The relationship between foraging efficiency, neural structure and learning in bumblebees (*Bombus terrestris*)

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Authorship declaration

I declare that the following work is my own, with the following exceptions.

Ellouise Leadbeater provided editing, corrected English language phrasing, and suggested material in all chapters. The development of the protocol presented in chapter 2 and used in chapter 3 and 4 was conducted in close collaboration with Dr Swidbert Ott (University of Leicester). Alberto Linguadoca provided his expertise on sulfoxaflor during experimental design stage and prepared the pesticide solution doses in chapters 4 and 5. Dr Christopher Pull participated in developing and building the radial-arm maze as well as the associated method used in chapter 5. Laboratory assistant Maddalena Vierbuchen contributed to 30% of the data collected on the radial-arm maze in chapter 5. Ash Samuelson and Zachary Chen-Wishart wrote the original R code I modified to run simulations in the radial-arm maze confirmation experiment in chapter 5. Matthew Hasenjager provided help on the R code and methodology for the analysis of the radial-arm maze validation experiment in chapter 5.

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Thesis abstract

In recent decades, social bee populations have been facing pressure from anthropogenic stressors such as habitat fragmentation and exposure to pesticides. In order to help inform policies to protect the ecosystem services that these species provide it is important to better understand the main behavioural driver of this service: foraging, and the proximate mechanisms that underlie it. Here I explore the topic of learning in foraging bumblebees *Bombus terrestris*, focusing on the relationship between foraging efficiency and neural plasticity, and how stressors such as pesticides can affect this relationship.

In Chapter 2, I begin by developing immunostaining methodology for brain tissues of *B. terrestris*, based on modification of existing protocols that were not reproducible. This technique allowed me to visualise and quantify the density of synaptic boutons (also known as microglomeruli; MG) within a neuropile of the insect central nervous system associated with learning, the mushroom bodies.

I then use this method to explore the relationship between foraging and MG density in Chapter 3, as learning abilities correlate with MG density and better learners are expected to be more efficient foragers. I measured the foraging efficiency of workers in 6 colonies during summer 2019 and quantified the MG of these foragers. While no significant relationship was detected, this chapter highlights the gaps in knowledge regarding foraging efficiency and offers routes to attempt to close them.

In the two following chapters (4 and 5) I present a case study on a novel neurotoxic insecticide, sulfoxaflor, that has documented negative sublethal effects on *B. terrestris* individuals and colonies. I investigated how this pesticide could influence the relationship between neural

structure, learning and foraging strategies. I first tested the effect of chronic exposure to sulfoxaflor on the synaptic density of the main centre for stimulus integration and important for foraging in bumblebees, the mushroom bodies (MB). No significant effect of the compound was detected either on MG density or volume of the MB. I then focused on testing short-term memory, which is less likely to have a direct neural correlate, using a radial-arm maze (RAM) mimicking within-patch foraging and for which I present a validation experiment of the paradigm. Once again, I found no significant effect of the insecticide on the foragers' performance in the RAM.

The results of this thesis show that the relationship between foraging, learning and neuroanatomy is less straightforward than traditionally believed and, additionally, that sulfoxaflor is unlikely to be causing negative effects to bumblebee colonies through a disruption of this relationship.

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Chapter 1: Introduction

Bumblebees (*Bombus spp.*) are key pollinators, and the service they provide is essential to the survival of wild plants as well as domesticated crops (Rader *et al*. 2016; Fijen *et al*. 2018). Unfortunately, due to the combined actions of anthropogenic factors such as the climate crisis, habitat fragmentation, agrochemicals and introduced pathogens, there is evidence that insect abundance is generally declining (Forister *et al*. 2019) and that many wild pollinators, including certain *Bombus* species, are undergoing range contractions which threaten the ongoing provision of this service (Biesmeijer *et al*. 2006; Colla and Packer 2008; Aizen and Harder 2009; Williams and Osborne 2009; Potts *et al*. 2010b; Cameron *et al*. 2011; Goulson *et al*. 2015; Kerr *et al*. 2015). While the western honeybee *Apis mellifera* is the most important single-species pollinator – owing to its wide distribution, generalist foraging behaviour and ability to pollinate – a large proportion of flowering plant species are not pollinated by honeybees (Garibaldi *et al*. 2013; Hung *et al*. 2018). Therefore, documented effects on wild bee populations are a major concern from the perspective of both crop and wild plant pollination.

Among the factors driving these declines, research into agrochemical stressors is important as there is a body of evidence linking such chemicals to negative effects on social bees (Goulson 2013; Bryden *et al*. 2013; Simon-Delso *et al*. 2015; Brown *et al*. 2016; Siviter *et al*. 2020b; Stuligross and Williams 2021). These impacts may be mediated in part through effects on the brain that impact foraging and other behaviours (van Tomé *et al*. 2012; Smith *et al*. 2020; for a general review of the effect of stressors on bee neural development see Klein *et al*. 2017). Stressors that affect neural development can affect learning abilities (Smith *et al*. 2020), and as learning abilities are expected to underlie foraging efficiency (Raine and Chittka 2008; Pull *et al*. 2022), it is possible that these individual effects scale up to impact food intake and

therefore fitness at the colony level (Stanley *et al*. 2015a). However, the role of negative effects on cognitive abilities as a driver of colony-level impacts brought about by agrochemical exposure is not yet fully established, because links between learning abilities and fitness are likely to be complex, and because some insecticides appear to elicit negative colony-level impacts without compromising learning (Siviter *et al*. 2018a, 2019).

This project explored (i) the relationship between learning and neural structure in the context of foraging in the model pollinator *Bombus terrestris* and (ii) how a neurotoxic pesticide can affect this relationship. I started by exploring the assumption that neural structure predicts foraging efficiency in *Bombus terrestris*, by customizing existing methods to visualize synaptic density in the bee brain, and using this method to relate neural structure to real-world foraging efficiency. I then investigated whether documented effects of a next-generation insecticide on colony productivity (Siviter *et al*. 2018a) could be driven by impacts on learning and memory, by exploring impacts on neural structure, and on behavioural assays of memory. In this introductory chapter, I will describe the tasks that face foraging social bees, the cognitive abilities that they apply within these tasks, and why negative impacts on these abilities are a cause for concern.

Animal cognition and natural selection

Cognition encompasses the mechanisms that allow animals to store, recall and act upon information gathered by interacting with their environment (Shettleworth 2001). These processes are integral to a number of key aspects of most animals' lives, such as mating or foraging, and thus, they are documented in some basic form even in species with the most rudimentary nervous system such as nematodes (*Caenorhabditis elegans*) or sea slugs (*Aplysia californica*) (Dukas 1998).

The evolutionary context that should promote investment in learning is generally accepted as an environment that sees a high level of unpredictability between generations but stays consistent within a generation (Boyd and Richerson 1988; Stephens 1991; Feldman *et al*. 1996; Kerr and Feldman 2003; Wakano *et al*. 2004; Dunlap and Stephens 2009; Fawcett *et al*. 2014). In other words, the environment must change enough between generations so that innate responses to stimuli alone cannot allow an individual to successfully transmit its genes to the next generation, and it must be stable enough that learning can be used reliably to grant an advantage to the individual engaging in it. More recent perspectives on the subject add that learning can evolve even in the absence of between-generational change if an environment is complex enough that innate behaviours simply cannot cover the range of responses necessary for an individual to pass on its genes to the next generation (Dridi and Lehmann 2016). As such, the ability to learn is assumed to have been shaped by selection, because an individual that is able to learn from experience could potentially interact with its environment more efficiently, which is likely to increase its survival and, potentially, its fitness (Dukas and Bernays 2000). Due to the ambiguous meaning of the word innate, it is important to note here that I have used it in the present thesis to designate documented behaviours observed in naïve individuals that have never encountered the stimuli concerned (Mameli and Bateson 2011).

Accordingly, although limited in number, studies that have investigated the link between cognitive traits and fitness proxies have found evidence in some contexts that efficient learners score higher in fitness-related tests. For example, Keagy *et al*. (2009) showed that male bowerbirds' (*Ptilonorhynchus violaceus*) problem-solving ability was positively correlated with mating success; Cole *et al* (2012) showed that female problem-solvers in great tits (*Parus major*) produced larger clutches, although this had no effect on overall fitness; Sonnenberg *et*

al. (2019) found that better spatial memory was associated with overwinter survival in mountain chickadees (*Poecile gambeli*); and Heathcote *et al*. (2023) demonstrated that spatial reference memory was positively correlated with home range but negatively correlated with predation risk in pheasants (*Phasianus colchicus*). However, evidence suggests that the potential benefits associated with better cognitive abilities also come at a cost, which likely constrains their evolution through trade-offs with other traits (Pennisi, 2014). For example, in *Drosophila melanogaster*, artificial selection for long-term memory has a negative impact on resistance to subsequent starvation and desiccation (Mery and Kawecki 2005) and the pathway necessary for long-term memory shuts down when the fly is starved (Plaçais and Preat 2013). Evidence that the process of memory formation itself can come at a cost can be found in *Drosophila*, where long-term memory appears to be triggered by an increase in metabolic rate (Plaçais *et al*. 2017). Thus, it seems likely that at the species level, cognitive traits are matched to ecological needs, representing a balance between efficient performance and costs of investment in learning and memory.

The task facing foraging bees

Non-parasitic *Bombus spp.* colonies have an annual life-cycle in temperate regions (Goulson 2003). The queen founds the colony alone after mating at the end of summer. She hibernates during winter and when early spring arrives, she finds a new nest site and starts collecting food and laying the first generation of eggs. As soon as this first generation reaches adulthood, they start foraging for the colony and taking care of the next generation of brood, which allows the queen to switch to egg-laying full-time. The colony will then grow in size (i.e. number of workers) until the summer comes and the queen starts producing gynes and males, which disperse. By the end of the colony cycle (late summer), the queen will die. This typically coincides with an initiation of worker reproduction and conflict within the nest (Goulson 2003).

Throughout this cycle, both queen (although only at the early nest-founding stage) and workers have to navigate a dynamic environment. Their food, nectar (which provides carbohydrates) and pollen (which provides proteins and fats), comes in minute quantities and its availability constantly changes as different plant species have their respective blooming periods and other pollinators deplete patches (Heinrich 2004). Bees have innate preferences for some flower qualities such as the colours blue and green in honeybees (Giurfa *et al*. 1995) or certain colour contrasts in bumblebees (Lunau *et al*. 1996), which may guide initial behaviour. However, such a complex environment requires them to be able to react to these changes and learn information such as colour, odour and shape of a flower as well as memorise the locations of food patches and their nest (Hammer and Menzel 1995).

This task has multiple different aspects that probably place demands on cognitive abilities. Firstly, bees are central place foragers so an obvious challenge is remembering where to find their nest upon return – a process that involves visual learning during orientation flights (Capaldi and Dyer 1999). Secondly, bees must find and identify rewarding flower patches. Flowers are distributed in patches that individuals visit repeatedly every day (Woodgate *et al*. 2016), and a typical foraging trip can involve a bee visiting several food patches (e.g. flowering bushes), all of them potentially containing many flowers. Between-patch decisions may draw upon long-term memories of patch locations or species (Menzel 1999). Furthermore, efficient navigation between multiple patches requires strategies to optimise foraging routes. A well-documented strategy is called traplining (Saleh and Chittka 2007), which involves the development of a stereotypical path. Traplining can allow the forager to minimise the path travelled on a foraging bout, and thus minimise the energy spent flying, by memorising a single route (Lihoreau *et al*. 2012). Traplining is a plastic strategy: bees can refine their route once it

has been established, and can readjust it if a new patch appears (Lihoreau *et al*. 2010). Within patches, short-term memory may be important in the decision about whether to stay or leave the patch (Menzel 1999), and there is also evidence that bees remember and avoid recently visited flowers (Brown and Demas 1994; Samuelson *et al*. 2016; Pull *et al*. 2022). They can also avoid revisits by learning to detect the presence of cuticular hydrocarbons (Stout and Goulson 2001; Saleh and Chittka 2006; Leadbeater and Chittka 2011). These molecules cover insects' bodies and leave a "footprint" on the flowers, providing an indirect cue regarding the current state of its nectar stores (Witjes and Eltz 2007).

Nectar foraging constitutes the bulk of the foraging efficiency literature, as well as the cognition literature, and this thesis is no exception (see chapter 3 and 4). However, it is important to note that pollen foraging – even if it is still under-researched – also involves associative learning and the longevity of the memory associated with pollen reward is comparable to that of nectar-based reward associations (Grüter *et al*. 2008; Muth *et al*. 2015a, 2016, 2017, 2018a). Foraging bees in the wild are exposed to both nectar and pollen rewards – which is especially true in bumblebees as they can forage for both resources within a bout (Goulson 2003; Hagbery and Nieh 2012; Muth *et al*. 2015a) – and these two reward systems have been shown to interact in interesting ways (Muth *et al*. 2017, 2018a). For example, while learning to associate nectar reward with flower features impairs the ability of bees to simultaneously learn to associate pollen reward with flower features (Muth *et al*. 2017), mixing a fatty acid present in the chemical composition of pollen with nectar appears to enhance abilities to learn about nectar associations (Muth *et al*. 2018a).

Cognitive repertoire of social bees

With such a wide array of information to process, it is perhaps unsurprising that the cognitive repertoire of bees is rich and includes apparently complex behaviour for such tiny brains (Giurfa 2013). Abilities that were once believed to be restricted to vertebrates such as numerosity, although limited to less than 4 objects (Chittka and Geiger 1995; Bortot *et al*. 2019; Howard *et al*. 2019; see also Giurfa 2019 for a review on the subject), or social learning, whereby an individual can use information from others to improve their performance on a task, (Chittka and Leadbeater 2005; Leadbeater and Chittka 2007; Grüter *et al*. 2010; Grüter and Leadbeater 2014) have been observed in bees. Nonetheless, the most widely used paradigm for testing learning and memory in bees is without a doubt the proboscis extension reflex paradigm (PER), which draws upon a basic classical conditioning task (see Kuwabara 1957 and Takeda 1961 for the original idea and creation of the paradigm; see Felsenberg *et al*. 2011 for the current standardised protocol). PER is an olfactory learning paradigm whereby a bee is trained to associate a specific scent with a reward by harnessing the innate proboscis extension reflex that occurs when the antennae detect sucrose (unconditioned response). At the first stage of the protocol, the immobilised bee will be exposed to a brief airflow containing a scent, immediately followed by a touch on the antenna with a sucrose solution. The bee consequently forms a Pavlovian association between the sucrose (unconditioned stimulus) and the scent (conditioned stimulus) and will, after sufficient training, respond to the scent by extending its proboscis in absence of any sucrose solution (conditioned response) (see Giurfa and Sandoz 2012 for a review of the paradigm). The protocol can be extended to include a second, unrewarded scent (differential conditioning) to which the bee should not respond. This method presents several advantages as the conditions are well controlled, the data output straightforward (learned/not learned) and throughput can be high – which consequently allows for larger sample sizes – compared with free-flying paradigms.

The PER has allowed for intense study of olfactory learning and scent discrimination in bees (Vareschi Ekkehard 1971; Guerrieri *et al*. 2005), including documentation of the effects of pesticides on olfactory learning (Stanley *et al*. 2015b; Muth and Leonard 2019; Smith *et al*. 2020 ; see also Siviter *et al*. 2018b for a meta-analysis on the topic). Nevertheless, the PER paradigm's simplicity makes it less useful to test traits beyond olfactory response. Additionally, it requires that the subjects be put in harnesses and starved overnight creating stressful conditions which may affect learning (Schwabe *et al*. 2012; Muth *et al*. 2015b). Studies comparing behaviours of free-moving bees to those tested using PER have led to criticisms regarding the ability to generalise results obtained with PER to foraging bees in the wild (Mujagic and Erber 2009; Ayestaran *et al*. 2010). To overcome this negative aspect of the protocol, a new method has recently been proposed that combines free-moving paradigms with the straightforwardness and higher throughput of PER (Muth *et al*. 2018b).

Free-flying paradigms have also been useful in exploring learning beyond classical conditioning paradigms. Delayed match-to-sample (reviewed in Lind *et al*. 2015) protocols make use of bees' ability to recognise visual patterns and allow for testing of a wider range of stimuli (including visual, acoustic as well as olfactory cues) than PER. Experiments using this paradigm first expose the subject to a sample stimulus with no direct ecological relevance (e.g. sounds, visual patterns, scent). Shortly afterwards, the subject is presented with a collection of secondary stimuli of a similar type that include the sample stimulus presented earlier. If the subject chooses the secondary stimulus that matches the sample one, it gets a reward. This paradigm has been used to explore varied cognitive tasks in bees, such as concepts of "sameness" and "difference" Giurfa *et al*. (2001). For bumblebees, the use of free-flying colour choice paradigms has become common, whereby bees must learn to identify the more

rewarding "flowers" (chips of a certain colour) within a small-scale artificial "meadow" (Ings and Chittka 2008, 2009; Wang *et al*. 2018).

Taken together, the cognitive mechanisms described above have the potential to contribute to efficient foraging. However, while there is some evidence to suggest they positively correlate with food intake at the colony level, the evidence is mixed, and recent work suggests that such effects may vary across foraging environments and even sometimes not be apparent at all (Raine and Chittka 2008; Evans *et al*. 2017; Pull *et al*. 2022). The remarkable cognitive repertoire of bees has inspired researchers to look under the proverbial hood, to investigate the neurobiology of these behaviours. As a result, social bees are among the insect groups where the relationship between neural structure and cognition is understood the best (Menzel 2012), and I will explore the findings that relate to foraging in the next section.

Neurobiology of learning and memory

(i) Memory phases

The neurobiology of learning and memory is particularly well-described in bees, or more specifically, in the honeybee (Menzel 2012). Across the vertebrates and invertebrates, memory is characterised by a number of distinct temporal processes that correspond to different cellular mechanisms and can be shut down separately (Izquierdo *et al*. 1999; Trannoy *et al*. 2011). In bees, initial exposure to a stimulus leads to the formation of short-term memories (STM) that last in the region of approximately 15 minutes. A second process, middle-term memory (MTM; lasting hours) is consolidated through several minutes of sustained neuronal activity leading to the activation of protein kinase M which is mediated by protein kinase C after a period in the range of an hour. Long-term memory (LTM; lasting days), on the other hand, requires the activity of protein kinase A, with early long-term memory (eLTM) depending on translation of

proteins and late long-term memory (lLTM) depending on both transcription of mRNA and translation (STM does not depend on these mechanisms; see Menzel 2012). Hourcade *et al.* (2010) have documented that LTM consolidation of an olfactory stimulus leads to a transcription-dependent increase in synaptic complexes, also known as microglomeruli, in the lip region of the mushroom bodies. The reliance on transcription and translation as well as synaptic reorganisation indicate that LTM is likely to be a costly investment, as evidenced by Plaçais and Preat's (2013) experiment on *Drosophila* where the pathway for LTM consolidation was shut down in response to starvation.

According to Menzel's model of memory dynamics (1999) between-patch movement (within the range of minutes to hour) involves late short-term memory (lSTM) while between-bout memory of the flower species present at a given patch (hours to days) would rely on MTM to LTM. At the level of within-patch decisions (in the range of seconds to minutes), foragers have to rely on early short-term memory (eSTM) to keep track of which flowers in the array they are currently visiting have already been depleted of the food they contain (Menzel 1999). Thus, in addition to differing in underlying physiology, the different memory phases may well play different ecological roles.

(ii) Neural structure

Among the different regions that comprise the insect brain, the mushroom bodies (MB) have piqued the interest of researchers since the late 19th century (Dujardin 1850) when they were first hypothesised to play a role in learning. More recent research has identified the MBs as an integrative centre for olfactory and visual stimuli (Heisenberg 1998), and in the Hymenoptera, the MBs are greatly expanded relative to many other insect groups – a process that appears to

have been associated with a parasitoid lifecycle that is basal within the group, pre-dating the evolution of sociality (Farris and Schulmeister 2011).

The MB comprise four calyces that are each subdivided into regions: the lip, the collar and the basal ring (Gronenberg 2001). In Hymenoptera, the lip receives olfactory afferent neurons from the antennal lobes, while the dense collar receives afferences from the optic lobes, and the basal ring both types of inputs (Gronenberg 2001). Growth and development of the MBs is limited to the larval and pupal stages: holometabolous insects go through large changes during metamorphosis during which the brain sees a consequent development particularly in the optic lobes, antennal lobes and the mushroom bodies (MB) (White and Kankel 1978; Truman 1990; Ito and Hotta 1992). MB development, precisely the proliferation of Kenyon cells, continues until the individual is ready to emerge from the pupa (Truman 1990; Ito and Hotta 1992), but after eclosion, neurogenesis stops and is not observed anymore during adulthood (Fahrbach *et al*. 1995). Adult bees can therefore only rely on synaptic pruning or redirection to adapt to their environment, rather than new growth.

In the MB, synaptic complexes known as microglomeruli (MG) can be observed through the use of histo-immunochemistry techniques, which have revealed that their density within the tissue varies with age and experience (Fahrbach and Van Nest 2016). In honeybees, young workers spend the first three weeks of their adult life tending to the brood and the nest itself, during which time MG density within the MBs will increase with age (Groh *et al*. 2012). It is typically only after this period that they start being active foragers. This change of task is preceded by a drop in MG density and MB volume, which increases again as the bee acquires experience on the new task (Withers *et al*. 1993; Farris *et al*. 2001; Ismail *et al*. 2006; Dobrin *et al*. 2011; Groh *et al*. 2012; Muenz *et al*. 2015; Cabirol *et al*. 2018). In *Bombus*, for which

foragers' task allocation is not dependent on age, body size strongly correlates with brain size and the size of the MBs correlates with foraging experience but not with age (Riveros and Gronenberg 2010). This suggests that such changes may be associated with the considerable increase in demands on learning and memory that are associated with foraging. Accordingly, variation in MG density is not exclusively observed in relation to life-history events. Laboratory experiments have also demonstrated a link between MG density and cognitive tasks. For example, Hourcade *et al*. (2010) have shown that the formation of long-term olfactory memory is correlated with an increase in MG density, while Li *et al*. (2017) have suggested a possible correlation between MG density and long-term visual memory formation. However, Li *et al*. (2017) failed to provide a convincing result when it comes to visual memory due to inconsistent methodology and Van Nest *et al*. (2017) even reported an absence of link between middle-term visual memory and MG density. Nonetheless, the finding above gives reason to expect that MG density may relate to learning and memory, and thus foraging efficiency. In the next section, I explore how insecticides may influence this relationship.

Pesticides

Neurotoxic systemic insecticides, such as neonicotinoids and sulfoximines, are widely used to treat crops (Simon-Delso *et al*. 2015) and provide protection against pest insects that can ravage fields and drastically reduce production. The active ingredient in both neonicotinoids and sulfoximines binds to nicotinic acetylcholine receptors within the insect central nervous system, which can inhibit neuronal development and can lead to cellular death (Palmer *et al*. 2013; Peng and Yang 2016). While they are intended to target specific groups of pest insect, an overwhelming amount of literature supports that insecticide exposure at field-realistic, legislatively approved levels can have sublethal effects on non-target species including pollinator species (Whitehorn *et al*. 2012; Godfray *et al*. 2014; Stanley *et al*. 2015a; Siviter *et*

al. 2018b). By definition, these sublethal effects do not lead to individual bees dying, but rather, affect individuals in sublethal ways that can reduce the ability of the colony to function optimally (Bryden *et al*. 2013) with effects ranging from increasing homing failure (Henry *et al*. 2012), diminishing worker immune response (Annoscia *et al*. 2020), to reducing egg-laying (Baron *et al*. 2017). These individual effects can impact colony-level survival and reproduction (Gill *et al*. 2012; Whitehorn *et al*. 2012; Siviter *et al*. 2018a).

As neonicotinoids have a neurotoxic effect on insects, it is perhaps unsurprising that they have been shown to negatively affect neural development and learning abilities. For example, Stanley *et al*. (2015b) found that chronic exposure to field-realistic doses of thiamethoxam (i.e. exposure to the product for a duration replicating the typical time-frame of flowering period of treated crops and at levels equivalent to those found in those plants' nectar stores) significantly impaired learning speed and MTM in bumblebees, tested using the proboscis extension reflex (PER) paradigm. Samuelson *et al*. (2016) showed that thiamethoxam exposure negatively impacted STM in *B. terrestris,* using a radial-arm maze paradigm. A meta-analysis of the literature looking at the effects of pesticides on bees' olfactory learning abilities and memory revealed a clear negative impact (Siviter *et al*. 2018b) although interestingly, bumblebees (*Bombus spp.*) may have been less affected or with more variance than the other genera. Testing bees in other learning contexts offered similar results; for example, *B. terrestris* chronically exposed to thiamethoxam learned to handle a new flower slower than their control counterparts (Stanley and Raine 2016). Importantly, there is evidence to suggest that at least some of these effects may be mediated through impaired neural development. For example, Smith *et al*. (2020) reported that chronic exposure to imidacloprid also leads to impaired olfactory learning abilities and, importantly, showed that this effect correlated with impacts on MB volume.

Likewise, Peng and Yang (2016) described negative effects of chronic neonicotinoid exposure in larvae on MG density.

The overwhelming body of evidence that neonicotinoids have harmful effects on bee colonies has led to a ban on three of these insecticides for outdoor agricultural use in the EU (EFSA 2018). Additionally, the development of resistance in target species has led to the emergence of new compounds onto the market (Brown *et al*. 2016). Among them, the novel insecticide sulfoxaflor acts as an agonist of nicotinic acetylcholine receptors, similarly to neonicotinoids, and has documented negative effects in *Bombus terrestris* on larval development (Siviter *et al*. 2020a), fecundity (Siviter *et al*. 2018a; Linguadoca *et al*. 2021), and egg-laying (Siviter *et al*. 2020b). However, in contrast to neonicotinoids, there is currently no documented evidence that sulfoxaflor impacts learning abilities, since the only study looking at the effect of acute exposure on *B. terrestris* did not detect any negative effect on STM or PER performances (Siviter *et al*. 2019).

At the colony level, sulfoxaflor has similar negative impacts on reproductive success to many neonicotinoids (Siviter *et al*. 2018a). Thus, its apparent lack of effect on learning and memory calls into question the idea that impacts on cognitive abilities are critically important mechanistic drivers of colony failure overall, in pesticide-exposed colonies, because sulfoxaflor-exposed colonies fail even though learning is apparently intact. Of course, the impacts of learning on fitness proxies are likely to be complex and not react uniformly to stressors. In the following chapters, I evaluate the relationship between foraging efficiency, neuroanatomy, learning, and sulfoxaflor exposure in detail. First, I tested the hypothesis that synaptic density in the MB is linked to foraging efficiency using a setup allowing for the monitoring of *Bombus* colonies' nectar intake from wild flowers (Chapter 3). Then I tested

whether a novel neurotoxic insecticide, sulfoxaflor, had an effect on synaptic density in the MBs (Chapter 4) and on short-term memory (Chapter 5) in *B. terrestris*. The work provides a key test of the relationship between neural anatomy and foraging efficiency, while also presenting a case study on the effects the novel insecticide sulfoxaflor can have on this relationship using the model pollinator *Bombus terrestris*.

Summary of chapters

Chapter 2: A reproducible immunostaining protocol for *Bombus terrestris*

An essential part of this thesis was the development of a protocol for immunostaining to use on bumblebee brains. Early on, I found that published methodology for this species showed logical inconsistencies that prevented its replication. In collaboration with Dr Swidbert Ott at the University of Leicester, we developed a protocol based on previous methods developed for honeybees and other insects that takes into account the anatomical particularities of bumblebees that are not observed in these other taxa. This protocol is summarised in Chapter 2.

Chapter 3: No evidence of a link between foraging efficiency and synaptic density in *Bombus terrestris*

It is typically assumed that microglomerulus (MG) density is related to learning abilities, because it (a) changes as a result of learning (Hourcade *et al*. 2010; Li *et al*. 2017), (b) correlates with aspects of learning ability (Li *et al*. 2017), (c) changes when bees start to forage. The outcome is expected to be a change in foraging efficiency, because foraging efficiency is expected to depend in part upon learning ability. Here, I provide the first test of this assumption, on a large scale, and in a key pollinator species. I measured the foraging efficiency of workers in 6 colonies following a staggered design during summer 2019 and quantified the MG density of these foragers. While no significant relationship was detected between foraging efficiency and MG density, this chapter highlights the gaps in knowledge regarding foraging efficiency and offers routes to attempt to close it.

Chapter 4: No evidence of a link between larval exposure to sulfoxaflor and mushroom body synaptic density in adult bumblebees (*Bombus terrestris*).

Previous studies on neonicotinoids showed an effect of chronic pesticide exposure at the larval stage on MB development in adult honeybees. However, this question had not yet been tested for sulfoxaflor, one of the most important insecticides in global markets outside the EU. Moreover, previous explorations of impacts of sulfoxaflor on learning and memory involved only brief acute exposures, whereas documented effects on colonies involved long-term chronic exposure. In this experiment, I tested the effect of chronic exposure to this novel neurotoxic insecticide on the synaptic density of the MB, an important centre for stimuli integration and learning in bumblebees. No significant effect of the compound was detected either on MG density or volume of the MB.

Chapter 5: Chronic exposure to sulfoxaflor during larval development does not affect bumblebees' performance in a radial arm maze.

In Chapter 4, I found no impact of chronic sulfoxaflor exposure on neural structure. However, not all cognitive processes are expected to have detectable neural correlates, and in particular, STM abilities are unlikely to have a distinct neural trace, since STM requires neither transcription nor translation. Nonetheless, STM has been shown to be compromised by insecticide exposure, and such effects may be important for within-patch foraging efficiency. Here, I tested whether chronic sulfoxaflor exposure impacts STM, making use of the radial-arm maze (RAM), a classic paradigm of cognitive psychology adapted for bees to mimic within-patch foraging.

Chapter 6: Discussion

This chapter discusses the implications of the results found in chapters 2 to 5 taken together, the gaps in knowledge it closes and further ways for new research.

Chapter 2: A reproducible immunostaining protocol for *Bombus terrestris* brain tissues.

Grégoire Pasquier, Swidbert Ott and Ellouise Leadbeater.

1. Abstract

Immunostaining techniques have been instrumental in the expansion of knowledge about the relationship between neuroanatomy, learning and foraging in insects. In honeybees, they have allowed closer study of the mushroom bodies (MB) and their micro-structures such as the synaptic complexes known as microglomeruli (MG). These technical advances have led to several discoveries such as the age and experience dependency of MG density and MB volume in workers, as well as an increase in MG density caused by long-term memory consolidation. As bumblebee models (*Bombus spp.*) present numerous advantages for laboratory experiments, recent studies have attempted to use the technique in this group, particularly in *Bombus terrestris*. Unfortunately, published methods currently present methodological inconsistencies that prevent replication of the protocol. Here I present the protocols I developed for a reproducible immunostaining of *B. terrestris* brain tissues with the aim of making the technique more accessible to future research.

2. Introduction

Immunofluorescence staining is a biochemical technique commonly used to highlight a protein of interest within a tissue. The tissue is put into contact with a molecule possessing a strong, specific binding affinity (usually a monoclonal antibody) for the protein of interest. The binding molecule can be conjugated to a fluorophore or require a secondary anti-body with this property.

Immunofluorescence approaches are particularly useful in insect neuroanatomy when used in conjunction with confocal microscopy to visualise structures in the brain tissue such as microglomeruli (MG). Each microglomerulus is formed of a synaptic bouton from an afferent neuron surrounded by dendrites local to the neuropile. The MG have been identified in different neuropils including the mushroom bodies (MB), a key region for stimulus integration in insects. The MG observed in the MB comprise afferent neurons coming from the optic lobes (bearing optical input projecting into the collar and basal ring region of the MB) and the antennal lobes (carrying olfactory input to the lip and basal ring regions), surrounded by the dendrites of the Kenyon cells (located at the surface of the MB neuropile). The proteins that are targeted in the immunostaining protocol are synapsin, which is a phosphoprotein found in the presynaptic bouton and modulates neurotransmitter release (Cesca *et al*. 2010), and f-actin, a structural protein of the cytoskeleton (Frambach *et al*. 2004). Immunolabelling of the former is possible through a primary antibody produced against the synapsin of *Drosophila melanogaster* followed by a secondary antibody conjugated to a fluorophore while the latter only requires phalloidin conjugated to a fluorophore (Groh and Rössler 2011).

In *Apis mellifera*, early experiments on neural development revealed that even though neurogenesis does not occur in the adult (Fahrbach *et al*. 1995), developmental processes were

contributing to an increase in the volume of foragers' MB compared to those of nurses (Withers *et al*. 1993). This change appears to be driven by the growth of the Kenyon cells that make up the neuropile (Farris *et al*. 2001; Dobrin *et al*. 2011). Indeed, honeybee workers typically undergo an occupational transition towards foraging around the third week of life (Allan *et al*. 1987; Robinson 2009). A decrease in both MB volume and MG density can be observed at the onset of foraging, followed by an increase again in both measurements as the forager accumulates experience (Withers *et al*. 1993; Ismail *et al*. 2006; Groh *et al*. 2012; Muenz *et al*. 2015; Cabirol *et al*. 2018). This mechanism is hypothesised to reflect neural changes that fine-tune the brain towards learning of new information and skills about the outside world such as the identification of food sources or navigation (Muenz *et al*. 2015; Cabirol *et al*. 2018). Note that exposure to light also produces a decrease in MG density in *A. mellifera* (Scholl *et al*. 2014) but this effect is not observed in *B. terrestris* (Kraft *et al*. 2019). Changes in MG density in honeybees are not limited to the transition to foraging as increased MG density in the MB has also been linked with long-term olfactory memory consolidation (Hourcade *et al*. 2010). It is fair to say that the study of MB volume and MG density has been instrumental in understanding the proximate aspects of the relationship between learning and foraging in social bees.

Recent work has attempted to expand this approach to *Bombus terrestris* owing to its experimental tractability and commercial availability all year round. Yet, the *B. terrestris* model presents several differences compared with *A. mellifera* when it comes to foraging. Bumblebees do not exhibit clear temporal polyethism (i.e. workers can start foraging at any age), foragers do not communicate food location via a dance language (although social cues can be used; Chittka and Leadbeater 2005; Leadbeater and Chittka 2007; Grüter *et al*. 2010; Grüter and Leadbeater 2014), colonies follow an annual life cycle at the end of which workers

can reproduce (Goulson 2003) and sexuals (queens and males) engage in foraging at some point in their life cycle. These differences between the two species offer new opportunities to study how learning abilities, foraging efficiency and neuronal plasticity interact with each other. A recent study by Li *et al*. (2017) has taken advantage of these aspects of working with *B. terrestris* but failed to provide a reproducible description of the immunostaining protocol. In this chapter, I describe and critique my development of a reproducible protocol for immunostaining for both wholemount and cryosections of *Bombus terrestris* brains.

A wholemount protocol allows the measurement of the volume of the brain's different neuropiles without compression of the tissue, but the protocol takes up to 9 days. Antibody penetration is more difficult in a tissue of thickness in the millimetre range and can lead to a gradient of penetration, with the intensity of the staining decreasing as it goes deeper into the tissue. This gradient adds to the opacity of the tissue itself and can reduce the consistency of the images across the tissue when scanning the tissue with a confocal microscope. Additionally, at the magnification typically required for looking at structures such as MG (60x), the depth of the tissue that can be scanned is dependent on the working distance of the objective and will usually be limited to the first 10 µm of the tissue. On the other hand, sectioning techniques allow for a quicker throughput (with only a few hours of antibody incubation for cryosections and the protocol taking up to 4 days) and allow for a deeper imaging of the tissue, with the caveat that volume measurement is usually not possible or less reliable due to compression of the tissue during sectioning and potential loss of material. The two method variants will be presented in detail in the following section.

My aims with this chapter were twofold: first, to provide the detail of the methods I used in the following chapter of this thesis and second, to offer a guide for future researchers looking to

use immunostaining techniques on *Bombus spp.* The development of this protocol was based on methods published in Ott (2008) and Groh *et al*. (2012) and was based in part at the University of Leicester research facilities.

3. Methods

The following immunostaining protocol first presents the preliminary tissue processing steps (i.e. sampling, fixation, dissection; section **3.1**) necessary for both a cryosection and a wholemount protocol. It will then proceed to present the immunostaining of cryosections (section **3.2**) and the immunostaining of wholemounts (section **3.3**) separately (see Figure 2.1).

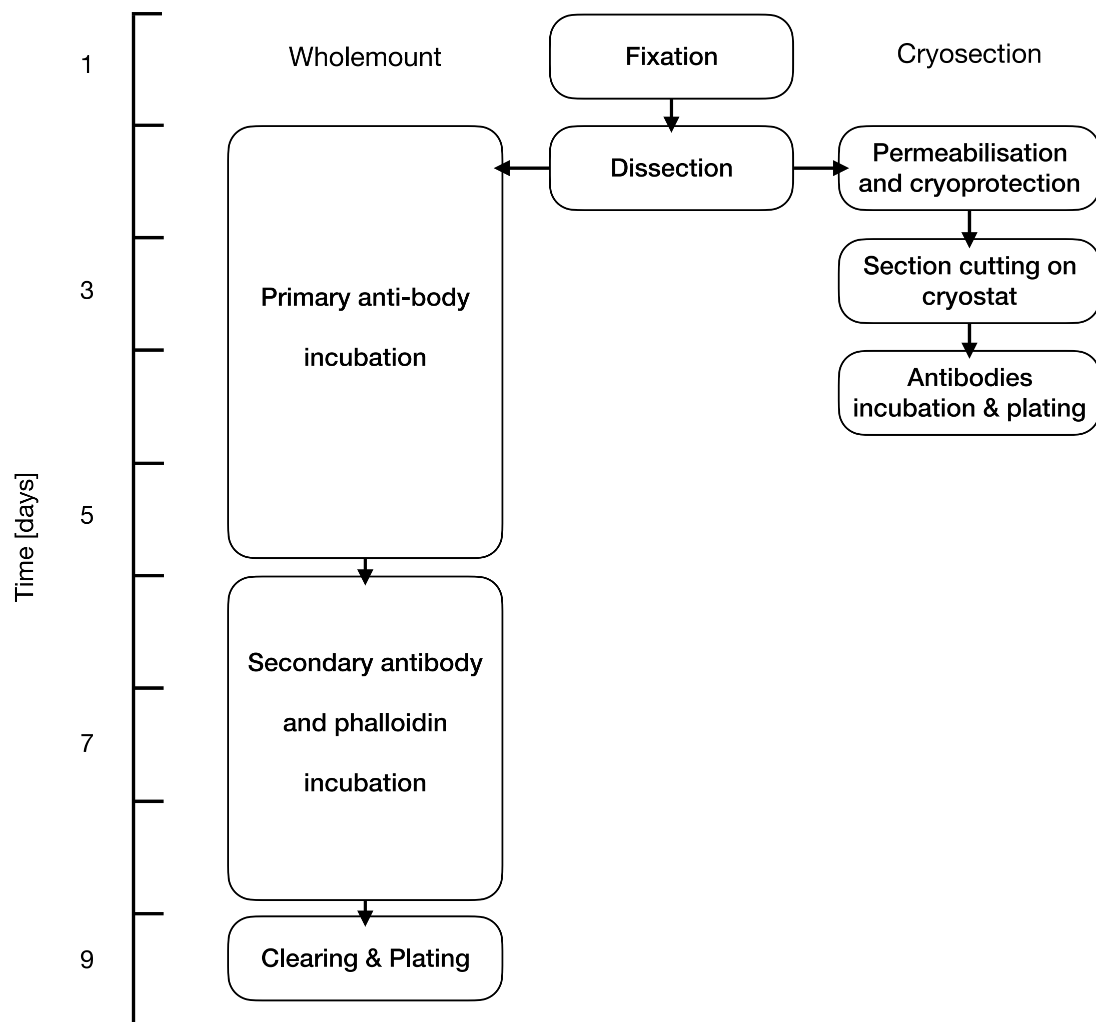

Figure 2.1: Flowchart comparing the time necessary to complete a wholemount or a cryosection immunostaining protocol on *B. terrestris* brain tissues.

3.1 Preliminary tissue processing

3.1.1 Tissue fixation

Bees were individually sampled from the colony in individually labelled 15 ml Eppendorf tubes and immediately placed on ice for anaesthesia. After 15 minutes on ice, the head was cut off, pinned onto a dissection plate (see Figure 2.2), immersed in Ringers solution and placed under a binocular microscope. Note that HEPES buffer saline or phosphate buffer saline (PBS) can be used here with no observed consequences. The antennae were removed to prevent them from interfering with dissection steps.

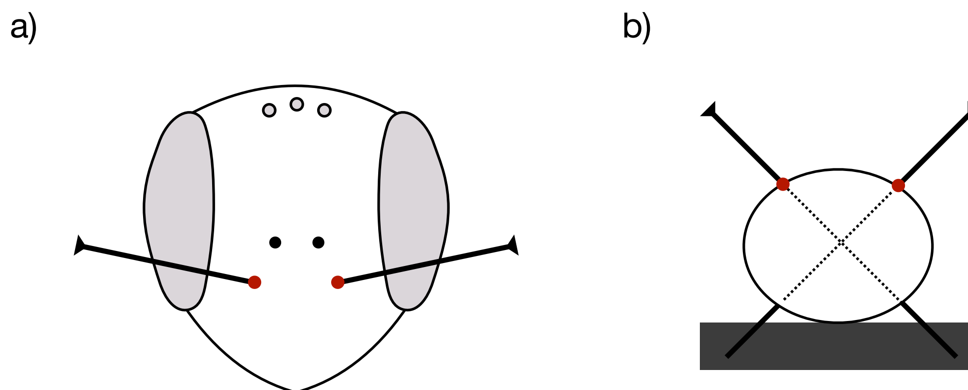

Figure 2.2: Schematic representation of *B.terrestris* head with recommendation on optimised angle to apply entomological nails on a dissection plate a) viewed from the top and b) in cross-section. The nails cross inside the head which limits movement and offer more support to the tissue than two nails pinned in parallel on each side would.

A large window was cut in the cuticle in the frontal part of the head capsule with a disposable scalpel. Shallow cuts in the cuticle were made along the two compound eyes, above the insertion of the antennae and at the back of the head, behind the three ocelli (see Fig. 2.3.a.-e.). The cuticle area resulting from the cut was discarded (Fig. 2.3.f.). The exposed air sacs (Fig. 2.3.g.) were gently removed with precision forceps (Fig. 2.3.h.), including the membrane

directly in contact with the brain. This step is essential to ensure the fixative will enter in contact with the tissue, otherwise a degradation of synapsin may occur and prevent visualisation of the MG under the confocal microscope. Additionally, great care should be observed at this stage to avoid damaging the tissue and particularly the area of interest. Any air bubble was blown out of the tissue using a Pasteur pipette. The head was then unpinned, removed from the dissection plate and placed in a snap-top glass vial containing an ice-cold solution of 4% formaldehyde in PBS (Fig. 2.3.i.). The vial was placed overnight in a cold room (4°C) on an orbital shaker. The speed of the shaker was adjusted to obtain a gentle bobbing of the head in the fixative solution.

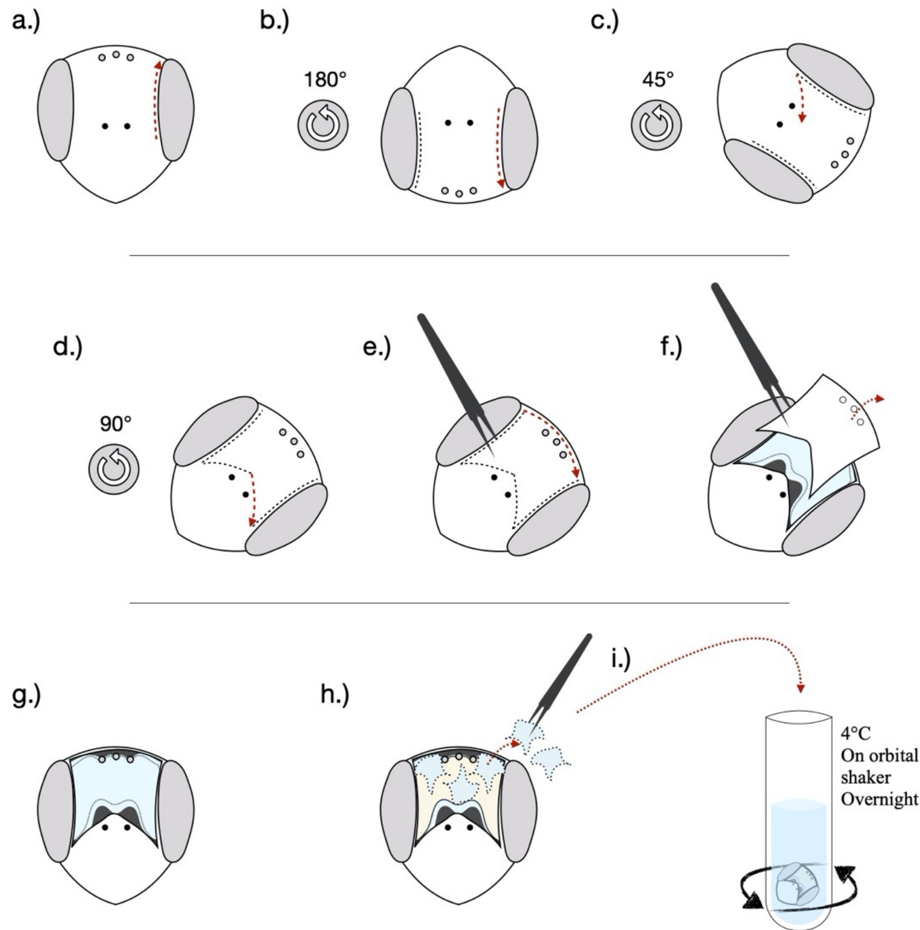

Figure 2.3: Schematic instructions on cutting a window in the cuticle of the head capsule of *B. terrestris* and removing the frontal part of the air sacs before fixation. Make shallow cuts in the cuticle, first along the left compound eye (a), then rotate 180° to cut along the right compound eye (b), rotate 45° to the left to cut from the bottom of the previous cut along the right compound eye to above the insertion of the antennae (c), rotate 90° on left and join the cut from above the insertion of the antennae to the bottom of the cut along the left compound eye (d). Hold the flap made from the 4 previous cuts with fine forceps and cut at the back of the head, behind the three ocelli (e). Remove the piece of cuticle (f) to expose the inside of the head capsule (g). Delicately peel-off the frontal part of the air sacs and discard the resulting membranes to expose the brain (h). Place the head with the exposed brain in a solution of 4% formaldehyde in PBS and leave on an orbital shaker overnight (i.).

3.1.2 Dissection

The vial was removed from the cold room and allowed to rise up to room temperature for 15 minutes. Avoiding sudden temperature differences diminishes the risk of finding air bubbles in the tissues at the imaging stage. The fixative was discarded and the head was quickly rinsed in PBS, then washed for 2 cycles of 5 minutes in PBS on an orbital shaker. The head was pinned again on a dissection plate (Fig. 2.2), covered in PBS and again placed under binocular lenses. Cuts were made in the compound eyes; the ocelli and remaining debris of the compound eyes were removed from the brain tissue (Fig. 2.4.a). The cuticle and compound eyes were trimmed off to completely expose the brain (Fig. 2.4.b). It is important at this stage to remove all the debris from the eyes as their pigments might reduce the transparency of the tissue in the final stages of the staining and cause some obscuring on the images produced by the confocal microscope. Finally, the brain was fully removed from the head capsule and the last pieces of membranes were gently cleaned off (Fig. 2.4.c). The fully dissected brain was then washed through three cycles of 10 minutes in PBS on an orbital shaker (Fig. 2.4.d). The brains were then ready for further processing either following a cryosection protocol or a wholemount protocol.

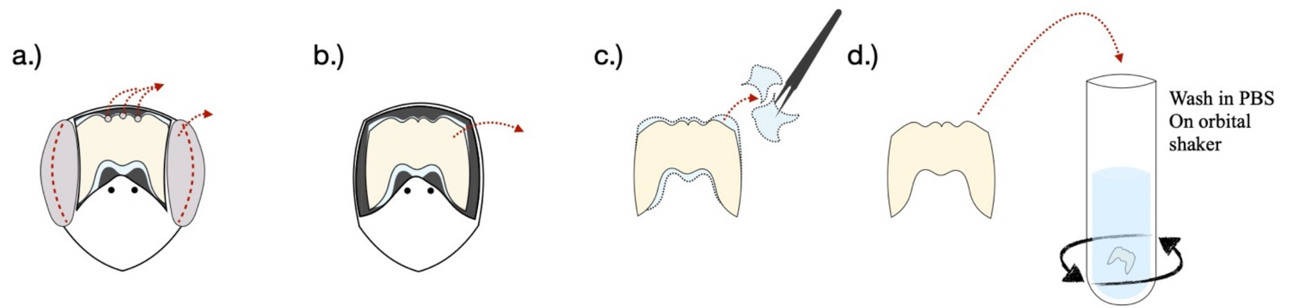

Figure 2.4: Schematic instructions on *B. terrestris* brain dissection. Make cuts in the compound eyes, then trim them off to completely and remove the ocelli (a). Scoop the brain out of the head capsule using fine forceps (b). Clean up any remaining membrane from the back of the brain (c). Place the fully dissected brain in PBS and wash on orbital shaker (d).

3.2 Immunostaining of cryosections

The following section describes the methods used to process, section at low temperature, immunostain and mount brain tissues of *B. terrestris*. Unless specified, all the following steps took place at room temperature.

3.2.1 Permeabilisation and cryoprotection

The fully dissected brain was permeabilised in a solution of methanol and dimethyl sulfoxide (DMSO; 80% methanol/20% DMSO) for 2 hours on an orbital shaker. It was then rehydrated through a graded series of methanol in 0.1 M Tris buffer solution (100%, 90%, 70%, 50%, 30% methanol then plain 0.1 M Tris buffer), with each step of this series lasting 15 minutes.
The brain was then cryoprotected through a graded series of sucrose solution (10%, 20% and 30% sucrose in 0.1 M PB with 0.005% sodium azide) for 1 hour each step. The brain was then stored at 4°C in fresh 30% sucrose in 0.1 M PB with 0.005% sodium azide solution overnight.

3.2.2 Embedding and freezing of the sample

The brain was removed from the sucrose solution, placed at the bottom of an aluminium mould and covered in 20% gelatine. The brain was arranged to have its frontal side facing down in the mould and a labelled cork disk was pressed on top of the gelatine. Here gelatine was preferred to the widely used TissueTek OTC as the embedding medium because the resistance to the blade was closer to the one of the brain tissues themselves and therefore led to a more consistent cutting on the cryotome. The mould was placed on dry ice until the medium was uniformly frozen. Fine pellets of dry ice were preferred for this step but coarse pellets did not lead to any significant change in the freezing process.

3.2.3 Section cutting on a cryostat

The mould was placed in the cryostat set at -18°C and left for 15 minutes to give time to the tissue and medium to reach temperature equilibrium with the chamber. The embedded tissue was removed from the mould and fitted the cork disk to a chuck of the cryotome using freezing demineralised water as glue. The entire brain tissue was cut on a cryotome set at -18°C in sections of thickness 30 µm which were each placed on pre-treated slides (Superfrost Plus™ Adhesion Slides, Thermo-Fisher). Typically, 2 to 3 slides were necessary to collect all sections of a single brain. Once full, the slides were placed on ice while the rest of the brain was being sectioned. This process reduces further the chances of observing the formation of air bubbles in the tissue. After sectioning was completed, the slides were left to dry overnight at 4°C.

3.2.4 Pre-incubation

The slides were dried at room temperature for 30 minutes then rehydrated in PBS and placed on a hot plate for 5 seconds in order to melt away the gelatine. From this point forward it was important to avoid letting the slides dry at all. The slides were rinsed in a solution of 0.2%

Triton X-100 in PBS (PBSTx) for 5 minutes and pre-incubated in a Coplin jar in a solution of 5% normal goat serum (NGS) in PBSTx for 45 minutes. This pre-incubation step is designed to saturate tissue with proteins before the antibody incubation to reduce occurrences of non-specific binding with the antibody in the following steps of the protocol.

3.2.5 Primary antibody incubation

The slides were framed using a PAP pen and placed in a moist chamber. 200 µl of a solution of primary antibody (mouse monoclonal anti-synapsin of *Drosophila* SYNORF1 1:50 in 5% NGS / PBSTx; Developmental Studies Hybridoma Bank, University of Iowa, USA) was pipetted and left to incubate in a closed moist chamber for 2.5 hours. The moist chambers can be built using large square Petri dishes with a flat sponge cut to fit the bottom of the Petri dish and made wet with demineralised water. Filter paper was also cut to fit the lid of the Petri dish and made wet with demineralised water allowing it to stick to the lid in the closed position. This allowed the slides to incubate at a constant level of humidity that prevented them from drying.

3.2.6 Secondary antibody and phalloidin incubation

The slides were quickly rinsed and washed for 4 cycles of 10 minutes to flush out the antibody that had not bonded to the synapsin of the tissue. The slides were then incubated in a solution containing the secondary anti-body (Jackson Immunoresearch, Cy2 Goat-anti-Mouse; diluted 1:100) and the phalloidin conjugated with an Alexa 488 fluorophore (diluted 1:200) in NGS/ PBSTx for 1.5 hours in the moist chamber. The moist chambers were shielded from the light using aluminium foil. This was done in order to protect the fluorophore from degradation. The slides were then quickly rinsed and washed for 4 cycles of 10 minutes in PBSTx in Coplin jars shielded from the light by aluminium foil. The slides were then mounted in a solution of 3%

n-propyl galate in glycerol, as anti-fade mounting medium equivalent to Vectashield, and were stored at -20°C.

3.3 Immunostaining of wholemounts

This section presents the methods used to process, immunostain and plate wholemount brains of bumblebees (*B. terrestris)*. Similar to the previous section, all steps took place at room temperature unless specified otherwise.

3.3.1 Permeabilisation, pre-incubation and primary anti-body incubation

The fully dissected brain was permeabilised in PBSTx for 2 cycles of 10 minutes. It was then pre-incubated in a solution of 2% NGS in PBSTx for 1 hour. The brain was then incubated for four nights in a cold room (4°C) on an orbital shaker in a solution of mouse anti-SYNORF1 antibody diluted 50:1 in 2% NGS in PBSTx.

3.3.2 Secondary antibody and phalloidin incubation

The brain was quickly rinsed and washed in PBS for 5 cycles of 10 minutes on an orbital shaker. It was then incubated for three nights in a cold room (4°C) on an orbital shaker in a solution of Cy3-conjugated goat anti-mouse antibody and AlexaFluor488-conjugated phalloidin that were both diluted 250:1 in 1% NGS in PBS.

3.3.3 Preparation for clearing and dehydration

During the next steps of the protocol, the brain was shielded from the light using aluminium foil. The brain was quickly rinsed and washed in PBS for 5 cycles of 10 minutes on an orbital shaker. I then added an extra step of fixation to ensure the AlexaFluor488-conjugated phalloidin would not be washed off during the subsequent dehydration in ethanol (EtOH) and

clearing in methyl salicylate. To do so, I fixed the brain in a solution of 1% formaldehyde in PBS for 1 hour on an orbital shaker. Following this fixation step, the brain was quickly rinsed and washed in PBS for 5 cycles of 10 minutes on an orbital shaker. The wholemount was then dehydrated in a graded series of EtOH (30%, 50%, 70%, 90%, 95%, 100%x2) for 10 minutes each step on an orbital shaker. 1 ml of EtOH was added to the vial to prepare the sample for the clearing step.

3.3.4 Clearing

Methyl salicylate was slowly poured in the sample's vial by pressing the mouth of the pipette against the wall of the vial. At this point, the methyl salicylate would sink to the bottom of the vial and the brain float on the interface between the two liquids. The brain was left without any agitation until it would sink at the bottom of the vial and become transparent. This process would usually take about 1.5 hour after which the EtOH supernatant was removed and replaced with fresh methyl salicylate.

3.3.5 Plating

In order to plate the brain, I fashioned a chamber by supergluing (cyanoacrylate, Loctite) a metal washer to a cover slip. The chamber was filled with methyl salicylate and the brain placed into the chamber. An additional cover slip was added to close the chamber while methyl salicylate was pipetted to remove any air bubble. The chamber was then sealed with superglue and the preparation stored at -20°C.

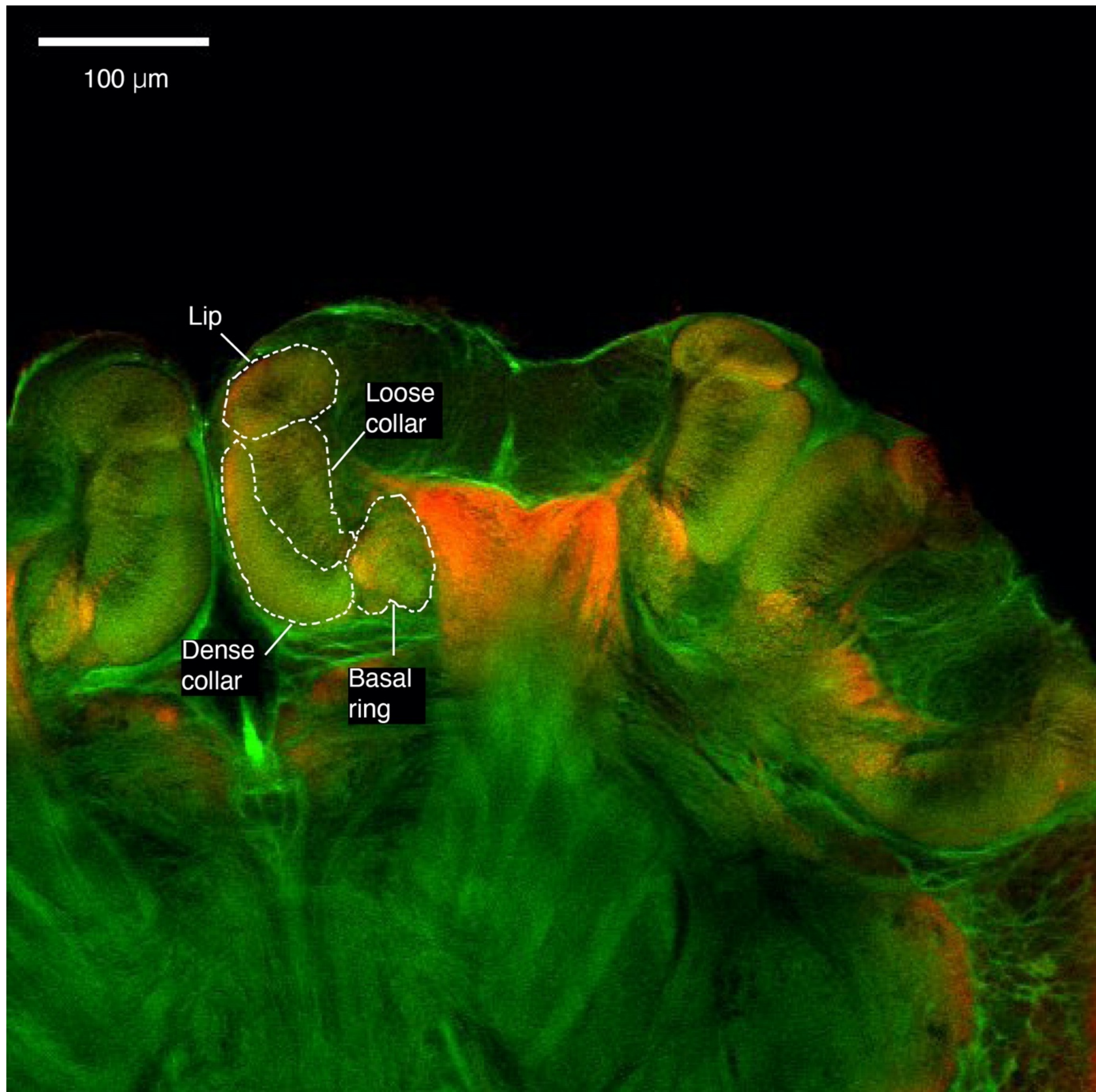

Figure 2.5: Confocal microscope image result of a wholemount staining of neural tissues of *Bombus terrestris* with subregion delineated. Synapsin is labelled in red, f-actin is labelled in green.

4. Discussion

Immunostaining of brain tissues constitutes a technique that has been instrumental in the exploration of the relationship between neuronal structures, learning and foraging in social insects. Here, I present reproducible methods aimed at making this technique more accessible to researchers looking to work with the *B. terrestris* model to further the knowledge on these aforementioned relationships and beyond.

The two technique variations developed here for brain tissue immunostaining present different advantages that I leveraged in the following chapters where they were used. Chapter 3 demanded a higher throughput and in this situation the use of cryosection was perfect, whereas chapter 4 required the ability to measure MB volume as well as MG density which called for the use of a wholemount technique. Overall, the wholemount technique is the most elegant, provides the addition of volume measurements, presents less potential risk of artifacts and is less labour-intensive than the cryosectioning method, albeit at the cost of 5 additional days of processing. The main issues that prevented the replication of the methods presented in Li *et al.* (2017) were related to the frontal part of the air sacs surrounding the brain. In *A. mellifera,* much like in locusts (*Schistocerca gregaria*), the air sacs loosely surround the brain and do not encase it which means that cutting a small window in the cuticle in the frontal part of the head capsule, between the two compound eyes, is enough to ensure the fixation of the brain tissue. Based on my personal observation, immunostaining of the synapsin is not possible in *B. terrestris* without the removal of this structure as the air sacs encase the brain. The air bubble contained in the air sacs, as well as the harder (possibly sclerotised) membrane of the air sacs in direct contact with the brain, would prevent the fixative to get in contact with the brain tissues. This absence of fixation would result in the degradation of synapsin, possibly due to the permeabilisation or dehydration steps or other degradation processes, to the point where it

would not be visible on the microscope. My protocol shows how to solve this issue without having to resort to additional reagents or longer fixation times.

Additionally, the intermediary steps in Li *et al.*'s (2017) protocol such as pre-incubation and washing between incubation steps were presented as each lasting a full day while my optimisation process during the development of this protocol revealed these steps could be completed much faster. The faster protocol I propose in this chapter could allow for greater flexibility for combining brain tissue immunostaining to other types of testing that can typically be time-consuming such as learning or foraging tests but could also be used in tandem with a wider variety of protocols such as gut microbiota analyses (Li *et al*. 2021) or immune challenge tests.

Despite its usefulness, immunostaining is outclassed by other techniques on some aspects. Micro-computed tomography (micro-CT) for example, provides a more reliable way to obtain volume measurements in insect brains, with minimal risk of creating processing artifacts, compared with a wholemount immunostaining (Smith *et al*. 2016, 2020). The micro-CT technique bypasses the most labour-intensive aspect of an immunostaining protocol such as the need for a complete dissection of the head-capsule, the two-step incubation of antibodies as well as the hurdles to avoid desiccation and other artifacts at the clearing stage. With regards to microscopy, micro-CT seems to require a similar amount of time when compared to laser-scanning confocal microscopy (Smith *et al*. 2016). The disadvantage of micro-CT is that it does not allow study of micro-structures such as MG. Density of MG in the MB is documented to correlate with foraging experience and memory consolidation in *A. mellifera* (Hourcade *et al*. 2010; Groh *et al*. 2012) and I wanted the ability to replicate these results in *B. terrestris* which is why I used immunostaining.

One the most time-consuming aspects of the study of MG density is the quantification of the density itself. At the time I developed my protocols, automated counting of MG had been criticised for underestimating MG density (Peng and Yang 2017), and I therefore decided to resort to manual counting which resulted in a large amount of time spent on this task. New techniques have since emerged that show good accuracy in showing no significant difference with manual counting (Cabirol and Haase 2019). A reliable automated counting technique is also important in making synapsin immunostaining more accessible to new research and would have been preferable with regards to time management in my thesis. Combining this automated counting method with my staining protocol could be promising for future research on bumblebees.

Finally, new techniques could prove useful in the future to circumvent the intensive and technical work needed to produce immunostained brain tissues while giving similar information on the neuronal processes at play in *B. terrestris*. One option could be to use quantitative polymerase chain reaction (qPCR) on MB extracts to quantify the levels of synapsin expression in this region of the brain (Blanco-Redondo *et al*. 2019). This technique could provide a proxy for MG density as these synaptic complexes are rich in synapsin but, as the sampling is destructive of the tissue, it would obviously preclude obtaining information on volume.

Chapter 3: No evidence for a link between foraging efficiency and synaptic density in *Bombus terrestris*.

Grégoire Pasquier, Christopher D. Pull, Swidbert Ott and Ellouise Leadbeater.

Abstract

The mushroom bodies are integrative structures in the insect brain that, in the Hymenoptera, contribute to both visual and olfactory learning. As neurogenesis does not occur in adult insects, neuroplasticity is implemented via changes in the network of connections between neurons and can be observed by quantifying the density of presynaptic boutons, also known as microglomeruli. In social bees, previous studies have linked changes in microglomerulus density to learning events, and to life stages where learning is important. Since learning is thought to be a key trait in determining bee foraging efficiency, allowing workers to remember the locations, identities and characteristic features of rewarding flower types, here we set out to directly test the relationship between foraging efficiency and microglomerulus density using a bumblebee model species, *Bombus terrestris*. We allowed lab-based bumblebee colonies, raised and maintained under controlled conditions, to forage naturally in the external environment, assaying the foraging efficiency of individual workers through mass measurement on entry and exit to the nest, before performing immunostaining to assess microglormerulus density. In contrast to our hypothesis that microglomerulus density may predict foraging efficiency, we found no significant correlation between the two variables. Our findings suggest that there is no simple direct relationship between neural structure, learning ability and foraging efficiency in bumblebees, highlighting the gap in knowledge regarding foraging and its relationship with learning abilities and neuroanatomy.

Introduction

The insect mushroom bodies (MBs) are integrative neural structures that have attracted attention since as early as the 19th century, owing to their association with learning and memory abilities (Dujardin 1850). Their function has been researched mainly in a handful of species (Heisenberg 1998; Zars 2000) including *Drosophila melanogaster* (Heisenberg *et al*. 1985; Wolf *et al*. 1998; Ito *et al*. 2013; Aso *et al*. 2014; Vogt *et al*. 2014; Owald *et al*. 2015), *Apis mellifera* (Vowles 1955; Mobbs 1982; Schürmann and Klemm 1984; Rybak and Menzel 1993) and *Periplaneta americana* (Li and Strausfeld 1997; Mizunami *et al*. 1998a, b; Okada *et al*. 1999; Strausfeld and Li 1999). The MB neuropils are formed of two lobes found on both sides of the insect brain located on the frontal section (Ito *et al*. 2014). Although it is not the case in all the aforementioned taxa (see Vogt *et al*. 2014), there is clear evidence that the MBs receive direct afference from the optic lobes in Hymenoptera (Gronenberg 2001). Specifically, the hymenopteran MBs comprise two regions of importance: the lip region is linked primarily to olfactory inputs while the collar region handles the visual inputs (Gronenberg 2001). In addition to roles in olfactory associative learning, the MB have been shown to contribute to higher-order cognitive tasks (Devaud *et al*. 2015) and to navigation (Kamhi *et al*. 2020; Buehlmann *et al*. 2020).

Intrinsic neurons in the MBs, the Kenyon cells, connect to dendrites coming from sensory neurons to form synaptic boutons also known as microglomeruli (MG) (Groh and Rössler 2011). Given that neurogenesis does not take place in the MBs of adult insects (Fahrbach *et al*. 1995), it is these structures that have been the focus of studies linking MB structural variation to learning performance in bees. For example, Hourcade *et al*. (2010) found that long-term olfactory memory formation is accompanied by changes in microglomerulus density in honeybees *Apis mellifera*, and Li *et al*. (2017) find a similar result for visual long-term memory

(but see also Van Nest *et al*. 2017 for absence of a link in a visual middle-term memory assay). Using immunocytochemistry techniques and with the help of laser-scanning confocal microscopy (Frambach *et al*. 2004; Fahrbach and Van Nest 2016), changes in MG density during an insect's life, occurring through events of significant reduction, have been shown to correlate with age, experience or exposure to light (Fahrbach *et al*. 1998; Groh *et al*. 2006; Ismail *et al*. 2006; Scholl *et al*. 2014). In honeybees, a significant decrease in MG density in the MB can be observed at the onset of foraging (Withers *et al*. 1993; Farris *et al*. 2001; Ismail *et al*. 2006; Dobrin *et al*. 2011; Muenz *et al*. 2015) and has been theorized to play a role in priming the brains for learning about floral rewards (Cabirol *et al*. 2018). Yet the role of the MBs play in wild foraging is poorly understood and only a handful of studies have explored their link directly. Notably, Withers *et al*. (1993) and Gronenberg *et al*. (1996) first showed that age and foraging experience caused a volume increase in the MBs of honeybees and *Camponotus floridanus* respectively while Farris *et al*. (2001) confirmed this change was due to the growth of dendrite branching in this region.

In studying foraging, social insects such as the buff-tailed bumblebee (*Bombus terrestris*, Linnaeus 1758) offer a formidable model as they are central place foragers that do not collect food directly for themselves but rather for their colony, which allows measurement of foraging activity (Raine and Chittka 2008; Evans *et al*. 2017; Pull *et al*. 2022). Bumblebee foraging trips present several cognitive challenges, such as memorising location of food patches or how to handle flowers (Dukas and Visscher 1994; Wehner 2003; Giurfa 2007; Collett *et al*. 2013; Grüter and Leadbeater 2014) which are likely to stimulate neuroplasticity (Cabirol *et al*. 2018). This species also presents the advantage of being easy to rear in the laboratory while having access to many of the same tools used to study honeybees such as radio frequency identification (RFID) systems to track foraging activity (Streit *et al*. 2003; Schneider *et al*. 2012; Nunes-

Silva *et al*. 2019; Costa *et al*. 2021) as well as immunocytochemistry protocols (Fahrbach *et al*. 1995; Groh and Rössler 2011; Fahrbach and Van Nest 2016).

Here we directly tested whether MG density predicted nectar foraging efficiency of bumblebee workers. Laboratory-reared colonies, all raised under identical conditions, were allowed to forage freely for 8 days using a hole-in-the-wall set-up (Raine and Chittka 2008; Evans *et al*. 2017; Pull *et al*. 2022), following a staggered design between June and August 2019. During this time their foraging efficiency was recorded as mass of nectar collected per minute, over all recorded foraging trips. After this period, foragers' brain tissues were processed following immunocytochemistry protocols, imaged using laser-scanning confocal microscopy, and their MG density was individually quantified. Foraging is theorised to represent a cognitive challenge likely to elicit neuroplasticity (Dukas and Visscher 1994; Wehner 2003; Giurfa 2007; Collett *et al*. 2013; Grüter and Leadbeater 2014; Cabirol *et al*. 2018), we therefore expected better learners to be more efficient at their task and, consequently, have a higher MG density in their MBs (Hourcade et al. 2010; Li et al. 2017). Our setup did not detect any evidence for a correlation between foraging efficiency and MG density in either the collar or lip region of the MBs. Instead, we found that the number of days since a bee started foraging was the only tested variable predicting foraging efficiency. This unexpected result highlights the need for more research into the links between foraging, learning abilities and neuroanatomy.

Methods

Overview

Six colonies of commercially-supplied *Bombus terrestris audax* were monitored over 4 months from June to September, following a staggered design. Work on each colony was spread across 30 days (see figure 3.1). After arrival, colony worker numbers were allowed to grow for the

first two weeks to allow the emergence of sufficient individuals of known age (Growth phase). The nest was then connected to the outside world, and bees were allowed to forage freely for eight days (Foraging phase), during which time the foraging efficiency of individuals was monitored. Finally, on the last day of the foraging phase, the surviving foragers were all collected and their brains were processed following a histo-immunochemistry protocol (Staining phase) in order to measure the density of pre-synaptic boutons in the mushroom bodies.

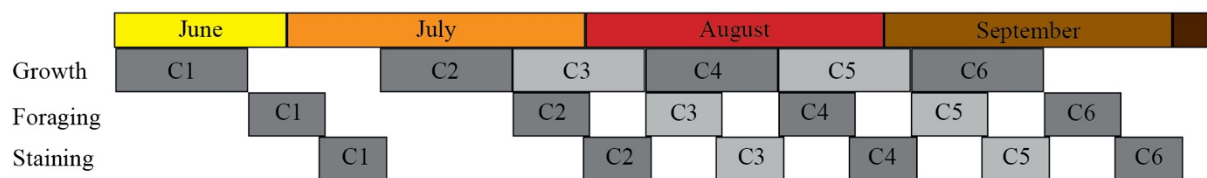

Figure 3.1: Timeline of the experiment. Each colony went through a "growth" phase to increase its size then they were left to forage for a week. At the end of the "foraging" week, the active foragers were sampled and their brain tissues were processed following a standard immuno-staining protocol. The time gap between C1 and C2 is due to a colony producing only 3 workers by the end of the growth phase which prompted its removal from the study.

Growth phase

For this experiment, we used six commercial colonies of *Bombus terrestris audax* (colony 1 to 5: supplied by Agralan, UK; colony 6: supplied by Koppert, Netherlands). Upon reception of a colony, we reduced the number of workers to 20 and individually tagged them by gluing (cyanoacrylate) plastic numbered disks on the dorsal part of their thorax. The queen and the brood were left untouched. In order to let as many new individuals emerge as possible, to produce bees of known age, the colony was allowed to grow in size in the laboratory for two

weeks. During this phase, the focal colony was kept in a dark room at 24°C with sugar solution (35% w/w) provided *ad libitum*. Three times a week, newly emerged bees were individually tagged with an RFID chip and a plastic numbered disk on the dorsal part of their thorax. Fresh-frozen honeybee-collected pollen containing multiple plant species (supplied by Agralan, UK) was provided three times a week.

Foraging phase

Two days before the end of the growth phase, we removed access to the sugar solution. This ensured that colonies were motivated to forage on the first day of being connected to the outside world while still having sufficient stores to avoid starvation. On the first day of the foraging phase, colonies were rehoused into a clean nest box made of grey Perspex (28x16x10.5 cm^3) opening to a clear Perspex tunnel fitted with a precision scale (Ohaus Advanced portable Balance Scout STX) and an RFID reader system (MAJA Bundle Bee Identification System iID2000, ISO15693 optimized, Micro-Sensys GmbH). This setup allowed us to measure the mass of nectar foragers brought to the colonies as well as keep track of their comings and goings. We excluded trips that were shorter than 4 minutes from our analysis as these are likely to represent orientation flights, waste disposal or defecation. The tunnel was connected to a clear plastic tube giving access to a hole cut in the window of the laboratory. During the foraging phase, the colony was allowed to forage freely in and around Royal Holloway's campus (Egham, Surrey, UK). The campus and private gardens in the surrounding area provided flowering plants to the colonies throughout the experiment and no additional sugar solution was required to feed the colonies during their "Foraging" phase.

Colonies were monitored for six hours (between 0815 and 1630 GMT) on five different days of the "Foraging" phase, during which time the mass on entry and exit of all foraging workers was recorded, alongside the time taken for each foraging trip.

Brain fixation and dissection

At the end of the 6 hours of foraging monitoring on the final monitoring day, all foragers were sampled upon returning from a foraging trip. The subjects were chilled on ice for 10 minutes and decapitated. The heads were then pinned on a dissection plate and submerged in 0.1 M HBS buffer. We cut a large square window in the frontal part of the head capsule and removed the frontal part of the air sacs. The heads were then transferred into an ice cold 4% Formaldehyde solution and fixed overnight at 4°C on an orbital shaker. The heads were then washed in HBS twice for 5 minutes, pinned again on a dissection plate and submerged in HBS. The compound eyes and the ocelli were removed as well as the air sac membranes remaining at the back of the brain. Finally, the last anchor points of the brain into the head capsule were severed by an incision under the antennal lobes and the brains were freed. The free-floating brains were then washed twice for 5 minutes at room temperature on an orbital shaker.

Cutting frozen sections

Before sectioning the tissues with the cryotome the brains were cryoprotected in sucrose to prevent the structures from being damaged by the low temperature. To do so they went through a graded series of sucrose solution: 10%, 20% and 30% in 0.1 M PB with 0.005% sodium azide (NaN_3). Each step of the graded series lasted an hour at room temperature until the brains sank at the bottom of the vial. The brains were then stored in 30% sucrose solution at 4°C for two days.

In order to cut tissue sections on the cryotome, the brains were embedded in a 20% gelatine solution in stainless steel moulds. The brains were oriented in the embedding medium with the frontal side facing down. The moulds were placed on dry ice in a Styrofoam container until the gelatine was uniformly frozen. The gelatine block was then fitted to a chuck and left in the

cryostat chamber for 15 min to give the object time to equilibrate its temperature with the chamber. The sections were cut at -18°C at a thickness of 30 µm, arranged on a pre-treated slide (Superfrost Plus™ Adhesion Slides, Thermo-Fisher) and stored overnight at 4°C to let them thaw and dry.

Antibody incubation and mounting

The slides were placed on a hot plate for approximately 5 seconds to melt the gelatine before being rehydrated for 10 minutes in 0.1 M PB in a Coplin jar. All subsequent washes were done in 0.1 M PBS with 0.2% Triton X-100 (PBSTx). Slides were rinsed in PBSTx and pre-incubated in a 5% normal goat serum in PBSTx solution (NGS/ PBSTx) for 45 minutes.
The bottoms of the slides were wiped and the edges framed with a hydrophobic pen after what the slides were placed flat in a moisture chamber. The primary antibody (mouse, anti-synapsin, SYNORF1, Developmental Studies Hybridoma Bank, University of Iowa, USA) was diluted 1:50 in NGS/ PBSTx and 200 µl was pipetted on each slide. The moisture chamber was closed and the slides incubated with the primary anti-body for 2.5 hours.

The slides were quickly rinsed in PBSTx before being washed 4 times in PBSTx for 10 minutes, and then incubated for 1.5 hours in a solution containing the secondary anti-body (Jackson Immunoresearch, Cy2 Goat-anti-Mouse; diluted 1:100) and the phalloidin (with Alexa 488 fluorophore; diluted 1:200) in NGS/ PBSTx. The slides were incubated in a similar fashion as for the primary antibody and were this time protected from direct light with an aluminium foil cover. Once again, they were quickly rinsed in PBSTx before being washed 4 times in PBSTx for 10 minutes, and were then mounted in a 3% n-propyl gallate solution in glycerol, as anti-fade mounting medium.

Confocal microscopy and microglomerulus density measurement

We created image stacks of the mushroom bodies of tested bees using a laser scanning confocal microscope (Olympus FV-10) with a 60x oil immersion objective. For each subject, we chose 4 contiguous sections and randomly assigned scanning of either the left lateral calix, the left medial calix, the right lateral or the right medial calix, between them. The stacks were then used to reconstruct 3D images in the software ImageJ (Rasband 2010). We used a random offset grid to place 3 cubes (8.2x8.2x8.2 μm^3) in the dense collar region and 3 cubes in the lip region of the mushroom bodies (see fig. 3.2). We then counted the number of presynaptic boutons contained in the cubes to quantify their density per μm^3.

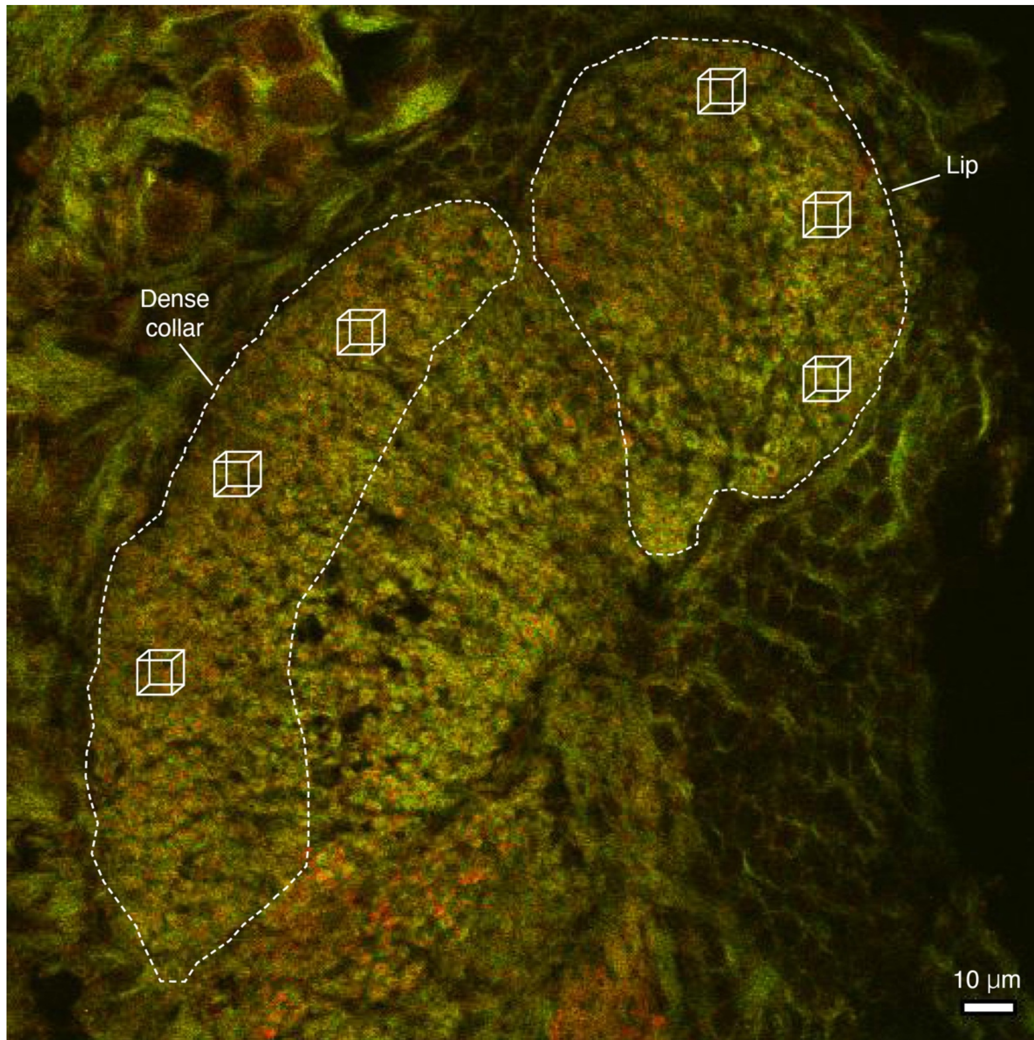

Figure 3.2: Confocal microscope image result of a cryosection staining of neural tissues of *Bombus terrestris* with subregion delineated. Synapsin is labelled in red, f-actin is labelled in green. In order to quantify MG density, we placed 3 cubes (8.2x8.2x8.2 μm^3) in the dense collar region and 3 cubes in the lip region of the MB.

Statistical analysis

Data were analysed using linear mixed-effect models (LMEs) (Zuur *et al*. 2009) built in R 4.1.2 (R Core Team 2021) with the lme4 package (Bates *et al*. 2014). We performed preliminary data exploration that revealed MG density in the collar and the lip region were correlated ($r = 0.63$), we therefore analysed them separately; none of the other variables were collinear.

First, we looked at whether MG density predicted foraging efficiency. To answer this question, we built an initial model containing foraging efficiency (mg of nectar/minute; ordered quantile normalization transformed with BestNormalize package, Peterson and Peterson 2020) as the response variable; age at the time of sampling (days), Julian date, individual body mass (average mass on the way out of the colony; mg) and number of days since first trip outside (days) as predictors. We included "colony" and "individual" as random effects to take into account the non-independence of repeated measures taken on the same individuals and on individuals hailing from the same colony. We then added MG density (microglomeruli/μm^3), with collar and lip analysed separately, to the list of predictors and compared the new model to the null model using ΔAIC method using a cut-off at >2. To test whether other predictors could have a significant relationship with foraging efficiency, we reused the null model presented above and produced every subset model possible including one containing only the intercept as predictor. We then selected the model with the lowest AIC.

We then looked at what predicted MG density. Data exploration revealed a non-linear effect of age sampled and total time spent outside on MG density in the lip region but not with MG density in the collar region. We therefore used a Generalised Additive Model (GAM) to analyse the data for the lip region. The initial model included MG density in the lip region as response variable, age sampled, body mass (log transformed), total time spent outside as predictors with

colony as a random factor. We generated all possible subsets of parameter combinations, including a model containing the intercept as the only predictor, and used ΔAIC to determine the best model. For the analysis of the predictors of MG density in the collar region, we built an initial linear model containing MG density in the collar region as the response variable, age sampled, body mass and total time spent outside as predictors with colony as a random factor. Once again, we used ΔAIC method to determine the best model.

Results

Over the course of the experiment, we recorded foraging efficiency (as mass of nectar collected per minute) for 2396 foraging trips made by 169 workers. Since not all bees survived until the end of the experiment, we obtained MG density estimates for 64 of these bees, who contributed 1339 foraging trips.

In contrast to our initial hypothesis that MG density might predict foraging efficiency, we found no correlation between foraging efficiency and MG density in either the collar (ΔAIC from null model= -1.95; fig. 3.3) or lip (ΔAIC = -0.643; fig. 3.4) region of the mushroom bodies. Instead, foraging efficiency was predicted solely by the number of days since the first trip outside (ΔAIC from intercept-only model = 71.68; parameter estimate = 0.093; SE = 0.011), indicating that bees became more proficient with experience. All other predictors had no significant effect.

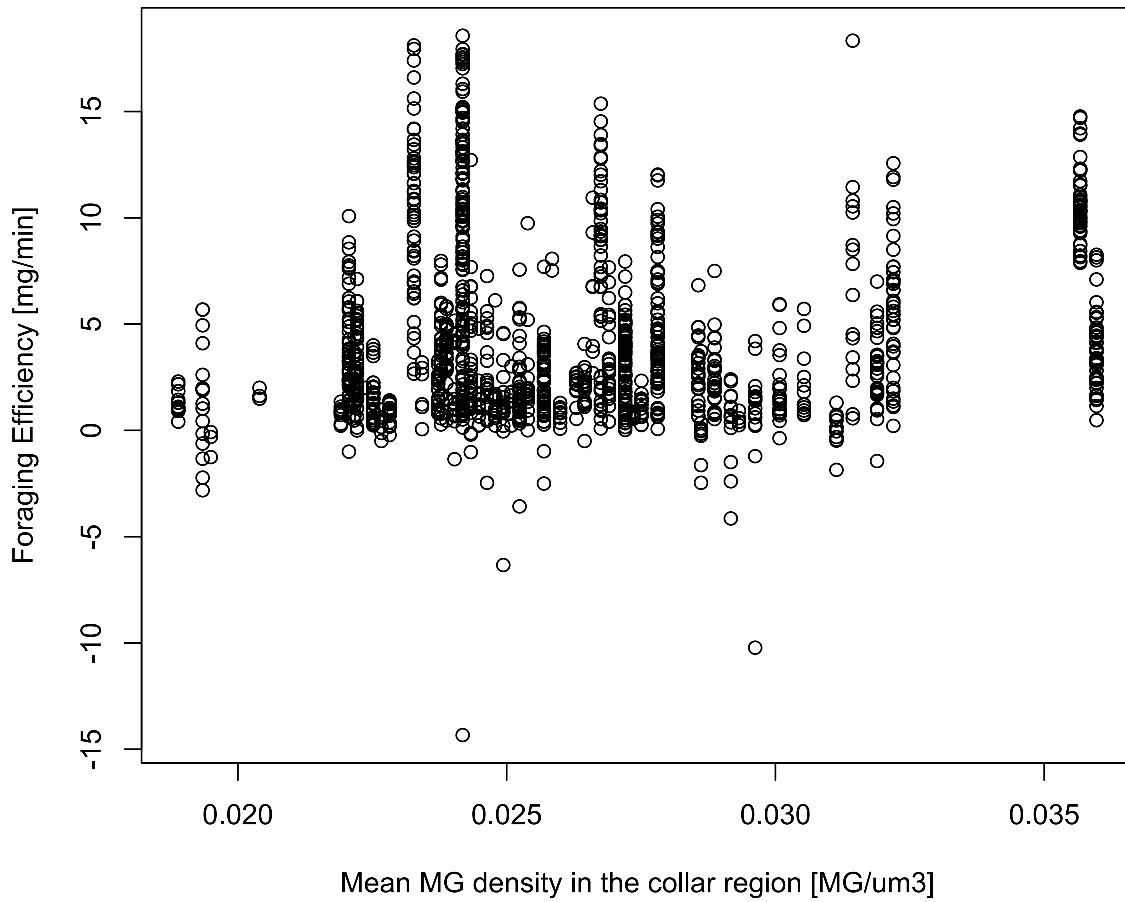

Figure 3.3: Plot representing no relationship between foraging efficiency and mean MG density in the collar region (n = 6 colonies, 66 individuals, 1339 foraging trips).

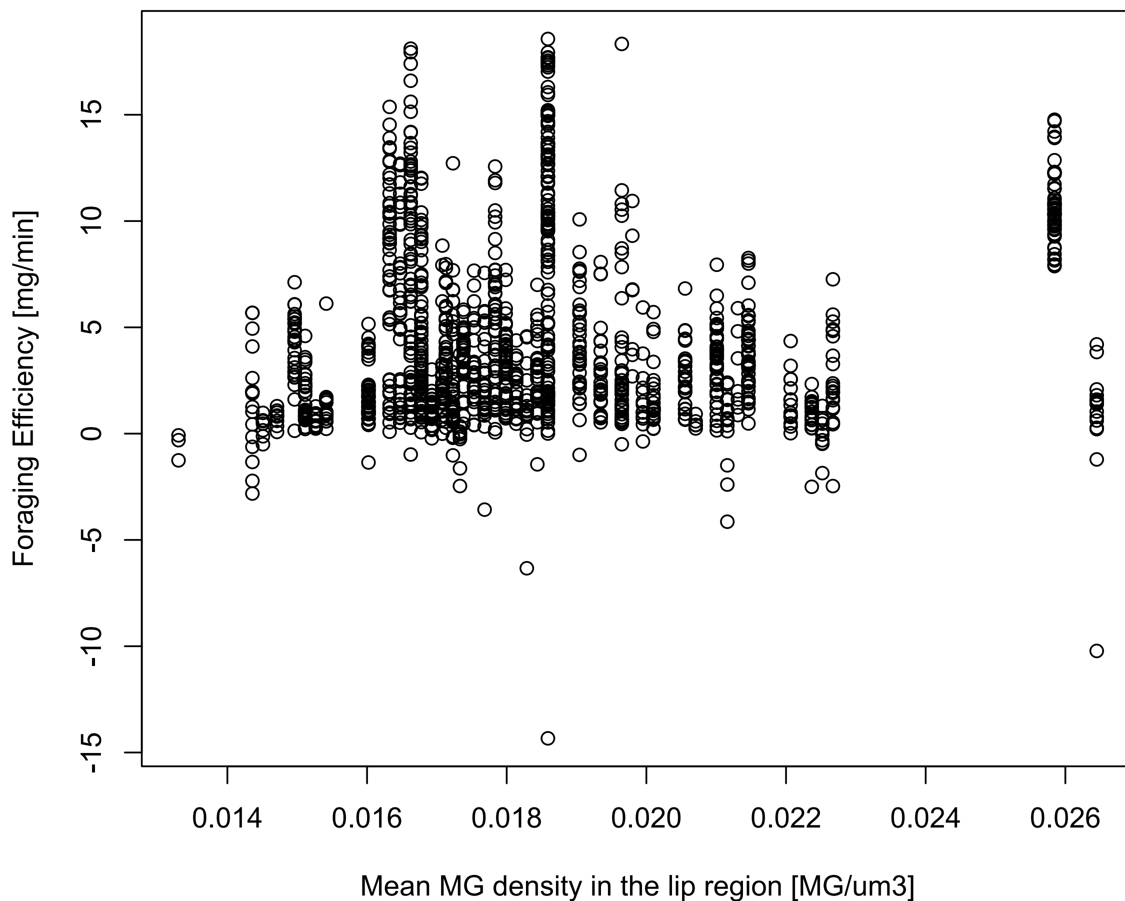

Figure 3.4: Plot representing no relationship between foraging efficiency and mean MG density in the lip region (n = 6 colonies, 66 individuals, 1339 foraging trips).

To test which variables predicted MG density, we first analysed the density in the lip region of the MB. We found the best model contained age when sampled as the sole predictor, and "colony" as random effect (GAM; ΔAIC with initial model = 3.39). This model showed that age when sampled significantly improved the model (fig. 3.5), and that colony (fig. 3.6) had a significant effect on MG density in the lip region of the MB. The inclusion of the other predictors, total time spent outside (GAM; ΔAIC with initial model = 1.67) and body mass (GAM; ΔAIC = 1.60), did not improve the final model.

The analysis of the predictors of MG density in the collar region showed the most parsimonious model was the intercept-only model with colony as random factor (*lme*; ΔAIC with initial model = 5.47). None of the other predictors – body mass (ΔAIC = 3.49) and total time spent outside (ΔAIC = 3.50) – allowed to build a significantly better model.

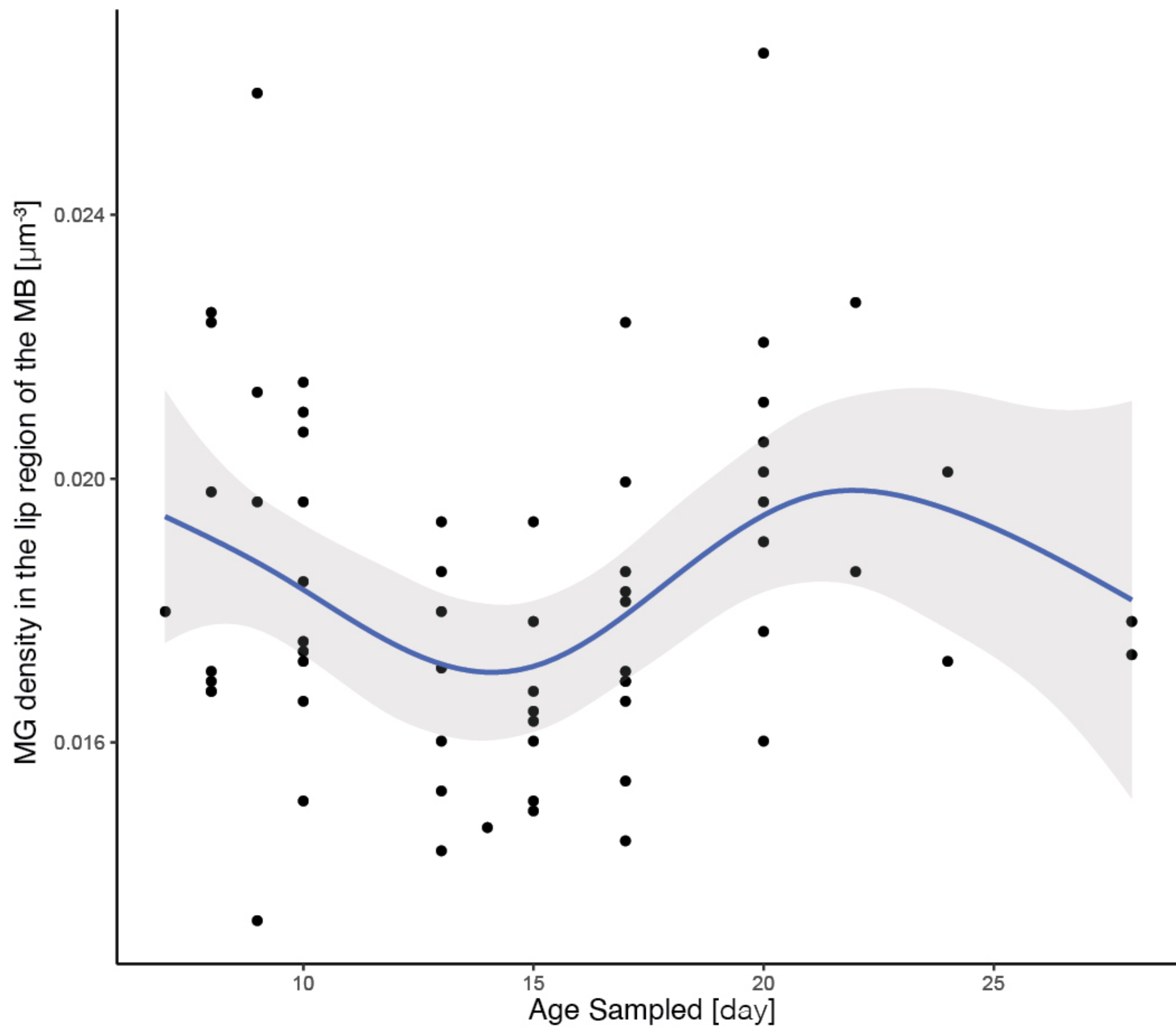

Figure 3.5: Non-linear relationship between MG density in the lip region of the MB and age at the time of sampling.

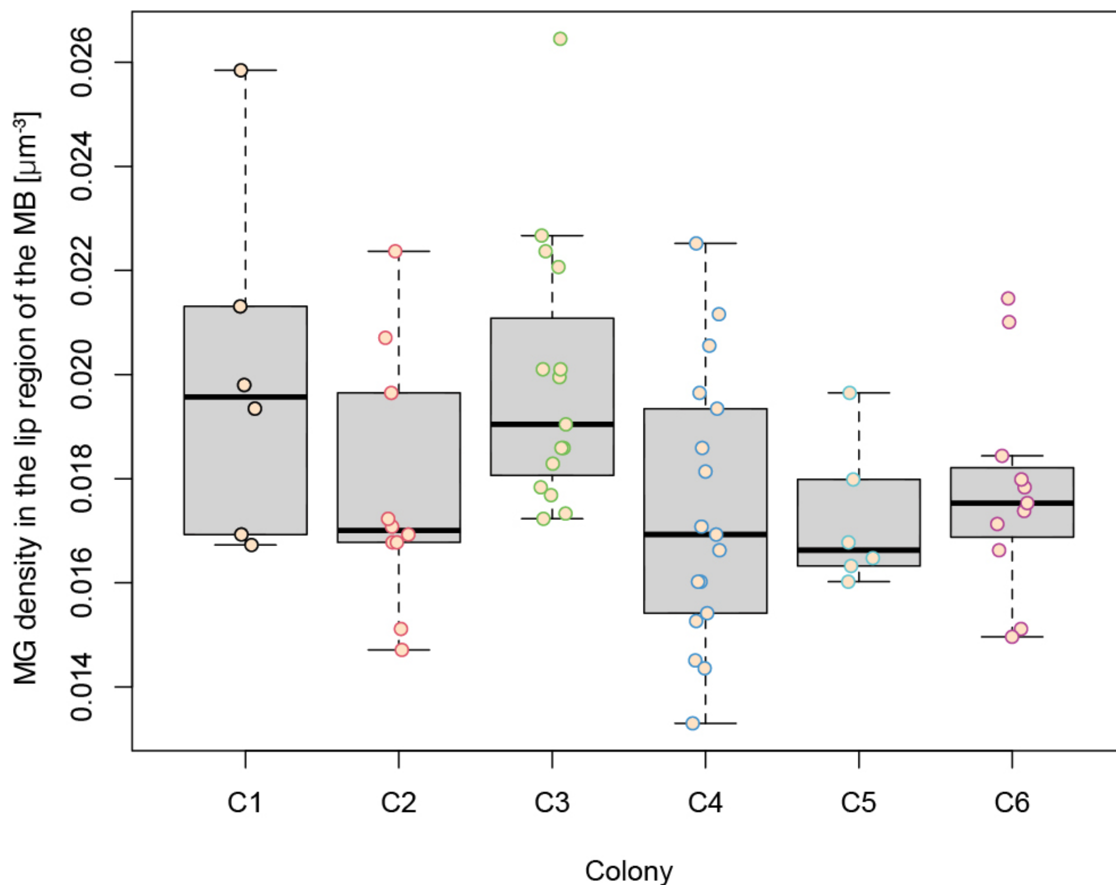

Figure 3.6: Boxplot of the relationship between MG density in the lip region of the MB and colonies.

Discussion

Our results did not reveal any correlation between MG density and foraging efficiency in bumblebees. Previous work has shown that MG density in the MBs changes in response to learning events (Hourcade *et al*. 2010), predicts learning ability (Cabirol *et al*. 2018), and coincide with the onset of foraging in honeybees (Muenz *et al*. 2015). However, our results suggest that these changes, if they affect learning ability, do not bring about improved foraging

efficiency, at least within the confines of our protocol. Instead, we found that the number of days between a focal foraging trip and the first outing of a bee (i.e. its experience of foraging) was the only predictor of foraging efficiency without our test cohort.

While this constitutes an unexpected result, the study itself offers a first attempt at exploring the link between neuroanatomy and foraging efficiency as the end product of learning in social insects. Our analysis revealed an effect of age on MG density in the lip but, surprisingly, not in the collar region. Age is documented to be one of the main factors influencing MG density in honeybees (Groh *et al*. 2006, 2012; Muenz *et al*. 2015) but many differences in the biology of the two species (e.g. temporal polyethism in honeybees, differences in colony life-cycle), as well as the design of our experiment compared to previous studies on honeybees, suggest that comparisons should only be made with caution. To our knowledge, only one study has looked at the relationship between age and MG density in *Bombus terrestris*. This was performed by Kraft *et al*. (2019) and revealed key differences with *A. mellifera*. First, the age-dependency of the relationship was only true at the beginning of adult development where a decrease of MG density was only observed in the first 3 days after eclosion. Additionally, light exposure did not have any effect on this trend. Taken together with this previous study (Kraft *et al*. 2019), our results suggest that foraging could indeed have an effect on neuroanatomy but as it stands, our results do not allow us to determine this. Future research should attempt to compare MG density in nest workers with foragers across a matched age range between the two groups to determine the effect foraging has on the MB of *B. terrestris*.

Contrary to previous studies looking at this relationship, our experimental setup allowed us to directly test foraging efficiency and therefore circumvent the need for a laboratory-based problem-solving task, conditioning assay or restricted array of flowers in the field. Colonies

were allowed to forage without restriction on flowers they could find in and around campus. To the best of our knowledge, this is the first study able to test directly MG density and foraging efficiency in social insects. However, the main strength of this experiment may also have been a weakness, because food availability and other environmental factors change across real-world environments. In particular, the flower dearth of summer (Leong and Roderick 2015) may have influenced our findings. The theoretical rational for this experiment was that if higher learning abilities had been found to separately correlate with MG density (Hourcade *et al*. 2010; Devaud *et al*. 2015) and foraging efficiency (Raine and Chittka 2008; but see also Evans *et al*. 2017 for a conterexample), we could expect to find a correlation between MG density and foraging efficiency. However, a recent study by Pull *et al*. (2022) that used an experimental setup and location almost identical to ours found that the relationship between short-term memory abilities and foraging efficiency depended upon ecological factors, namely food availability. While individuals with higher short-term memory scores were more efficient when food was plentiful, the relationship reversed in the summer to show a negative effect of short-term memory abilities on foraging efficiency during the flower dearth between July and September. This result is highly relevant here because the colonies we used were allowed to forage exactly during this time period (between 28th June and 26th September 2019). While our analysis did not reveal any negative effect of MG density on foraging, it is safe to say that at least one of the theoretical relationships on which our hypothesis hinged (i.e. learning abilities and foraging efficiency are correlated) might not have entirely be true at the time of the year that our experiment took place and could therefore explain why we found no relationship between MG density and foraging efficiency. However, Pull *et al*. (2022)'s results likely reflect the ecological importance of short-term memory rather than cognitive abilities as a whole. There is indeed evidence of a positive effect of long-term memory on foraging in the middle of summer in *Bombus terrestris* (Raine and Chittka 2008) and long-term memory training has

been shown to increase MG density in the MB (Hourcade *et al*. 2010). Alternatively, it is possible that training a certain type of memory (be it long-term or short-term) prior to starting a foraging career causes the bees to invest in a certain type of memory regardless of its real-world use in foraging. Future studies could answer this question by testing experienced foragers on both long-term and short-term memory tasks from spring to autumn, to see how the relevance of each for foraging efficiency changes depending on the ecological conditions.

Another explanation for our results could be that cognitive abilities play little role in foraging under harsher ecological conditions where food is scarce and exploratory activity would be of greater importance (Pasquier and Grüter 2016). Fidelity to a route decreases the likelihood of discovering a new, more profitable food source by chance, and might be detrimental in a poor and changing environment. Furthermore, a recent study failed to find neural correlates between MB extrinsic neurones and exploratory activity in bumblebees (Jin *et al*. 2020) which suggest exploratory activity is unlikely to be linked to MG density. Nevertheless, exploration is expected to lead to substantial energetic costs that colonies might find harder to balance in a poorer environment. Similarly, increased cognitive abilities also come at a cost and have been shown to trade-off with survival (Mery and Kawecki 2005). More research is therefore needed to determine the trade-offs involved with exploratory activity alone and comparison with cognitive abilities. Overall, our experiment highlights the gaps in knowledge on foraging and its relationship with cognition and neuroanatomy.

Chapter 4: No evidence of link between larval exposure to sulfoxaflor and mushroom body synaptic density in adult bumblebees (*Bombus terrestris*).

Grégoire Pasquier, Alberto Linguadoca, Swidbert Ott and Ellouise Leadbeater.

Abstract

Sulfoxaflor is a novel insecticide that acts as an agonist of nicotinic acetylcholine receptors and currently occupies an important part of the insecticide market worldwide. Much like neonicotinoids, which have a similar mode of action, sulfoxaflor has documented sublethal effects on non-target wild pollinator species. Effects on individuals of social species, such as *Bombus terrestris*, have downstream repercussions on the colony and sulfoxaflor has been shown to negatively impact the reproductive output of colonies foraging in the wild. As sulfoxaflor is neurotoxic, the effects observed at the colony level could be due to the insecticide affecting neural development which in turn could prevent workers from performing essential tasks such as foraging. Here we investigated the effect that sulfoxaflor has on a region of the brain of *B. terrestris* associated with foraging and learning, the mushroom bodies. We compared volume of the mushroom bodies, as well as the synaptic density, between individuals from colonies chronically exposed at the larval stage and unexposed controls. While our analysis failed to detect any effect of sulfoxaflor on either of those variables, this study highlights the gap in knowledge there is about insecticides and the individual-level mechanisms that underlie colony-level effects.

Introduction

Neurotoxic insecticides are commonly used in agriculture to prevent herbivorous insects from damaging crops, which would lower production and revenue (Simon-Delso *et al*. 2015). Compounds such as neonicotinoids act as agonists of the nicotinic receptors within insect neural tissue, to which they bind strongly, causing overstimulation and death to the target insect pest at sufficiently high doses (Tomizawa and Casida 2005). Given that non-target insects may also be exposed, through flower visitation, herbivory, run-off or soil residues, legislation typically requires that impacts on non-target organisms are tested prior to licensing, particularly on pollinators (see Siviter *et al*. 2023 for an in-depth guide on pesticide licensing in the European Union). The ecosystem services that insect pollinators offer in the context of agriculture, as well as their key role in plant and ecosystem conservation, make domesticated and wild bees critically important in maintaining food security (Rader *et al*. 2012, 2016; Garibaldi *et al*. 2014; Hung *et al*. 2018).

While the lower tiers of pre-licensing testing typically focus on mortality of individual bees they are unlikely to capture the full extent of effects caused by the widespread use of neurotoxic insecticides (Siviter *et al*. 2023). Consequently, there is now a substantial body of evidence that sublethal effects not tested at lower-tier pre-licensing levels cause harm to pollinators (Thompson and Maus 2007; Sgolastra *et al*. 2020; Siviter *et al*. 2021), because when scaled up to the colony level, they have significant impacts on colony fitness (Bryden *et al*. 2013). The main reason why these effects are usually not detected at the lower tiers of pre-licensing testing is because a pesticide must cause a mortality rate of over 10% of a honeybee colony to trigger higher-tier studies looking at sublethal effects (Siviter *et al*. 2023). Affected traits can include locomotion (Williamson *et al*. 2014), navigation (Henry *et al*. 2012), immune responses (Annoscia *et al*. 2020) and olfactory learning and memory (Siviter *et al*. 2018b). The latter has

been a source of particular concern, as the ability to associate floral scents with reward is thought to be an important contributor to individual foraging efficiency (Raine and Chittka 2008; Pull *et al*. 2022), which in turn should influence colony food stores (Klein *et al*. 2017). Moreover, there is evidence that neurotoxic neonicotinoid pesticides affect neural development by reducing the volume of the bee mushroom bodies (MB) (Smith *et al*. 2020), a key area of the insect brain for stimulus integration and learning, as well as the density of synaptic complexes, also known as microglomeruli (MG), in this same area (Peng and Yang 2016).

The MG found in the MB are formed of chemosensory projection neurons (notably from the optic lobe and antennal lobe) surrounded by Kenyon cell dendrites (Yusuyama *et al*. 2002). The signal input from afferent neurons coming from the optic lobe can be found in the collar region of the MB while the lip region receives input from the antennal lobe (Gronenberg 2001). The MG of both regions can be visualised by microscopy through the use of immunofluorescence techniques targeting the proteins synapsin and f-actin (Klagges *et al*. 1996; Frambach *et al*. 2004; see also Fahrbach and Van Nest 2016 for an in-depth review of the technique). Synapsin is a protein found in the membrane of presynaptic boutons and responsible for modulating the release of neurotransmitters (Johnson *et al*. 1972; see also Cesca *et al*. 2010 for a review of the history of the discovery of synapsin's function). Evidence from *Drosophila* also suggests that synapsin is a key component of neuroplasticity in insects as mutants lacking the protein perform significantly worse than wild types on olfactory learning assays, despite showing no other detectable differences in terms of brain morphology or function (Godenschwege *et al*. 2004; Kleber *et al*. 2016). In addition, restoring synapsin expression in Kenyon cells of mutants restored learning and memory performances (Niewalda *et al*. 2015).

In honeybees, changes in MG density and MB volume are associated with the onset of foraging (Fahrbach and Van Nest 2016), and have been correlated with learning abilities in both honeybees and bumblebees (Gronenberg and Couvillon 2010; Hourcade *et al*. 2010; Li *et al*. 2017). Accordingly, neonicotinoid exposure is associated with negative impacts on learning abilities in adult bees (Stanley *et al*. 2015; Muth *et al*. 2019; see also Siviter *et al*. 2018b for a meta-analysis on this subject).

Evidence documenting negative impacts of neonicotinoids on bees, mediated through sublethal effects, has led to a ban on the agricultural use of three neonicotinoids (imidacloprid, thiamethoxam and clothianidin) outside of greenhouses within the EU (the European Union, EFSA 2018). Together with evidence for the development of resistance to neonicotinoids (Bass *et al*. 2015), novel insecticide products are now obtaining considerable market share. One such product is sulfoxaflor, a chemical of the sulfoximine family with a similar mode of action to neonicotinoids (Brown *et al*. 2016). Here, we test the sublethal effect sulfoxaflor has on the brain development of a model pollinator, *Bombus terrestris* (Linnaeus, 1758). Negative sublethal effects of sulfoxaflor on colony reproductive success and fecundity have been shown in previous studies (Siviter *et al*. 2018a; Linguadoca *et al*. 2021), but surprisingly and contrary to neonicotinoids (Siviter *et al*. 2018b), no influence of exposure on learning ability was detected (Siviter *et al*. 2019). Instead, other mechanisms such as documented effects on egg-laying (Siviter *et al*. 2020b) and larval development (Siviter *et al*. 2020a) may mediate these effects. We propose that this may reflect contrasting exposure regimes across studies, whereby colony-level effects were documented following chronic exposure of entire colonies over time periods designed to match bloom periods in the field, while previous learning experiments had only involved a single exposure administered to an adult bee. While this previous acute exposure study reproduces the conditions a worker would experience on a single bout of

foraging on treated flowers, the chronic exposure regime we propose here captures the effects on larval development a colony experiences when foraging during the flowering period of treated crops.

In the following study, we expose bees to sulfoxaflor chronically as larvae, at a concentration known to elicit colony-level effects, following a protocol that has previously been used to demonstrate effects of neonicotinoid exposure on MB volume in bumblebees (Smith *et al*. 2020). Based on documented results for neonicotinoids, we expected to find a significantly lower MG density and volume in the MB of emerging adult bees that had been exposed to sulfoxaflor as larvae, compared to the control group.

Material and methods

Animal rearing

We used twelve queenright colonies of bumblebees (*Bombus terrestris audax*; obtained from Agralan, UK), standardised at 20 workers upon arrival. Colonies were assigned randomly to either an insecticide exposure treatment or to control conditions, within two time-blocks (Colonies 1-6 in block 1 and colonies 7-12 in block 2; see fig. 4.1). Three colonies had to be removed from the experiment: colony 6 (control group) was removed after the queen died 3 days before the end of the pesticide exposure phase and a majority of males started to emerge; colony 10 (control group) was removed for identical reasons at the end of the second week of pesticide exposure; colony 11 (sulfoxaflor exposure group) was not used because its queen was dead on arrival.

The experimenters were blind to the treatment assignments throughout the whole experiment and until data analysis was completed. Newly emerged bees were tagged three times a week in

each colony in order to individually record their age within a 2-day margin. Colonies were maintained under red light throughout, at a temperature of 24°C.

Insecticide exposure regime

The colonies in the insecticide exposure treatment received food in the form of a sucrose solution (30% w/w) containing sulfoxaflor (concentration of 5 ppb, dissolved in acetone) over 3 weeks, followed by a 4th week where food consisted of a sucrose solution (30% w/w) with no added insecticide. The three-week exposure regime was chosen to cover the average time of 14 days that *Bombus* pupae take to emerge (Alford 1975) which ensured all individuals emerging on the third week had been exposed to sulfoxaflor during their whole larval development. It was also chosen to enable direct comparison with work using neonicotinoid insecticides (Smith *et al*. 2020). We chose a concentration of 5ppb following the low-end of concentrations observed in nectar collected post-spray by foragers on a cotton crop (EPA 2016). Although residues in nectar and pollen can be higher than this (Linguadoca *et al*. 2021), *ad libitum* chronic exposure to sulfoxaflor at this concentration has been shown to have negative impacts at the colony level (Siviter *et al*. 2018a). Colonies assigned to the control condition were fed using a sucrose solution (30% w/w) with matched added acetone but no insecticide for 3 weeks followed by a 4th week where food consisted of a sucrose solution (30% w/w) with no added acetone. An untreated mixed-species load of honeybee-collected pollen (approximately 8g) was provided three times a week to colonies in both groups.

Sampling and immunolabelling

For each colony, three individuals that emerged after the 3rd week were randomly sampled at the end of the 4th week. These individuals are expected to have been exposed to sulfoxaflor throughout their larval period since bumblebee pupae take an average 14 of days to emerge

(Alford 1975). Their brains were dissected, processed and scanned using a laser-scanning confocal microscope following a whole-mount immunolabelling protocol inspired by Groh *et al.* (2012) and Ott (2008).

Each bee was chilled on ice for 15 minutes, after which the head was cut and a square window was opened in the cuticle of the frontal part of the head capsule, between the two compound eyes above the antenna, under Ringers solution. The exposed parts of the air sacs were removed and the head was fixed overnight at 4°C with a solution of 4% formaldehyde in PBS. The head was then washed in PBS and the brain was dissected from the head capsule. We then permeabilised the brains in a solution of 0.2% Triton-X in PBS before blocking it in 2% normal goat serum (NGS) in PBS-Tx for an hour. We incubated the brains in a solution of 50:1 mouse anti-SYNORF1 antibody in 2% NGS in PBS-Tx for 4 nights at 4°C. Then, after cycle of washing, we incubated the brains a solution of 250:1 Cy3-conjugated goat anti-mouse antibody and 250:1 AlexaFluor488-conjugated phalloidin in 1% NGS in PBS for 3 nights at 4°C. Samples were then washed and fixed using 1% Formaldehyde in PBS for 1 hour at room temperature to prevent degradation of the f-actin staining during subsequent steps. The samples were then dehydrated in a graded concentration of EtOH (30%, 50%, 70%, 90%, 95%, 100% x2; 10 minutes each step). The brains were then cleared in methylsalycylate and plated for microscopy. In total, 26 individuals were sampled and processed this way.

Microscopy

To quantify MG density, we took optical sections with a confocal microscope (Olympus FV10) at 0.5 μm interval with a 60x oil immersion objective (Olympus UPLSAPO 60xo, NA = 1.35) and for calyx volume measurement, we took optical sections of the left medial calyx at an interval on 5 μm with a 10x air objective (Olympus UPLANSAPO 10x, NA = 0.40). The image

stacks were analysed using ImageJ (Rasband 2010). For MG density quantification, we counted the number of synaptic boutons contained within manually placed cubes of dimensions 10 µm ×10 µm ×10 µm. For each stack, three of these cubes were placed in the dense collar and three in the lip region (see fig. 4.1). This gave us a measure of MG density in MG/µm^3 in the dense collar region as well as for the lip region of the MB for each bee. To measure the volume of the MB neuropile we manually detoured the dense collar, loose collar, lip and basal ring region on each frame of each stack. This procedure gave us a measurement of calyx volume in µm^3.

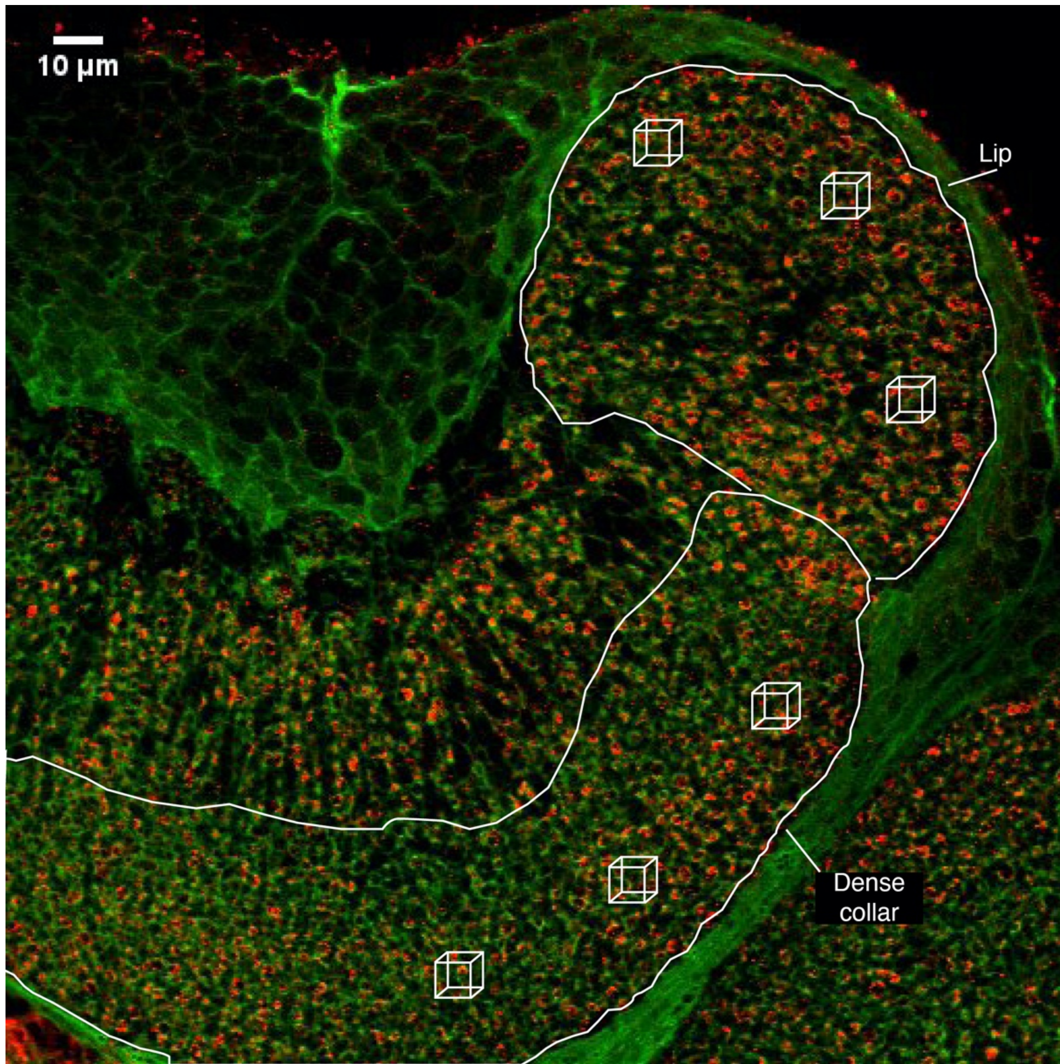

Figure 4.1: Confocal microscope image result of a wholemount staining of neural tissues of *Bombus terrestris* with subregion delineated. Synapsin is labelled in red, f-actin is labelled in green. In order to quantify MG density, we placed 3 cubes (10x10x10 µm^3) in the dense collar region and 3 cubes in the lip region of the MB.

Statistical analysis

Data was analysed in R 4.1.2 (R Core Team 2021) where we used the package *lme4* (Bates *et al*. 2014) to construct models.

To analyse the effect of the pesticide treatment on MG density, we built two initial null models (one for MG density in the collar region and one for MG density in the lip region) each containing the relevant predictors body size, age at time of sampling and block number with colony and individual as random factors. We then performed AIC-based model selection to select the best null models for each MB region. The null models for both the collar region and the lip region contained MG density (log-transformed) as the response variable and solely the intercept as predictor number with colony and individual as random factors. For each model, we then added treatment as a fixed factor, and assayed the effect using ΔAIC method with a cut-off >2 to determine which model was better for each pair.

We applied the same methodology to analyse the effect of treatment on the volume of the MB. The initial model had total volume of the left medial calyx as response variable; body size, age at time of sampling and block as predictors; colony and individual as random factors. The null model obtained after AIC-based model selection contained body size and age as predictors with colony and individual as random factors. We then added treatment as a fixed factor, and assayed the effect using ΔAIC method with a cut-off >2 to determine which model was better.

Results

We tested the effect of chronic larval exposure to the neurotoxic pesticide sulfoxaflor on MG density in two regions of the MB of adult bumblebees. We sampled a total of 27 individuals but, due to a problem during immunostaining, we were unable to take any measurements on the neural tissues of one individual from colony 7. Our total sample size for MG density was thus n = 26 individuals, 11 in the control group, 15 in the pesticide treatment group. For the calyx volumes, we were unable to measure 11 image stacks due to poor image resolution in the neural tissue depths and led to a sample size n = 15 individuals, 8 in the control group, 7 in the pesticide treatment group. Although this sample size is lower than we had planned (please see COVID impact statement), it is comparable to previous experiments that used wholemount immunostaining of neural tissues of *B. terrestris*. Li *et al*. (2017) tested the correlation between learning speed and MG density in the collar region of the MB (experiment 2) with n = 10; Kraft *et al*. (2019) studied the effect the effect of light exposure with n = 14 for their group of 3 days old bees reared in the dark and n = 15 for the other groups.

We found no effect of sulfoxaflor treatment on MB density in the lip region (ΔAIC to null model = -1.91; Fig. 4.3.a) with the null model being the most parsimonious. None of the other predictors from the initial model had any significant effect in improving the model; age (ΔAIC to null model = -1.99), body size (ΔAIC to null model = -1.27) and block (ΔAIC to null model = -0.04) were therefore discarded during the selection of the null model.

Similarly, the analysis of density in the collar region revealed no effect of treatment on MG density where the null model was found to be the better one (ΔAIC = -1.94; Fig. 4.3.b). The predictors from the initial model had no significant effect in improving the model. The

predictors age (ΔAIC to null model = -1.05), body size (ΔAIC to null model = -1.99) and block (ΔAIC to null model = -1.98) were therefore discarded during null model selection.

We also tested whether the pesticide exposure regime had an impact on the volume of the MB neuropile and found no effect of treatment on left medial calyx volume (ΔAIC = -1.25; Fig. 4.3.c). However, as expected, we found a significant positive relationship between body size and calyx volume (Estimate = $8.42 \cdot 10^6$, SE = $8.71 \cdot 10^5$; Fig. 4.3.d). Age contributed to the null model's lowest AIC score but the parameter did not have a significant effect in the model (r = 0.47; *lme*, p = 0.17; Fig. 4.4).

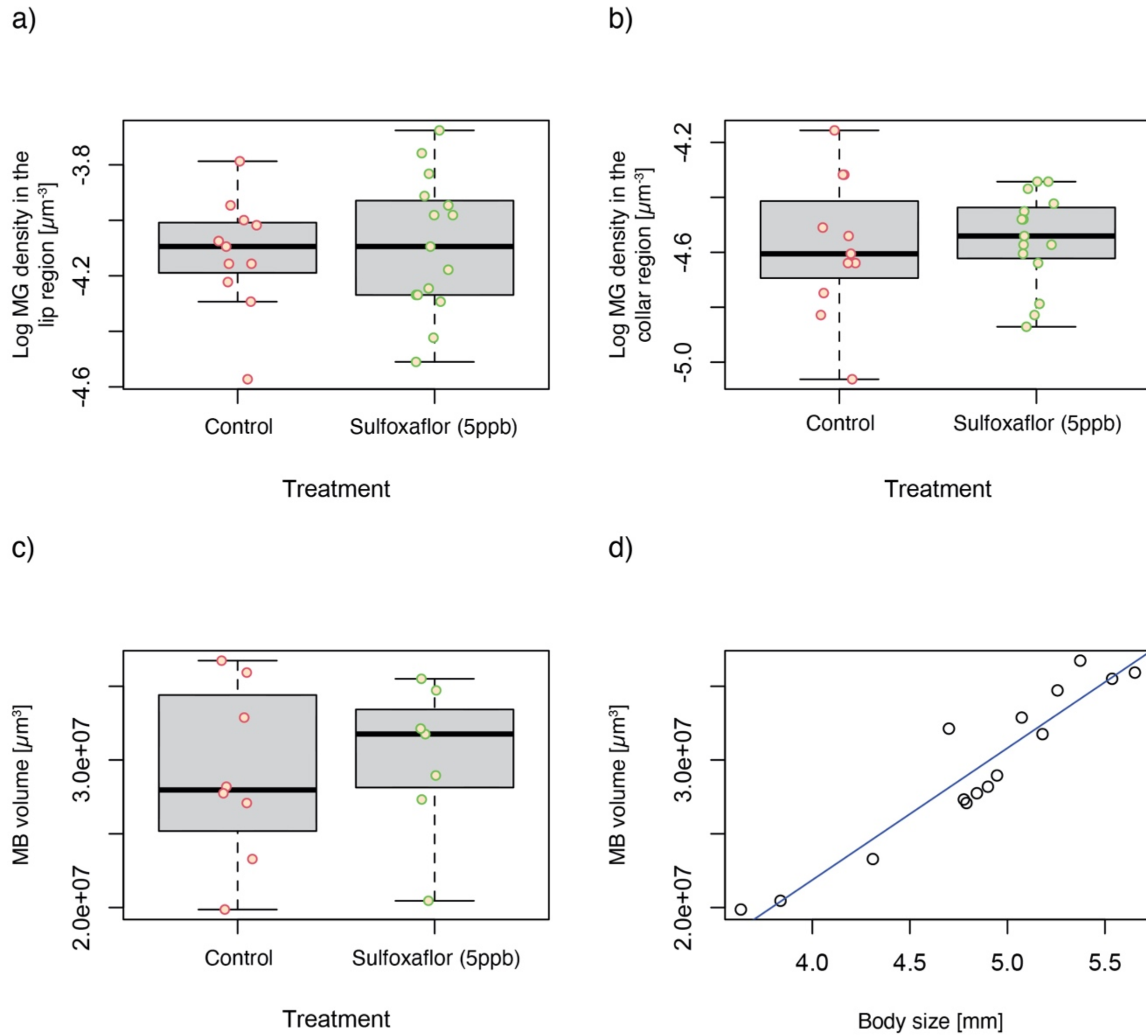

Figure 4.3: a) Boxplots comparing the log MG density in the lip region of the MB in the control and sulfoxaflor (5 ppb) treatment group (*lme*, ΔAIC = -1.91); b) Boxplots comparing the log MG density in the dense collar region of the MB in the control and sulfoxaflor (5 ppb) treatment group (*lme*, ΔAIC = -1.94); c) Boxplots comparing the volume of the left medial calyx of the MB in the control and sulfoxaflor (5 ppb) treatment group (*lme*, ΔAIC = -1.25); d) Plot representing the relationship between body size and volume of the left medial calyx in both treatments (*lme*, Estimate = $8.42 \cdot 10^6$, SE = $8.71 \cdot 10^5$).

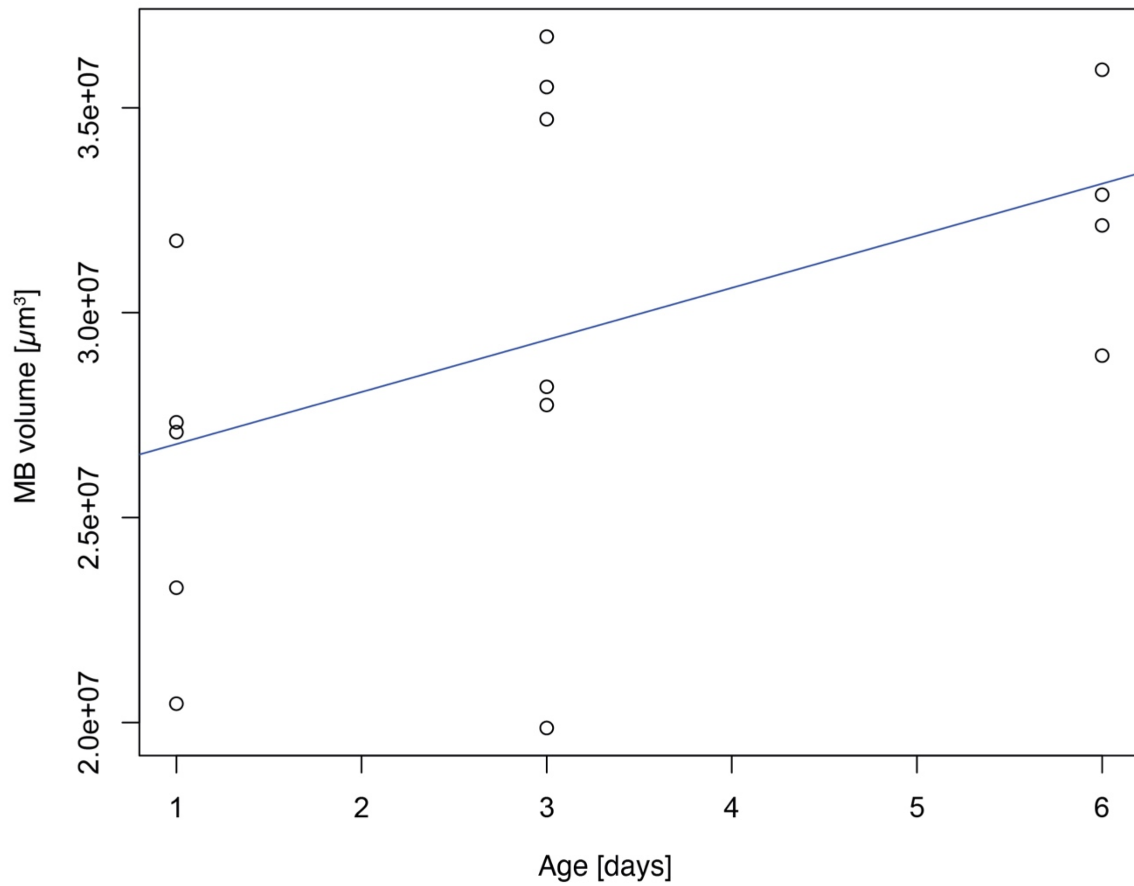

Figure 4.4: Plot representing the relationship between age and volume of the left medial calyx in both treatments combined (r = 0.47; *lme*, p = 0.17).

Discussion

Our analysis did not detect an effect of sulfoxaflor on the volume of the MB or MG density in the lip or the collar region of the MB of *B. terrestris* adult workers that had been chronically exposed during larval development. These results contradict the effect we expected from sulfoxaflor based on the documented effects of neonicotinoids on MB volume (Smith *et al*. 2020) and MG density (Peng and Yang 2016), but are in line with previous findings that suggest the mechanism by which sulfoxaflor elicits colony-level effects on *Bombus terrestris* colonies does not involve impacts on learning and memory (Siviter *et al*. 2019).

Similar to neonicotinoids, sulfoxaflor is a neurotoxic insecticide that acts as an agonist of the nicotinic acetylcholine receptors (nAChR) in insect brain tissues (Sparks *et al*. 2013) and does have documented evidence of negative sublethal effects on *B. terrestris* colonies. Yet, based on our results it appears that the individual-level effects of sulfoxaflor is not identical to those of neonicotinoids. This discrepancy could be explained by differences in the 3D structure of sulfoxaflor and neonicotinoids, as this is fundamentally important in determining receptor binding as well as how new active molecules interacting with these receptors are designed (Kubinyi 1998). As the molecules are not identical, differences in binding affinity are expected, but the question is to determine the significance of these differences regarding the mode of action of the insecticide. Studies in green peach aphids (*Myzus persicae*), which are among the primary targets of insecticides, suggest that sulfoxaflor has a lower binding affinity to their nAChR compared with the neonicotinoid imidacloprid (Watson *et al*. 2011; Cutler *et al*. 2013). Further research should determine whether this difference in binding could be the cause for the absence of effect on brain development we found in *B. terrestris*.

When discussing toxicology, it is important to remember the maxim devised by 16th Century Swiss physician and alchemist Paracelsus: "*Allein die Dosis macht, dass ein Ding kein Gift ist.*" (the dose alone makes it so something is not a poison; Paracelsus 1538). In this experiment, we replicated the dosage of insecticide used in a previous experiment that found sublethal effects of sulfoxaflor on *B. terrestris* colonies (Siviter *et al*. 2018a). Additionally, the chronic exposure regime we used reproduced the methodology of a previous experiment on imidacloprid that found effects of the insecticide on MB volume in *B. terrestris*. However, a recent publication modelled its exposure levels on micro-colonies of *B. terrestris* after new data reporting the dosage and gradient of degradation observed on crops treated with sulfoxaflor (Linguadoca *et al*. 2021). Their exposure regime started at a higher concentration and diminished rapidly afterwards while, in contrast, our exposure level was conservatively low and stable during the exposure period. This difference means that the colonies we tested might have experienced an initial concentration much lower than what a colony foraging on a treated field would be subjected to. Consequently, our experiment might have used too low a concentration to produce detectable effects on the MB of *B. terrestris* as well as using concentration that underestimated the ones found in the field. Future research should use the promising new methodology presented by Linguadoca *et al*. (2021) to implement pesticide exposure regimes that more closely reflect those observed in the field.

In addition to the nectar concentration, wild pollinators can also get exposed to pesticides via the plants' pollen (Laurent and Rathahao 2003). In this experiment, we provided colonies with untreated pollen to replicate the conservative conditions in which a previous studies found an effect of chronic exposure to sulfoxaflor on colony reproductive output (Siviter *et al*. 2018a), larval growth (Siviter *et al*. 2020a) and egg-laying (Siviter *et al*. 2020b). Yet, the concentration of sulfoxaflor found in the pollen of treated crop ranges between 50.12 and 510.95 ppb (EPA

2019) and pollen consumption is most important for larval development (Goulson 2010). Additionally, it is unclear what dosage larvae receive as the volume of pollen they consume is unknown yet and would constitute an interesting avenue for new research.

Together, these two factors could have led us to create exposure condition too conservative to detect any effect of sulfoxaflor on the development of brain tissues of *B. terrestris*. These shortcomings should be addressed in future studies by including exposure through pollen consumption, possibly through full-factorial designs. Such a setup could also shine a light on the comparative importance pollen plays in larval exposure to pesticides.

Reduction in individual learning abilities has been put forward as a major driver of the negative sublethal effects of neonicotinoids observed at the colony level in eusocial pollinators (Siviter *et al*. 2018b). The results presented here and by Siviter *et al*. (2019) seem to suggest that, while sulfoxaflor has similar negative sublethal effect to neonicotinoids at the colony level (Siviter *et al*. 2018a), these effect are not mediated by a reduction in individual learning abilities. This questions the importance of the role individual learning really plays in driving the documented effects of insecticides at the colony level. For sulfoxaflor, the main drivers appear to be linked with larval development (Siviter *et al*. 2020a), egg-laying (Siviter *et al*. 2020b) and fecundity (Linguadoca *et al*. 2021). However, not all aspects of learning have neuroanatomical correlates and processes such as short-term memory could still be affected by sulfoxaflor. This possibility will be explored in the next chapter of this thesis.

Chapter 5: Chronic exposure to sulfoxaflor during larval development does not affect bumblebee performance in a radial arm maze.

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Abstract

Anthropogenic stressors are currently regarded as the main drivers for range decline in insect pollinator populations. Among these stressors, systemic insecticides have documented sublethal effects on workers of eusocial pollinators such as *Apis mellifera* and *Bombus terrestris* that produce downstream effects impacting colony growth and reproductive output. Previous research on neonicotinoids has demonstrated the negative impact that these neurotoxic insecticides can have on individual learning abilities, and suggested that this could be contributing to the negative effects observed at the colony level. Because neonicotinoids have been banned from use outside of greenhouses in the European Union and the primary targets of the pesticides have developed resistance to their effects, novel insecticides have been taking over substantial shares of the market. One of these new agrochemicals is sulfoxaflor, a neurotoxic insecticide which has a similar mode of action to neonicotinoids in targeting nicotinic acetylcholine receptors of insects. As sulfoxaflor has documented negative sublethal effects on *B. terrestris* reproductive output and fertility, we investigated whether these effects could be driven by impacting individual foragers' learning ability. Here we chronically exposed a group of *B. terrestris* colonies to sulfoxaflor and compared their performance with a control group of colonies on a free-flying short-term memory assay. We tested bumblebees' short-term memory using a radial-arm maze, a paradigm classically used in rodents to test

drugs effects on memory. We did not find any effect of sulfoxaflor on performance in the radial-arm maze.

Introduction

An abundance of evidence attributes the current decline of pollinator populations to anthropogenic stressors such as climate change, loss of habitat, increase in pathogen prevalence or the use of agricultural pesticides (Brown and Paxton 2009; Winfree *et al*. 2009; Potts *et al*. 2010a; Cameron *et al*. 2011; Goulson *et al*. 2015; Kerr *et al*. 2015; Woodcock *et al*. 2017). Among them, neurotoxic insecticides such as neonicotinoids and sulfoximines have attracted particular interest as they are systemic pesticides i.e. the insecticide is present in the whole plant including the nectar and pollen (Bonmatin *et al*. 2015; Rundlöf *et al*. 2015; Wood *et al*. 2019). The mode of action of these insecticides is to target the nervous system by disrupting the nicotinic acetylcholine receptors of neurons within the central nervous system which can lead to inactivity and compromise development (Palmer *et al*. 2013; Peng and Yang 2016). In many cases, at field realistic levels, the resultant effects may not be lethal to individual bees, but instead are more subtle, affecting traits such as learning ability (Siviter *et al*. 2018b), ability to navigate (Henry *et al*. 2012), locomotion (Williamson *et al*. 2014) or immune response (Annoscia *et al*. 2020).

The ecological risk assessment protocols upon which agrochemical licensing processes are based rarely take into account sub-lethal effects on non-target species at the lower tiers (Thompson and Maus 2007; Sgolastra *et al*. 2020; Siviter *et al*. 2021, 2023). Yet, a large body of evidence now points to the negative effects that sub-lethal doses can have on social bees when scaled up to the colony level (Bryden *et al*. 2013; Klein *et al*. 2017). In bumblebees (*Bombus spp.*), which are key pollinators of numerous important crop species such as tomatoes or peppers, in addition to their important role in natural ecosystems, such impacts can be seen on several close proxies of fitness such as colony growth or reproductive output (Whitehorn *et al*. 2012; Feltham *et al*. 2014; Rundlöf *et al*. 2015; Siviter *et al*. 2018a).

In 2018, the agricultural use of three neonicotinoid insecticides outside of greenhouses was banned in the European Union (EFSA 2018). Although neonicotinoids remain widely-used globally, this ban, together with an increase in neonicotinoid resistance amongst pest species (Bass *et al*. 2015) prompted other insecticides to emerge on the market (Simon-Delso *et al*. 2015; Brown *et al*. 2016). Sulfoximines, of which sulfoxaflor is the single marketed product, share a mode of action with neonicotinoids as agonists of nicotinic acetylcholine receptors and were identified as likely to replace the prohibited substances (Brown *et al*. 2016). Although sulfoximines degrade rapidly post-spray (Linguadoca *et al*. 2021), which might be argued as a factor diminishing its threat to bee populations, recent studies have already highlighted the negative impact that chronic exposure to sub-lethal doses can have on larval development and colony reproductive output in bumblebees even at field-realistic exposure (Whitehorn *et al*. 2012; Feltham *et al*. 2014; Rundlöf *et al*. 2015; Tsvetkov *et al*. 2017; Siviter *et al*. 2018a, 2020b).

In the wild, *Bombus terrestris* (Linnaeus 1758) colonies follow an annual life cycle in which initial cohorts of workers forage to build a workforce that eventually raises the gyne and male offspring that will produce colonies for the next year (Goulson 2003). Because they cover large foraging ranges to collect minute quantities of food, there is great pressure on foragers to forage efficiently, so any negative impacts of stressors on traits that determine foraging efficiency have the potential to be particularly damaging (Klein *et al*. 2017). Such traits are thought to include cognitive abilities such as learning and memory. For example, Raine and Chittka (2008) showed that the average learning speed of workers within a colony, assayed through a colour-learning task, correlated with that colony's foraging performance in the wild, while (Pull *et al*. 2022) found that short-term memory (STM) predicted foraging success in rich, complex environments only. It is therefore important to point out that neurotoxic insecticides

such as thiamethoxam have been shown to impact learning abilities and impair foraging: chronic exposure to field-realistic doses of the compound slows down olfactory associative learning as well as memory recall 3 hours later (Stanley *et al*. 2015b), slows down learning of new flower handling in addition to changing flower preferences (Stanley and Raine 2016) and reduces flower visitation rate with the direct consequence of reducing seed concentration in the resulting fruits (Stanley *et al*. 2015a). Additionally, acute exposure to thiamethoxam also has a negative effect on STM (Samuelson *et al*. 2016). STM in insects is a process that involves sustained neuronal firing but, contrary to long-term memory, does not rely on transcription or translation. Importantly, the different phases of memory are independent from one another (Trannoy *et al*. 2011; for a review specific to honeybees, see Menzel 2012) and this mechanistic independence appears to be conserved between insects and vertebrates (Izquierdo *et al*. 1999). These negative effects of neonicotinoids can be seen across species, as exposed in a recent meta-analysis that looked at learning scores and memory in studies using the proboscis extension reflex paradigm (Siviter *et al*. 2018b).

While the effect that neonicotinoids have on learning seem clear and could be driving the effects observed at the colony level (Whitehorn *et al*. 2012; Feltham *et al*. 2014), this relationship is not clear for sulfoxaflor. Where previous work has shown the detrimental consequences of chronic exposure at the colony level (Siviter *et al*. 2018a), the effects of sulfoxaflor on cognitive ability do not appear as straightforward as for neonicotinoids. Indeed, early work looking at the effect of acute sulfoxaflor exposure on PER and STM performance has not revealed any effect of the pesticide (Siviter *et al*. 2019). However, while studies on acute exposure are useful to simulate the effect of the neurotoxic substance on a single foraging bout, they cannot capture the effect of long-term exposure to a compound that pollinators are likely to face in cases such as the whole flowering period of a nearby treated crop (Tsvetkov *et*

al. 2017). As chronic exposure regimen to neonicotinoids have been shown to reduce the development of neural regions as key to learning as the mushroom bodies (Smith *et al*. 2020) it is reasonable to expect that reproducing similar exposure conditions to sulfoxaflor, given its similar mode of action, may yield similar results.

In testing the effect of stressors on animal behaviour, classic psychology paradigms such as the radial arm maze (RAM) can be useful to provide a well-established standardised assay (Olton and Samuelson 1976). The RAM is traditionally used to assay short-term working memory, because individuals must remember the location of visited arms within a foraging bout, but not between bouts, when the maze is rebaited. It has been used in the past to evaluate the effect of drugs in rodent models (Foreman and Ermakova 1998) and has been used to test the effects of acute exposure to thiamethoxam on bumblebees (Samuelson *et al*. 2016). We also present here a validation experiment of this paradigm in bumblebees (also published in Pull *et al*. 2022), confirming that bees can use memory to improve performance in the RAM, rather than simply relying on stereotypical movement rules.

Here we tested the effect chronic exposure to sulfoxaflor during larval development has on the RAM performance of adult bumblebee foragers. We expected to find a negative effect of sulfoxaflor exposure across all our measurements of performance in the RAM, in line with results obtained with other neurotoxic insecticides but instead found no difference between our cohort exposed to the insecticide and the control group.

Material and Methods

Overview

Twelve commercially-obtained early-stage queenright colonies of bumblebees (*Bombus terrestris audax*; Agralan, UK) were assigned randomly to either a chronic insecticide exposure treatment (5ppb sulfoxaflor in *ad libitum* sucrose) or to control conditions, within three staggered blocks (block 1: Colony number 1-4; block 2: Colony number 5-8; block 3: Colony number 9-12; see figure 5.1). Newly emerged bees were tagged three times a week in each colony in order to individually record their age within a two-day margin, and workers that emerged as adults during the week following the exposure phase were tested in a Radial Arm Maze (RAM) paradigm.

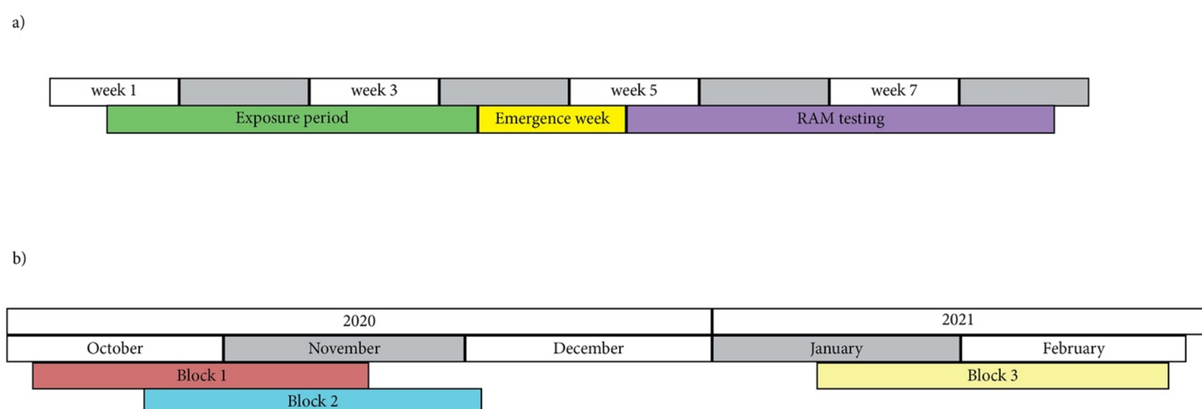

Figure 5.1: Sequence of the phases of an experimental block (a) and the time frame during which the experiment was conducted (b).

Insecticide exposure

The experimenters were blind to the treatment assignments throughout the whole experiment and until data analysis was completed. Upon arrival, colonies were kept at 24°C in a dark room and their size was standardised at 20 workers each. The colonies in the insecticide exposure treatment received food in the form of a sucrose solution (30% w/w) containing sulfoxaflor

(Chem Service Inc., USA; concentration of 5 ppb, dissolved in acetone) over 3 weeks followed by a 4th week where food consisted of a sucrose solution (30% w/w) with no added insecticide. This methodology allowed us to expose bees during early larval development stages but not during adulthood; previous work following this same regime detected effects on mushroom body development as well as cognitive traits (Smith *et al*. 2020). Untreated pollen was provided 3 times a week (8 g). The colonies assigned to the control condition were fed using a sucrose solution (30% w/w) with an acetone concentration matched with the food in the insecticide exposure treatment but without insecticide for 3 weeks followed by a 4th week where food consisted of a sucrose solution (30% w/w) with no added acetone.

Colony number 4 was removed from the experiment before the RAM testing phase as it had only produced males and no new workers until that point. This is unlikely to have been caused by our treatment regimen as the pattern was recorded from 2 days after arrival and remained unchanged until 25 days later when RAM testing began. In total, we reared 1980 workers; 530 emerged during the 4th week after arrival and were therefore available for testing in the RAM. The daily consumption of food for the control group was 26.45 g/day (sd: 9.18) and 25.99 g/day (sd: 9.22) for the sulfoxaflor treatment group.

Radial-arm maze test

In each colony, we tested 10 bees that emerged during the 4th week of their respective experimental block in a RAM (see fig. 5.2). The RAM used in this experiment was an octagonal box with a clear lid and baffles dividing the space into eight arms all connected with each other through the centre of the maze. We used a classic RAM paradigm where the subject tested was required to visit each arm of the maze once within a foraging bout in order to obtain the hidden reward they contained, which was a 10 µl drop of 45% w/w sugar solution. The number of

times a subject attempted to extract the reward of an arm it had already depleted (revisit) was recorded for each bout and used as a measure of short-term memory.

An experimental day for each colony would start with a group training where the nest box was connected to the maze. The whole colony was allowed to forage for an hour during which we identified motivated foragers. Bees selected for testing underwent, on the same day, 10 training bouts in the RAM immediately followed by 3 test bouts. We recorded each bout and transcribed the sequence of behaviour observed. A landing corresponded to the focal bee resting on a platform for any amount of time and a revisit was recorded when the focal bee landed on a platform on which it had already landed during the ongoing bout. Bees' performances were based on two measurements: total number of revisits, and landings before first revisit. The former is a straightforward measurement of how well a subject did in a given bout and the latter is a measurement of how early in the bout an error is made. A typical subject trained repeatedly on the RAM typically sees total number of revisits decreasing while landings before first revisit increases (Dubreuil *et al*. 2003; Samuelson *et al*. 2016). We also recorded the number of times a bee would enter an arm without landing on a platform (approaches). This variable was added as an attempt to measure indecisiveness in our subjects. Body size was measured on the frozen individuals after the experiment was completed.

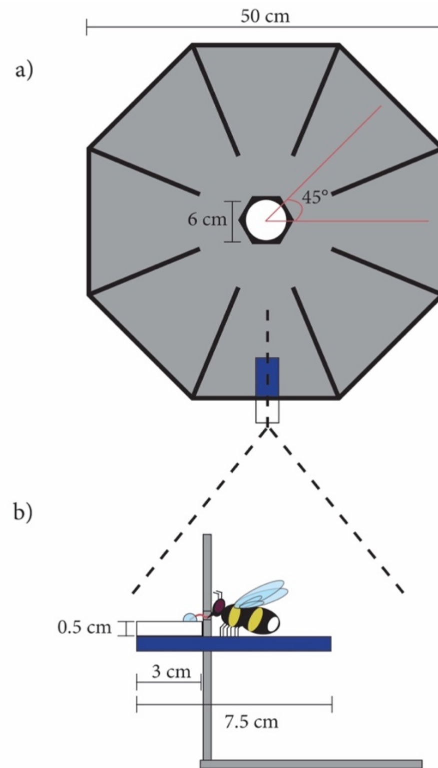

Figure 5.2: Schematic view of the radial arm maze (RAM) used for the experiment. a) The RAM viewed from above. The maze consists of an octagonal grey Perspex™ box (50x50x10 cm^3) with a clear UV-transparent lid. It contains 8 baffles, which divide the internal space of the maze into 8 arms. The walls and the floor of the box were sealed together with grey aquarium-grade fungicide-free silicon joints (Bond It, Grey HA6 RTV). Access is through the central hole (6 cm diameter) in the floor of the maze. The end of each arm features a rectangular slot (3x0.5 cm^2) where a platform could be inserted. b) Sectional view of an end-point of the RAM with a bee shown feeding on a 10 µl drop of sucrose solution. The food reward is only accessible by the subject via the extension of its proboscis through the hole situated 0.5 cm above the platform. A platform consisted of a rectangular 7.5x3x0.5 cm^3 blue Perspex chip glued to a square 3x3x0.5 cm^3 white Perspex chip. The blue part of the platform acts as a landing area whereas the white section of the platform (inaccessible to the bee) serves only for placement of the reward droplet.

Validation experiment: do bumblebees use STM to solve a RAM?

Before using the methods for RAM testing presented above, we performed a validation experiment of the RAM paradigm by analysing the movement patterns of 20 bumblebees from 4 colonies that underwent 10 individual training bouts and 10 test bouts in the RAM. This protocol is adapted from (Brown *et al*. 1997; Samuelson *et al*. 2016), but customised such that possible variation in individual movement rules between bees is taken into account.

In order to establish whether bumblebees were achieving better performance than could be explained by the use of stereotypic movement rules or pure chance, we compared the observed data with 10,000 simulated datasets using a randomisation analysis (Farine and Whitehead 2015). This statistical method can be used to compare a test statistic derived from observed data with a distribution of the equivalent test statistic obtained from several simulated data sets, created based on a null hypothesis. In this case, the two null hypotheses were (a) random movement between arms (b) the use of stereotypical movement rules to solve the RAM.

We derived an individual transition matrix (see table 5.1) for each bee based on the decision it made over 10 test bouts. Each cell expressed the percentage of transitions that occurred from one platform to another (e.g. from platform 1 to platform 2) and allowed us to determine the preferred movement rule of each bee. This approach allowed us to capture variation between individuals in movement rules as opposed to using one general transition matrix of all the tested individuals (Samuelson *et al*. 2016) and thus provided us with a more accurate modelling tool.

From\To	1	2	3	4	5	6	7	8
Tunnel	10%	0%	0%	0%	10%	20%	60%	0%
1	0%	12%	6%	0%	0%	6%	24%	53%
2	57%	0%	0%	0%	14%	21%	0%	7%
3	8%	69%	0%	8%	0%	15%	0%	0%
4	4%	13%	46%	4%	13%	8%	8%	4%
5	13%	8%	13%	54%	0	4%	4%	4%
6	10%	0%	0%	20%	65%	0%	5%	0%
7	5%	0%	0%	23%	23%	41%	9%	0%
8	0%	0%	0%	0%	11%	11%	78%	0%

0%	20%	40%	60%	80%	100%

Table 5.1: Example of individual transition matrix derived from the percentage of movements between platforms made by a focal bee over 10 foraging bouts in the RAM.

The statistical analysis for this validation was performed in R (version 3.5.1, R Core Team 2017) using the *glmer* function (Bates *et al*. 2014). We fitted a GLMM (Poisson distribution; with head width and age as fixed effects; colony and individual as random factors) to the observed data for each of three response variables of interest (i.e. total number of revisits, number of landings before the first revisit and number of revisits in the first 8 landings) and extracted the intercept estimate for the three resulting models. We then used the transition matrices to simulate a new data set that was structurally identical to the observed data. Every subject had their 10 foraging bouts replaced by the decisions of a virtual bee, which were determined by the transition matrix of its observed counterpart. We then fitted the same model we used for the observed data on the new simulated one and extracted the intercept estimate. This process was repeated 10,000 times, which gave me a set of 10,000 estimates extracted from the simulated datasets. Finally, we calculated p-values based on the percentile of the simulated distribution in which the intercept for the observed data fell.

We followed the same procedure to compare the observed data with 10,000 simulated data sets under the hypothesis that the subjects were choosing the sequence of platforms based on pure chance.

Statistical methods for assessing impact of insecticide

Statistical analysis was carried out in R 4.1.2 (R Core Team 2021). Data exploration showed that the total number of revisits was correlated with number of approaches ($r = 0.6$) and we therefore analysed these separately. We used the *glmer* function from the *glmm* package (Christina *et al*. 2022) to build the models presented below.

To analyse the effect of the pesticide treatment on the three performance measurements in the RAM (total number of revisits, number of landings before first revisit and number of approaches) we built three initial null models, one for each of the three performance measurements as the response variable. In each null model, age (days) and body size (mm) were included as predictors with bee ID and colony as random factors to account for non-independence.

We then performed AIC-based model selection to select the best null model. For total number of revisits as the response, the null model selected used a Poisson family with body size as predictor and bee ID and colony as random factors. The null model selected for number of landings before first revisit as response used a Poisson family, had only the intercept as predictor and bee ID and colony as random factors. The null model selected for number of approaches as the response contained body size as predictor with bee ID and colony as random factors. For each model, we then added treatment as a fixed factor, and assayed the effect using ΔAIC method with a cut-off >2 to determine which model was better for each pair.

Finally, a linear model was used to test the effect of treatment on body size. For the initial model we used body size as the response variable and age as predictors with colony as a random factor. After performing AIC-based model selection the null model contained body size as the response variable, only the intercept as the predictor and colony as a random factor. We used ΔAIC method with a cut-off >2 to determine whether adding treatment to the model significantly improved it.

Results

Validation experiment: do bumblebees use STM to solve a RAM?

Overall, we found that observed bees performed significantly better on the RAM than expected under random choice. We found a significant difference between the observed data and the random decision simulations across all three measures of performance in the maze (total number of revisits, $p < 0.001$; fig. 5.3.a ; number of landings before the first revisit, $p < 0.001$, fig. 5.4.a ; revisits in the first 8 landings, $p < 0.001$, fig. 5.5.a).

Regarding the null hypothesis that subjects were using individual stereotypical movement rules while foraging in the maze, we found a significant difference between the simulations and the observed data for total number of revisits ($p < 0.001$; fig. 5.3.b) and number of landings before the first revisit ($p = 0.0012$; fig. 5.4.b). The subjects tested in the RAM made fewer errors in total and visited more flowers before their first error than the simulated bees. However, the revisits in the first 8 landings did not differ significantly from the distribution of simulated estimates ($p = 0.43$; fig. 5.5.b), suggesting that the subjects might have been relying more prominently on movement rules during the first few decisions in each bout.

Additionally, we tested whether the three measurements of performance in the maze were correlated using Spearman's rank test. We found all three measurements were significantly correlated with each other (total number of revisits and number of landings before the first revisit, $p < 0.001$, $r = -0.51$; total number of revisits and number of revisits in the first 8 landings, $p < 0.001$, $r = 0.59$; number of landings before the first revisit and number of revisits in the first 8 landings, $p < 0.001$, $r = -0.61$).

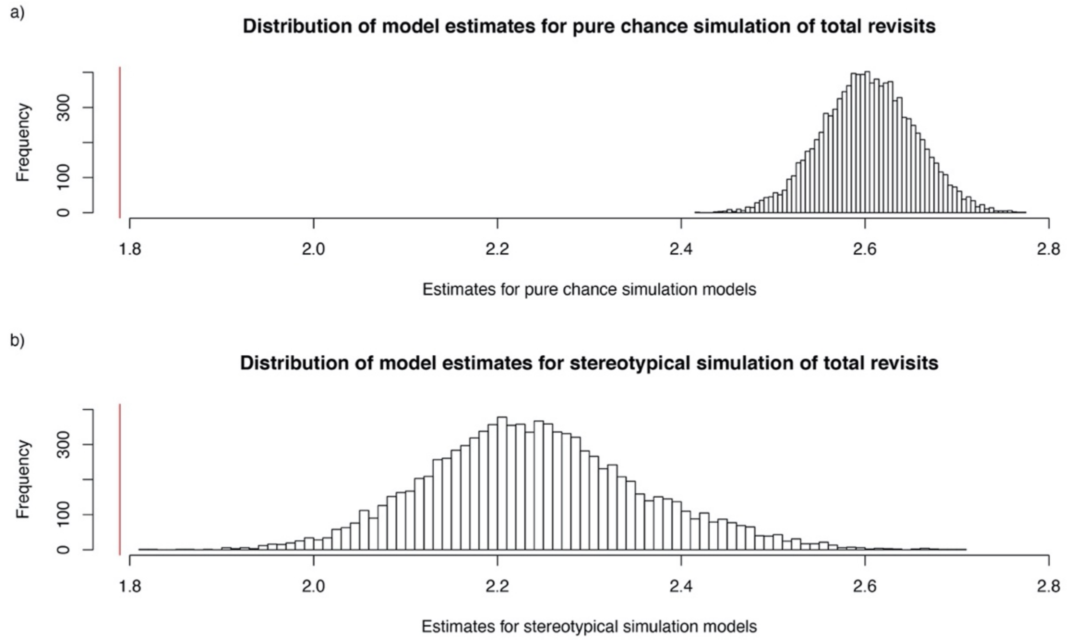

Figure 5.3: The distribution of intercept estimates for a) the pure chance simulations for total revisits and b) the stereotypical movement simulations for total revisits. The red line represents the intercept estimate of the model using the observed data.

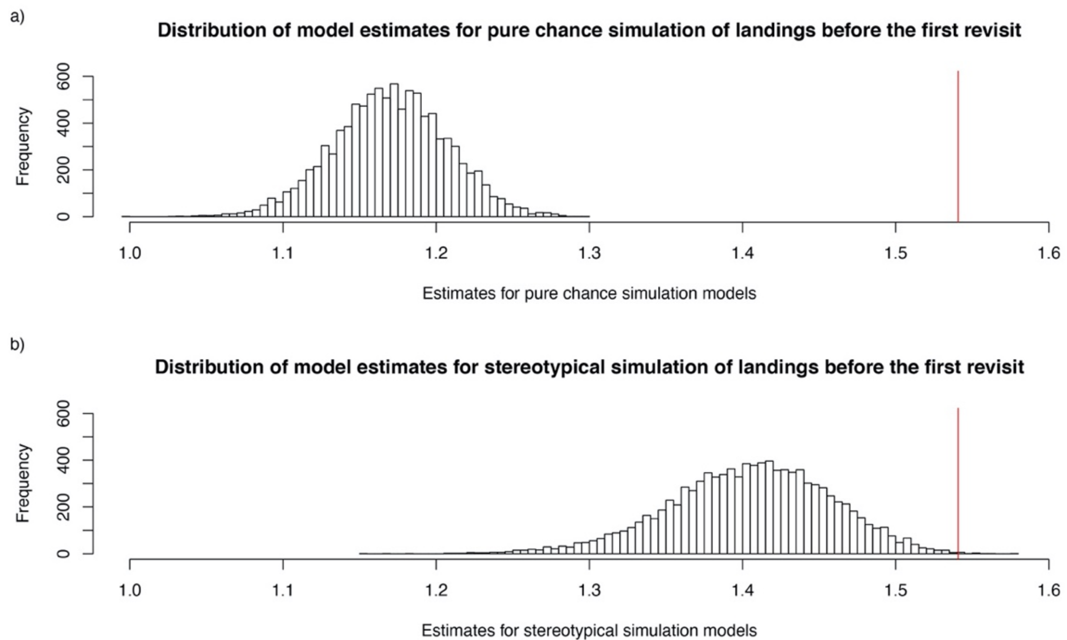

Figure 5.4: The distribution of intercept estimates for a) the pure chance simulations for number of landings before the first revisit and b) the stereotypical movement simulations for number of landings before the first revisit. The red line represents the intercept estimate of the model using the observed data.

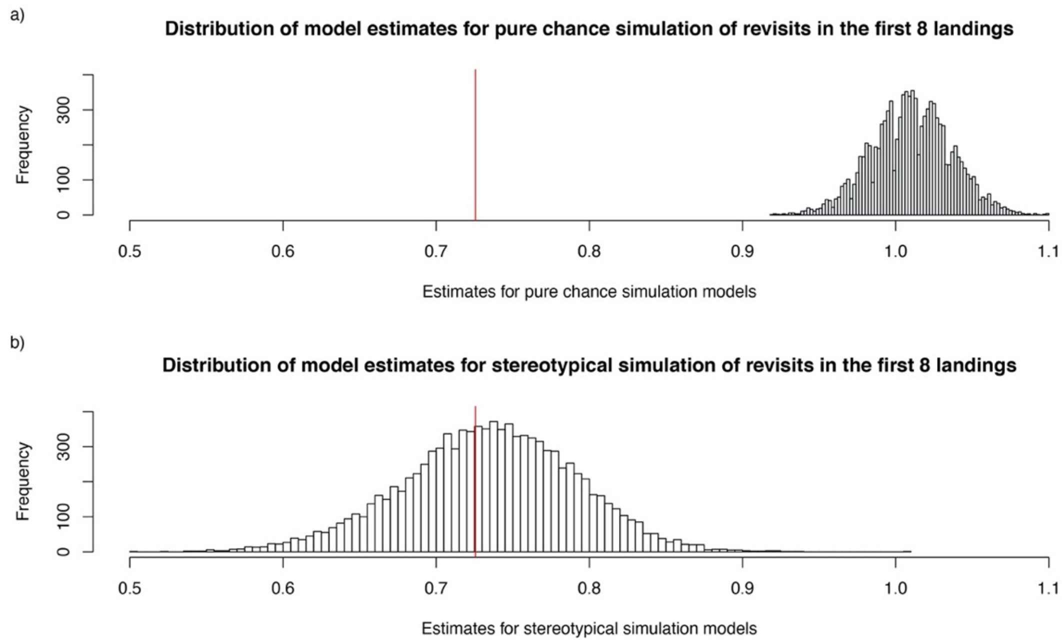

Figure 5.5: The distribution of intercept estimates for a) the pure chance simulations for number of revisits in the first 8 landings and b) the stereotypical movement simulations for number of revisits in the first 8 landings. The red line represents the intercept estimate of the model using the observed data.

Does chronic exposure to sulfoxaflor affect performance in the RAM?

When looking at the effect total number of revisits, we found no significant effect of insecticide treatment with the null model being the most parsimonious (*glmm*, ΔAIC with null model = -1.63; fig. 5.6.a). Nevertheless, selection of the null model showed a significant positive effect of body size on total number of revisits (estimate = 0.49, SE = 0.21; fig. 5.7). Age did not improve the model significantly and was removed during selection for the null model (ΔAIC with null model = -0.84).

For number of landings before first revisit, we found that treatment did not improve the model significantly (ΔAIC with null model = -1.83; fig. 5.6.b). Neither age (ΔAIC with null model = -1.97) nor body size (ΔAIC with null model = -0.63) had any effect and were discarded during the selection of the null model.

Finally, treatment had no significant effect on the number of approaches (ΔAIC with null model = -1.99; fig. 5.6.c). Yet, the selection of the null model revealed that body size had a significant positive effect on number of approaches (estimate = 0.75, SE = 0.37; fig. 5.8). Once again, the predictor age did not improve the model significantly and was removed during the null model selection step (ΔAIC with null model = -1.62).

As body size had a significant effect on both total revisits and approaches, we tested whether treatment predicted body size. We found no effect of treatment (*lme*, ΔAIC with null model = -2.87; fig. 5.9) or age (ΔAIC with null model = -9.82) on body size.

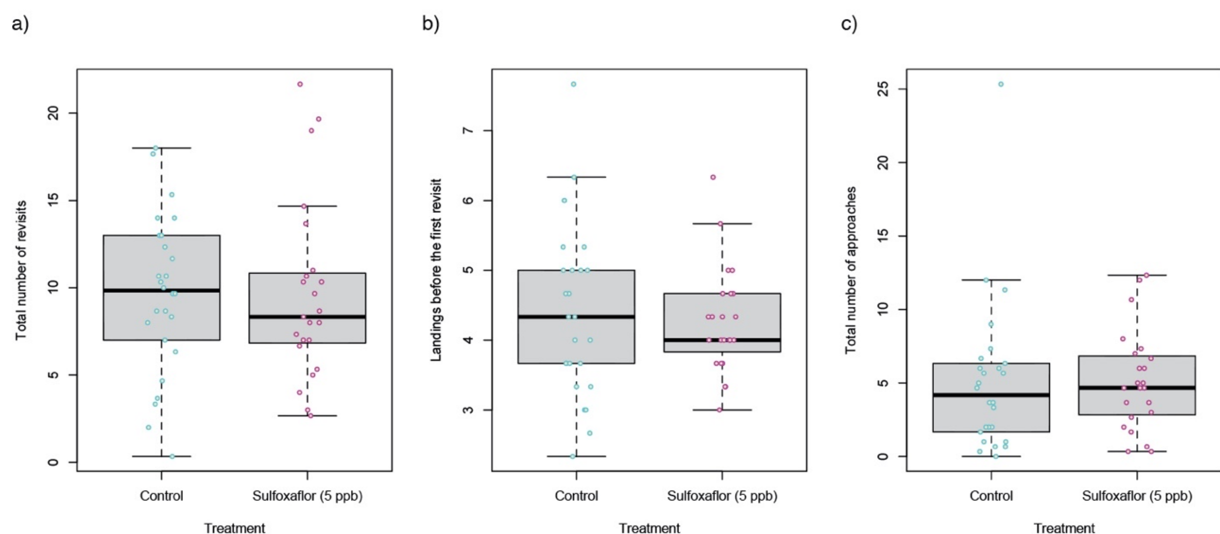

Figure 5.6: Boxplots showing no effect of chronic sulfoxaflor exposure (5 ppb) on performance in the RAM (n = 10 colonies, 49 individuals): a) total number of revisits (*glmm*, ΔAIC with null model = -1.63), b) landings before the first revisits (ΔAIC = -1.83) and c) Total number of approaches (ΔAIC = -1.99).

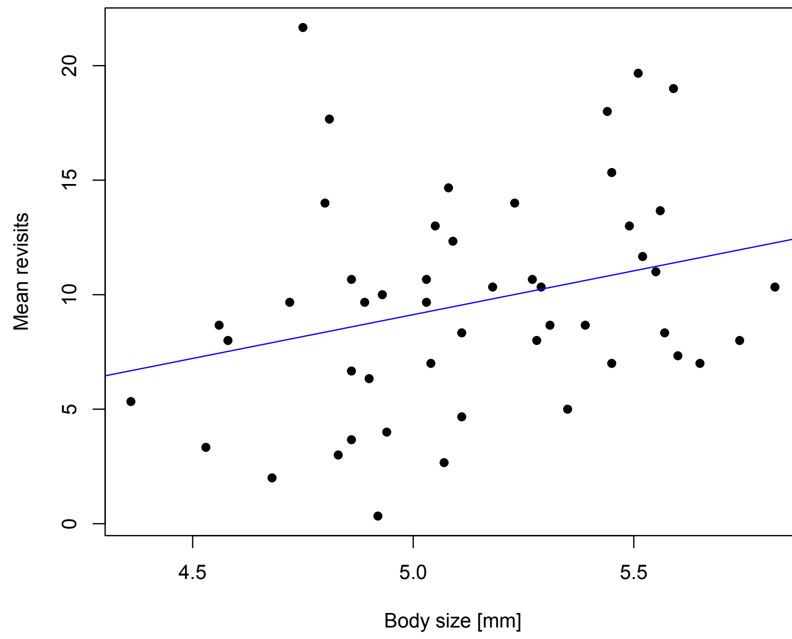

Figure 5.7: The relationship between total revisits and body size (*glmm*, parameter estimate = 0.49, SE = 0.21)

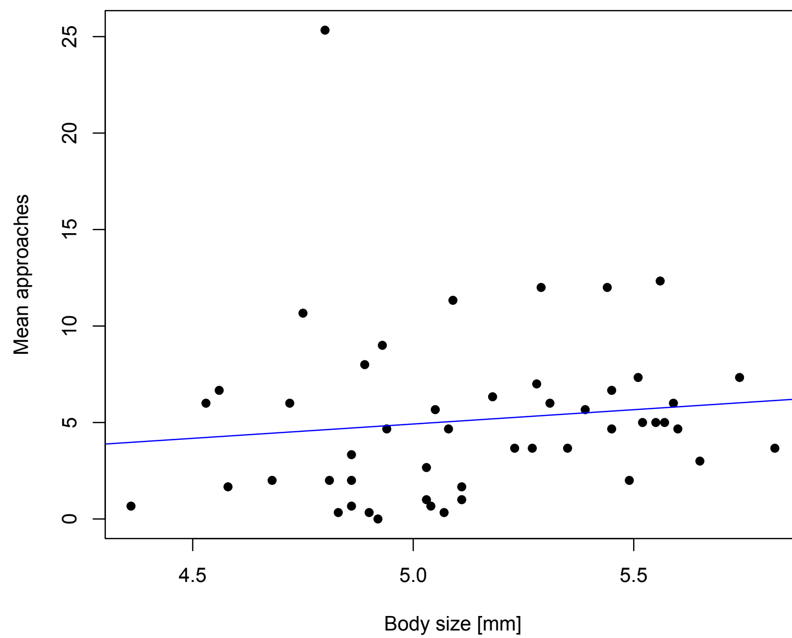

Figure 5.8: The relationship between total revisits and body size (*glmm*, parameter estimate = 0.75, SE = 0.37)

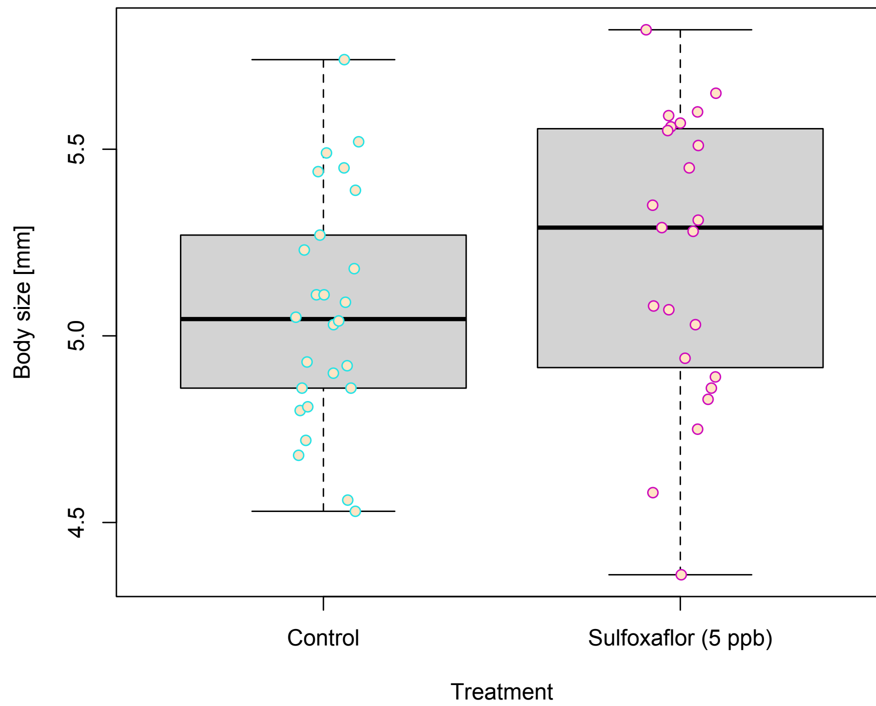

Figure 5.9: Boxplot showing no significant effect of sulfoxaflor on body size (*lme*, ΔAIC with null model = -2.87)

Discussion

We tested the effect of chronic exposure to sulfoxaflor on STM performance in a RAM by *B. terrestris* workers, and found no evidence that this insecticide significantly affected STM performance. This result is in line with a previous experiment performed under the same RAM paradigm using acute exposure to sulfoxaflor as the insecticide treatment (Siviter *et al*. 2019). The same publication also reported an absence of effect on olfactory conditioning in the proboscis extension reflex paradigm (Siviter *et al*. 2019) which, together with the results presented here, suggest that sulfoxaflor may not have sublethal negative effects on learning in *B. terrestris*.

Despite our result, sulfoxaflor does have documented negative sublethal effects detectable at the colony level in *B. terrestris* (Siviter *et al*. 2018a; Linguadoca *et al*. 2021). Our results suggest that these documented effects are unlikely to be mediated through a negative impact on learning abilities. Instead, the negative sublethal effects caused by sulfoxaflor in *B. terrestris* may be solely driven by impacts on reproductive output (Siviter *et al*. 2018a), egg-laying (Siviter *et al*. 2020b), larval development (Siviter *et al*. 2020a) and fertility (Linguadoca *et al*. 2021).

Interestingly, we found an effect of body size on RAM performance. Our data indicates that individuals with larger body size made more revisits to already empty platforms and made more approaches (i.e. they entered arms without landing on the platform) which suggests that these individuals performed worse in the RAM than their smaller sized counterparts. This is a surprising result as alloethism, whereby the task performed by workers is determined by their size, is associated with better adaptation to nectar foraging (Goulson *et al*. 2002). One key factor that has been proposed as an explanation for larger workers being better adapted to

foraging tasks is their larger compound eyes and ocelli (Kapustjanskij *et al*. 2007). This gives larger workers enhanced visual perception of light which allows them to fly under lower intensity light, i.e. at dawn and dusk, thus making it possible to forage for a longer time period every day (Kapustjanskij *et al*. 2007). 3D reconstruction and modelling have also shown that larger eyes also gives larger *B. terrestris* better image resolution and larger visual field, allowing them to perceive smaller objects better (Taylor *et al*. 2019). In their study on the effect of thiamethoxam on RAM performance, Samuelson *et al*. (2016) found a size-dependent effect of the pesticide where larger bees were more affected by the pesticide. Here we found no evidence that pesticide treatment was related to body size. If larger body sizes are better adapted to foraging tasks, a possible explanation for our result could be that avoiding revisiting flowers within a patch may not be important to foraging efficiency and, consequently, this trait was selected. Further research on within-patch strategies employed by *B. terrestris* is therefore necessary.

A caveat of our experiment is the dosage of pesticide we used. We chose a conservative level of concentration of sulfoxaflor corresponding to the low-end of observed concentration on a treated cotton crop (EPA 2019) and kept this concentration stable throughout 3 weeks of exposure. More recently, a study made use of new data on concentration of sulfoxaflor on multiple treated crops and its degradation rate over time (Linguadoca *et al*. 2021). This new method proposes starting concentrations much higher than those used here, followed by rapid diminishment ones over time. In comparison, our colonies might thus have been exposed to a concentration of sulfoxaflor that is lower than field-realistic levels (Linguadoca *et al*. 2021). Reproducing this method of chronic exposure informed by these newer data on exposure regimes, either using the RAM paradigm again or another learning paradigm used in *B.*

terrestris, might help answer the question of sulfoxaflor's effect on learning and memory in this species.

In the field, nectar is not the only vector of pesticide exposure for pollinators foraging on treated crops as most systemic insecticides, including sulfoxaflor, find their way into the pollen post-treatment (when exposed as a seed treatment) or during spray. Here we elected to only treat nectar during the chronic exposure period but in the field, a *B. terrestris* colony foraging on treated crop would also feed on contaminated pollen (Laurent and Rathahao 2003), potentially exposing them to much higher concentration than the nectar we used (between 50.12 and 510.95 ppb; EPA 2019), at least immediately post-spray. In this regard, we attempted to replicate the conditions in which prior research had found a significant effect of sulfoxaflor at the colony level and those experiments relied exclusively on nectar as their exposure vector (Siviter *et al*. 2018a, 2020b, a). However, pollen consumption is most important during larval development, it is therefore possible that, by not treating the pollen, we further underestimated the impact sulfoxaflor could have on wild *B. terrestris* colonies foraging on treated crop. As it is not a method commonly used in exposure protocols, further research would be needed to determine how exposure to pesticide via the pollen influences the development of *B. terrestris* larvae.

In summary, we found no evidence that sulfoxaflor has a negative effect on STM, which is in line with previous results and strongly suggest that the documented negative sublethal effects may not be achieved by impacting learning abilities, with the caveat that a low dosage may have influenced the results. We also found that larger sized workers performed more poorly in our STM assay which questions current knowledge on the adaptiveness of alloethism in *B. terrestris*.

Chapter 6: General discussion

The aim of this thesis was to expand knowledge on the relationships between learning, neuroanatomy and foraging in *Bombus terrestris* and present a case study on how a novel insecticide – sulfoxaflor – could influence them.

Key findings

MG density in the mushroom bodies does not predict real-world foraging efficiency

I found no evidence of a relationship between foraging efficiency and MG density in the MB of bumblebees. As efficient foraging is assumed to require learning (Raine and Chittka 2008; Pull *et al*. 2022), and learning and retention ability have been linked to MG density (Hourcade *et al*. 2010; Li *et al*. 2017), I expected that foraging efficiency would correlate with MG density. Instead, I did not find such a relationship to be true in my setup. A previous laboratory study looking at this relationship has reported that the experience of learning leads to changes in MG density using the PER paradigm (Hourcade *et al*. 2010). Li *et al*. (2017) also reported an effect of the experience of learning on MG density, and showed that overall, faster learners had higher MG density. The results obtained by Li *et al*. (2017) should be considered with caution though, because there was no significant difference between the group of bees that experienced learning and a group that experienced simply the same colours, and because the report contained inconsistencies. I was not able to replicate the staining methods they described in their supplementary materials section (Li *et al*. 2017).

Why did I find no relationship between MG density and foraging efficiency? Firstly, although the results of Hourcade *et al*. (2010) suggest that the *experience* of learning may lead to an increase in MG density, this effect may be relatively small and only detectable in the highly

specific and controlled PER paradigm. These effects may be undetectable when bees forage freely in the wild. Accordingly, Van Nest *et al*. (2017) found no relationship between learning ability and MG density in honeybees trained on artificial flowers that had been foraging in natural environments, which meant they were exposed to a wide range of visual and olfactory stimuli. These could have added noise to the data and prevented the analysis from detecting any relationship. In honeybees, exposure to a light source has been demonstrated to cause a decrease in MG density in the collar region (Scholl *et al*. 2014) which suggest that simply spending time in daylight would be enough to cause changes in neuroanatomy. Surprisingly, this relationship was not observed in *B. terrestris* where exposure to light was not found to cause any change in MG density (Kraft *et al*. 2019). It seems therefore unlikely that exposure to light was responsible for the variation or added noise to the data in the experiment presented in chapter 3. Age, on the other hand, has documented effects on MG density in both *B. terrestris* and *A. mellifera* and could have been a source of variation. According to a study by Kraft *et al*. (2019), the effect of age alone on *B. terrestris* MG density is mainly observed between 1 and 7 days of age while it appears to stabilise after 7 days, remaining so until the limit of the age tested of 28 days. The youngest individuals in the data presented in chapter 3 were 7 days old when sampled so, once again, it is difficult to argue for this variable adding noise to the data. The interesting non-linear effect I found between age and MG density in the lip region, taken together with the results obtained by Kraft *et al*. (2019), suggest that engaging in foraging itself could produce changes in MB. It is not clear, though, whether exposure to olfactory stimuli alone could have been the driver of the changes in MG density as the relationship has not yet been investigated *B. terrestris*.

Another explanation for these negative results could be linked to how ecological conditions shape the need for learning abilities. As demonstrated by Pull *et al*. (2022), the importance of

learning abilities depends on resource availability throughout the foraging season. When comparing their results with the time period during in which I ran my experiment, it appears that I collected data during a time when STM (short-term memory) does not play a role in foraging efficiency, which could potentially explain my results. Nonetheless, it is important to point out that, as presented in my introduction chapter, MG density is linked to LTM consolidation which relies on protein expression (Hourcade *et al*. 2010) whereas Pull *et al*. (2022) tested STM and so their results may not extrapolate to my findings. The importance of LTM across different ecological contexts, at different times of the year, remains to be explored but according to Menzel (1999, 2012) STM and LTM play different roles in foraging, which means that they might not follow the same patterns.

Chronic exposure to sulfoxaflor has no detectable effect on MG density

I also tested whether sulfoxaflor had an effect on neuroanatomy. I found no evidence of an effect of the insecticide on either MB volume or MG density. I expected to find an effect of sulfoxaflor on both variables based on results of previous studies obtained using neonicotinoids (Peng and Yang 2016; Smith *et al*. 2020) and given that sulfoxaflor has a similar mode of action to that of neonicotinoids (Sparks *et al*. 2013). However, a similar mode of action does not mean an exact reproduction of this mode of action. Differences in 3D structure exist between sulfoxaflor and neonicotinoids (Sparks *et al*. 2013), which potentially make for differences in the active binding site on nACh receptors, and/or affinity to them. In support of this conjecture, the only documented case of cross-resistance to both neonicotinoids and sulfoxaflor so far is the FRC-R strain of green peach aphid (*Myzus persicae*) which possesses a mutation of the structure of its nACh receptors (Bass *et al*. 2011; Cutler *et al*. 2013) and could suggest that a different interaction between the 3D structures of sulfoxaflor and the nACh receptors of *B. terrestris* is unlikely to be responsible for the non-significant effect we

observed. However, more research would be needed to establish whether this is the case, and in most species documented to have evolved resistances to neonicotinoids, oxidative metabolism underlies the resistance (Karunker *et al*. 2008; Wen *et al*. 2009; Roditakis *et al*. 2009; Gilbert and Gill 2010; Markussen and Kristensen 2010; Philippou *et al*. 2010; Puinean *et al*. 2010). In these species, sulfoxaflor evades the resistance thanks to its greater stability in oxidative condition (Sparks *et al*. 2012), not because binding sites are different.

It is possible that, while sulfoxaflor had no effect at the dosage I chose to expose the colonies, it could have effects on neural development at a different concentration. The dosage used in this thesis was chosen to replicate previous experiments on *B. terrestris* in which sulfoxaflor was found to have an effect on colonies (Siviter *et al*. 2018a) and was conservatively based on the low-end of observed concentrations in the nectar of a treated cotton crop (EPA 2019). The exposure regime that I used corresponded to previous research in which a neonicotinoid insecticide was found to have an effect on neural development (Smith *et al*. 2020). Since then, a recent paper (Linguadoca *et al*. 2021) modelled their sulfoxaflor exposure regime based on degradation data and attempted to replicate the dosage gradient over time observed on crops. While the dosage starts very high it degrades rapidly, in contrast with the exposure regime that I applied, which was conservatively low throughout the exposure period. Had I used a similar exposure gradient, the larvae would have been exposed to greater concentration in their early stages of development which may have had a different effect to that which I observed in chapter 4.

Additionally, bees were only exposed to sulfoxaflor through nectar consumption, which means that larval exposure may have been low compared to what could have been experienced in the field. Indeed, treated crops also contain pesticide residues in their pollen (Laurent and Rathahao

2003) often at concentrations much higher than nectar (between 50.12 and 510.95 ppb; EPA 2019). Pollen consumption is also particularly important for larval development (Goulson 2010) which might suggest that by concentrating on exposure via nectar consumption, I may have missed a key aspect of pesticide exposure in the wild. Nevertheless, previous studies have detected effects of sulfoxaflor and other pesticides with only nectar as the source of pesticide exposure while pollen remained untreated (Siviter *et al*. 2018a, 2020b, a). It is therefore unclear whether the setup I used failed to capture the effect or that sulfoxaflor truly has no effect on the neuroanatomy of *B. terrestris* at field realistic concentrations.

Chronic exposure to sulfoxaflor did not affect RAM performance

The absence of evidence that sulfoxaflor affected neuroanatomy documented in Chapter 4 did not exclude the possibility that the insecticide affected learning abilities. Short-term memory – defined by Menzel (1999, 2012) as the phase of memory lasting in the range of seconds to minutes and relying on sustained neuronal activity – does not rely on transcription or translation to function and therefore does not have obvious or established neuroanatomical correlates compared to long-term memory (LTM).

A previous study, in which bees were acutely exposed to sulfoxaflor, reported no effect of the pesticide on STM as assayed through RAM (Radial Arm Maze) performance (Siviter *et al*. 2019), but employed an acute exposure regime which cannot capture effects on developing larvae. In the experiment presented in chapter 5, which employed a chronic exposure regime that was a closer mimic of that which had previously been shown to impact colony-level productivity (Siviter *et al*. 2018a), I found no effect of chronic exposure to sulfoxaflor on performance in a RAM assay. There is therefore little evidence that sulfoxaflor affects STM. To my knowledge, the only documented effect of a pesticide on STM in bees was through

acute exposure to thiamethoxam in *B. terrestris* (Samuelson *et al*. 2016). Intuitively, it would make more sense that acute exposure might have greater impacts on STM than chronic exposure, as this phase of memory involves sustained neuronal activity in the MB mediated by nACh receptors (Menzel 2012) and agonist pesticides would lower the efficiency of this system. Alternatively, chronic exposure may somehow affect development of the receptors but there is no documented evidence of this yet. However, the results obtained by Siviter *et al*. (2019) demonstrate at the very least that this relationship is not straightforward and the impacts of on STM differ from those of thiamethoxam.

Finally, it is important to discuss what the RAM paradigm actually tests. In this thesis, I made the choice to refer to Menzel's model of the phases of memory in honeybees to characterise the cognitive trait I was testing in the RAM as STM. This decision was motivated mainly by the description of STM as lasting within the range of seconds to minutes. In order to efficiently solve repeated bouts of foraging in the RAM, a bee needs to remember which platform has already been depleted within a bout (capped at 10 minutes in my experiment) but this information also needs to be overwritten by the time the bee starts a subsequent bout (here I enforced a 10 minutes gap minimum between bouts) in order to reset representation of the current status (full or depleted) of any given platform. In chapter 5, I described a validation experiment that I ran prior to using the RAM paradigm to test the effect of sulfoxaflor on STM. This experiment demonstrated that *B. terrestris* achieved significantly better performance levels in the RAM than would have been expected if they had been making random decisions between platforms, or had used individual movement rules (e.g. always shift clockwise) to collect the rewards in the RAM, suggesting that they used memory. This provides evidence that RAM performance draws upon shorter-term memory, but there are also some likely aspects

of longer-term memory at play as the bees needed to remember the nature of the task for 6 to 9 hours during testing.

Broader implications

Does learning ability always translate into more efficient foraging?

One of the key findings of this thesis was that, even though I found individual variation in the MG density and MB volume data, I did not detect any relationship with foraging efficiency. As discussed above, this might be because MG density is neither a product nor a driver of learning ability, or that variation in other drivers of MG density hides such effects. However, I could alternatively infer that learning and LTM were not important for foraging efficiency at the time of year I ran my experiment. This result, together with Pull *et al*. (2022), Evans *et al*. (2017) in the same species, and consistent with the laboratory experiment Pasquier and Grüter (2016) performed in ants, suggests investment in learning may not always be the more adaptive strategy for an animal. In situations where food is scarce, such as the middle of summer in the south of the United Kingdom, foragers might be better off relying essentially on their innate preferences to find flower patches. This is because Menzel's model suggests that LTM is most important in the decision as to whether to accept or reject a new flower patch (Menzel 2012). In a situation of low food availability, such patches might be few, and be the source of heavy competition between different pollinator species (Balfour *et al*. 2015; Wignall *et al*. 2020) so foragers may not have the "luxury" of rejecting new patches. A counter-argument is that the area where my experiment took place (Royal Holloway campus and surrounding area) is full of suburban private gardens that display flowering plants all summer long and could have provided a rich buffet to my bees. However, Pull *et al*. (2022) showed that the diversity of available forage decreased during summer which confirms that food was harder to find at that time of year.

Are the effects of pesticides on colony reproductive success driven by effects on learning?

Given the results found in the literature, it appears that sulfoxaflor does have a negative effect on *B. terrestris* colonies (Siviter *et al*. 2020b, a; Linguadoca *et al*. 2021). Yet, the results of this thesis, in combination with those of others, provide no evidence that this effect is driven by individual learning ability (Siviter *et al*. 2019). Siviter *et al*. (2018) also measured foraging performance and did not find differences between the control group and the treatments which could suggest that, unlike with neonicotinoids (Gill and Raine 2014), another mechanism is at play here, as the effect of sulfoxaflor only became apparent when the cohort exposed during their larval stage eclosed. It is therefore clear that the individuals chronically exposed during early development were driving the effects documented (Siviter *et al*. 2018a). Processes that are reportedly affected by sulfoxaflor include egg-laying (Siviter *et al*. 2020b), larval development (Siviter *et al*. 2020a) and fecundity (Siviter *et al*. 2018a; Linguadoca *et al*. 2021).

The link between a neurotoxic and reproductive mechanism does not appear straightforward at first glance until one considers the bigger picture of cholinergic systems. As stated several times before in this thesis, sulfoxaflor is an agonist of nicotinic acetylcholine receptors. While the non-neuronal functions of acetylcholine are not fully understood (Grünewald and Siefert 2019), some studies have linked it with functions linked to reproduction in both vertebrates and honeybees (Wessler and Kirkpatrick 2017). In honeybees, the food produced in the hypopharyngeal glands of nurses contains acetylcholine and artificially lowering its concentration increases larval mortality (Wessler *et al*. 2016). As non-neuronal production of acetylcholine is observed, it is not unreasonable to expect that non-neuronal nACh receptors are present in the honeybee and that they play a role in developmental pathways but this has yet to be investigated as the current knowledge on this topic is limited to only two non-neuronal receptors for nACh identified in *D. melanogaster* and *T. castaneum* (Collin *et al*. 2013). Given

the apparent absence of effect of sulfoxaflor on bee cognition and neural development compared to neonicotinoids, sulfoxaflor could be a good candidate to study more specifically the non-neuronal aspect of the impacts of pesticide on bees and ultimately lead to a better understanding of non-neuronal cholinergic systems in insects.

Future research directions

What are the knowledge gaps that remain regarding the relationship between learning, MG density and sulfoxaflor in *Bombus terrestris*? First of all, while my experiments investigated the relationship between foraging and neuroanatomy (chapter 3) and touched on the link between foraging efficiency and learning (chapter 5), I was not able to test how learning and neuroanatomy interact in my setup. I originally planned an experiment aimed at doing just this by exploring how MG density responds to constant demands on STM. This experiment aimed to compare MG density in three groups: (1) bees that constantly foraged in the RAM over an extended period of several days ("STM" group, following a similar testing protocol as presented in chapter 5), (2) bees from the same colonies tested at the same time on a randomly baited version of the RAM where they could not use STM to predict whether an arm was rewarding ("No STM" group) and (3) bees from the same colonies that never left their colony and therefore had no foraging experience ("No Foraging" group). While this experiment had to be curtailed due to lockdown restrictions during the COVID-19 pandemic (after extensive investment in development of the methods and training of the bees; see COVID statement), the design could have allowed the identification of any impacts of STM on MG density, discriminating between the changes in MG density occurring through simply interacting with the maze and those caused by the use of memory. This experiment would be a key next step in the continuation of the work I presented here.

Another important step would be to replicate my experiment on foraging efficiency and extend it to the whole foraging season, from early spring to autumn. This would allow further investigation of the variation in importance of learning and memory in relation to seasonality in pollinators. If the importance of memory consolidation was dictated by the environment, I would expect to find differences in MG density across the foraging season. Tracking behavioural pattern changes in how bees interact with flower patches would also be important, because it could allow testing of the hypothesis that LTM is important when between-patch movements are rare, but could present some technical limitations. Traditionally, harmonic radar tracking techniques (Lövei *et al*. 1997; Capaldi *et al*. 2000; Woodgate *et al*. 2016) can only track bees over a small range on a flat landscape but recent developments of this method indicate that new technology may soon be able to solve these issues (Maggiora *et al*. 2019; Lioy *et al*. 2021). Additionally, it would be helpful to evaluate the nectar production and ease of handling of the flowers available based on their species or subspecies. For example, pollinators are not attracted to most ornamental flowers widely available in garden centres (Garbuzov *et al*. 2017). Overall, it remains unclear which periods of the year place the highest demands on memory.

Relatedly, these demands may vary between different aspects of memory, which may play different ecological roles which change in importance in response to environmental factors. Pull *et al*. (2022) have set this in motion by demonstrating that the link between STM and foraging efficiency is dependent on ecology, particularly food abundance, so it would be particularly interesting to compare MTM, LTM and STM performances in their relationship with foraging efficiency. MTM is expected to be used to remember which patches have already been visited in a foraging bout (between-patch decisions), LTM is more closely associated with revisits or avoidance of patches in the time range of days, whereas STM is expected to reflect

within-patch decisions (Menzel 2012). As MG density is associated with LTM consolidation (Hourcade *et al*. 2010), the results presented in chapter 3 seem to suggest that LTM is unlikely to correlate with foraging efficiency in the summer. However, the results of chapter 3 do not constitute direct evidence for a lack of importance of LTM in foraging efficiency in the summer and neither does it inform on variations throughout the foraging season. A more definitive answer to these questions through a replication of the setup used by Pull *et al*. (2022), this time replacing the STM assay with a test of LTM, would therefore be most useful to the understanding of the relationship between memory, foraging and how it relates to ecology.

A step further in this direction – although potentially further in the future – would be to selectively prevent the use of a certain phase of memory and analyse the effect of the treatment on foraging efficiency. A local inhibition of protein kinase M (PKM) would knock out MTM (Grünbaum and Müller 1998) while doing the same to protein kinase A (PKA) would prevent consolidation of LTM (Friedrich *et al*. 2004). The combined use of the harmonic radar technique for tracking the movements of a bee on a foraging bout (Capaldi *et al*. 2000; Woodgate *et al*. 2016) with memory phase inactivation could allow experimenters to identify the patches visited by each individual bee as well as detect patterns such as the likelihood to revisit a patch in the case of MTM inhibition, for example. This kind of setup could also be used to look at the role the MB play in foraging by anesthetising them with procaine injections (Devaud *et al*. 2015; Buehlmann *et al*. 2020). The downside of these methods is that they are highly invasive and it would currently be impractical to perform micro-injections in the mushroom bodies of bumblebees because of the layout of their air sacs which encase their brain (see chapter 2) and would likely be pierced by the injector. To the best of my knowledge, no experiment has reported a successful micro-injection in the MB of bumblebees although it is possible that this technical challenge could be overcome in the future. Nevertheless, such an

injection can be performed in honeybees but it is difficult to evaluate what is the typical life expectancy of a bee after the injection as most study report the sacrifice of the treated individual minutes after the intervention (Plath *et al*. 2017; Kamhi *et al*. 2020; Buehlmann *et al*. 2020). These experiments may require additional research and development in technology or methodology in order to happen but they would represent an important contribution to better understanding the role of memory in foraging.

Of course, the relationship between memory and foraging is unlikely to be straightforward and testing it precisely is difficult, but using *B. terrestris* as model species can reduce the limitations of models such as *A. mellifera*. Colony maintenance in *B. terrestris* is, contrary to *A. mellifera*, minimal, does not require the use of specialised tools, and the nest itself can be housed in a container smaller than a shoebox which makes this species easy to maintain all year round in a laboratory setting. As for their foraging period, *B. terrestris* can forage in temperate regions from early spring to the beginning of autumn, a time period that is longer than for *A. mellifera* in the same climate. Additionally, it is possible to run the entirety of a laboratory-based experiment with *B. terrestris* using only an indoor flight arena. These reasons make *B. terrestris* the ideal model for testing the relationship between foraging and learning.

Synthesis

The aims of this thesis were to better understand the importance of learning in foraging efficiency, how it relates to neuroanatomy, and how the novel insecticide sulfoxaflor could impact these relationships. The results show that the relationship between MG density and foraging efficiency is not straightforward, and I suggest that this may reflect changes in the value of learning and memory with additional ecological factors such as food availability. My case study did not reveal any effect of sulfoxaflor on STM or on neuroanatomy, suggesting

that the results of previous studies showing detrimental effects at the colony level are unlikely to be explained by effects on individual learning abilities.

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