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Causes and consequences of individual forager variability in social bees

Simon Klein

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THÈSE

En vue de l'obtention du

DOCTORAT DE L'UNIVERSITÉ DE TOULOUSE

Délivré par :

Université Toulouse 3 Paul Sabatier (UT3 Paul Sabatier)

Cotutelle internationale avec l'Université de Macquarie à Sydney (Australie)

Présentée et soutenue par :
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“’L'esprit de la ruche’, où est-il, en qui s'incarne-t-il? [...] Il dispose impitoyablement, mais avec discrétion, et comme soumis à quelque grand devoir, des richesses, du bonheur, de la liberté, de la vie de tout un peuple ailé.”

- Maurice Maeterlinck (La Vie des Abeilles, Livre II l'Essaim, Chap. II; 1901)

The spirit of the hive, where is it, in who is it embodied?
It pitilessly manage the wealth, the happiness, the freedom and the life of a whole winged people; but discreetly, as dictated by a great duty.

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Jean, Emeline, Arthur, Lilou, Aglaë: oui
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Oncles et tantes, vous, cousines et cousins,
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A regarder la nature, la chérir, l'aimer.*



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Summary:

In social insects, such as bees, different individuals specialise in the collection of different resources, and it is assumed that natural behavioural variability among foragers contributes to a self-organised optimisation of colony performance. The causes and consequence of the inter-individual variability for colonies of social bees are not yet well understood.

In this thesis in cognitive ecology, I examined the different foraging strategies for the different macronutrient sources, pollen and nectar, and the inter-individual variation in bee foraging performance. I also looked at how stressors, such as pesticides, can impact on bee foraging efficiency. I compared two social Hymenoptera that vary in their level of social complexity: the European honey bee (*Apis mellifera* L.) and the buff-tailed bumblebee (*Bombus terrestris* L.).

I used Radio Frequency Identification (RFID) to automatically track the foraging behaviour of bees throughout their life. I found that honey bee and bumblebee colonies rely on a subset of very active bees to supply the whole colony needs. In honey bees, these foragers are more efficient and collect more pollen. I also identified different strategies for pollen or nectar collection in both species.

Using manipulative experiments, I then showed that bees exhibit consistent inter-individual different behaviours in a spatial learning task and that pesticides impair visual learning.

My thesis aims at better explaining the causes of inter-individual variability of foraging behaviour and its implication for the colonies. The results highlight the need for considering behavioural diversity as an adaptation for social insects, as well as a potential dimension of colony-level vulnerability to environmental stressors that can affect the nutritional balance of whole colony.

Résumé :

Chez les pollinisateurs sociaux, comme l'abeille domestique (*Apis mellifera* L.) et le bourdon terrestre (*Bombus terrestris* L.), mes deux modèles d'étude, différents individus sont spécialisés dans différentes tâches. Il est admis que différents types de comportement de butinage contribuent à une optimisation des performances de la colonie. Actuellement, les populations de pollinisateurs sont exposées à des stress environnementaux, qui sont connus pour perturber le comportement des individus en visant directement leur cognition. Il est ainsi crucial de mieux comprendre comment les colonies d'abeilles et de bourdons maintiennent une activité de butinage efficace, et quels sont les effets de stress environnementaux sur les butineuses.

Dans cette thèse, j'ai donc examiné les différentes stratégies de butinage pour différentes sources de nourriture, pollen et nectar, et les variabilités interindividuelles dans le comportement de butinage. Je me suis aussi intéressé à l'impact de stress tels que les pesticides sur l'efficacité de butinage.

J'ai utilisé la technologie RFID pour suivre le comportement des abeilles tout au long de leur vie. J'ai trouvé que les colonies d'abeilles et de bourdons reposent sur un petit groupe d'individus très actifs qui fournissent la majorité de la nourriture pour la colonie. Chez les abeilles, ces individus très actifs sont aussi plus efficaces pour collecter nectar et pollen. J'ai aussi identifié l'existence de différentes stratégies pour la collecte de pollen ou de nectar.

Ensuite, j'ai pu montrer que les bourdons ont des différences interindividuelles très marquées dans un test de navigation, une tâche cruciale dans le comportement de butinage. Finalement, j'ai testé l'effet néfaste de pesticides sur l'apprentissage visuel chez l'abeille.

Cette thèse a pour but de mieux comprendre les causes de vulnérabilité des pollinisateurs aux stress environnementaux. Mes résultats soulignent le besoin de considérer la diversité comportementale comme une adaptation des espèces de pollinisateurs sociaux, mais aussi comme une potentielle cause de vulnérabilité de la colonie vis-à-vis des stress.

Preface:

The chapters of this thesis constitute an ensemble of papers ready for submission, submitted, accepted or published in peer-reviewed journals.

Publications included in this thesis:

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

- Included as Chapter 1.

AC, ABB and JMD reviewed the behavioural and brain literature – SK, ABB and ML reviewed the behavioural and ecological literature – SK and AC wrote the first version of the review, which was then corrected and improved by all authors.

Klein S., Pasquaretta C., Barron AB., Devaud JM. & Lihoreau M. (2017). **Inter-individual variability in the foraging behavior of traplining bumblebees.** *Scientific Reports* 7, 4561

- Included as Chapter 4.

SK and ML designed the experiment – SK conducted the experiment – SK and CP analyzed the data – SK, PC, ABB, JMD and ML wrote the paper.

Bordier C., Klein S., Le Conte Y., Alaux C., Barron AB. (submitted). **Stress decreases pollen forager performance in honeybees.** *Journal of Experimental Biology*

- Included as Appendix 1.

SK and CB designed the experiment – SK and CB conducted the experiment – CB, CA and ABB analyzed the data – CB, CA, YLC and ABB wrote the manuscript.

Conference presentations during the candidature:

Klein S., Devaud JM. & Lihoreau M. (2015) **Unrevealing the foraging strategies of honeybees along their life.** *Oral presentation, 28th Conference of the French section of the International Union for the Study of Social Insects (UISSI), Tours, France.*

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- Included as Appendix 2.

Klein S., Vine P., Bordier C., Devaud JM., Lihoreau M. & Barron AB. (2016). **Why social bees are so vulnerable to environmental stressors?** *Oral presentation, 6th European Conference of the UISSI, Helsinki, Finland.*

Klein S. & Barron AB. (2017). **Pollen foraging: specificity, plasticity and anticipation in honey bees.** *Oral presentation delivered at ASSAB'17: the 45th meeting of the Australasian society for the study of animal behaviour, Mooroolbark, VIC, Australia.*

Specific contribution for non-published chapters:

Chapter 2: **Individual and experience-dependent variation in foraging performance in honey bees** Klein S., Xu Jiang H, Perry C., Søvik E., Devaud JM., Lihoreau M. & Barron AB.

SK ES and CP designed the experiment – SK and XJH conducted the experiment – SK analyzed the data – SK, ABB, JMD and ML wrote the chapter

Chapter 3: **Inter-individual variability in the lifelong foraging activity of bumblebees** Klein S., Pasquareta C., Barron AB., Devaud JM. & Lihoreau M.

SK and ML designed the experiment – SK conducted the experiment – SK and CP analyzed the data – SK, PC, ABB, JMD and ML wrote the chapter

Chapter 5: **Sublethal effects of miticides on honey bee visual learning**

Klein S., Vine P., Devaud JM., Lihoreau M. & Barron AB.

SK a designed the experiment – SK and PV conducted the experiment – SK analyzed the data – SK, ML, ABB and JMD wrote the chapter

Credit for the drawings: S. Klein, based on personal pictures (except *B. terrestris* forager in chapter 4, picture kindly provided by T. Gomez)

General introduction:

The European honey bees (*Apis mellifera*) and buff-tailed bumblebees (*Bombus terrestris*) are ecologically (Ollerton & Waser 2006) and economically (Gallai *et al.* 2009) vital pollinators and are currently manifesting population declines due to a range of current environmental stressors (Potts *et al.* 2010). This thesis explores the causes of inter-individual variation in foraging behaviour in these two species and investigates how this new knowledge of foraging performance and behaviour can help us understand the responses of bees and their colonies to environmental stressors.

Comparing social foraging in bumblebees and honey bees

While many of the almost 20,000 bee species are solitary or display low levels of social organization (Michener 2000), the European honey bee (*Apis mellifera*) and the buff-tailed bumblebee (*Bombus terrestris*) are among those displaying the most complex form of animal social organization, termed eusociality (Wilson & Holldobler 2005). A eusocial society is defined as a society in which there is reproductive division of labour, distinct morphological castes aligned to the different reproductive roles, and an overlap of generations with adult offspring contributing to raising the next generation of adults (Wilson 2000; Wilson & Holldobler 2005). Eusociality is found in a few animal taxa, primarily in insects such as ants (Hölldobler & Wilson 2009), termites (Thorne 1997), bees and wasps (Hölldobler & Wilson 2009), some shrimp (Emmett 1996) and mammals (naked mole rats (Jarvis 1981)).

Honey bees are advanced eusocial insects. They live in colonies formed by one reproductive individual (the queen) and up to 80,000 usually non-reproductive workers who are made up of several generations of daughters of the queen, and there is a clear morphological difference between the large queen and the smaller female workers (Wilson 2000; Nowak, Tarnita & Wilson 2010). Bumblebees, such as *Bombus terrestris* are primitively eusocial bees: there is a reproductive division of labour, with one reproductive female (the queen) and up to 300 of her non-reproductive daughters (Goulson 2010). Bumblebee colonies have an annual cycle, with queens single-handedly founding nests in spring (Goulson 2010). There are no truly distinct female castes in bumblebees, rather the queen is usually the largest in a continuous variation of female size (Goulson 2010). Both honey bee and bumblebee species also produce short-lived males with the sole purpose of reproduction (Winston

1991; Goulson 2010). One of the most striking differences between honey bees and *B. terrestris* societies is their scale. A *B. terrestris* colony at its largest is a few hundred individuals whereas honey bee colonies of 80,000 individuals have been documented. As a consequence of this the collective functions of the colony can be supported by far more workers in honey bees than bumblebees (table 1).

Honey bee queens are polyandrous (Winston 1991). Consequently, the workers are genetically diverse due to their mixed paternity. Genetic variation within the bee colony is recognised as a cause of behavioural variation (Jones *et al.* 2004; Myerscough & Oldroyd 2004). This has been argued to support colony-level functions and homeostasis by increasing inter-individual variation between workers (Oldroyd & Fewell 2007). In *B. terrestris*, by contrast, each queen mates with a single male (Goulson 2010) and, thus, the degree of genetic diversity, and possibly inter-individual variation, is lower in bumblebees than it is in honey bee workers.

In both species, the non-reproductive workers are divided into behavioural castes, defined as groups of individuals performing a common task (Page & Erber 2002). The greatest division is between nurses or in-hive bees, which stay in the hive and take care of the brood and the nest, and foragers that travel outside to collect food on flowers such as nectar (carbohydrate source) and pollen (protein and lipids) for their nestmates (Winston 1991; Michener 2000; Goulson 2010). For honey bees, division of labour among workers is primarily age-based. Every worker starts its adult life as a nurse and then eventually starts to forage, performing different tasks successively across its life (Winston 1991). All honey bee workers are morphologically very similar (Winston 1991) whereas in *B. terrestris* there is marked variation in size between workers (Goulson 2010). In *B. terrestris* division of labour is mainly based on these morphological differences: small individuals will be nurses and may stay in the colony all their life, whereas larger individuals will be foragers, and may start foraging on the day of adult emergence (Goulson 2010). In bumblebees, only some individuals will ever forage whereas some others will never leave the nest. These three differences in social organisation between honey bees and bumblebees all suggest that very different levels of inter-individual variability might be present in the two societies, and such variation could have different consequences for the function of honey bee and bumblebee societies.

Honey bee and bumblebee workers are both central-place foragers, as they exploit the environment around their nest, but must always return to the nest. It has been proposed that the challenges imposed by this particular lifestyle has led them to evolve very strong cognitive capacities to learn and remember environmental features in order to navigate, locate rewarding flowers and find their way back to the colony (Giurfa 2015). The two species are also both floral generalists, deriving their nutrition from nectar and pollen (Michener 2000). Honey bee colonies are perennial (table 1) and survive winters by stocking nectar stored as honey (Winston 1991). Bumblebee colonies, on the other hand, last only for one year, from spring to autumn (Goulson 2010) and do not stock nectar for more than a few days in their cells (Goulson 2010). In both species, pollen is harvested during the flowering season and stocked for only a few days (Winston 1991; Goulson 2010). Maintaining colony nutritional balance requires a fine adjustment of pollen and nectar stocks according to changes in colony needs (Plowright & Silverman 2000; Pankiw 2007).

Studying these two species allows us to analyse the role of individual variability in two species with different social structures but sharing a similar ecological niche. The causes of division of labour are very different between the two species allowing us to contrast mechanisms of colony inter-individual variability.

Within caste inter-individual variability

There is now an abundant library of literature focusing on inter-individual variability across the animal kingdom (Nettle 2006; Montiglio *et al.* 2015) and also in social insects (Jeanson & Weidenmüller 2014; Jandt *et al.* 2014). This literature has established important concepts in behavioural ecology, such as behavioural syndromes (a suit of correlated behavioural tendencies in different contexts specific to an individual) (Sih, Bell & Johnson 2004), or keystone individuals (a very influential individual in a group) (Modlmeier *et al.* 2014). In this thesis, I use these concepts to consider the consequences of variability in foraging behaviour for colony dynamics and nutritional balance in both honey bees and *B. terrestris*.

Across the social insects, inter-individual variability has been noted in different aspects of foraging behaviours. First of all, not all individuals contribute in the same way to the colony. Foragers differ in their level of activity, and frequently, a large proportion of foragers are relatively inactive (e.g. in ants (Charbonneau, Hillis & Dornhaus 2014), or bees (Tenczar *et al.* 2014)). By contrast, some foragers are

extremely active, which are considered keystone, or elite, individuals for the colony as they contribute disproportionately to the foraging effort (Tenczar *et al.* 2014).

Bee foragers also individually differ in their foraging strategies: for instance, some bees have a greater propensity to collect nectar or pollen (Robinson & Page 1989; Hagbery & Nieh 2012). In honey bees, this has been partly explained by genetic differences (Robinson & Page 1989). Bumblebee foragers show differences in foraging performance that are related to differences in morphology (Spaethe & Weidenmüller 2002), adopting different strategies in navigating or choosing flowers depending on their size (Chittka *et al.* 2003). Evidence suggests that there are relatively clear individual differences within a caste of supposedly quite similar individuals sharing the same social role in the colony.

Bee foragers collect food from several types of flowers surrounding the colony for most of the flowering season (Michener 2000; Ollerton & Waser 2006) to provide the whole colony with pollen and nectar (Vaudo *et al.* 2015). Foraging behaviour is crucial for maintaining the nutritional balance of the colony (Vaudo *et al.* 2015). Carbohydrates derived from nectar are an essential source of energy for the workers while proteins and lipids derived from pollen are mainly consumed by larvae and are required for a normal development (Winston 1991; Goulson 2010). For instance, larvae that have been starved with pollen will be less efficient as foragers (Scofield & Mattila 2015). Thus, keeping a nutritional balance is very important for colony survival, and unbalanced nutrition is one of the factors involved in colony failure (Vaudo *et al.* 2015). Understanding the different strategies of social bee foragers when collecting pollen and nectar is then a key element of determining colony health.

Bee population declines

Bee pollinators are key species in most terrestrial ecosystems (Ollerton & Waser 2006). Animals, including bees, pollinate 78% (in temperate environment) to 94% (in tropical environment) of the flowering plants on the planet (Ollerton, Winfree & Tarrant 2011).

Human societies are heavily reliant on pollinators as more than half of crop plants (Klein *et al.* 2007), and even coffee (Roubik 2002), are, to some extent, pollinated by animals. The economic value of pollination, worldwide, has been evaluated as €153 billion annually, i.e. 9.5% of the value of world agricultural production (Gallai *et al.* 2009). Among pollinator species, the honey bee plays a

particularly important role as it is one of just a few domesticated pollinators (Bloch *et al.* 2010). They also provide honey and other bee products (such as wax, pollen, propolis, royal jelly) and are an important part of the rural economy (Crane 1999; Chauzat *et al.* 2013). Bumblebees (e.g. *Bombus terrestris* or *Bombus impatiens*) are also managed to provide pollination to green houses or orchards in some countries (Velthuis & van Doorn 2006), especially in Europe and North America.

Currently, an increase in environmental stressors, mainly driven by human activities, is negatively affecting bee populations (Potts *et al.* 2010). Many countries have been reporting high losses of honey bee colonies over recent years (Laurent *et al.* 2014; Steinhauer *et al.* 2014) and more bumblebee species have become listed as endangered (Williams & Osborne 2009; Cameron *et al.* 2011). This could have huge consequences on farming economies in the future (Steffan-Dewenter, Potts & Packer 2005). Diverse causes, such as parasites and viruses (Francis, Nielsen & Kryger 2013), pesticides (Rundlöf *et al.* 2015), pollutants (Hladun *et al.* 2012), nutrition (Vaudo *et al.* 2015), habitat fragmentation (Goulson, Lye & Darvill 2008), farming and beekeeping practices (Simone-finstrom *et al.* 2016), and even climate change (González-Varo *et al.* 2013) are implicated in bee population declines (Barron 2015). The myriad interacting causes of pollinator declines can justifiably be described as a wicked problem.

Given this imperative, it is crucial to better understand the mechanisms of bee declines. To do this we must first gain a better understanding of how social pollinators operate in their environment. Colony function is reliant on the collective contributions of individuals, and it is therefore necessary to have a better understanding of individual performance and inter-individual variability within colonies.

Thus, studying the nature and the potential causes and consequences of inter-individual variation in foraging behaviour in honey bees and bumblebees will help us understand the dynamics of colonies of two important social bee species.

Thesis prospectus:

Fundamentally, this thesis addresses a simple question: what is the nature of variation in foraging behaviour of social bees in their environment? To answer this question, I examined the behaviour of individual foragers in detail and how it is modulated by experience, using a combination of field observations and laboratory experiments.

In **chapter 1**, I argue, in a review paper published in *Trends in Ecology and Evolution*, that bees are very sensitive to environmental stressors because of their cognitive capacities. Such cognitive capacities that are essential for foragers navigating between flowers and back to the nest require an optimally functioning neuronal system in order to recognise and handle rewarding flowers. I discuss how different stressors can easily impair the neuronal basis of foragers' cognition. I also stress that foragers are key elements in the social structure of colonies, and that losses of foragers can have dramatic effects on colony survival.

In **chapters 2 and 3** I used a specially developed automated methodology to record forager activity and performance and examined inter-individual variability in honey bee and bumblebee colonies. Different aspects of foraging behaviour such as foraging activity and homing behaviour can be addressed by Radio Frequency Identification technology (RFID) (Gill, Ramos-Rodriguez & Raine 2012; Henry *et al.* 2012; Tenczar *et al.* 2014). RFID technology is based on the recognition by a sensor at the entrance of the colony, of unique individual tags that are glued on the back of the bees. Here I coupled RFID technology with camera sensors and a weighing device. In addition to monitoring foraging activity of a large number of individuals of the same colony, I was able to monitor their foraging efficiency, by looking at the amount and type of resources collected. This allowed automatic monitoring of foraging performance of a large number of different individuals from the same colony.

In **chapter 2**, I explored the differences in behaviour between different foragers of two honey bee colonies and found that a subset of very active honey bees (the elite foragers) did the majority of foraging and that these bees were also the most efficient at collecting both pollen and nectar. Variation in foraging efficiency in the colony was found to be related to individual experience. This finding presents a potential vulnerability of the entire colony as only a subset of bees collect the majority of the food supply.

In **chapter 3**, I investigate the question of elite foragers in bumblebee colonies. I also found that a subset of very active individuals performed the majority of the colony's foraging activity. Contrary to the honey bees, variation in bumblebees' pollen collection was not influenced by individual experience, but was mostly driven by external factors.

In **chapters 4 and 5**, I looked more closely at particular capacities involved in foraging tasks, such as spatial and visual learning. First, I tested if foragers of the same nest consistently performed similarly in a navigation task under semi-controlled foraging conditions (**chapter 4**). I found that foragers showed a continuum of constant foraging behaviours: the same colony contained a mix of efficient or less efficient foragers for this navigation task. This chapter has been published in *Scientific Reports*.

In **chapter 5**, I assessed the performance of foragers in a laboratory task involving associative learning of visual cues. In particular, I studied the possible impact on such abilities of chronic exposure to chemicals used for pest control within colonies.

In **Appendix 1** I present a paper by lead author Celia Bordier examining the impact of a controlled non-pathogenic stressor on forager behaviour and brain neurochemistry. I have chosen to present this as an appendix because for this work C. Bordier was both instigator and intellectual lead. My contribution was the RFID method used in this chapter, the methods for the analysis and the operation of the RFID experiment. For all other experimental chapters I was the intellectual leader and instigator of the projects.

By studying individual variation in foraging performance I was able to gain a better understanding of the collective foraging behaviour of two social bees. This work helps us to better understand the consequences of individuality for the social group and highlight important differences between two closely related species that vary in their social organization.

Table 1: Different ecological properties of honey bee (*Apis mellifera* L.) and bumblebee (*Bombus terrestris*) colonies.

	<i>Honey bee</i>	<i>Bumblebee</i>
Colony size	20,000 – 50,000 workers	300 – 400 workers
Queen / colony lifespan	5 – 6 years (perennial)	1 year (annual)
Mating system	polyandrous	monandrous
Morphological differences	Between queen and workers / No difference between workers	Between queen and workers / differences between workers
Polyethism type	Based on worker's age	Based on worker's size
Pheromone communication	yes	yes
Waggle dance	yes	no
Foraging type	generalist	generalist
Foraging home-range	10 km	3 km

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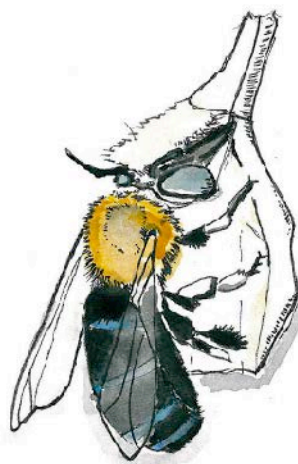
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Chapter 1: Why bees are so vulnerable to environmental stressors



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Chapter 1: Why bees are so vulnerable to environmental stressors

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Abstract:

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

Keywords: pollinators; central-place foraging; cognition; environmental stressors; sublethal effects; pesticides.

Bees are exposed to multiple environmental stressors

Bees are ecologically and economically vital pollinators for both wild and cultivated flowers. Presently many populations are in decline [1–4]. While demand for pollination dependent crops continues to rise, generating understandable alarm and debate about the possibility of an emerging ‘pollination crisis’ [5]. Many causal factors have been identified, including a range of pathogens and parasites [6,7], human-induced stressors such as pesticides [8–10] and forms of environmental degradation [11]. Very few of these stressors can be considered new, but many have increased in intensity over the last decade in much of the industrialised world. Our objective in this review is to consider why bees are particularly sensitive to these environmental stressors at doses that would be considered sublethal, and why their populations are now declining.

Bees, with exception of parasitic species, raise their brood in a single defensible nest [12]. We argue that in these insects, central-place foraging on ephemeral, dispersed and highly variable floral resources places particularly heavy demands on cognitive capacities. Bees must learn to forage at an energetic profit, locate high quality feeding sites, efficiently handle flowers and navigate back to the nest to provision nest mates with the right mix of nectar and pollen. The cognitive capacities underpinning these complex behaviours require optimal development and function of central brain structures, and precisely regulated plasticity of brain circuits necessary for learning, memory and navigation [13,14]. These brain systems are very easily disrupted, and it is especially problematic that many pesticides found in floral resources directly target key neural pathways [15,16]. Pathogens and nutritional deficits also compromise cognitive functions [17,18]. Even quite mild damage to the brain can significantly reduce foraging performance, thus rendering bees especially vulnerable to the sublethal effects of stressors. In social species, such as honey bees, bumblebees and stingless bees, efficient division of labour and coordination of tasks across nest mates, should provide buffering against environmental stressors, since individuals share a fortress-factory stocked with stored resources [19]. However, this buffering capacity has limits, which can be exhausted by chronic exposure to stressors. Once this occurs the result is a catastrophic colony decline [20–22].

Here we develop a neurobiological, ecological and evolutionary thesis to explain why central place foraging bees are particularly sensitive to environmental stressors. First we describe the complex cognitive challenges bees face when foraging and the neural substrates supporting these abilities. Next we review evidence that these essential cognitive abilities are impaired by

a range of stressors, ultimately threatening colony function and survival. Finally, we discuss how understanding the mechanisms of action of the different stressors and their consequence on individuals and colonies can help better manage and protect these vital pollinators.

Central-place foraging on flowers imposes high cognitive challenges

Bees must gather large volumes of highly dispersed pollen and nectar, and return with it to the nest to feed their brood [12]. Accordingly, these insects have evolved excellent memory and navigation skills enabling them to exploit complex and variable foraging environments, and more than a century of research has identified the underlying neural circuits [13,14]. Although most studies have focused on few economically important social species, such as honey bees and bumblebees, solitary bees show similar behaviours [12], cognitive capacities [23] and overall brain organisation [24]. In the bee brain (Figure 1), visual and olfactory stimuli are first processed by their respective sensory lobes (for detailed reviews see [25,26]), which then convey information to multisensory integration centres, such as the mushroom bodies (MBs) and the central complex (CX), that are specialised for learning and memory and spatial navigation tasks, as we describe below.

Learning the most rewarding flowers

Despite a large variety of available floral species, individual bees tend to forage on the same type of flower as long as it provides sufficient nectar or pollen [27]. This floral constancy demonstrates the abilities of foragers to learn the association between food rewards and particular floral cues (odour, colour, shape, temperature etc.) [28]. In many cases, bees learn more complex associations by generalising specific floral cues to learn conceptual features common to a range of flowers [13]. The amount of reward offered by flowers can change very rapidly, and bees can update their learned flower preferences accordingly [29,30]. Bees can also use combinations of cues (second-order cues), such as the presence of conspecifics or other bee species on flowers, to locate and learn rewarding flowers [31].

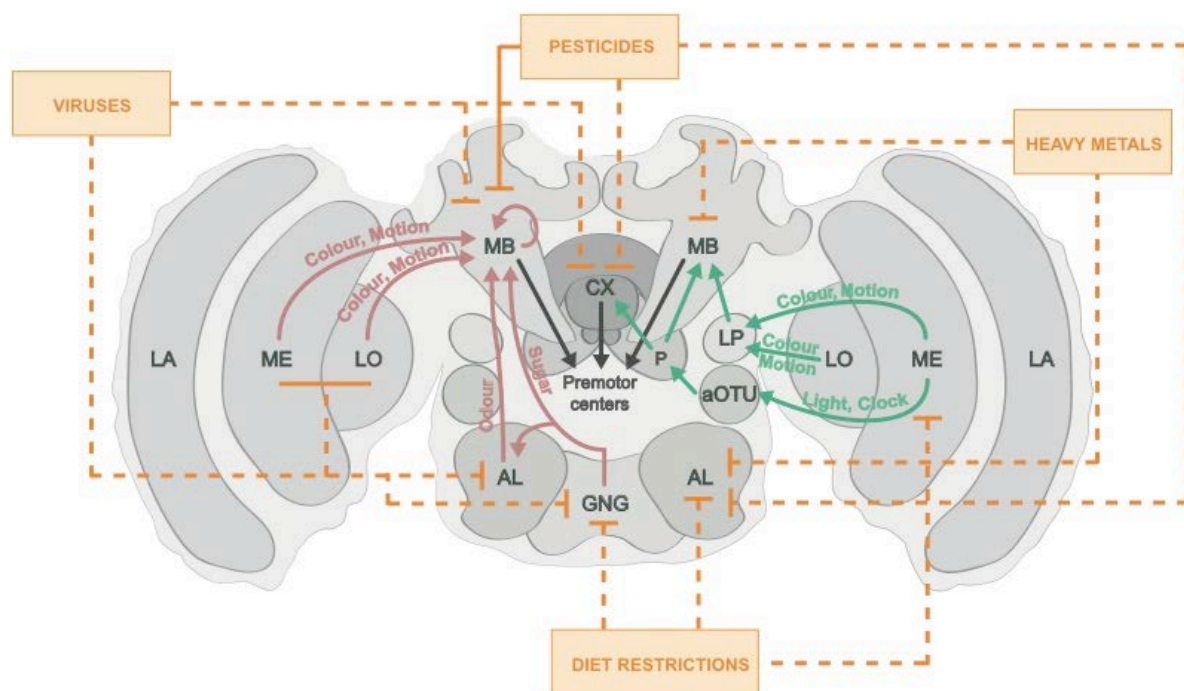


Figure 1. Brain structures supporting the cognitive capacities needed for foraging and how they are impacted by stressors. Schematic frontal view of a bee brain. Sensory information from the environment is first processed in specialised brain structures. The antennal lobes (AL) process olfactory information. The lamina (LA), medulla (ME) and lobula (LO), as part of the optic lobes, process visual information. The gnathal ganglion (GNG) receives gustatory information, and is sensitive to sugar. Sensory signals are then conveyed to higher-order centers (arrows). The mushroom bodies (MB) are involved in stimulus classification (odour, colour), complex associative learning and memory. They receive information directly from the sensory centers or indirectly through the lateral protocerebrum (LP) and the protocerebrum (P). The central complex (CX) receives processed visual input through the structures of the protocerebrum including the anterior optic tubercle (aOTU) and bulbs. The central complex locates the bee in space using celestial information and visual landmarks and is key for orientation and navigation. Environmental stressors (orange boxes) alter functions of various systems in the brain, and can alter the neural pathways supporting learning (purple arrows) and navigational capacities (green arrows). Dashed orange lines indicate impacts of stressors that have not been directly demonstrated for bees, but can be inferred by behavioural observations or has been observed in other insects.

These mechanisms of learning and memory have been examined in details using laboratory assays (Box 1). Acquisition of associative memories linking floral cues with food rewards relies on changes in neural activity induced by locally coincident activity in neural networks processing such cues and those signalling food detection [32]. Plastic changes in connectivity in either the antennal lobes (ALs) or the MBs (Figure 1) can support associative learning about odorants, and both structures modify their activity following learning [26]. In particular, the MBs are required for some complex forms of olfactory learning as well as for the formation of olfactory long-term memory [33,34]. Although less is known about visual learning, there is visual input from optic lobes (OLs) to the MBs (Figure 1), and it is increasingly likely that associative learning of visual features and colour also involves the MBs [35]. Memorising simple odour-food associations involves excitatory signalling through acetylcholine in the ALs and MBs (Figure 1) [13], a neurotransmitter system specifically targeted by major pesticides, as for instance neonicotinoids and organophosphate miticides [15].

Orienting, navigating and learning places

Bee foragers use multiple different sources of information to orient [35]. Path integration requires storing information on distances and directions travelled during the outward journey, in order to plot a direct return path to the nest [36]. Distance is estimated from optic flow [37], which is the movement of the image of the environment across the eye during flight. Direction is determined using the position of the bee relative to the sun [38] and/or the pattern of polarised light in blue sky [39]. Bees possess specialised mechanisms to compensate for the apparent movement of the sun (and the polarisation pattern it generates) across the sky during the day [40]. They are sensitive to other global sources of navigational information such as fine magnetic field variations, and can learn to relate them to local landmarks so that they can still navigate when celestial cues are blocked by cloud [41].

Bees can also learn locations by memorising the visual panorama. They use these stored ‘snapshots’ for navigation by positional image-matching [36], which compares their current view of the environment with a visual memory of the goal. The degree of matching provides a cue for guidance [42]. Bees form snapshot memories of the nest surroundings on their first foraging attempts outside the nest and also of the location of food sources [43]. For visual matching, individuals use salient objects (flower patches, trees, buildings), which can be either local cues or panoramic landmarks [36]. Honey bees can also perform ‘optic flow matching’, using the direction of optic flow caused by major landmarks as a navigational cue

[44]. Processing information on optic flow and landmarks while flying demands integrating visual and proprioceptive input with a temporal component. Responses to motion stimuli and colour are displayed by neurons connecting the OLs to central areas (lateral protocerebrum (LP) and MBs) [45] (Figure 1), and some of these neurons are involved in visual landmark detection [46].

The functions of the central complex (CX) (Figure 1) are presently poorly understood, but data from other insect species suggest that it is crucial for navigation [35]. Besides being a likely substrate for a sky compass [38], the CX could also support visual short-term (working) memory and spatial memory [47]. A study using a virtual reality assay (Box 1) in *Drosophila* showed that activity of the ellipsoid body neurons of the CX represented the orientation of the fly relative to visual landmarks [48]. Thus it is increasingly likely that activity in the CX contributes to internal representation of position for path integration [48].

Learning optimal routes

Bees can use their spatial memories dynamically to establish and optimise foraging routes. In nature, bees must sometimes visit hundreds of patchily distributed flowers to collect sufficient nectar and pollen in a foraging trip [27], and many species revisit familiar patches over consecutive hours or days in stable sequences called ‘traplines’ [49]. Recordings of bumblebee flight paths, using harmonic radar in the field (Box 1), shows that foragers attempt to minimise the overall travel distances between discovered flower patches, a complex optimisation task akin to the Travelling Salesman Problem [50]. On each new foraging trip, bees try different visitation sequences, ultimately approximating (or finding) the shortest possible path to visit all patches once, starting and ending at the nest [51]. Route optimisation is therefore an iterative improvement process based on learning and memory of flight vectors between feeding locations, supported by path integration and visual guidance [52]. This process allows for route flexibility and rapid adjustment of trapline geometry in response to changes in spatial distribution of floral resources, for instance when a patch becomes depleted or a more rewarding one is discovered [53].

Foraging performance improves with foraging experience

Most bees do not engage in foraging right upon emergence [12]. In honey bees the transition from in-hive activities (e.g. brood nursing) to foraging depends on a complex developmental program regulated by social signals [54]. On their first flights from the hive honey bees make orientation flights without collecting food to systematically acquire information about the hive

location [55]. Foraging performance then improves over the first week of foraging, likely due to learned flower identification and handling, and route optimisation [56,57].

Dramatic changes in the structure of the adult brain are seen during the period of orientation flights and the first week of foraging [58]. Foraging activity is reflected by allometric increase in MB volume [59,60]. In honey bees this expansion is caused by increased dendritic arborisation of the MBs intrinsic neurons receiving visual and olfactory input accompanied by the pruning of microglomeruli (synaptic boutons) [58,61], partly due to the activation of cholinergic receptors [62]. The selective localisation of these structural changes suggests activity-dependent synaptic plasticity as an underlying mechanism [58]. Dendritic growth can provide a substrate for the formation of new synapses to support stable memories [63]. At the same time selective growth and pruning of connections is thought to optimise the performance of brain centres in the rich visual and olfactory environments experienced during foraging [58].

Stressors affect brain functions, cognition and behaviour

Successful foraging is based on the precise integration of information processed across the major brain networks, as well as dynamic structural modifications of such networks. Therefore even subtle disturbances of neural function could have dramatic consequences on individual cognitive abilities and hence foraging performance. From this perspective it is a major concern that most of the stressors presently impacting on bees target the brain. The range of stressors has been well reviewed previously [1,11]. Here we emphasise how many of these impair cognitive abilities and foraging performance at exposure levels far below those that kill the bee.

Pesticides and heavy metals

Many pesticides impair the cognitive functions required for foraging. In recent years, neonicotinic insecticides have drawn the most attention [64]. These insecticides disrupt cholinergic transmission, the main excitatory pathway in the insect brain, vital for effective learning and synaptic plasticity [13,26]. While acute exposure to very small doses of neonicotinoids has been shown to inactivate MB neurons [15], chronic exposure can impair the whole MB development [16,65]. These effects almost certainly explain the dramatic impacts of sublethal doses of neonicotinoids on learning and memory in honey bees [66], bumblebees [67], and solitary bees [23], which can be linked to deficits in MB plasticity [16]. Pesticide exposure also disrupts visuo-spatial memory and navigation [9,68,69], most likely

through disruption of processing in the corresponding pathways (Figure 1), but this has yet to be demonstrated. Alarming, bees learn to prefer nectar containing neonicotinoids over non-contaminated nectar because of incidental actions of pesticides on the nicotinic receptors involved in reward processing [70].

Fipronil, a widely used insecticide and acaricide, targets neuronal receptors involved in inhibitory transmission by GABA and glutamate [71]. In honey bees GABA signalling is vital for normal MB function, particularly for complex learning [72]. Acute fipronil treatment severely reduces olfactory learning and memory performance [73]. Additional indications of neuronal cell death in the MBs following fipronil exposure suggest possible long-term cognitive impairments in honey bees [74] and stingless bees [75].

Some pesticides contain manganese, which induces precocious foraging in honey bees [76]. Its effect on sucrose responsiveness suggests that it interferes with signalling pathways important for associative learning, as indicated by the abundant expression of a manganese transporter in MBs and ALs [76] (Figure 1). Selenium, another heavy metal found in crop treatments, has been found to change sucrose responsiveness, olfactory learning and long-term memory [77].

Parasites and viruses

Human activities have intensified the pathogen pressures on social bees through dispersion of pathogens across the world. While few parasites or pathogens act directly on the brain, many have a strong impact on the behaviour of bees [6]. Part of this can be explained by the activation of immune mechanisms, which might interfere with energy supply or signalling mechanisms. Even an immune response induced by non-pathogenic molecules can reduce olfactory associative learning abilities [78,79].

The microsporidian *Nosema cerana* and the mite *Varroa destructor* are two major parasites of honey bees. Exposure to either of them induces specific but overlapping patterns of altered gene expression in their hosts' brain [80]. *Varroa* infection alters brain expression of many genes involved in neurotransmitter signalling, including through GABA [80]. These impacts on the brain are thought to induce poor navigation performances by infected bees [81,82].

Varroa carries many viruses, and a *Varroa* infection of a colony is a complex syndrome of many co-associated pathogens. Part of the effects of varroensis is due to viral infections [7,80]. For example, the deformed wing virus (DWV) impacts on olfactory learning, possibly by targeting brain areas of importance for foraging [18]. Although there is

no known impact of DWV on bee visual learning and navigation, other viruses, such as the Israeli acute paralysis virus (IAPV), affect homing behaviour [83].

Malnutrition

Intensive farming and the expansion of monocultures have imposed constraints on the dietary diversity of bees since only rather few food resources are available to them, often in limited flowering seasons [11]. Bee nutrition is partitioned between nectar, the main source of carbohydrates, and pollen, which provides proteins, lipids, vitamins and other micro-nutrients [84]. Limited food intake reduces performance in a simple learning task [79], but having enough food is not necessarily sufficient for optimal cognitive processing. In honey bees, olfactory associative learning is disrupted by qualitative changes in essential lipids [17] or amino acids [85]. Pollen shortage during development can also lead adults to forage earlier and for a shorter period [86], whereas nectar deprivation increases impulsive, suboptimal, food choices [87].

From reduced foraging performances to colony collapse

Few of the stressors we have considered would kill bees outright at ecological levels. Nonetheless, impairment of the cognitive abilities and food collection performance of foragers by sublethal stressors can have extremely severe consequences on populations.

Comparative research on bee declines suggests bees are more or less resilient to stressors depending on their social lifestyle [2,88], although this needs to be confirmed by more studies (Box 2). In principle, solitary bees are the most vulnerable since the reduced foraging efficiency of the female following stress exposure immediately jeopardises the development of its entire brood. These species lack the profusion of specialised group behaviours observed in social bees (e.g. corpses and diseased brood removal, social fever, collection of antimicrobial and antiviral plant resins) that mitigate the impact of stressors on colonies [89].

However, the stress tolerance of a colony is not without limits and sublethal stressors can also have extremely severe consequences on colonies. In the most socially advanced species, such as honey bees, foraging is undertaken by middle-aged adults that have completed a period of orientation flights and brain maturation to prepare them for the cognitive demands of foraging [58,59]. Stressors not only disrupt foraging performance, but also the process of preparing for foraging. For honey bees, a very common response to many stressors is to begin foraging prematurely [21] (Figure 2). It has been argued that delaying

high-risk tasks to later in life is an effective strategy to extend mean longevity of workers and increase the total contribution of workers to the colony [90]. But if worker lifespan is reduced, workers react by proportionally compressing their time allocation to each task, and commence foraging early. This is likely an adaptive response to acute stress, since it would temporarily compensate the foraging effort of the colony. However, in conditions of chronic stress, this response can accelerate colony decline since bees that commence foraging precociously complete fewer trips in their lifetime [91] and live less long [21].

Simulation models suggest that chronic environmental stressors can create a situation in which the foraging force is dominated by precocious foragers [21,92], and becomes so inefficient that it can no longer support the colony, at which point the colony population dramatically collapses (Figure 2). Stressed bumblebee colonies, though smaller and socially simpler than honey bee colonies, also show highly non-linear responses to chronic environmental stressors [10,20]. Various impairments of colony function (including foraging, but also thermoregulation, defence and hygienic behaviour) can generate changes in population dynamics via feedback loops affecting rates of hatching and adult death, sometimes leading to colony collapse [20]. These complex dynamics might explain why widespread declines in wild and managed bee populations have been observed recently [1–4]. The known stressors of social bees are not new, and many populations have been in a steady decline for decades, but the accelerated declines described recently [2–4] suggest that we are now reaching the point at which the cumulative stress on colonies is exceeding their capacity to tolerate it.

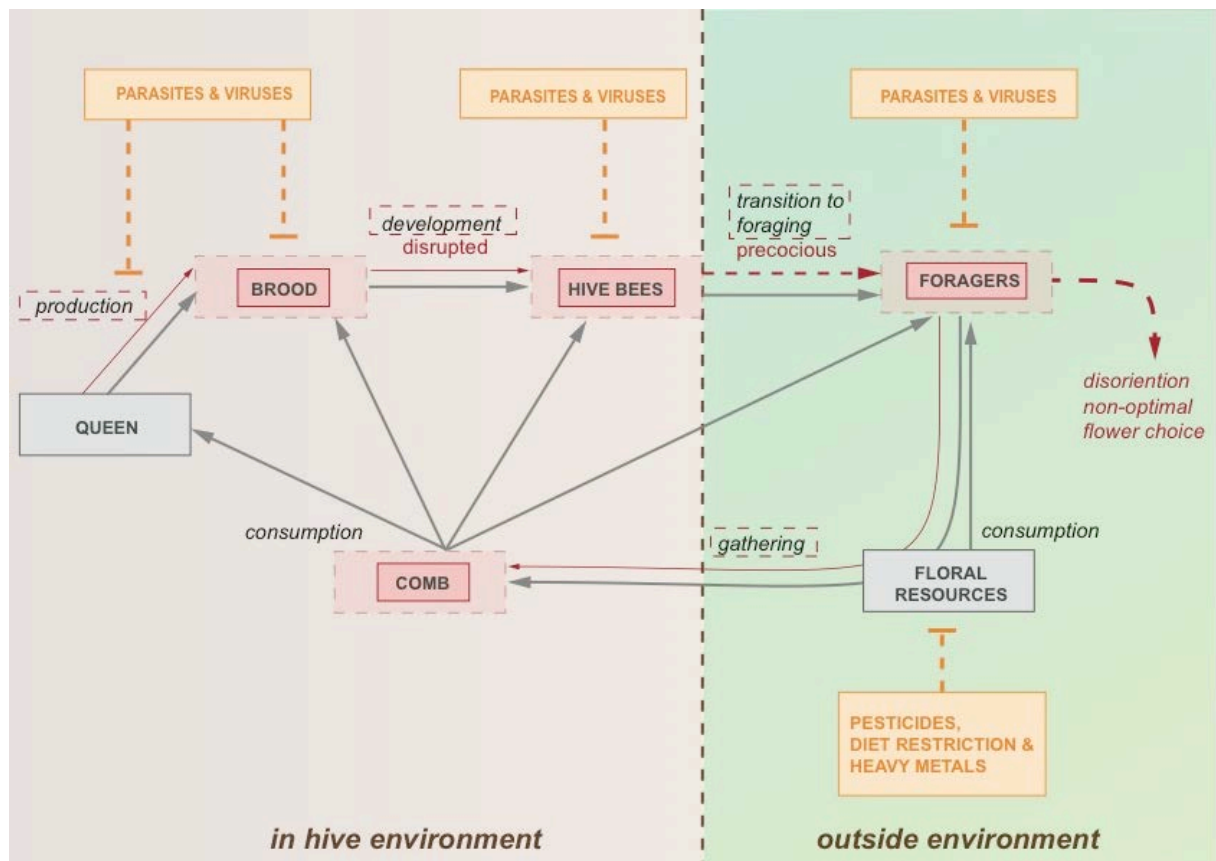


Figure 2. Sublethal effects of stressors on honey bee colony dynamics. In a non-stressed colony (grey arrows), the brood (eggs, larvae and pupae) develops into hive bees (e.g. nurses) that begin to forage two weeks later. Foragers gather nectar and pollen from floral resources for storage in the hive (comb). The food stock is consumed by the queen, the larvae, the hive bees and the foragers. Individual bees can be exposed to environmental stressors (orange boxes) at different stages, potentially disrupting the whole colony dynamics. Stressors reduce brood production, alter development, induce a precocious foraging onset in hive bees and affect the cognitive performances of foragers, leading to disorientation and less efficient food gathering (red arrows). The synergistic action of stressors at different levels of this complex system can lead to dramatic colony collapse. Plain red arrows indicate quantitative changes. Dashed red arrows indicate qualitative changes. Adapted from [22].

Summary and future prospects

Bees are particularly vulnerable to the sublethal effects of many current environmental stressors. These insects have evolved refined cognitive abilities to enable them to effectively exploit complex and changing foraging environments from a central nest. Such capacities demand the optimal function and coordination of major systems in the small bee brain. Many stressors disrupt brain function with the consequence of reduced foraging performance, ultimately compromising brood or whole colonies. These gradual and pervasive effects might explain why eco-toxicological studies, alone, have failed to provide accurate predictions of how stressors can damage bee colonies. We therefore argue that more integrated research that considers actions of the different stressors on bee behaviour, cognition and colony function is urgently needed to understand the declines of these major pollinators and manage their populations (Box 2).

Pesticides provide an informative case in point. Agriculture has become increasingly reliant on the ‘next generation’ neonicotinoid pesticides because they are so effective at killing pest insects at low doses by directly targeting the insect central nervous system [8]. Growing research describing the neural impacts, behavioural impairments and changes in colony dynamics at field contamination levels by pesticides [8–10,56,69] has forced a re-evaluation of the ‘safe-level’ of pesticide exposure for individual bees and colonies [64]. Using this new knowledge we must now determine how pesticides can be managed in the agricultural landscape in a manner that is compatible with sustaining bee populations. Many other stressors contribute to colony decline [1,11], for which the precise mechanisms of action need to be unravelled (Box 2).

As discussed above the stress tolerance of a colony is not without limits, and given the increase in bee declines seen in the last decade it would appear we are very close to exhausting those limits for some key pollinating bee species. But this is far from a hopeless story. Combining conceptual and methodological advances in neurosciences, ecology and evolutionary biology can bring considerable insights into how specific stressors affect bee behaviour and colony dynamics, and help identify ecological interventions to ameliorate stress on bees. Most of the stressors damaging bee populations are human induced, and can be reduced or eliminated from the environment if there is sufficient will, or economic imperative.

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BOX 1. Studying the mechanisms of learning and memory in bees

Experimental work addressing the fine scale neural and behavioural bases of bees' cognitive capacities has relied on Pavlovian conditioning, where an individual is trained to associate an initially neutral stimulus (the conditioned stimulus, CS) with an unconditioned stimulus (US) that elicits an innate response [13,14]. Learning the CS-US association leads the animal to respond to the CS. Historically, the dominant paradigm has been the appetitive conditioning (using a sugar solution as the US) of the proboscis (tongue) extension reflex (PER) using a restrained bee (Figure IA) [32] although aversive paradigms exist [93]. This method allows study of elemental associations between two prescribed events, and also non-elemental associations (when individuals respond in an adaptive manner to novel stimuli using learned information in a new context). In recent years considerable progress has been made by combining PER conditioning with pharmacological treatments, electrophysiological recordings and brain functional imaging, to unravel mechanisms of learning and memory, especially for olfactory learning [34].

So far, attempts at associative conditioning of visual CS in PER conditioning with restrained bees has yielded low performance levels [94]. By contrast, impressive visual learning capacities have been shown using free-flight assays, in which bees obtain a sugar reward if they make a correct choice when learning to navigate in a maze (Figure IB) [95] or foraging in arrays of artificial flowers (Figure IC) [31]. Automated tracking systems, such as harmonic radars (Figure ID) [53,68], radio frequency identification (RFID) (Figure IE) [9,10,21], or computer vision [96] allow precise quantification of behavioural data lab and semi-field conditions. These approaches have revealed bees' cognitive abilities for learning complex visual features and relational properties between stimuli [13]. Fast developing virtual reality assays, in which tethered bees walk on a locomotion compensator (Figure IF) [97] or fly [98] to make foraging decisions in response to stimuli displayed on a screen, hold considerable promises to explore the neural mechanisms of visual learning and navigation.

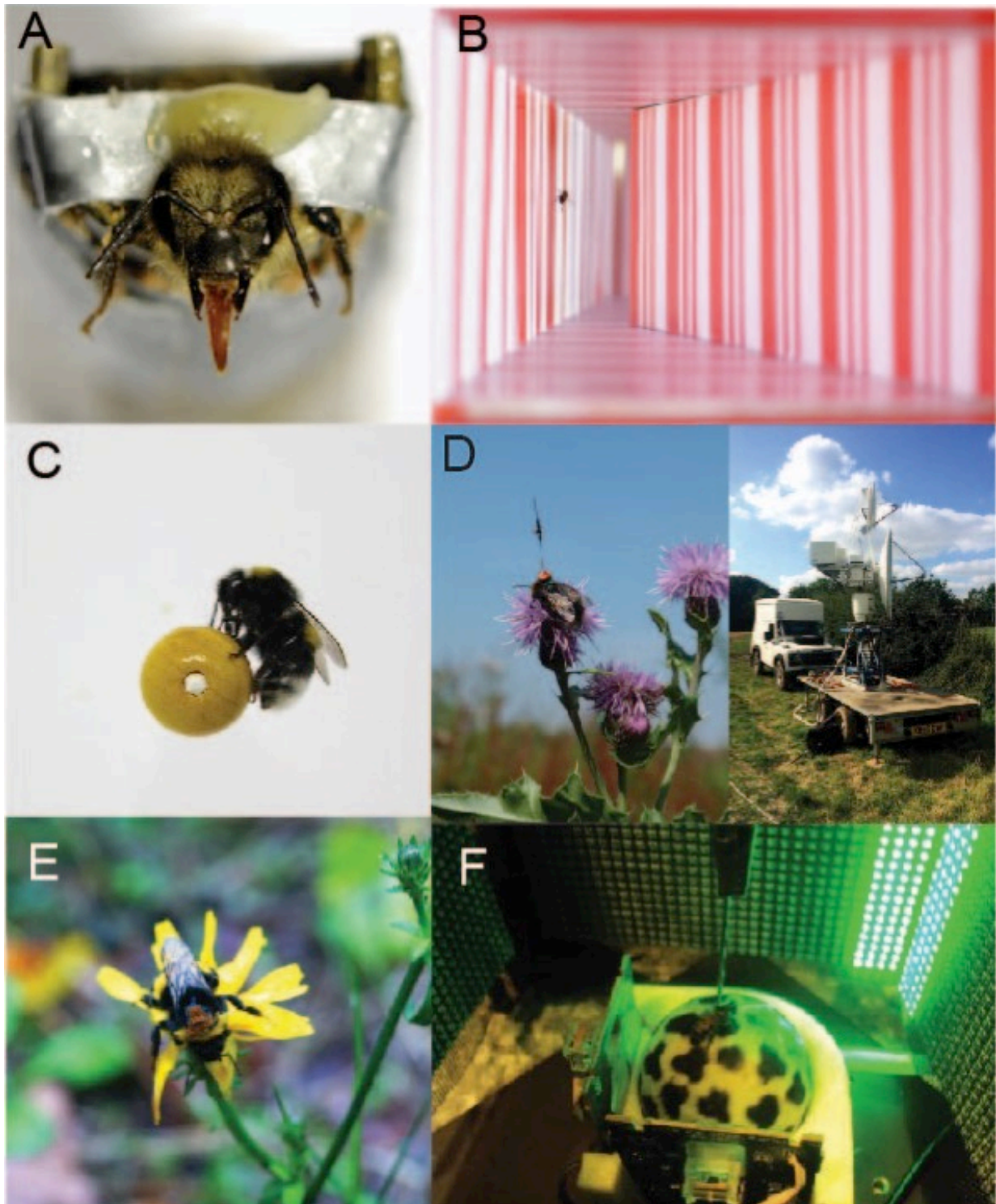


Figure I. Methods for studying bee learning and memory. (A) Restrained honey bee showing proboscis extension reflex (PER) (C. Fresillon/CNRS). (B) Free-flying honey bee in a flight tunnel covered with visual patterns generating optic flow (F. Vrignaud/DGA). (C) Bumblebee foraging on an artificial flower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

Box 2: Outstanding questions: research to sustain social bee populations

- 1.** What are the points of greatest vulnerability in the bee brain? Neurogenomic profiling has started to provide a broad but coarse picture of the gene expression changes occurring in the brain in response to pathogens [80], but more integrative work is now needed to identify precisely how stressors damage the brain to reduce foraging performance. This must couple genomic studies with functional analyses of changes in circuit performance and behaviour. If the points of vulnerability in the developing and adult brain can be identified, it would help the development of neuroprotective treatments to improve the resilience of managed bees.
- 2.** Are all bee species similarly vulnerable to stressors? Bees greatly vary in their social organisation (from solitary to eusocial), feeding ecology (nutrient needs, dietary niche breadth) and habitats (temperate, tropical) [12]. While most attention has focused on managed populations of generalist species with a social lifestyle, such as honey bees and bumblebees, comparative research is needed to assess the general impact of stressors on the wide diversity of pollinators.
- 3.** How can pesticides and bees be managed to keep colonies at a ‘safe level’ of exposure? A key issue is determining what cocktails and levels of pesticide exposure a bee colony can tolerate and maintain a healthy population. Often there are multiple different pesticides at use in the landscape. We need more information on how pesticides might accumulate and persist in colonies, and how they interact to impact bee physiology and change colony function.
- 4.** How then can the agricultural environment be managed to ensure bees receive adequate nutrition from diverse floral sources? Can we design nutritionally optimised plant assemblages to preserve bee populations? Crops provide huge amounts of foods but these plants that have been selected to optimise production and typically yield poor quality diets to bees [99]. Research is needed to quantify the precise nutrient needs of bees, how they vary across colony developmental stages, species and in the face of specific stressors, and their impact on behaviour and cognition.
- 5.** Can the pollination performance of managed bees be sustainably improved by manipulating colony composition? Within a colony, social bees show high levels of behavioural and cognitive variability [100]. In honey bees a small number of individuals complete a disproportionately high number of foraging trips [101]. Characterising this variability between bees, what causes it, and how it changes under stress conditions is needed to understand the consequences of environmental stressors on the resilience of colonies.

Chapter 2: Individual and experience-dependent variation in foraging performance in honey bees



Chapter 2: Individual and experience-dependent variation in foraging performance in honey bees

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Abstract:

For honey bees, macronutrients are supplied from pollen (proteins and fat) and nectar (sugars). The foraging force must supply its colony with a precise balance of both resources to support colony growth. Inter-individual variability within the foraging force has long been observed, in terms of nectar or pollen collection and differences in the foraging activity level. But so far, the nature, the cause and the consequence of such inter-individual variability are not well understood. To explore this here we automatically recorded the weight of bees on departing the hive, their trip durations and videoed returning bees to visually score pollen collection. Only a subset of foragers collected pollen, and no bee foraged exclusively for pollen across their lifetime. A subset (19% of the foragers) of very active bees performed 50% of the colony's foraging trips. Those individuals were more efficient at collecting pollen and nectar, and their efficiency was linked with their foraging experience. Our data bring new information on how a social insect collectively achieves nutritional balance.

Keywords: *Apis mellifera*, division of labour, pollen foraging, radio frequency identification (RFID), behavioural plasticity

Introduction:

A honey bee (*Apis mellifera*) colony is reliant on a subset of active bees, the caste of foragers, to supply nutrients and resources for the whole colony [1]. However, not all foragers make a similar contribution to the provisioning of the colony: some bees will collect more pollen than others [2–6] and some bees are more active than others [7]. What causes inter-individual variability in the foraging behaviour of honey bees is not yet well understood. To analyse the possible causes of variation in foraging activity and efficiency among foragers, we used a novel radio frequency identification (RFID) system to monitor individual bees foraging activity and performance throughout their foraging life.

Inter-individual variability is a commonly documented phenomenon in a wide range of animal species (*e.g.* mammals [8,9], birds [10] or insects [11–17]). In honey bees, inter-individual differences contribute to a highly structured division of labour [18]. A typical honey bee colony is composed of one reproductive female (the queen), a few hundred reproductive males and approximately 50,000 non-reproductive females; the workers who undertake all tasks needed to support and maintain the colony. Workers adopt different tasks according to their age, and thus belong to different behavioural castes at different stages of their adult life, such as in-hive nurse bees, guards and foragers [19].

Even within the forager behavioural caste there is marked inter-individual variation in resource specialisation. Such differences between foragers have also been discussed in terms of different tasks or subcastes, or even personality traits [13,15,16,20]. The most discussed form of variation between foragers relates to the type of resource they return to their colony. Adequate colony nutrition is reliant on a supply of both pollen (protein and lipids) and nectar (carbohydrate) [21]. Because of the differences in the spatial distribution of these major nutritional resources, and the different behavioural skills and strategies required to collect them it may be most efficient for a colony to have different individuals specialised on collection of nectar or pollen [22,23]. This is generally assumed to be the case for bees where pollen and nectar foragers are often described as behavioural castes [2–6] that have been found to differ in their brain neuropeptide profile [24], sucrose response threshold [25], ovary size [26] and levels of vitellogenin (a yolk precursor protein) [27]. Together with evidence for genetic variation and heritability of pollen and nectar collection [3,28–30],

these observations suggest that pollen and nectar collection are different evolved specialisations within the foraging force [29,31,32].

Recent studies, however, have reported that the distinction between the pollen and nectar collection foraging forces is far from absolute. In honey bees, a large proportion of the foragers seem to collect both resources, or may change specialisation as they age [7,25,33]. These studies, however, were either investigating foraging behaviour over a short period of time, often less than the natural foraging lifetime of an individual [25], or were looking at few individuals only [33], or were operating in controlled environments with fixed pollen or nectar sources [7]. Thus, so far, no study has analysed the long-term foraging preferences of a large cohort of honey bees in the natural environment, and thus, data on how foragers partition their effort between pollen and nectar collection across their lifetime are limited.

Some studies of social insects have argued that not all foragers make an equal contribution to the collective foraging effort [34–36], with the observation of extreme skews in the foraging activity of a few very active individuals that can be referred as keystone individuals, or “elite” foragers [7]. Tenczar et al. [7] identified that around 20% of the foragers performed 50% of the colony’s collective foraging activity. Those individuals were more active as they performed more trips compared to the rest of the colony. It is intriguing to observe such skew in the distribution of foraging activity, and one would argue that it would be better for a hive, for all bees to perform at the level of the elite minority. Tenczar et al [7] proposed that non-elite bees could act as a back-up pool of individuals that enable replacement of removed elite bees within a few days after removal, but this further raises the question of why a colony should support an underutilised foraging force when it would be more successful and competitive to bring all foragers into effect. In their study, Tenczar et al [7] were not able to measure bees foraging efficiency and profitability in terms of nectar or pollen collection. Without these data it is impossible to know if a more active bee is more useful to a colony, or simply more active and costly. To understand the nature of variation in foragers’ activity, it is crucial to understand the foraging efficiency of the different individuals to understand the potential benefits of a skew in the distribution of the collective foraging effort.

One possible cause of inter-individual variation in foraging performance is the amount of experience foragers have gained. Studies have shown that individuals

improve their foraging performance with experience [33,37]. Net nectar income has been shown to increase over the first four to five days of the foraging life of a bee [33,37]. Dukas [33] observed that after the fourth day of foraging, foraging efficiency for nectar (measured as milligrams of nectar collected per minute) started to decline. Both of these studies focused on nectar resources only and did not analyse the effects of experience on pollen collection. Moreover, both studies looked at a rather limited number of bees (less than 30 individuals) and presented only general trends. Studying more individuals would enable consideration of individual variation.

More recently the use of radio frequency identification (RFID) technology has made it possible to follow the foraging activity of a much larger number of bees. This technology has been used for more than a decade to address several questions about bee foraging activity, such as the impact of environmental stressors on different aspects of bee foraging behaviour [38–43], or individual foraging strategies [7,44]. RFID systems are based on the detection of passive chips that have to be in close contact with a radio wave emitter to emit back their identity. This constrains the detection to only registering when the back of a bee is in contact with the RFID reader. Thus, most of the studies have focussed on detecting bees at the entrance of the colony. This is then a very efficient way to detect bees leaving and entering the hive, and enables an interpolation of individuals foraging trips. Using a RFID system on its own, would not give information about either the type of resource a bee collected on a given trip, or its efficiency of collecting resources, or where in the environment they went.

In order to analyse variation in forager performance with greater detail, here we developed a use of RFID technology in combination with video analyses and measures of weight of foragers. With these data we analysed how bees differed in foraging efficiency, their lifetime foraging behaviour and also compared their pollen and nectar collection strategies to better understand the causes and consequences of individual variation in forager performance.

Material and Methods:

Experimental hive:

Honey bees (*Apis mellifera*) were obtained from the research apiary of Macquarie University (Sydney, NSW, Australia). The experimental hive was a four-frame nucleus (small wooden box of 56x23x28 cm) hive, placed in a dark room at the constant temperature of 24°C and connected to the outside environment via a specially designed entrance (figure 1). The hive contained two frames of honey and pollen, one frame of capped brood and one frame of polystyrene to fill the remaining space in the hive box.

Entrance board:

The entrance to the hive operated to force bees to exit the hive along one path and to enter using a different path (figure 1). Each path was made of transparent plastic tubing of 1cm of diameter that passed across an RFID antenna (Invengo, Guangzhou, China) and a microbalance pan (A&D company, supplied by National Weighing & Instruments Pty Ltd, Australia). To prevent bees to go out or in through the wrong tube, we attached inwardly tapering plastic bristles at the end of each tube. Along the entrance path bees also passed beneath a webcam (Logitech), placed in a plastic box lit with white LED light, in order to video record the entrance tunnel. Motion detection video recording software (Netcam Studio X, Moonware Studios and ZoneTriger, Omega Unfold Inc. Canada) was used to capture video footage of returning bees, thus allowing us for visual assessment of the resources they carried (*i.e.* presence of pollen or not on the bee legs).

Automatic gates (micro-controlled servos connected to infrared emitter/receiver) regulated the traffic of bees within each path. The gates were placed at the beginning of the entrance and exit tubes. When a bee walked through the tubes and broke the beam of an infrared emitter/receiver, the connected gate would close behind the bee for 10 seconds. This time was an estimation of the maximum time needed for a bee to cross the RFID antenna and the balance, as the gating system would slow down the bees, but without disturbing the traffic. The infrared beams and gates were all connected and monitored via Arduino technology (Arduino, Adafruit and little birds electronics, Hornsby, Australia).

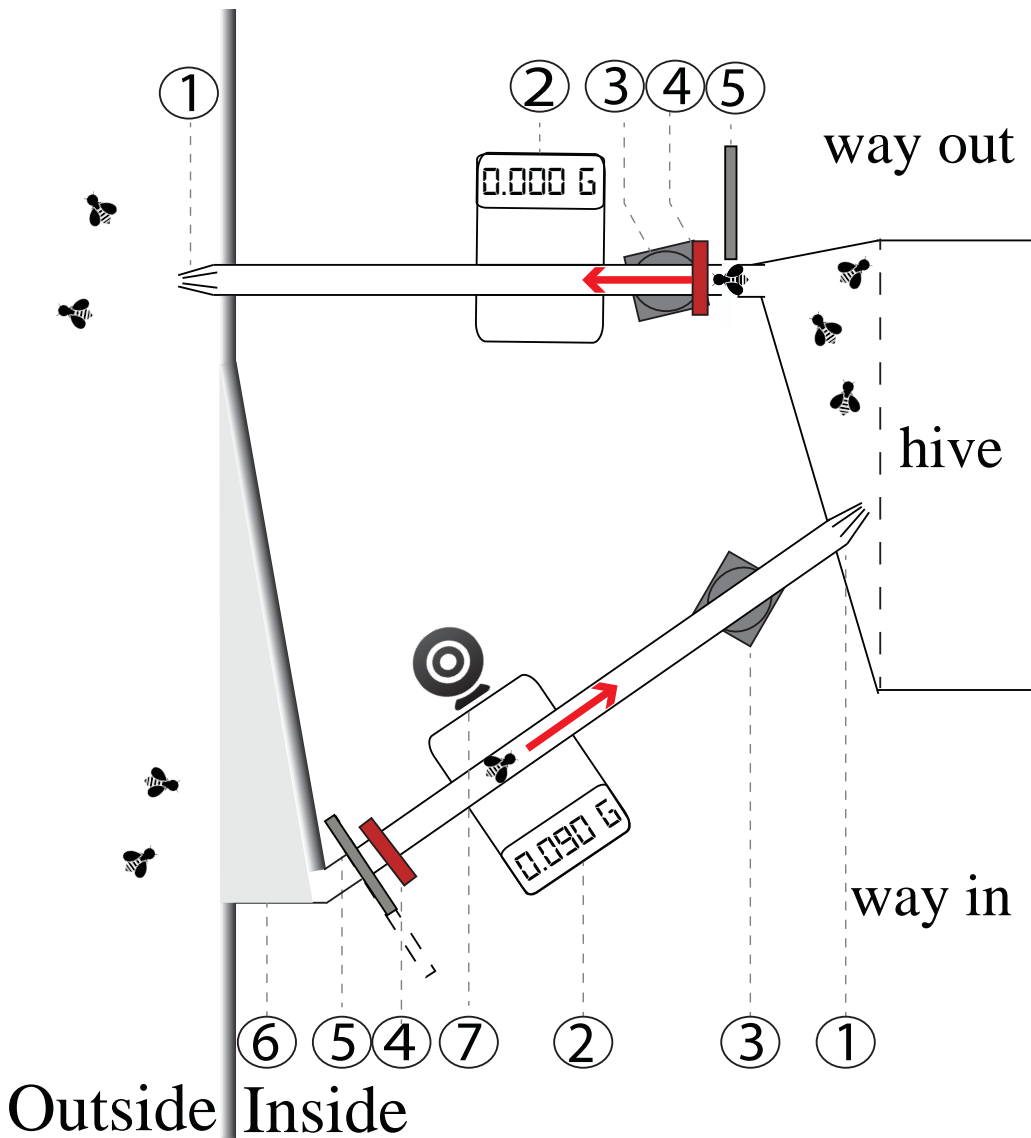


Figure 1: Colony entrance with sensors. The entrance and exit tubes were 1cm diameter transparent plastic tubing. 1: plastic bristles (forcing the passage of a bee from one direction only), 2: balance, 3: RFID antennae, 4: infrared emitter/receiver, 5: automatic gate, 6: landing platform (open on the outside), 7: motion detection webcam.

Radio Frequency Identification (RFID) detection:

RFID tags were obtained from Invengo (Guangzhou, China). Each circular tag had a diameter of 4mm and a weight of 1mg and could be fixed to the bees' dorsal thorax with glue (Loctite, Gel Super Glue). Each RFID tag had a unique 12-byte hexadecimal identifier that allowed us to track individual bees as they were detected by each antenna on exiting and entering the hive.

Balances:

Sections of the entry and exit tunnels ran across dynamic micro balances of 10 mg sensitivity (figure 1). The balances captured the weights of bees as they entered and exited the hive. Sometimes several bees crossed the balance at the same time and thus, the weight did not reflect the weight of just one individual. For this reason, we retained only values between 60 and 150 mg (we evaluated the coherence of individual weigh values as fitting in a realistic individual weight range of each bee [45]). Examination of the videos of the entrance tunnel indicated that these limits were realistic as it showed only one bee walking in the tubes for such range of weights.

Experimental bees:

The hive was established with about 500 background (untagged) bees of mixed ages and a queen taken from a single colony. To this hive newly emerged bees carrying RFID tags were successively added. To source newly emerged bees we collected brood frames from up to eight different colonies over the course of the two experimental trials to provide a diverse source of brood for the experiment. For each brood collection frames were collected from two or three of the eight colonies. Brood frames were stored overnight in an incubator maintained at 37°C. The next morning we glued individually programmed RFID tags to the thorax of the newly emerged bees with super-glue.

Bees were added successively to the colony at different times and we defined a cohort as a group of bees tagged and added to the colony on up to six successive days. Each cohort came from a combination of different hives from the apiary in order to increase the diversity of added bees. 500 tagged bees were added every day for the four first days of the experiment (first cohort). From day 21 of the experiment a further 2,000 tagged bees were added over four days (500 bees a day, second cohort). From day 35 of the experiment a further 800 bees were added over four days (200 bees a day, third cohort). Thus over the five weeks of the experiment, the hive received 4,000 bees in total distributed within three cohorts. The experiment was repeated twice: colony 1, from April-May 2015 (Australian Autumn); colony 2, from November-December 2015 (Australian Spring). In colony 1, the queen died after two weeks and was replaced with a queen mandibular pheromone substitute (BeeBoost, Hornsby beekeeping supply, Australia).

Bees foraged in the surrounding suburban Australian environment including several public reserves and private gardens. Such an environment provided nectar and pollen flow during the two seasons of the experiment, with a predominance of flowering native trees and shrubs such as different eucalyptus species (personal observations).

Collating data on bee trips:

RFID data for each trip included the date and time the bee left the hive, the date and time the bee returned to the hive, and the RFID number for that bee, enabling us to calculate the duration of all individual trips. Trips that lasted less than 10 s were removed from the dataset as they are considered as non-foraging trips [46]. RFID readings were time-matched with readings from the balances. Videos taken up to 20 s before an entry RFID detection were inspected to score whether the tagged returning bee carried pollen (P), no pollen (NP) or could not be reliably scored (NA) due to multiple bees in the tube or a bee moving through the tube at an angle where their body occluded view of their legs. 20 s was visually assessed as the maximum time for a bee to travel from the webcam to the RFID antenna.

Data reliability:

A total of 8,640 bees were tagged during the two runs (4,390 for colony 1 and 4,250 for colony 2). We excluded from the dataset bees that had only 'NA' as load type over all their trips and bees that performed only one trip. In the final dataset, 3,432 bees (1,728 for colony 1 and 1,704 for colony 2) were kept. We speculate that the discrepancy between final bee counts and initial bees tagged is due to many bees losing their tags within the hive, some of the tags being damaged during the tagging process and rendered unreadable, and some bees not returning to the hive during their first trip, for reasons of health, physical ability to return or being rejected by hive mates. For each bee, we excluded the first five trips, which are more likely orientation flights than foraging flights [46]. A summary of our trip dataset is given in Table 1. The high number of 'NA' trips was mainly due to camera software issues, which was unfortunately most problematic for colony 2.

Data analyses:

Data were analysed in R version 3.2.3 [47] (operating via Rstudio, version 1.0.136 [48]) using the packages lme4 [49] and lmerTest [50]. We define here elite bees as the subset of bees that completed more than 50% of all the trips of each colony. Differences in age at first foraging trip and differences in average weight on departure between elite and non-elite foragers were tested with a Wilcoxon rank sum test. The proportion of mixed foragers in elite or non-elite bees groups has been tested with a chi-squared test. The correlation between the average weight difference for non-pollen trips and bee activity was tested with a general linear model (GLM). The correlation between the total weight differences for non-pollen trips and bee activity was tested with a general quadratic model. Changes in weight difference for non-pollen trips according to the number of trips was tested with a general logarithmic model. Changes in the number of foraging trips per day according to the number of foraging days were tested with a general linear model (GLM). We analysed the probability of foraging for pollen according to experience (number of foraging trips) using a binomial generalized linear mixed model (GLMM). Differences in foraging durations and weights on departure between pollen and non-pollen trips were investigated using a Poisson generalized linear model (GLM). Trip durations were normalised (natural log). Differences in weights on departure for non-pollen trips between non-pollen foragers (bees that did not perform any pollen trip) and mixed foragers (bees that collected pollen at least once) were analysed using a Poisson GLM with colony identity as a random factor. The regressions between trip durations and weights on departure were investigated with linear models (LMs).

For all models, colony identity was included as a covariate and bee identity nested in cohort identity was included as a random factor. Day identity was included as a random factor to control for environmental variation between days. The colony of origin of the bees was found to have no effect when included as a fixed factor on the models. We thus did not include colony of origin as a potential explanatory variable in our analyses. We did not include day identity as a random factor in models that also included trip number as an explanatory variable, because of the complete confound of day and trip number. All minimum adequate models were selected by comparing their Akaike Information Criterion (AIC) to null models [51] (table S1).

Results:

Inter-individual variability in the foraging behaviour of honey bees

Overview of the data

We analysed a total of 564 bees that foraged at least once and for which we have at least information of the resource type for one trip (table S1). As an overview of our data: on average, bees performed a total of 19 trips (mean \pm SE, colony 1: 17 ± 1 trips, $N = 295$; colony 2: 21 ± 1 trips, $N = 269$) and foraged for less than a week (colony 1: 4.20 ± 0.18 days, $N = 295$; colony 2: 4.85 ± 0.18 days, $N = 269$).

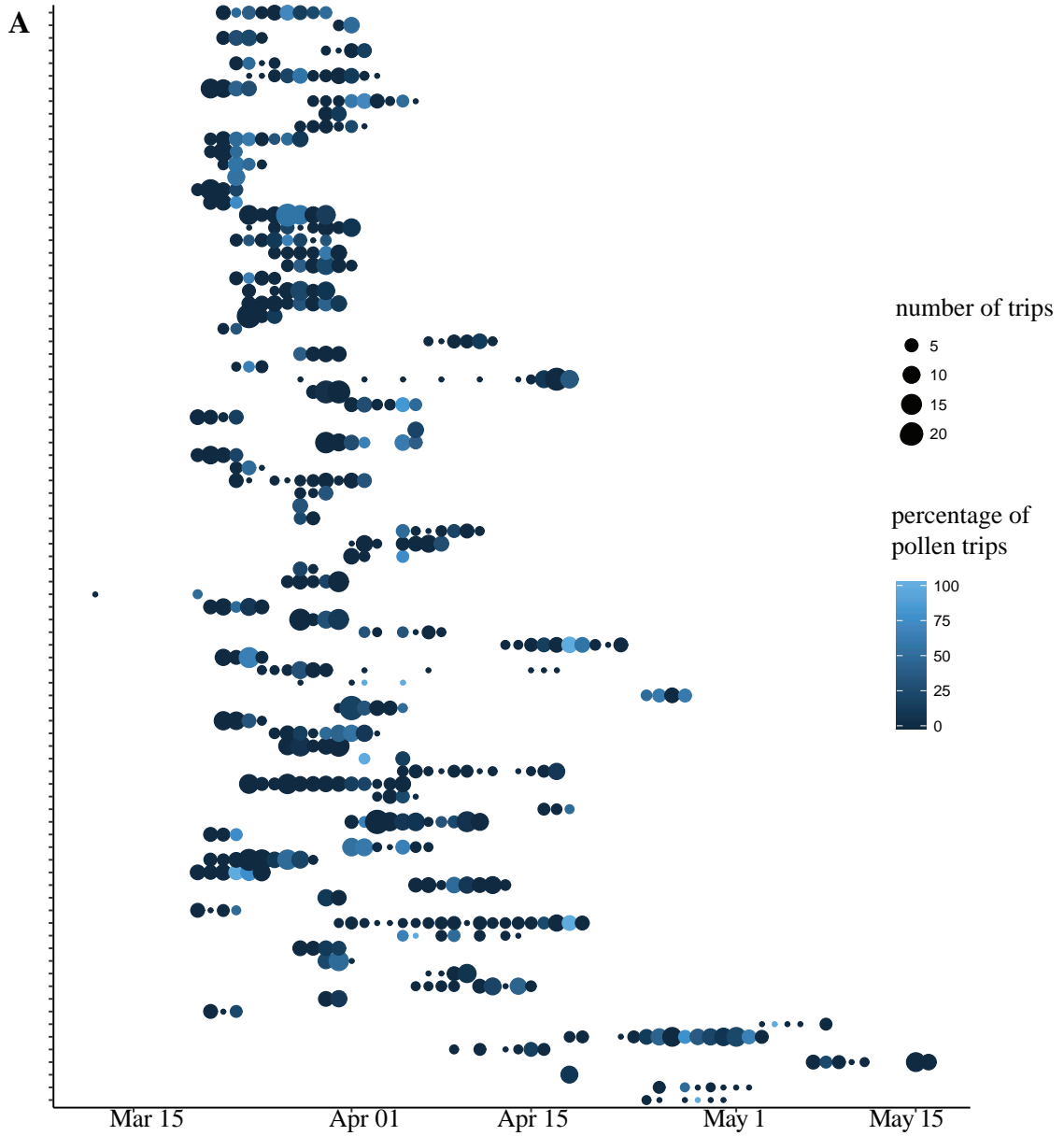
On average 27% of the bees foraged at least once for pollen (29% for colony 1, figure 2A, and 25% for colony 2, figure 2B, details in table S1). None of these bees exclusively foraged on pollen. All of them were ‘mixed’ foragers, performing a combination of pollen and non-pollen trips (a non-pollen trip can be nectar, water or a trip with no resource). Incidences of pollen collection were distributed throughout the experiment and foragers varied in the number of trips as well as percentage of pollen trips they performed on different days (figure 2). Pollen collection activity differed between each bee but was also dependent on the environment as we noted pollen collection to be more frequent on some days than others (figure 2). For instance on the 24th of March, 16% of the total number of trips (246) were pollen trips; whereas on the 4th of April none of the bees collected pollen (68 trips). Figure S1 shows examples of the foraging history for particularly active bees. This illustrates the high inter-individual variability both in resource collection and in foraging activity.

Extreme variation in foraging activity

We examined the proportion of foraging trips performed by each bee relative to all the foraging trips of their colony. The Lorenz curve [52] shown in figure 3A, presents a visualisation of the inter-individual variation in foraging effort seen in each colony. It shows the proportion of the total activity of the hive performed by a certain proportion of individuals, and indicates that around 19% of the tagged foragers performed 50% of the total number of trips recorded (17.29% for colony 1, 20.45% for colony 2). Following the terminology of Tenczar *et al* [7], we defined elite bees as the group of bees that cumulatively accounted for 50% of the total foraging trips performed by the colony.

We then examined the skew of foraging activity between all the foragers by computing a Gini coefficient [53]. The Gini index varies between 0 (all individuals contributed equally to the common task) and 1 (very few individuals performed the vast majority of the task). We obtained a Gini index of 0.49 colony 1 and 0.46 colony 2, meaning that all individuals did not contribute equally to the common foraging task.

We then looked at the relative activity per day of each forager compared to the overall hive activity on that day (figure 3B). We found that 8.81% of the foragers took 50% of the daily activity for colony 1 and 16.73% for colony 2. On average, 13% of the bees accounted for 50% of the colony daily activity.



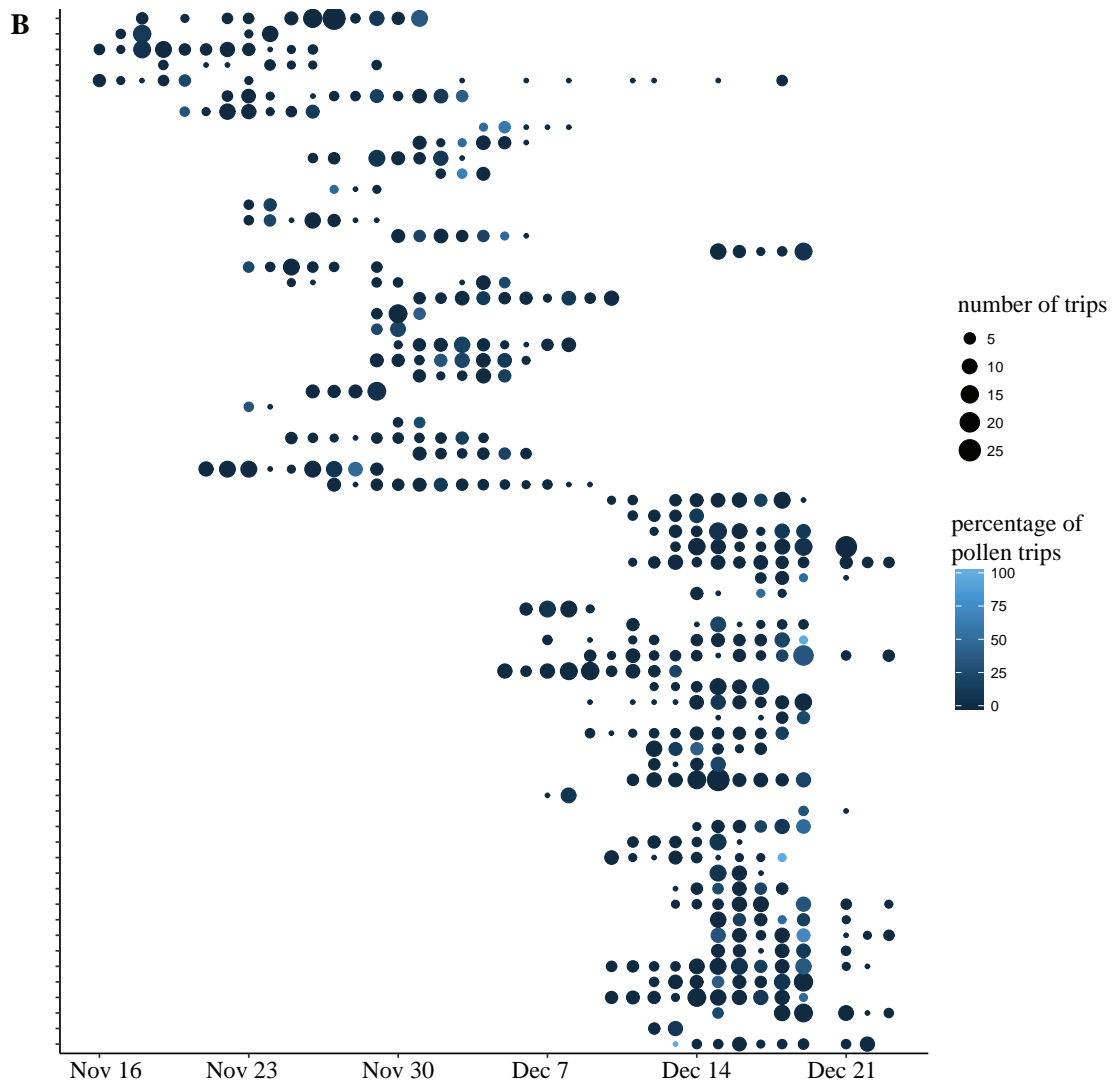


Figure 2: Heat maps representing the number of trips per day as well as the percentage of pollen trips a given bee performed on each day. Only the mixed foragers are shown. Each line represents the foraging activity of a given bee for all its foraging life. A. Mixed foragers of colony 1. B. Mixed foragers of colony 2.

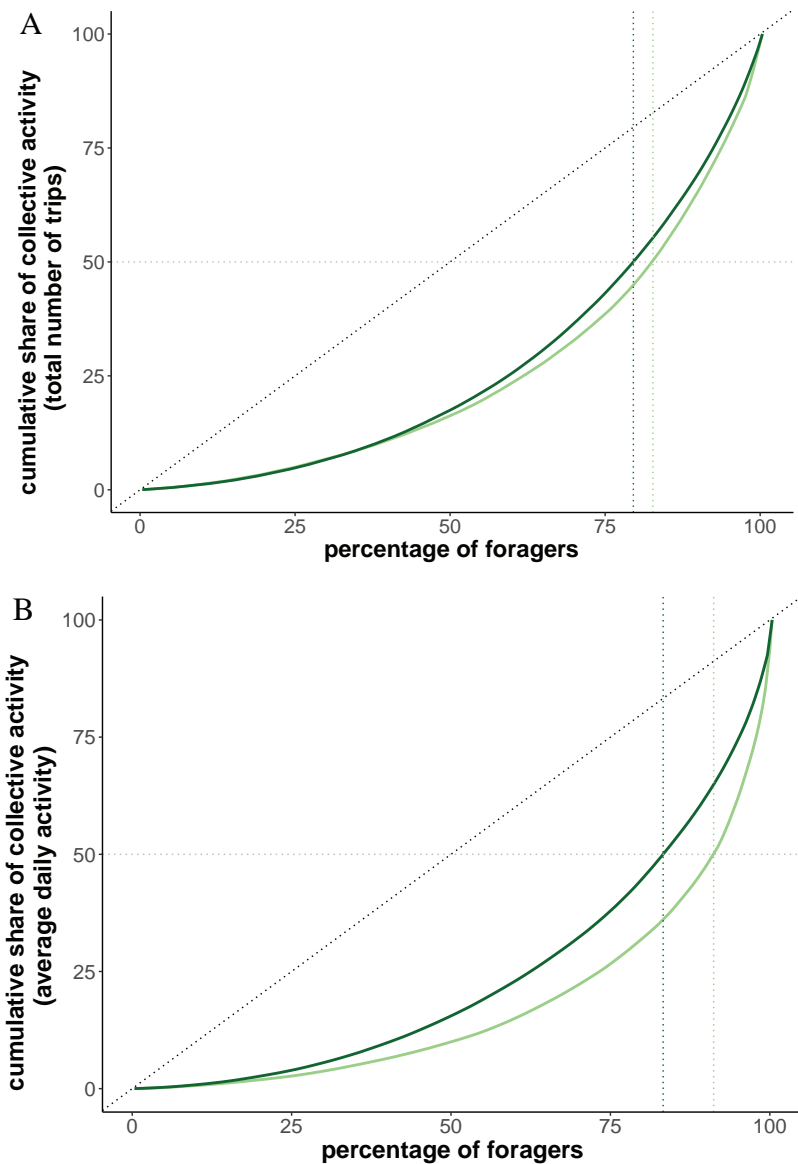


Figure 3: Lorenz curves of relative individual contributions to the colony foraging activity. Such curves present, for each colony, the proportion of foragers that contribute to a certain amount of the total number of trips (**A**) or to the number of trips per bee per day compared to the total number of trips performed by the colony that day (**B**) [7]. For each colony (light green: colony 1, dark green: colony 2), bees were ranked by the lifetime number of trips (**A**) or relative daily activity (**B**) they performed in ascending order, and the fraction of each bee's contribution to the was cumulatively plotted in the Y axis. Black dotted lines represent the distribution predicted by an evenly distributed contribution of each individual. Grey dotted horizontal lines indicate the threshold of a contribution to 50% of the total activity. Vertical green dotted lines represent the fraction of foragers, for each colony, for which this threshold was reached. **A.** In colony 1 (N = 296 foragers in total): 17.29% of the total of bees performed 50% of the total number of trips. In colony 2 (N = 270 foragers in total): 20.45% of the total of bees performed 50% of the total number of trips. **B.** Here, 8.81% (colony 1) and 16.73% (colony 2) of the foragers contributed to the total colony foraging activity.

Effects of age and weight on inter-individual variability

Considering the age of first foraging trip (figure S2A) elite bees from colony 1 started foraging earlier than non-elite bees (mean \pm SE, elite = 20.21 ± 0.71 days; non-elite = 25.1 ± 0.63 days; Wilcoxon rank test: $W = 8\ 368$, $P = 0.0005$). This difference was not significant in colony 2 (mean \pm SE, elite = 18.43 ± 0.56 days; non-elite 18.92 ± 0.30 days; Wilcoxon rank test: $W = 6\ 717$, $P = 0.194$). In terms of average weight on departure (figure S2B), there was no difference between the elite bees and the non-elite bees (GLMM, $N = 1\ 870$, $F_{(1,143.3)} = 1.52$, $P = 0.390$, model selection in table S4E). We found no difference in weight on departure between the mixed and non-pollen foragers when collecting non-pollen resources (figure S3, GLMM, $N = 1\ 870$, $P = 0.45$, model detailed in table S2, model selection in table S4F).

Factors contributing to inter-individual variability

Pollen collecting trips took longer than non-pollen trips

Foraging trip duration was longer when bees returned with pollen than when they returned with other resources (GLMM, $N = 1,913$, $P < .0001$, figure 4, table S2A, example in figure S1).

Bees weight on departure from the hive varied with trip type and duration

For pollen trips we observed a positive correlation between the weight on departure and the duration of the trip (GLMM, $N = 1,061$, $P = 0.008$, figure 5, table S2B). By contrast, for non-pollen trips there was a negative correlation between the weight on departure and the duration of the trip (GLMM, $N = 1,061$, $P = 0.001$, figure 5, table S2B). There was no correlation between the weight on departure during the first pollen trip of a given day and weight on departure of any other pollen trips for this same day (figure 6).

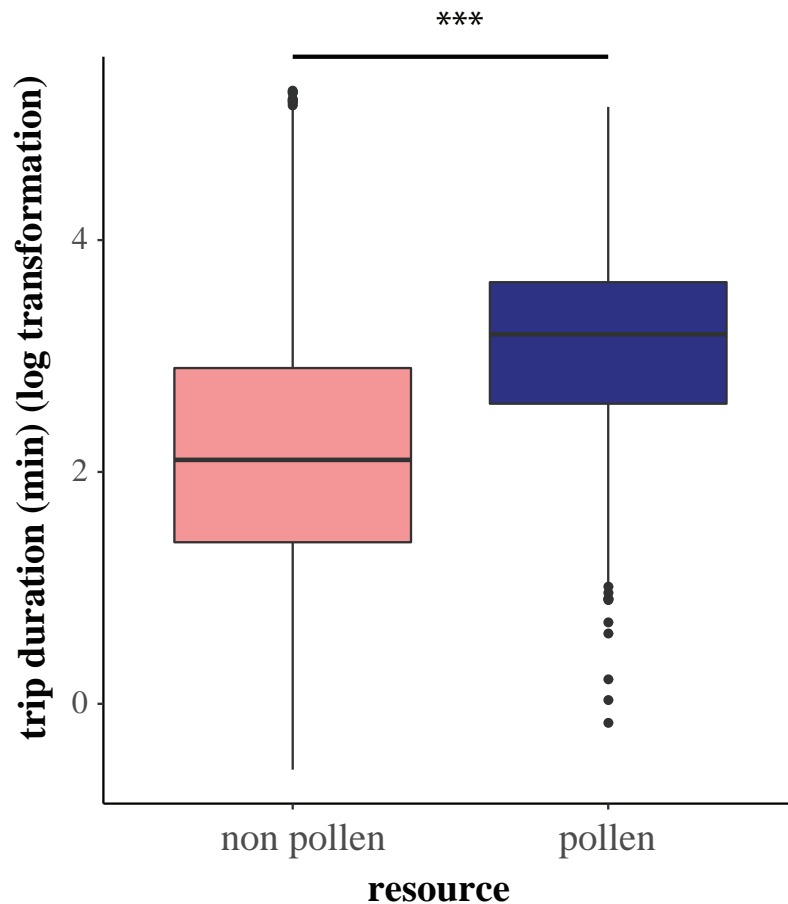


Figure 4: Average trip duration according to the resource collected. Difference in duration of non-pollen (pink) and pollen (blue) trips, for bees that foraged at least once for pollen. Only the trips shorter than 200 min were included (98.55% of all 1,941 trips). N = 154 bees. Non-pollen trips = 1,409. Pollen trips = 504. Boxplot: the line shows the median, boxes and the whiskers represent interquartile ranges; dots represent outliers (data greater than third quartile + 1.5*(interquartile range), or less than first quartile – 1.5*(interquartile range)). Pollen trips take more time to perform than non-pollen trips. Stars represent significant difference (GLMM, N = 1,913, P < .0001, table S2A, model selection in table S4A).

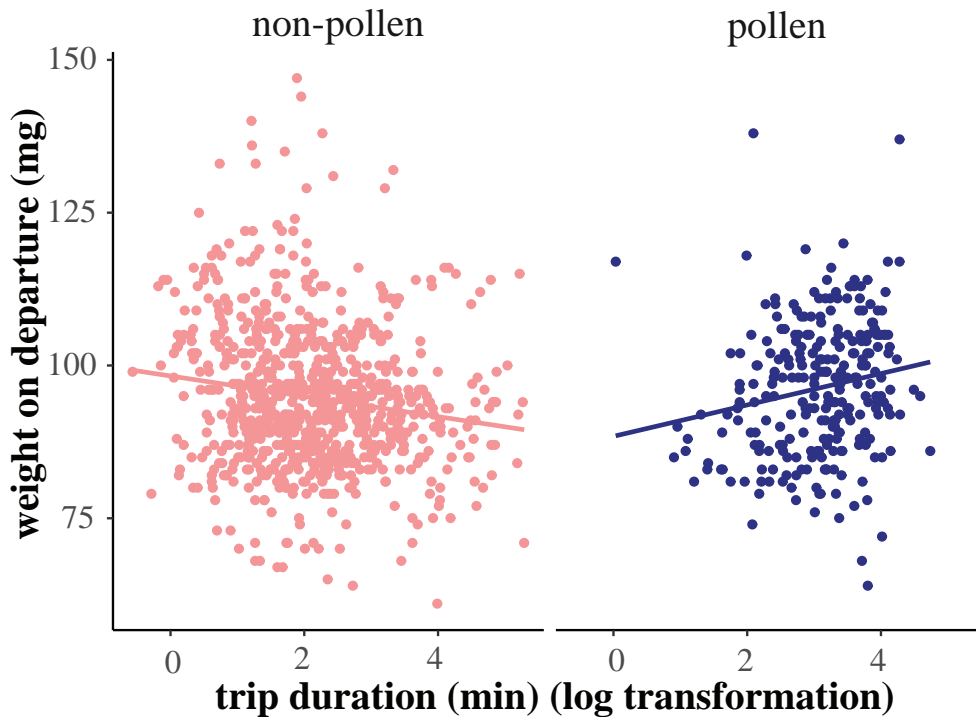


Figure 5: Correlation between weight on departure and trip duration. Weight on departure plotted against trip duration (natural log transformation), for individuals that foraged for non-pollen (pink dots) and pollen (blue plots) resources. For pollen trips (blue points) bees leave the hive heavier for a longer trip. For non-pollen trips (pink points) bees leave the hive lighter for a longer trip. Only trips shorter than 200 min have been included (54.66% of all 1,941 trips). N = 106 individuals. Non-pollen trips = 411. Pollen trips = 129. GLMMs are summarised in table S2B, model selection in table S4B).

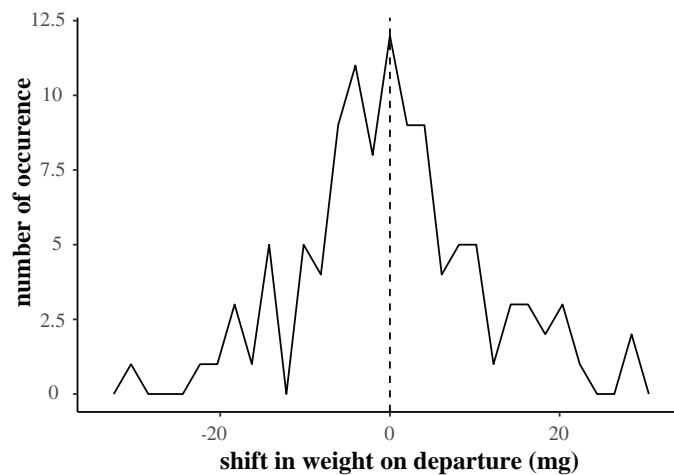


Figure 6: Shift in weight on departure between the first pollen trip and the other pollen trips of a given day. The distribution shows the average change in weight on departure between first pollen trip and other pollen trips during a given day for the mixed foragers. Since the distribution is centred in 0 there was no change in weight difference between the first pollen collecting trip of a day and subsequent trips, from which we infer there was no refinement of weight on departure following discovery of a pollen resource.

Bees improve their foraging performance with experience

The probability of collecting pollen on a foraging trip increased with the number of trips already performed (binomial GLMM, $N = 2,215$, $P < 0.0001$, figure 7; and figure 2 and figure S1 for examples) indicating that bees were more likely to collect pollen as they accumulated experience.

Foragers changed their foraging activity pattern as they gained experience. Bees progressively performed more trips per day (figure 8, $N = 2,539$, $F_{(1,179,01)} = 26.19$, $P < 0.001$).

Bees increased their efficiency with experience: there is an increase in weight difference for non-pollen trips with successive trip number for both elite and non-elite foragers (figure 9, GLMM, $N = 925$, $F_{(1,31,27)} = 6.45$, $P = 0.016$). Finally, bees decreased the time spent inside the hive between two trips, with the number of trips they performed (Figure 10, GLMM, $N = 2,539$, $F_{(1,12,92)} = 9.89$, $P = 0.008$).

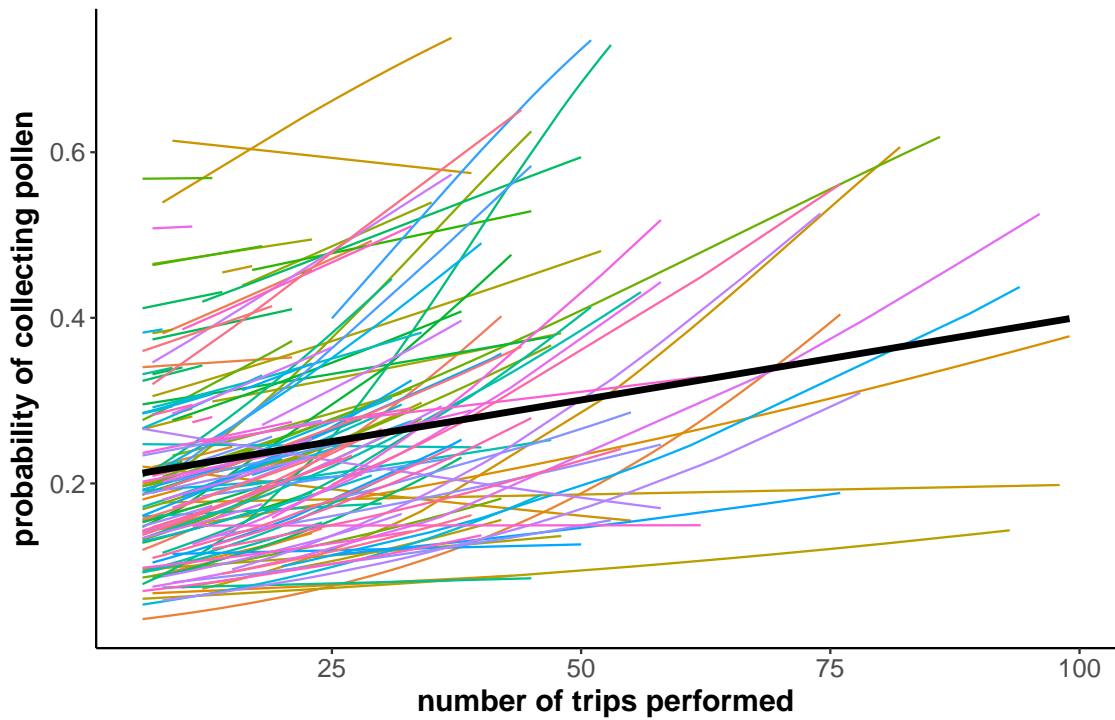


Figure 7: Probability of collecting pollen increases with experience. The probability for an individual to collect pollen on a given trip increased with the number of trips performed (pollen and non-pollen trips). Each coloured line represents the fitted estimators of each bee according to a binomial GLMM (N = 154 bees. Non-pollen trips = 1,432. Pollen trips = 509, intercept: -1.68 ± 0.24 , $T = -6.99$, $P < 0.0001$, slope: 0.02 ± 0.006 , $T = 3.47$, $P < 0.0001$). The black line represents the average of the fitted estimators. Model selection table S4G.

Number	62	388	224	122	58	26	12	3	2	2
of foragers:	503	308	177	79	38	16	6	3	3	2

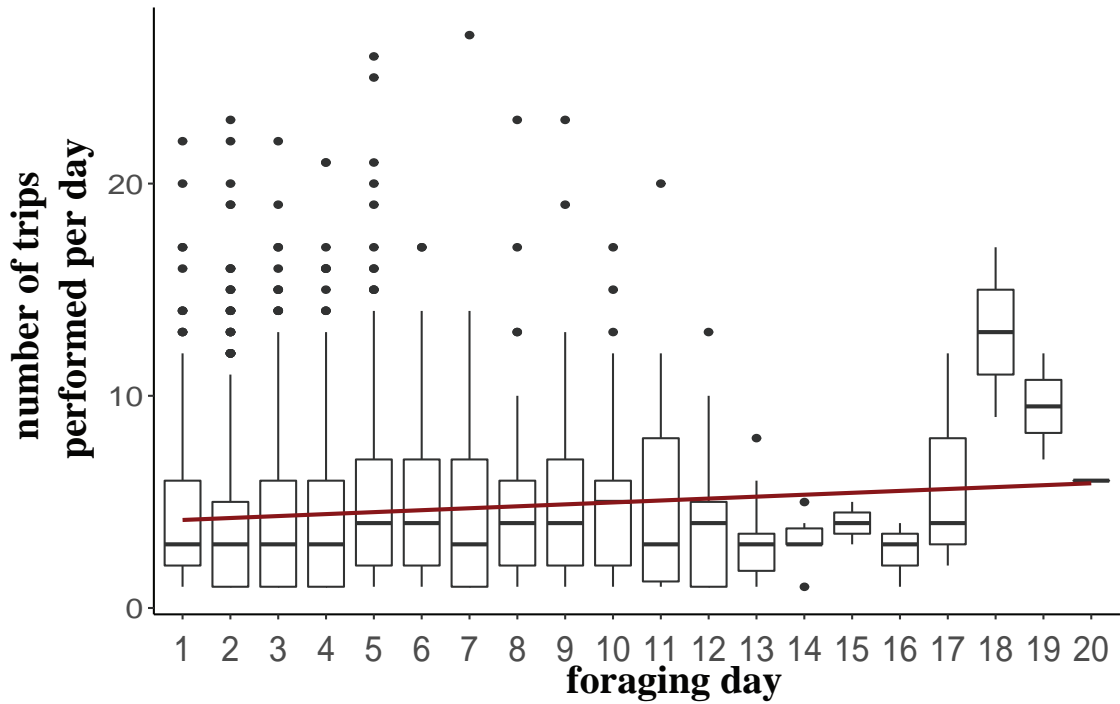


Figure 8: Changes in foraging frequency with experience. Relation between the number of trips performed per day and consecutive foraging days. Boxplot: the line shows the median, boxes and the whiskers represent interquartile ranges; dots represent outliers (data greater than third quartile + 1.5*(interquartile range), or less than first quartile - 1.5*(interquartile range)). Red line indicates the best fitted linear model. There is an increase of foraging activity with experience. GLMM: intercept = 3.38 ± 0.20 , DF = 112.33, T = 17.20, P < 0.0001; slope = 0.19 ± 0.04 , DF = 179.01, T = 5.12, P < 0.0001. Model selection table S4H.

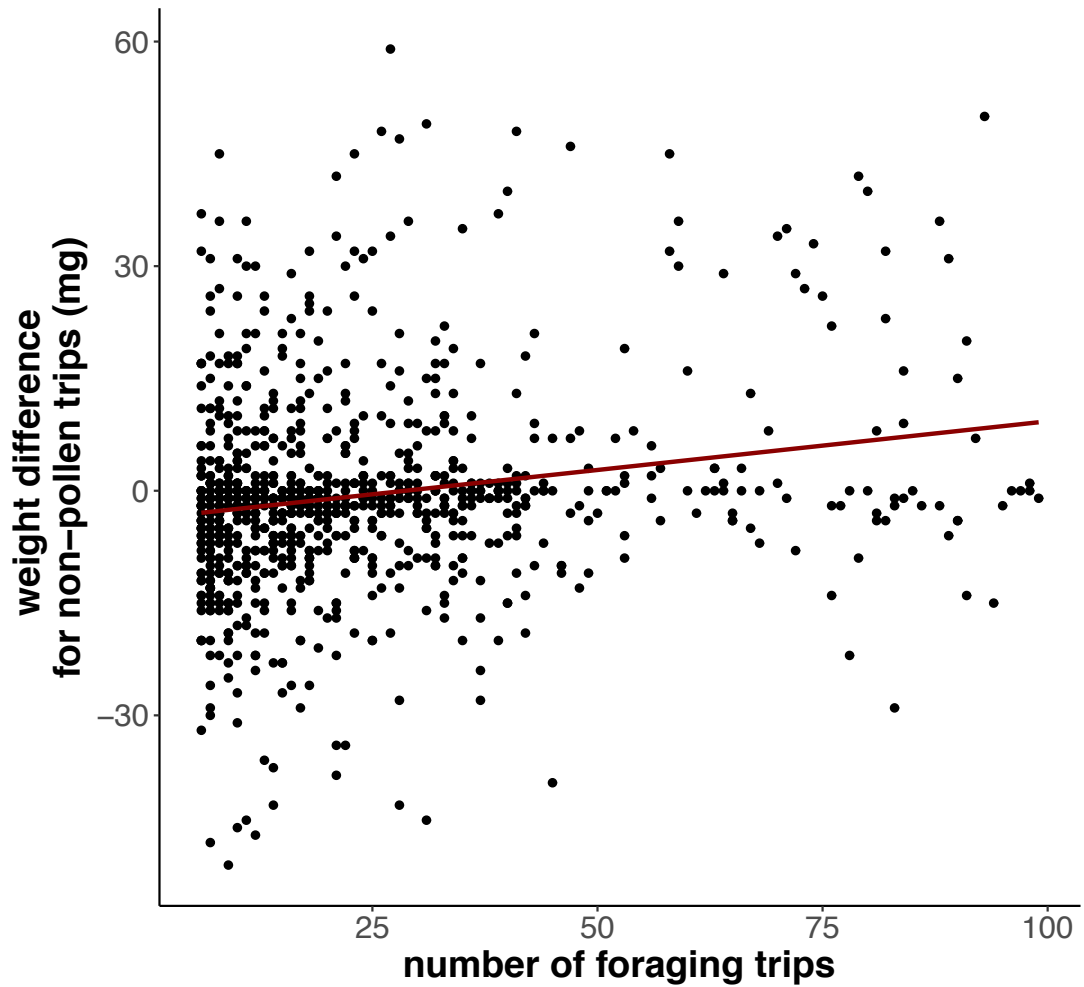


Figure 9: Foraging efficiency according to the number of trips performed. Only the foraging trips recognised as non-pollen trips are analysed here (N = 925 trips, for 277 bees). Bees tend to be more efficient when they are more experienced. GLMM: intercept = -3.83 ± 1.19 DF = 3.12, T = -3.22, P = 0.046; slope = 0.12 ± 0.04 , DF = 31.27, T = 2.54, P = 0.016. Model selection in table S4I.

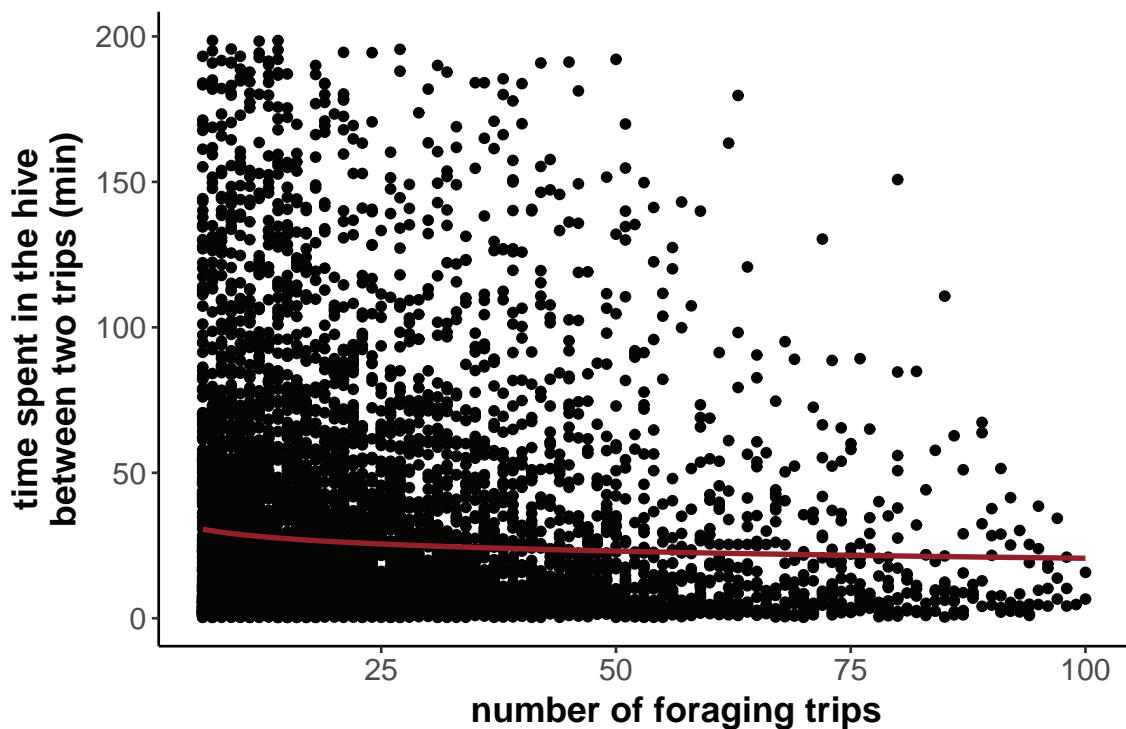


Figure 10: Time spent in the hive between two consecutive trips. Only inter-trip durations less than 200 min have been kept in this analysis. The time spent inside the hive between two consecutive trips decreases with individual experience (N = 8,199 inter-trips, for 545 bees). GLMM: intercept = 31.16 ± 2.82 DF = 4.5, T = 11.06, P = 0.0002; slope = -0.13 ± 0.04 , DF = 12.92, T = -3.14, P = 0.007. Model selection in table S4J.

Elite bees are more efficient foragers than non-elite bees

There was a larger proportion of mixed foragers (foragers that have been recorded at least once with pollen on their legs as they returned to the hive) within the elite group of bees than within the rest of the forager population (figure S4, colony 1: elite bees: 72% of mixed foragers, non-elite bees: 20% of mixed foragers: $\chi^2 = 55.68$, $df = 1$, $P < .0001$; colony 2: elite bees: 60% of mixed foragers, non-elite bees: 15% of mixed foragers: $\chi^2 = 46.99$, $df = 1$, $P < .0001$).

More nectar collected by the more active bees (figure S5A). Finally, elite bees reduced their time spent inside the hive between two trips (figure S5B). Therefore, the more active bees tended to collect more of both resources (pollen and non-pollen, *i.e.* nectar or water) through their lifespan.

The differences in performance of elite and non-elite bees in our data could be explained by the differences in experience between these two groups, as models show no significant effect when considering elite bees as a fixed factor (figure S5).

Discussion

High inter-individual variability in honey bee colony foragers

We recorded the lifetime foraging activity of a large cohort of foragers in two honey bee colonies using an RFID system, video cameras and balances as they gathered pollen and nectar resources in the field. We found high inter-individual variability in foraging performance. A minority of very active bees (the elite bees) undertook the majority of the colony's foraging activity (figure 3). A minority of foragers collected pollen (table S1), and none of them did it exclusively (figure 2). There was no relationship between inter-individual variability in foraging performance and weight on departure or the age when the individual started foraging (figure S2 and S3). We found, however, that individuals adapted their behaviour depending on the resource they collected. Pollen collection trips took longer (figure 4), and foragers adapted their weight on departure from the colony according to the duration of the trip and the type of resource collected (figure 5). Finally, bees increased their foraging efficiency with individual foraging experience: their probability to collect pollen on a given trip increased with their experience (figure 7), they foraged more actively day after day (figure 8), became more efficient at collecting non-pollen resources (figure 9) and finally spent less time in the

hive between two trips (figure 10). The elite bees, because they had more experience, were also the most efficient foragers (figures S4 and S5).

In our data, scoring bees that returned with pollen loads was unambiguous, but it was impossible to judge the crop content of returning foragers. For bees that returned to the hive without pollen we could not discriminate between an unsuccessful trip, a successful nectar collection or a successful water collection, and thus we classified trips as simply pollen or non-pollen collection. Using this approach, our data clearly demonstrate that the distinction between the pollen and nectar collection foraging forces in bees is far from absolute, thereby confirming recent short-term observations [7,25,33,54–56]. Pollen collection was undertaken by a minority and later in life, contributing to a diversity of individual foraging profiles.

Our experimental design was constrained to the use of small colonies, with a minimum amount of brood because of the limitations on forager traffic imposed by the RFID sensors at the entrance. The foraging behaviour we have seen might be different from that of a full size commercial honey bee colony. Bees often start foraging at a younger age when in a small colony, and this might also explain the relatively short average lifetime of our individuals (5 days) [46].

Elite foragers

In our study 19% of the foragers completed more than 50% of the total number of foraging trips in their colonies (figure 3A). This is comparable to the skew in foraging effort reported by Tenczar et al [7] in the same species. These authors compared individual activity with total colony activity on a day-by-day basis because of a limit in the accuracy of their RFID sensor detection. Applying Tenczar *et al.*'s [7] particular metric to our data (figure 3B), we found an even greater skew in the foraging distribution: 12% of the workers performed more than 50% of the colony's daily activity [7].

Bees increase their efficiency with experience

Our study indicates a clear connection between performance and experience (figures 7 to 10). Bees are incredible learners [57]. They learn the features of their environment and accordingly enhance their foraging skills [58]. With experience, bees improve in navigating to their food sources [17,59–61], and flower discrimination (based on colours of odours or shape discrimination [57]) and flower handling [62,63]. Individual

experience in foraging tasks has been reported to influence individual's foraging strategies in other insect species. For example, ants (*Cerapachys biroi*) that are successful at foraging during their first attempts are more likely to continue foraging later in life [64]. Bumblebee (*Bombus impatiens*) workers that start off foraging collecting pollen collect more pollen during their lifetime than the ones that started collecting nectar [65]. Thus the early experience, during first foraging trips, influences the rest of an individual's foraging life.

Previous studies of honey bees that have observed a skew in foraging activity of social insects either failed to identify any particular link with higher foraging efficiency in the active individuals [7] or reported that the most active foragers were also the most efficient [66,67] but did not analyse the link between activity and efficiency. In our case, the more active foragers were also the more efficient because bees increased their efficiency with accumulated experience [33,37,68,58].

Variation between pollen and non-pollen collection trips

Bees also finely adapted their behaviour according to the resource they were collecting. We observed slight adjustments of weight on departure with both trip duration and the type of resource they would collect from the forthcoming trip (figure 4). Bees weighed more on departure when foraging for longer pollen collection trips indicating that they left the hive carrying the content in their crop (presumably honey) when performing long pollen collection trips. By contrast, bees' weight on departure decreased with duration of non-pollen trips. These findings could be interpreted in two ways. Either foragers anticipate the nature of their forthcoming trip and depart the hive loaded with honey to support their estimated energetic cost of the foraging trip. Alternatively, because the crop content on departure limits the energy a bee can allocate to its foraging flight, pollen collection trips may be shortened if bees leave the hive with little nectar. For non-pollen trips, if bees were collecting nectar or water, trip duration may be limited by the crop capacity and hence bees departing the hive with a partially full crop will have a limited foraging capacity.

While the latter hypothesis seems the most parsimonious, Harano and colleagues [69,70] suggested that bees adjust their weight on departure from the hive in anticipation of their foraging trip. Similar to our study, these authors found that bees left their hive with more sugar (larger amounts of more concentrated nectar) when collecting pollen than nectar [69,70]. More remarkably, perhaps, they also found that

dance followers filled their crop according to their interpretation of the waggle dance [71], and left the hive with more sugar after witnessing a longer waggle dance (indicating a food source further away) than when witnessing a short dance [71]. This suggests that bees are able to anticipate the energetic cost of the journey they are about to undertake according to their personal experience or the social information shared by the waggle dance [69–72]. Nevertheless, we found that there is no positive correlation between the weight on departure of a first pollen trip on a given day and the weight on departure of any other pollen trips on the same given day for a given bee (figure 5). This suggests that, bees do not seem to adjust their load on departure according to their prior individual foraging experience within a given day.

Inter-individual variability in foraging in a stressful environment

Our results reveal an important point of vulnerability in a colony's nutritional balance. For more than a decade now, there have been concerns over increased rates of honey bee colony failure [73], due to diverse environmental stressors that impair the lifespan and the cognition of the foragers [74]. Our data have shown that foraging performance improves with experience. As a consequence the most active bees in the colony also tend to be the most efficient. This promotes a skew in performance toward a minority of high-performing bees. These high performers will be the bees most frequently exposed to environmental stressors. Stressors that shorten foragers' lifespan [43,75–85] may prevent bees accumulating enough experience to maximise their foraging efficiency. Since foraging efficiency takes time to learn, if a colony loses its experienced and efficient foragers it may be able to replace them in terms of number, but it cannot immediately replace them in terms of performance. Perry *et al.* [86] showed that an accelerated maturation of individual foragers, that lead to precocious foragers, will end up reducing the foraging lifetime of such individuals and can impair the whole colony dynamic. Our results suggest that not only the foraging activity, depicted as the number of trips performed by a bee by Perry *et al.* but also foraging efficiency would be severely impacted.

As a consequence of pollen being more likely to be collected by more experienced bees, we propose that a stressor on the forager population could cause a significant nutritional imbalance to a colony leading to an excess of nectar (carbohydrates) collection and a deficit of pollen (protein and lipids) which is detrimental for bee survival under normal [87,88] and stressed conditions [89]. There is

some evidence supporting this hypothesis, as some stressors reduce pollen collection: honey bees infected by *Nosema ceranae* collect less pollen than non-infected bees [42] so do immune-stressed bees (Célia Bordier *et al.* submitted in JEB, manuscript in appendix 1). Also, bumblebee colonies exposed to realistic doses of neonicotinoids collect less pollen than control colonies [40,41]. A pollen deficit in a colony could establish a vicious cycle of nutritional imbalance since pollen starved larvae become poor foragers themselves [90], potentially compounding a nutritional imbalance for another generation.

Modelling colony growth dynamics would be a useful way to explore the interaction between the features of the pollen forager force and the colony-level response to stressors. Models have proven extremely useful for understanding the problem of honey bee colony collapse [91–96]. So far, however, few models have considered pollen and nectar fluxes separately through a colony [93,95]. Lihoreau *et al.* [97,98] proposed an approach to model the complex nutritional system of insect colonies based on nutritional geometry, but their model does not incorporate the fine understanding of the diversity of individual foraging strategies among foragers. Our study emphasises the need to realistically address both pollen and nectar fluxes in models of colony nutrition, and provides the field data needed to begin modelling of these phenomena.

Authors' contributions:

SK, ES, CJP and ABB conceived the study and designed the experiment. SK and XJH conducted the experiments. SK and ABB analysed the data. SK, XJH, ES, CJP, ML, JMD and ABB wrote the manuscript.

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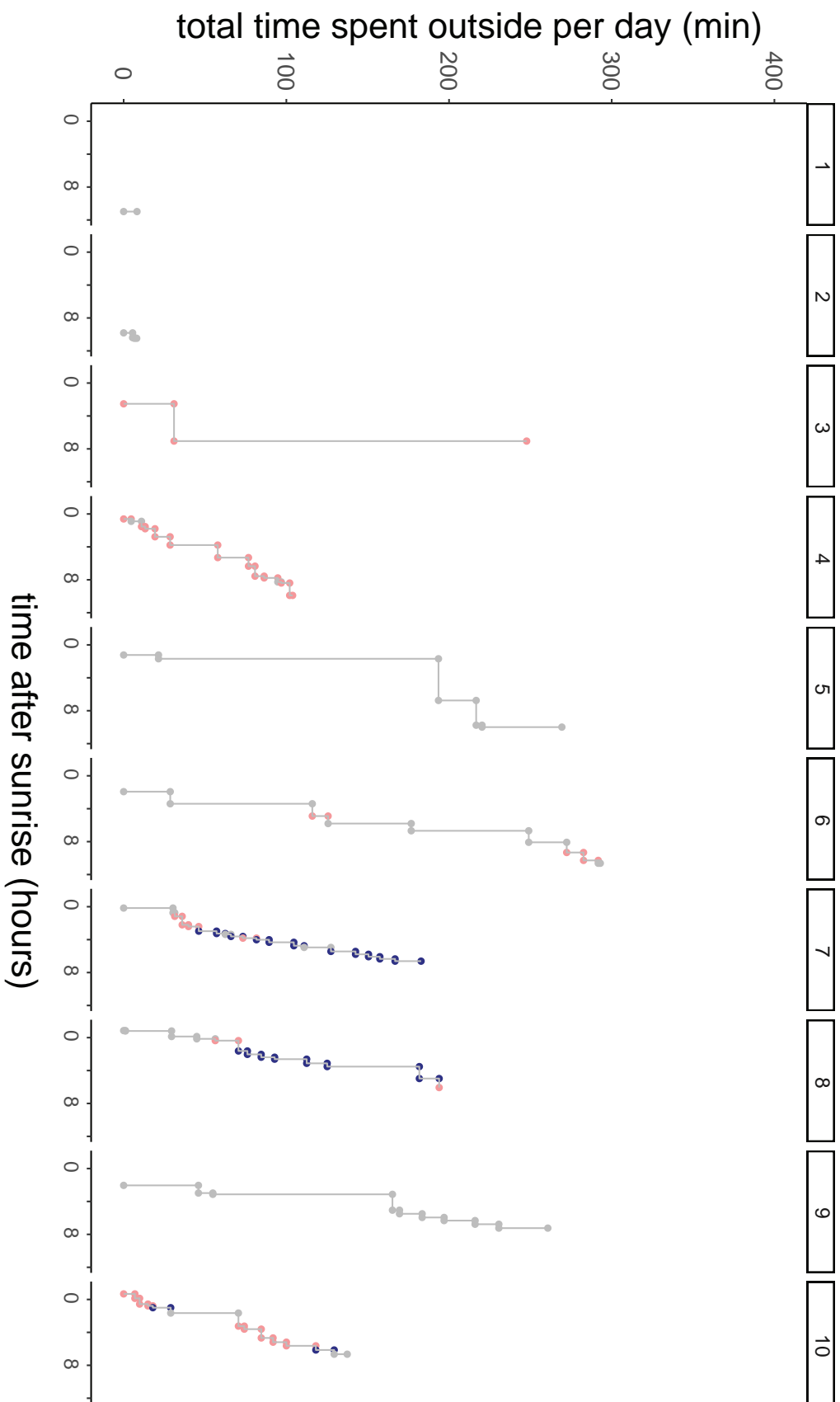
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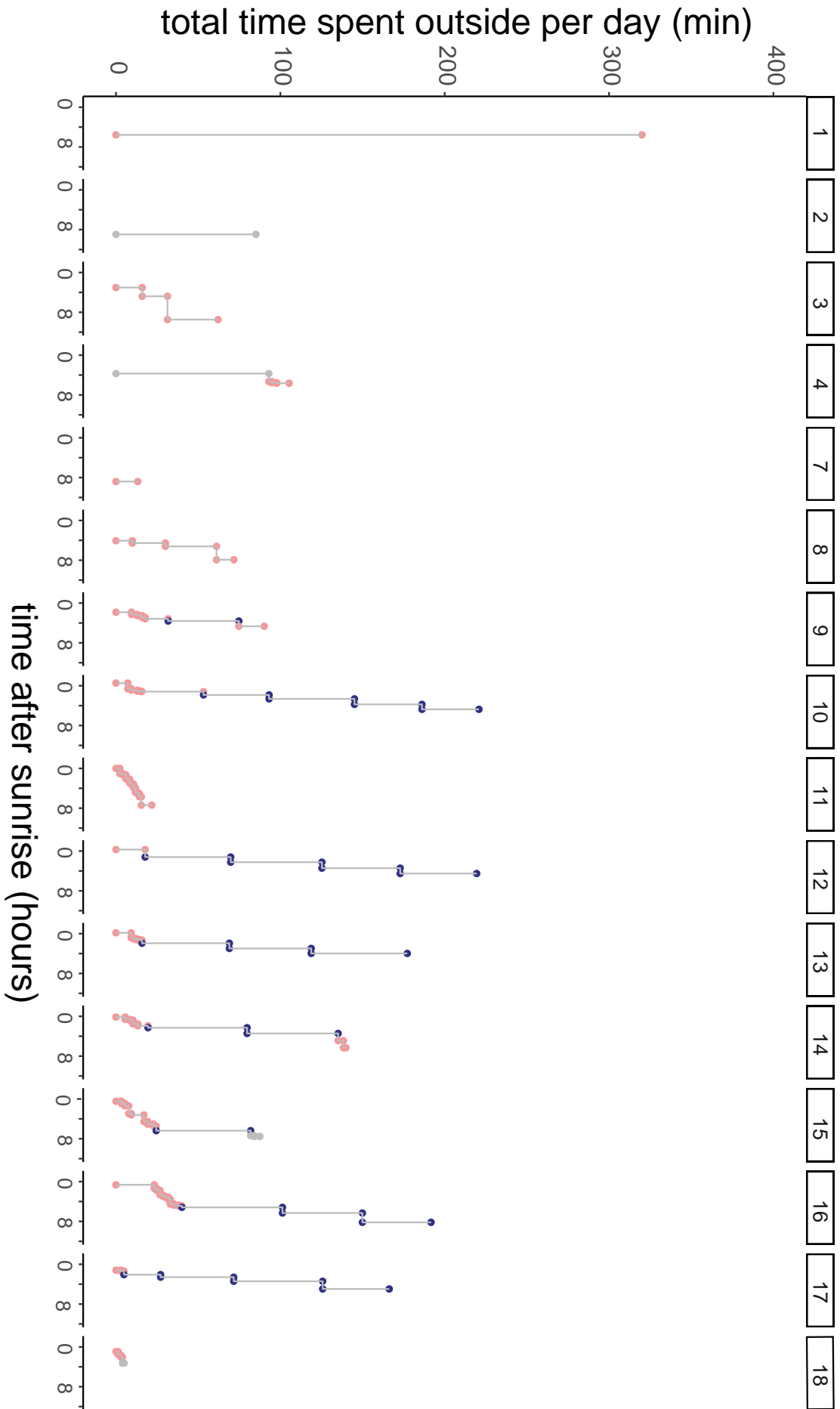
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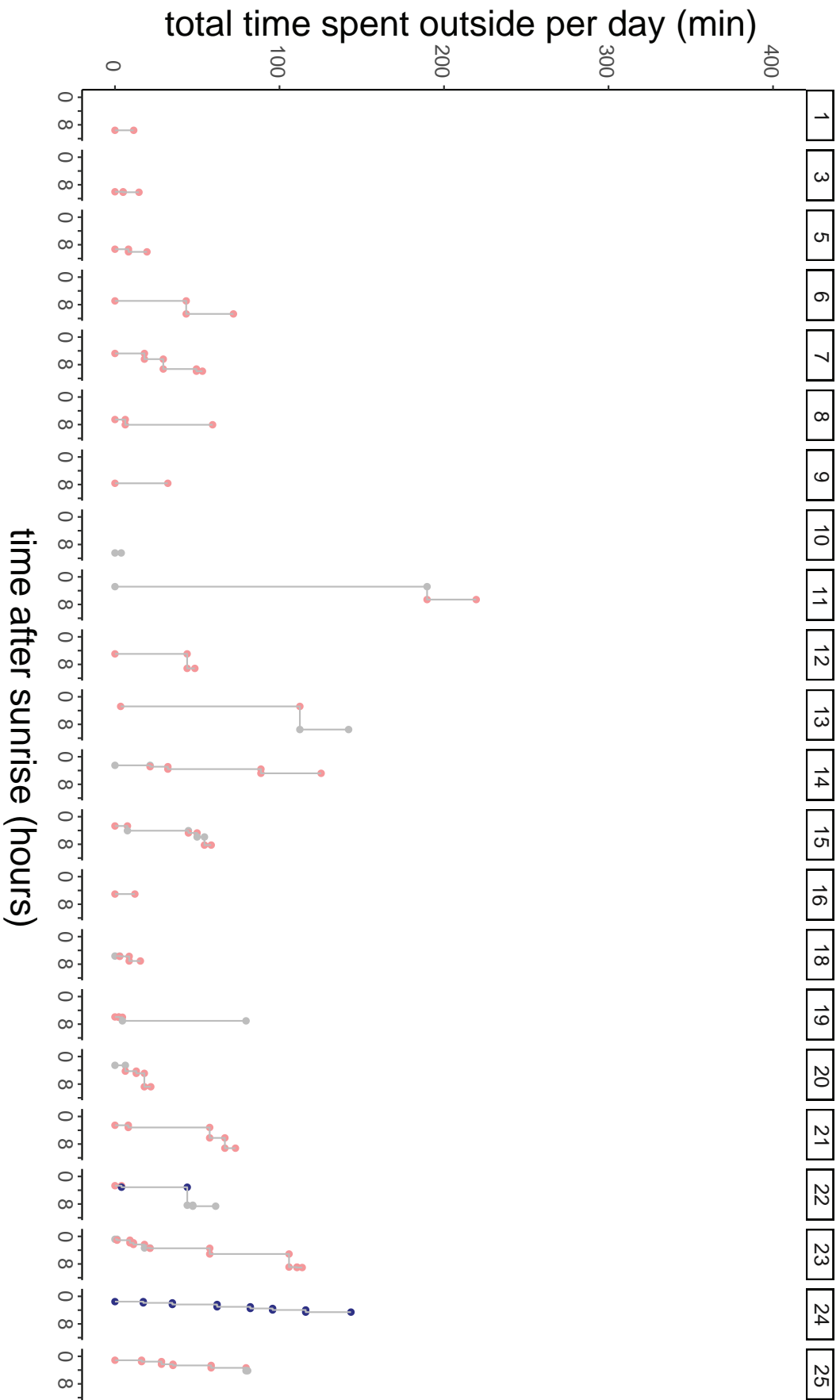
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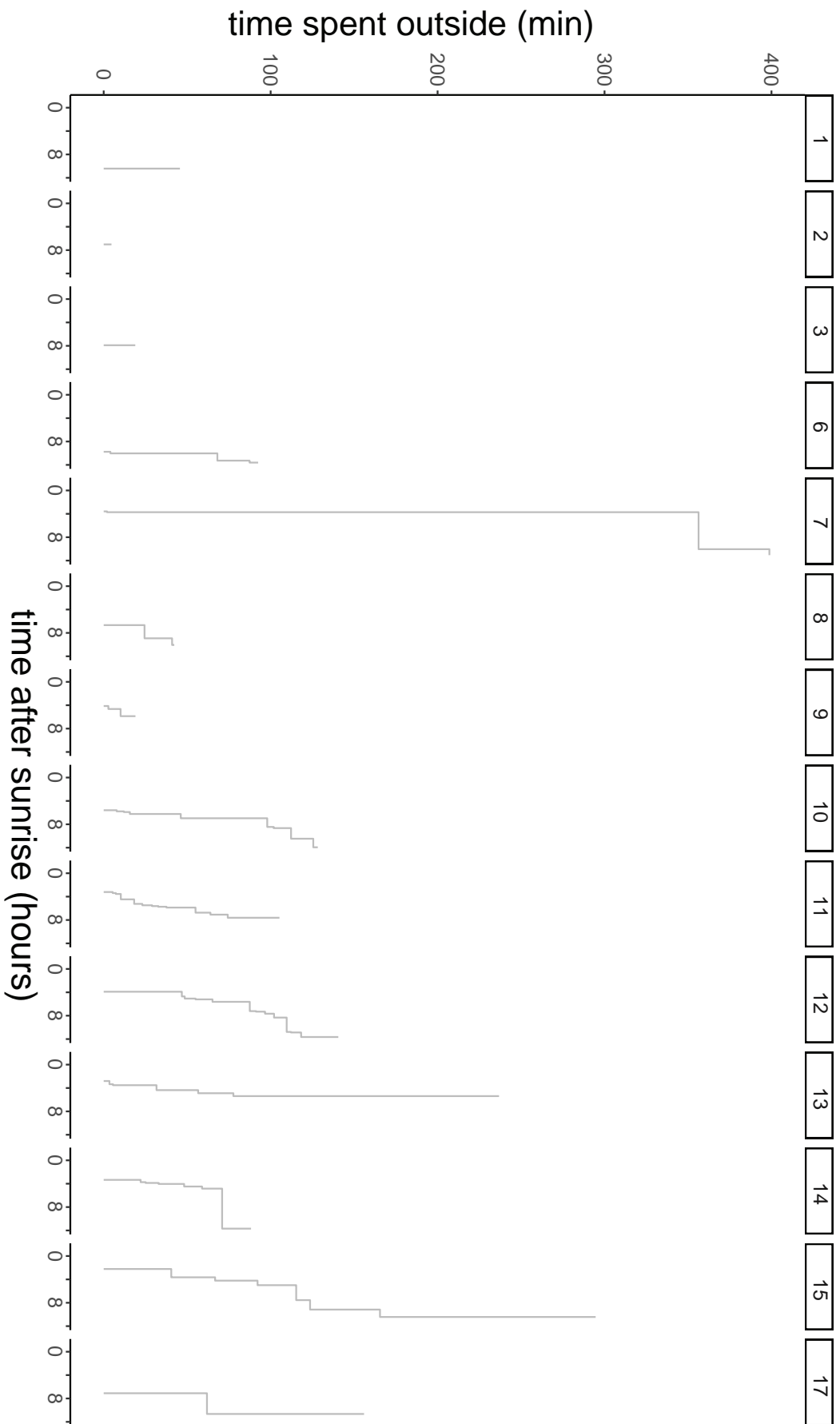
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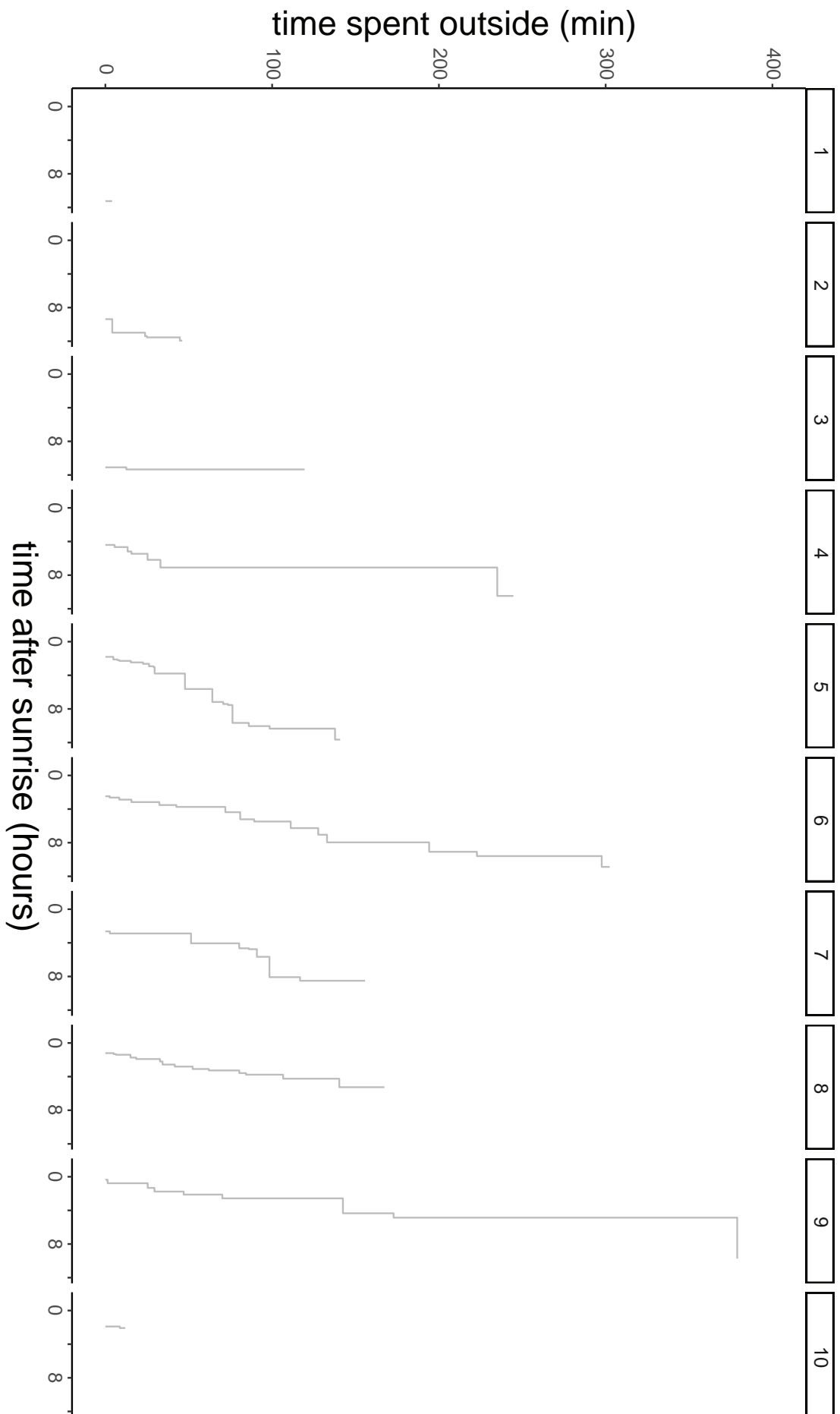
SUPPLEMENTARY MATERIAL:











<- **Figure S1:** Example of foraging trips patterns for two particularly active pollen foragers. Pink points delimit duration of non-pollen trips; blue represent pollen trips and grey represent trips with no information about the resource collected (NA). X axis represents, on each particular foraging day, time after sunrise in hour. The Y axis represent the cumulative time the bee spent outside the hive on a particular day.

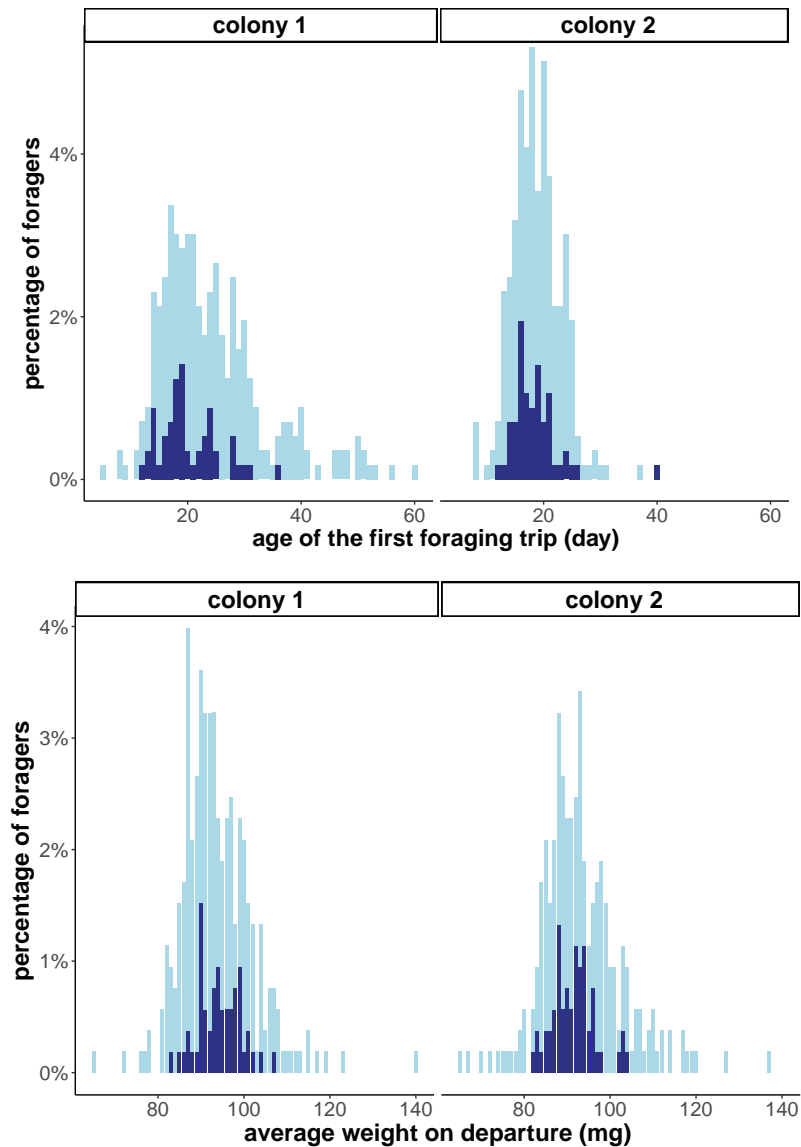


Figure S2: A. Proportion of bees of a given colony that started foraging at a given age (in days since emergence). Left: colony 1, right: colony 2. Dark blue represents the elite bees and light blue represents the rest of the foragers. Elite bees of colony 1 started to forage earlier than the other bees. Wilcoxon rank test: $W = 8\ 368$, $P = 0.0005$. There was no difference of distribution for the age of first foraging between the elite bees group and the rest in colony 2. Wilcoxon rank test: $W = 6\ 717$, $P = 0.194$. **B.** Proportion of bees of a given colony (panel 1: colony 1 and panel 2: colony 2) for a given average weight on departure (in mg). Dark blue represents the elite bees and light blue represents the rest of the foragers. There is no difference of distribution for the average weight on departure (for pollen or non-pollen foraging trips) (GLMM, $N = 1\ 870$, $F_{(1,143.3)} = 1.52$, $P = 0.390$, Table S4 for model selection).

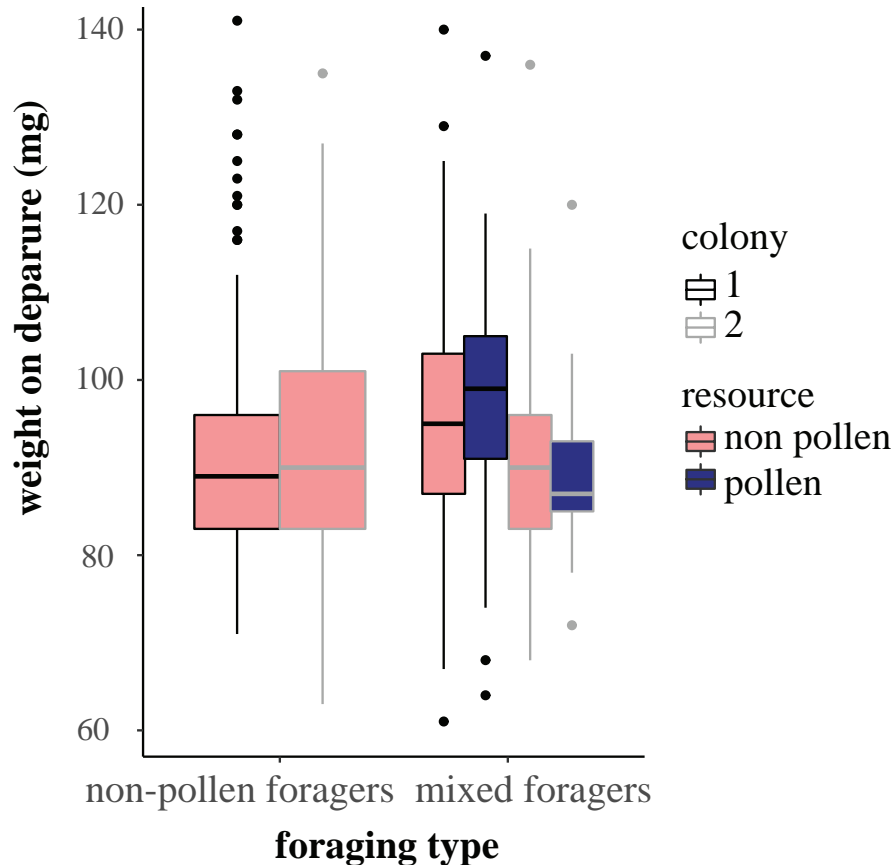


Figure S3. Difference of weight on departure for the different types of foragers and for non-pollen (pink boxplots) or pollen (blue boxplots) trips. There was no difference in weight on departure between non-pollen and mixed foragers when collecting non-pollen resources in both colonies. Mixed foragers of colony 1 (black lines and dots) left the hive heavier when collecting pollen than when departing to collect nectar. Mixed foragers of colony 2 (grey lines and dots) left the hive lighter when collecting pollen than when collecting nectar. Non-pollen foragers = 410 individuals (889 trips). Mixed foragers = 154 individuals (727 non-pollen trips, 254 pollen trips). Boxplot: the line shows the median, boxes and the whiskers represent interquartile ranges, dots represent outliers (data greater than third quartile + 1.5*(interquartile range), or less than first quartile - 1.5*(interquartile range)). Stars indicate significant differences (GLMM, non-pollen foragers difference: N = 889, P = 0.45, mixed foragers: Colony 1: N = 809, P = 0.019, Colony 2: N = 172, P = 0.032, table S3).

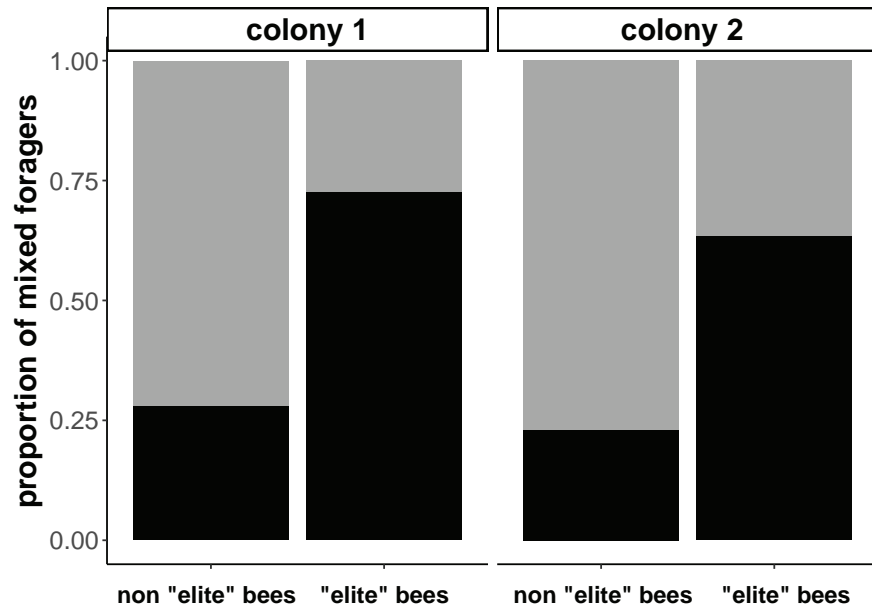


Figure S4: Relative proportions of mixed foragers among elite bees and other foragers. While non-pollen foragers (in grey) represent the majority of non-elite bees, elite bees are mostly mixed foragers (i.e. those that performed at least one trip collecting pollen, in black). (Left: colony 1: $\chi^2 = 55.68$, DF = 1, P < .0001; right: colony 2: $\chi^2 = 46.993$, DF = 1, P < .0001).

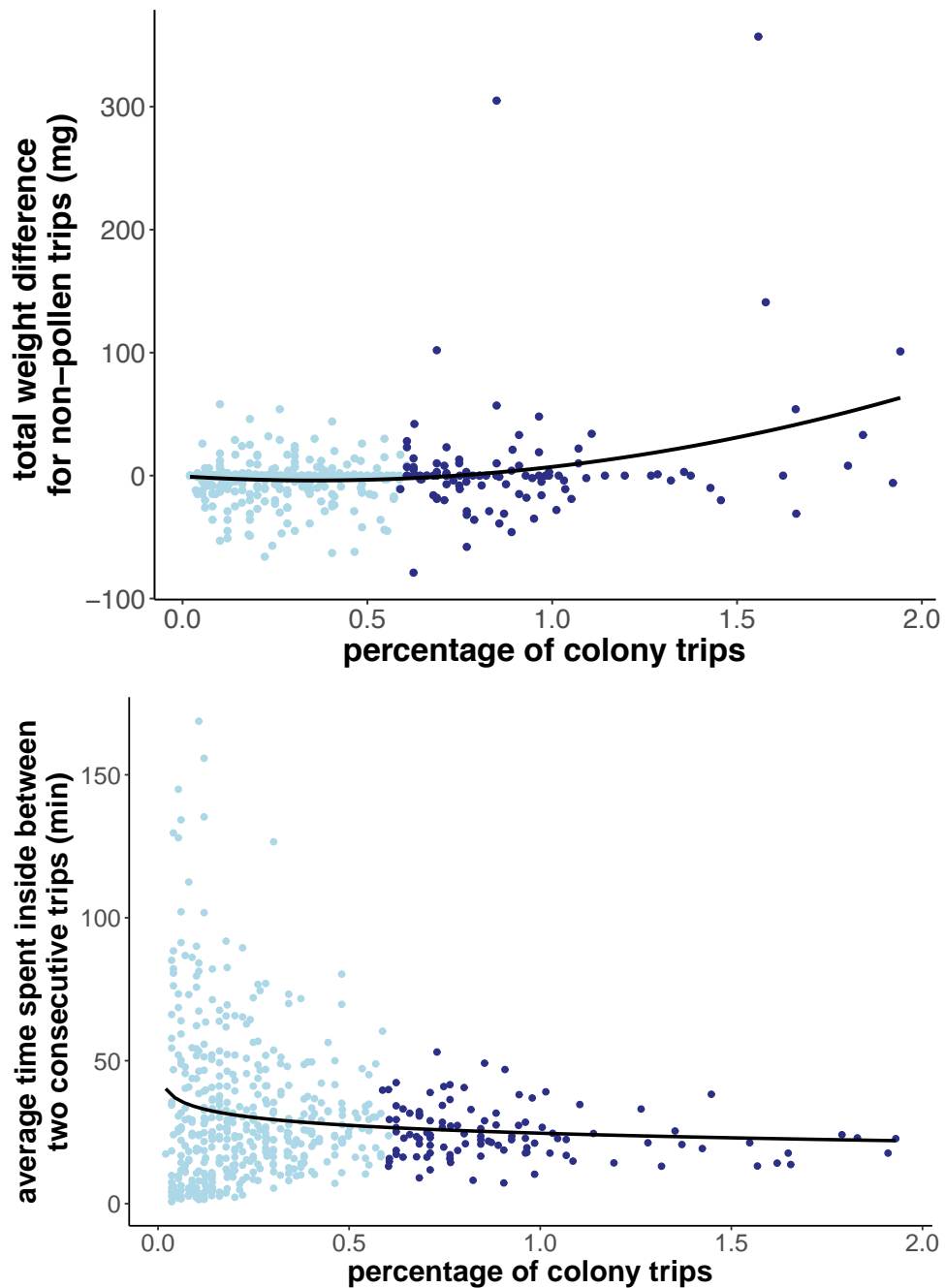


Figure S5: Comparative foraging efficiency of elite (*dark blue*) and other foraging bees (*light blue*) (N = 527 individuals, colony 1: 281, colony 2: 246). **A.** Accumulated weight difference for each of the bees. The most active bees accumulated larger amounts of food. General quadratic model: $y \sim ax^2 + bx + c$: $a = 25.34 \pm 5.54$, DF = 20.16, $T = 4.57$, $P < .0001$; $b = -19.27 \pm 10.55$, DF = 1887, $T = -1.85$, $P = 0.068$; $c = 1.05 \pm 15.69$, DF = 835, $T = 0.067$, $P = 0.947$, elite: 1.85 ± 4.81 , DF = 2062, $T = 0.38$, $P = 0.699$. Model selection in table S4C. **B.** Average time spent in the hive between two consecutive trips (inter-trips duration). Only the inter-trips duration inferior to 200min have been kept in this analysis. The more active the bees are, the shorter their inter-trip durations are. General logarithmic model: intercept: 26.51 ± 5.69 , DF = 508, $T = 4.65$, $P < 0.0001$; slope: -3.03 ± 1.07 , DF = 579, $T = -3.27$, $P = 0.039$, elite: -1.91 ± 3.40 , DF = 579, $P = 0.693$. Model selection in table S4D.

Table S1: Number of trips and foragers recorded per colony. The first five trips for each bee (orientation flights) were excluded. An individual is considered ‘non-pollen forager’ if it has never collected pollen. An individual is considered ‘mixed forager’ if it has performed at least one trip for pollen. NA designed a trip where the kind of resource was not identified; bees recorded with only NA flights were excluded (see material and methods).

		Colony 1	Colony 2	All
Number of foragers	<i>Non-pollen foragers</i>	208	202	410
	<i>Mixed foragers</i>	87	67	154
	<i>Total</i>	295	269	564
Number of foraging trips	<i>Non-pollen</i>	2,159	1,076	3,235
	<i>Pollen</i>	371	138	509
	<i>NA</i>	2,399	4,380	6,779
	<i>Total</i>	4,929	5,594	10,523

Table S2: A. Summary of linear model representing the trip duration according to the kind of resources collected for mixed foragers. Trip duration has been natural log transformed to obtain a Gaussian distribution. Bees anticipate their journey: when they are leaving the hive for pollen, they leave heavier for a longer trip. When they are leaving the hive to collect non-pollen resource, they leave lighter for a longer trip. Significant effects are in bold. Model selection in table S4A. **B.** Summary of linear model representing the weight on departure according to the trip duration for mixed foragers. Trip duration has been natural log transformed to obtain a Gaussian distribution. Bees anticipate their journey: when they are leaving the hive for pollen, they leave heavier for a longer trip. When they are leaving the hive to collect non-pollen resource, they leave lighter for a longer trip. Significant effects are in bold. Model selection in table S4B.

	Estimate (SE)	df	t	P
<i>a. log(trip duration) ~ resource + (1+resource day) + (1+resource cohort/ID)</i>				
Intercept	97.49 (1.39)	7.67	38.20	<.0001
Pollen trips	0.82 (0.07)	81.75	-3.27	<.0001
<i>b. weight on departure ~ log(trip duration) * resource + colony + (1+log(trip duration) day) + (1+log(trip duration) cohort/ID)</i>				
NP trip intercept	97.49 (1.39)	59.90	70.00	<.0001
NP trip slope	-1.22 (0.37)	141.60	-3.27	0.001
P trip intercept	92.01 (2.95)	952.50	-1.87	0.063
P trip slope	1.44 (0.95)	879.80	2.82	0.005
Colony 2	93.24 (1.44)	68.20	-2.95	0.004

Table S3: a. Summary of linear model looking at the difference of weight on departure between non-pollen foragers and mixed foragers for non-pollen trips. There is no difference in the weight on departure between mixed and non-pollen foragers for non-pollen trips (see details of model selection in table S4). **b.** Summary of linear model looking at the difference of weight on departure between non-pollen trips and pollen trips for the mixed foragers. (see details of model selection in table S4).

	Estimate (SE)	df	t	P
a. weight on departure ~ forager type * colony + (1+forager type/day)+ (1+forager type cohort/ ID)				
Non pollen foragers colony 1	94.56 (1.77)	3.36	53.3	<.0001
Mixed foragers colony 1	95.01 (1.53)	2.06	0.31	0.786
Non pollen colony 2	93.31 (2.77)	3.41	-0.31	0.777
Mixed foragers colony 2	92.02 (2.53)	2.98	-1.00	0.390
b. weight on departure ~ resource * colony + (1+resource day) + (1+resource cohort / ID)				
Intercept colony 1	93.86 (0.84)	6.70	111.2	<.0001
Resource colony 1	98.99 (2.72)	1.84	1.85	0.215
Intercept colony 2	91.27 (1.50)	17.55	-1.72	0.102
Resource colony 2	90.83 (4.29)	197.02	-1.86	0.186

Table S4: Selection of the best binomial model representing the probability to collect pollen (resource) with experience (number of trips performed). Best model is highlighted in bold.

	df	AIC	Loglik	Chi²	P
a. Log(trip time) ~ resource	3	17592	-8793		
Log(trip time) ~ resource + (1+resource) day + (1+resource) cohort/ID	12	5245	-2610	12364	<.0001
Log(trip time) ~ resource + colony + (1+resource) day + (1+resource) cohort/ID	13	5245	-2609	2.1	0.15

	df	AIC	Loglik	Chi ²	P
Log(trip time) ~ resource * colony + (1+resource) day + (1+resource) cohort/ID	14	5247	-2609	0.1	0.746
b. Weight on departure ~ log(trip duration) * resource	3	7495	-3744		
Weight on departure ~ log(trip duration) * resource + (1+log(trip duration)) day identity + (1+log(trip duration)) cohort/ID	9	7334	-3657	173.6	<.0001
Weight on departure ~ log(trip duration) * resource + colony + (1+log(trip duration)) day identity + (1+log(trip duration)) cohort/ID	11	7330	-3654	7.6	0.022
Weight on departure ~ log(trip duration) * resource * colony + (1+log(trip duration)) day identity + (1+log(trip duration)) cohort/ID	14	7398	-3651	6.3	0.097
c. Total non-pollen income ~ percentage of colony trips + elite	3	5327.1	-2660.5		
Total non-pollen income ~ percentage of colony trips + elite + (1+ percentage of colony trips) cohort/ID	5	5165.1	-2577.6	198.3	<.0001
Total non-pollen income ~ exp(percentage of colony trips) + elite + (1+ exp(percentage of colony trips)) cohort/ID	5	5153.4	-2560.6	34.5	<.0001
d. Intertrips duration ~ percentage of colony trips + elite	3	5049.7	-2521.8		
Intertrips duration ~ percentage of colony trips + elite + (1+ percentage of colony trips) cohort/ID	5	4873.7	-2431.9	189.3	<.0001
Intertrips duration ~ log(percentage of colony trips) + elite + (1+ log(percentage of colony trips)) cohort/ID	5	4853.6	-2421.8	20.13	<.0001
e. Weight on departure ~ elite	9	12338	-6135		

	df	AIC	Loglik	Chi ²	P
Weight on departure ~ elite + (1+ elite 9 cohort/ID)		14182	-7082	15.49	0.003
Weight on departure ~ elite + colony + 10 (1+elite cohort/ID)		14181	-7080	3.27	0.071
Weight on departure ~ elite * colony + 11 (1+elite cohort/ID)		14183	-7080	0.08	0.777
Weight on departure ~ elite * colony + 14 (1+elite Day) + (1+elite) cohort/ID		12284	-6128	13.4	0.004
f. Weight on departure ~ forager type	3	12530	-6204		
Weight on departure ~ forager type + (1+forager type cohort/ID)	9	12338	-6135	132.2	<0.0001
Weight on departure ~ forager type + colony + 10 (1+forager type cohort/ID)		12290	-6135	1.9	0.169
Weight on departure ~ forager type * colony + 11 (1+forager type cohort/ID)		12291	-6135	1	0.319
Weight on departure ~ forager type * colony + (1+forager type Day) + (1+forager type) cohort/ID	14	12284	-6128	13.4	0.004
g. Resource ~ experience	2	2235.1	-1110.0		
Resource ~ experience + 8 (1+experience) cohort/ID	8	2177.5	-1050.8	118.51	<.0001
Resource ~ experience + colony + 9 (1+experience) cohort/ID		2117.8	-1049.9	1.68	0.19
Resource ~ experience * colony + 10 (1+experience) cohort/ID		2119.6	-1049.8	0.30	0.58
h. Trip per day ~ day foraging	3	13798	-6896.3		
Trip per day ~ day foraging + (1+day foraging) day identity + (1+day foraging) cohort/ID	12	13366	-6670.9	119.6	<.0001

	df	AIC	Loglik	Chi ²	P
Trip per day ~ day foraging + colony + (1+day foraging) day identity + (1+day foraging) cohort/ID	13	13365	-6669.5	2.82	0.092
i. Weight difference ~ experience	5	7414.0	-3702.0		
Weight difference ~ experience + (1+experience) cohort/ID	9	7403.0	-3692.5	14.54	<.0001
Weight difference ~ experience + colony + (1+experience) cohort/ID	10	7405.0	-3692.5	0.03	0.862
Weight difference ~ experience * colony + (1+experience) cohort/ID	11	7406.9	-3692.5	0.03	0.856
j. Intertrips ~ experience	5	75966.4	-37980		
Intertrips ~ experience + (1+experience) cohort/ID	9	75433	-37707	30.31	<.0001
Intertrips ~ log(experience) + (1+log(experience)) cohort/ID	9	75416	-37699	16.44	<.0001
Intertrips ~ log(experience) + colony + (1+log(experience)) cohort/ID	10	75417	-37699	0.81	0.367
Intertrips ~ log(experience) * colony + (1+log(experience)) cohort/ID	11	75419	-37698	0.87	0.350

Chapter 3: Inter-individual variability in the lifelong foraging activity of bumblebees



Chapter 3: Inter-individual variability in the lifelong foraging activity of bumblebees

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Abstract:

The bumblebee (*Bombus terrestris*) is a primitively eusocial species. A subset of the colony members forage to collect nectar and pollen from flowers. Bumblebee foragers vary in size and exhibit strong inter-individual differences in various aspects of foraging behaviour. Whether all foragers contribute in the same way to the colony's foraging force has not yet been examined. Here we studied the inter-individual variability in the foraging activity and performance of bumblebees using radio frequency identification and motion detection cameras at the nest entrance to automatically monitor the complete foraging history of every individual in a colony. We found that the majority of foraging trips were undertaken by a small group of individuals (elite foragers) that did not differ morphologically from the other foragers. Elite foragers completed similar proportions of pollen and non-pollen trips to the less active foragers. Bees showed stable inter-individual variability in their tendency to forage for pollen and non-pollen resources through time. Foragers that collected both resources typically made longer trips that occurred later in the day for pollen. Our study adds to the growing evidence of high levels of behavioural inter-individual variability within castes of social insects.

Keywords: *Bombus terrestris*, foraging, behavioural plasticity, inter-individual variability, pollen, division of labour.

Introduction:

Animals, from mammals [1] to birds [2] and insects [3–5], show high levels of inter-individual behavioural variability. In social species, behavioural diversity can provide benefits at the group level, for instance through enhanced overall foraging performances or defence against predators [6,7].

In colonies of social insects, inter-individual variability results in a division of labour where different individuals perform different tasks for their entire life, or a period of it [8–10]. Individuals with different behavioural roles are often described as pertaining to different castes that structure the social organisation [11–13]. However, growing evidence indicate that members of the same caste can also show high levels of behavioural variation ([4,5], e.g. ants [14,15]; bees [16–18]; wasps [19,20]). In honey bees, for instance, workers specialised in food collection (the foragers) collecting nectar (carbohydrates) and pollen (protein and lipids) from flowers to supply the nutritional demands of all other colony members [21–23]. Foragers of the same colony do not equally contribute to this task ([24] and chapter 2). Rather, a subset of very active honey bees, described as “elite” foragers [24,25], make a disproportional contribution to the colony foraging effort (e.g. 20% of foragers contribute to 50% of all colony trips, see chapter 2). These elite individuals are not only the most active foragers [24] but also the most efficient at collecting pollen and nectar (chapter 2).

While inter-individual behavioural variability has been well-described in the advanced eusocial honey bee [8], where division of labour occurs as a pattern of age-based behavioural development [26–28], little is known about behavioural variability in other eusocial species. The bumblebees are described as primitively eusocial because colonies are smaller, annual rather than perennial and there are no discrete morphological castes in the females [11,12]. Very little is known about the extent of inter-individual variability in primitively eusocial species.

Primitively eusocial bumblebees are an interesting study case. Bumblebee colonies are composed of a reproductive queen, up to three hundred non-reproductive worker females [21], and variable numbers of males and virgin females towards the end of the season [29]. Only a handful of the workers (typically less than 10) engage in foraging tasks each day [21]. Non-foraging workers (nurses) stay inside the colony

to take care of the larvae and nest maintenance [9,21]. Unlike honey bees who progress between nurse and forager castes as they age [27,28], the distinction between nurses and foragers in bumblebees is influenced by body size as only the largest individuals tend to forage [9,21,30]. Bumblebee foragers show some degree of inter-individual behavioural variability. For instance, many foragers collect both pollen and nectar (mixed foragers), whereas others tend to specialise on nectar only [30–34]. Bumblebees also show consistent differences in route-following behaviour [18] and in olfactory learning performances [35]. While these studies bring clear evidence of behavioural variability among bumblebee foragers, whether such variability leads to differences in foraging activity and efficiency is still an open question.

To address this question, we developed an automated tracking system based on radio frequency identification (RFID) and motion detection cameras to monitor the complete foraging history of every bumblebee of a colony. RFID systems have been increasingly used to access the foraging activity of social insects (e.g. honey bees [24,36], ants [37], bumblebees [33,38–40]). This approach has enabled recording bee foraging behaviour in laboratory conditions [33,41] or testing the effects of environmental stressors, such as pesticides, on colony dynamics [38–40,42,43]. Here we analysed the complete foraging history of all individuals in a bumblebee colony exploiting natural resources. We investigated: (1) the variation in foraging activity between individuals; (2) whether foragers specialise in the collection of pollen or non-pollen resources (nectar), and; (3) whether pollen and non-pollen trips differ in duration or time during the day.

Material and methods:

Bee colony:

The experiment was performed from 20/09/2016 to 22/11/2016 at the apiary of the University Paul Sabatier (Toulouse, France). The bumblebee colony (*Bombus terrestris*) was purchased from Biobest (Westerlo, Belgium). Upon arrival, the queen and the workers (N = 50 bees) were transferred into a two-chamber wooden nest box. The colony was maintained in a dark room at the constant temperature of 24°C, and connected to the outside environment via a specially designed entrance tube (figure 1). Bees foraged on the university campus and the surrounding areas (figure S1).

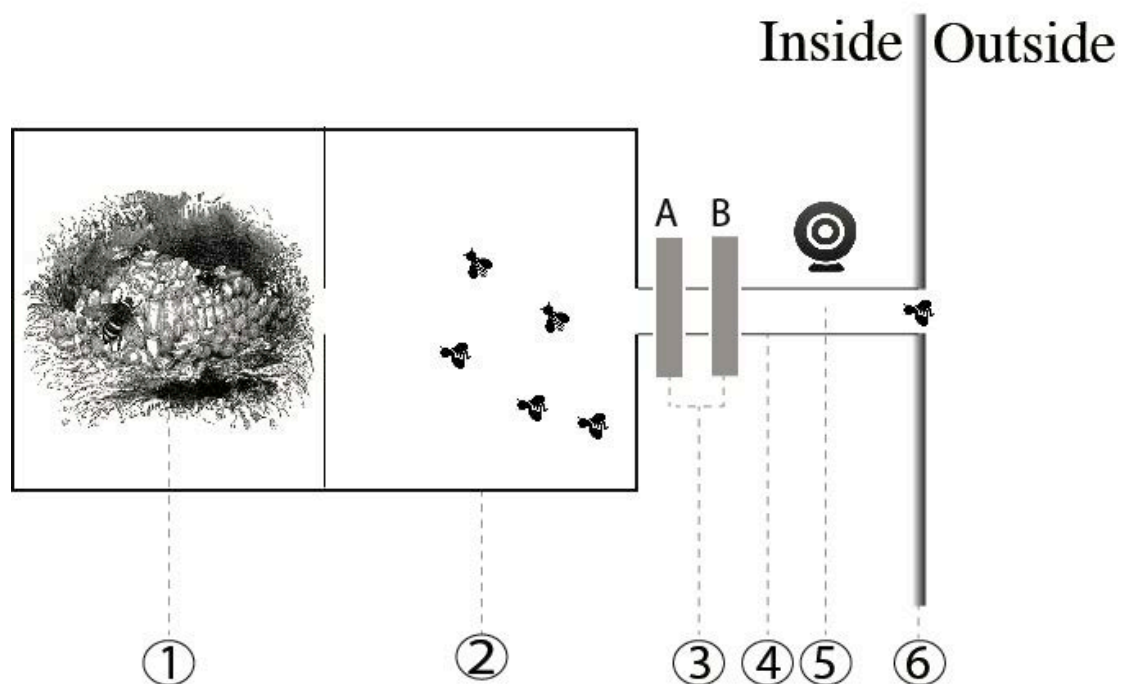


Figure 1: Experimental nest box. 1&2. Two-chambered wooden nest-box (15x28x11 cm). 1. Bumblebee nest. 2. Litter compartment. 3. RFID antennae. Detection of a bee at the two antennae A and B enabled inference of the direction of the bee in the colony entrance tube: AB means that a bee departed from the colony, BA means that a bee arrived at the colony. 4. Transparent plexiglass tunnel (\varnothing 3 cm). 5. Webcam. 6. Outside wall. Images from © The Graphics Fairy 2007.

Radio Frequency Identification (RFID) detection:

Bees could access the outside environment via a transparent entrance tube equipped with two RFID antennae (MAJA system, Microsensys GmbH, Erfurt, Germany) connected to a RFID reader. RFID chips were obtained from Microsensys (mic3 ® RFID transponders, Microsensys GmbH, Erfurt, Germany). Each square chip was 2x2mm long and weighed 2 mg (less than 2% of the bee's weight) and could be fixed to the bees' dorsal thorax. Each RFID chip had a unique 8-byte hexadecimal identifier that allowed us to track individual bees as they were detected by RFID antennae on exiting and entering the hive. Each RFID antenna recorded the time and the ID of a bee passing the antenna. Data were stored in a .xml file on a SD card in the RFID reader.

A webcam (Logitech, Lausanne, Swiss) was placed on a 30cm tall retort stand midway in the transparent tunnel, and a desk lamp (Ikea, France, light bulb: 15 W,

1250 lm, Ilight, Italy) was placed beside the tube to guarantee enough light for video capture. A motion detection video recording software (ZoneTriger, Omega Unfold Inc. Canada) was used to capture video footages of returning bees, allowing visual assessment of the resources they carried (*i.e.* presence/absence of pollen on the bee legs).

Experimental procedure:

We started the observations soon after the arrival of the colony and stopped when the queen died (63 days later). The colony was checked every day. Every new-born bee was gently extracted from the colony, placed along a ruler, and photographed (Fairphone 2 camera module, f/2.2, 8 megapixel). A RFID tag was then fixed on the thorax of the bee using a toothpick and super-glue (Loctite, Henkel AG&Co. KGaA, Düsseldorf, Germany), before replacing the bee in the colony.

Collating data on bee trips:

For every arrival and departure at the colony entrance, the bees had to pass through the two RFID antennae (figure 1). Antennae were labelled A and B to allow us to reconstruct foraging trips from the RFID record file. The sequence AB was interpreted as a bee leaving the colony. The sequence BA was interpreted as a bee returning to the colony. The time latency between two successive AB and BA sequences for the same bee corresponded to the duration of a single trip. Trips that lasted fewer than 60 seconds were unlikely to be foraging trips [33] and were thus removed from the dataset.

We estimated the maximum time for a returning foraging bee to cross the tunnel from the outside to the RFID gate B at 10s. Videos taken up to 10s before an entry RFID detection were inspected to score whether the tagged returning bee carried pollen (P), did not carry pollen (NP), or could not be reliably scored (NA) due to the presence of multiple bees in the tube or a bee moving in the tube at an angle where its body occluded the view of their legs. With this approach, we were not able to discriminate if a bee, returning to the hive without pollen (NP), performed a successful nectar foraging trip or an unsuccessful pollen or nectar foraging trip.

Data reliability:

A total of 335 bees were tagged. We identified foragers as bees that made at least one foraging trip (N = 223) and excluded individuals that had only 'NA' as load type over

all their trips (N = 124 bees). The final dataset contained 13,243 foraging trips performed by 99 bees.

We recorded the date of emergence from the pupa of each bee in order to keep track of their age (except for the 50 bees that were originally in the colony upon arrival in the lab). We also measured the inter-tegular span (IT span = distance between the two wing bases on the thorax) from pictures of each bee using imageJ (Mac OS X version, Wayne Rasband, Maryland, USA). IT span is a commonly used proxy of bumblebee body size [44].

Data analyses:

Unless specified, results are provided as mean values \pm SE. Data were analysed in R version 3.2.3 [45] (operating via Rstudio, version 1.0.136 [46]).

Differences in IT span between groups of bumblebees (foragers vs. non foragers; elite vs. non-elite; mixed vs. non-pollen foragers) were tested using Wilcoxon ranked tests for non-paired data.

The proportion of the total activity of the hive performed by a given proportion of individuals, in terms of number of trips, was examined using a Lorenz curve [47]. The Gini index [48] was used to assess the skew in the contribution of individuals in a common task: here, the number of foraging trips performed. The Gini index varies between 0 (all individuals contributed equally to the common task) and 1 (one individual performed all of the task).

The difference in trip duration and in time of the day when trips occurred between non-pollen and pollen trips was tested with Wilcoxon ranked test for paired data. The probability to engage in a pollen trip with foraging experience (number of foraging trips completed) was tested with a binomial Generalised Linear Mixed Model (GLMM) with bee IT span as covariate and bee identity as random factor, using the packages lme4 [49] and lmerTest [50].

We studied individual consistency in the proportion of pollen foraging using a binomial GLMM with Julian date, experience and IT span as fixed factors and bee identity as random factor. To estimate whether individuals presented different trends across days we compared this model with another model including Julian date as a random slope and bee identity as a random intercept, with a likelihood-ratio test (LRT) [51], using the packages lme4 [49] and lmerTest [50]. To quantify inter-

individual variability, we calculated individual repeatability as the percentage of total variance explained by the individual difference [51].

Results:

Inter-individual variability in foraging activity:

Among the 335 bees tagged during the 63 days of the experiment, 99 (30%) were seen foraging at least once. These foragers did not differ in body size from the non-foragers (non-foragers, N = 236, IT span (\pm SE) = 5.22 ± 0.05 mm, foragers, N = 99; 5.01 ± 0.16 mm, Wilcoxon rank test: W = 12749, P = 0.608).

On average the foragers performed a total of 134 trips (134 ± 19.32) for 10 days (9.95 ± 0.96), *i.e.* around 9 trips per day (8.97 ± 0.73). This activity was not equally distributed among foragers. A subset of 12 bees (11.11% of all foragers), hereafter called ‘elite’ foragers, completed more than 50% of the total number of the foraging trips in the colony (figures 2 and S2, Gini index = 0.67). This skew in the distribution of the foraging trips is even higher if we consider all the bees of the colony, including nurses that never foraged. In that case, 1.81% of the workers contributed to 50% of the total number of foraging trips (Gini index = 0.93).

All elite bees were ‘mixed foragers’ that collected pollen at least once, but never exclusively foraged for pollen. We found no difference in the proportion of pollen trips (proportion of individual foraging trips resulting in pollen collection) between elite and non-elite foragers (elite bees: 34.24 ± 5.86 %, non-elite bees: 24.81 ± 3.07 %, Wilcoxon rank test: W = 374, P = 0.105, figure S1). They did not differ either in body size as measured by IT span (elite: 5.26 ± 0.16 mm; non-elite: 4.61 ± 0.21 mm, Wilcoxon rank test: W = 448, P = 0.199).

Therefore the colony contained a few elite foragers that performed most of the foraging trips but were neither specialised on one type of resource, nor were they larger than the other foragers. In the absence of obvious difference between elite and non-elite bees, in what follows we analysed the behaviour of all the foragers together.

Specialisation for pollen and/or non-pollen trips:

Two thirds of the foragers completed both pollen and non-pollen trips (mixed foragers: N = 63, *i.e.* 63% of all foragers). One third of the foragers exclusively completed non-pollen trips (non-pollen foragers: N = 36, *i.e.* 36% of all foragers). One forager exclusively completed pollen trips (figure S4).

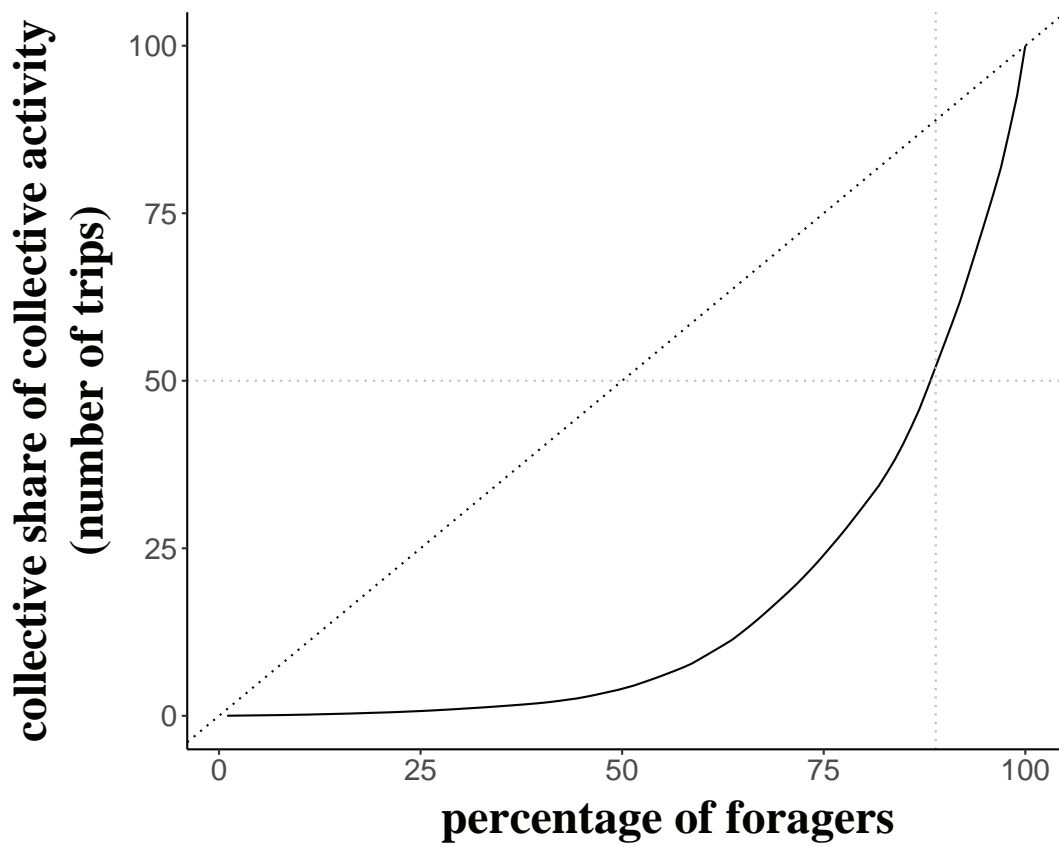


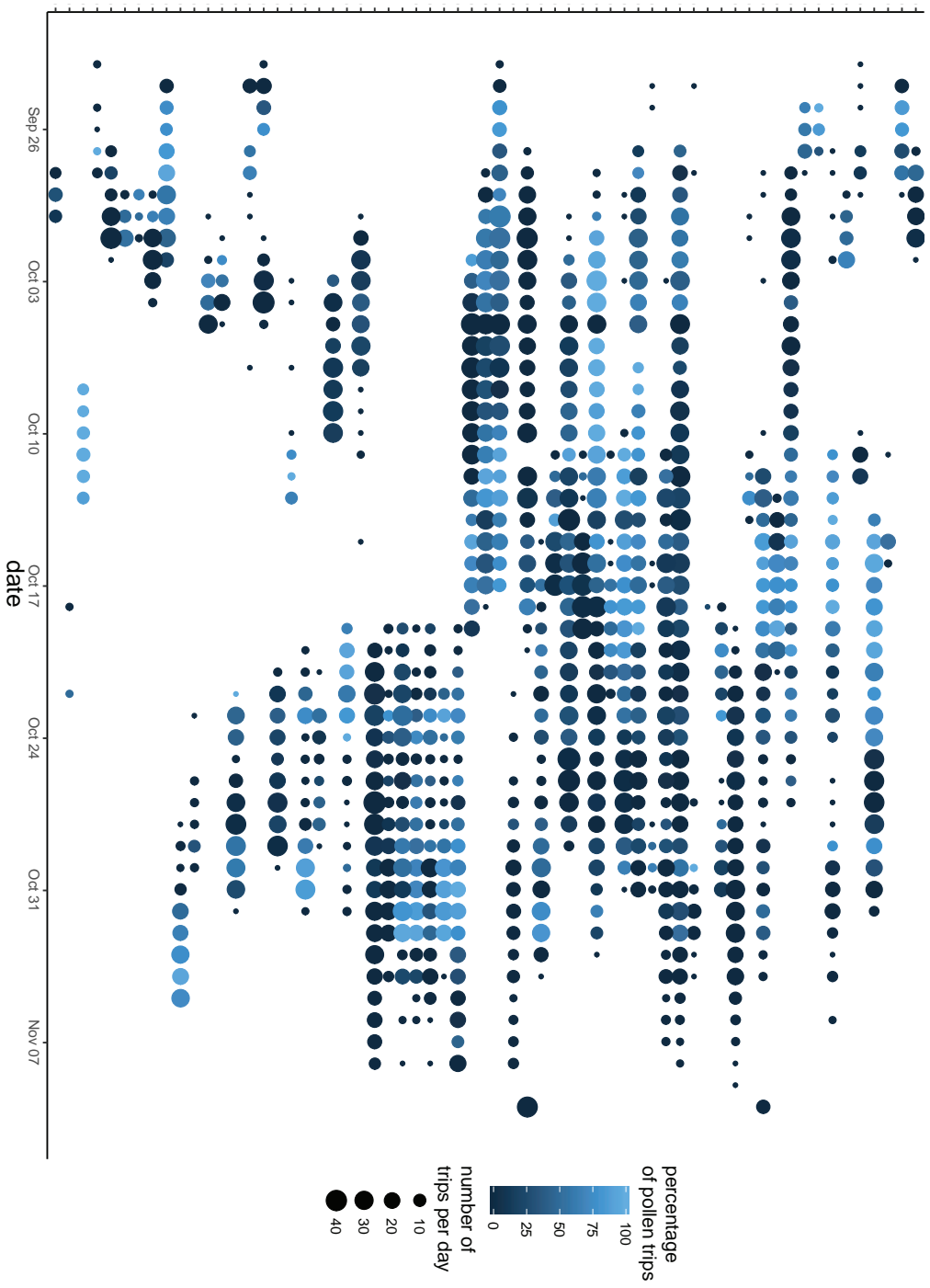
Figure 2: Lorenz curve representing the relative contribution of the foragers (X axis) to the collective effort of all foragers (Y axis). 11.11% of the foragers performed up to 50% of the total number of trips of the colony.

On average, non-pollen foragers performed fewer trips than mixed foragers (non-pollen foragers: $N = 36$, number of trips = 11.27 ± 4.18 , mixed foragers, $N = 63$, 204.39 ± 26.51 trips, Wilcoxon rank test: $W = 91$, $P < 0.0001$). On average, non-pollen foragers were larger than mixed foragers (non-pollen foragers, $N = 36$, IT span = 5.83 ± 0.27 mm, mixed foragers, $N = 63$; 4.76 ± 0.11 mm, Wilcoxon rank test: $W = 1138.5$, $P = 0.005$). Therefore, the specialisation of foragers for one type of resource was partially explained by morphological differences.

Differences between pollen and non-pollen trips in mixed foragers:

When considering mixed foragers only, the variability in the proportion of pollen foraging trips between individuals and between days was very high (figure 3). Our analyses of inter-individual variability indicated that bumblebees differed consistently in the proportion of pollen trips completed across days (verified with a likelihood ratio test between model with and without bee identity as random effect: $\chi^2 = 18.29$; $P < 0.001$), suggesting relatively stable behavioural profiles through time. Although significant, inter-individual variability explained 31% of the total variance of the proportion of daily pollen foraging trips (analysed as repeatability index from GLMM).

Interestingly, a similar level of variability in their proportion of pollen foraging trips could be attributed to variation between days (verified with a likelihood ratio test between model with and without Julian date as random slope effect: $\chi^2 = 3.84$; $P = 0.146$). This suggests that there is significant daily variation in resource availability, perhaps due to changes in environmental conditions (weather, competition). While some bumblebees ($N = 28$) specialised on pollen or non-pollen resources on different days, others (35 bees) foraged on both resources during the same day (figure 3 and S4). During days of mixed foraging, foragers typically started to forage for non-pollen and then switched for pollen later in the day ($N = 35$ bees, for 2,432 non-pollen trips, median time of the day: 13:00:02; and 3,298 pollen trip: 14:21:09. Wilcoxon paired rank test: $W = 1124700$, $P < 0.0001$, figures 4B, S4 and S5). Overall, bumblebees made longer pollen trips than non-pollen trips (figure 4A, Wilcoxon rank sum test $W = 4999100$, $P < 0.0001$).



<- **Figure 3: Heat map of all mixed foragers showing the proportion of pollen trips completed on a given day and the number of trips performed on that day.** The heat maps indicates that the foraging activity is highly variable between individuals (Y axis) and varies with time (date, X axis). N = 63 individuals.

Table 1: Binomial GLMM examining the temporal distribution of pollen collection, individual experience (as number of foraging days) and individual body size (inter-tetragular span). The model includes individual identity as random factor.

	Estimate (SE)	DF	z	P
<i>a. proportion of pollen collected ~ day identity (Julian date) + experience + IT span + (1 + day identity ID)</i>				
Intercept	-0.17 (0.10)	428	-1.69	0.09
day identity	-0.29 (0.19)	428	-1.51	0.132
experience	-0.12 (0.17)	428	-0.69	0.489
IT span	0.40 (0.21)	51	1.89	0.059

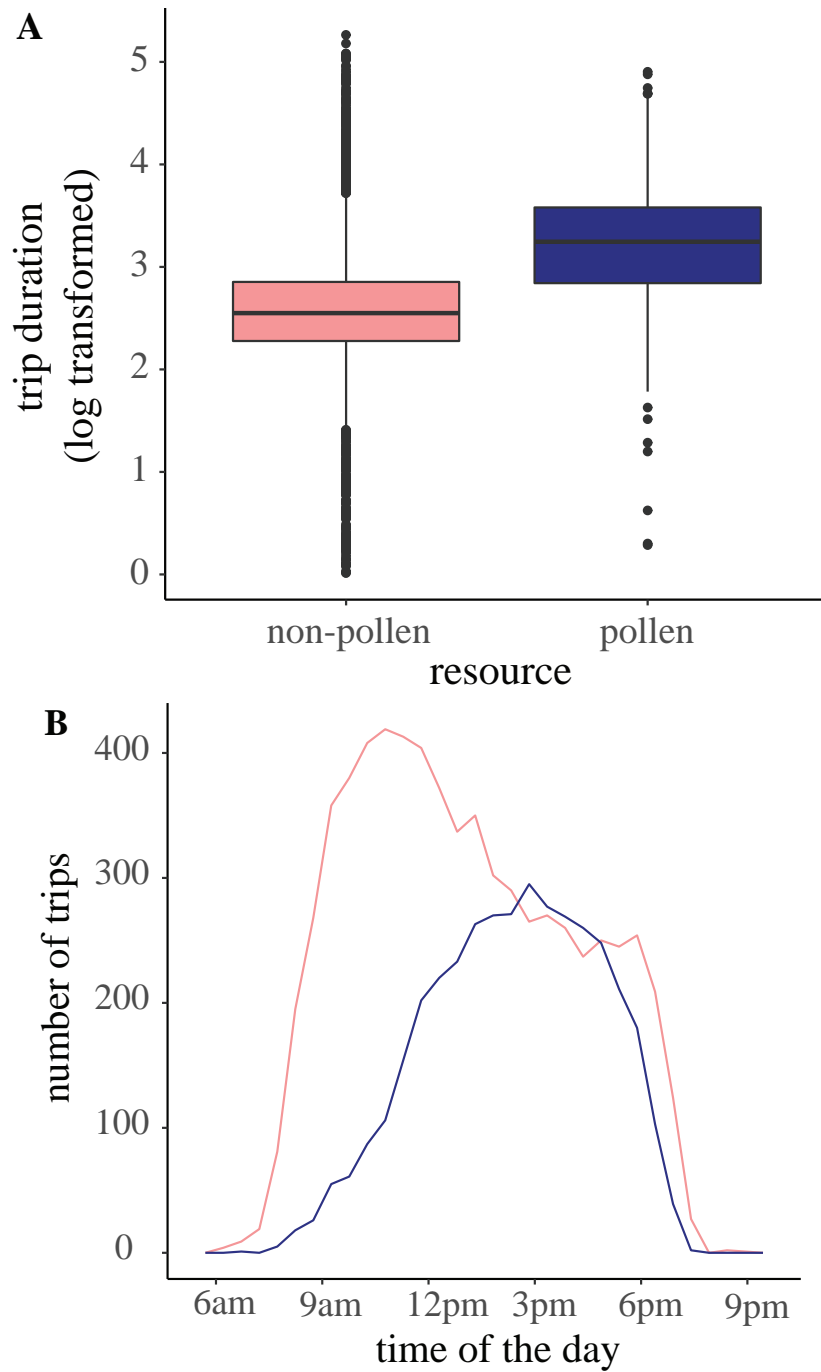


Figure 4: **A.** Trip duration for non-pollen (pink) and pollen trips (blue). Only trips shorter than 200 min were kept in the analyses (98.72% of the total number of trips). The duration of the trips was log transformed for normalisation of the data. Boxplot: the line shows the median, boxes and the whiskers represent interquartile ranges, dots represent outliers (data greater than third quartile + 1.5*(interquartile range), or less than first quartile - 1.5*(interquartile range)). N = 35 bees. Non-pollen trips = 7,063. Pollen trips = 3,858. Wilcoxon paired test: $W = 4999100$, $P < 0.0001$. **B.** Daily distribution of pollen (pink line) and nectar (blue line) trips. N = 35 bees. Non-pollen trips = 7,120. Pollen trips = 3,874. Wilcoxon paired test: $W = 9373500$, $P < 0.0001$.

Discussion

We monitored the foraging activity of all individuals of a bumblebee colony for two months. Our results revealed a high levels of behavioural variability within the forager caste. The degree of inter-individual variation in this primitively eusocial species with a small foraging force was similar to that recently described in advanced eusocial species (e.g. honey bees [24], ants [52–54] and wasps [19]), suggesting that the existence of elite foragers is a common property for cooperatively foraging eusocial hymenoptera. In our colony, most of the bumblebee foragers collected both pollen and nectar. Fewer foragers specialised on nectar only, and only one forager specialised on pollen. Overall, only a small subset of foragers (11%), which we consider as elite foragers, performed most of the foraging trips in the colony.

Previous studies have shown that the foraging performance of bumblebees increases with body size [30,55,56]. For instance, large bumblebees develop foraging routes faster and more efficiently than smaller ones (chapter 4, [18]). Larger bumblebee foragers are able to bring more nectar per time unit than smaller individuals [30]. Here we did not find any significant effect of body size on the activity level of bumblebee foragers. In our data therefore, elite bees occupied one end of a continuous distribution of individual foraging activity in the colony (figure S1), and do not form a sub-caste of foragers based on morphological differences. In this regard our findings for bumblebees were similar to what has previously been shown in honey bees (chapter 2) and ants [54].

Importantly, our study in a primitively eusocial bumblebee highlights interesting differences with more advanced eusocial insects in the behaviour of elite foragers, possibly reflecting differences in their social organisation. Elite bumblebees did not differ from other foragers in the type of resources they collected. Unlike honey bees (chapter 2), very active bumblebees did not collect more pollen. Moreover, whereas in honey bee colonies the proportion of mixed foragers is very low compared to exclusive non-pollen foragers (in chapter 2, only a subset of 27% of the foragers collected pollen at least once); in our bumblebee colony, 63% of the foragers collected pollen and non-pollen resources at least once. A possible explanation is that bumblebees, in contrast to honey bees, are non-perennial social

insects that do not need to store large amounts of nectar-derived honey for nourishing the colony over winter [21].

Further, in honey bees experience was a major cause of variation in resource collection with more experienced bees more likely to collect pollen (chapter 2). This was not the case for bumblebees, who did not increase their probability of collecting pollen with foraging experience (table 1). In honey bees, division of labour is based on age [57], and honey bees show a long process of brain maturation (in the nest and during early foraging [57,58] emphasising the role of behavioural plasticity in shaping foraging role in this species. In bumble bees, division of labour is mainly based on morphological differences, and large individuals can start foraging on their first day upon emergence [21]. Thus, bumblebees may be less prone to experience-dependent adjustments of their foraging behaviour.

The vast majority of bumblebee foragers specialised on pollen collection on certain days only (figure 2), as previously suggested by studies performed in laboratory conditions [32,33], and in the field [59,60]. Bumblebees primarily collected pollen in the afternoon, supporting previous observations that pollen collection is dependent on temperature, occurring mostly during the warmest hours on dry days [60], as dry conditions seem to favour anther dehiscence and thus pollen availability [60]. Bumblebees took more time to perform pollen trips than non-pollen trips, a result also obtained in honey bees (e.g. chapter 2). Since we have no reason to expect that nectar and pollen resources were differently distributed in the natural environment around the colony, this difference is therefore unlikely to be caused by differences in travel distances to obtain pollen and nectar. Instead, time required for flower handling and food collection is potentially higher for pollen than for nectar. Bumblebees exhibit various strategies to access food from the flowers such as robbing nectar by biting a hole in the corolla [61], or buzzing behaviour to release pollen from flower anthers [62]. Foragers load nectar in their crops [21], but need to pack pollen on their legs as pellets that they carry back to the colony [21,62]. These behavioural differences may explain the observed difference of trip duration when foraging for one or the other resources [62].

Our results suggest that inter-individual behavioural variability among foragers is a shared characteristic of insect colonies exhibiting different levels of social complexity. Whether inter-individual variability is on average higher in species

with lower levels of social integration (e.g. primitively eusocial insects) still needs to be confirmed by additional analyses with more colonies and more species. Like in honey bees [24], elite bumblebees represent less than 20% of the pool of foragers. In social wasps [19], elite individuals have been estimated to represent 23% of the workers. In ants such skews have also been reported [52–54,63]. I. For instance, in a *Theremothorax* species, active foragers represent between 31 (in small colonies, median of 57 workers) and 58% (in large colonies, median of 165 workers) of individuals [63].

In these insects, behavioural diversity may constitute an efficient way for colonies to cope with harsh environmental conditions [6], whereby inactive and less active foragers serve as a backup pool of individuals to replace elite foragers that suffer a high mortality as they are more exposed to various stressors [24]. Indeed, this reserve of less active foragers can be rapidly allocated to the forager workforce and replace missing elite individuals, as shown by experimental removal of active foragers from honey bee and ant colonies [24,64]. A continuum between inactive foragers that can be quickly mobilized and a taskforce of rather versatile elite bees (i.e. collecting both nectar and pollen) may therefore provide a high degree of flexibility improving resilience at the colony level [10,65].

Finally, similarly to honey bees, bumblebee foragers show constant inter-individual variability, as well as a plastic behaviour when they switch from pollen to nectar collection. We also highlighted differences in foraging specialisation and degree of activity that reflect the specificity of the smaller and primitively eusocial bumblebee societies compare to the honey bee colonies. Our work is a first step for understanding the behavioural differences featured by foragers of species showing different degrees of sociality, and how this can be link to the specific evolution of division of labour. The next step will be to investigate the degree of variability of solitary bee foragers.

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Author contributions:

SK and ML conceived the experimental design. SK collected the data. SK, CP and ML analysed the data. SK, CP, JMD, ABB and ML wrote the manuscript.

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Supplementary materials

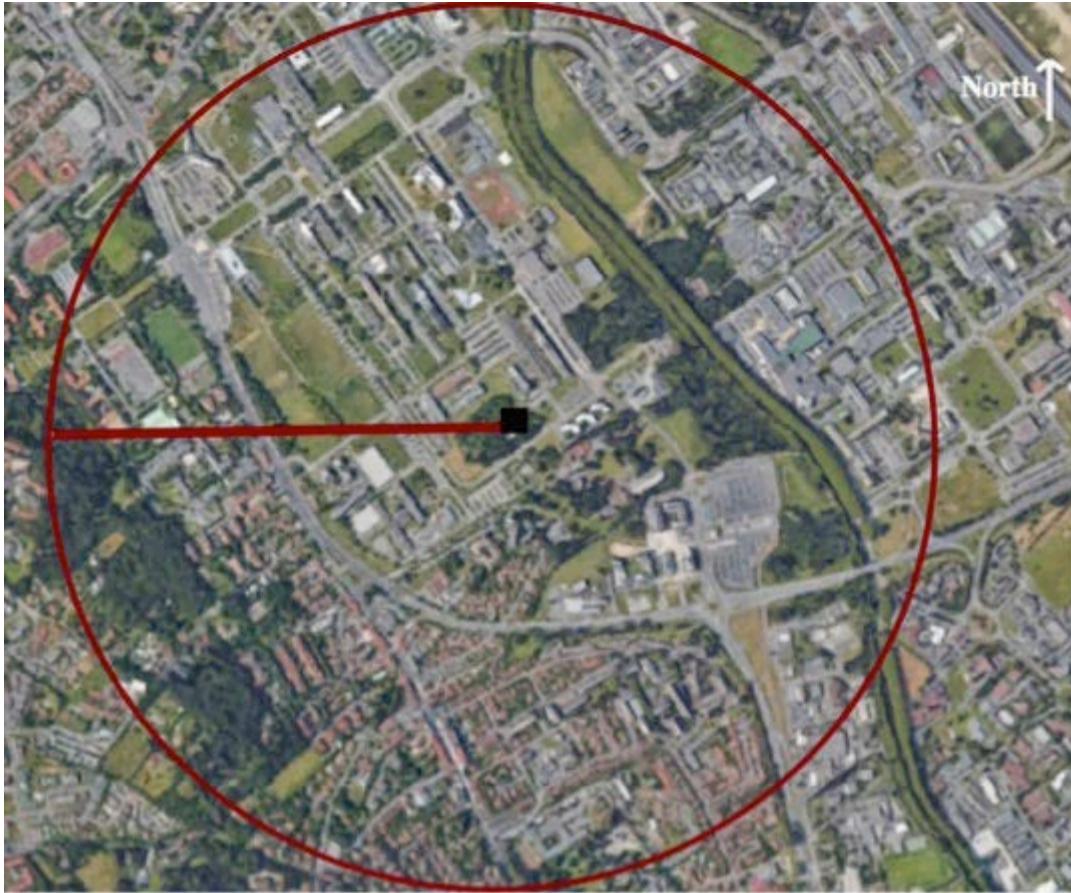


Figure S1: View of the experimental apiary and the surrounding environment. The black square indicates the location of the apiary where the bumblebee colony was set up. Red line indicates a radius of 1km around the colony. Bees were able to forage in a suburban and rural area with a mix of private garden, campus green space, forests and small agricultural fields.

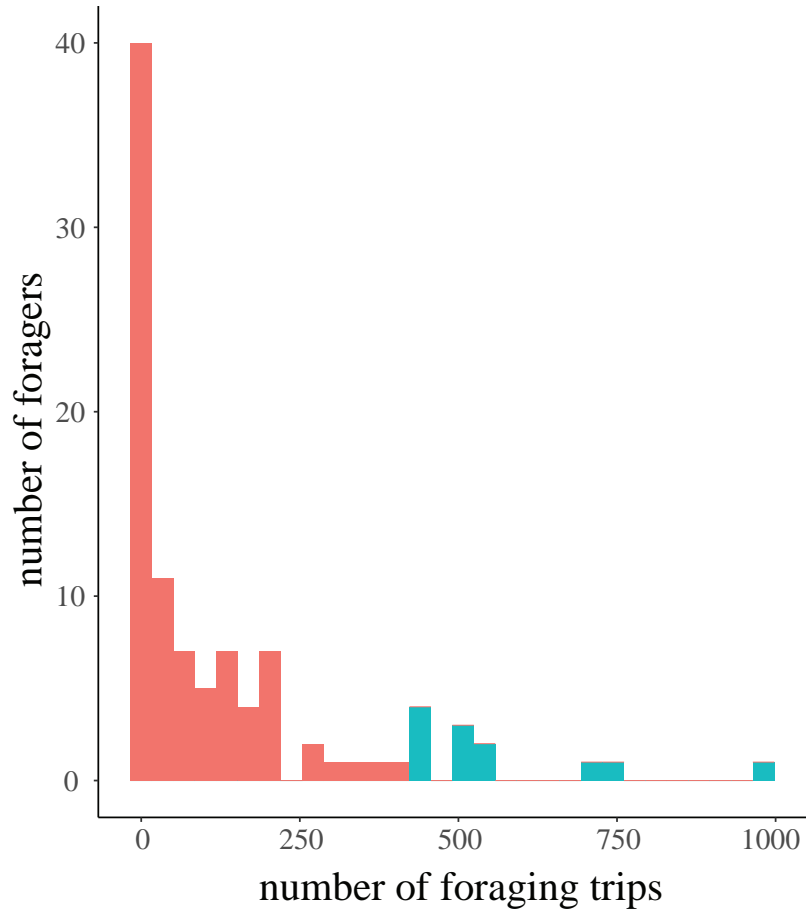


Figure S2: Distribution of non-elite (red) and elite (blue) bumblebees in term of foraging activity.

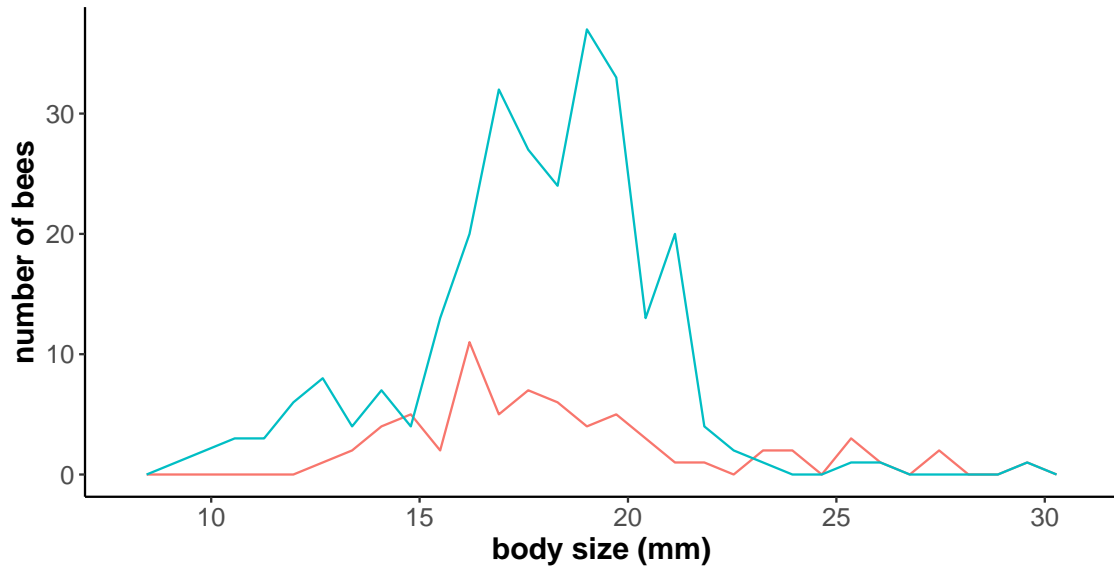
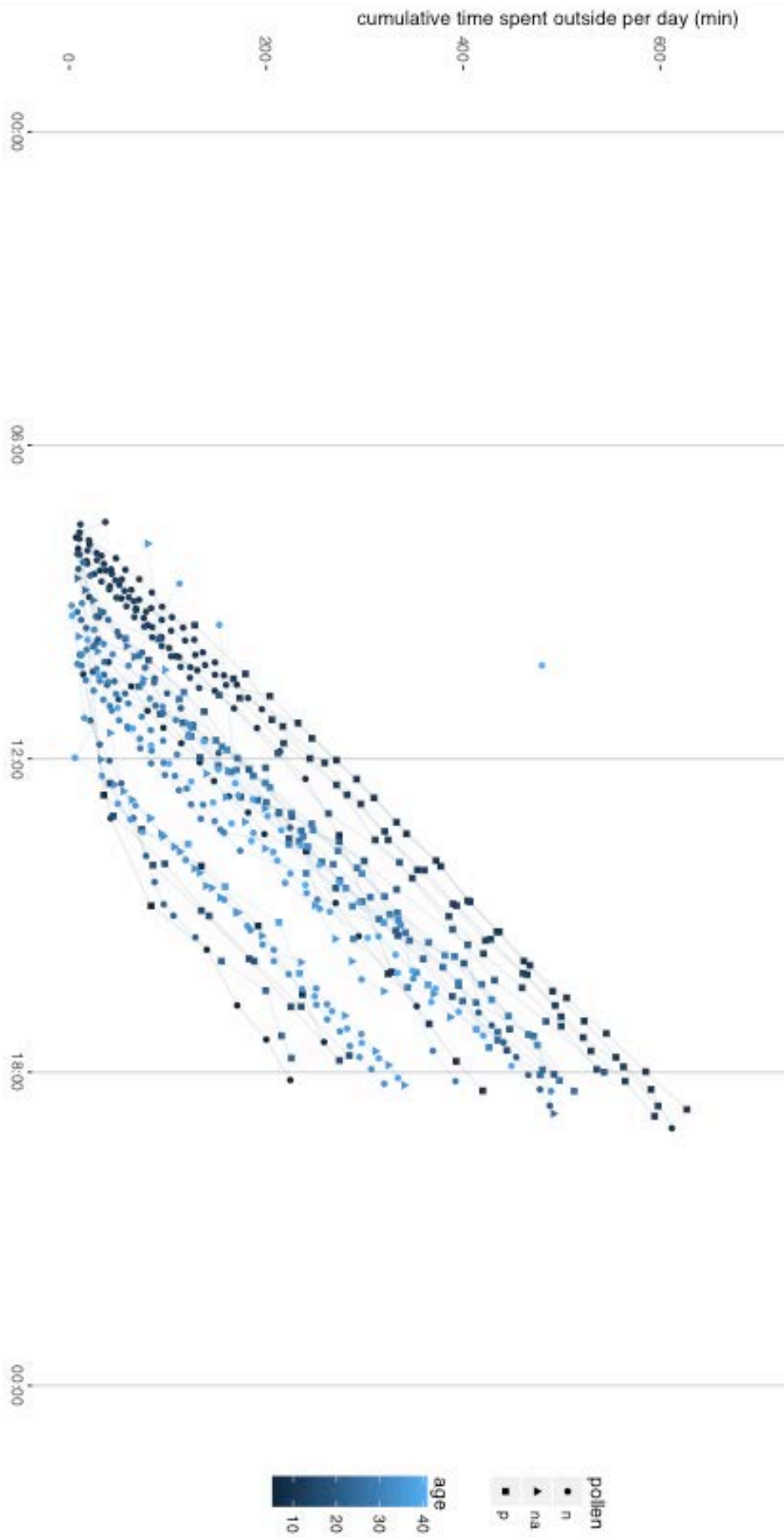
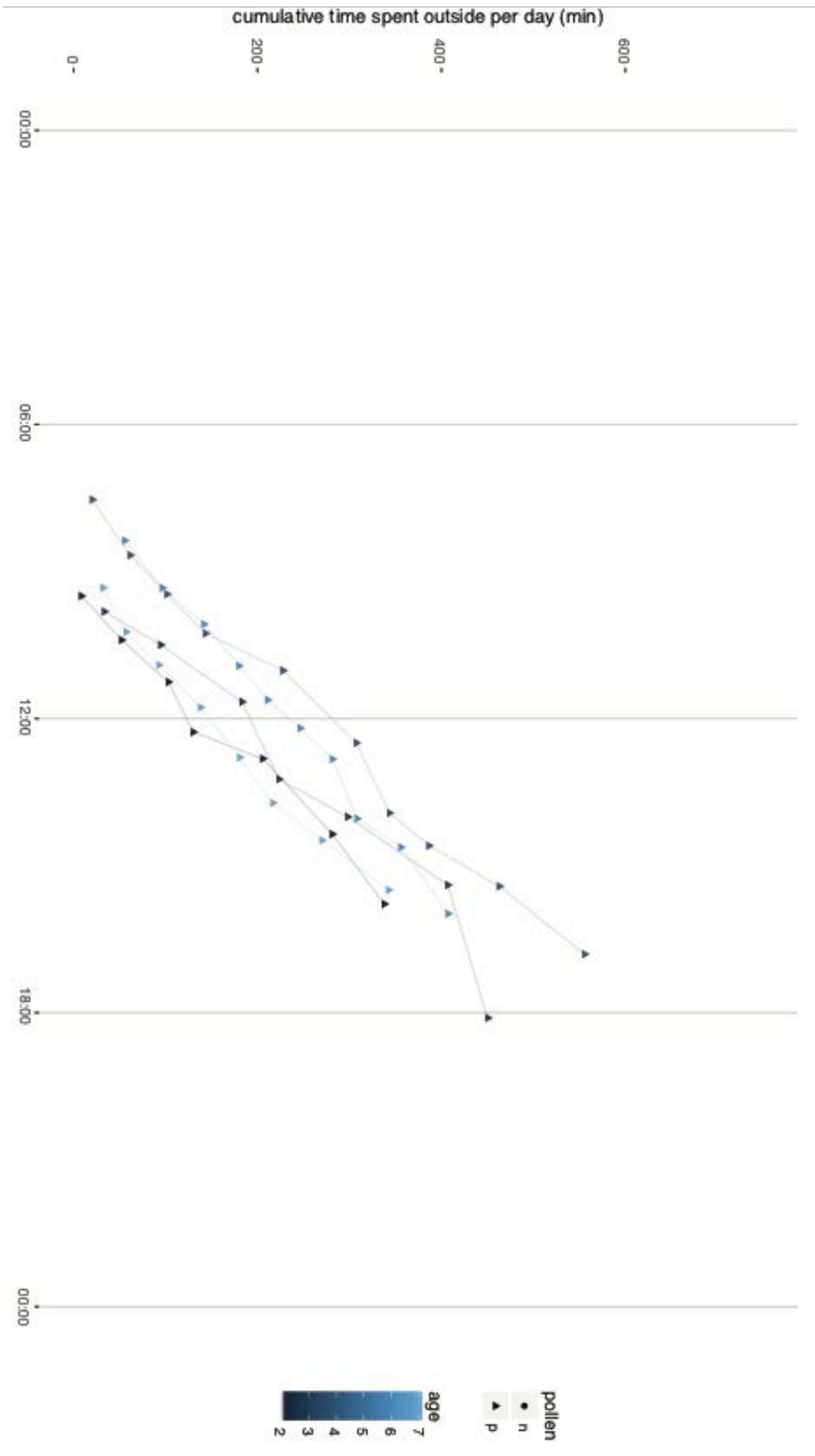
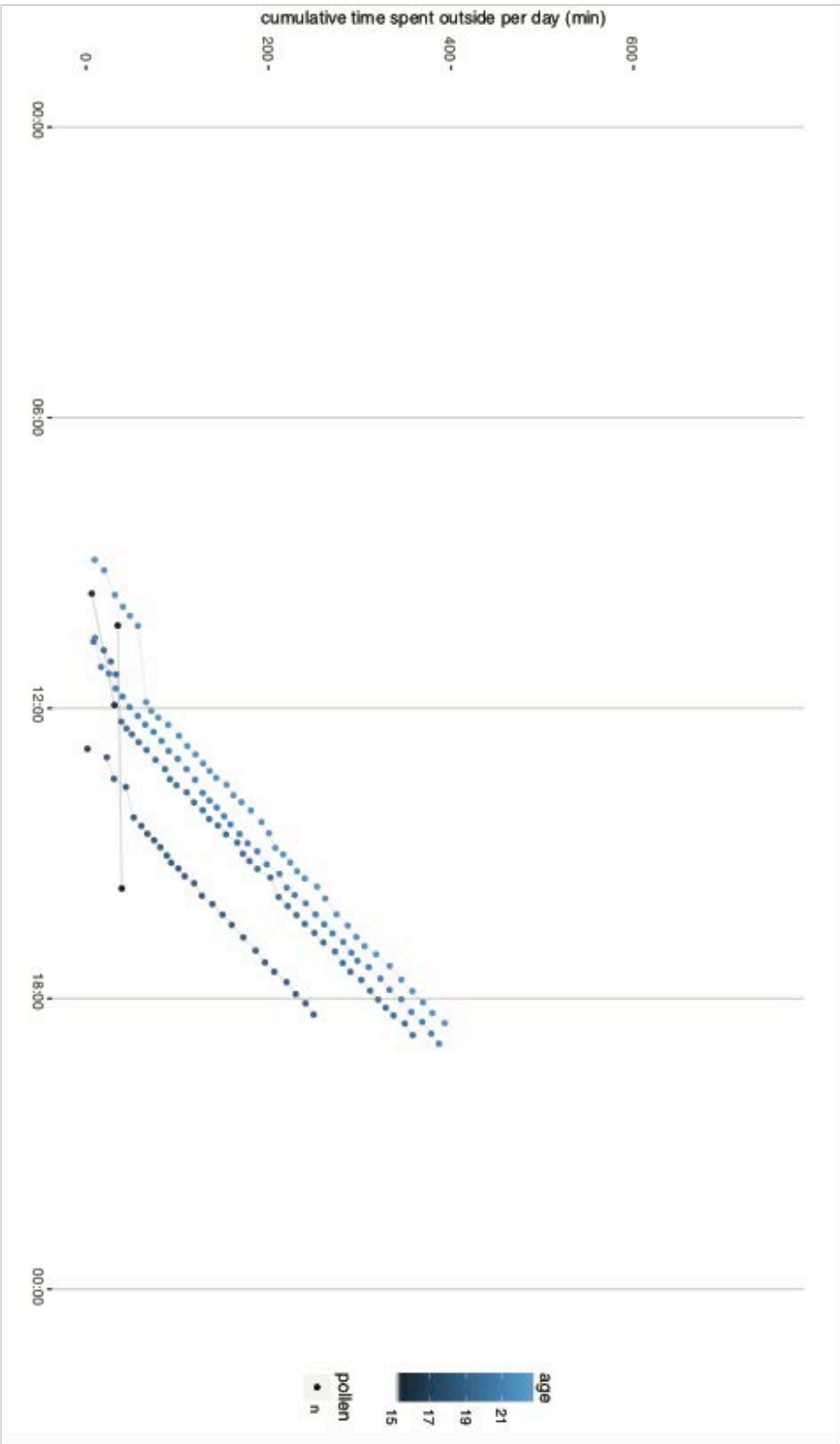
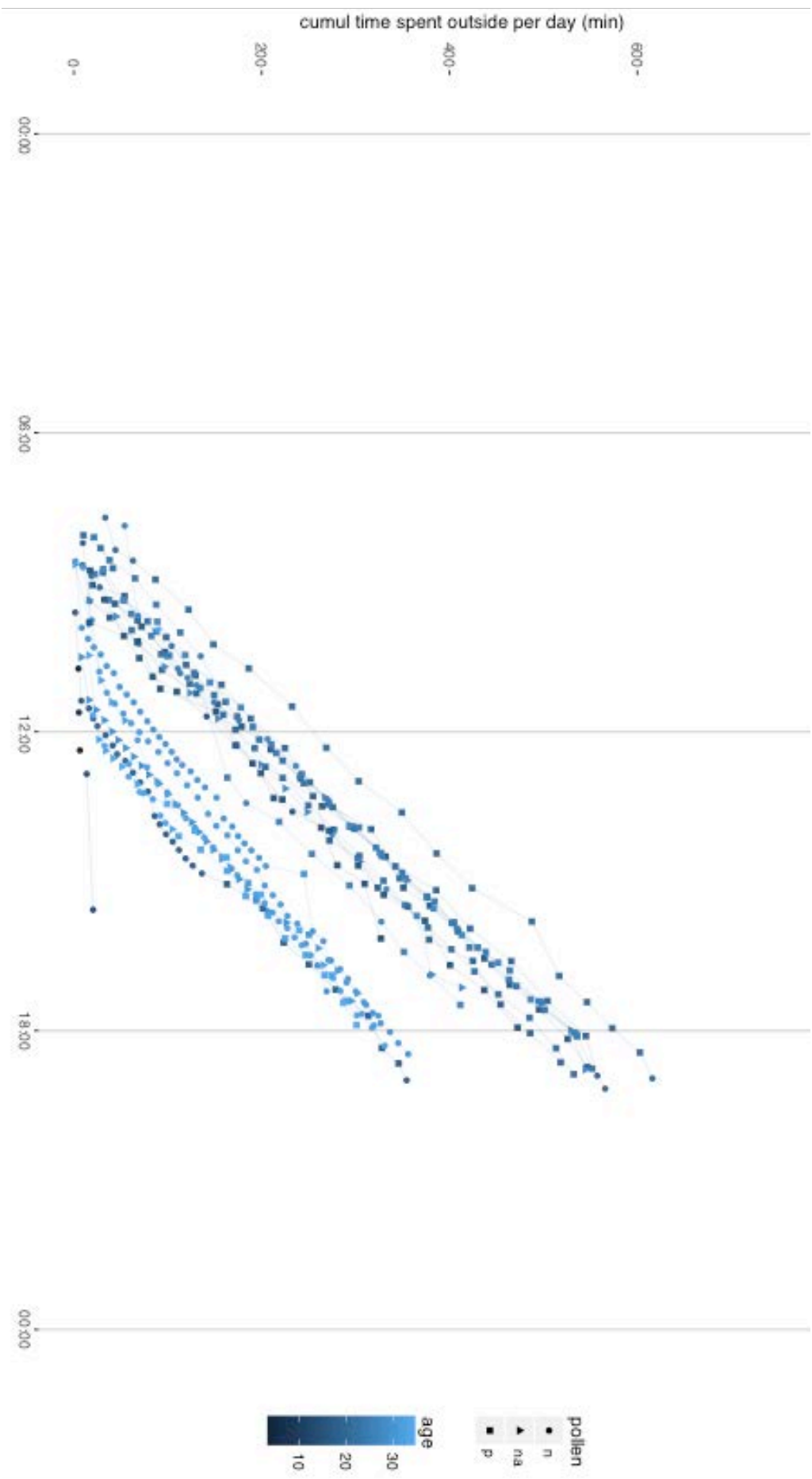


Figure S3: Distribution of the total body length for bumblebees that never foraged (blue) and for foragers (red).









<- **Figure S4:** Examples of foraging activity for some active bees. Plots show the cumulative time spent outside the colony-nest on a given day according to the time of the day. Shades of blue refer to the age of the individual on a given day. The shape of the points refers to the type of resource collected (n = non-pollen, p = pollen, na = non applicable) **A.** Pollen specialists. **B.** Mixed foragers collecting pollen or non-pollen for entire days. **C.** Mixed foragers collecting pollen only for part of the day. **D.** Non-pollen specialists.

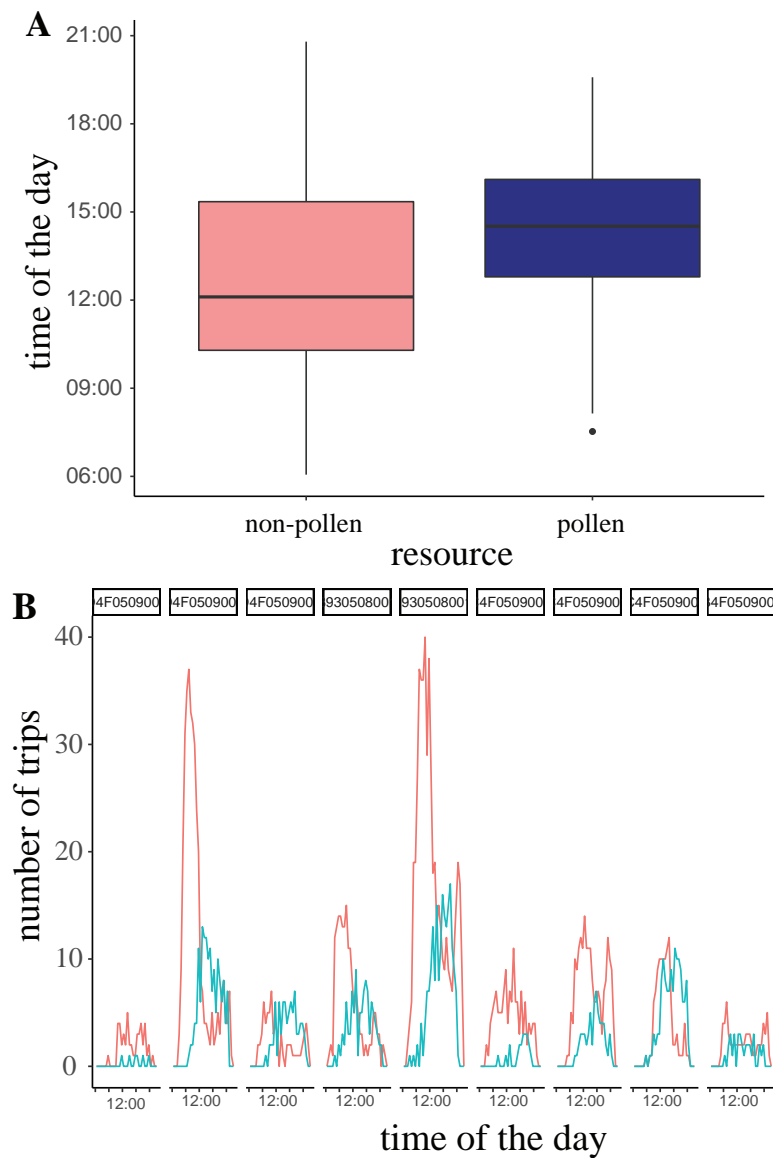


Figure S5: **A.** Difference in time of the day between non-pollen and pollen trips for days when individual bumblebees foraged for both resources. $N = 35$ bees, for 2,432 non-pollen trips and 1,3298 pollen trip. Wilcoxon paired rank test: $W = 1124700$, $P < 0.0001$. Pink is for non-pollen and blue for pollen trip. **B.** Example of the distribution of pollen and non-pollen trips over the days for days when bees foraged for both resources on the same day, only 9 active bumblebees are represented (foraged more than 200 trips).

Chapter 4:

Inter-individual variability in the foraging behaviour of trapping bumblebees



Chapter published in Scientific Reports

Chapter 4: Inter-individual variability in the foraging behaviour of traplining bumblebees

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Abstract:

Workers of social insects, such as ants, bees and wasps, show some degree of inter-individual variability in decision-making, learning and memory. Whether these natural cognitive differences translate into distinct adaptive behavioural strategies is virtually unknown. Here we examined variability in the foraging patterns of bumblebees establishing routes between artificial flowers. We recorded all flower visitation sequences performed by 29 bees tested for 20 consecutive foraging bouts in three environments, each characterised by a unique spatial configuration of artificial flowers and three-dimensional landmarks. All bees started to develop efficient routes as they accumulated foraging experience in each environment, and showed consistent inter-individual differences in their levels of route fidelity and foraging performance, as measured by travel speed and the frequency of revisits to flowers. While the tendency of bees to repeat the same route was influenced by their colony origin, foraging performance was correlated to body size. The largest bees travelled faster and made less revisits to empty flowers. We discuss the possible adaptive value of such inter-individual variability for optimisation of colony-level foraging performances in social pollinators.

Introduction:

In recent years, behavioural ecologists have become increasingly interested by the fact that animals often exhibit consistent behavioural traits that vary between individuals from the same group, population or species, irrespective of time or context [1–3]. Inter-individual behavioural variability has been described in a wide range of taxa, from invertebrates (nematodes [4], cnidarians [5], molluscs [6], insects [7,8]) to mammals [9], including humans [10]. The existence of such individualistic behavioural traits may have different adaptive values depending on the ecology of the species [11–13].

Social insects, such as ants, some bees and wasps, show extreme cases of inter-individual behavioural variability [14]. In these animals, division of labour typically implies that specific individuals reproduce (the queens and the males), whereas others work to support their reproductive outputs (the workers) [15]. Among the workers different individuals specialise on different roles. Some take care of the brood (the nurses), while others defend the colony entrance (the guards and the soldiers) or collect food (the foragers). These behavioural specialists exhibit specific behavioural repertoires that can be associated with differences in morphology (e.g. bumblebees [16]), physiology and genetics (e.g. honey bees [17,18]), age (e.g. honey bees [19]) or experience (e.g. ants [20]), together defining the caste phenotype. Growing evidence indicates that some level of behavioural variability also exists between individuals of the same caste [21–23]. Bumblebees, for instance, show consistent inter-individual differences in decision speed and accuracy in flower discrimination tasks [24,25]. When having to choose between a rewarding flower and an empty flower in a laboratory decision chamber, some foragers always make slow but accurate decisions, while others are consistently fast and inaccurate [24]. Bee foragers also show inter-individual variability in learning performance [21,26]. Bumblebee colonies containing foragers with high visual learning speeds have a higher foraging efficiency [27].

Whether such cognitive variability translates into distinct foraging strategies in the more complex and ecologically relevant task of exploiting patchily distributed floral resources remains virtually unexplored. In nature, bees often develop stable foraging routes (sometimes called traplines in analogy to trappers checking their traps along fixed routes [28]) to exploit multiple feeding locations from their central nest

[29,30]. Manipulative experiments on bumblebees [31,32] and honey bees [33] foraging for sucrose solution in simple arrays of artificial flowers (equivalent to natural flower patches) show how foragers often find the shortest possible route to visit all flowers once and return to the nest using an iterative improvement strategy based on learning and memory that is different from just linking nearest neighbour locations [31,34].

Thus far empirical research on trapline foraging has been aimed at describing this behaviour at the species level, using relatively small sample sizes (four to seven individuals per experiment), without characterising variation among individuals [31–33,35–38]. In principle however, some level of variation in the foraging behaviour of the workers of a colony could improve the colony foraging efficiency [39]. For instance, regular trapliners that accurately follow the same route across multiple hours or days may perform better in stable environments when resources are highly predictable, while irregular trapliners that sample new locations at each foraging bout may be advantaged in more variable environments. Consequently, colonies containing foragers of different behavioural profiles may differ in performance in similar environmental conditions. Ultimately, understanding how natural behavioural variability affects the foraging performances of colonies may help evaluate the adaptability of bees in the face of environmental changes, such as natural climatic events, human-induced habitat degradations or the introduction of predators and parasites [40]. This approach may also help refine predictions of current pollination models based on bee movement patterns [34,38,39,41,42].

Here we explored the level of inter-individual variability in the foraging behaviour of bumblebees (*Bombus terrestris*) by comparing the movement patterns of foragers from two colonies collecting sucrose solution in three different arrays of artificial flowers and landmarks in a controlled flight room.

Material and methods:

Bees and flight room:

We used two colonies of *Bombus terrestris* (Biobest, Westerlo, Belgium). Only one colony was tested at a time (colony 1: November-December 2015, colony 2: May-June 2016). We did not anticipate seasonal effects when working with commercially reared bumblebees in controlled laboratory conditions [27]. The colony was maintained in a two-chamber wooden nest box placed in an experimental flight room with white walls (length: 683 cm, width: 516 cm, height: 250 cm; Figure 1). Controlled illumination was provided by 12 wide-spectrum light-emitting diode bulbs mimicking sunlight (15 W, 1250 lm, Ilight, Italy), with a 10 h : 14 h day : night photoregime (light on at 8:00 AM GMT+1). Temperature was maintained at 20°C. Bees were individually marked with numbered-colour tags (Opalith tags, Christian Graze KG, Germany) on their thoraces upon emergence from the pupae. The colony nest entrance was equipped with a transparent colourless Perspex tube with a series of shutters to control the traffic of foragers. Honey bee collected pollen was provided every two days directly into the colony nest box. Foragers collected sucrose solution (50% [w/w]) from artificial flowers in the flight room.

Artificial flowers and landmarks:

Each flower was made of a cylindrical plastic container (height: 7.5 cm, diameter: 6.2 cm) with a blue lid acting as a landing platform (Supplementary Figure S1A). The platform was held 30 cm above ground by a clamp stand. We used two versions of this general flower design. “Pre-training” flowers provided bees with *ad libitum* reward through a cotton wick soaked in the flower’s container filled with sucrose solution (Supplementary Figure S1B). “Training” flowers provided bees with a controlled volume of sucrose solution specific to each bee (range: 24–52 μ L, N = 29 bees, see calculation of nectar crop capacity below). This volume was placed in the middle of the landing platform using an electronic micropipette (Handystep) (Supplementary Figure S1C). We used nine three-dimensional landmarks made of cardboard and paper. Landmarks were uniquely defined by their shape and coloured patterns (Supplementary Figure S2).

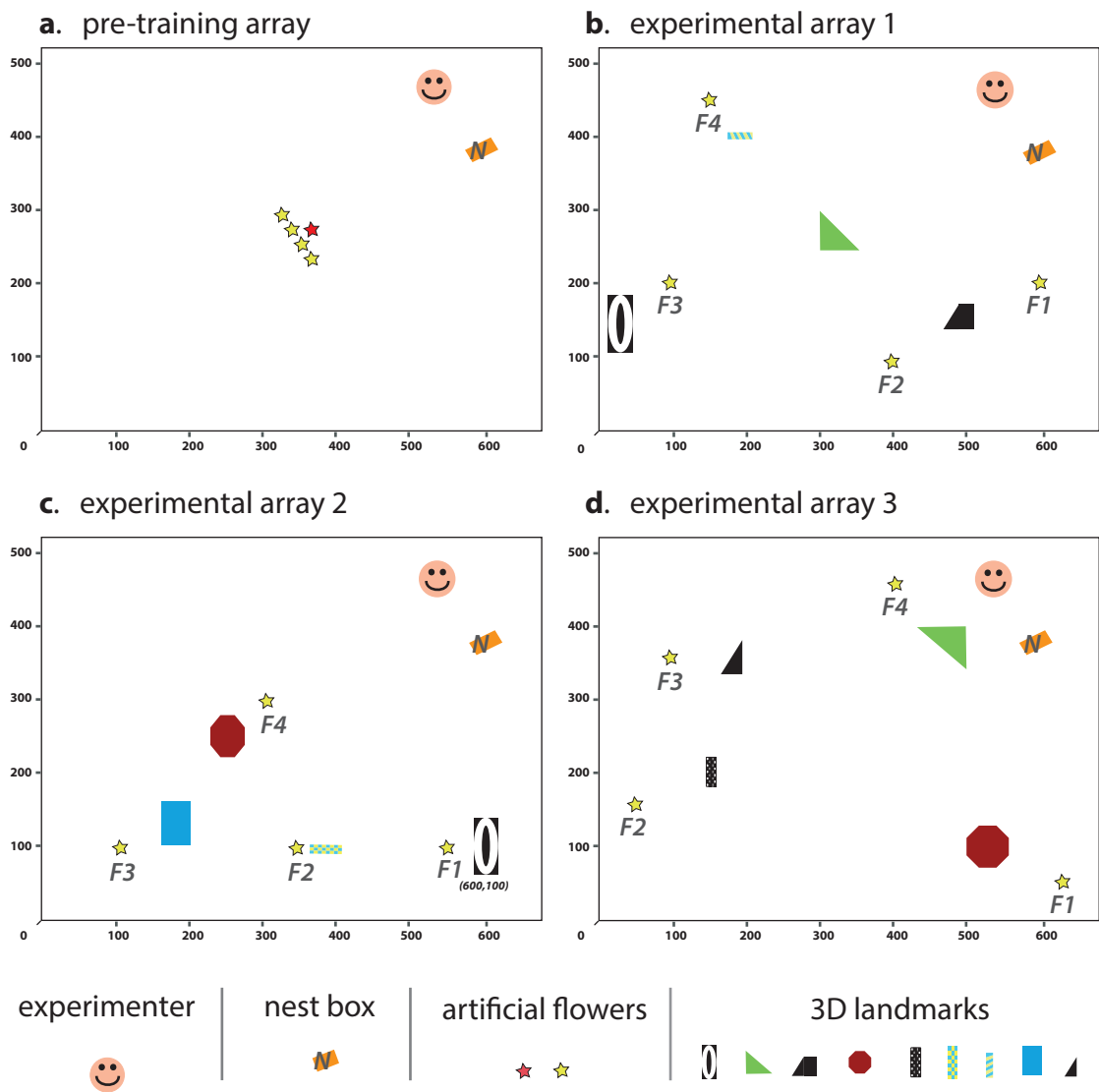


Figure 1: Experimental arrays of flowers and landmarks. **a.** Pre-training array. Bees were allowed to forage on a pre-training flower (red star) in a landmark-free environment for one hour. A selected bee was then observed foraging on four training flowers (yellow stars) during five foraging bouts to estimate its nectar crop capacity. **b, c,** and **d** show the first, second and third experimental arrays used for testing. Each array was characterised by a unique combination of four training flowers (F1-F4) and three to four landmarks (coloured shapes). Detailed descriptions of the artificial flowers and the 3D landmarks are given in Figure S1 and S2. X- and Y-axis graduations represent the distance to the origin (down left corner) in cm.

Experimental procedure

Bees were allowed to forage collectively on a pre-training flower placed in the middle of the flight room (Figure 1A). A regular forager that made at least five foraging bouts within one hour (flower visits followed by returns to the colony nest box) was selected for testing. The bee was first observed foraging on four training flowers arranged in a patch in the middle of the room (Figure 1A). Each flower was refilled with 10 μL of sucrose solution by the experimenter immediately after being visited, until the bee returned to the nest. The average volume of sucrose solution collected by the bee over five foraging bouts was used to estimate its nectar crop capacity (range 48–208 μL , $N = 29$ bees) [31,36–38].

The bee was then tested for 20 consecutive foraging bouts in each of three experimental arrays on the same day (60 foraging bouts, ca. 6 h of observation per bee). Each array was characterised by a unique combination of four flower locations and four different landmarks (see details Figure 1). All bees were tested in the same sequence (arrays 1, 2, 3). During the test, each flower provided a quarter of the bee's crop capacity and was refilled by the experimenter between foraging bouts, so that the bee had to visit all flowers to fill its crop and return to the colony nest box. Because bumblebees drink sucrose rewards until their crop is full, any revisit to a flower within the same foraging bout was unrewarded [35–38,43]. All flower visits, detailing the time when the bee landed on a flower and departed, and the time when the bee arrived and departed from the nest, were recorded using the software Ethom v.1.0 [44] (the complete flower visitation sequences are available in the Supplementary Dataset S1). Flowers were cleaned with ethanol solution (90% v/v) between changing arrays to preclude potential scent marks from influencing the bee's flower choices in the new experimental array[45]. At the end of the test, the bee was freeze-killed and its body size (top of head to end of abdomen) measured with a digital calliper ($\pm 0.01\text{mm}$). A total of 29 bees were tested (14 workers from colony 1, 15 workers from colony 2). Bees from colony 1 were younger (age since emergence from the pupae (mean \pm SE); colony 1: 14.2 ± 8.66 days; colony 2: 24.5 ± 5.67 days, t-test: $t = 6.61$, $df = 76$, $P < 0.001$) and smaller (body length (mean \pm SE); colony 1: 13.41 ± 1.44 mm; colony 2: 16.13 ± 1.44 mm, t-test: $t = 8.67$, $df = 82$, $P < 0.001$) than bees from colony 2.

Data analyses

Average foraging behaviour:

All analyses were performed in R (version 3.2.3 [46]). We used regression models to describe changes in the average number of immediate revisits to flowers (two successive visits to the same flower), the average number of non-immediate revisits to flowers (two non-successive visits to the same flower), the average number of different flowers visited, and the average travel speed (flight duration divided by the Euclidian distance between all successively visited flowers), across the 20 foraging bouts of each bee in each experimental array. For each behavioural measure we ran both linear and logarithmic models and retained the model that had the highest R^2 (Supplementary Table S1). We built a linear regression model using number of foraging bouts, identity of experimental arrays and the interaction between them as fixed effects. We examined the differences between experimental arrays using post-hoc Tukey tests (« multcomp » R package[47]).

To assess the overall similarity between all flower visitation sequences of each bee in a given experimental array we used a determinism index (DET) derived from recurrence quantification analyses [48]. We compared the DETs calculated on the observed sequences to DETs calculated on 1000 randomly simulated sequences of 154 flowers - corresponding to the average number of flowers visits and nest returns over the 20 foraging bouts for all bees in each experimental array (mean \pm SE: 153.5 \pm 33 visits, range = 107-286, N = 29 bees). The R code for generating random flower sequences is available in Supplementary Text S1. Observed and simulated DETs were compared using an analysis of variance (ANOVA) followed by a post-hoc Tukey test («multcomp» R package [47]). To compare the three observed DETs of the same bee (1 per experimental array), we applied a least-square means test («lsmeans» R package [49]) on a linear mixed effect model (LMM) including the experimental array as fixed effect and individual identity as random effect («nlme» R package [50]).

To examine whether some routes were more often used than others by the same bee, we focused on four-flower visitation sequences excluding revisits to flowers[31,36–38]. We calculated the frequency of use of the primary route (highest proportion of foraging bouts in which the same four-flowers visitation sequence — excluding revisits to flowers — was used by a bee). Assuming that there are 24 ($4! = 4 \times 3 \times 2 \times 1$) possible routes to visit four flowers once and return to the nest, we used

a binomial test with a random probability of 0.042 (1/24) to use each route in a given foraging bout. Because each bee was tested for 20 foraging bouts in an experimental array, routes that were used at least four times by the same bee were used significantly more often than expected by chance (at the 5% level).

Intra- and inter-individual variability in foraging behaviour:

We compared the foraging behaviour of individual bees using a principal component analysis (PCA). This PCA aimed to reduce our predictors (i.e. travel speed, number of different flowers visited, non-immediate revisits to flowers, immediate revisits to flowers, proportion of primary route usage, DET) to compound behavioural axes. We applied the Kaiser-Guttman criterion to select the number of principal components (PCs) to retain [51]. We then run the PCA function from the «psych» R package [52] with only the retained PCs. We extracted the PC scores for each bee and used them as dependent variables in the subsequent analyses. To identify the effect of inter-individual (amount of variation among individuals around the average behaviour) and intra-individual (phenotypic plasticity of each individual across arrays) variability on the two PC components over the three experimental arrays of flowers, we ran mixed linear models (LMMs) with individual identity nested within colony identity as random effects. To do this, we ran both a random intercept (inter-individual variability) and slope (intra-individual variability) mixed effect model. We used individual age, body size and experimental array as fixed effects in order to evaluate their respective influence on both PCs. To assess inter-individual differences we tested for the significance of random intercept effects by applying a likelihood ratio test (LRT), comparing the LMM with individual identity nested within colony, the LMM with only colony as random effect and the linear model (LM) excluding both individual and colony identity. To quantify inter-individual variability, we calculated individual repeatability as the percentage of total variance explained by both colony origin and individual differences [53]. We also ran these two analyses on the slope models in order to assess the level of intra-individual variability over the three arrays.

Results:

We tested 29 bees (N = 15 from colony 1, N = 14 from colony 2). Each bee was successively observed for 20 consecutive foraging bouts (flower visits followed by returns to the colony nest box) in three experimental arrays each characterised by four flower locations and four different landmarks (Figure 1, Supplementary Figure S1 and

S2). At every foraging bout, each flower contained a volume of sucrose solution equivalent to one quarter of the bee's nectar crop (stomach) capacity so that the task for the bee was to visit the four flowers to fill its crop to capacity and then return to the nest.

Bees developed routes in the three experimental arrays

We first considered the overall foraging behaviour of bees in all three experimental arrays. On average bees increased by $154.5 \pm 48.3\%$ (mean \pm SE) their travel speed (flight duration divided by the Euclidian distance between all successively visited flowers) between the first and the last foraging bout in the same array (Figure 2A, Table 1). Although we used an indirect measure of travel speed, there is clear evidence that bumblebees rapidly develop straight flight trajectories to join known flower locations [38,54]. As they gained experience in an array, bees also increased by $6.3 \pm 3.8\%$ (mean \pm SE) the average number of different flower locations they visited per bout (Figure 2B, Table 1), decreased by $85.3 \pm 3.5\%$ (mean \pm SE) the average number of immediate revisits to flowers (two successive visits to the same flower; Figure 2C, Table 1), and decreased by $58.0 \pm 8.0\%$ (mean \pm SE) the average number of non-immediate revisits (two non-successive visits to the same flower; Figure 2D, Table 1).

We estimated the tendency of bees to follow regular routes over repeated foraging bouts by calculating the frequency of use of a primary route (highest proportion of foraging bouts in which the same four-flowers visitations sequence — excluding revisits to flowers — was used by a bee) [36]. Each bee established a primary route that it used on average in $27.5 \pm 2.2\%$ (mean \pm SE) of all its foraging bouts for a given array (Figure 2E). This proportion of primary route usage was similar in the three experimental arrays (Kruskall-Wallis test: $\chi^2 = 1.47$, $P = 0.478$). We calculated the level of similarity between the 20 complete flower visitation sequences for each bee in each experimental array using a determinism index (DET). This index is derived from recurrence quantification analyses that reflect the amount of repeated sequences in a dataset [48]. DET varies between 0 (the bee never repeats the same flower visitations sequence) and 1 (the bee always repeats the same flower visitations sequence).

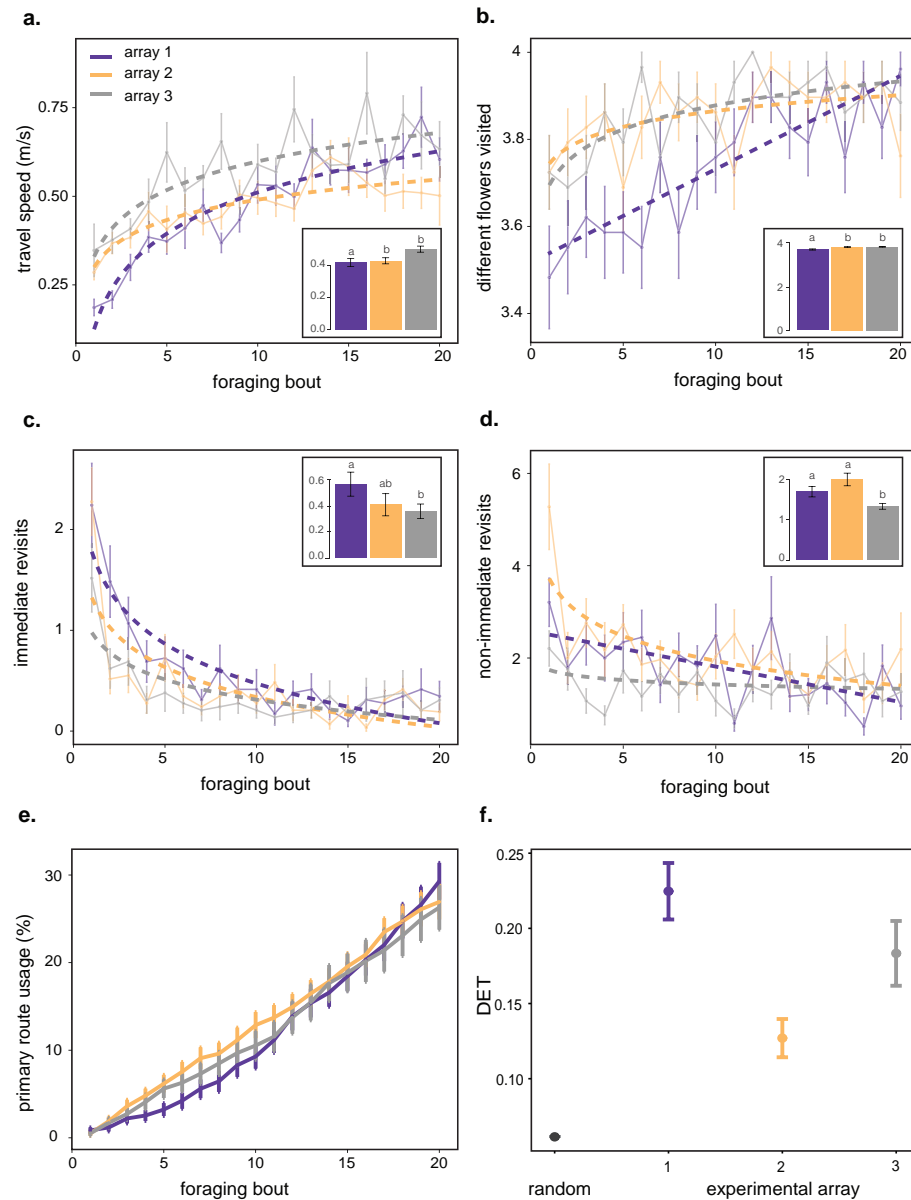


Figure 2: Average behavioural measures in the three experimental arrays (array 1: purple, array 2: orange, array 3: grey, see details of flower and landmark configurations in Figure 1). **a.** Travel speed per foraging bout (flight duration divided by the Euclidian distance between all successively visited flowers). **b.** Number of different flower visited per foraging bout. **c.** Number of immediate revisits to flowers per foraging bout (when the bee visited the same flower twice in a row). **d.** Number of non-immediate revisits per foraging bout (when the bee revisited a flower after having visited one or more different flower locations). **e.** Cumulative frequency of primary route usage per foraging bout. **a** to **e**: plain lines show means \pm (N = 29 bees), dashed lines show regression models (see details in Table 1 and Supplementary Table S1). **f.** Comparison between simulated random determinism index (DETs, N=1000 simulations) and observed DETs (N = 29 bees) in each experimental array (mean \pm SE). **a** to **d**: Bar plots show means \pm se for each array of flowers. Tukey post-hoc analysis: different letters above bars represent significant differences between arrays (see details in Supplementary Table S2).

Table 1: Regression coefficients of average behavioural measures for the three experimental arrays. Significant effects are highlighted in bold.

	Type of regression	Estimate (SE)	t	P
Travel speed				
Array 1	logarithmic	0.16 (0.01)	11.04	<0.001
Array 2	logarithmic	0.09 (0.02)	4.35	<0.001
Array 3	logarithmic	0.64 (0.11)	-1.23	<0.001
Different flowers visited				
Array 1	linear	0.02 (0.003)	7.80	<0.001
Array 2	logarithmic	0.05 (0.02)	2.71	0.014
Array 3	logarithmic	0.08 (0.02)	4.57	<0.001
Immediate revisits to flowers				
Array 1	logarithmic	-0.57 (0.06)	-9.33	<0.001
Array 2	logarithmic	-0.43 (0.09)	-4.73	<0.001
Array 3	logarithmic	-0.29 (0.06)	-5.13	<0.001
Non-immediate revisits to flowers				
Array 1	linear	-0.08 (0.02)	-3.42	0.003
Array 2	logarithmic	-0.77 (0.18)	-4.34	<0.001
Array 3	logarithmic	-0.14 (0.11)	-1.25	0.228

For all three arrays, observed DETs were consistently higher than theoretical DETs calculated on simulated random flower visitations sequences (Figure 2F; post-hoc Tukey test, array 1: $\beta = 0.16 \pm 0.01$, $t = 30.41$, $P < 0.001$; array2: $\beta = 0.07 \pm 0.01$, $t = 12.22$, $P < 0.001$; array 3: $\beta = 0.12 \pm 0.01$, $t = 22.72$, $P < 0.001$). This indicates that bee movement patterns were more repeatable than expected by chance. Thus, overall bees increased their foraging efficiency and began to develop traplines as they accumulated foraging experience in each array, irrespective of the spatial distribution of flowers and the nature and arrangement of three-dimensional landmarks.

Nonetheless, some behavioural differences were observed for all bees between the three arrays. For instance, in array 1 bees tended to travel slower (Figure 2A, Supplementary Table S2), visited fewer flowers (Figure 2B, Supplementary Table S2) and tended to perform more immediate revisits (Figure 2C, Supplementary Table S2), while they performed fewer non-immediate revisits in array 3 (Figure 2D, Supplementary Table S2). This suggests that foragers continuously improved their foraging performance throughout the experiment, as they accumulated experience from the first to the third array. We cannot exclude that the changes of foraging performance of the bees reflect the changes in the different navigational challenges offered by the three arrays of flowers. Bees also appeared to have lower DETs in

array 2 (least-squares means post-hoc test: array 2 vs. array 1: $P < 0.001$; array 1 vs. array 3: $P = 0.072$; array 2 vs. array 3: $P = 0.031$). Presumably specific changes in the spatial arrangement of flowers and landmarks, inherent to our choice of experimental arrays and their sequences of presentation, induced these behavioural differences. For example in array 2, flower 2 may have been particularly difficult to locate as it was hidden behind a tall landmark.

Bees showed strong variability in route fidelity and foraging performance

Having described the average foraging behaviour of bees in the three arrays, we next explored the level of inter-individual variability among the different foragers. We ran a principal component analysis (PCA) based on the mean per array of the six behavioural measures described above (i.e. travel speed per foraging bout (flight duration divided by the Euclidian distance between all successively visited flowers); number of different flowers visited per foraging bout; number of immediate revisits to flowers per foraging bout (when the bee visited the same flower twice in a row); number of non-immediate revisits per foraging bout (when the bee revisited a flower after having visited one or more different flowers); cumulative frequency of primary route usage per foraging bout; determinism index (DET, level of similarity between the 20 flower visitation sequences) for each experimental array; Figure 3, Supplementary Figure S3). We retained two PCs using the Kaiser-Guttman criterion (Supplementary Figure S4). PC1 and PC2 were not correlated with each other (Spearman's correlation test: $r = 0.01$, $S = 108460$, $P = 0.915$). PC1 explained 54% of the proportion and PC2 46%. PC1 was positively associated with the frequency of use of a primary route and the DET, but negatively associated with the number of non-immediate revisits to flowers (Figure 3, Supplementary Table S3). We interpreted PC1 as a "route fidelity" variable. Accordingly individuals with a high PC1 score were regular route-followers characterised by highly repeatable flower visitation sequences and occasional non-immediate revisits to flowers. PC2 was positively associated with the number of immediate and non-immediate revisits to flowers, and negatively associated with travel speed and the number of different flowers visited (Figure 3, Supplementary Table S3). We interpreted PC2 as a "foraging performance" variable. Individuals with a high PC2 score were slow and inaccurate foragers, characterised by slow movements between flowers and frequent revisits to empty flowers. Variance along PC1 and PC2 defined a continuum between four behavioural

extremes (Figure 3): fast accurate and regular route followers (high PC1/low PC2 scores), fast accurate and irregular route-followers (low PC1/low PC2 scores), slow inaccurate and regular route-followers (high PC1/high PC2 scores), and slow inaccurate and irregular route-followers (low PC1/high PC2 scores). While foragers of colony 2 were uniformly distributed across the entire PC space, 50% of the foragers of colony 1 were nested within the area defined by high PC1 and low PC2 scores (slow inaccurate and irregular route-followers; Figure 3).

Variability was expressed both at the inter- and intra-individual levels

We next explored the effects of inter- and intra-individual variability on PC1 and PC2, using linear mixed effect models (LMMs) with individual identity nested within colony identity as random effects and both intercept (inter-individual variability) and random slope (intra-individual variability) structures.

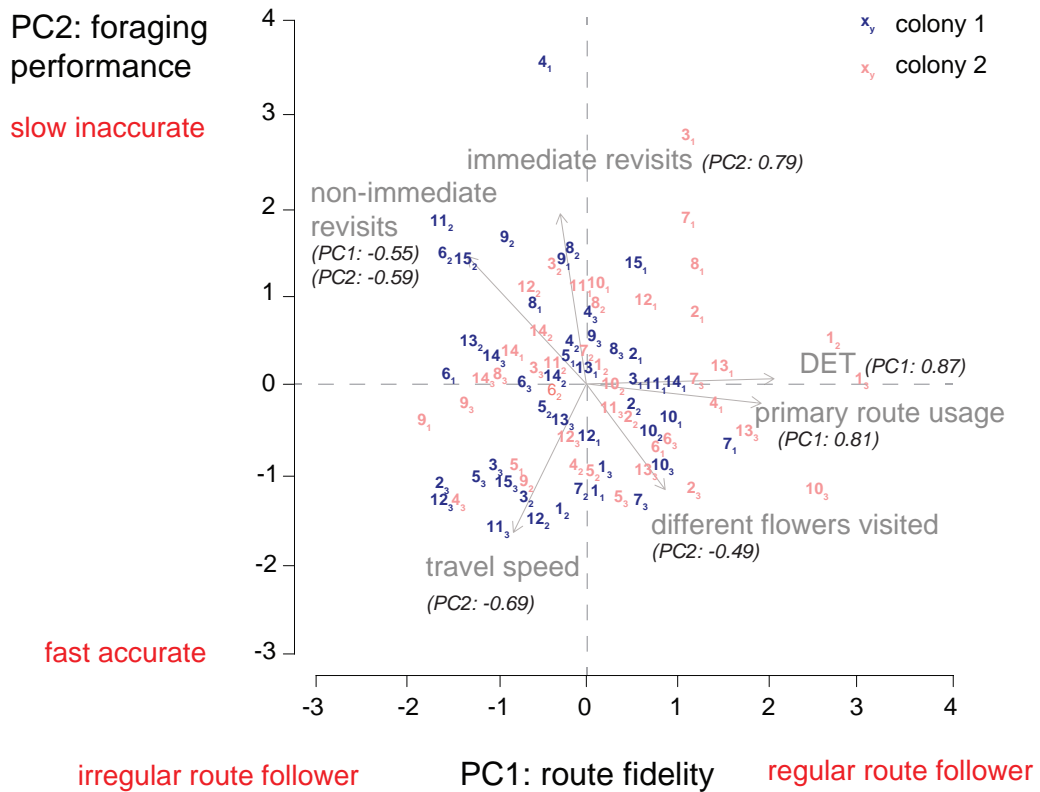


Figure 3: Correlations between the two first components (PCs) of the principal component analysis (PCA). Grey arrows represent the six behavioural measures on PC1 (route fidelity) and PC2 (foraging performance). PC loadings are in brackets. Only loadings $> |0.4|$ were retained (see Supplementary Table S3 for the complete PCA loadings). Each data point represents the PC1 and PC2 scores of a given bee in each experimental array. The PCs define a continuum between four behavioural extremes: fast accurate and regular route followers, fast accurate and irregular route followers, slow inaccurate and regular route followers, slow inaccurate and irregular route followers. Blue: colony 1 (N=15 bees, 45 data points), red: colony 2 (N=14 bees, 42 data points). Numbers refer to individual bees (same number code as in Figure 4 and 5). Subscripts refer to experimental arrays (1-3).

Table 2: Log-likelihood Ratio tests to estimate inter- and intra-individual variability on the two principal components (PCs) of the principal component analysis (PCA). **a.** To study inter-individual variability we compared a linear model (LM) built using each PC as a response variable and age, body size and experimental array as fixed variables with two mixed effect models (LMEs) using colony or individual nested in colony as random effects. **b.** To study intra-individual variability we compared the random intercept model (LME_1|colony/ID) previously built using each PC with a random intercept and slope model (LME_0+array|colony/ID). Degree of freedom (df), Akaike Information Criterion (AIC), Log-likelihood values (Loglik) and Log-likelihood ratio test (L.Ratio) are presented with the corresponding p-values. Significant effects are highlighted in bold.

	df	AIC	Loglik	L.Ratio	P
a.					
<i>Random intercept model PC1</i>					
LM	5	262.67	-126.34		
LME_1 colony	6	228.64	-108.32	7.08	0.008
LME_1 colony/ID	7	254.48	-120.24	5.11	0.024
<i>Random intercept model PC2</i>					
LM	5	239.54	-114.77		
LME_1 colony	6	237.84	-112.92	3.70	0.054
LME_1 colony/ID	7	225.13	-105.57	14.72	<0.001
b.					
<i>Random slope model PC1</i>					
LME_1 colony/ID	7	242.57	-114.29		
LME_0+array colony/ID	6	235.93	-111.96	4.64	0.031
<i>Random slope model PC2</i>					
LME_1 colony/ID	7	201.92	-98.46		
LME_0+array colony/ID	6	227.93	-107.92	19.00	<0.001

Variability in PC1 was significantly explained by inter-individual differences (Table 2A; 27% of variance explained), meaning that bees showed consistent differences in their average level of route fidelity across arrays. Bees also differed in their level of intra-individual variability (Table 2B; 11% of variance explained) so that some individuals consistently increased their route fidelity in each array while others did not. Variability in PC1 was also explained by differences between colonies (Table 2A; 38% of variance explained). Overall bees from colony 2 were more regular at following a route than bees from colony 1, irrespective of the experimental array (Figure 4A).

Variability in PC2 was significantly explained by inter-individual differences (Table 2A; 46% of variance explained). Therefore bees showed consistent differences in their average level of route performance across arrays. Bees did not present intra-individual variability in their response to the different arrays (Table 2B; 5% of variance explained), meaning that all bees tended to increase their foraging performance as they gained experience in a given array. Colony origin had no effect on PC2 (Table 2A; 26% of variance explained).

Body size partly explains inter-individual variability in foraging performances

We used LMMs to examine whether experimental factors (spatial configuration of flowers and landmarks) or biological characteristics of bees (body size and age) explained both PCs (Table 3). PC1 was neither explained by experimental arrays, body size or age (Table 3). By contrast PC2 was negatively correlated with body size, so that larger bees tended to travel faster and make fewer revisits to flowers than smaller bees (Figure 5). We also found a significant influence of the experimental arrays on PC2 (Table 3), indicating that bees similarly increased their foraging performance as they moved from array 1 to array 2 and array 3 (Figure 4B). This gradual improvement of foraging performances supports the hypothesis of a continuous learning process.

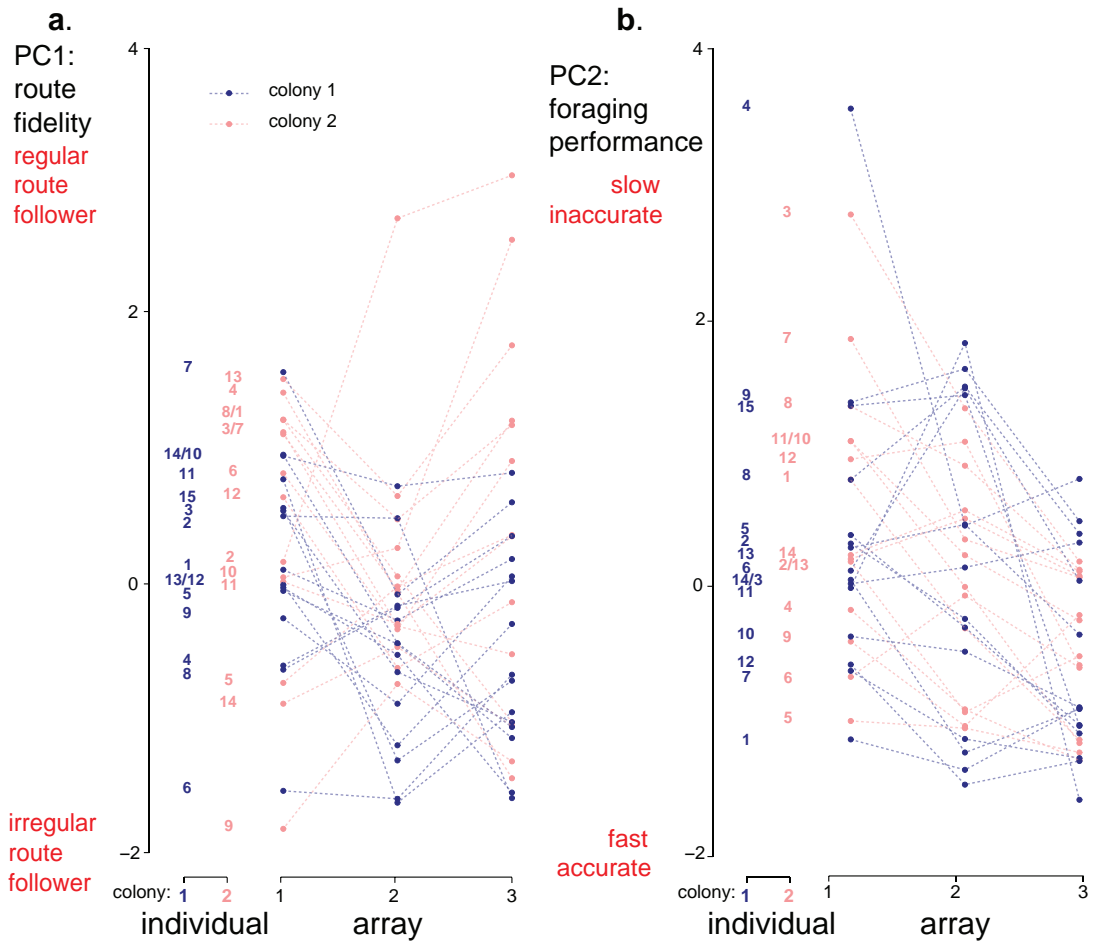


Figure 4: Intra- and inter-individual behavioural variance across experimental arrays. **a.** Route fidelity (PC1). **b.** Foraging performance (PC2). Data points connected by a dashed-line represent the scores of the same individual over the three arrays. Blue: colony 1 (N=15 bees), red: colony 2 (N=14 bees). Numbers refer to individual bees (the same number code was used in Figure 3 and 5).

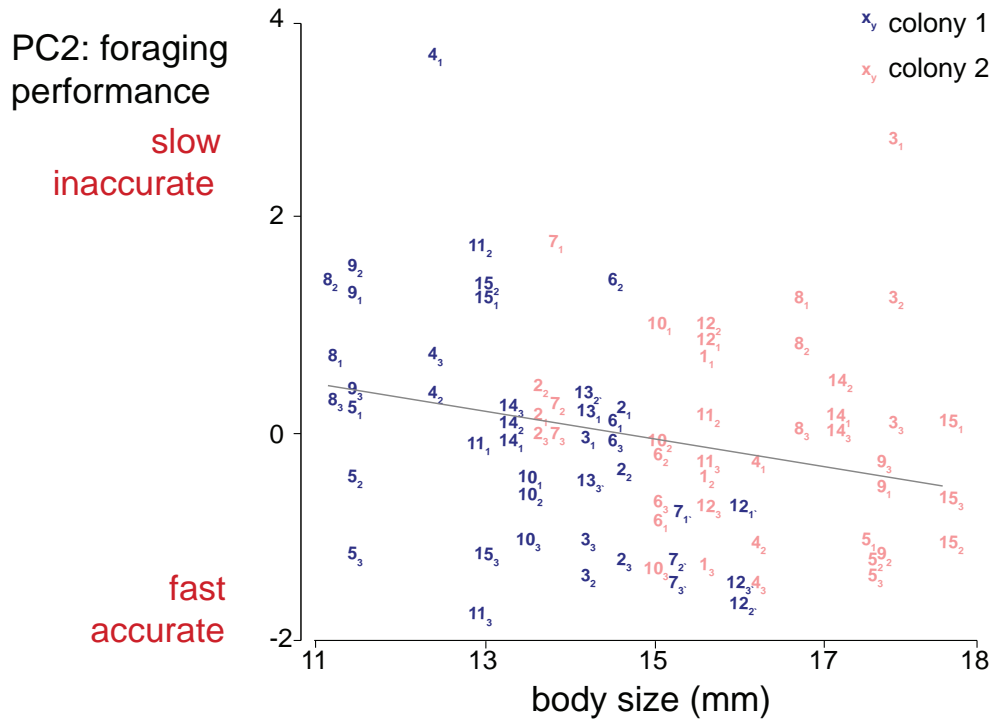


Figure 5: Inter-individual variance in foraging performance (PC2) is partly explained by body size (length from top of head to end of abdomen). Each data point represents the average score of an individual in an experimental array (three values per individual). Blue: colony 1 (N=15 bees), red: colony 2 (N=13 bees). Numbers refer to individual bees (the same number code was used in Figure 3 and 4). Subscripts refer to experimental arrays (1-3). Marginal $R^2 = 0.12$, conditional $R^2 = 0.44$.

Table 3: Linear mixed models (LMMs). LMMs were run on the two principal components (PCs) of the principal component analysis (PCA), using individual identity nested within colony identity as random variables and age, body size and experimental array as fixed variables. Significant effects are highlighted in bold.

	Estimate (SE)	df	t	P
<i>Route fidelity (PC1)</i>				
Body size	-0.12 (0.09)	24	-1.38	0.190
Age	-0.01 (0.02)	24	-0.37	0.709
Array	-0.18 (0.11)	55	-1.23	0.116
<i>Foraging performance (PC2)</i>				
Body size	-0.21 (0.09)	24	-2.36	0.03
Age	-0.01 (0.02)	24	-0.53	0.60

Discussion:

Understanding inter-individual behavioural variability in complex societies, such as colonies of social insects, may offer unique insights into how and why relatively high levels of inter-individual behavioural variability are observed in animal groups and populations [21,55]. Here we compared the movement patterns of all foragers from two bumblebee colonies exploiting arrays of stable feeder locations, and report consistent inter-individual differences in their spatial foraging behaviour. Rather than defining distinct behavioural profiles of foragers, this natural variability follows a continuum along two behavioural dimensions. Some bees were always more faithful to a route and/or faster and more accurate in their spatial foraging decisions than others.

Bees showed consistent inter-individual variability in their tendency to follow stable routes between flowers. This variability was neither explained by the characteristics of our experimental arrays of flowers and landmarks, nor the body size or the age of bees. Interestingly, degrees of route fidelity differed between our two colonies, meaning that foragers from one colony were more regular in following a route than those from the other colony. These results are not due to differences in the average body size or age between the foragers of each colony. Behavioural variability between individuals of different groups or colonies is a widespread phenomenon in social animals [55], including insects [23,55–58]. Inter-colonial behavioural variability has been reported previously in bees, for inter-colonial differences in aggression [59] or for both vision- and olfaction-related cognitive tasks, and these differences are correlated with the foraging success of colonies [26,27]. In bumblebees, high genetic relatedness between colony members, due to female monandry (single mating) and haplo-diploidy (haploid males, diploid females), may favour strong inter-colony variability [26,60]. Other non-genetic factors may also contribute to phenotypic variability between colonies, such as changes in the pre-imaginal environment. For instance variation in temperature [61] and nutrition [62] during the larval stage can lead to differences in olfactory learning in adult honey bees. Further studies using more colonies with known genetic relatedness are needed to test the existence of a genetically determined inter-colony variability for traplining.

In the present spatial task, bees also showed some level of inter-individual variability in their ability to make fast and accurate spatial decisions, so that fast

travelling bees made fewer revisits to empty flowers. This result is consistent with the observation that goal-directed flights in experienced bees, for instance between the nest and familiar flowers, are faster than exploration flights, in which naïve bees scan the environment to search for flowers and acquire spatial memories [38,54]. Thus potentially bees showed inter-individual variability in their tendency to make exploitation and exploration flights. Differences in foraging performance among bees were partly explained by their body size, so that larger foragers tended to travel faster and make fewer revisits than smaller foragers. Because we tested only naturally motivated foragers, we describe here variability within the foragers' caste. This observation is consistent with previous studies showing that the largest bumblebees make more foraging trips [63], take less time [16] and collect more nectar in natural conditions [16]. Large bumblebees also tend to learn faster in visual discrimination tasks [64]. These inter-individual behavioural and cognitive differences may be explained by differences in the sensory equipment of small and large bees. For instance, larger bees have bigger compound eyes and may thus be more accurate at finding small objects [65]. Size polymorphism in bumblebees is primarily determined by the frequency of feeding so that larvae raised in the middle of the nest area (where workers are more active) tend to become the largest adults [66]. Therefore it is very likely that the diversity of body sizes and their associated behavioural traits within bumblebee colonies is a self-organised process, regulated by population densities and structural constraints within the nest at a given time during the colony cycle.

Our description of inter-individual variability in the spatial foraging behaviour of bumblebees is in line with recent observations that foragers of social bees show high variability to their contribution to the global colony foraging effort [63,67], suggesting that some behavioural traits may support higher foraging success. It has been suggested that behavioural diversity in a social group or population can be an advantageous trait at the collective level [7,8]. Honey bee colonies showing higher genetic variability (and thus inter-individual behavioural variability) perform better in group tasks such as nest thermoregulation [68]. Colonies of *Thermothorax* ants showing high variability in the aggressiveness of workers are more productive [13]. In the social spider *Anelosimus studiosus*, mixed colonies composed of aggressive (asocial) and docile (social) individuals capture more prey than colonies with high proportion of only one type of individuals [69]. Accordingly, maintaining a diversity

of behavioural profiles among foragers of a colony may allow the colony to locate and exploit a larger diversity of resources in fast changing environments [1,24,70,71]. For instance, artificial bumblebee colonies containing individuals with different foraging profiles along a speed-accuracy trade-off have a more constant nectar collection rate than homogenous colonies [24]. Further investigation of the correlates of inter-individual behavioural and cognitive differences among members of a social group, such as bees, holds considerable promise for better assessing plastic collective responses and the adaptability of groups to stressful environmental conditions.

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Supplementary materials:

Table S1: Choice of regression models. R^2 were calculated for linear and logarithmic models for each behavioural measure in each experimental array. The model with the highest R^2 (bold) was retained for the analyses.

Experimental array	1		2		3	
	<i>linear</i>	<i>logarithmic</i>	<i>linear</i>	<i>logarithmic</i>	<i>linear</i>	<i>logarithmic</i>
Travel speed	0.802	0.871	0.390	0.513	0.459	0.636
Different flower locations visited	0.773	0.740	0.215	0.289	0.419	0.547
Immediate revisits	0.514	0.829	0.297	0.555	0.268	0.594
Non-immediate revisits	0.394	0.388	0.291	0.512	0.017	0.079

Table S2: Differences in behavioural measures between experimental arrays. Post-hoc Tukey tests. Results in bold represent significant differences.

	β (SE)	t	P
<i>Travel speed</i>			
Array 1 vs array 2	0.15 (0.06)	2.60	0.032
Array 1 vs array 3	0.20 (0.06)	3.49	0.003
Array 2 vs array 3	0.05 (0.06)	0.89	0.65
<i>Different flowers visited</i>			
Array 1 vs array 2	0.33 (0.06)	5.32	<0.001
Array 1 vs array 3	0.28 (0.06)	4.53	<0.001
Array 2 vs array 3	-0.05 (0.06)	-0.79	0.711
<i>Immediate revisits</i>			
Array 1 vs array 2	-0.46 (0.23)	-2.01	0.124
Array 1 vs array 3	-0.80 (0.23)	-3.45	0.002
Array 2 vs array 3	-0.35 (0.23)	-1.53	0.285
<i>Non-immediate revisits</i>			
Array 1 vs array 2	0.77 (0.49)	1.57	0.372
Array 1 vs array 3	-1.27 (0.49)	2.57	0.034
Array 2 vs array 3	-2.04 (0.49)	-4.14	<0.001

Table S3: Principal Components Analysis (PCA) loadings. For each individual in each experimental array, the average travel speed, number of immediate revisits to flowers, number of non-immediate revisits to flowers, number of different flowers visited per foraging bout were included in the PCA. The cumulated frequency of primary route usage and the determinism index (DET) of each bee in each array were also included. The correlation matrix of the six behavioural measures is showed in Supplementary Fig. S3.

	Route fidelity (PC1)	Foraging performance (PC2)
Travel speed	-0.34	-0.69
Immediate revisits to flowers	-0.12	0.79
Frequency of primary route usage	0.81	-0.09
DET	0.87	0.02
Non-immediate revisits to flowers	-0.55	0.59
Different flower locations visited	0.36	-0.49
Proportion explained	0.54	0.46

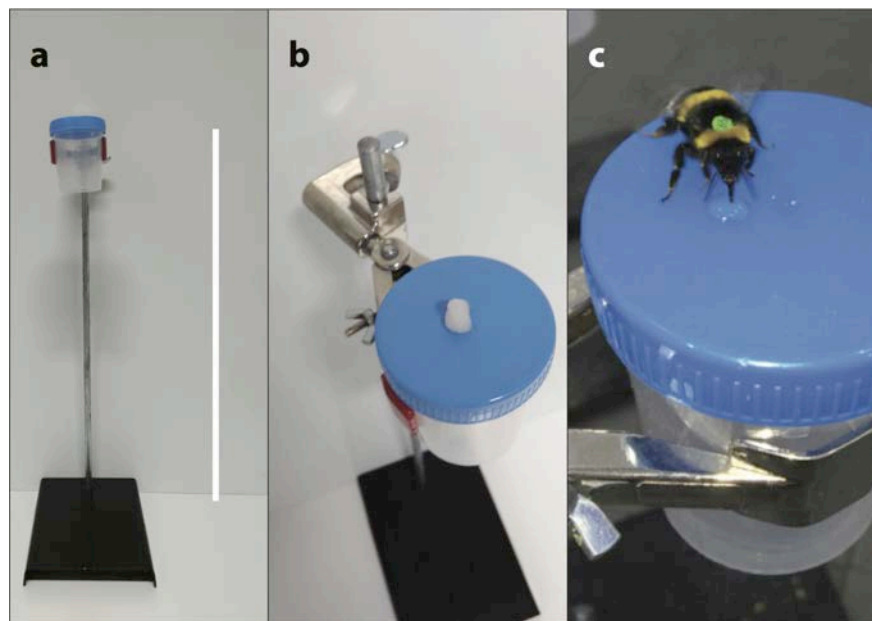


Figure S1: Photos of the artificial flowers. **a.** General flower design. The flower is made of a blue circular landing platform on top of a transparent, colourless, cylindrical reservoir of sucrose solution held by a stand clamp. White bar = 30 cm. **b.** Pre-training flower. Bees can drink *ad libitum* sucrose solution through the cotton wick connecting the landing platform to the sucrose reservoir. **c.** Training flower. A bee with a coloured numbered tag is drinking a controlled volume of sucrose solution placed in the middle of the landing platform. The bee cannot access the sucrose reservoir below. Pictures by S. Klein.

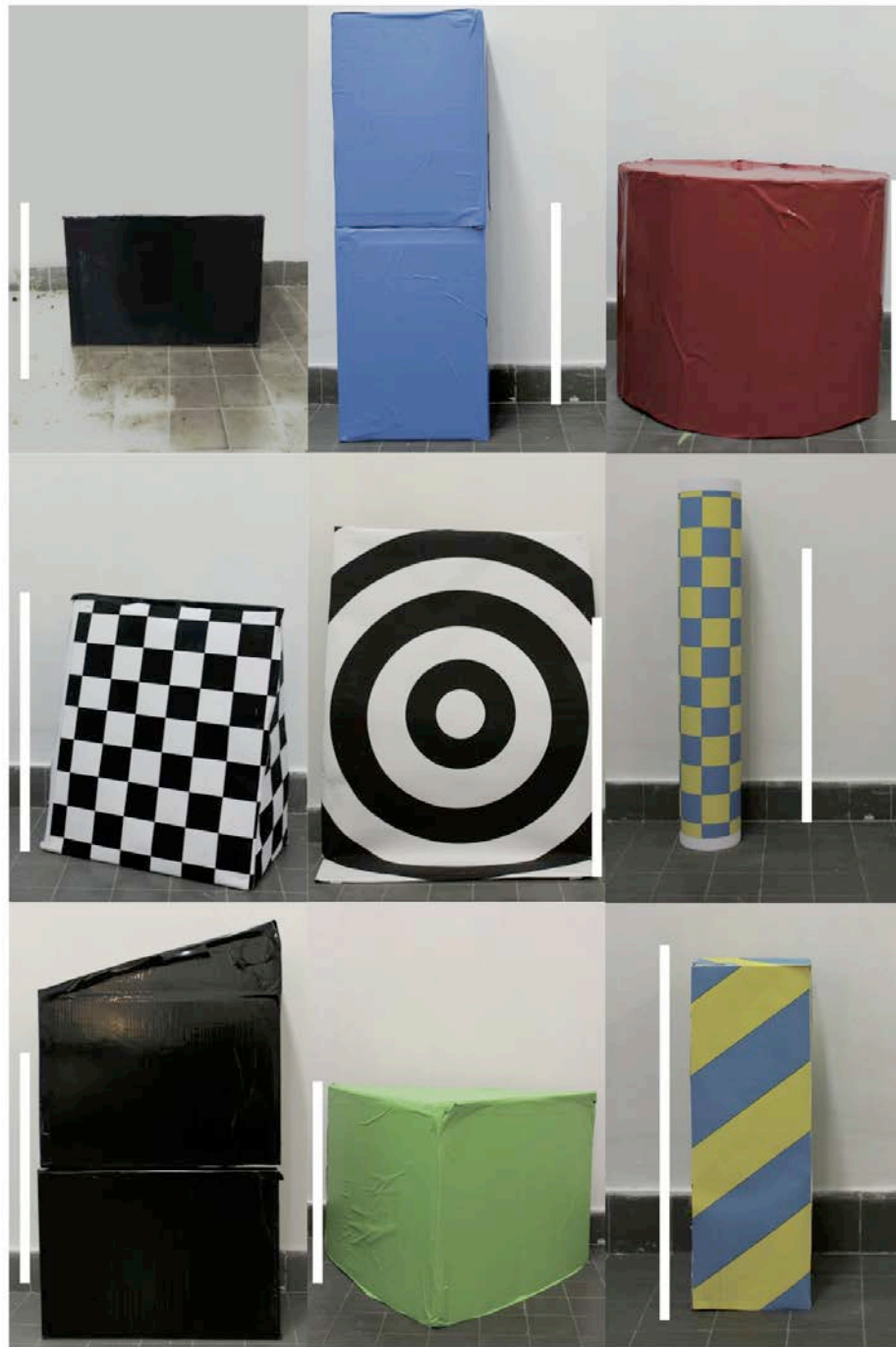


Figure S2: Photos of the three-dimensional landmarks. We used nine items made of cardboard and paper that could be used by bees as visual landmarks to assist their navigation. Each landmark was uniquely defined by its shape and colour pattern. White bar = 30 cm. The spatial arrangements of landmarks in the flight room are showed in Fig. 1. Pictures S. Klein.

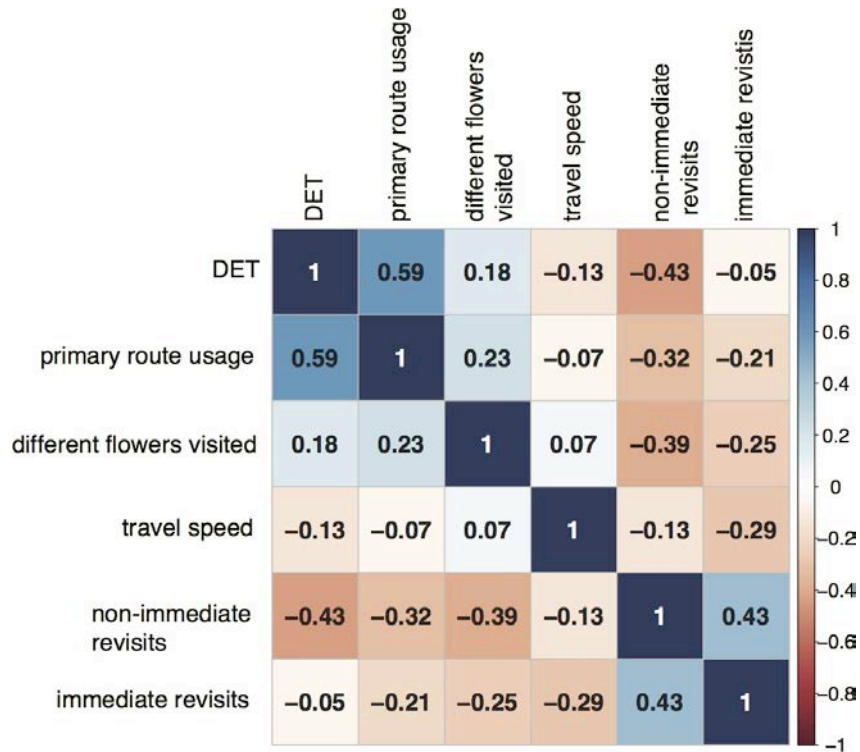


Figure S3: Correlation matrix of the six behavioural variables included in the principal component analysis. Travel speed per foraging bout (flight duration divided by the Euclidian distance between all successively visited flowers); number of different flowers visited per foraging bout; number of immediate revisits to flowers per foraging bout (when the bee visited the same flower twice in a row); number of non-immediate revisits per foraging bout (when the bee revisited a flower after having visited one or more different flowers); cumulative frequency of primary route usage per foraging bout; determinism index (DET, level of similarity between the 20 flower visitation sequences) for each experimental array.

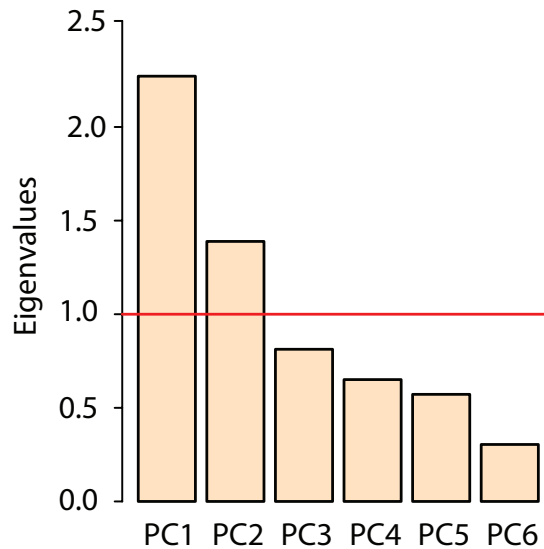


Figure S4: Selection of the principal components (PCs) based on the Kaiser-Guttman criterion. Two PCs with an eigenvalue higher than the average (red line) were retained to construct the principal component analysis.

Dataset S1: Data file (csv.file) containing mean values for the number of different flowers visited (*nb_flower*), the number of immediate revisits (*imm_revisit*), the number of non-immediate revisits (*non_imm_revisit*), the travel speed (*speed*, in m/s), the proportion of primary route usage (*prop*) and

the determinant (*DET*) for each individual (*bee_ID*) in the three experimental arrays of flower (*array*). SE are provided for each variable (*se_variablename*). This file also contains information about the colony origin (*colony*), age (*age*) and body size (*body*) of bees. NA = non-available value.

Dataset S2: Data file (csv.file) containing raw values for the number of different flowers visited (*nb_flower*), the number of immediate revisits (*imm_revisit*), the number of non-immediate revisits (*non_imm_revisit*), the travel speed (*speed*, in m/s), the proportion of primary route usage (*prop*) and the sequence of flower visits (*sqce_tot*) for each foraging bout (*bout*) of each individual (*bee_ID*) in the three experimental arrays of flowers (*array*). This file also contains information about colony origin (*colony*), age (*age*) and body size (*body*) of bees. NA = non-available value.

Text S1: R scripts used for generating random flower visits sequences and calculate random DET.

```
#####  
#-This R code creates a simulation of 1000 individuals visiting --#  
#- flowers in random sequences and calculates their determinism index  
--#  
#####  
  
# load the 2 functions developed by Ayers et al. 2015.  
source(file                                     =  
"~/Documents/bumble_expe/scripts/functions/determinism.R")  
source(file                                     =  
"~/Documents/bumble_expe/scripts/functions/removeperpdiag.R")  
  
# generate 1000 artificial bees  
det_rdm <- 0  
for (j in 1:1000){  
  seq_rdm=0  
  for (i in 1:158) # 158 is the mean number of flowers visited by the  
  29 bumble bees in our dataset  
  {  
    TMP1=sample (1:4,1) # we arbitrarily assigned successions of 3  
    visited flowers. The 4th visit can be either nest return or another  
    flower visit.  
    TMP2=sample (c(1:4),1)  
    TMP3=sample (c(1:4),1)  
    TMP4=sample (c(0:4),1)  
    seq_rdm=c(seq_rdm,c(TMP1,TMP2,TMP3,TMP4))  
  }  
  seq_rdm <- c(seq_rdm,0)  
  print(seq_rdm)  
  det_rdm <- rbind(det_rdm,determinism(seq_rdm,4))  
  
#generate DET values for each of the 1000 artificial bees  
}  
det_rdm2 <- det_rdm[-1]  
  
write.csv(det_rdm2,file="det_rdm.csv")
```


Chapter 5: Sublethal effects of miticides on honey bee visual learning



Chapter 5: Sublethal effects of miticides on honey bee visual learning

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Abstract:

The varroa mite (*Varroa destructor*) has been implicated as one of the major causes of the global rise in honey bee colony failure. In order to control this parasite, beekeepers use miticides to treat hives. The effect of chronic exposure to such hive treatments on bee behaviour and cognition is not well known. Here we used an aversive associative visual learning assay to test the impact of two widespread miticide treatments (tau-fluvalinate and thymol) on bee visual cognition. We found that thymol has no detectable effect on visual learning. By contrast, exposure to tau-fluvalinate reduced the ability of bees to learn the association between a colour and a mild electric shock. Our results highlight the need to consider harmful effects of in-hive chemicals on bee cognition, which could result in impaired foraging performance and altered colony function.

Keywords: *Apis mellifera*, cognition, visual learning, aversive learning, miticides, chronic stress, *Varroa destructor*.

Introduction:

In order to treat managed honey bee hives against the parasitic mite *Varroa destructor*, beekeepers are using several different miticides as treatments applied inside the bee hive. Whereas these miticides can be efficient at reducing the parasite loads in the colonies, some evidence suggests that they also affect the bees in different ways. Here we analyse the effect of two miticides on honey bee visual learning ability, as it is a crucial element in foraging behaviour.

The honey bees' (*Apis mellifera*) capacity for learning is vital for their foraging and navigation in complex environments [1,2]. However a current increase in environmental stressors impacting bees is damaging bee cognitive capacities [3] and negatively affecting bee populations [4]. One of the main stressors of the honey bee is the mite *Varroa destructor*, a widely-distributed honey bee pest that has been recognised as a major driver of honey bee colony losses [5–7]. This ectoparasite feeds on the haemolymph of larvae and adult bees [8], thus weakening them through physical injury, deprivation of proteins and associated weight loss, spread of viruses, and, in larvae, disruption of development. As a consequence, many infested individuals exhibit a reduced body size with morphological abnormalities, and have reduced longevity.

In addition, infected bees often show impaired foraging behaviour, such as altered olfactory learning [9], reduced flight duration and homing capacity [10]. These effects are likely to decrease foraging efficiency and thus reduce colony performance [11]. varroa is also a vector for several highly pathogenic viruses such as the deformed wing virus (DWV) and the acute bee paralysis virus complex [12–14]. Indeed, infection with viruses, such as these appears to be responsible for some of the morphological defects induced by *Varroa* [15], and is sufficient to cause learning impairment in the absence of the mite [16]. All in all, coincident varroa infestation and viral infection are a major cause of a high risk of colony failure [5,7,17].

Miticides have been developed to control varroa levels in hives [8,18,19]. These chemicals are used directly in the hive so that their active compounds are in contact with the brood and the adult bees. It is thus possible that they might have undetected sublethal effects on honey bees. Prolonged exposure to low levels of the miticides might particularly alter the development and activity of the nervous system and thereby reduce cognitive performance, as has been shown for sublethal field

doses of pesticides using learning and memory assays [20–23]. The effects of chronic exposure to in-hive miticides have not been studied in detail, even though the presence of chemicals in the hive environment is an increasingly common situation for honey bee colonies [24–28]. Here, we propose to study the effect of miticides on bee visual learning ability.

We focus here on two of the most commonly-used miticides: tau-fluvalinate and thymol. Tau-fluvalinate (hereafter FL, commercialized as *Apistan*®) is a synthetic pyrethroid compound [29]. This class of compounds acts as arthropod neuron excitotoxins, which prevent the closure of voltage-gated sodium channels of axonal membranes, leading to prolonged membrane depolarization and thus to paralysis or death [29–31]. It also suppresses neuron excitability in adult bee brain structures [32] and impairs olfactory memory [21]. Thymol (TH) is a monoterpene extracted from essential oil of thyme (*Thymus vulgaris*) and has been used for varroa control under the commercial product *Apiguard*® [18]. Though considered more environment-friendly than other miticides as it is based on plant products [33], thymol may have negative effects on bees. Indeed, chronic TH exposure has been shown to reduce phototactic behaviour [34]: in a walking assay, treated bees were less efficient at reaching the source of light in a limited time. Effects on cognition have also been studied [18,35–37], and topical applications of high TH doses (10 – 100ng / bee) reduce long-term memory of a food-odour association measured with the classic proboscis extension assay [35]. However, these latter studies used only olfactory appetitive learning as a behavioural output. Varying the behavioural task is necessary to conclude whether miticides have a general impact on learning abilities. Can effects be seen in other cognitive tasks, such as in visual discrimination or in an aversive learning paradigm?

In-hive pest-control products can be detected in the wax, honey and pollen for years after treatment [24–28]. Fluvalinate for instance has a half-life of five years in wax [38] and thymol can still be found in the wax a year after the treatment stopped [39]. Therefore we expect developing larvae and in-hive bees to undergo chronic low-level exposure following treatment of a hive [26]. An additional difficulty is that none of the studies looking at the effects of miticides on bee cognition controlled for the potentially confounding effect of a change in varroa mite load, as they have been conducted on populations also exposed to the varroa mite [21,32]. Here we examined

the effects of miticide treatment of bees in Australia [40] where there currently is no varroa in order to examine the effect of the miticide on bee cognition with no confounding varroa infection.

We analysed the effect of in-hive chronic exposure to tau-fluvalinate or thymol on bee forager learning abilities. Because olfactory appetitive learning tasks were used in previous studies, we aimed at determining if miticides could alter performance in an aversive learning task involving a different sensory modality; vision. For this, we used a recently developed visual aversive learning assay called Automatic Performance Index System (APIS, [41,42]). The APIS assay consists of an automatic tracking chamber where a walking individual has to learn to associate one of two different coloured environments (provided by LEDs in the chamber's walls), with electric shocks (provided by a metal grid on the floor of the chamber). Based on their locomotor responses, we were able to assess individual bee's ability to learn to specifically avoid the light associated with the electric shock, and thus to capture cognitive deficits in adult foragers from hives treated with miticides.

Material and methods:

Animals:

We sourced bees from six European honey bee (*Apis mellifera*) colonies from the Macquarie University experimental apiary, North Ryde, Australia. The colonies were kept in two-box Langstroth hives. Two hives were treated with thymol (TH group) using *Apiguard*® (Vita Europe Ltd, Basingstoke, UK), two other hives with tau-fluvalinate (FL group) using *Apistan*® (Vita Europe Ltd, Basingstoke, UK), and two non-treated hives were used as controls (NT group). Both miticides were applied following suppliers' instructions. Two *Apistan*® tau-fluvalinate soaked plastic strips were placed in each of the fluvalinate treated hives, between two brood frames, for six weeks. One *Apiguard*® thymol gel container was placed on top of the frames of the top box of each of the thymol-treated hives for three weeks. After two weeks, the treatment containers were replaced by another thymol gel container in each of the two hives for a further three weeks. Treatments started on the 1st of February 2016. We performed hive inspections every week to check for the presence of a queen and eggs. Six weeks after we started the treatments we began testing the bees (14th of March 2016). Considering the time required for larval development (21 days) [43] and the

worker transition from in-hive work to foraging (around two weeks) [43], we are confident that we could test foragers that had been passively exposed to the chemicals during their development and adult time spent in the hive. The experiment lasted for a month (14th of March 2016 to 16th of April 2016).

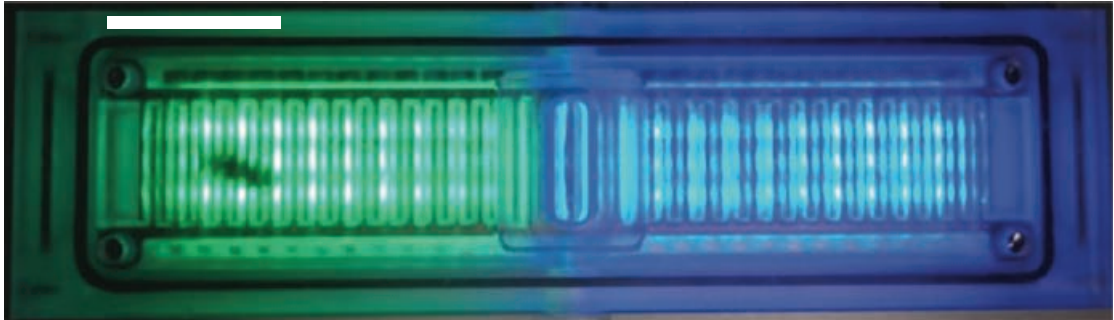
A total of 146 bees were tested in two APIS chambers in parallel (NT group: 48 bees, TH group: 48 bees, FL group: 50 bees, see details in table S1). The order of testing bees in the APIS chamber was reandomised with respect to source colony and treatment, and we made sure to have an approximately equal representation from all hives on each sampling day.

Foragers were collected when leaving the hive, using a hand-held aspirator (BioQuip Products, Inc., CA, USA). Bees were captured less than 15 minutes prior to testing and stored for a minimum of 10 minutes in the dark, at approximately 24°C in 50mL Falcon™ conical centrifuge tubes. Since foragers were selected randomly, their age and foraging experience were unknown, but was expected to be distributed similarly within each treatment group.

Testing apparatus (APIS box):

We used an aversive visual conditioning apparatus (figure 1A) developed and manufactured at Konstanz University, Germany [41,42,44]. The apparatus is a conditioning box 148mm long, 20mm wide, and 6mm high. Within the chamber bee motion is automatically tracked with 26 infra-red LED sensors lining the walls. The registered parameters, with a frequency of 5 Hz, are position, direction of movement and distance. Any turn made by the animal is only registered by the sensors if it is a complete reversal of direction. The apparatus uses sensor feedback to determine the bee's location and initiates delivery of appropriate visual stimuli according to the bee's location in the chamber. These are provided by tricolour (Blue-Green-Yellow) LEDs along the chamber walls. During the experiment, blue light LEDs ($\lambda = 465$ nm, luminous intensity: 105 mcd) were switched on in the half of the chamber where the bee was located, and green light LEDs ($\lambda = 525$ nm, Luminous intensity: 119 mcd) illuminated the opposite half. For the training trials, electric shock pulses (10 V, 4Hz, 100ms) were delivered to the tarsi of the bee through the metal grid as long as movement sensors on the blue side were triggered.

A.



B.

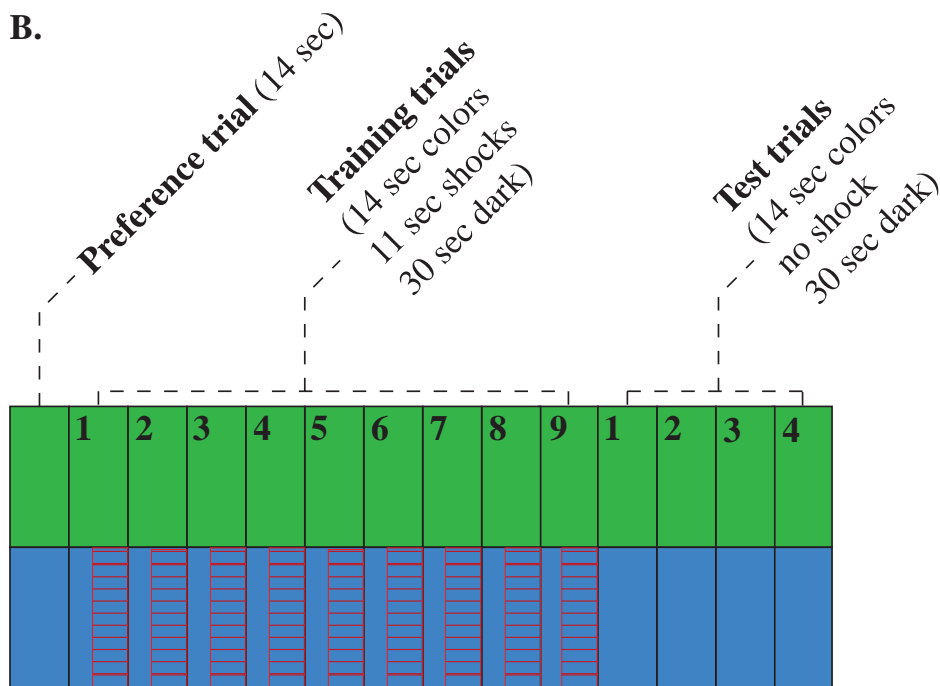


Figure 1: Assay protocol. **A.** APIS chamber (white bar = 20 mm). Either side of the chamber can be illuminated with two different light fields: light appearing green ($\lambda = 525$ nm) or blue ($\lambda = 465$ nm) to humans. The chamber is equipped with an electrified grid delivering 10 V shocks to the bee's tarsi, and with infrared sensors to automatically track the bee's movements. **B.** Visual learning assay protocol. After a habituation period of 7 min with no light, the bee was exposed to 14s of both green and blue illumination to determine its spontaneous preference. The bee was then subjected to nine training trials in which, after 3s of illumination, the bee experienced shocks (red rectangles) on the blue side for another 11s, but not while on the green side. Subsequently, the bee was tested four times with 14s of illumination without shocks to determine the post-training response to blue and green light fields.

Prior to each assay, all inner surfaces of the chamber were cleaned with 70% ethanol solution [41], and left to air dry, in order to avoid any remaining alarm pheromones, which are known to impair learning [45].

Conditioning protocol:

Almost all individual bees transferred themselves into the aversive conditioning chamber (figure 1A) by walking from the Falcon™ tube (kept in the dark) into the chamber entrance, which was illuminated from within with yellow light. Some bees (N = 14) were excluded from trials if an assisted transfer was required, and prolonged buzzing was induced. Following testing, bees were euthanised by freezing.

Bees were conditioned to avoid a blue light environment (conditioned stimulus: CS) associated with electric shocks (unconditioned stimulus: US). They could avoid being shocked by moving into a safe and unshocked zone (the green light environment, for which bees also exhibited a spontaneous preference [44]). This protocol has been successfully applied in previous studies [42,44]. The conditioning protocol consisted of one unreinforced preference test followed by nine reinforced training trials, and ended with four unreinforced test trials (figure 1B). All trials lasted 14 s, with an inter-trial interval of 30 s. For the training trials, electric shock pulses were activated 3 s after light onset.

Statistical analysis:

Analyses were adapted from Plath et al. [42]. We used a Performance Index (PI) as a metric representing the learning performance of the bees:

$$PI = \frac{t(\text{green}) - t(\text{blue})}{t(\text{green}) + t(\text{blue})}$$

Where $t(\text{green})$ is the time spent on the green (safe) side of the chamber, and $t(\text{blue})$ is the time spent on the blue (shocked) side of the chamber. PI varies between – 1 and 1, where the positive values indicate that the bee spent more time in the safe side than on the shocked side, and negative values indicate the opposite. A bee that has learned the association between the blue light and the shocks is expected to run away from the blue side shortly after light-onset and avoid returning to the blue side, and thus have high PI values. By contrast a bee that has not learned the association is expected to have low PI values.

We used different metrics to assess the degree of learning during the training phase. First, we measured the crossing latency, i.e. the delay to cross over to the green side from the blue side after light-onset. If the bee managed to cross over in less than 3 s, it could completely avoid being shocked due to the delay of the shock-onset after light-onset, assuming the bee would not then return to the blue side. If the crossing

latency was higher than 3 s the bee would experience shocks on the blue side. We thus recorded the number of electric shocks delivered to each bee, during each training trial, which was highly negatively correlated with the PI values (correlation: Pearson's ρ [CI]= -0.84 [-0.85,-0.82], $df = 1378$, $t = -56.82$, $P < .0001$).

The number of reversals of direction were used to calculate a reversing difference: number of reversals performed on the blue side subtracted from the number of reversals performed on the green side of the chamber divided by the total number of reversals:

$$\text{reversing difference} = \frac{\text{reversals}(\text{green}) - \text{reversals}(\text{blue})}{\text{reversals}(\text{green}) + \text{reversals}(\text{blue})}$$

If a bee did not return to the blue side after having received shocks, it would make frequent reversals on the green side and thus its reversing difference index would be positive. Otherwise, a bee that did not react to shocks would do reversals in both the green and the blue side and have a less positive or negative reversing difference index. This index is another indicator of learning ability.

In order to address the locomotion aspect in more detail, we also calculated the average speed per trial as well as the total number of direction changes (or reversals) per trial in both the green and the blue side. A high number of direction changes can indicate an agitated behaviour, and can be correlated with higher speed. A low number of direction changes can indicate slow movements or stationary periods.

Data were analysed and graphed using R version 3.2.3 [46] (operating via Rstudio, version 1.0.136 [47]). Statistical analyses of PI for all trials of the assay, were investigated with Generalised Linear Mixed Models (GLMMs) with bee identity as a random factor to correct for repeated measurements (lme function from nlme package [48]). The same statistics have been applied for the other metrics for the second the last training trial. Minimum adequate models were identified among other models following the Akaike Index Criterion (AIC) selection method [49] (table S2). For all metrics, we found that hive origin did not have any significant effect on variance (table S2). Differences between the treatments groups were then assessed with Tukey post-hoc tests, using the R multcomp package [50].

Results:

Since bees moving slowly could not perform well in this movement-based performance assay, animals with average speeds lower than 2.1 cm/s were excluded from the analysis [42,44] (8 of the 146 tested bees, table S1). Thus, 138 bees were kept for our analyses (NT: 44, FL: 49, TH: 45). We found reduced associative learning capabilities in FL-treated bees compared to NT bees. This was not the case when comparing TH-treated bees and NT bees.

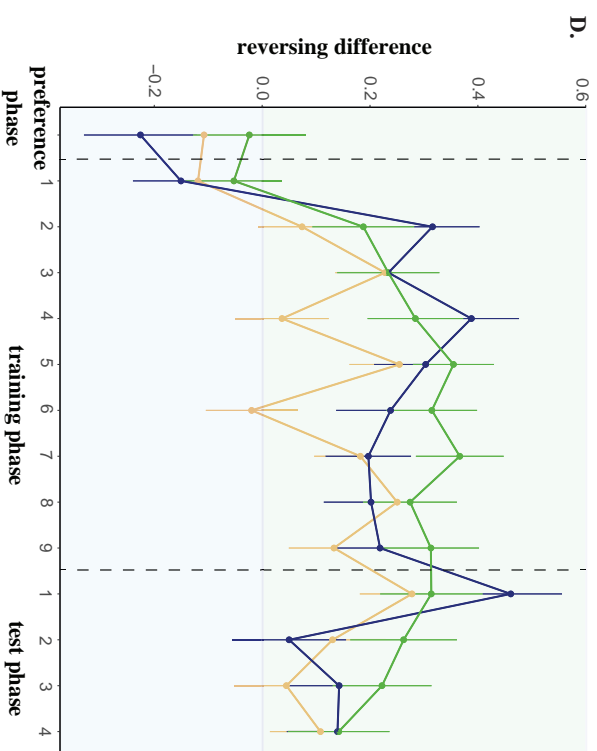
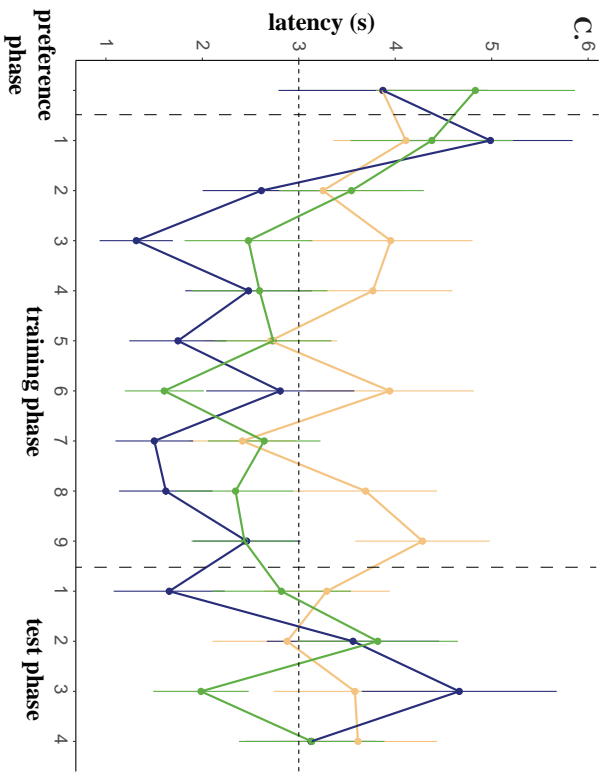
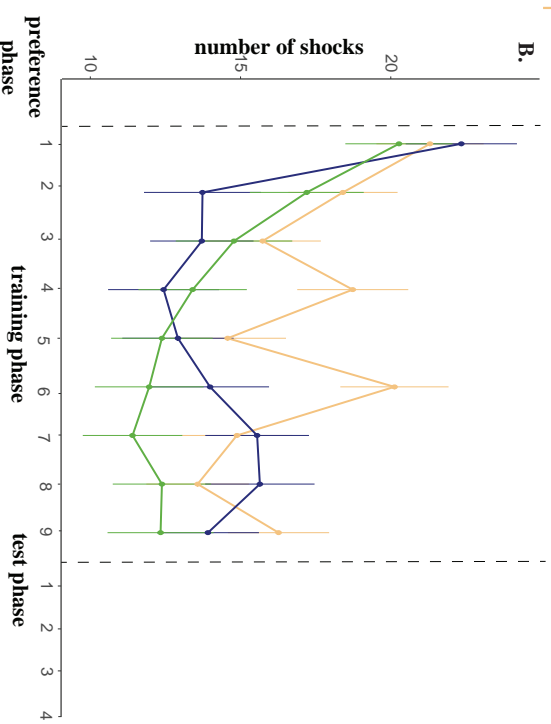
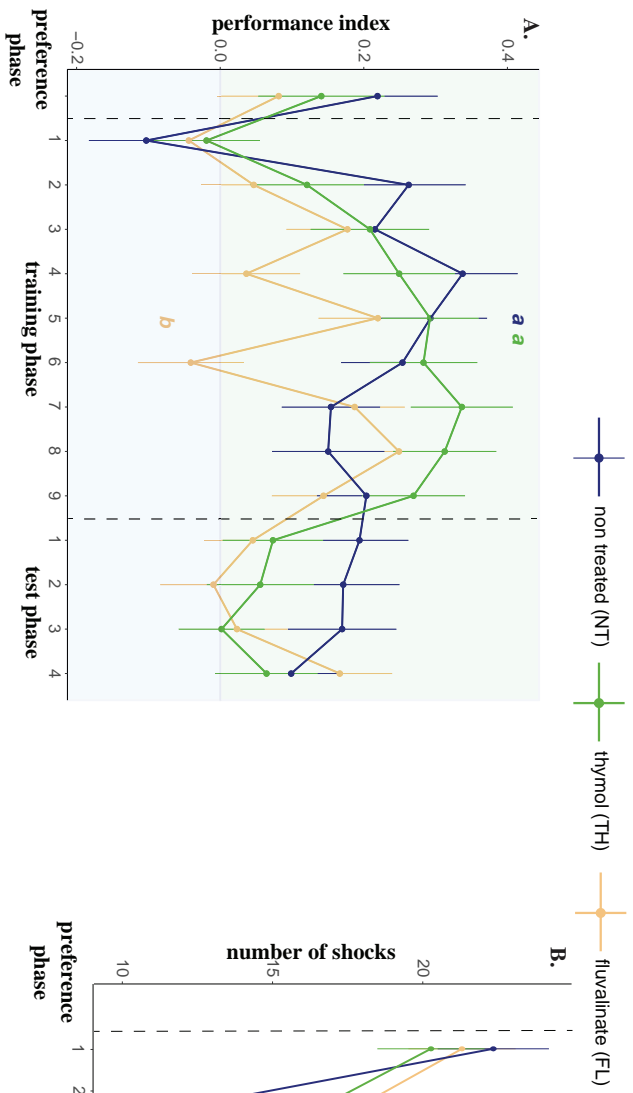
Tau-fluvalinate-treated bees showed reduced learning performance:

Treatment groups differed in the change in PI with across trial (figure 2A; GLMM, $N = 138$, $F_{(2,135)} = 3.64$, $P = 0.029$, HDS Tuckey post-hoc test, NT – FL = 0.096 ± 0.038 , $Z = 2.50$, $P = 0.037$).

Considering the overall changes in performance index over training (figure 2A), we found that NT bees and TH bees learned to avoid the blue side of the chamber. For both groups, PI significantly increased over the training phase (last vs. first trial, paired t test: NT group: $df = 43$, $t = -2.54$, $P = 0.0145$, TH group: $df = 44$, $t = -2.92$, $P = 0.005$). This increase in PI values over trials was accompanied by a decrease in the number of electric shocks received by the bees (figure 2B, last vs. first trial: NT, paired t test, $df = 43$, $t = 2.99$, $P = 0.005$; TH, $df = 44$, $t = 3.23$, $P = 0.002$). In parallel, the crossing time latency decreased (figure 2C; last vs. first trial: NT, paired t test, $df = 43$, $t = 2.65$, $P = 0.013$; TH, $df = 44$, $t = 2.31$, $P = 0.026$), and the reversing difference showed increasingly positive values, indicating bees turning more often in the green side (figure 2E; last vs. first trial: NT, paired t test, $df = 43$, $t = -2.87$, $P = 0.006$; TH, $df = 44$, $t = -3.06$, $P = 0.004$).

FL bees spent similar amounts of time in the green zone during the first (48%) and the last (57%) training trials and thus did not change their PI over training (figure 2A, last vs. first trial, t test: $df = 48$, $t = -1.78$, $P = 0.081$). They did receive marginally fewer shocks in the last training trial when compared to the first (figure 2B, last vs. first trial, t test: $df = 48$, $t = 2.03$, $P = 0.048$). They maintained a relatively high crossing latency towards the green side between the first (4.11 s) and the last training trails (4.28 s) (figure 2C, last vs. first trial, t test: $df = 48$, $t = -0.17$, $P = 0.866$).

Finally, they reversed direction more frequently in the green side over successive trials (figure 2D, last vs. first trial, t test: $df = 48$, $t = -2.12$, $P = 0.039$)



<- **Figure 2: Indicators of learning performance.** Means \pm SE are plotted for all variables. Blue: non-treated (NT, N = 44). Green: thymol (TH, N = 45). Orange: tau-fluvalinate (FL, N = 49). Bees were subjected to one preference trial, nine training (reinforced) trials and four test (unreinforced) trials. **A. Preference Index.** There was a significant difference between the treatments (GLMM, N = 138, $F_{(2,135)} = 3.64$, $P = 0.029$). Treatment comparison with a Tukey HSD post-hoc test showed differences in PIs of NT and FL groups (0.096 ± 0.038 , $Z = 2.50$, $P = 0.037$). Significant treatment effects determined with a GLMM ($p < 0.05$) are indicated with letters a and b. **B. Number of shocks received during the training phase.** There was no significant difference between the treatments (GLMM, N = 138, $F_{(2,135)} = 2.88$, $P = 0.059$). **C. Crossing latency to move towards the safe side (green side) after the light onset.** During the 3 first seconds there was no shock. During the training phase, after the first training trial, tau-fluvalinate bees have a longer latency than non treated bees (GLMM, N = 138, $F_{(2,135)} = 3.36$, $P = 0.037$, Tukey HSD, NT vs. FL, $est = -1.43 \pm 0.57$, $Z = -2.53$, $P = 0.034$). **D. Reversing differences.** After the first training phase, all bees increase their reversing difference towards the green side. After the first training trial, tau-fluvalinate treated bees had a lower reversing difference than the thymol treated bees (GLMM, N = 138, $F_{(2,135)} = 3.40$, $P = 0.036$, Tukey HSD, TH – FL, $Est = 0.13 \pm 0.05$, $Z = 2.39$, $P = 0.050$).

No effect of treatments on short-term memory

Overall, there was no significant difference between groups for PI values (figure 2A, GLMM, N = 138, $F_{(2,135)} = 2.32$, $P = 0.10$) or crossing latencies during test trials (figure 2C, GLMM, N = 138, $F_{(2,135)} = 0.15$, $P = 0.86$), indicating no effect of treatments on the test phase: therefore all groups recalled the learned paradigm and thus their short-term memory was not significantly affected.

NT bees learned to avoid the blue side and remembered it during the test phase. Their performance index did not change over the four successive unreinforced test trials (figure 2A, ANOVA, N = 138, $F_{(1,413)} = 0.02$, $P = 0.896$).

Effects of treatment on speed and turning in the APIS chamber:

Bees from all groups showed similar speeds before the training started (preference phase: GLMM, N = 138, $F_{(2,135)} = 0.08$, $P = 0.926$). They then significantly increased their speed after they received shocks for the first time (second vs. first trial: t test: NT: $df = 43$, $t = -5.99$, $P < 0.0001$; TH: $df = 44$, $t = -4.98$, $P < 0.0001$; FL: $df = 48$, $t = -4.41$, $P < 0.0001$), thus indicating that the shocks elicited a clear behavioural response in all three treatment groups (figure 2D). However, on average FL bees were slower (figure 3A, GLMM comparing speed of treatment groups in training trials 2-9,

$N = 138$, $F_{(2,135)} = 4.60$, $P = 0.012$, Tukey HSD, NT vs. FL, Est = 0.56 ± 0.23 , $Z = 2.50$, $P = 0.033$, TH vs. FL, Est = 0.60 ± 0.22 , $Z = 2.54$, $P = 0.033$).

When looking at the total number of changes of direction (figure 3B) we found that, during the preference phase (i.e. before receiving the shocks), but not after, NT bees did fewer reversals than the two treated groups (figure 3B, GLMM comparing reversal number in treatment groups during the preference test, $N = 138$, $F_{(2,135)} = 4.46$, $P = 0.013$, Tukey HSD: NT vs. TH Est = 2.33 ± 0.99 , $Z = 2.35$, $P = 0.038$, NT vs. FL, Est = -2.72 ± 0.97 , $Z = -2.79$, $P = 0.016$). This indicates a potential effect of both FL and TH treatment on bee locomotor activity in the APIS chamber prior to shock.

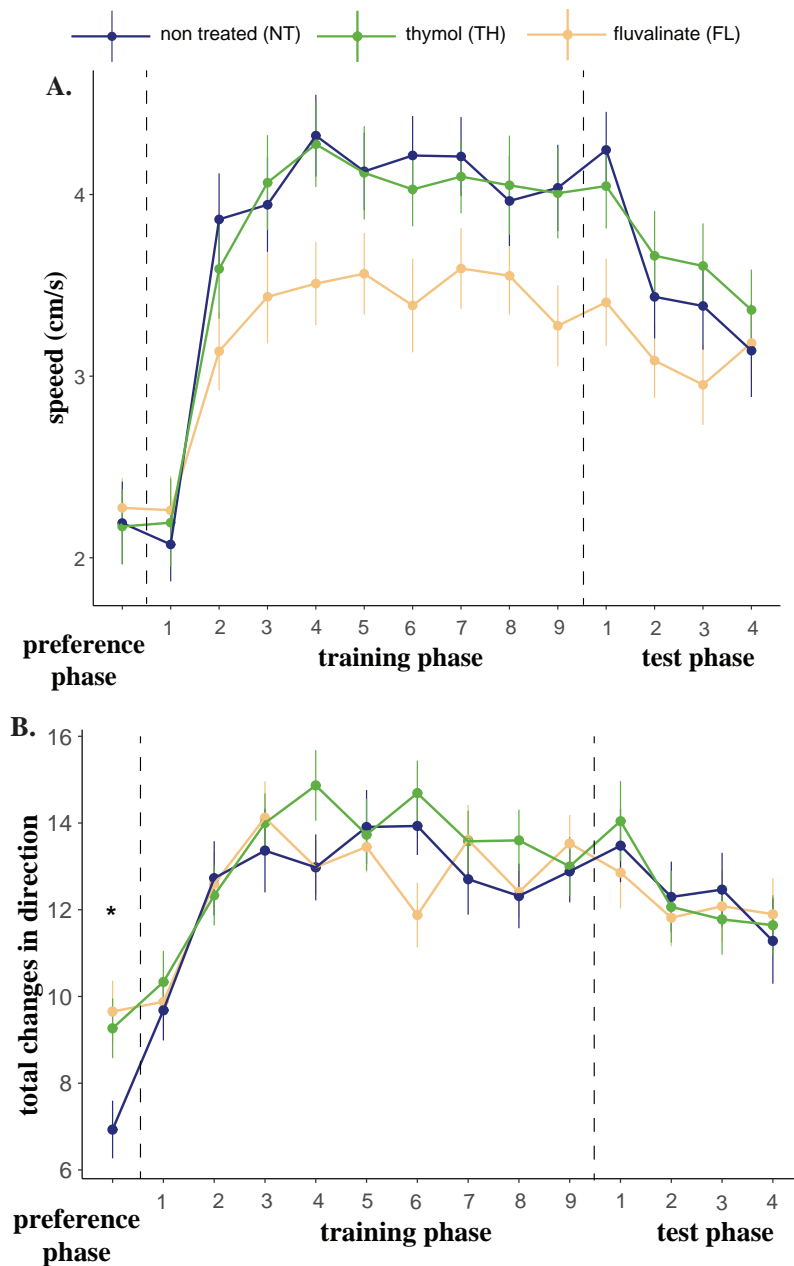


Figure 3: Indicators of locomotor response. Means \pm SE are plotted for all variables. Blue: non-treated (NT, N = 44). Green: thymol (TH, N = 45). Orange: tau-fluvalinate (FL, N = 49). **A.** Average speed at trial onset Speed values (cm/s) were averaged over the first 3 seconds of each trial, i.e. before bees could receive shocks. After the first training trial, FL-treated bees were slower than the two other groups (GLMM, N = 138, $F_{(2,135)} = 3.72$, $P = 0.027$, Tukey HSD, NT vs. FL, Est = 0.56 ± 0.23 , $Z = 2.50$, $P = 0.033$, TH vs. FL, Est = 0.60 ± 0.22 , $Z = 2.54$, $P = 0.033$). **B. Total number of changes of direction during each trials.** The total number of reversal on both green and blue sides is smaller for the NT bees than the two treated groups during the preference phase (GLMM, N = 138, $F_{(2,135)} = 4.46$, $P = 0.013$, Tukey HSD, NT vs. TH Est = 2.33 ± 0.99 , $Z = 2.35$, $P = 0.038$, NT vs. FL, Est = -2.72 ± 0.97 , $Z = -2.79$, $P = 0.016$).

Discussion:

We compared the effect of two miticides frequently used to reduce varroa loads in honey bee colonies – tau-fluvalinate and thymol – on foragers' cognition. Miticide treatments were specifically applied to colonies never exposed to the varroa parasite, in order to avoid possible confounding effects due to varroa infestation itself. We found that whereas thymol did not affect bee visual aversive learning abilities, bees that were treated with tau-fluvalinate were less efficient learners than non-treated bees in this particular visual aversive learning task.

Our assay was able to capture visual learning behaviour in honey bee foragers, as clearly shown by non-treated bees which recapitulated the findings from a previous study using the same protocol [42]: foragers started with a low performance index that increased during the training phase as more bees associated blue light with electric shocks (figure 2A). Concomitantly, foragers progressively learned to avoid receiving electric shocks, as indicated by their increasing tendency to reverse direction while still in the safe green side, as well as by the decreasing number of shocks received per trial (figure 2B,D). In addition, these learned changes in behaviour persisted during the test phase without shocks, thus indicating retention of the associative memory.

While bees from thymol-treated hives showed a very similar learned change in behaviour to that of controls (figure 2A-D), tau-fluvalinate-treated bees demonstrated less evidence of learning over the course of the training trials (figure 2A,C). Thus, our study indicates that the miticide tau-fluvalinate can impair aversive learning performance, when administered according to manufacturers' instructions. By contrast, short-term memory performance did not seem to be affected by miticide treatments as there is no difference between the three groups for the unshocked post training tests phase.

These results are partly consistent with those of several studies addressing the impact of such miticides on appetitive olfactory learning, using a standard protocol in which odorants were associated with a food reward [35,51]. Indeed, negative effects were reported on learning performance for tau-fluvalinate [21], but not for thymol [35,51].

What could be the mechanisms by which fluvalinate impairs aversive learning? Interestingly, we observed that the change in speed in response to shocks by fluvalinate-treated bees, was lower than in other groups (figure 3A). This suggests

that the miticide did not affect overall locomotion, but rather the level of shock-induced activity, thus suggesting possible impacts on the animal's sensitivity to the shock. This might thus be an explanation of the poorer learning performance, i.e. if the valence of the unconditioned stimulus (the shocks) was decreased in fluvalinate-treated bees.

Interestingly, fluvalinate also affects sucrose responsiveness [21], thus having potentially general effects on modulatory circuits and/or attentional processes. Alternatively, learning may be normal but not its behavioural manifestation, if chronic fluvalinate exposure impedes the maintenance of high activity levels such as those triggered by the shocks. Our results confirm that fluvalinate-treated bees were slower than the non-treated and the thymol-treated group after the second training trial (figure 3A). More studies, using other visual learning paradigms requiring no locomotor response [52], are needed at this point to address such a question. Interestingly, in the absence of shocks during the preference test, both treated groups were more active than the non-treated group, as they performed more reversals (figure 3B). This may indicate a potential higher basal level of stress for miticide-treated bees, which needs to be explored further. Simple locomotion tests in an arena might confirm such a finding.

Our experimental design allowed us to capture the impact of chemical stressors on foragers' visual cognition when chronically applied at field realistic doses (suppliers' instructions) during the development and the in-hive phase of bees from varroa-free colonies. In fact, this may explain why we observe more subtle effects than in previous studies done using infested colonies [21,35,49]. One can then suspect that the stronger effects seen in those studies, particularly on short-term memory, can be due to the combination of miticide treatment and varroa infestation. Our study importantly calls for more studies on effects of miticides on different aspects of cognition in bees that should be conducted in Australia where there is currently no varroa mite presence.

Control of varroa mite is one of the major stakes of modern beekeeping. So far our study has highlighted the fact that, without being in contact with the varroa mite, Australian bees reacted negatively to the synthetic chemical treatment tau-fluvalinate, but that using thymol was not impairing the visual learning abilities of adult bees. Since we used foragers in these studies, the apparent learning and/or locomotor

impairments found here suggest that fluvalinate might reduce individual foraging performance and thus the overall efficiency to collect food, at the colony level. Further experiments, e.g. based the monitoring of foraging performance (chapter 2) and its impact on colony fitness, should be conducted to test these hypotheses.

Authors contribution:

SK, PV and ABB design the experiment. PV and SK collected the data. SK analysed the data. SK, ABB, JM and ML wrote the manuscript.

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Supplementary material:

Table S1: Size samples. The total number of bees in each group (*bottom row*), is the one used for analysis after removing individuals that were too slow ($< 2\text{cm}\cdot\text{s}^{-1}$) [42]

Treatment	Non-treated (NT)		Fluvalinate (FL)		Thymol (TH)	
	E	J	G	H	A	B
Tested bees	24	24	26	24	24	24
Excluded bees	0	4	0	1	2	1
Analysed bees	24	20	26	23	22	23
Total	44		49		45	

Table S2: Model selections based on AIC comparisons. For each parameter (preference index, number of shocks, crossing latency, reversing difference, speed, total number of reversals), the best model is in bold. Hive origin seems to have no effect on the variability of either of the analysed parameters.

	df	AIC	Loglik	Chi ²	P
a. Preference Index (PI)					
PI ~ treatments + trials	5	2959.4	-1474.7		
PI ~ treatments + trials + 1 ID	6	2930.5	-1459.2	0.87	<.0001
PI ~ treatments + trials + 1 hive/ID	7	2932.4	-1459.2	0.07	0.79
b. Number of shocks					
Shocks ~ treatments + trials	5	14983.4	-7486.7		
Shocks ~ treatments + trials + 1 ID	6	14958.8	-7473.4	26.64	<.0001
Shocks ~ treatments + trials + 1 hive/ID	7	14960.7	-7473.3	0.09	0.77
c. Crossing latency					
Latency ~ treatments + trials	5	11724.5	-5857.2		
Latency ~ treatments + trials + 1 ID	6	11545.6	-5766.8	180.8	<.0001
Latency ~ treatments + trials + 1 hive/ID	7	11546.9	-5766.4	181.6	0.567
d. Reversing difference					
Reversing difference ~ treatments + trials	5	3116.6	-1553.3		
Reversing difference ~ treatments + trials + 6 1 ID	6	3051.9	-1519.9	66.45	<.0001

Reversing difference ~ treatments + trials + 1 hive/ID	7	3053.6	-1519.8	66.79	0.654
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e. Speed

Speed ~ treatments + trials	5	14749.1	-7815.3		
Speed ~ treatments + trials + 1 ID	6	14705.3	-7345.6	937.2	<.0001
Speed ~ treatments + trials + 1 hive/ID	7	14705.5	-7345.6	2.25	0.134

f. Total number of reversals

reversals ~ treatments + trials	5	11946.3	-5968.2		
reversals ~ treatments + trials + 1 ID	6	11679.0	-5833.5	269.3	<.0001
reversals ~ treatments + trials + 1 hive/ID	7	11680.1	-5833.0	0.87	0.349

General discussion:

In this thesis, I investigated the foraging behaviour of individuals from two economically and ecologically important eusocial bee species: the European honey bee (*Apis mellifera*) and the buff-tailed bumblebee (*Bombus terrestris*). I developed novel experimental designs that allowed me to analyse individual behaviour of foragers in different contexts, from natural environments (**chapters 2 and 3**), or in laboratory conditions (**chapter 4**, in a free-flying assay, and **chapter 5** in a walking assay). I found a certain degree of individual variation in the behaviour and performance of both honey bee and bumblebee foragers. In this thesis, I have explored the factors contributing to this variation. My thesis has recognised the importance of considering individual responses to environmental stressors to understand the impacts of stressors on bees and their colonies.

For **chapters 2 and 3** of this thesis, I developed a novel and powerful method by combining Radio Frequency Identification (RFID) technology with microbalances and video recording. This system allowed automated reconstruction of foraging trips and an estimation of the foraging efficiency of many individual foragers within a given colony. This method enabled me to follow hundreds of individuals for their entire foraging life in a natural environment and reconstruct their lifelong contribution to the colony. Using this technique, I gathered far more and higher quality data than any prior study of individual bee foraging efficiency in either the lab or the wild.

Prior to this research the most comprehensive analysis of bumblebees was that of Peat and Goulson (2005) who studied the effects of experience on bumblebee foraging efficiency (Peat & Goulson 2005). Their data set contained data from 473 individuals from three colonies each followed for two weeks, but each bee only was observed for a few hours each day and therefore foraging histories were not complete. By contrast my bumblebee studies (**chapter 3**) included detailed foraging histories of 99 bees from one colony. Each of these 99 bees was followed for its complete foraging lifetime. The most active bee in my dataset completed 983 trips and on average I documented 134 complete foraging trips for each bee.

Prior to this study, ironically there has been less data on individual foraging performance for honey bees than bumblebees. Dukas (2008) exhaustively followed 26 bees and documented changes in their foraging efficiency across their lifespan (Dukas

2008). Tenczar et al. (2014) employed an RFID system to monitor individual variation in honey bees and followed up to 400 bees per colony for their number of foraging trips but with no information about the food collected (Tenczar *et al.* 2014). In a flight cage, they followed 200 bees from two colonies with RFID feeding at artificial nectar and pollen feeders, which allowed an estimation of what resource bees collected in this simplified environment. By contrast my studies in **chapter 2** included data from 574 bees that were followed for their complete foraging lifespan including the trip efficiency in the natural environment.

The theme linking **chapters 2 to 5** has been an investigation of the causes of inter-individual variation in foraging behaviour. Several factors have been shown to influence bee foraging performance, which are discussed below and my major findings are summarised in Table 1.

Causes of inter-individual variation in foraging behaviour

Foraging experience

Bees gain foraging experience progressively as they complete foraging trips. With experience, bees learn about their environment and improve their behaviour accordingly. This is particularly true when bees learn the spatial features and landmarks surrounding the hive (Giurfa 2013). In **chapters 4 and 5**, when I looked at the behaviour of bumblebees in a spatial task and honey bees in a visual learning test, I observed an improvement in performance in successive trials. In **chapter 4**, bees were trained to find the most efficient route between artificial flowers; they sped up and made fewer navigation errors with experience. In **chapter 5** control and thymol-treated bees learned to avoid the colour associated with electric shocks, by repeated exposure to the colour associated with shock.

For honey bees, the benefits of experience for task performance were also evident in foraging behaviour. I found, in **chapter 2**, that consistent with existing reports (Dukas & Visscher 1994; Dukas 2008), foraging efficiency for nectar increased with experience. I showed in **chapter 2** that pollen foraging is more likely to be performed by more experienced honey bees. Thus, differences in foraging efficiency in honey bees were strongly influenced by forager experience.

By contrast, I did not find any effect of experience on bumblebee foraging performance for pollen (**chapter 3**). But earlier studies have reported that, similar to

honey bee foragers, bumblebees increase their nectar foraging collection rate with foraging experience (Peat & Goulson 2005; Durisko, Shipp & Dukas 2011). It seems likely that if we are able to measure a rate of pollen collection in our study, or a rate of nectar collection we may also have detected an experience-dependent improvement in performance in bumblebees.

Age

Division of labour in honey bees is an age-related phenomenon (Huang & Robinson 1996). Each individual starts foraging typically around two weeks after eclosion as an adult, following a period working inside the hive as a nurse bee (Huang & Robinson 1996). But variability in age of onset of foraging occurs naturally in most colonies (Huang & Robinson 1996). Age of onset of foraging can also heavily be impacted by environmental stressors that can induce precocious foraging (reviewed in **chapter 1**, and in **appendix 2 Figure 1a**, I present data showing miticide treatment can induce a precocious foraging onset). Precocious foragers show poor performance in both short and long range spatial tasks, and have lower survival rates in the wild (Ushitani *et al.* 2015) and have poor olfactory learning and memory (Cabirol 2017) when compared to normal age foragers. Precocious foraging can also have dramatic consequences for the whole colony as mathematical models of colony dynamics show that a high proportion of precocious foragers in the foraging force can contribute to a colony collapse as a consequence of reduced forager survival and performance (Perry *et al.* 2015).

I did not find any effect of age of onset of foraging on whether the bees were either mixed or only nectar foragers, or elite bees or non-elite bees in either bumblebees or honey bees **chapter 2 and 3**. In the analyses in this thesis we did not attempt to induce precocious foraging by either a social manipulation or applying stressors. For the honey bees (**chapter 2 Figure S2A**) more than 90% of the bees monitored in our sample would not have been considered as precocious foragers (beginning when less than 14 days old (Perry *et al.* 2015)) and therefore precocious foragers were not strongly represented in our samples.

I did not find any effect of age of the first foraging on bumblebees' performance in the navigation assay (**chapter 4**). For bumblebees it is far less clear whether age at onset of foraging has any impact on foraging performance since in bumblebees division of labour is not an age-related phenomenon. In bumblebees

division of labour is more strongly based on morphological variation between bumblebee workers than age (Goulson 2010). Large bumblebee workers have been documented foraging on their first day post eclosion as adults. While honey bees show a prolonged period of brain maturation as adults in the hive (Fahrbach *et al.* 1998) bumblebees show a more rapid brain maturation (Jones *et al.* 2013), but the development of the bumblebee brain is still sensitive to the social environment.

Morphology

Honey bee foragers are considered monomorphic, and I did not find any evidence of an influence of weight of the individual on honey bee foraging activity and performance (**chapters 2, figure S2B**). By contrast, bumblebee workers vary in both size and weight, and within a colony of *Bombus terrestris*, some individuals can be three times bigger than others (Goulson 2010). In bumblebees size seems to influence foraging behaviour as I found that larger foragers performed better at the traplining spatial learning task (**chapter 4**). I also found that bigger foragers were less likely to collect pollen (**chapter 3**). These results are consistent with many studies showing that larger individuals are more efficient at foraging for nectar rather than pollen (Goulson *et al.* 2002; Worden, Skemp & Papaj 2005).

Impact of stressors

Exposure to different stressors at different stages of development may shape adult performance. In **chapter 5** I show that chronic exposure to a miticide during larval development and during the time adult bees spent inside the hive directly can impact honey bee forager's visual learning. Rearing environment during young adulthood also influences honey bee cognitive ability (Cabirol *et al.* 2017). Honey bees deprived of the sensory and social stimuli of the hive environment during the early weeks are poor performers at a learning task that relies on the mushroom body, the high-order sensory integrative centre in insect brains.

In this thesis I also demonstrated that when a bee was stressed with a non-pathogenic stressor that activated the bees immune system during its nurse stage, it is less likely to collect pollen when it becomes forager (**appendix 1**). In the same way, it has been reported that parasitized honey bees forage less for pollen (Lach, Kratz & Baer 2015). Such reduction of pollen foraging due to environmental stressors has also

been observed in bumblebee colonies: bumblebees that are exposed to neonicotinoid insecticides collect less pollen (Gill & Raine 2014).

Contrasting social foraging in honey bees and bumblebees

Foraging for pollen or nectar

When comparing the foraging behaviour of honey bees and bumblebees, one of the most striking differences was the distribution of mixed foragers (that collected pollen and nectar) in the forager population. In my data honey bee colonies contained around 27% of mixed foragers (**chapter 2**) whereas the bumblebee colony contained 63% of mixed foragers (**chapter 3**).

There could be a number of different factors contributing to this difference. This observation is in line with theories of specialisation with sociality: larger and more specialised societies (such as honey bees) contain more specialised individuals than smaller and/or less advanced societies (such as bumblebees) (Anderson & McShea 2001; Fewell, Holbrook & Kukuk 2013).

Demand for carbohydrate relative to protein may be higher in a honey bee rather than a bumblebee colony given that honey bees transform nectar into a stockpile of honey to survive the winter, whereas bumblebees, as non perennial species, store a supply of nectar for a few days only (Goulson, Lye & Darvill 2008).

Intra-individual variability and inter-individual variability are beneficial for colony function

In an animal society, several individuals can collectively achieve the flexibility required for a task that might be too complex to be achieved by a single individual, such as colony food regulation (Jeanson, Dussutour & Fourcassié 2012). Further, it has been argued that in a society both efficiency and collective task performance can be improved if the society contains diverse specialists rather than uniform generalists (Robson & Traniello 1999). For instance more genetically diverse honey bee colonies have a higher fitness (Mattila & Seeley 2007) and better colony thermoregulation (Jones *et al.* 2004), ants colonies showing a high variability of behavioural types are more productive (Modlmeier, Liebmann & Foitzik 2012). Argentine ants show individual differences in their exploratory behaviour that enhance the nest hunting process (Hui & Pinter-Wollman 2014). Social spider *Anelosimus studiosus* show different consistent degrees of aggressiveness towards their prey (Pruitt & Riechert

2011) that enhance colony productivity. Burns & Dyer (2008) suggest that variability of speed-accuracy trade off between individuals would benefit bumblebee societies (Burns & Dyer 2008), especially in complex and challenging environments (Muller & Chittka 2008).

Nevertheless, bees rely on their flowering environment for their food supply, and this environment is patchily distributed in space and highly unpredictable in time. In order to guarantee food stability for the colony, individuals must collectively adjust food intake to compensate for changes in the demand, the stock in the colony, and supply theorised by collective nutritional ecology concepts (Lihoreau et al., 2014). In order to accomplish such a complex task, social insect groups might benefit from both inter-individual variation in foraging preferences (Muller & Chittka 2008) as well as individual plasticity (or intra-individual variation) for resource preference and resource persistence.

In this thesis, I have shown that honey bee and bumblebee individuals show strong individual behavioural consistency as well as a degree of individual plasticity in their foraging strategies.

I observed clear and consistent differences in foraging preferences in bumblebees in **chapters 3 and 4**. Bumblebees that foraged for pollen were constantly either high pollen gatherers or low pollen gatherers during their foraging life (**chapter 3**). When considering their traplining behaviour, some bumblebees were consistently more efficient at traplining than others (**chapter 4**). These kinds of inter-individual differences in route following also have been observed in homing pigeons (Meade, Biro & Guilford 2005), hummingbirds (Tello-Ramos, Hurly & Healy 2015) and ants (Jayatilaka *et al.* 2014); different individuals have consistent idiosyncratic route preferences.

At the individual level, mixed foragers in both species displayed a high behavioural plasticity regarding the choice of collected resources. This was seen as differences in foraging duration and a specific time of collection for pollen and non-pollen resources (**chapters 2 figure 4 and chapter 3 figure 4A**). Honey bees also show behavioural plasticity as they leave the colony with different crop quantity according to the trip duration and the type of resource they are about to collect (**chapter 2, figure 5**). Such plasticity in foragers towards one or the other resource is beneficial for regulating the nutritional needs of the colony.

Applying these methods and findings to other social insect societies

The new RFID technique used in **chapters 2 and 3**, allowed me to analyse the distribution of foraging activity (**chapters 2 and 3**) and performance (**chapter 2**) of the workers of bumblebee or honey bee colonies. I found that both social bees show a skew in the foraging activity distribution, with around 17% of the honey bee foragers (**chapter 2, figure 3**) and 11% of the bumblebee foragers (**chapter 3, figure 1**) performing more than half of the total colony activity. Moreover, I found that the elite honey bees were more productive than the non-elite ones (**chapter 2, figure 5**). Thus, this technique is a powerful tool to assess the importance of inter-individual variability of foraging activity and efficiency in the collective foraging force of a colony.

Nevertheless, how foraging efficiency is distributed between the workers in natural conditions has not yet been studied in detail in other social insect species. So far, some studies have explored either foraging activity or drifting behaviour via RFID technology in ants (Robinson *et al.* 2009), wasps (Sumner *et al.* 2007) or stingless bees (Oystaeyen *et al.* 2013), but none of these studies has reported on efficiency as no information on the food collected was provided,

Similarly, some other studies have documented the degree of inter-individual variation in foraging in other social insect species, but such studies often relied on controlled or laboratory conditions (e.g. ants: (Gordon *et al.* 2005; Robinson *et al.* 2009)) or focused on inside-colony specific tasks (e.g. ants: (Pinter-Wollman 2011)) or just looked at foraging activity but not foraging performance (Beverly *et al.* 2009). Even though these studies found high inter-individual variability in the workers' tasks, so far none of them have been able to analyse extensively the individual foraging contribution of workers in natural conditions during their whole foraging life, as I reported for bees in **chapter 2**. Applying the methods developed here to other social insect species would then allow us to extend the analysis of the consequences of inter-individual variation for colony function to other systems that do not share the same ecological niche and/or show different degrees of sociality for a much richer comparative analysis.

The keystone individual concept and bee societies

During this thesis, I showed that the high variability in foraging activity results in the existence of a subset of high contributors to the foraging force: the elite bees

(chapter 2, figure 3 and 3, figure 2). I have shown that elite bees, in honey bee colonies, are not only the most active but also the most efficient in food collection and therefore are by far the most valuable foragers for the colony. Such high contributors to a social group have been coined as keystone individuals. In many other social groups (like other insects (Chang & Sih 2013), fish (Cote *et al.* 2010), or mammals (Lusseau & Newman 2004)) keystone individuals are often disproportionately important for maintaining group integrity (Modlmeier *et al.* 2014), or can have a strong detrimental effect on a group or a population. For instance in several taxa, individuals that both have high disease load and are hubs in a social network (superspreaders) can propagate disease would have a strong negative impact on a group or a population by spreading quickly diseases (Paull *et al.* 2012). In the majority of the cases, the removal of keystone individuals will have a severe and long-lasting impact on the group (be it positive or negative) (Modlmeier *et al.* 2014).

Nevertheless, in social insects, it has been argued that highly active individuals can be easily replaced by less active ones, thus increasing resiliency of the colonies (e.g. ants (Charbonneau, Sasaki & Dornhaus 2017), bumblebees (Jandt *et al.* 2012) or honey bees (Tenczar *et al.* 2014)). Tenczar *et al.* (2014) argued that if elite bees are lost to a colony they can be replaced rapidly by the less active bees that in fact serve as a back-up pool. They then argue that this reserve of less active bees to replace the most active (and apparently expendable) elite bees increases the resilience of the group.

I disagree with this interpretation. My new data shows that elite honey bees are more efficient than less active bees, and that they gained this efficiency through extensive individual foraging experience gained over time. Given the active elites are also the most efficient, one could expect that their removal will have a greater negative impact on the colony nutritional balance than what Tenczar *et al.* (2014) were able to measure. Moreover, Tenczar *et al.* (2014) argued that replacement of elite bees is fast (less than two days in their study), but I demonstrated that foraging efficiency requires the workers to gain experience over a successive number of trips. Therefore, while the number of foragers might be rapidly replaced the performance of the foraging force would not recover as quickly.

The method developed in this thesis could be used in a removal experiment to evaluate the actual impact on the colony of removing more efficient and active

foragers that developed their foraging efficiency over days. Such an approach, if applied to both bumblebees and honey bees under similar conditions would then enable an investigation of how inter-individual variation in performance might interact with resiliency in different species. Rundlöf et al. (2015) reported that, in a similar context of exposure to neonicotinoid-treated crops, bumblebee colonies survival was lower than honey bee colonies (Rundlöf *et al.* 2015). Does this suggest a bumblebee colony has lower intrinsic resilience than a honey bee colony?

Perspectives

My work brings some new insights to the sociobiology and behavioural ecology of social insects, which has led to several questions of interest.

Integrating environmental and social conditions in an understanding of foraging diversity

Given the amount of data I collected and its precision at the individual as well as the collective level, it may help to build more accurate and sensitive models of both honey bee and bumblebee colony dynamics, incorporating both individual behaviour and weather conditions.

Theoretical models have been used to investigate honey bee colony dynamics (Khoury, Myerscough & Barron 2011; Khoury, Barron & Myerscough 2013; Becher *et al.* 2013, 2014) without taking into account individual variation. Other models have approached the role of inter-individual variation for different aspects of colony function (honey bee colony pollen collection (Bertram, Gorelick & Fewell 2003), colony response to environmental change (Myerscough & Oldroyd 2004) or colony thermoregulation (Jones *et al.* 2004)). Finally, some predictive models have been used to investigate how insect societies achieve their nutritional balance (Behmer 2009; Lihoreau *et al.* 2015). But so far, those models lack validation against field data. It is also important to be able to include inter-individual variability in general models of colony growth and social nutritional framework modelling.

By including inter-individual variation into a model of colony dynamics and nutritional regulation, I can potentially create a more powerful predictive model of colony functions. My data can give baseline indicators of foraging efficiency and foraging variation in natural environments for a simulation of a colony in which one can explore the consequences of varying the degree of the skew of the distribution of

the foraging force. Another simulation could consider how variation in sensitivity of foraging efficiency to individual experience might influence colony growth dynamics.

Importance of variability for the resilience towards stressors

I have suggested that individual variation confers resiliency in changing and/or stressful environments. To validate such a statement, I first would need to address the importance of foraging diversity in stressful environments. Experimentally it would be interesting to artificially create less diverse colonies (considering monandrous single drone inseminated (SDI) honey bee colonies, or bumblebee colonies with artificially-controlled variation in size of the workers), and compare their resilience to more diverse colonies.

Second, it would be interesting to test whether, under stressful conditions, the properties of the foraging force are changed. This could be tested quite easily by using some experimental stressor (such as the pathogen free stress condition, used in **appendix 1**) and measuring the impact of the stressors on the extent of foraging variability and forager performance. Does a stressed colony increase inter-individual foraging variation in order to cope with the stress, or is this diversity too costly under stressful conditions? Fundamentally, is a colony able to adjust its foraging diversity according to the presence of stress, and if so, what is the consequences of such plasticity for the superorganism?

Importance of foraging diversity for pollination services:

I perceived idiosyncratic variation in the foraging behaviour of different individuals that could potentially impact pollination outcomes. In the field, the patchiness, incompleteness or idiosyncrasy in pollination are still puzzling growers and pollination biologists (Cunningham *et al.* 2016).

My data have highlighted how floral visits in a local area might be dominated by a relatively small number of individuals (**chapter 2 and 3**) each with idiosyncratic floral preferences, schedules and behaviour. In **chapter 4** I showed how different individuals adopted and stuck to idiosyncratic foraging paths. In bumblebees, only a handful of foragers forage for the colony in a given period of time. It is then important to consider what plants that particular individuals will forage on, as they will be the main pollinating agents in the system. I also recognise the variation of navigation skills in bumblebee foragers that can have different impacts on plant-pollination

ecology (Pasquaretta *et al.* 2017). Thus, by analysing the individual foraging dynamics of the two most commercially important pollinating bees we can better address recommendations for crop management (Klein *et al.* 2007).

In conclusion, this thesis provides a better understanding of the sociobiology of two common social insect species. This work also, by exploring the diversity of foraging behaviour of economically important pollinators, provides knowledge that we can use to mitigate threats to pollinators and therefore protect global food security.

Individual level	
Foraging performance increase with experience	Honey bees foragers are more active and collect more nectar and pollen with experience (Chap. 2)
	Bumblebee foragers are better at traplining with experience (Chap. 4)
Environmental stressors affect bee cognition	Non-treated and thymol treated honey bees are better at associate colour with shock with experience (Chap. 5)
	Environmental stressors can directly affect brain functions involved in different bee cognition skills (Chap. 1)
	Miticide tau-fluvalinate impairs visual learning (Chap. 5)
Environmental stressors affect bee foraging behaviour	Immune-challenged honey bee foragers show changes in biogenic amines concentration in their central brain (Appendix 1)
	Environmental stressors can affect bee homing behaviour and navigation (Chap. 1)
High inter-individual variability in foraging behaviour	Immune-challenged honey bee foragers collect less pollen than non stressed bees (Appendix 1)
	Some honey bee foragers collect pollen and non-pollen resources whereas other collect only non-pollen (Chap. 2)
	Some honey bee foragers collect pollen and non-pollen resources whereas other collect only non-pollen (Chap. 3)
	Some honey bees are more active and more efficient than others (Chap. 2)
	Some bumblebees are more active than other (Chap. 3)
Behavioural plasticity	Some bumblebees foragers are better at traplining than other (Chap. 4)
	Honey bees take more time for pollen foraging, they also adapt their weight on departure according to the resource collected (Chap. 2)
Difference in morphology	Bumblebees take more time for pollen foraging and forage more for pollen in the afternoon (Chap. 3)
	Bigger bumblebees are better at traplining (Chap. 4)
	Slightly bigger bumblebees forage less for pollen (Chap. 3)

Colony nutritive balance in supported by a small subset of foragers		A few individuals (elite bees) performed more trips in honey bees. They are also more efficient and collect more pollen (Chap. 2)
		A few individuals (elite bees) performed more trips in bumblebees. (Chap. 3)
No clear sub-caste of active and efficient foragers		Elite honey bees do not differ in weight or in age of onset of foraging (Chap. 2)
		Elite bumblebees do not differ in body size from the rest of the foragers (Chap. 3)
Foraging variability is a point of vulnerability for the colony		Foragers are key elements in colony structure, if they are impaired by stressors the colony can collapse, according to models (Chap. 1)
		If the small subset of mixed foragers is hit by stressors, the whole colony can be impacted (Chap. 2)
		Stressed bee collect less pollen (Appendix 1)
Inter-individual variability can be beneficial for the colony		If the elite bumblebees are hit by stressors, the whole colony can be impacted (Chap. 3)
Inter-individual variability can be beneficial for the colony		Bumblebee colony show equal proportion of foragers with different navigation skills that can be useful in a changing environment (Chap. 4)

Table 1: Table summarizing the different results as they appear in the thesis. The different chapters approached transversal questions on both individual and collective levels. This sum-up also highlights the differences and the common features between honey bees and bumblebee colonies.

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Appendices



Appendix 1: Stress decreases pollen foraging performance in honeybees

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SHORT COMMUNICATION

Stress decreases pollen foraging performance in honeybees

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ABSTRACT

Foraging in honeybees is energetically demanding. Here, we examined whether stressors, which generally increase metabolic demands, can impair foraging performance. A controlled non-pathogenic stressor (immune challenge) resulted in a change in the foraging preferences of bees. It reduced pollen foraging and increased the duration of trips in pollen foragers. Stress also reduced the amount of octopamine in the brain of pollen foragers (a biogenic amine involved in the regulation of foraging and flight behaviour in insects). According to the literature, flight metabolic rate is higher during pollen foraging than during nectar foraging, and nectar gives a higher energetic return relative to the foraging effort when compared with pollen. We thus propose that stress might be particularly detrimental to the performance of pollen foragers, and stressed bees prefer the energy-rich resource of nectar. In conclusion, stress, even at low levels, could have consequences for bee foraging behaviour and thereby the nutritional balance of the colony.

KEY WORDS: Immune challenge, Flight, Biogenic amine, Radio-frequency identification device

INTRODUCTION

For honeybees, which are central-place foragers relying on pollen and nectar from flowers, foraging behaviour places demands on both cognitive capacity (Klein et al., 2017) and metabolic capacity: indeed, insect flight is known to be among the most intense and energy-demanding physiological processes in the animal kingdom (Dudley, 2000). The metabolic rates of flying insects, mainly fuelled by carbohydrates, can be up to 170 times higher than those of resting individuals (Bartholomew and Casey, 1978). As a consequence, it is expected that environmental stressors (e.g. parasites and temperature changes), which often impose increased metabolic demands (Bordier et al., 2017a; Johnson and White, 2009), could compromise foraging performance. Deciphering how stress impacts honeybee foraging performance might therefore help us better understand the mechanisms underlying colony decline and failure, which continues to be an issue of widespread concern (Goulson et al., 2015; Potts et al., 2010).

Stressors may directly limit bees' energetic reserves and thus reduce foraging performance. Indeed, there are several reports of a reduction of global flight activity in parasitized bees due to energy

depletion (Kralj and Fuchs, 2010; Alaux et al., 2014; Naug, 2014; Wolf et al., 2014). Stressors may also affect forager decision-making processes as a consequence of the energetic challenges of the stressor, in which case bees may show a preference for carbohydrate-rich resources to supply their own energy needs. The finding that the gene coding for the pheromone biosynthesis-activating neuropeptide, a neuropeptide known to be present at higher levels in nectar foragers than in pollen foragers (Brockmann et al., 2009), is over-expressed in parasitized bees (McDonnell et al., 2013) provides some indirect support for this hypothesis. Stress can decrease sucrose responsiveness (Pankiw and Page, 2003), which is lower in nectar foragers than in pollen foragers (Pankiw and Page, 2000), suggesting that stress might cause a change in foraging preference. In addition, it has been shown that parasitized bees are less likely to forage for pollen (Lach et al., 2015). Together, these findings suggest that stressed bees may favour nectar over pollen foraging. This could have consequences for the nutritional balance and development of the colony, as the majority of larva protein intake indirectly comes from pollen supply (Brodshneider and Crailsheim, 2010; Pernal and Currie, 2000). Moreover, pollen nutrition promotes immunocompetence and parasitism tolerance of adult bees (Alaux et al., 2010; Di Pasquale et al., 2013).

To test the hypothesis that stress can induce a change in foraging performance, without any potential effects of parasite manipulation of host metabolism (Adamo, 2012; Biron and Loxdale, 2013), we exposed bees to a non-pathogenic immune challenge. Immune responses are energetically costly, and even simple responses, like encapsulation, can raise metabolic rate by up to 28% in insects (Ardia et al., 2012; Freitak et al., 2003). We then tracked their foraging behaviour throughout their life with a radio-frequency identification device (RFID), and a camera at the colony entrance to identify whether they carried pollen loads. Finally, we assessed the influence of stress on brain biogenic amine levels, which are known to be involved in the regulation of bee behaviour (Schulz and Robinson, 2001; Schulz et al., 2002).

MATERIALS AND METHODS

Experiments were performed from January to April 2016 with honeybees (*Apis mellifera* Linnaeus 1758) obtained from the research apiary at Macquarie University (Sydney, NSW, Australia). We tested the influence of stress on foraging behaviour (experiment 1) and brain biogenic amine signalling (experiment 2). Frames containing late-stage pupae were collected from three donor colonies and placed into an incubator overnight at 34°C. Newly emerged bees were marked on the thorax with either a RFID tag for experiment 1 or a paint mark for the experiment 2, and released into host colonies. They were then re-captured when 7 days old and placed in plastic cages with *ad libitum* sugar solution (50% w/v). Half of the bees were given an immune challenge, which consisted of piercing the cuticle between the third and the fourth tergites of the abdomen using a 0.15 mm needle. If a haemolymph drop was released after the pin prick, the bee was discarded. Previous studies have shown that the bee's immune system is activated by this

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wounding alone, without pathogen infection (Alaux et al., 2014; Evans et al., 2006; Siede et al., 2012). Control bees did not receive any pin prick. Handled bees (control and immune challenged) were given an additional paint mark on the abdomen to identify them by their treatment group before they were released back into their colony. This procedure was repeated three times with different bees.

Experiment 1: impact of immune challenge on foraging performance

Following the stress treatment at 7 days, 380 control and 370 stressed bees in total ($n=3$ trials) were released into a small nucleus hive equipped with a modified entrance. Bees had to use a specific path to exit the hive and another one to enter the hive. Each path was made of transparent 1 cm diameter plastic tubing (Bunnings, Gordon, NSW, Australia). To avoid bees using the wrong path, a plastic gate with plastic bristles, which bees could use in only one direction, was placed at the end of each path. The traffic of bees was also regulated using infrared-activated gates placed at the beginning of each path (Arduino Technology, Arduino, Adafruit and Little Bird Electronics, Hornsby, NSW, Australia). Each time a bee broke the infrared beam, the linked gates were closed behind the bee for 10 s, which was the time needed for bees to cross the path and RFID system. Each path was equipped with a RFID reader (Invengo, Guangzhou, China; Perry et al., 2015; Søvik et al., 2015) to monitor each of the entrance and exit channels. Each RFID tag (diameter 4 mm, mass 1 mg) had a unique digital identifier read by the antennae at the entrance and exit. The entrance path was also equipped with a digital video camera (Logitech, Lausanne, Switzerland) and a white LED light enclosed in a plastic box. Motion detection video recording software (ZoneTriger, Omega Unfold Inc., Montreal, QC, Canada) was used to visually identify whether bees carried pollen or not.

Experiments continued until the last recording of the last bee, i.e. 55 days. RFID data, i.e. bee ID, direction (entry into or exit from the hive) and time (day, hours, minutes and seconds), were recorded in .csv files. From these data, we were able to reconstruct trips outside the hive for each bee. RFID readings were time matched with readings from the camera, and videos taken from 10 s before RFID detection were inspected to identify the resource for the returning bees (pollen or not pollen). Only data for bees with an RFID tag and paint marks on their abdomen were analysed. Trips shorter than 30 s were not considered as foraging flights and were excluded from the study. As in Perry et al. (2015), trips longer than 8 h were also removed.

Of the 380 control and 370 immune-challenged bees, a completed foraging flight was recorded at least once from 96 and 74 bees, respectively. This loss of bees could be due to the loss of tag prior to leaving the hive, ejection from the colony by nestmates or death of the bee during its first flight. In total, 979 flights identified as pollen ($n=154$) or non-pollen (which can be nectar, water or an empty crop; $n=825$) foraging flights were recorded. The number of foraging flights appeared to be relatively low for a total of 170 bees, but was probably explained by the fact that the experimental device contained only one entry and one exit path (one bee at a time could use the path), and that many bees completed a very low number of flights (median, first and third quartiles: 4, 2, 8 foraging trips per bee, respectively). A maximum of 83 completed foraging trips per bee was recorded and 20 bees completed more than 20 trips.

Experiment 2: impact of immune challenge on brain biogenic amine levels

After the stress treatment on day 7, 637 control and 695 immune-challenged bees in total were introduced into a normal Langstroth

colony ($n=3$ trials). Bees returning to the colony when they were between 24 and 28 days old were sampled and immediately flash-frozen in liquid nitrogen. Whether they carried pollen or not was also noted. Frozen heads were freeze-dried for 60 min at a pressure below 300 mTorr (~ 40 Pa; VirTis Benchtop™) and -35°C and then stored at -80°C until brain dissection and biogenic amine analysis. Brain dissections (including optic lobes, antennal lobes, the central brain and gnathal ganglion) were performed on dry ice.

Brain biogenic amine (octopamine, OA; dopamine, DA; tyramine, TYR; and serotonin, 5-HT) levels were measured using high-pressure liquid chromatography (HPLC) following the protocol described by Søvik et al. (2013) and also used later (Scheiner et al., 2014; Søvik et al., 2015). Briefly, the HPLC system was composed of a pump and an autosampler (Agilent 1200 Series, Agilent Technologies, Santa Clara, CA, USA), coupled to an electrochemical detector (ESA Coulochem III, Chelmsford, MA, USA) connected to an analytical cell (ESA 3011A). A 100 mm Hypersil BDS octadecylsilane column was used to separate samples (ThermoFisher Scientific, Waltham, MA, USA). Signals were integrated using ChemStation software (Agilent Technologies) with reference to a standard curve obtained from perchloric acid solutions containing $10\text{ pg }\mu\text{l}^{-1}$ of dihydroxybenzylamine and varying amounts of OA, DA, TYR and 5-HT (Sigma-Aldrich).

In total, we obtained information on brain levels of biogenic amines for 94 control bees (32 with pollen and 62 without pollen) and 50 immune-challenged bees (12 with pollen and 38 without pollen). TYR was detected in only 14% of brains, and thus was not analysed.

Statistical analysis

All statistics were performed using the statistical software R version 3.2.1 (<http://www.R-project.org/>). For experiment 1, the last day any individual bee was detected using RFID was assumed to mark the date of bee death. We then compared the probability of survival between stressed and control bees using the Kaplan–Meier test ('surfit' function of the survival package in R) (Therneau and Lumley, 2014).

Aspects of the foraging performance of bees were analysed using mixed models. The choice of best-fit model was based on the smaller sample size-corrected Akaike's information criterion (AICc) (Burnham and Anderson, 2004). Variation in total number of completed foraging flights per bee, the collected resource (pollen or not pollen) and foraging trip duration were each analysed using different mixed models and fitted with a Poisson, binomial and Gaussian distribution, respectively (based on the distributions of our experimental data). To analyse the number of trips and the collected resource, the treatment (immune challenged or control) and trial were set as fixed and random explanatory variables, respectively. To analyse foraging trip duration, collected resource and honeybee identification were added as fixed and random explanatory variables, respectively.

The normality and the homoscedasticity of brain biogenic amine levels were such that parametric analyses were appropriate for these data. Biogenic amine amounts were analysed using a repeated measures ANOVA followed by Tukey's *post hoc* comparison. Treatment and the resource collected (pollen or not pollen) were analysed as fixed factors, while trial was analysed as a random factor.

RESULTS AND DISCUSSION

Experiment 1: impact of immune challenge on survival and foraging performance

Survival probability did not differ between the control and immune-challenged groups (Kaplan–Meier test, $P=0.42$; Fig. 1A).

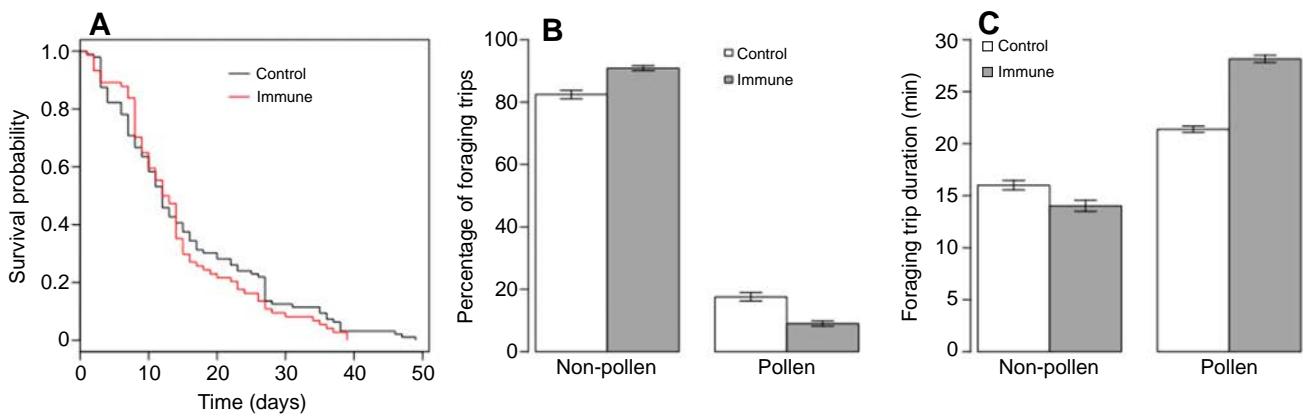


Fig. 1. Survival probability and foraging trip characteristics according to treatment. (A) Survival over 49 days for control bees and immune-challenged bees. Day 0 was the day of stress exposure. Bees from the two treatment groups did not differ in survival probability (Kaplan–Meier test, $P=0.42$). (B) Percentage of pollen and non-pollen foraging trips. (C) Foraging trip duration. For B and C, the mean and 95% confidence intervals predicted by the model (Table 1) are shown according to the collected resource and the treatment: control ($n=100$ pollen and 401 non-pollen foraging trips) and immune challenge ($n=54$ pollen and 424 non-pollen foraging trips). Immune-challenged bees performed fewer but longer pollen foraging trips than control bees.

The best-fit model explaining the variability in the number of trips per bee (lowest AICc) included a significant effect of treatment (Table 1). Immune-challenged bees completed slightly more flights than control bees [mean predicted values with 95% confidence interval: 6.46 (6.12–6.80) versus 5.22 (4.95–5.49), respectively].

A significant switch in foraging preference was detected, with immune-challenged bees performing 1.9 times fewer pollen foraging trips [9.14% (8.32%–9.96%)] than control bees [17.56% (16.20%–18.91%); Fig. 1B and Table 1].

Considering foraging trip duration, the best-fit model included a significant interaction between treatment (immune challenged or control) and the collected resource (pollen or not pollen) (Table 1). Pollen foraging trips were longer than non-pollen foraging trips (Fig. 1C), but trip duration for each collected resource also varied with treatment. Immune-challenged bees performed slightly shorter non-pollen foraging trips than control bees (Fig. 1C), but when foraging for pollen, immune-challenged bees performed 30% longer trips than control bees (Table 1).

Experiment 2: impact of immune challenge on brain biogenic amine levels

Brain DA and 5-HT levels did not differ significantly between treatment groups (ANOVA: $P=0.67$ and $P=0.14$, respectively) or the collected resource (ANOVA: $P=0.75$ and $P=0.27$, respectively; Fig. 2A,B). However, we found a significant treatment×resource

interaction for brain OA levels (ANOVA: $P=0.02$; Fig. 2C). No difference in brain OA levels was found in non-pollen foraging bees (Tukey's *post hoc* tests: $P=1$); however, when sampled on return to the hive carrying pollen, immune-challenged bees had significantly less OA in the brain than control bees (~27% less, Tukey's *post hoc* tests: $P=0.032$; Fig. 2C).

Experimental findings

In this study, we have provided experimental evidence for a stress-induced decrease in pollen-foraging performance in honeybees. The non-pathogenic immune challenge stress applied did not affect bee survival, as has been found previously (Alaux et al., 2014), but did induce a shift in resource collection. An increase in non-pollen foragers (water foragers, nectar foragers and/or bees with empty crops) was observed at the expense of pollen foragers. As more than 90% of non-pollen foragers are nectar foragers and bees with empty crops (Bordier et al., 2017b) and these bees have lower sucrose responsiveness than pollen foragers (Pankiw and Page, 2003), we could reasonably assume that stress decreased bee sucrose responsiveness. Stressed bees may prefer to forage for resources that are rich in carbohydrates to overcome the energetic cost of the stress, as has been observed with parasitism of honeybees (Lach et al., 2015). Indeed, compared with pollen, nectar gives a higher energetic return relative to the foraging effort (8:1 gain with pollen versus 10:1 gain with nectar; Winston, 1987). Similarly,

Table 1. Summary of best-fit mixed models to analyse the impact of immune challenge on foraging behaviour

Dependent variable	Explanatory variables		No. of statistical units	d.f.	AICc
	Fixed	Random			
No. of foraging trips	Treatment	Trial	170 bees from 3 trials	3	1594.7
	Null			2	1606.1
Foraging trip duration	Treatment×resource	Trial/bee	979 observations of 170 bees from 3 trials	6	9120.3
	Treatment+resource			5	9129.0
	Treatment			4	9148.3
	Resource			4	9130.9
	Null			3	9150.2
Foraging preference	Treatment	Trial	170 bees from 3 trials	3	379.2
	Null			2	391.3

Three models were fitted to analyse the number of foraging trips, foraging trip duration and foraging preference (pollen or not pollen). Only summaries of the best-fit models are shown. For each model, fixed and random explanatory variables, the number of statistical units, degrees of freedom (d.f.) and corrected Akaike's information criterion (AICc) are detailed. For each dependent variable, the selected model, i.e. the one with the lowest AICc, is indicated in bold.

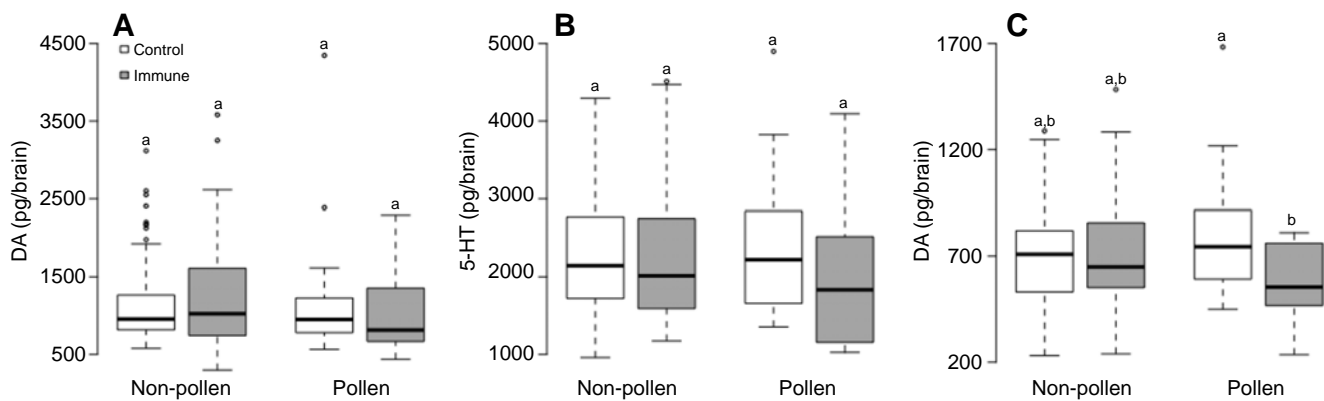


Fig. 2. Brain biogenic amine levels in response to immune challenge in different forager groups. Dopamine (DA, A), serotonin (5-HT, B) and octopamine (OA, C) levels are shown for control ($n=32$ pollen and 62 non-pollen foragers) and immune-challenged bees ($n=12$ pollen and 38 non-pollen foragers), according to the collected resource. Boxes show the first and third interquartile range with the line denoting the median. Whiskers encompass 90% of the individuals, beyond which each outlier is represented by a circle. Plots that do not share a common letter are statistically different ($P<0.05$, ANOVA followed by Tukey's *post hoc* comparisons).

bumblebees exposed to pesticides were found to exhibit lower pollen foraging performance (Feltham et al., 2014; Gill and Raine, 2014).

Such changes in foraging decision making could cause a nutritional imbalance with a pollen deficit at the colony level, and thereby affect colony development. Indeed, pollen shortage may have detrimental effects on brood care, resulting in undernourished larvae (Blaschon et al., 1999) and emerging adults with behavioural deficiencies (Scofield and Mattila, 2015). Moreover, pollen nutrition during the adult stage is essential for stress tolerance (DeGrandi-Hoffman et al., 2010; Di Pasquale et al., 2013; Wahl and Ulm, 1983). Finally, under extreme pollen shortage, nurse bees may reduce the number of larvae that need to be fed, and cannibalize eggs and young larvae (Schmickl and Crailsheim, 2001).

Pollen foraging trips were also 30% longer for immune-challenged bees, suggesting a significant effect of the stressor on foraging capacity. It has been found that the thorax temperature differs between different classes of foragers, in the order pollen>nectar>water foragers (Feuerbacher et al., 2003). These differences were linked to flight metabolic rate, with pollen foragers exhibiting a 10% higher hovering metabolic rate than nectar foragers, regardless of their loads (Feuerbacher et al., 2003). The authors suggested that pollen foragers require more power output to generate the same vertical lift as nectar foragers. We therefore propose that immune-challenged bees spend more time on pollen-collecting trips because it is the most energetically demanding resource to collect (Feuerbacher et al., 2003) and the stressor probably decreases the energy budget of the bees. The increase in foraging trip duration may simply reflect more time resting rather than any other changes in flight characteristics (e.g. distance, speed, etc.) (Wolf et al., 2014). It is also possible that a lower energy budget induced by the stressor caused cognitive impairment in pollen foragers and thus affected their navigation capacities (Jaumann et al., 2013), lengthening their trip times.

Finally, we found that brain OA level was depressed in immune-challenged pollen foragers. OA is known to increase sucrose responsiveness in bees (Scheiner et al., 2002) and stimulate flight activity (Fussnecker et al., 2006), and therefore the drop in OA level is in accordance with the behavioural changes observed in pollen foragers after stress exposure. A previous study reported a rapid decrease in OA and DA but not 5-HT levels in response to stress exposure (chilling anaesthesia and vertical spin; Chen et al., 2008).

We did not find variation in DA levels after our stress exposure. However, to conclude on the nature of the causal role of biogenic amines in honeybee stress responses, functional studies involving manipulation of OA, DA and 5-HT signalling would be required.

Conclusion

Our study suggests that the highly energy-demanding foraging activity of pollen foragers makes them susceptible to stress, even at low levels, which could potentially affect the colony nutrient balance (pollen versus nectar). Therefore, future studies on whether stress narrows the colony foraging flexibility in response to environmental changes might help us to better understand colony decline.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: C.B., A.B.B., C.A.; Methodology: C.B., S.K.; Formal analysis: C.B., S.K.; Resources: A.B.B.; Writing - original draft: C.B., C.A.; Writing - review & editing: C.B., S.K., Y.L.C., A.B.B., C.A.; Supervision: A.B., C.A.

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Appendix 2: Impact of miticide treatments on honeybee foraging performance.

Poster presented at the 2016 International Conference on Pollinator Biology, Health and Policy. July 2016, Penn State University, State College, PA, USA.

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Beekeepers frequently use chemicals against the parasite mite *Varroa destructor*, which accumulate at a low level in the wax and the food stores. The impact of miticides on the foraging behaviour of the adult bees from a treated hive is unknown. Moreover, all the studies conducted so far on this subject, investigated the effect of the miticide on varroa-infected hives, therefore any negative effects of the miticide on bees were confounded with indirect positive effects of the miticide on bees via Varroa knockdown. We conducted our experiments in Australia, which is the last varroa-free country.

We examined how larval exposure to hive treatments with the miticides thymol or tau-fluvalinate affected adult foraging efficiency. We (i) accessed to the foraging activity of the bees via RFID technology and (ii) looked at the ability of the bees to associate a specific colour with electric shocks, via a laboratory visual associative learning essay.

Larval tau-fluvalinate exposure affected the learning speed of the bees, but none of the miticide treatments affect their memory when tested after training. Results from the RFID survey indicate that bee were more susceptible to be lost on their first trip when they have been exposed to miticides.

Impacts of miticides on honey bee foraging behaviour and visual learning

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Introduction: Miticides are now used routinely in apiculture to hold the destructive parasitic mite *Varroa destructor* in check, but what effects do miticides have on honey bees? Here, we examined the effects of two common miticides on bee behaviour in a varroa-free honey bee population. Two hives were treated with **Apistan™ (τ -fluvalinate)**, two with **Apiguard™ (thymol)**, and two were **controls**. Three weeks post treatments bees were collected for behavioural analyse

Impacts of miticides on foraging behaviour:



Cohorts of day old bees were tagged with radio frequency identification (RFID) tags and placed in a common nucleus hive equipped with a RFID reader and a camera. All flights were recorded and sorted as 'pollen' or 'non-pollen' foraging trips.

- Bees treated with thymol started to forage younger than the control groups (fig. 1a).
- Bees treated with thymol performed longer 'non-pollen' foraging trips (fig. 1b).

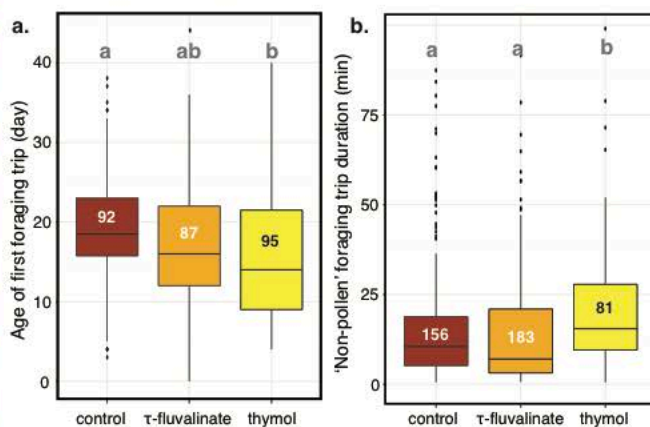


Fig. 1a: Age of the first foraging trip. Kruskal-Wallis test: $\chi^2 = 8.6421$, p-value = 0.013. Post-hoc Conover test: ctl-rflu = 0.124, ctl-thy = 0.011, thy-rflu = 0.314. b: 'Non-pollen' foraging trip duration. Kruskal-Wallis test: $\chi^2 = 16.8705$, p-value = 0.0002. Post-hoc Conover test: ctl-rflu = 0.15, ctl-thy = 0.007, thy-rflu = 0.0001.

Impact of miticides on visual learning:

Foragers were caught when departing the experimental hives. Visual learning was tested using the APIS chamber (Kirkerud et al. 2013) (fig. 2a) which presented green and blue light fields. During training phases bees learned to avoid the blue light: the blue field was illuminated for 3 sec and then electrified (pulses of 10V) for 11 sec.

- Bees treated with τ -fluvalinate move less to the safe side (green) than the two other groups (fig. 2b).

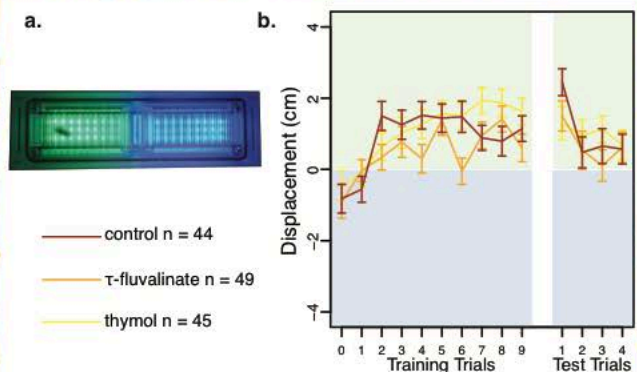


Fig. 2a: APIS chamber. b: Relative displacement of the bees during the first three sec. after the light is on. Bars indicate ± 1 SEs. Mean of displacement: Kruskal-Wallis $\chi^2 = 8.859$, p-value = 0.02. Post-hoc Conover test: ctl-rflu = 0.032, ctl-thy = 0.929, thy-rflu = 0.032.

Discussion: Thymol and τ -fluvalinate both changed the behaviour of bees:

- Thymol caused precocious and less efficient foraging.
- τ -fluvalinate influenced visual cognition.

Our work highlights that miticides, while effective in controlling *Varroa* may themselves be a stressor of honey bees. This should be a consideration for optimal bee management practice.



Appendix 3: Why bees are so vulnerable to environmental stressors.

*Published version of Chapter 1 in Trends in Ecology and Evolution.
The article have made the cover of the journal issue.*



Review

Why Bees Are So Vulnerable to Environmental Stressors

Simon Klein,^{1,2,3} Amélie Cabirol,^{1,2,3} Jean-Marc Devaud,¹
Andrew B. Barron,² and Mathieu Lihoreau^{1,*}

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

Bees Are Exposed to Multiple Environmental Stressors

Bees are ecologically and economically vital pollinators for both wild and cultivated flowers. Presently many populations are in decline [1–4], while demand for pollination-dependent crops continues to rise, generating understandable alarm and debate about the possibility of an emerging ‘pollination crisis’ [5]. Many causal factors have been identified, including a range of pathogens and parasites [6,7], human-induced stressors such as pesticides [8–10], and other forms of environmental degradation [11]. Very few of these stressors can be considered new, but many have increased in intensity over the past decade in much of the industrialized world. Our objective in this review is to consider why bees are particularly sensitive to these environmental stressors, even at low levels, and why their populations are now declining.

Bees, with the exception of parasitic species, raise their brood in a single defensible nest [12]. We argue that, in these insects, central-place foraging on ephemeral, dispersed, and highly variable floral resources places particularly heavy demands on cognitive capacities. Individuals must learn to forage at an energetic profit, locate high-quality feeding sites, efficiently handle flowers, and navigate back to the nest to provision their brood with the right mix of nectar and pollen. The cognitive capacities underpinning these complex behaviors require optimal development and function of central brain structures as well as precisely regulated plasticity of brain circuits necessary for learning, memory, and navigation [13,14]. These brain systems are very easily disrupted, and it is especially problematic that many pesticides found in floral resources directly target key neural pathways [15,16]. Pathogens and nutritional deficits also compromise cognitive functions [17,18]. Even mild damage to the brain can significantly reduce foraging performance, thus rendering bees especially vulnerable to these environmental stressors. In social species, such as honey bees, bumblebees, and stingless bees, efficient division of labor and coordination of tasks across nest mates provide buffering against environmental stressors because individuals share a fortress-factory stocked with stored resources [19]. However, this

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buffering capacity has limits which can be exhausted by frequent stressors. Once this occurs the result is a catastrophic colony decline [20–22].

We develop here a neurobiological, ecological and evolutionary thesis to explain why central-place foraging bees are particularly sensitive to environmental stressors. First we describe the complex cognitive challenges that bees face when foraging, and the neural substrates supporting these abilities. Next we review evidence that these essential cognitive abilities are impaired by a range of stressors, ultimately threatening brood development as well as colony function and survival. Finally, we discuss how understanding the mechanisms of action of the different stressors and their consequences for individuals and colonies can help to better manage and protect these vital pollinators.

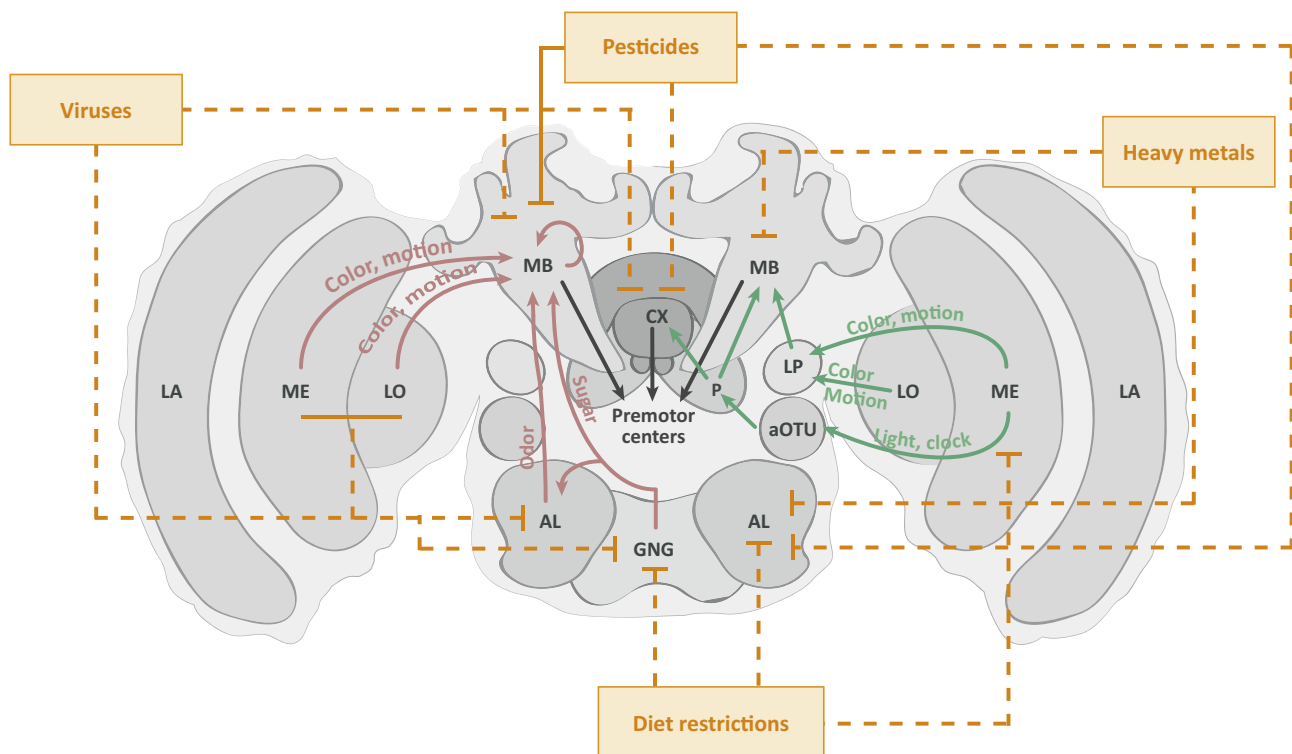
Central-Place Foraging on Flowers Imposes Significant Cognitive Challenges

Bees must gather large volumes of highly dispersed pollen and nectar, and return with them to the nest to feed their brood [12]. Accordingly, these insects have evolved excellent memory and navigation skills enabling them to exploit complex and variable foraging environments, and more than a century of research has identified the underlying neural circuits [13,14]. Although most studies have focused on a few economically important social species, such as honey bees and bumblebees, solitary bees appear to show similar behaviors [12], cognitive capacities [23], and overall brain organization [24]. In the bee brain (Figure 1), visual and olfactory stimuli are first processed by their respective sensory lobes ([25,26] for detailed reviews), which then convey information to multisensory integration centers, such as the mushroom bodies (MBs) and the central complex (CX), that are specialized for learning, memory, and spatial navigation tasks, as we describe below.

Learning To Recognize Flowers

Despite a large variety of available floral species, individual bees tend to forage on the same flower type as long as it provides sufficient nectar or pollen [27]. This floral constancy demonstrates the abilities of bees to learn the association between food rewards and particular floral cues (odor, color, shape, temperature etc.) [28]. In many cases, bees learn more complex associations by generalizing specific floral cues to learn conceptual features common to a range of flowers from the same species [13]. The amount of reward offered by flowers can change very rapidly, and bees can update their learned flower preferences accordingly [29,30]. Bees can also use combinations of floral and social cues, including the presence of conspecifics or other bee species on flowers, to locate and learn rewarding flowers [31].

Many of these mechanisms of learning and memory have been examined in details using experimental approaches (Box 1). For instance, the acquisition of associative memories linking floral cues with food rewards relies on changes in neural activity induced by locally coincident activity in the neural networks that process such cues and in those signaling food detection [32]. Plastic changes in connectivity in either the antennal lobes (ALs) or the MBs (Figure 1) can support associative learning about odorants, and both structures modify their activity following learning [26]. In particular, the MBs are required for some complex forms of olfactory learning as well as for the formation of olfactory long-term memory [33,34]. Although less is known about visual learning, there is visual input from optic lobes (OLs) to the MBs (Figure 1), and it is increasingly likely that associative learning of visual features and colors also involves the MBs [35]. Memorizing simple odor–food associations involves excitatory signaling through acetylcholine in the ALs and MBs (Figure 1) [13], a neurotransmitter system specifically targeted by many common pesticides such as neonicotinoids and organophosphate miticides [15].



Trends in Ecology & Evolution

Figure 1. Brain Structures Supporting the Cognitive Capacities Needed for Foraging and How They Are Affected by Stressors. Schematic frontal view of a bee brain. Sensory information from the environment is first processed in specialized brain structures. The antennal lobes (AL) process olfactory information. The lamina (LA), medulla (ME) and lobula (LO), as part of the optic lobes, process visual information. The gnathal ganglion (GNG) receives gustatory information, and is sensitive to sugar. Sensory signals are then conveyed to higher-order centers (arrows). The mushroom bodies (MB) are involved in stimulus classification (odor, color) and in complex associative learning and memory. They receive information directly from the sensory centers or indirectly through the lateral protocerebrum (LP) and the protocerebrum (P). The central complex (CX) receives processed visual input through the structures of the protocerebrum, including the anterior optic tubercle (aOTU) and bulbs. The central complex locates the bee in space using celestial information and visual landmarks, and is crucial for orientation and navigation. Environmental stressors (orange boxes) alter the functions of various systems in the brain, and can alter the neural pathways supporting learning (purple arrows) and navigational capacities (green arrows). Broken orange lines indicate the impacts of stressors that have not been directly demonstrated for bees, but can be inferred from behavioral observations or have been observed in other insects.

Orienting, Navigating, and Learning Places

Bees use multiple different sources of information to orient [35]. Path integration requires storing of information about distances and directions traveled during the outward journey to be able to plot a direct return path to the nest [36]. Distance is estimated from optic flow [37], which is the movement of the image of the environment across the eye during flight. Direction is determined using the position of the bee relative to the sun [38] and/or the pattern of polarized light in blue sky [39]. Bees possess specialized mechanisms to compensate for the apparent movement of the sun (and the polarization pattern it generates) across the sky during the day

Box 1. Studying the Mechanisms of Learning and Memory in Bees

Experimental work addressing the fine-scale neural and behavioral bases of cognitive capacities in bees has relied primarily on Pavlovian conditioning in which an individual is trained to associate an initially neutral stimulus (the conditioned stimulus, CS) with an unconditioned stimulus (US) that elicits an innate response [13,14]. Learning the CS-US association leads the animal to respond to the CS. Historically, the dominant paradigm has been the appetitive conditioning (using a sugar solution as the US) of the proboscis (tongue) extension reflex (PER) using a restrained bee (Figure 1A) [32], although aversive paradigms also exist [92]. This method allows study of elemental associations between two prescribed events, and also non-elemental associations (when individuals respond in an adaptive manner to novel stimuli using learned information in a new context). In recent years considerable progress has been made by

combining PER conditioning with pharmacological treatments, electrophysiological recordings, and brain functional imaging to unravel mechanisms of learning and memory, especially for olfactory learning [33].

So far, attempts at associative conditioning of visual CS in PER conditioning with restrained bees have yielded low performance levels [93]. By contrast, impressive visual learning capacities have been described using free-flight assays in which bees obtain a sugar reward if they make a correct choice when learning to navigate in a maze (Figure 1B) [94,95] or foraging in arrays of artificial flowers (Figure 1C) [30,31,51,77]. Automated tracking systems such as harmonic radars (Figure 1D) [53,67,80,81], radio-frequency identification (RFID) (Figure 1E) [9,10,21,55,68], and computer vision [96] allow precise quantification of behavioral data in laboratory or semi-field conditions. These approaches have revealed the cognitive abilities of bees in learning complex visual features and relational properties between stimuli [13]. New developments in virtual-reality assays, in which tethered bees walk on a locomotion compensator (Figure 1F) [97] or fly [98] to make foraging decisions in response to stimuli displayed on a screen, hold considerable promise to explore the neural mechanisms of visual learning and navigation.

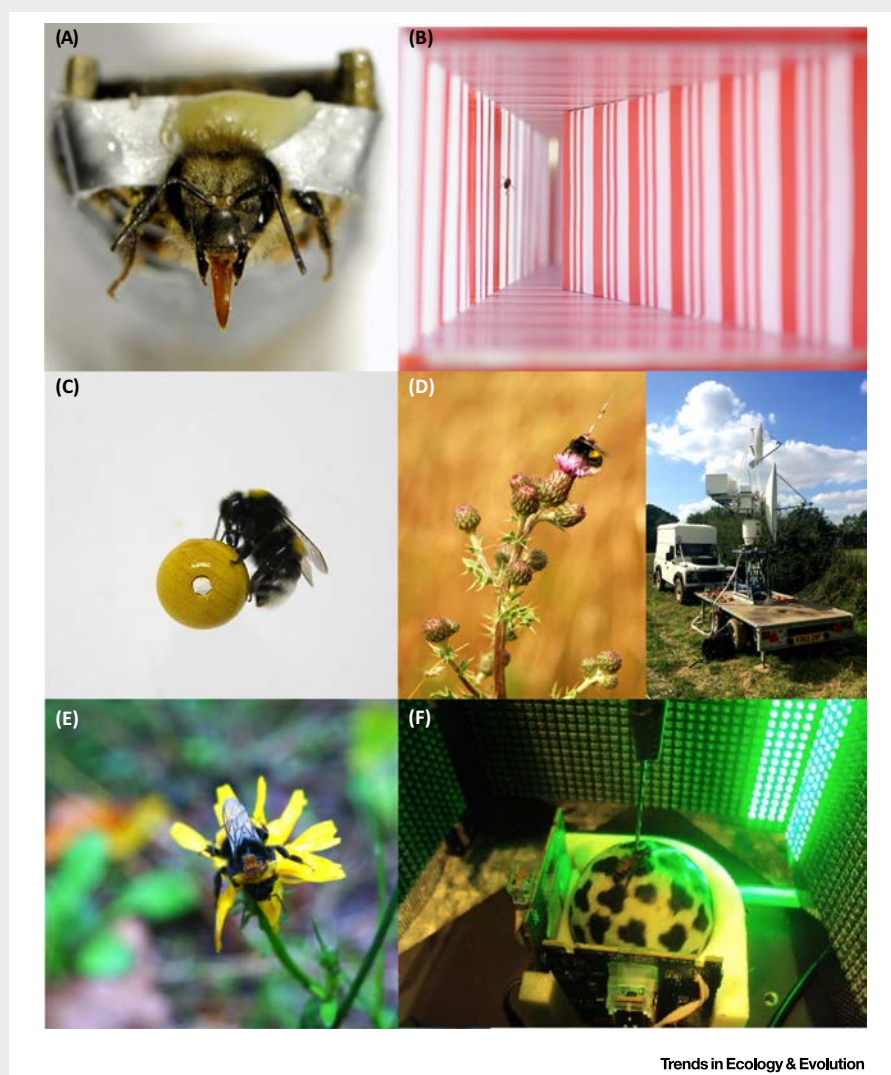


Figure 1. Methods for Studying Bee Learning and Memory. (A) Restrainted honey bee showing proboscis extension reflex (PER) (C. Fresillon/CNRS). (B) Free-flying honey bee in a flight tunnel covered with visual patterns generating optic flow (F. Vignal/DGA) [95]. (C) Bumblebee foraging on an artificial flower (M. Lihoreau). (D) (Left) Bumblebee with a radar transponder in the field (J.L. Woodgate); (Right) harmonic radar (J.C. Makinson). (E) Bumblebee with an RFID tag in the field (S. Klein). (F) Tethered honey bee walking on a locomotion compensator in a controlled visual environment displayed onto LED panels (G.J. Taylor) [97].

[40]. Bees are also sensitive to other global sources of navigational information, such as fine magnetic field variations, and can learn to relate them to local landmarks such that they can still navigate when celestial cues are blocked by cloud [41].

Bees can also learn locations by memorizing visual scenes. They use these stored 'snapshots' for navigation by positional image-matching [36], which compares their current view of the environment with a visual memory of the goal. The degree of matching provides a cue for guidance [42]. Bees form snapshot memories of the nest surroundings on their first foraging attempts outside the nest, and also of the location of food sources [43]. For visual matching, individuals use salient objects (flower patches, trees, buildings), which can be either local cues or panoramic landmarks [36]. Honey bees can also perform optic flow matching, using the direction of optic flow caused by major landmarks as a navigational cue [44]. Processing information on optic flow and landmarks while flying demands integrating visual and proprioceptive input with a temporal component. Responses to motion stimuli and color are displayed by neurons connecting the OLs to central areas, the lateral protocerebrum (LP) and the MBs [45] (Figure 1), and some of these neurons are involved in visual landmark detection [46].

The functions of the central complex (CX) (Figure 1) are presently poorly understood, but data from other insect species suggest that it is crucial for navigation [35]. In addition to being a likely substrate for a sky compass [38], the CX could also support visual short-term (working) memory and spatial memory [47]. A recent study using a virtual-reality assay (Box 1) in *Drosophila* showed that activity of the ellipsoid body neurons of the CX represented the orientation of the fly relative to visual landmarks [48]. Thus it is increasingly likely that neural activity in the CX contributes to internal representation of position for path integration [48].

Learning Foraging Circuits

Bees can use their spatial memories dynamically to establish and optimize foraging routes. In nature, foragers must sometimes visit hundreds of patchily distributed flowers to collect sufficient nectar and pollen in a single trip [27], and many species revisit familiar patches over consecutive hours or days in stable sequences called 'trappelines' [49]. Recordings of bumblebee flight paths using harmonic radar (Box 1) show that foragers attempt to minimize the overall travel distances between discovered flower patches, a complex optimization task akin to the Traveling Salesman problem [50]. On each new foraging trip, bees try different visitation sequences, ultimately finding (or approximating to) the shortest possible path to visit all patches once, starting and ending at the nest [51]. Route optimization is an iterative improvement process based on learning and memory of flight vectors between feeding locations, supported by path integration and visual guidance [52]. This process allows route flexibility and rapid adjustment of trapline geometry in response to changes in the spatial distribution of floral resources, for instance when a patch becomes depleted or a more rewarding one is discovered [53].

Foraging Performance Improves with Foraging Experience

On their first foraging attempts, bees make orientation flights to systematically acquire information about the nest location without collecting food [54]. Foraging performance then improves over the first week of foraging, likely owing to learned flower identification and handling, and route optimization [55,56]. Dramatic changes in the structure of the adult brain are seen during this period [57]. Foraging activity is reflected by an allometric increase in MB volume [58,59]. In honey bees this expansion is caused by increased dendritic arborization of MB intrinsic neurons receiving visual and olfactory input accompanied by pruning of microglomeruli (synaptic boutons) [57,60], partly due to the activation of cholinergic receptors [61]. The selective localization of these structural changes suggests activity-dependent synaptic

plasticity as an underlying mechanism [57]. Dendritic growth can provide a substrate for the formation of new synapses to support stable memories [62]. At the same time selective growth and pruning of connections is thought to optimize the performance of brain centers in the rich visual and olfactory environments experienced during foraging [57].

Stressors Affect Brain Functions, Cognition, and Behavior

Successful foraging is based on the precise integration of information processed across the major brain networks, as well as dynamic structural modifications of such networks. Therefore even subtle disturbances of neural function could have dramatic consequences on individual cognitive abilities and hence foraging performance. From this perspective it is a major concern that most of the stressors presently impacting on bees target the brain. The range of stressors has been well reviewed previously [1,11]. We emphasize here how many of these impair cognitive abilities and foraging performance at exposure levels far below those that kill the bee.

Pesticides and Heavy Metals

Many pesticides affect bee cognition. In recent years, neonicotinoid insecticides have drawn the most attention [63]. These insecticides disrupt cholinergic transmission, the main excitatory pathway in the insect brain, vital for effective learning and synaptic plasticity [13,26]. While acute exposure to very small doses of neonicotinoids has been shown to inactivate MB neurons [15], chronic exposure can impair development of the entire MB [16,64]. These effects almost certainly explain the dramatic impacts of sublethal doses of neonicotinoids on learning and memory in honey bees [65], bumblebees [66], and solitary bees [23], which can be linked to deficits in MB plasticity [16]. Pesticide exposure also disrupts visuospatial memory and navigation [9,67,68], most likely through disruption of processing in the corresponding pathways (Figure 1), but this has yet to be demonstrated. Alarmingly, bees learn to prefer nectar containing neonicotinoids over non-contaminated nectar because of incidental actions of pesticides on the nicotinic receptors involved in reward processing [69].

Fipronil, a widely used insecticide and acaricide, targets neuronal receptors involved in inhibitory transmission by γ -aminobutyric acid (GABA) and glutamate [70]. In honey bees GABA signaling is vital for normal MB function, particularly for complex learning [33,71]. Acute fipronil treatment severely reduces olfactory learning and memory performance [72]. Additional indications of neuronal cell death in the MBs following fipronil exposure suggest possible long-term cognitive impairments in honey bees [73] and stingless bees [74].

Some pesticides contain manganese, which induces precocious foraging in honey bees [75]. Its effect on sucrose responsiveness suggests that it interferes with signaling pathways important for associative learning, as indicated by the abundant expression of a manganese transporter in MBs and ALs [75] (Figure 1). Selenium, another heavy metal found in crop treatments, has been found to change sucrose responsiveness, olfactory learning, and long-term memory [76].

Parasites and Pathogens

Human activities have intensified the pressures of parasites and pathogens on bees through dispersion of bacteria, viruses, fungi, and mites across the world [11]. While few parasites or pathogens act directly on the brain, many have a strong impact on the behavior of bees [6]. Part of this can be explained by the activation of the immune system, which might interfere with energy supply or signaling mechanisms. Even an immune response induced by non-pathogenic molecules can reduce olfactory associative learning abilities [77,78].

The microsporidian *Nosema cerana* and the mite *Varroa destructor* are two major parasites of honey bees. Exposure to either parasite induces specific but overlapping patterns of altered

gene expression in the brain of their host [79]. *Varroa* infection alters brain expression of many genes involved in neurotransmitter signaling, including through GABA [79]. These impacts on the brain are thought to induce poor navigation performance by infected bees [80,81].

Varroa carries many viruses, and a *Varroa* infection of a colony is a complex syndrome of many co-associated pathogens. Some the effects of varroensis are due to viral infections [7,79]. For example, the deformed wing virus (DWW) impacts on olfactory learning, possibly by targeting brain areas of importance for foraging [18]. Although there is no known impact of DWW on bee visual learning and navigation, other viruses, such as the Israeli acute paralysis virus (IAPV), affect homing behavior [82].

Malnutrition

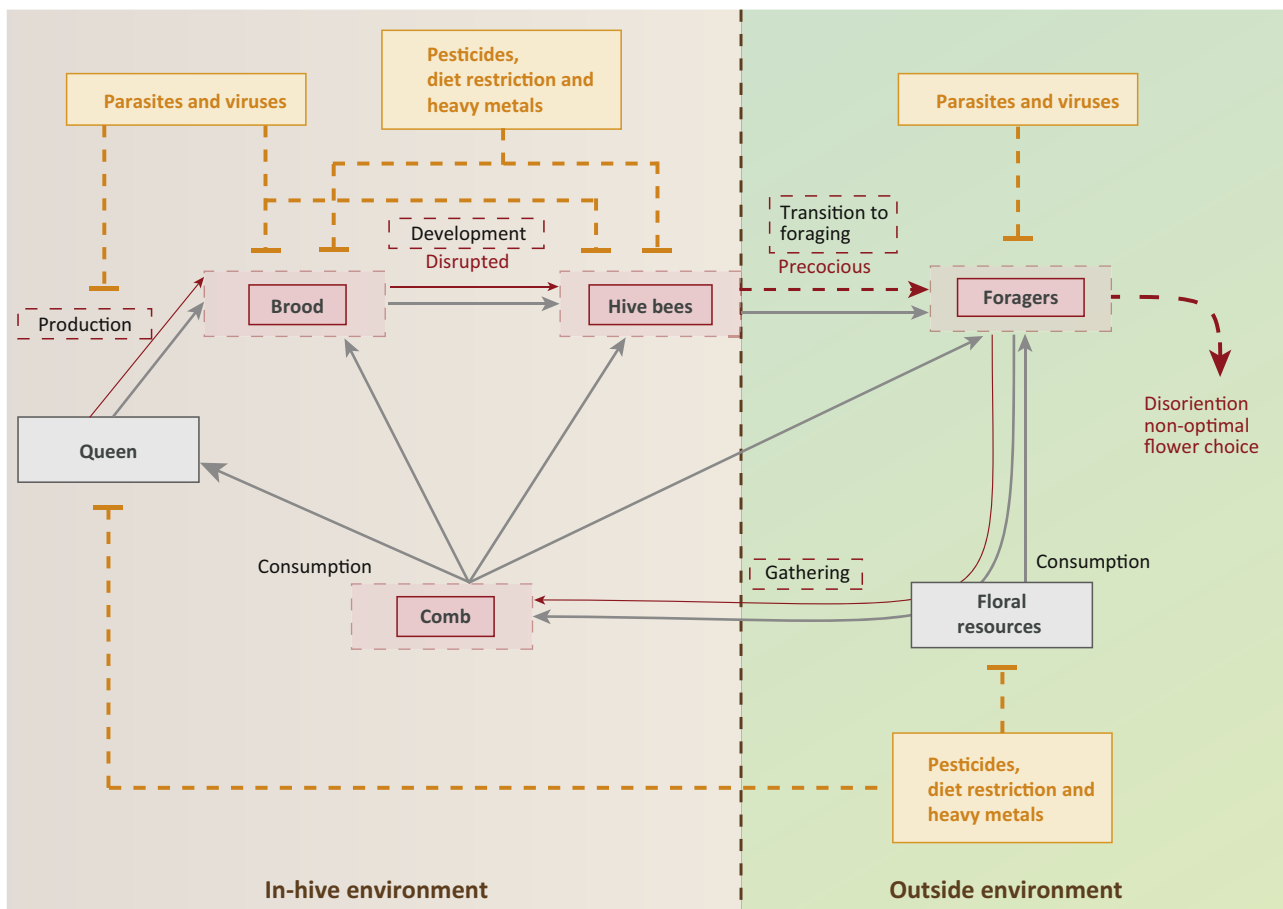
Intensive farming and the expansion of monocultures have imposed strong constraints on the dietary diversity of bees because significantly fewer food resources are available to them, often during limited flowering seasons [11]. Bee nutrition is partitioned between nectar, the main source of carbohydrates, and pollen, which provides proteins, lipids, vitamins, and other micronutrients [83]. Limited food intake reduces performance in a simple learning task [78], but having enough food is not necessarily sufficient for optimal cognitive processing. In honey bees, olfactory associative learning is disrupted by qualitative changes in essential lipids [17] or amino acids [84]. Pollen shortage during development can also lead adults to forage earlier and for a shorter period [85], whereas nectar deprivation increases impulsive, suboptimal food choices [86].

From Reduced Foraging Performance to Colony Collapse

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

Comparative research on bee declines suggests that the resilience of bees to stressors depends on their level of sociality [2,87], although this needs to be confirmed by further studies (see Outstanding Questions). In principle, solitary bees are the most vulnerable because reduced foraging efficiency of the female following stress exposure immediately jeopardizes the development of her brood. These species lack the profusion of specialized group behaviors observed in social bees (e.g., corpses and diseased brood removal, social fever, collection of antimicrobial and antiviral plant resins) that can mitigate the impact of pathogen stressors on colonies [88].

However, the stress tolerance of social bees is not without limits – stressors, even at low levels, can also have extremely severe consequences on colonies. In the most social species, such as honey bees, foraging is undertaken by middle-aged adults that have completed a period of orientation flights and brain maturation to prepare them for the cognitive demands of foraging [58,59]. Stressors not only disrupt foraging performance, but also the process of preparing for foraging. For honey bees, a very common response to many stressors is to begin foraging prematurely [21] (Figure 2). It has been argued that delaying high-risk tasks to later in life is an effective strategy to extend mean longevity of workers and increase their total contribution to the colony [89]. Nevertheless, if worker lifespan is reduced, workers react by proportionally compressing their time allocation to each task, and commence foraging early. This is likely to be an adaptive response to acute stress because it would temporarily compensate for the diminished foraging effort of the colony. However, in conditions of prolonged stress, this response can accelerate colony decline because bees that start foraging precociously complete fewer trips in their lifetime [90] and live less long [21].



Trends in Ecology & Evolution

Figure 2. Effects of Stressors on Honey Bee Colony Dynamics. In a non-stressed colony (grey arrows), the brood (eggs, larvae, and pupae) develops into in-hive bees (e.g., nurses) that begin to forage 2 weeks later. Foragers gather nectar and pollen from floral resources for storage in the hive (comb). The food stock is consumed by the queen, the larvae, the in-hive bees, and the foragers. Individual bees can be exposed to environmental stressors (orange boxes) at different stages, potentially disrupting the dynamics of the whole colony. Stressors reduce brood production, alter development, induce precocious foraging onset of in-hive bees, and affect the cognitive performance of foragers, leading to disorientation and less-efficient food gathering (red arrows). The synergistic action of stressors at different levels of this complex system can lead to dramatic colony collapse. Plain red arrows indicate quantitative changes. Broken red arrows indicate qualitative changes. Adapted from [22].

Simulation models suggest that continuous stress can create a situation in which the foraging force is dominated by precocious foragers [21,91], and then becomes so inefficient that it can no longer support the colony, at which point the colony population dramatically collapses (Figure 2). Stressed bumblebee colonies, although smaller and socially simpler than honey bee colonies, also show highly non-linear responses to environmental stressors [10,20]. Various impairments of colony function (including foraging, but also thermoregulation, defense, and hygienic behavior) can generate changes in population dynamics via feedback loops affecting rates of hatching and adult death, sometimes leading to colony collapse [20]. These complex dynamics might explain the observed widespread declines of wild and managed bee populations [1–4]. The known stressors of bees are not new, and many populations have been in a steady decline for decades, but the accelerated declines described recently suggest that we are now reaching the point at which the cumulative stress on colonies is exceeding their tolerance capacity [11].

Concluding Remarks and Future Prospects

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

Pesticides provide an informative case in point. Agriculture has become increasingly reliant on 'next-generation' neonicotinoid pesticides because they are so effective at killing pest insects at low doses by directly targeting the insect central nervous system [8]. Recent research describing the neural impacts, behavioral impairments, and changes in colony dynamics at field contamination levels by pesticides [8–10,55,68] has forced a re-evaluation of the 'safe-level' of pesticide exposure for individual bees and colonies [63]. Using this new knowledge we must now determine how pesticides can be managed in the agricultural landscape in a manner that is compatible with sustaining bee populations. Many other stressors contribute to colony decline [1,11], for which the precise mechanisms of action need to be unraveled.

As discussed above, the stress tolerance of a colony is not without limits and, given the increase in bee declines seen in the past decade, it would appear we are very close to exhausting those limits for some key pollinating bee species. Even so, this is far from a hopeless story. Combining conceptual and methodological advances in neuroscience, ecology, and evolutionary biology can bring significant insights into how specific stressors affect bee behavior and colony dynamics, and help to identify ecological interventions to ameliorate stress on bees. Most of the stressors damaging bee populations are human-induced, and can be reduced or eliminated from the environment if there is sufficient will and/or economic imperative.

Acknowledgments

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Outstanding Questions

What are the points of greatest vulnerability in the bee brain? Neurogenomic profiling has started to provide an overview of the gene expression changes occurring in the brain in response to pathogens [79], but we have yet to understand the signaling pathways involved and the functional relevance of these changes. More integrative work is now necessary to identify precisely how stressors damage the brain to reduce foraging performance. This must couple genomic studies with functional analyses of changes in circuit performance and behavior. Understanding these fundamental processes in the developing and adult brain will help designing neuroprotective treatments to improve the resilience of managed bees.

Are all bee species similarly vulnerable to stressors? Bees greatly vary in their social organization (from solitary to social), feeding ecology, and habitats [12]. While most attention has focused on managed populations of generalist species with a social lifestyle, such as honey bees and bumblebees, comparative research is now needed to assess the general impact of stressors on the wide diversity of pollinators.

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

How then can the agricultural environment be managed to ensure bees receive adequate nutrition from diverse floral sources? Can we design nutritionally optimized plant assemblages to preserve bee populations? Crops provide huge amounts of foods, but these plants that have been selected to optimize production typically yield poor quality diets to bees [99]. Research is necessary to quantify the precise nutrient needs of bees, how they vary across colony developmental stages, species, and in the face of specific stressors, and their impact on behavior and cognition.

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Can the pollination performance of managed social bees (honey bees and bumblebees) be sustainably improved by manipulating colony composition? Within a colony, social bees show high levels of interindividual behavioral and cognitive variability. In honey bees a small number of individuals complete a disproportionately high number of foraging trips [100]. Characterizing this variability between bees, what causes it, and how it changes under stress conditions will be necessary to understand the consequences of environmental stressors on the resilience of colonies.

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Appendix 4: Inter-individual variability in the foraging behaviour of traplining bumblebees

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Inter-individual variability in the foraging behaviour of traplining bumblebees

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Workers of social insects, such as bees, ants and wasps, show some degree of inter-individual variability in decision-making, learning and memory. Whether these natural cognitive differences translate into distinct adaptive behavioural strategies is virtually unknown. Here we examined variability in the movement patterns of bumblebee foragers establishing routes between artificial flowers. We recorded all flower visitation sequences performed by 29 bees tested for 20 consecutive foraging bouts in three experimental arrays, each characterised by a unique spatial configuration of artificial flowers and three-dimensional landmarks. All bees started to develop efficient routes as they accumulated foraging experience in each array, and showed consistent inter-individual differences in their levels of route fidelity and foraging performance, as measured by travel speed and the frequency of revisits to flowers. While the tendency of bees to repeat the same route was influenced by their colony origin, foraging performance was correlated to body size. The largest foragers travelled faster and made less revisits to empty flowers. We discuss the possible adaptive value of such inter-individual variability within the forager caste for optimisation of colony-level foraging performances in social pollinators.

In recent years, behavioural ecologists have become increasingly interested by the fact that animals often exhibit consistent behavioural traits that vary between individuals from the same group, population or species, irrespective of time or context^{1–3}. Inter-individual behavioural variability has been described in a wide range of taxa, from invertebrates (nematodes⁴, cnidarians⁵, molluscs⁶, insects^{7,8}) to mammals⁹, including humans¹⁰. The existence of such individualistic behavioural traits may have different adaptive values depending on the ecology of the species^{11–13}.

Social insects, such as ants, some bees and wasps, show extreme cases of inter-individual behavioural variability¹⁴. In these animals, division of labour typically implies that specific individuals reproduce (the queens and the males), whereas others work to support their reproductive outputs (the workers)¹⁵. Among the workers different individuals specialise on different roles. Some take care of the brood (the nurses), while others defend the colony entrance (the guards and the soldiers) or collect food (the foragers). These behavioural specialists exhibit specific behavioural repertoires that can be associated with differences in morphology (e.g. bumblebees¹⁶), age (e.g. honey bees¹⁷), physiology and genetics (e.g. honey bees^{18,19}), or experience (e.g. ants²⁰), together defining the caste phenotype. Growing evidence indicates that some level of behavioural variability also exists between individuals of the same caste^{21–23}. For instance in bumblebees, foragers show consistent inter-individual differences in decision speed and accuracy in flower discrimination tasks^{24,25}. When having to choose between a rewarding flower and an empty flower in a laboratory decision chamber, some foragers always make slow but accurate decisions, while others are consistently fast and inaccurate²⁴. Foragers also show inter-individual variability in learning performance^{22,26} and colonies containing foragers with high visual learning speeds have a higher foraging efficiency²⁷. These differences are independent of body size or any other measurable morphological attributes²⁷.

Whether such cognitive variability translates into distinct foraging strategies in the more complex and ecologically relevant task of exploiting patchily distributed floral resources remains virtually unexplored. In nature, bees often develop stable foraging routes (sometimes called traplines in analogy to trappers checking their traps along fixed routes²⁸) to exploit multiple feeding locations from their central nest^{29,30}. Manipulative experiments

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on bumblebees^{31,32} and honey bees³³ foraging for sucrose solution in simple arrays of artificial flowers (equivalent to natural flower patches) show how foragers often find the shortest possible route to visit all flowers once and return to the nest using an iterative improvement strategy based on learning and memory that is different from just linking nearest neighbour locations^{31,34}.

Thus far empirical research on trapline foraging has been aimed at describing this behaviour at the species level, using relatively small sample sizes (four to seven individuals per experiment), without characterising variation among individuals^{31–33,35–38}. In principle however, some level of variation in the foraging behaviour of the workers of a colony could improve the colony foraging efficiency³⁹. Regular trapliners that accurately follow the same route across multiple hours or days may perform better in stable environments when resources are highly predictable, while irregular trapliners that sample new locations at each foraging bout may be advantaged in more variable environments. Consequently, colonies containing foragers of different behavioural profiles may differ in performance in similar environmental conditions. Understanding how natural behavioural variability affects the foraging performances of colonies may help evaluate the adaptability of bees in the face of environmental changes, such as natural climatic events, human-induced habitat degradations or the introduction of predators and parasites⁴⁰. Ultimately, this approach may also help refine predictions of current pollination models based on bee movement patterns^{34,38,39,41,42}.

Here we explored the level of inter-individual variability in the foraging behaviour of bumblebees (*Bombus terrestris*) by comparing the movement patterns of foragers from two colonies collecting sucrose solution in three different arrays of artificial flowers and landmarks in a controlled flight room.

Results

We tested 29 bees from two colonies (N = 15 from colony 1, N = 14 from colony 2). Each bee was successively observed for 20 consecutive foraging bouts (flower visits followed by returns to the colony nest box) in three experimental arrays each characterised by four flower locations and four different landmarks (Figs 1, S1 and S2). The experimental arrays were chosen in order to maximise the level of dissimilarity between them while keeping a simple design. Bees were tested successively following the same order of arrays presentation. At every foraging bout, each flower contained a volume of sucrose solution equivalent to one quarter of the bee's nectar crop (stomach) capacity so that the task for the bee was to visit the four flowers to fill its crop to capacity and then return to the nest.

Bees developed routes in the three experimental arrays. We first considered the overall foraging behaviour of bees in all three experimental arrays. On average bees increased by $154.5 \pm 48.3\%$ (mean \pm SE) their travel speed (flight duration divided by the Euclidian distance between all successively visited flowers) between the first and the last foraging bout in the same array (Fig. 2A, Table 1). Although we used an indirect measure of travel speed, there is clear evidence that bumblebees rapidly develop straight flight trajectories to join known flower locations with training^{38,43}. As they gained experience in an array, bees also increased by $6.3 \pm 3.8\%$ (mean \pm SE) the average number of different flower locations they visited per bout (Fig. 2B, Table 1), decreased by $85.3 \pm 3.5\%$ (mean \pm SE) the average number of immediate revisits to flowers (two successive visits to the same flower; Fig. 2C, Table 1), and decreased by $58.0 \pm 8.0\%$ (mean \pm SE) the average number of non-immediate revisits (two non-successive visits to the same flower; Fig. 2D, Table 1).

We estimated the tendency of bees to follow regular routes over repeated foraging bouts by calculating the frequency of use of a primary route (highest proportion of foraging bouts in which the same four-flowers visitations sequence — excluding revisits to flowers — was used by a bee)³⁶. Each bee established a primary route that it used on average in $27.5 \pm 2.2\%$ (mean \pm SE) of all its foraging bouts for a given array (Fig. 2E). This proportion of primary route usage was similar in the three experimental arrays (Kruskall-Wallis test: $\chi^2 = 1.47$, $P = 0.478$). We calculated the level of similarity between the 20 complete flower visitation sequences for each bee in each experimental array using a determinism index (DET). This index is derived from recurrence quantification analyses that reflect the amount of repeated sequences in a dataset⁴⁴. DET varies between 0 (the bee never repeats the same flower visitations sequence) and 1 (the bee always repeats the same flower visitations sequence). For all three arrays, observed DETs were consistently higher than theoretical DETs calculated on simulated random flower visitations sequences (Fig. 2F; post-hoc Tukey test, array 1: $\beta = 0.16 \pm 0.01$, $t = 30.41$, $P < 0.001$; array 2: $\beta = 0.07 \pm 0.01$, $t = 12.22$, $P < 0.001$; array 3: $\beta = 0.12 \pm 0.01$, $t = 22.72$, $P < 0.001$). This indicates that bee movement patterns were more repeatable than expected by chance. Thus, overall bees increased their foraging efficiency and began to develop traplines as they accumulated foraging experience in each array, irrespective of the spatial distribution of flowers and the nature and arrangement of three-dimensional landmarks.

Nonetheless, some behavioural differences were observed for all bees between the three arrays. For instance, in array 1 bees tended to travel slower (Fig. 2A, Supplementary Table S2), visited fewer flowers (Fig. 2B, Supplementary Table S2) and tended to perform more immediate revisits (Fig. 2C, Supplementary Table S2), while they performed fewer non-immediate revisits in array 3 (Fig. 2D, Supplementary Table S2). This suggests that bees continuously improved their foraging performance throughout the experiment, as they accumulated experience from the first to the third array. However we cannot exclude that these changes of foraging performance also reflect differences in the degree of navigational challenge offered by each array and their sequences of presentation. For instance bees appeared to have lower DETs in array 2 (least-squares means post-hoc test: array 2 vs. array 1: $P < 0.001$; array 1 vs. array 3: $P = 0.072$; array 2 vs. array 3: $P = 0.031$). In this case flower 2 may have been particularly difficult to locate as it was hidden behind a tall landmark.

Bees showed strong variability in route fidelity and foraging performance. Having described the average foraging behaviour of bees in the three arrays, we next explored the level of inter-individual variability among the different foragers. We ran a principal component analysis (PCA) based on the mean for

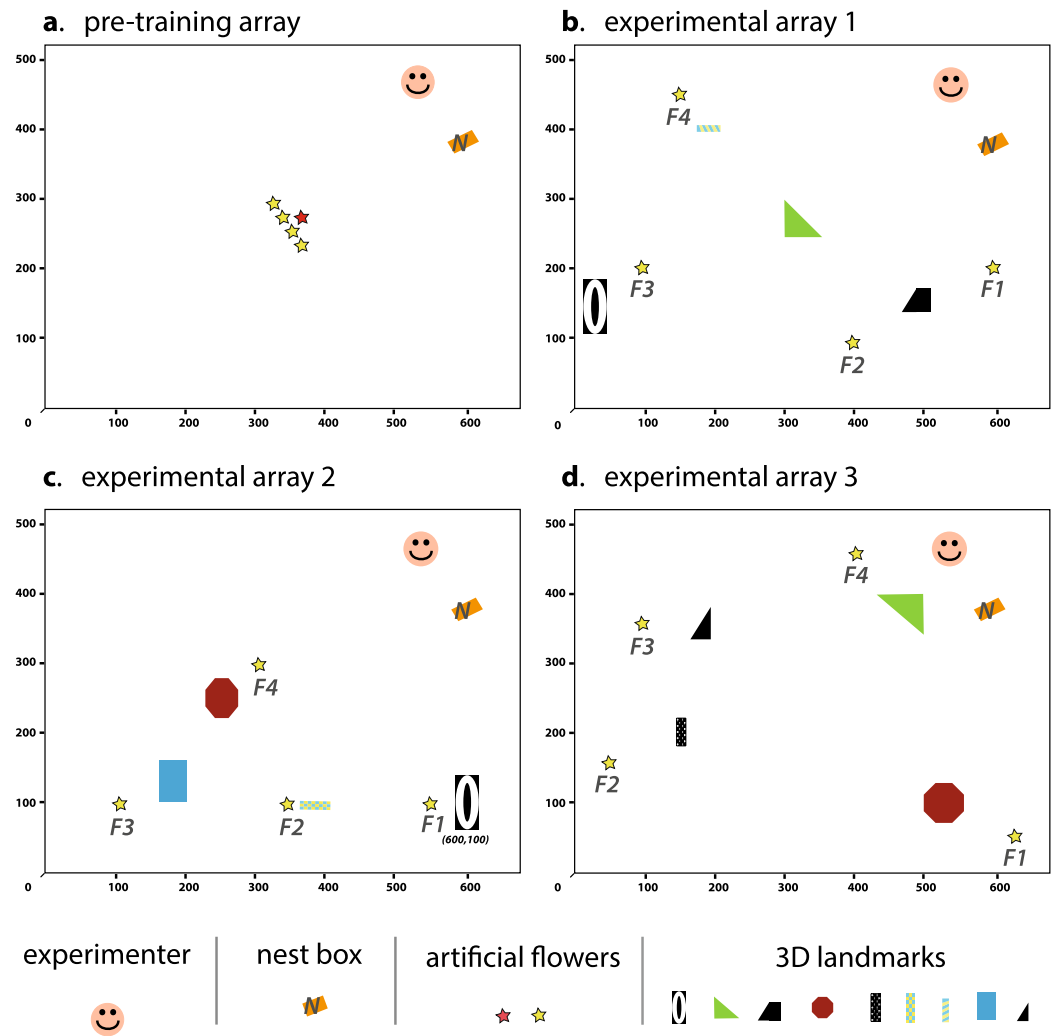


Figure 1. Experimental arrays of flowers and landmarks. **(a)** Pre-training array. Bees were allowed to forage on a pre-training flower (red star) in a landmark-free environment for one hour. A selected bee was then observed foraging on four training flowers (yellow stars) during five foraging bouts to estimate its nectar crop capacity. **(b–d)** show the first, second and third experimental arrays used for testing. Each array was characterised by a unique combination of four training flowers (F1–F4) and three to four landmarks (coloured shapes). Detailed descriptions of the artificial flowers and the 3D landmarks are given in Figs S1 and S2. X- and Y-axis graduations represent the distance to the origin (down left corner) in cm.

each individuals per array for the six behavioural measures described above: (1) travel speed per foraging bout (flight duration divided by the Euclidian distance between all successively visited flowers); (2) number of different flowers visited per foraging bout; (3) number of immediate revisits to flowers per foraging bout (when the bee visited the same flower twice in a row); (4) number of non-immediate revisits per foraging bout (when the bee revisited a flower after having visited one or more different flowers); (5) cumulative frequency of primary route usage per foraging bout; (6) determinism index (DET, level of similarity between the 20 flower visitation sequences) for each experimental array; Figs 3 and S3). We retained two PCs using the Kaiser-Guttman criterion (Supplementary Fig. S4).

PC1 and PC2 were not correlated with each other (Spearman's correlation test: $\rho = 0.01$, $S = 108460$, $P = 0.915$). PC1 explained 54% of the proportion and PC2 46%. PC1 was positively associated with the frequency of use of a primary route and the DET, but negatively associated with the number of non-immediate revisits to flowers (Fig. 3, Supplementary Table S3). We interpreted PC1 as a "route fidelity" variable. Accordingly individuals with a high PC1 score were regular route-followers characterised by highly repeatable flower visitation sequences and occasional non-immediate revisits to flowers. PC2 was positively associated with the number of immediate and non-immediate revisits to flowers, and negatively associated with travel speed and the number of different flowers visited (Fig. 3, Supplementary Table S3). We interpreted PC2 as a "foraging performance" variable. Individuals with a high PC2 score were slow and inaccurate foragers, characterised by slow movements between flowers and frequent revisits to empty flowers. Variance along PC1 and PC2 defined a continuum between four behavioural

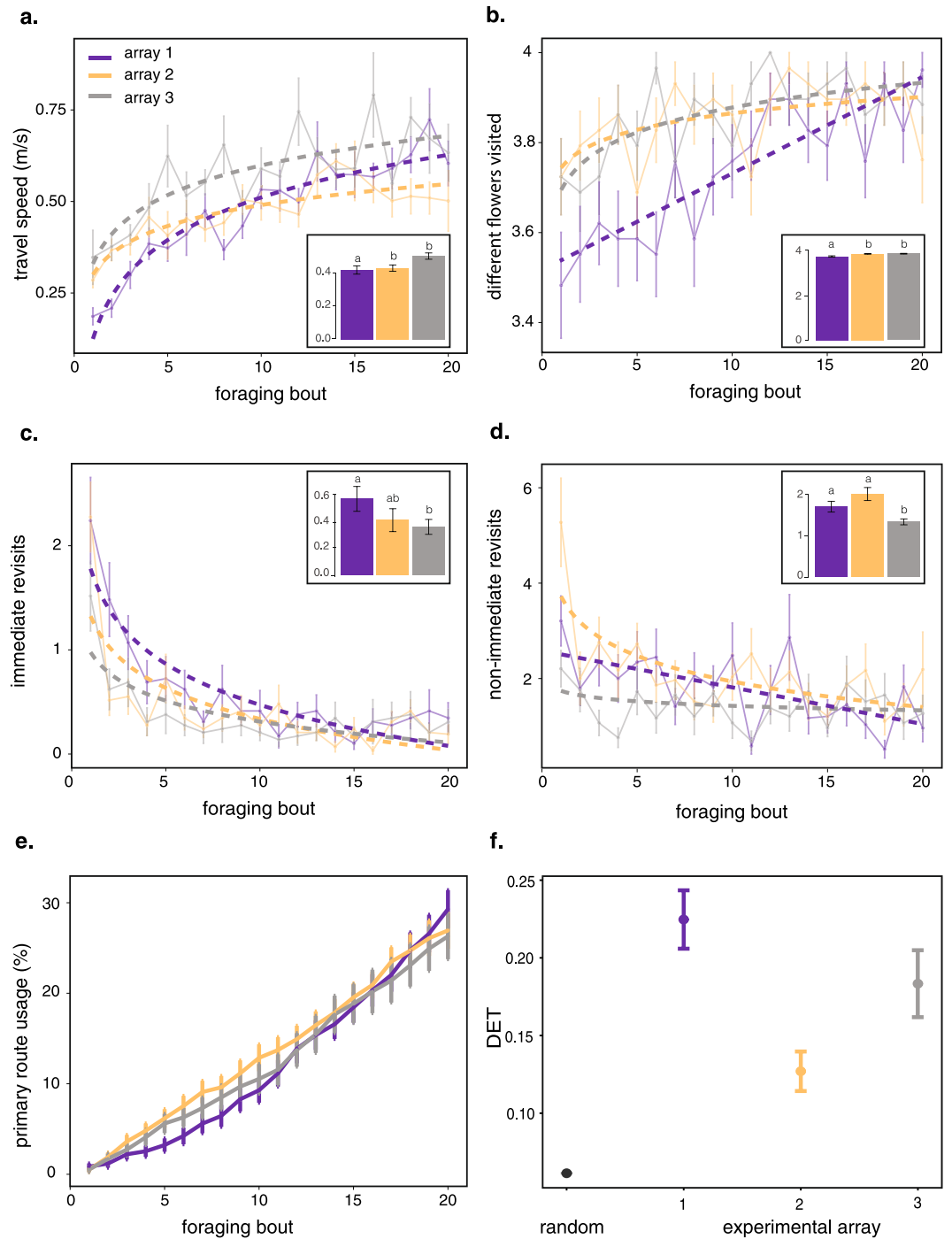


Figure 2. Average behavioural measures in the three experimental arrays (array 1: purple, array 2: orange, array 3: grey, see details of flower and landmark configurations in Fig. 1). **(a)** Travel speed per foraging bout (flight duration divided by the Euclidian distance between all successively visited flowers). **(b)** Number of different flower visited per foraging bout. **(c)** Number of immediate revisits to flowers per foraging bout (when the bee visited the same flower twice in a row). **(d)** Number of non-immediate revisits per foraging bout (when the bee revisited a flower after having visited one or more different flower locations). **(e)** Cumulative frequency of primary route usage per foraging bout. **(a–e)** plain lines show means \pm SE ($N = 29$ bees), dashed lines show regression models (see details in Table 1 and Supplementary Table S1). **(f)** Comparison between simulated random determinism index (DETs, $N = 1000$ simulations) and observed DETs ($N = 29$ bees) in each experimental array (mean \pm SE). **(a–d)** Bar plots show means \pm SE for each array of flowers. Tukey post-hoc analysis: different letters above bars represent significant differences between arrays (see details in Supplementary Table S2).

	Type of regression	Estimate (SE)	t	P
Travel speed				
Array 1	logarithmic	0.16 (0.01)	11.04	<0.001
Array 2	logarithmic	0.09 (0.02)	4.35	<0.001
Array 3	logarithmic	0.64 (0.11)	-1.23	<0.001
Different flowers visited				
Array 1	linear	0.02 (0.003)	7.80	<0.001
Array 2	logarithmic	0.05 (0.02)	2.71	0.014
Array 3	logarithmic	0.08 (0.02)	4.57	<0.001
Immediate revisits to flowers				
Array 1	logarithmic	-0.57 (0.06)	-9.33	<0.001
Array 2	logarithmic	-0.43 (0.09)	-4.73	<0.001
Array 3	logarithmic	-0.29 (0.06)	-5.13	<0.001
Non-immediate revisits to flowers				
Array 1	linear	-0.08 (0.02)	-3.42	0.003
Array 2	logarithmic	-0.77 (0.18)	-4.34	<0.001
Array 3	logarithmic	-0.14 (0.11)	-1.25	0.228

Table 1. Regression coefficients of average behavioural measures for the three experimental arrays. Significant effects are highlighted in bold.

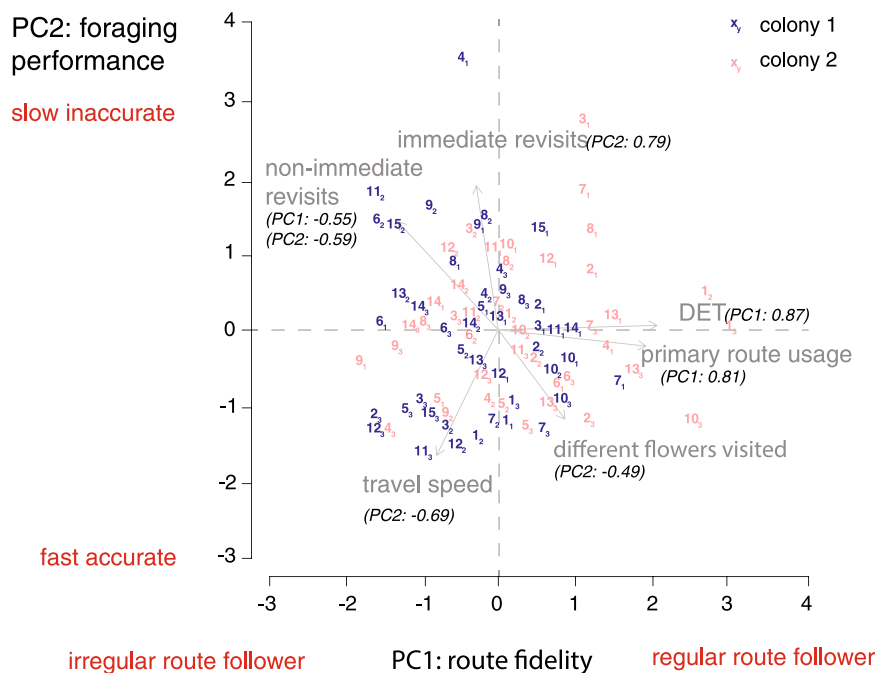


Figure 3. Correlations between the two first components (PCs) of the principal component analysis (PCA). Grey arrows represent the six behavioural measures on PC1 (route fidelity) and PC2 (foraging performance). PC loadings are in brackets. Only loadings $>|0.4|$ were retained (see Supplementary Table S3 for the complete PCA loadings). Each data point represents the PC1 and PC2 scores of a given bee in each experimental array. The PCs define a continuum between four behavioural extremes: fast accurate and regular route followers, fast accurate and irregular route followers, slow inaccurate and regular route followers, slow inaccurate and irregular route followers. Blue: colony 1 (N = 15 bees, 45 data points), red: colony 2 (N = 14 bees, 42 data points). Numbers refer to individual bees (same number code as in Figs 4 and 5). Subscripts refer to experimental arrays (1–3).

extremes (Fig. 3): fast accurate and regular route followers (high PC1/low PC2 scores), fast accurate and irregular route-followers (low PC1/low PC2 scores), slow inaccurate and regular route-followers (high PC1/high PC2 scores), and slow inaccurate and irregular route-followers (low PC1/high PC2 scores). While foragers of colony 2

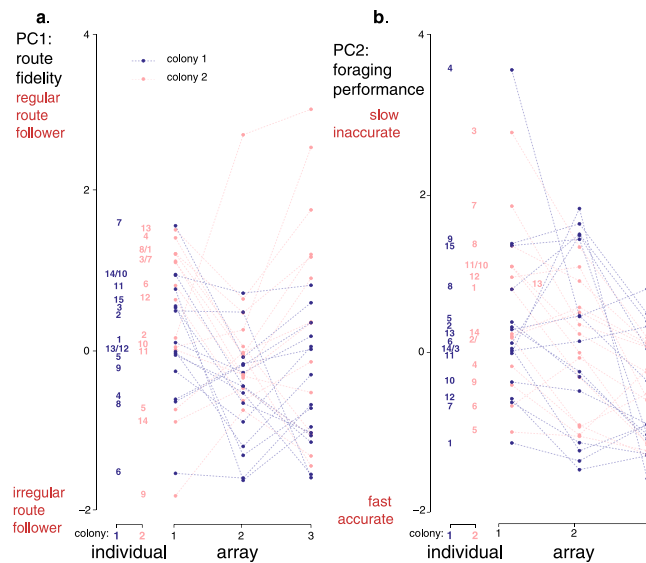


Figure 4. Intra- and inter-individual behavioural variance across experimental arrays. (a) Route fidelity (PC1). (b) Foraging performance (PC2). Data points connected by a dashed-line represent the scores of the same individual over the three arrays. Blue: colony 1 (N = 15 bees), red: colony 2 (N = 14 bees). Numbers refer to individual bees (the same number code was used in Figs 3 and 5).

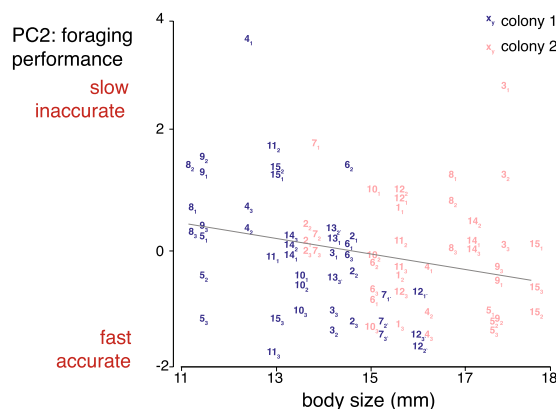


Figure 5. Inter-individual variance in foraging performance (PC2) is partly explained by body size (length from top of head to end of abdomen). Each data point represents the average score of an individual in an experimental array (three values per individual). Blue: colony 1 (N = 15 bees), red: colony 2 (N = 13 bees). Numbers refer to individual bees (the same number code was used in Figs 3 and 4). Subscripts refer to experimental arrays (1–3). Marginal $R^2 = 0.12$, conditional $R^2 = 0.44$.

were uniformly distributed across the entire PC space, 50% of the foragers of colony 1 were nested within the area defined by high PC1 and low PC2 scores (slow inaccurate and irregular route-followers; Fig. 3).

Variability was expressed both at the inter- and intra-individual levels. We next explored the effects of inter- and intra-individual variability on PC1 and PC2, using linear mixed effect models (LMMs) with individual identity nested within colony identity as random effects and both intercept (inter-individual variability) and random slope (intra-individual variability) structures.

Variability in PC1 was significantly explained by inter-individual differences (Table 2A; 27% of variance explained), meaning that bees showed consistent differences in their average level of route fidelity across arrays. Bees also differed in their level of intra-individual variability (Table 2B; 11% of variance explained) so that some individuals consistently increased their route fidelity in each array while others did not. Variability in PC1 was also explained by differences between colonies (Table 2A; 38% of variance explained). Overall bees from colony 2 were more regular at following a route than bees from colony 1, irrespective of the experimental array (Fig. 4A).

Variability in PC2 was significantly explained by inter-individual differences (Table 2A; 46% of variance explained). Therefore bees showed consistent differences in their average level of route performance across arrays. Bees did not present intra-individual variability in their response to the different arrays (Table 2B; 5% of variance

	df	AIC	Loglik	L.Ratio	P
(a)					
<i>Random intercept model PC1</i>					
LM	5	262.67	−126.34		
LME_1 colony	6	228.64	−108.32	7.08	0.008
LME_1 colony/ID	7	254.48	−120.24	5.11	0.024
<i>Random intercept model PC2</i>					
LM	5	239.54	−114.77		
LME_1 colony	6	237.84	−112.92	3.70	0.054
LME_1 colony/ID	7	225.13	−105.57	14.72	<0.001
(b)					
<i>Random slope model PC1</i>					
LME_1 colony/ID	7	242.57	−114.29		
LME_0+array colony/ID	6	235.93	−111.96	4.64	0.031
<i>Random slope model PC2</i>					
LME_1 colony/ID	7	201.92	−98.46		
LME_0+array colony/ID	6	227.93	−107.92	19.00	<0.001

Table 2. Log-likelihood Ratio tests to estimate inter- and intra-individual variability on the two principal components (PCs) of the principal component analysis (PCA). **(a)** To study inter-individual variability we compared a linear model (LM) built using each PC as a response variable and age, body size and experimental array as fixed variables with two mixed effect models (LMEs) using colony or individual nested in colony as random effects. **(b)** To study intra-individual variability we compared the random intercept model (LME_1|colony/ID) previously built using each PC with a random intercept and slope model (LME_0+array|colony/ID). Degree of freedom (df), Akaike Information Criterion (AIC), Log-likelihood values (Loglik) and Log-likelihood ratio test (L.Ratio) are presented with the corresponding p-values. Significant effects are highlighted in bold.

	Estimate (SE)	df	t	P
<i>Route fidelity (PC1)</i>				
Body size	−0.12 (0.09)	24	−1.38	0.190
Age	−0.01 (0.02)	24	−0.37	0.709
Array	−0.18 (0.11)	55	−1.23	0.116
<i>Foraging performance (PC2)</i>				
Body size	−0.21 (0.09)	24	−2.36	0.03
Age	−0.01 (0.02)	24	−0.53	0.60

Table 3. Linear mixed models (LMMs). LMMs were run on the two principal components (PCs) of the principal component analysis (PCA), using individual identity nested within colony identity as random variables and age, body size and experimental array as fixed variables. Significant effects are highlighted in bold.

explained), meaning that all bees tended to increase their foraging performance as they gained experience in a given array. Colony origin had no effect on PC2 (Table 2A; 26% of variance explained).

Body size differences partly explain inter-individual variability in foraging performances. We used LMMs to examine whether experimental factors (spatial configuration of flowers and landmarks) or biological characteristics of bees (body size and age) explained both PCs (Table 3). PC1 was neither explained by experimental arrays, body size or age (Table 3). By contrast PC2 was negatively correlated with body size, so that larger foragers tended to travel faster and make fewer revisits to flowers than smaller foragers (Fig. 5). We also found a significant influence of the experimental arrays on PC2 (Table 3), indicating that bees similarly increased their foraging performance as they moved from array 1 to array 2 and array 3 (Fig. 4B). This gradual improvement of foraging performances supports the hypothesis of a continuous learning process throughout the experiment.

Discussion

Understanding inter-individual behavioural variability in complex societies, such as colonies of social insects, may offer unique insights into how and why relatively high levels of inter-individual behavioural variability are observed in animal groups and populations^{22,45}. Here we compared the movement patterns of all foragers from two bumblebee colonies exploiting arrays of stable feeder locations, and report consistent inter-individual differences in their spatial foraging behaviour. Rather than defining distinct behavioural profiles of foragers, this natural variability follows a continuum along two behavioural dimensions. Some bees were always more faithful to a route and/or faster and more accurate in their spatial foraging decisions than others.

Bees showed consistent inter-individual variability in their tendency to follow stable routes between flowers. This variability was neither explained by the characteristics of our experimental arrays of flowers and landmarks, nor the body size or the age of bees. Interestingly, degrees of route fidelity differed between our two colonies, meaning that foragers from one colony were more regular in following a route than those from the other colony. These results are not due to differences in the average body size or age between the foragers of each colony. Behavioural variability between individuals of different groups or colonies is a widespread phenomenon in social animals⁴⁵, including insects^{21, 46–48}. Inter-colonial behavioural variability has been reported previously in bees, (e.g. aggression in honey bees⁴⁹ or for both vision- and olfaction-related cognitive tasks in bumblebees²⁷) and suggested to be correlated with the foraging success of colonies^{26, 27}. In bumblebees, high genetic relatedness between colony members, due to female monandry (single mating) and haplo-diploidy (haploid males, diploid females), may favour strong inter-colony variability^{26, 50}. Other non-genetic factors may also contribute to phenotypic variability between colonies, such as changes in the pre-imaginal environment. For instance variation in nest temperature⁵¹ and nutrition⁵² during the larval stage can lead to differences in olfactory learning in adult honey bees. Further studies using more colonies with known genetic relatedness are needed to test the existence of a genetically determined inter-colony variability for traplining.

In the present spatial task, bees also showed some level of inter-individual variability in their ability to make fast and accurate spatial decisions, so that fast travelling bees made fewer revisits to empty flowers. This result is consistent with the observation that goal-directed flights in experienced bees, for instance between the nest and familiar flowers, are faster than exploration flights, in which naïve bees scan the environment to search for flowers and acquire spatial memories^{38, 43}. Thus potentially bees showed inter-individual variability in their tendency to make exploitation and exploration flights. Interestingly, differences in foraging performance among bumblebee foragers were partly explained by differences in their body size, so that larger foragers tended to travel faster and make fewer revisits than smaller foragers. Bumblebees show a continuous variation in body size that is primarily determined by the frequency of feeding so that larvae raised in the middle of the nest area (where workers are more active) tend to become the largest adults⁵³. Size polymorphism is considered a main factor of caste determinism in bumblebees, such that only the largest individuals tend to undertake foraging the tasks⁵⁴. Our novel results suggest that natural size variations also influence within caste behavioural variance among foragers. This observation is consistent with previous studies showing that the largest bumblebees make more foraging trips⁵⁵, take less time¹⁶ and collect more nectar in natural conditions¹⁶. Large bumblebees also tend to learn faster in visual discrimination tasks⁵⁶. These inter-individual behavioural and cognitive differences may be explained by differences in the sensory equipment of small and large bees. For instance, larger bees have bigger compound eyes and may thus be more accurate at finding small objects⁵⁷. Size polymorphism in bumblebees is primarily determined by the frequency of feeding so that larvae raised in the middle of the nest area (where workers are more active) tend to become the largest adults⁵³. Therefore it is very likely that the diversity of body sizes and their associated behavioural traits between and within castes of bumblebee colonies is a self-organised process, regulated by population densities and structural constraints within the nest at a given time during the colony cycle.

Our description of inter-individual variability in the spatial foraging behaviour of bumblebees is in line with recent observations that foragers of social bees show high variability to their contribution to the global colony foraging effort^{55, 58}, suggesting that some behavioural traits may support higher foraging success. It has been suggested that behavioural diversity in a social group or population can be an advantageous trait at the collective level^{7, 8}. Honey bee colonies showing higher genetic variability (and thus inter-individual behavioural variability) perform better in group tasks such as nest thermoregulation⁵⁹. Colonies of *Theremothorax* ants showing high variability in the aggressiveness of workers are more productive¹³. In the social spider *Anelosimus studiosus*, mixed colonies composed of aggressive (asocial) and docile (social) individuals capture more prey than colonies with high proportion of only one type of individuals⁶⁰. Accordingly, maintaining a diversity of behavioural profiles among foragers of a colony may allow the colony to locate and exploit a larger diversity of resources in fast changing environments^{1, 24, 61, 62}. For instance, artificial bumblebee colonies containing individuals with different foraging profiles along a speed-accuracy trade-off have a more constant nectar collection rate than homogeneous colonies²⁴. Further investigation of the correlates of inter-individual behavioural and cognitive differences among members of a social group, such as bees, holds considerable promise for better assessing plastic collective responses and the adaptability of groups to stressful environmental conditions.

Material and Methods

Bees and flight room. We used two colonies of *Bombus terrestris* (Biobest, Westerlo, Belgium). Only one colony was tested at a time (colony 1: November–December 2015, colony 2: May–June 2016). We did not anticipate seasonal effects when working with commercially reared bumblebees in controlled laboratory conditions²⁷. The colony was maintained in a two-chamber wooden nest box placed in an experimental flight room with white walls (length: 683 cm, width: 516 cm, height: 250 cm; Fig. 1). Controlled illumination was provided by 12 wide-spectrum light-emitting diode bulbs mimicking sunlight (15 W, 1250 lm, Ilight, Italy), with a 10h: 14h day: night photoregime (light on at 8:00 AM GMT + 1). Temperature was maintained at 20 °C. Bees were individually marked with numbered-colour tags (Opalith tags, Christian Graze KG, Germany) on their thoraces upon emergence from the pupae. The colony nest entrance was equipped with a transparent colourless Perspex tube with a series of shutters to control the traffic of foragers. Honey bee collected pollen was provided every two days directly into the colony nest box. Foragers collected sucrose solution (50% [w/w]) from artificial flowers in the flight room.

Artificial flowers and landmarks. Each flower was made of a cylindrical plastic container (height: 7.5 cm, diameter: 6.2 cm) with a blue lid acting as a landing platform (Supplementary Fig. S1A). The platform was held 30 cm above ground by a clamp stand. We used two versions of this general flower design. “Pre-training” flowers

provided bees with *ad libitum* reward through a cotton wick soaked in the flower's container filled with sucrose solution (Supplementary Fig. S1B). "Training" flowers provided bees with a controlled volume of sucrose solution specific to each bee (range: 24–52 μL , $N = 29$ bees, see calculation of nectar crop capacity below). This volume was placed in the middle of the landing platform using an electronic micropipette (Handystep) (Supplementary Fig. S1C). We used nine three-dimensional landmarks made of cardboard and paper. Landmarks were uniquely defined by their shape and coloured patterns (Supplementary Fig. S2).

Experimental procedure. Bees were allowed to forage collectively on a pre-training flower placed in the middle of the flight room (Fig. 1A). A regular forager that made at least five foraging bouts within one hour (flower visits followed by returns to the colony nest box) was selected for testing. The bee was first observed foraging on four training flowers arranged in a patch in the middle of the room (Fig. 1A). Each flower was refilled with 10 μL of sucrose solution by the experimenter immediately after being visited, until the bee returned to the nest. The average volume of sucrose solution collected by the bee over five foraging bouts was used to estimate its nectar crop capacity (range 48–208 μL , $N = 29$ bees)^{31,36–38}.

The bee was then tested for 20 consecutive foraging bouts in each of three experimental arrays on the same day (60 foraging bouts, ca. 6 h of observation per bee). Each array was characterised by a unique combination of four flower locations and four different landmarks (see details Fig. 1). All bees were tested in the same sequence (arrays 1, 2, 3). During the test, each flower provided a quarter of the bee's crop capacity and was refilled by the experimenter between foraging bouts, so that the bee had to visit all flowers to fill its crop and return to the colony nest box. Because bumblebees drink sucrose rewards until their crop is full, any revisit to a flower within the same foraging bout was unrewarded^{35–38,63}. All flower visits, detailing the time when the bee landed on a flower and departed, and the time when the bee arrived and departed from the nest, were recorded using the software Ethom v.1.0⁶⁴ (the complete flower visitation sequences are available in the Supplementary Dataset S1). Flowers were cleaned with ethanol solution (90% v/v) between changing arrays to preclude potential scent marks from influencing the bee's flower choices in the new experimental array⁶⁵. At the end of the test, the bee was freeze-killed and its body size (top of head to end of abdomen) measured with a digital calliper (± 0.01 mm). A total of 29 bees were tested (14 workers from colony 1, 15 workers from colony 2). Bees from colony 1 were younger (age since emergence from the pupae (mean \pm se); colony 1: 14.2 ± 8.66 days; colony 2: 24.5 ± 5.67 days, t-test: $t = 6.61$, $df = 76$, $P < 0.001$) and smaller (body length (mean \pm se); colony 1: 13.41 ± 1.44 mm; colony 2: 16.13 ± 1.44 mm, t-test: $t = 8.67$, $df = 82$, $P < 0.001$) than bees from colony 2.

Data analyses. Average foraging behaviour. All analyses were performed in R (version 3.2.3). We used regression models to describe changes in the average number of immediate revisits to flowers (two successive visits to the same flower), the average number of non-immediate revisits to flowers (two non-successive visits to the same flower), the average number of different flowers visited, and the average travel speed (flight duration divided by the Euclidian distance between all successively visited flowers), across the 20 foraging bouts of each bee in each experimental array. For each behavioural measure we ran both linear and logarithmic models and retained the model that had the highest R^2 (Supplementary Table S1). We built a linear regression model using number of foraging bouts, identity of experimental arrays and the interaction between them as fixed effects. We examined the differences between experimental arrays using post-hoc Tukey tests (`<<multcomp>>` R package⁶⁶).

To assess the overall similarity between all flower visitation sequences of each bee in a given experimental array we used a determinism index (DET) derived from recurrence quantification analyses⁴⁴. We compared the DETs calculated on the observed sequences to DETs calculated on 1000 randomly simulated sequences of 154 flowers - corresponding to the average number of flowers visits and nest returns over the 20 foraging bouts for all bees in each experimental array (mean \pm se: 153.5 ± 33 visits, range = 107–286, $N = 29$ bees). The R code for generating random flower sequences is available in Supplementary Text S1. Observed and simulated DETs were compared using an analysis of variance (ANOVA) followed by a post-hoc Tukey test (`<<multcomp>>` R package⁶⁶). To compare the three observed DETs of the same bee (1 per experimental array), we applied a least-square means test (`<<lsmmeans>>` R package⁶⁷) on a linear mixed effect model (LMM) including the experimental array as fixed effect and individual identity as random effect (`<<nlme>>` R package⁶⁸).

To examine whether some routes were more often used than others by the same bee, we focused on four-flower visitation sequences excluding revisits to flowers^{31,36–38}. We calculated the frequency of use of the primary route (highest proportion of foraging bouts in which the same four-flowers visitation sequence — excluding revisits to flowers — was used by a bee). Assuming that there are 24 ($4! = 4 \times 3 \times 2 \times 1$) possible routes to visit four flowers once and return to the nest, we used a binomial test with a random probability of 0.042 (1/24) to use each route in a given foraging bout. Because each bee was tested for 20 foraging bouts in an experimental array, routes that were used at least four times by the same bee were used significantly more often than expected by chance (at the 5% level).

Intra- and inter-individual variability in foraging behaviour. We compared the foraging behaviour of individual bees using a principal component analysis (PCA). This PCA aimed to reduce our predictors (i.e. travel speed, number of different flowers visited, non-immediate revisits to flowers, immediate revisits to flowers, proportion of primary route usage, DET) to compound behavioural axes. We applied the Kaiser-Guttman criterion to select the number of principal components (PCs) to retain⁶⁹. We then run the PCA function from the `<<psych>>` R package⁷⁰ with only the retained PCs. We extracted the PC scores for each bee and used them as dependent variables in the subsequent analyses. To identify the effect of inter-individual (amount of variation among individuals around the average behaviour) and intra-individual (phenotypic plasticity of each individual across arrays) variability on the two PC components over the three experimental arrays of flowers, we ran mixed linear models (LMMs) with individual identity nested within colony identity as random effects. To do this, we ran both

a random intercept (inter-individual variability) and slope (intra-individual variability) mixed effect model. We used individual age, body size and experimental array as fixed effects in order to evaluate their respective influence on both PCs. To assess inter-individual differences we tested for the significance of random intercept effects by applying a likelihood ratio test (LRT), comparing the LMM with individual identity nested within colony, the LMM with only colony as random effect and the linear model (LM) excluding both individual and colony identity. To quantify inter-individual variability, we calculated individual repeatability as the percentage of total variance explained by both colony origin and individual differences⁷¹. We also ran these two analyses on the slope models in order to assess the level of intra-individual variability over the three arrays.

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Author Contributions

S.K. and M.L. conceived the study and designed the methodology; S.K. collected the data; S.K. and C.P. analysed the data; S.K., C.P., A.B.B., J.M.D. and M.L. wrote the manuscript.

Additional Information

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Appendix 5: Ten years after the crisis, what is happening to the world's bees?

Popular science paper published in The Conversation.
<https://theconversation.com/ten-years-after-the-crisis-what-is-happening-to-the-worlds-bees-77164>

THE CONVERSATION

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Ten years after the crisis, what is happening to the world's bees?

May 8, 2017 5.38am AEST

Bees have been living with the mysterious Colony Collapse Disorder for a decade. Simon Klein, Author provided

Ten years ago, beekeepers in the United States raised the alarm that thousands of their hives were mysteriously empty of bees. What followed was global concern over a new phenomenon: Colony Collapse Disorder.

Since then we have realised that it was not just the US that was losing its honey bees; similar problems have manifested all over the world. To make things worse, we are also losing many of our populations of wild bees too.

Losing bees can have tragic consequences, for us as well as them. Bees are pollinators for about one-third of the plants we eat, a service that has been valued at €153 billion (US\$168 billion) per year worldwide.

Ten years after the initial alarm, what is the current status of the world's bee populations, and how far have we come towards understanding what has happened?

The current status of bees worldwide

Authors



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Since the alarm was first raised, many countries have created new monitoring methods to judge the status of their bee stocks. As a result we have much more data on bee populations, although coverage is still patchy and differences in survey methods make it hard to compare between continents.

It is clear that bees in the United States are still struggling. Beekeepers can tolerate up to 15% losses of colonies over winter, but the US is massively above this threshold, having lost 28.1% of colonies over the 2015-16 winter.

Canada, by contrast, reported 16.8% losses. This is better, but still above the level of losses at which beekeepers can easily restock.

Only recently have we had data from central Europe. There, honey bees seem to be doing better: 11.9% losses in 2015-16. Meanwhile, in New Zealand surveys only began in the last year and have reported winter loss of 10.7%. Australia does not yet have a countrywide survey of the state of bee colonies.



Fortunes are mixed for bees around the world. Simon Klein, Author provided

Honey bees are not the only bees that we should care about: wild bees are vital pollinators too. Some plants are pollinated by only one wild bee species, such as the macropis bees that forage on the loostrife plant.

Unsurprisingly, we have much less data on wild bees than honey bees, and those data we do have point to bigger concerns. For our wild bees we only really have good data for populations that are endangered or that have completely disappeared. Between 2008 and 2013, wild bee diversity in the US dropped by 23%, and a previously common bumblebee species was recently listed as endangered.

Do we understand why?

The good news is that the past decade has seen plenty of progress in understanding the mystery of Colony Collapse Disorder. The bad news is that we now recognise it as a **complex problem with many causes**, although that doesn't mean it is unsolvable.

For all bees, foraging on flowers is a hard life. It is energetically and cognitively demanding; bees have to travel large distances to collect pollen and nectar from sometimes hard-to-find flowers, and return it all to the nest. To do this they need finely tuned senses, spatial awareness, learning and memory.

Anything that damages such skills can make bees struggle to find food, or even get lost while trying to forage. A bee that cannot find food and make it home again is as good as dead.

Because of this, bee populations are very vulnerable to what we call “sublethal stressors” – factors that don't kill the bees directly but can hamper their behaviour.



For solitary species such as the blue-banded bee, difficulty foraging can be a very serious problem. Simon Klein, Author provided

In a recently published review, we argue that modern agriculture and industry have created a host of sublethal stressors that damage bees' cognition. For example, **diesel fumes and neonicotinoid pesticides** both reduce bees' foraging efficiency by disturbing chemical communications in their brains. Modern intensive agriculture **disturbs bee nutrition**, which impairs their brain. **Climate change** interferes with the relationship between bees and the plants on which they feed.

In addition, managed honey bees are afflicted by a range of pests, viruses and predators that have been spread around the world as a side-effect of international trade. The worst is the ominously named *Varroa destructor* mite, which causes brain development disorders.

What can we do?

At the global level, to preserve our bees we have to improve the environments in which they collect food. Every small action can make a difference. Planting flower borders with **bee-friendly flowers** in your garden can provide food for both wild and domestic bees. You can reduce or eliminate the use of herbicides or pesticides when gardening. Even **mowing the lawn less often** can help bees out.

You could install a **native bee hive** or **insect hotel**. Another tempting option is to buy local honey, which often has a more distinctive flavour than mass-produced versions.

In Australia, we are fortunate in that our bees seem to be doing better than many other parts of the world. The Varroa mite has not yet invaded our shores, and in many areas bees can access pesticide-free bushland (although unlike Europe, Australia has **not yet banned use of neonicotinoids in agriculture**).

Australia also has an incredibly rich diversity of wild native bees: **up to 1,600 different species**, including our emblematic stingless bees. Even so, to protect this diversity we need better surveys of how these species are doing.

Ten years on from the alarm over disappearing bees, it is fair to say we now know the nature of the problem and what can be done to fix it. It's up to us to take the steps needed to sustain these precious pollinators of our food for the future.

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Appendix 6: Vingt ans après le début de l'effondrement des colonies,
comment se portent les abeilles ?

Popular science paper published in the French version of The Conversation.
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THE CONVERSATION

L'expertise universitaire, l'exigence journalistique

Vingt ans après le début de l'effondrement des colonies, comment se portent les abeilles ?

13 juin 2017, 22:57 CEST



Les abeilles sauvages et domestiques pollinisent un tiers des plantes que nous consommons. Simon Klein, CC BY-NC-ND

C'était il y a vingt ans déjà : les apiculteurs français alertaient sur l'utilisation de pesticides comme le **Gaicho**, responsable selon eux d'une mortalité accrue dans les ruches ; on parle à l'époque de pertes annuelles entre **300 000** et **400 000** abeilles, entraînant une **chute de 50 %** de la production de miel aux abords de champs de tournesols traités avec ce produit phytosanitaire. Cet épisode a constitué la première prise de conscience du danger auquel sont exposés ces insectes dans nos sociétés industrialisées.

Dix ans plus tard, c'est au tour des apiculteurs américains de tirer la sonnette d'alarme, après avoir observé des milliers de ruches soudainement vidées de leurs occupantes. Sur **2,4 millions** de ruches au total, **1,5 million** disparaissent en effet en quelques mois dans une petite trentaine d'États. Ce phénomène appelé « **syndrome d'effondrement des colonies** » a provoqué une nouvelle prise de conscience planétaire. Contrairement à l'épisode du **Gaicho**, les pertes concernées sont plus importantes et leurs causes bien moins claires.

Une préoccupation mondiale

Depuis, nous avons réalisé que ces pertes ne concernaient pas seulement la France ou les États-Unis : des **problèmes similaires** ont été observés un peu partout en Europe, en Asie et en Australie.

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Préoccupation supplémentaire, les abeilles domestiques ne sont pas les seules atteintes : de nombreuses espèces sauvages (comme les abeilles solitaires et les bourdons) sont désormais en danger. Or certaines plantes ne sont pollinisées que par ces espèces, à l'image de certaines Méllitidés qui butinent uniquement les fleurs de lysimaques.

La perte des abeilles peut avoir de graves conséquences pour la biodiversité et l'humanité. Car les abeilles sauvages et domestiques **pollinisent** environ un tiers des plantes que nous consommons, participant ainsi à un service écologique évalué à **153 milliards d'euros par an** à travers le monde (dont **2,9 milliards d'euros en France**).

Deux décennies après les premiers signalements d'effondrement des colonies, dans quel état se trouvent les populations d'abeilles dans le monde ?



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Les abeilles aujourd'hui

Depuis les premiers symptômes de déclin, nombre de pays ont développé des méthodes de recensement des colonies d'abeilles domestiques et nous avons accès aujourd'hui à un ensemble conséquent de données ; mais ces études demeurent souvent incomplètes et il persiste de réelles disparités entre les méthodes de comptage, rendant délicate la comparaison entre les pays ou les continents.

Au sortir de l'hiver 2016, l'évaluation des pertes pour la France variait par exemple entre **13 et 20 %** en fonction des méthodes de comptage.

Aux États-Unis, les chiffres indiquent une situation préoccupante avec **28,1 %** de colonies vidées durant l'hiver 2015-2016. On estime en général que les apiculteurs peuvent tolérer jusqu'à **15 %** de pertes naturelles en hiver. Au Canada, les pertes atteignent **16,8 %**, ce qui est mieux mais ce chiffre dépasse encore le seuil à partir duquel il est difficile de repeupler les cheptels.

Si nous ne disposons que de peu de recul pour l'Europe centrale, les abeilles semblent résister assez bien dans cette zone, avec **11,9 %** de pertes en 2015-2016.

Du côté de la Nouvelle-Zélande, les comptages n'ont débuté que l'an dernier, montrant une perte faible de **10,7 %**. Il faut souligner que dans nombre de pays, comme l'Australie et la plupart des pays asiatiques, africains ou sud-américains, les comptages nationaux réguliers font toujours défaut.

Pour ce qui est des espèces non domestiques, les données demeurent à ce jour insuffisantes mais celles dont nous disposons sont alarmantes. En Europe, 9,2 % des 1965 espèces d'abeilles sauvages recensées sont en danger d'extinction.



Les bourdons, pollinisateurs sauvages, sont tout autant menacés que les abeilles domestiques. Tamara Gomez, CC BY-NC-ND

Les causes de l'effondrement

Ces dix dernières années, la recherche s'est intensifiée et a fait d'énormes progrès dans la compréhension de l'effondrement des colonies. Nous savons désormais qu'il s'agit d'un problème complexe et multi-causal... mais pas insoluble.

Pour toutes les abeilles, butiner est une tâche complexe : elles doivent parcourir de longues distances pour récolter pollen et nectar sur des fleurs pas toujours faciles à localiser. Puis il leur faut retourner au nid pour nourrir leur colonie. L'accomplissement de ces tâches nécessite des systèmes sensoriels et d'apprentissage performants pour s'orienter correctement, reconnaître les fleurs et apprendre à les manipuler.

Tout ce qui endommage leurs systèmes cognitifs peut ainsi désorienter les abeilles et les empêcher de trouver des fleurs ou leur nid. Or une abeille dans une telle situation est considérée comme morte pour sa colonie.

Les abeilles sont ainsi très vulnérables aux stress dits « sublétaux », qui ne provoquent pas directement leur disparition mais perturbent leur comportement. Dans un article publié récemment dans *Trends in Ecology & Evolution*, nous avançons l'idée que l'industrialisation toujours plus grande de nos sociétés est à l'origine de la multiplication des stress sublétaux, qui restent toutefois difficiles à identifier.

La pollution automobile ou les pesticides réduisent par exemple l'efficacité de butinage en perturbant les communications nerveuses dans le cerveau des insectes. L'agriculture intensive et le réchauffement climatique altèrent également la nutrition des abeilles, en réduisant la diversité des plantes disponibles ou leurs périodes de floraison.

Les abeilles domestiques sont d'autre part sujettes à de nombreux parasites, virus ou prédateurs qui se sont répandus au niveau mondial au gré des échanges commerciaux et autres transports humains incessants. *Varroa destructor*, le plus répandu de ces parasites provoque ainsi chez les abeilles des problèmes de développement cérébral.



Butiner le pollen, une activité exigeante au niveau cognitif. Simon Klein, CC BY-NC-ND

Quelles actions pour sauver les abeilles ?

La préservation des populations d'abeilles dépend de la qualité de leur environnement. Et la moindre petite action peut faire la différence ! Fleurir son jardin ou son balcon de variétés riches en nectar permettra de nourrir les abeilles. Réduire, voire éliminer, l'utilisation d'herbicides et de pesticides constitue une autre bonne pratique, de même que passer la tondeuse moins fréquemment pour fournir de nombreuses plantes à fleurs locales aux abeilles sauvages.

S'initier à l'apiculture en rejoignant un club ou construire un hôtel à insectes sur votre balcon ou dans votre jardin sont d'autres initiatives à explorer. Enfin, l'achat de miel de production locale et l'approvisionnement auprès de circuits courts ou d'une agriculture respectueuse de l'environnement pourront contribuer à protéger les colonies.

Bricolage au jardin : comme fabriquer un hôtel à insectes



Comment fabriquer un hôtel à insectes (Rustica, 2015).

Sur le plan législatif, la France aura été l'un des premiers pays à prendre position en faveur de l'interdiction des pesticides neonicotinoïdes, dont de **nombreuses recherches** ont prouvé l'effet néfaste sur la cognition des abeilles. La loi, entrée en vigueur récemment, prévoit une interdiction de leur utilisation à partir de **septembre 2018**, avec cependant des dérogations possibles jusqu'en **2020** (un recul par rapport au **premier rapport de loi** qui témoigne de l'influence des industries pétrochimiques sur les parlementaires).

Au niveau européen, la forte **mobilisation citoyenne** grâce à une vaste **pétition** aura sans doute poussé l'Union européenne à statuer prochainement sur l'**interdiction de ces insecticides**.

De la même manière, il a été montré que le glyphosate, cet herbicide commercialisé par Monsanto sous le nom de Round Up, **constituait un agent perturbateur** du comportement des pollinisateurs (et tout aussi inquiétant pour la santé humaine). Malgré cela, l'Europe a signé l'autorisation de commercialisation de ce produit. Une **initiative citoyenne européenne** lancée en février 2017 tente d'infléchir cette position.

Deux décennies après les premières constatations d'un déclin massif des abeilles, nous pouvons affirmer que nous connaissons la nature des problèmes qui affectent les colonies et qu'il est possible de l'enrayer. Il nous incombe à tous de protéger ces précieux pollinisateurs, acteurs clés de notre environnement et de celui des générations futures.

 biodiversité extinction pollution Monsanto insectes phytosanitaires Roundup abeilles pollinisateurs fleurs

