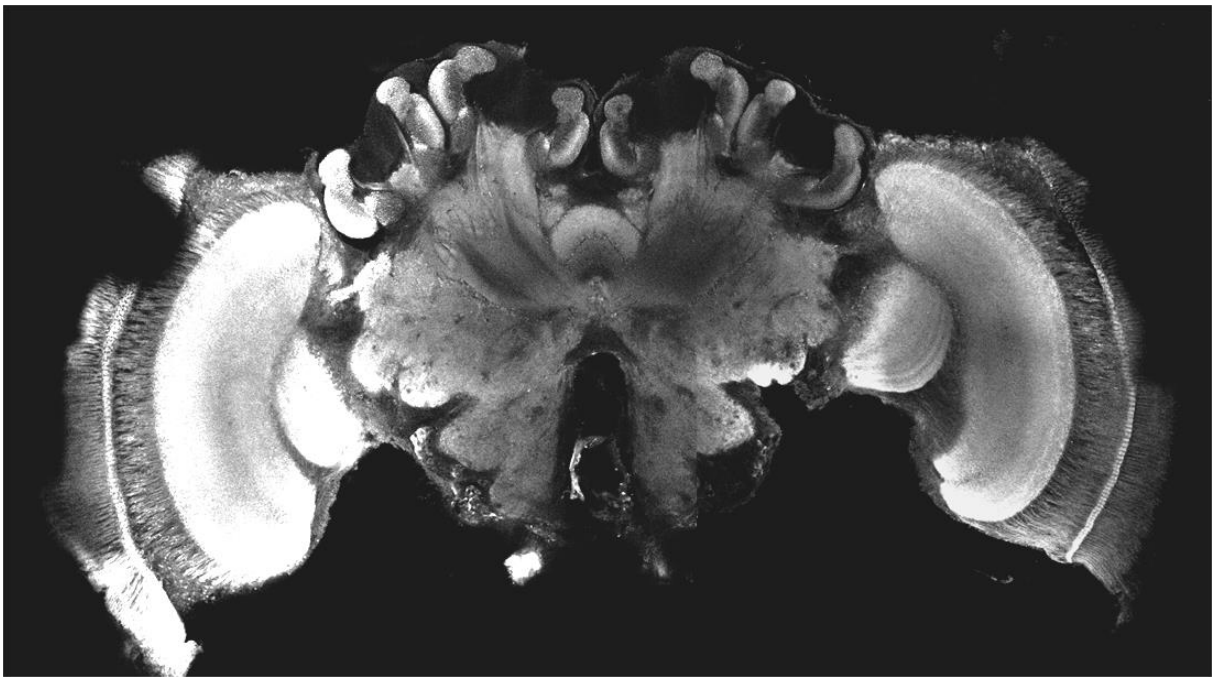
# Chapter 6.

General discussion



This thesis has highlighted the possible influence of the connectivity at the input region of the MB on performance in MB-dependent learning tasks **(Figure 6.1)**. Experience of a rich environment, whether it be in the hive during early adulthood or at the transition to outdoor activities, was beneficial for learning performance in a MB-dependent task, by reducing the number of synaptic boutons in the MB neuropil. Foraging activity, by contrast, increased synaptic bouton number, which had a negative impact on MB-dependent learning capacities. My data suggests that this foraging-induced cognitive decline might be partly mediated by the cholinergic pathway. I also provided evidence for the role of environmental stressors on bees' cognitive decline. Stressed bees, starting to forage before a normal age, were more sensitive to the foraging-induced cognitive decline.

## Synaptic bouton number in the MB affects performance in MB-dependent learning tasks

I have demonstrated a negative relationship between synaptic bouton number in the MB olfactory neuropil (lip) and performance in reversal learning, either by comparing directly MB structure of learners and non-learners (Chapter 3), or by correlating experience-dependent variations in both parameters (Chapters 2, 3) **(Figure 6.1)**. Although the method used to quantify synaptic boutons has some limitations (assuming a homogeneous repartition of synaptic boutons within the MB calices, including variation in signal intensity) (Fahrbach and Van Nest 2016), it is a well-established protocol that allowed us comparisons with previous studies (Groh et al. 2012; Muenz et al. 2015). Also, as all the brains were analysed blind to condition, I am confident about the reliability of the results presented in this manuscript. Thus, I observed that a high number of synaptic boutons had a detrimental effect on reversal learning performance. Also, in chapter 4 I observed that experienced foragers, who were previously shown to have a high number of synaptic bouton in the lip (Chapter 3), had a decreased performance in positive patterning (another MB-dependent task (Devaud et al. 2015)). Although this still needs to be clarified, a high number of synaptic boutons in the lip seems to be also deleterious for olfactory positive patterning performance (Chapters 3, 4). Our model of MB function proposes a hypothesis why this might be so (Chapter 3). A relatively low number of boutons in the lip would be associated with a sparse activation of MB intrinsic neurons in response to individual odours which would be critical for success in reversal learning and positive patterning. Sparse activation of MB neurons in response to odorants has been reported in bees and flies and is thought to improve the ability of flies to discriminate similar odours by reducing overlap between odour representation (*honey bees*: Szyszka et al. 2005; *flies*: Perez-

orive et al. 2002; Lin et al. 2014). Sparse coding is however not required to discriminate dissimilar odours in flies (Lin et al. 2014), which might explain why synaptic bouton number in the lip did not affect the first discriminative phase of reversal learning in my study (Chapter 2, 3). Many mathematical models have also highlighted the benefits of sparse coding of sensory information for associative memory storage (Palm 2013). In bees and flies, sparseness of odour representation in the lip is maintained by GABAergic PCT neurons, which connect the MB output to the input and are required for the resolution of a reversal learning task (*honey bees*: Grünewald 1999; Froese et al. 2014; Boitard et al. 2015; *flies*: Wu et al. 2012; Lin et al. 2014). In our model, reducing input from the GABAergic PCT neurons in the MB neuropil also resulted in decreased sparseness and altered reversal learning performance (Chapter 3). Therefore, maintaining a relatively low number of synaptic boutons in the lip, possibly through GABAergic inhibition, seems critical to solve MB-dependent learning tasks.

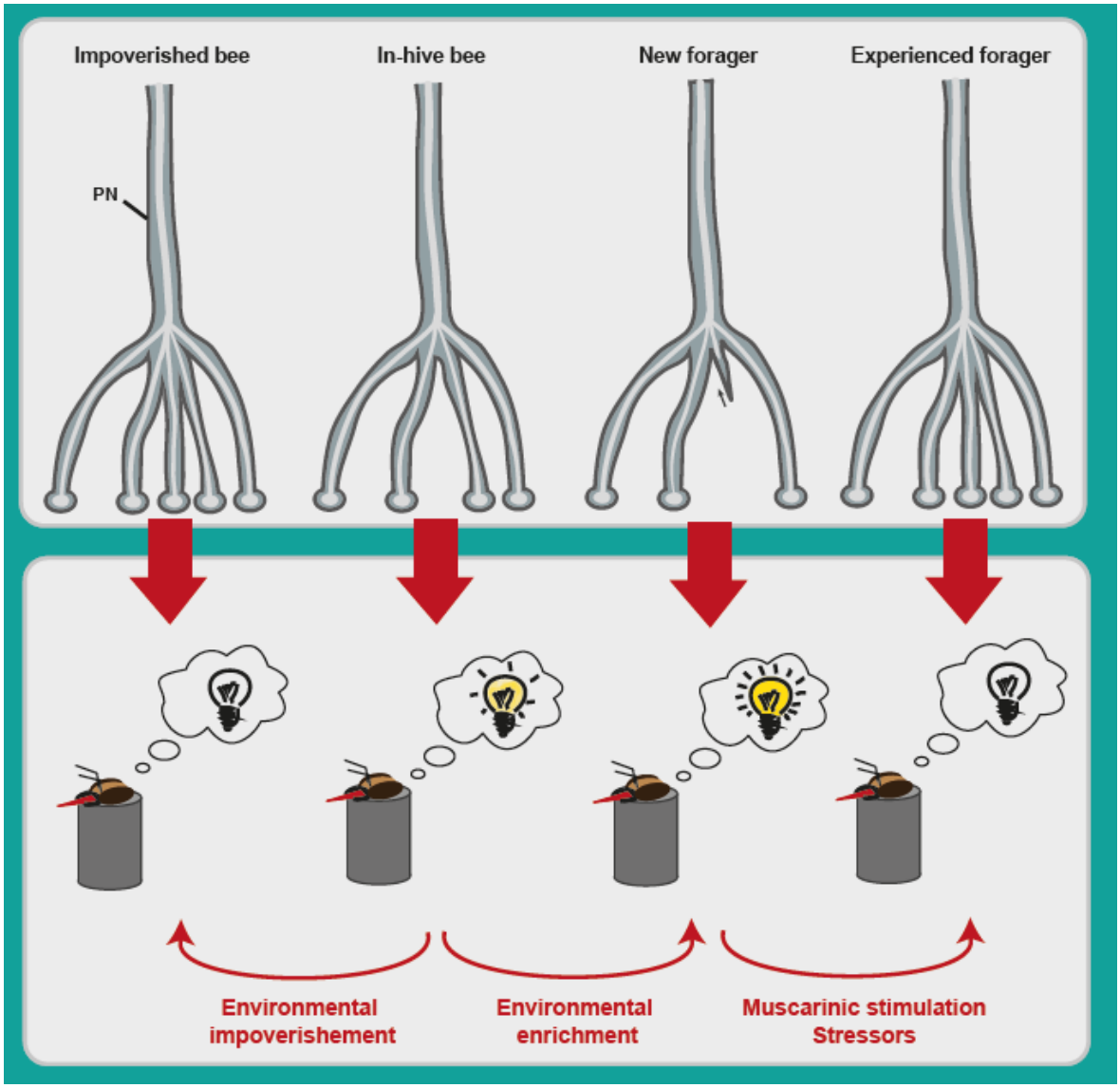


**Figure 6.1. Experience-dependent plasticity of synaptic boutons in the lip of the mushroom bodies and consequences on learning capacities.** Axon terminals of projection neurons (PNs) in the MB lip (*upper panel*) and performance in MB-dependent learning paradigms based on the conditioning of the PER (*lower panel*) are represented for bees from an impoverished environment, in-hive bees, new foragers, and experienced foragers (*from left to right*). Potential mechanisms are represented in red.

## Possible neurobiological mechanisms regulating synaptic bouton number

Two general mechanisms of structural plasticity have been described that can explain variation in synaptic bouton number: Hebbian structural plasticity and homeostatic structural plasticity (Fauth and Tetzlaff 2016). The former is based on Hebb's theories of synaptic plasticity stating that coincident activity in the pre- and post-synaptic compartment increases synapse efficacy, while low activity levels weaken the connection (Hebb 1949). Thus, Hebbian structural plasticity refers to increased connectivity (number of synapses) between activated neurons and vice versa. Homeostatic structural plasticity, by contrast, stabilizes the neural network by decreasing, or increasing, the number of synapses when neuronal activities are high, or low, respectively (Butz et al. 2009; Fauth and Tetzlaff 2016). In honey bees, long term memory formation of olfactory information induces an increase in the number of synaptic boutons in the MB lip (Hourcade et al. 2010). It is unknown whether such an increase is stable in bees, but it has been shown to be transient in ants (Falibene et al. 2015). These changes in the number of synaptic boutons upon memory formation might reflect an interaction between Hebbian (increases bouton number) and homeostatic structural plasticity (decreases bouton number back to physiological levels) (Hebb 1949; Fauth and Tetzlaff 2016).

Inhibitory signalling via GABAergic PCT neurons, which innervate synaptic boutons (*honey bees*: Ganeshina and Menzel 2001; *flies*: Leiss et al. 2009), and excitatory cholinergic signalling via PNs might regulate bouton activity and therefore structural plasticity in the pre- and post-synaptic compartments of the boutons. In *Drosophila*, preventing PNs from firing induces an increase in synaptic bouton number and size in the MB (Kremer et al. 2010). In chapter 4, I observed a decreased performance in positive patterning after a chronic stimulation of the muscarinic receptors to acetylcholine. If, as discussed above, a high number of synaptic boutons in the lip is also detrimental to positive patterning performance, it suggests that the chronic stimulation of muscarinic receptors increased the number of boutons in my study. This would be consistent with the reported increase in MB volume and dendritic branching in response to the same treatment (Ismail et al. 2006; Dobrin et al. 2011). This contradicts, however, the principles of homeostatic structural plasticity as a chronic activation of neurons should decrease connectivity in the network (Fauth and Tetzlaff 2016). In mice, a chronic

stimulation of muscarinic receptors induced their depletion of the plasma membrane of neurons, thus reflecting an homeostatic plasticity process (Decossas et al. 2003). One hypothesis, which still needs to be tested, could be that the mechanisms of homeostatic plasticity, in particular pruning processes (elimination of boutons) are altered in foragers. Even so, a balance between cholinergic excitation and GABAergic inhibition seems to regulate bouton number, sparse coding in the MB lip, and performance in MB-dependent learning tasks.

## Experience of varied environments improves learning capacities and decreases synaptic bouton number in the MB

I have demonstrated that environmental enrichment has a positive impact on learning capacities in honey bees (Chapter 2), as observed in other vertebrate (*rodents*: van Praag et al. 2000; *fishes*: Salvanes et al. 2013) and invertebrate species (*cuttlefishes*: Dickel et al. 2000; *crickets*: Mallory et al. 2016). At early stages of adult life, a rich natural environment was required for the acquisition of the ability to solve reversal learning. I then demonstrated that reversal learning performance could be even more improved at the transition to foraging (Chapter 3). Therefore, in older bees, environmental enrichment also improves olfactory learning capacities. Further studies should investigate the impact of environmental enrichment on other learning paradigms and modalities, under laboratory and natural conditions, as well as the neural bases of this experience-dependent plasticity.

The richness of the environment experienced by bees also influenced the number of synaptic boutons in their MB neuropil. This number was increased by environmental impoverishment (Chapter 2), and decreased by environmental enrichment (Chapter 3). The high number of synaptic boutons in the lip of bees experiencing impoverished environments (Chapter 2) might be explained by a compensatory mechanism for the decreased sensory stimulation and therefore the low levels of activation of PNs (Fauth and Tetzlaff 2016). As mentioned earlier, inhibiting firing from cholinergic PN increases synaptic boutons number in *Drosophila* (Kremer et al. 2010). Such homeostatic structural plasticity to compensate for sensory deprivation has also been reported in mice, for which monocular deprivation increased spine number of neurons in the visual cortex (Hofer et al. 2009). Compensation might be undertaken by the GABAergic pathway since sensory deprivation decreases inhibitory GABAergic synapses in the brain of different mammals (*review*: Flores and Méndez 2014). At foraging onset, by contrast, the richness and novelty of sensory stimulations might induce a high level of activation of PNs resulting in enhanced synaptic pruning (elimination of boutons) (Chapter 3). Indeed, repeated visual and olfactory stimulations are known to induce synaptic pruning in the collar and lip

respectively (*honey bees*: Scholl et al. 2014; *ants*: Stieb et al. 2010; Falibene et al. 2015), which reflects homeostatic structural plasticity. Again, GABAergic signalling might be a compensatory mechanism for the enhanced activation of cholinergic PNs. Indeed, an increase in the number of GABAergic inhibitory synapses has been observed in response to sensory stimulation in mammals (Knott et al. 2002; Flores and Méndez 2014), for which environmental enrichment is also known to increase cholinergic signalling (van Praag et al. 2000). Therefore, I highlighted the effects of environmental enrichment on brain structure and learning capacities, and discussed the role of the balance between brain excitation and inhibition for the first time in an insect.

## Foraging experience decreases MB-dependent learning capacities and increases MG number

The cognitive improvement at foraging onset was transient. I demonstrated that greater foraging experience decreased MB-dependent capacities assessed under laboratory conditions (Chapter 3, 4). Whether such changes in learning performance reflect a cognitive variation in the bee natural environment still needs to be demonstrated but it is relevant with the transient improvement in foraging performance reported by Dukas (2008) in the field. Contrary to what was observed in young bees in Chapter 2, placing foragers in cages in an incubator had a beneficial impact on learning capacities (Chapter 4). Therefore, the decreased performance in foragers does not seem to result from a decreased time spent inside the hive but to foraging activity itself. A lack of sleep due to intense foraging, which is known to affect learning and memory in insects (Hussaini et al. 2009; Donlea et al. 2011; Beyaert et al. 2012), might also be responsible for the decreased learning performance. Yet, this hypothesis still needs to be tested.

One hypothesis for this foraging-induced cognitive decline could be the negative influence of environmental stressors (natural or human-induced) on brain structure and function (Chapter 5). Some stressors directly affect the balance between cholinergic excitation and GABAergic inhibition, and subsequently learning capacities. For instance, organophosphate pesticides are known to increase cholinergic neurotransmission and therefore alter olfactory learning capacities (Williamson and Wright 2013). Other pesticides, such as fipronil, directly target the GABAergic pathway, resulting in decreased learning performance in bees exposed to that compound (Simon-Delso et al. 2015; Bernadou et al. 2009). It is therefore possible that pesticides targeting cholinergic or GABAergic signalling also affect the number of synaptic boutons in the brain of foragers. Cholinergic signalling has been shown to be higher in the brain

of foragers compared to nurses (Shapira et al. 2001). I have highlighted the potential role of an elevated stimulation of the muscarinic receptors in foraging-induced cognitive decline (Chapter 4). It remains to be investigated whether foraging increases cholinergic signalling, independent of any impact from exposure to organophosphate pesticides.

Although most stressors affect the whole colony, old bees have been shown to be more sensitive to physiological stressors (starvation, heat, oxidative stress) than young bees (Remolina et al. 2007). I also observed that precocious foragers are more affected than normal-age foragers by the foraging-induced cognitive decline (Chapter 3). They are also less efficient in foraging than normal-age foragers (Chang et al. 2015; Perry et al. 2015; Ushitani et al. 2016). Precocious foragers often appear in stressed colonies (Goblirsch et al. 2013; Perry et al. 2015) and, although this still has to be demonstrated, they might be more sensitive to additional stressors.

In mammals, exposure to one stressor affects the response to subsequent stressors (Bhatnagar and Dallman 1998; Armario et al. 2008). In the foraging environment, repeated exposure to stressors such as predators or sudden weather changes, might cause a chronic stress responsible for the decreased cognitive capacities. Stress is known to alter learning and memory capacities in different species (*rodents*: Cazakoff et al. 2010; Conrad 2010; Hurtubise and Howland 2016; *zebrafishes*: Gaikwad et al. 2011) and has already been observed in invertebrates (*review*: Anderson and Adolphs 2014; *crayfishes*: Fossat et al. 2014; *flies*: Enell et al. 2010; Mohammad et al. 2016). Although stress has never been clearly demonstrated in honey bees, bees that have been vigorously shaken exhibit a pessimistic cognitive bias regarding ambiguous olfactory stimuli (Bateson et al. 2011). In addition, stress has been associated with altered GABAergic signalling in many species (*crayfishes*: Fossat et al. 2014; *flies*: Mohammad et al. 2016; *humans*: Goddard 2016; *rodents*: Depino et al. 2008; Bains et al. 2015), and GABAergic inhibition is critical for behavioural flexibility assessed in reversal learning paradigms in rodents (Morellini et al. 2010; Powell et al. 2015; Hurtubise and Howland 2016). As mentioned earlier, the GABAergic PCT neurons projecting onto the MB are required for success in reversal learning in bees and flies (*bees*: Boitard et al. 2015; *flies*: Ren et al. 2012; Wu et al. 2012), and a stress-induced alteration of this pathway might therefore be deleterious for MB-dependent learning capacities. Also, caging bees in isolation with a dead bee has been shown to increase the volume of their MB (Maleszka et al. 2009), which could be explained by a stress-induced decrease in GABAergic signalling. Similarly, an alteration of the GABAergic pathway could be responsible for the high number of synaptic boutons reported in the MB of

experienced foragers, and for their poor performance in reversal learning (Chapter 3). Further experiments are needed to test this hypothesis.

## Conclusion

In cognitive neuroscience, learning performance often differs between individuals, but the causes of such variability are rarely examined when comparing groups of individuals. I demonstrated that variability in cognitive capacities is partly linked to prior individual experience, positive or negative, which affected synaptic connectivity in the brain regions involved in learning processes in honey bees. I hope this thesis will convince the neuroscientific community of the relevance of the honey bee as a model to study experience-dependent plasticity in brain structure and function. This might also help managing honey bee populations by understanding and reducing the negative impact of foraging on cognitive capacities.

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# Appendix A

Klein S*, Cabirol A*, Devaud JM, Barron AB, Lihoreau M (2017) Why bees are so vulnerable to environmental stressors. *Trends in Ecology and Evolution* 32, 268-278

[* These authors contributed equally to the work]

Appendix A of this thesis has been removed as it contains published material. The published version of this article can be found at:

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