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Causes and consequences of individual forager variability in social bees

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

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Centre de Recherches sur la Cognition Animale

ED BSB

“’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)

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Table of contents:

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. Currently, bee populations are facing an increasing number of environmental stressors, known to disturb the behaviour of individuals, presumably upon their impact on cognitive capacities. Hence it is important to learn more about how stressors impact on individual foraging behaviour to understand how a colony maintains effective nutrition and development.

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 buffed-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 vulnerability of pollinators to sublethal pesticides and other environmental stressors. 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 impair the whole colony nutritional balance.

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. Therefore, some repetitions were unavoidable, but I still consider this format to be the more efficient to highlight my work.

Publications included in this thesis:

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.

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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.

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- Included as Appendix 2.

Klein S., Vine P., Bordier C., Devaud JM., Lihoreau M. & Barron BA. (2016). **Why social bees are so vulnerable to environmental stressors?** *Oral presentation delivered at the 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.*

Credit for the drawings: S. Klein, based on personal pictures
(except *B. terrestris* forager in chapter 5, picture kindly provided by T. Gomez)

General introduction:

Advanced social bee colonies have been described as superorganisms (Hölldobler & Wilson, 2009); because the collective efforts of the colony members manifest a range of functions such as thermo and nutritional regulation that operate at the level of the colony. Unlike cells that constitute organisms, individuals in a colony of social bees are extremely individualistic and can be highly variable. Community ecologists focus on how such individual variation can influence group structure and the nature of collective and cooperative behaviours. My thesis is an exploration of the degree of inter-individual variation in European honey bees (*Apis mellifera*) and Buffed-tailed bumblebees (*Bombus terrestris*) societies and how individual actions can influence collective behaviour. Given that these two species are ecologically (Ollerton & Waser, 2006) and economically (Gallai, Salles, Settele, & Vaissière, 2009) vital but also threatened by a range of current environmental stressors (Potts et al., 2010), my thesis also explores how this new perspective on colony function can help us understand the responses of bee colonies to environmental pressure and stress.

A comparative analysis 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 highest degree of animal social organization, termed eusociality (Wilson & Holldobler, 2005). Eusociality is found in a few animal taxa, primarily in insects such as ants (Hölldobler & Wilson, 2009), termites (Thorne, 1997), bees (Hölldobler & Wilson, 2009) and wasps, some shrimps (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 50,000 usually non-reproductive workers who are made up of several generations of daughters of the queen (Nowak, Tarnita, & Wilson, 2010; Wilson, 2000).

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).

Both species also produce short-living males with the sole purpose of reproduction (Wilson, 2000). Honey bees and *B. terrestris* differ in their sociality level and as a major consequence, honey bees rely on lot more workers than bumblebees for ensuring the collective functions of the colony (table 1).

Honey bee queens are polyandrous (Winston, 1991). Consequently, the workers will be genetically diverse due to their mixed paternity. This can explain some degree of inter-individual variability (Oldroyd & Fewell, 2007). In bumblebees, each queen mates with a single male (Goulson, 2010) and the degree of genetic diversity is lower in bumblebees than it is in honey bees.

In both species the non-reproductive workers are divided into behavioural castes, as groups of individuals performing different tasks (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: nectar (carbohydrate source) and pollen (protein and lipids), for their nestmates (Goulson, 2010; Michener, 2000; Winston, 1991).

For honey bees, division of labour among workers is primarily age-based. Every worker bee will start its adult life as a nurse and then eventually start to forage, performing different tasks successively across its life (Winston, 1991). All honey bee workers have a similar morphology (Winston, 1991). Division of labour in *Bombus terrestris* is mainly based on morphological differences, however: 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 emergence (Goulson, 2010). In this case, only some individuals will ever forage whereas some others will never leave the nest.

Honey bee and bumblebee workers are central-place foragers, as they exploit the environment around their nest, but must always return to the nest. This particular lifestyle 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, 2011). The two species are, nevertheless, both floral generalists, deriving their nutrition from nectar and pollen (Michener, 2000). Honey bee colonies 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 (Goulson, 2010; Winston, 1991) and maintaining colony nutritional balance requires a fine adjustment of pollen and nectar stocks according to changes in colony needs (Pankiw, 2007; Plowright & Silverman, 2000).

Studying these two species allow us to analyse the role of individual variability in two species with different social structures but sharing the same 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

Growing evidence suggests that different individuals do not react similarly to the same stimulus, and that foragers from the same colony can display different cognitive traits (Jandt et al., 2014; Jeanson & Weidenmüller, 2014). There is now an abundant library of literature focusing on inter-individual variability across the animal kingdom (Montiglio, Sih, Mathot, Wolf, & Dingemanse, 2015; Nettle, 2006), and also in social insects (Jandt et al., 2014; Jeanson & Weidenmüller, 2014). This literature has established important concepts in behavioural ecology, such as behavioural syndromes (as a suit of correlated behavioural tendencies in different contexts specific to an individual) (Sih, Bell, & Johnson, 2004) or keystone individuals (as a very influential individual in a group) (Modlmeier, Keiser, Watters, Sih, & Pruitt, 2014). In this thesis I use these concepts to analyse the variability in foraging strategies and the consequences of this variability 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, Lutz, Rao, Goldenfeld, & Robinson, 2014)). By contrast, some foragers are extremely active, which are considered as keystone individuals for the colony as they contribute disproportionally to the foraging effort (Tenczar et al., 2014).

Bee foragers also individually differ in their foraging strategies: for instance, in their propensity to collect nectar or pollen (Hagbery & Nieh, 2012; Robinson & Page, 1989). In honey bees, this has been 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, Dyer, Bock, & Dornhaus, 2003). Finally, honey bees exhibit strong inter-individual variability in their investment in different tasks (Walton & Toth, 2016). For instance, some bees are consistently more interactive with others, while others are consistently less interactive (Walton & Toth, 2016). 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.

One fundamental yet still unanswered question is whether and how behavioural variability among foragers helps the colony to optimise food collection. 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, Tooker, Grozinger, & Patch, 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 full and correct development (Goulson, 2010; Winston, 1991). 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.

Behavioural variability and bee population declines

Pollinators are key species in every global ecosystem. They pollinate from 78% to 94% (Ollerton, Winfree, & Tarrant, 2011) of the flowering plants on the planet.

Human societies heavily rely 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 cost 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 (Chauzat et al., 2013; Crane, 1999). Bumblebees (e.g. *Bombus terrestris* or *Bombus impatiens*) are managed to provide pollination to green houses or orchards in some countries (Velthuis & van Doorn, 2006), especially in Europe and North America.

But, a current increase in environmental stressors, mainly driven by human activities, is affecting bee populations (Potts et al., 2010). Many countries have been reporting high losses of honey bee colonies over recent years (Laurent, Hendrikx, Ribiere-chabert, & Chauzat, 2014; Steinhauer et al., 2014) and more bumblebee species have become listed as endangered (Cameron et al., 2011; Williams & Osborne, 2009). This could have huge consequences on farming economy 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, Smith, Mustard, Morton, & Trumble, 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). Even if a lot of work is dedicated to analysing the causes of this crucial issue, we still do not fully understand all the repercussions of such a complex problem.

Against this background, it is now crucial to better understand the mechanisms of pollinator declines. By studying the nature and consequences of variation in foraging behaviour in two important social bees we can ask whether individual behavioural variability can be an advantage in the face of mounting environmental stressors?

Thesis prospectus:

Fundamentally, this study addressed a simple question: what makes a good bee forager, and how this is relevant for colonies responding to environmental stressors? 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, 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 a fully functioning neuronal system in order to recognise and handle rewarding flowers. I demonstrate that 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, 3 and 4** 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 the 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 between pollen and non-pollen foraging in honey bees, and found that only a subset of foragers collected pollen and that they did it later in their lives. This presents a potential vulnerability of the entire colony as only a subset of bees collect all the pollen. In **chapter 3,** I 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. It proves that, in honey bees, foraging

activity and efficiency are linked by experience. In **chapter 4**, I used a similar approach to 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. This inter-individual variability was not correlated with differences in the foragers' morphology.

In **chapters 5 and 6**, 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 perform similarly in a navigation task under semi-controlled foraging conditions (**chapter 5**). 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. In **chapter 6**, 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 access 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.

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

	Honey bee	***Bumblebee***
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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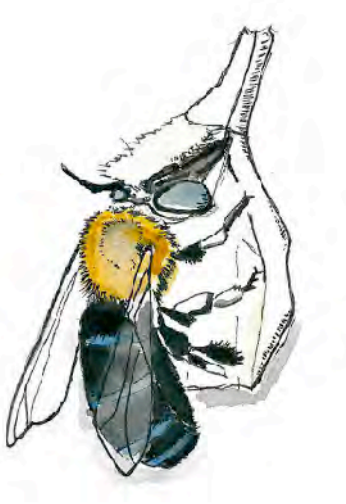
CHAPTER 1: Why bees are so vulnerable to environmental stressors



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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.

Bee 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 understandible 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].

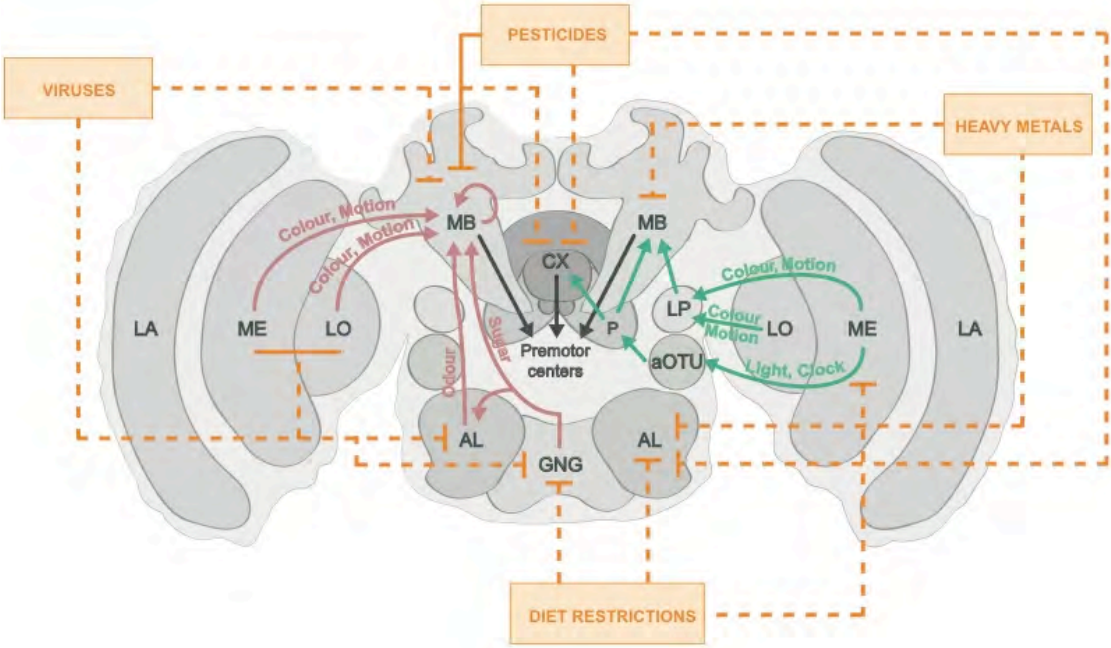

Figure 1. Brain structures supporting the cognitive capacities needed for foraging and how they are impacted by stressors. Schematic frontal view of a bee brain. Sensory information from the environment is first processed in specialised brain structures. The antennal lobes (AL) process olfactory information. 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 is it 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. 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 [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 microsporodian *Nosema cerana* and the mite *Varroa destructor* are two major parasites of honey bees. Exposure to either of them induces specific but overlapping patterns of altered gene expression in their 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.

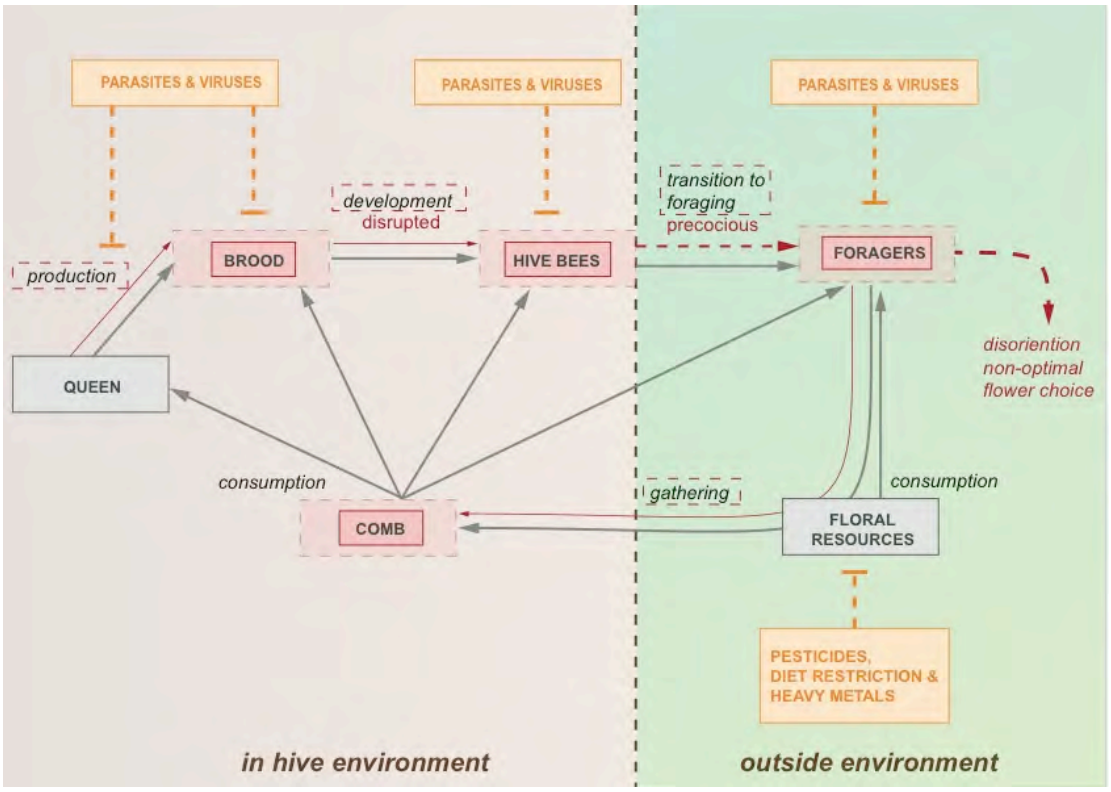

Figure 2. Sublethal effects of stressors on honey bee colony dynamics. In a non-stressed colony (grey arrows), the brood (eggs, larvae and pupae) develops into hive bees (e.g. nurses) that begin to forage two weeks later. Foragers gather nectar and pollen from floral resources for storage in the hive (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.

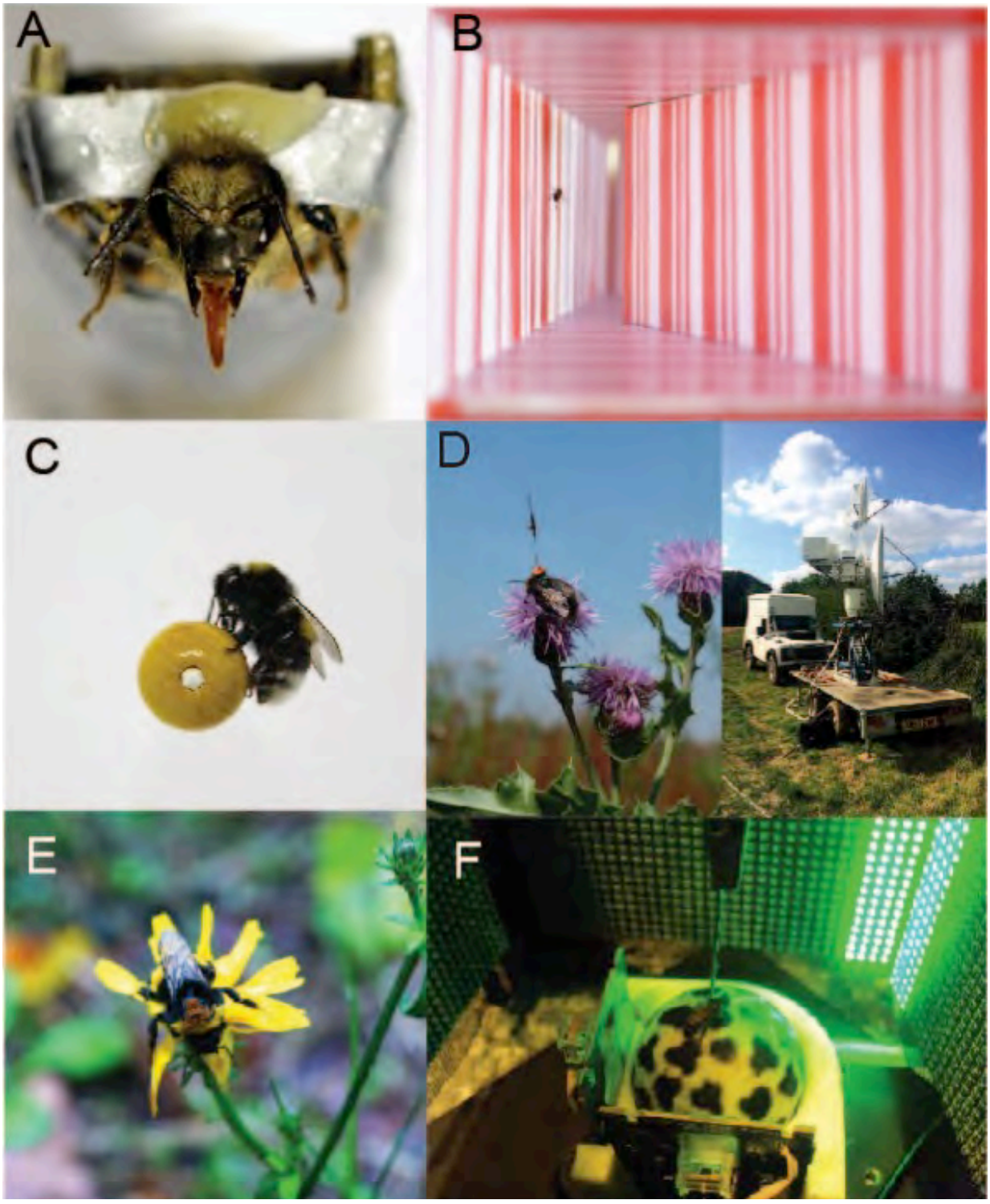

Figure I. Methods for studying bee learning and memory. (A) Restrained honey bee showing proboscis extension reflex (PER) (C. Fresillon/CNRS). (B) Free-flying honey bee in a flight tunnel covered with visual patterns generating optic flow (F. Vrignaud/DGA). (C) Bumblebee foraging on an artificial flower (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 breath) 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:

The properties of pollen and nectar collection in a honey bee colony

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Chapter 2: The properties of pollen and nectar collection in a honey bee colony

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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. These resources require different collecting methods, but the strategies of individual foragers for pollen and nectar collection remain poorly understood. Here we analysed the foraging behaviour of individual bees throughout their life using Radio Frequency Identification, dynamic balances and video cameras. We automatically recorded the weight of bees on departing the hive, 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. Pollen trips were longer than non-pollen trips and performed by more experienced foragers. Bee weight on departing the hive varied between pollen and non-pollen trips, and with trip duration, indicating a relationship between crop load and resource collection. Our data bring new information on how a social insects collectively achieve nutritional balance and point toward pollen collection as the greatest point of vulnerability for bee colonies.

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

Introduction:

Social insects face the challenge of collectively exploiting different food resources in order to regulate nutrient influx at the colony level [1–3]. For honey bees, the division of labour between nectar and pollen collection has been a subject of intense study [4–6]. It is often assumed that different foragers in a colony collect different resources, and pollen and nectar foragers are distinct behavioural specialists or from distinct behavioural castes [7,8]. Here we used a new Radio Frequency Identification (RFID) application to examine the within- and between-individual variation in nectar and pollen collection strategies in order to better understand bee colony nutrient balance.

Recently, foraging decisions of bees have been studied using contemporary theories of nutritional ecology [9–11] that emphasise on how animals precisely and dynamically regulate their food intake to meet their needs for different macronutrients [12]. The influential nutritional geometry framework models the current nutritional state of an animal as a point in a multidimensional nutrient space relative to its optimal nutritional target [13]. A social insect colony can be considered to operate as a single physiological entity (or superorganism [14,15]) trying to reach its own nutritional target composed of the different targets of all colony members, including the workers, the reproductive adults and the brood [16,17]. Considering social insect nutrition in this way emphasises how both the assessment of nutritional demands of the colony and the nutrient supply are a decentralised and collective process, which adds a layer of complexity to their foraging ecology.

A honey bee colony contains a single reproductive queen supported by up to ~50,000 of non-reproductive females workers, of which only a subset forages to supply food to the whole colony [18]. As a group, the foraging force changes its collective behaviour in response to changes in colony nutritional needs [14]. For example, honey bees rapidly increase pollen collection following depletion of the colony pollen stores [19], and after rainy days when pollen collecting is compromised the foraging force increases pollen foraging [20]. Foragers are sensitive to protein demand by the colony and adjust protein intake according to the amount of developing brood [15,21,22]. For honey bees, brood pheromone is a key signal stimulating foragers to collect pollen [21]. Foragers also adjust the type of pollen they collect in order to increase pollen diversity for the colony [10].

Pollen and nectar are often distributed differently in the environment, which further complicates the challenge of balancing colony nutrition. Flowers do not always produce pollen and nectar synchronously, if indeed they produce both resources at all [23]. Foragers must therefore distribute themselves across different resources in order to reach nutritional balance [24]. Pollen and nectar are also collected differently. Pollen grains are packed into pellets held by specialised hairs on the hind legs [25] whereas nectar is ingested by the bee and carried in the crop (its social stomach) [25]. Some of the nectar can be consumed to fuel the bee during foraging [26].

Because of the difference in the spatial distribution of these major nutritional resources and the behavioural skills required to collect them it may be most efficient for a colony to have different individuals to specialise on nectar or pollen [23]. This is generally assumed to be the case for bees where pollen and nectar foragers are often described as behavioural castes [4,27–30] that differ in their brain neuropeptide profile [31], sucrose response threshold [32], ovary size [5] and levels of vitellogenin (a yolk precursor protein) [33]. Together with evidence for genetic variation and heritability of pollen and nectar collection [7,28,34,35], these observations suggest that pollen and nectar collection are different evolved specialisations [7,36,37]. However, recent studies have reported that the distinction between the pollen and nectar collection foraging forces is far from absolute. In honey bees and bumblebees, a large proportion of the foragers seem to collect both resources, or may change specialisation as they age [32,38–42]. But so far, no study has analysed the long-term foraging activity of social bees in natural environment, and thus, data on how foragers partition their effort between pollen and nectar collection are limited.

Here we addressed this question using radio frequency identification technology (RFID) at the hive entrance combined with weight and video sensors to monitor the foraging activity, the nature and the amount of food collected by individual bees. In recent years, RFIDs have been increasingly used to track bee foraging activity [39,43–45]. Our new design allowed us to monitor bees' lifetime foraging behaviour and compare their pollen and nectar collection strategies to better understand the collective process of nutrient supply to a bee colony.

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. The infrared beams and gates were all connected and monitored via Arduino technology (Arduino, Adafruit and little birds electronics, Hornsby, Australia).

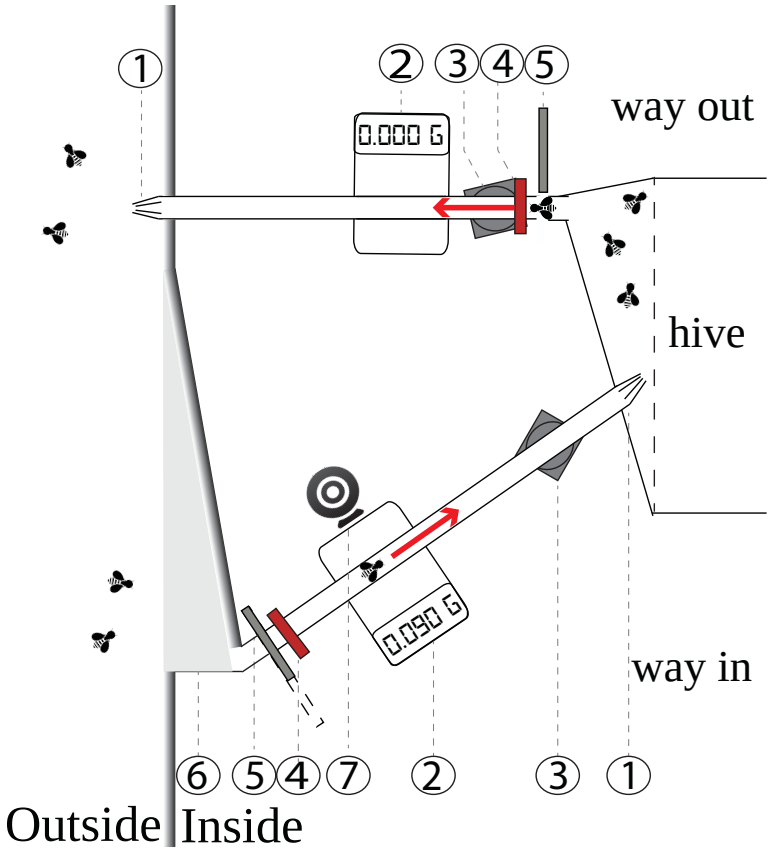

Figure 1: Colony entrance with sensors. The entrance and exit tubes were 1cm diameter transparent plastic tubing. 1: plastic bristles (forcing the passage of a bee from one direction only), 2: balance, 3: RFID antennae, 4: infrared emitter/receiver, 5: automatic gate, 6: landing platform (open on the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

[46]). Examination of the videos of the entrance tunnel indicated that these limits were realistic.

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 where successively added. To source newly emerged bees we collected brood frames from five different colonies and stored them overnight in an incubator maintained at 37ºC. The next morning we glued individually programmed RFID tags to the thorax of the newly emerged bees with super-glue.

500 tagged bees were added every day for the four first days of the experiment. From day 21 of the experiment a further 2,000 tagged bees were added over four days (500 bees a day). From day 35 of the experiment a further 800 bees were added over four days (200 bees a day). Thus over the five weeks of the experiment, the hive received 4,000 bees in total. 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 bushes 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 consider as non-foraging trips [47]. 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 (electronic supplementary material videos S1). 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 hivemates. For each bee, we excluded the first five trips, which are more likely orientation flights than foraging flights [47]. 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. The complete data set can be found in Supplementary Material Dataset S1.

Data analyses:

Data were analysed in R version 3.2.3 [48] (operating via Rstudio, version 1.0.136 [49]) using the packages lme4 [50] and lmerTest [51]. We analysed the probability of foraging for pollen according to experience (number of foraging trips) using a binomial generalized linear mixed model (GLMM), with bee identity (ID) as random factor. Differences in foraging durations and weights on departure between pollen and non-pollen trips were investigated using a Poisson generalized linear mixte model (GLMM). Trip durations were normalised (natural log). Colony identity was included as a covariate and bee identity as a random factor. 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 GLMM with colony identity as a covariate. The regressions between trip durations and weights on departure were investigated with linear models (LMs).

Results:

Pollen collection was performed by a minority of foragers

On average 27% of the bees foraged at least once for pollen (29% for colony 1 and 25% for colony 2, details in table 1). 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). Electronic material figure S1 shows examples of the foraging history for particularly active bees.

More experienced bees foraged for pollen

In average, bees foraged for less than 5 days (mean ± SE = 4.65 ± 0.73 days, N = 1 244, colony 1: 4.97 ± 0.79 days, N = 650 and colony 2: 4.04 ± 0.65 days, N = 594). The probability of collecting pollen on a foraging trip increased with the number of trips already performed (binomial GLMM, N = 2 215, P < .0001, figure 2, table 2*a*, and electronic supplementary material figure S1 for examples) indicating that bees were more likely to collect pollen as they accumulated experience.

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

Table 2: a. Summary of binomial GLMM with logistic link function of the probability of a bee collecting pollen on a given trip in its life. Variable definition: *resource* is the trip load (pollen or non-pollen), *number of trips performed|ID* represents individual as random effect. Probability of pollen collection increased with trip number. **b.** Summary of linear model exploring variation in trip duration according to the type of resource collected. Trip duration was natural log transformed to obtain a Gaussian distribution. Pollen collection trips were longer than non-pollen trips. Minimum adequate model selection methods are reported in electronic supplementary material Tables S1 and S2. Significant effects are in bold. *Resource|ID* represents individual as random effect.

	Estimate (SE)	**df**	**t**	**P**
a. *resource ~ number of trips performed + (number of trips performed |ID)*				
Intercept	**-1.63 (0.17)**	**2210**	**-9.70**	**<.0001**
Experience	**0.021 (0.006)**	**2210**	**3.69**	**<.0001**
b. *log(trip duration) ~ resource * colony + (resource|ID)*				
Intercept colony 1	**2.21 (0.04)**	**304.1**	**59.26**	**<.0001**
Resource colony 1	**0.88 (0.08)**	**147.1**	**10.91**	**<.0001**
Intercept colony 2	**2.45 (0.06)**	**428.9**	**4.23**	**<.0001**
Resource colony 2	**1.72 (0.13)**	**236.5**	**-3.65**	**0.0003**

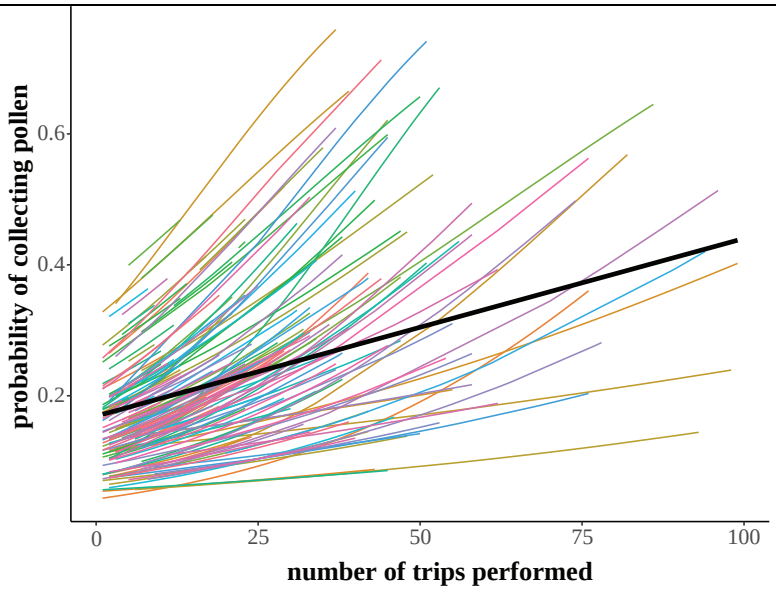

Figure 2. The probability for an individual to collect pollen on a given trip increased with the number of trips performed (pollen and non-pollen trips). Each coloured line represents the fitted estimators of each bee according to a binomial GLMM (N = 1,941, P <.0001, table 2*a*). The black line represents the average of the fitted estimators. N = 154 bees. Non-pollen trips = 1,432. Pollen trips = 509.

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 3, table 2*b*, example in electronic supplementary material figure S1).

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

We divided our dataset into foragers that were never recorded collecting pollen (non-pollen foragers) and foragers that were recorded collecting pollen at least once (mixed foragers) (table 1). We found no difference in weight on departure between these two types of foragers when collecting non-pollen resources (GLMM, N = 1,870, P = 0.45, table 3*a*). Non-pollen foragers' weight on departure did not differ according to the colony origin (GLMM, N = 889, P = 0.62). When we considered the mixed foragers only, however, bees of colony 1 departed the hive heavier for pollen trips than for non-pollen trips (GLMM, N = 820, P = 0.013, figure 4, table 3*b*). Bees of colony 2 departed the hive lighter for pollen trips than for non-pollen trips (GLMM, N = 174, P = 0.021, figure 4, table 3*b*). Therefore, bees' weight on departure from the hive varied depending on the resource they were about to collect in that trip, and the nature of the adjustment differed between our two colonies.

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 4). 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 4).

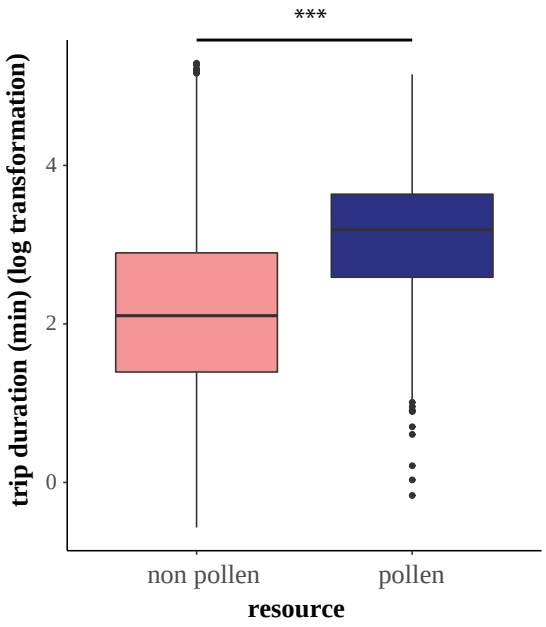

Figure 3: Difference in duration of non-pollen (pink) and pollen (blue) trips, for bees that foraged at least once for pollen. Only the trips shorter than 200 min were included (98.55% of all 1,941 trips). N = 154 bees. Non-pollen trips = 1,409. Pollen trips = 504. Boxplot: the line shows the median, 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 2*b*).

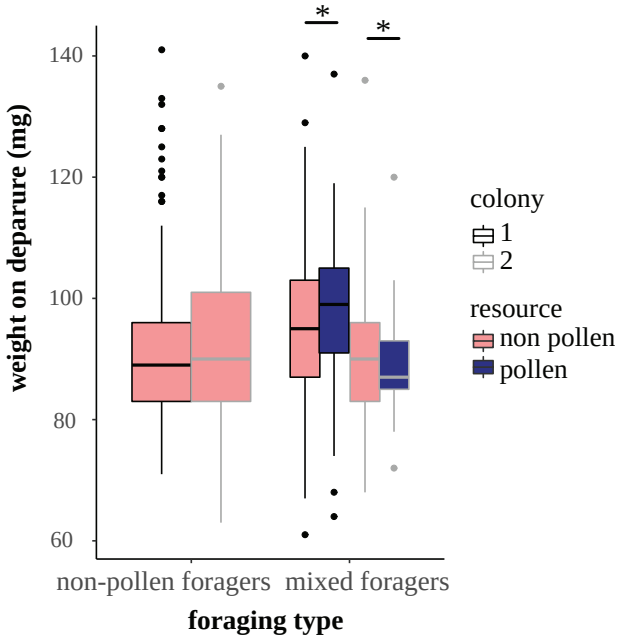

Figure 4. Difference of weight on departure for the different types of foragers and for non-pollen (pink boxplots) or pollen (blue boxplots) trips. There was no difference in weight on departure between non-pollen and mixed foragers when collecting non-pollen resources in both colonies. Mixed foragers 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 3).

Table 3: 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 electronic supplementary material table S3). *Foraging type|ID* indicates the individuals as random effect. **b.** Summary of linear model looking at the difference of weight on departure between non-pollen trips and pollen trips for the mixed foragers. In colony 1, bees left the hive heavier when collecting pollen. In colony 2, bees left the hive lighter when collecting pollen. (see details of model selection in electronic supplementary material table S4). *Resource|ID* indicates the individuals as random effect. Significant effects are in bold.

	Estimate (SE)	**df**	**t**	**P**
a. *weight on departure ~ forager type + (forager type | ID)*				
Intercept	**93.65 (6.06)**	**219.6**	**154.51**	**<.0001**
Forager type	-4.32 ($9.27.10^{-4}$)	299.0	-0.49	0.618
b. *weight on departure ~ resource * colony + (resource | ID)*				
Intercept colony 1	**93.67 (0.82)**	**73.05**	**114.02**	**<.0001**
Resource colony 1	**2.49 (1.02)**	**58.09**	**2.42**	**0.019**
Intercept colony 2	91.27 (1.47)	105.61	-1.63	0.106
Resource colony 2	**-2.71 (2.41)**	**197.02**	**-2.16**	**0.032**

Discussion:

We recorded the lifetime foraging activity of a subset of foragers in two honey bee colonies exploiting natural pollen and nectar resources in the field using RFIDs and video cameras. We found that only a few foragers collected pollen and none of them did it exclusively. Pollen collection was most likely to be performed by experienced foragers and took longer than collection of other resources.

Monitoring of lifetime foraging activity of honey bee foragers shows that the distinction between the pollen and nectar collection foraging forces in bees is far from absolute, thereby confirming recent short-term observations [32,38–42]. 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 pollen collection is not exclusive, is undertook by a few individuals and comes later in life.

We observed slight adjustments of weight on departure with both trip duration and the type of resource they would collect from the forthcoming trip. 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. Or, because the crop content on departure limits the energy a bee can allocate to its foraging flight, pollen collection trips are short 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 [52,53] suggested that bees adjust their weight on departure from the hive in anticipation of their foraging trip. Like in our study, these authors found that bees left their hive with more sugar (larger amounts of more concentrated nectar) when collecting pollen than nectar [52,53].

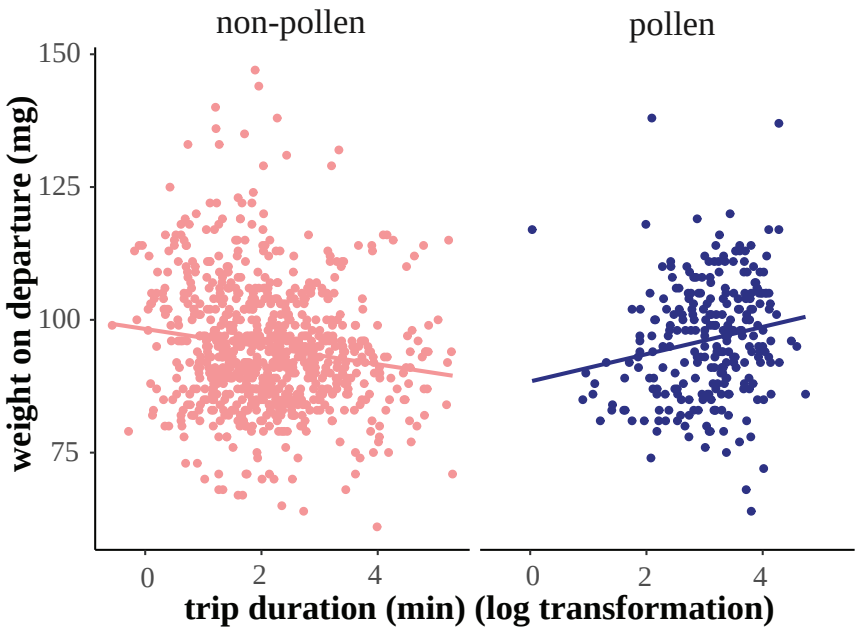

Figure 5. Weight on departure plotted against trip duration (natural log transformation), for individuals that foraged for non-pollen (pink dots) and pollen (blue plots) resources. For pollen trips (blue points) bees leave the hive heavier for a longer trip. For non-pollen trips (pink points) bees leave 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 4.

Table 4: 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. *log(trip duration) |ID* indicates the individuals as random effect. Significant effects are in bold.

	Estimate (SE)	**df**	**t**	**P**
a. *weight on departure ~ log(trip duration) * resource + (log(trip duration) | ID)*				
NP trip intercept	**95.06 (1.09)**	**126.00**	**87.22**	**<.0001**
NP trip slope	**-1.08 (0.36)**	**618.30**	**-3.05**	**0.002**
P trip intercept	90.13 (2.98)	962.70	-1.65	0.098
P trip slope	**1.51 (0.96)**	**906.30**	**2.71**	**0.007**

More remarkably perhaps, they also found that dance followers filled their crop according to their interpretation of the waggle dance [54], 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 [54]. 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 [52–55].

Our tagged bees were genetically diverse as they were sourced from five different colonies, each headed by a naturally mated queen. Therefore, it is possible that there have been genetic differences between bees identified as mixed foragers and non-pollen foragers, as has been reported previously [7,28,34,35]. A telling finding from our data, however, was that for almost all the bees the likelihood of foraging for pollen increased with foraging experience. Thus, in our dataset individual experience was the strongest factor influencing pollen foraging behaviour.

Our results reveal an important point of vulnerability in a colony's nutritional balance. Foraging is itself stressful for bees [56–58], and the foraging period is short: the foraging life time (foraging longevity, excluding the orientation phase) for the bees of our two colonies was around five days, which is similar to other studies [59]. In our colonies the time spent collecting pollen by any given forager was short (it represented only a small proportion of their total foraging trips), and pollen was collected by bees nearer to the end of their foraging life than to the beginning, after their physiological and cognitive optimum [38,60]. Our work also suggests that pollen foraging trips take longer, either because of flight distances (bees would fly further away for a pollen source), flower discrimination or flower handling time (bees usually take more time collecting pollen on their hind legs [25]), which may increase the predation risk for the bees. Since the nature of non-pollen trips is not precise, non-pollen trip duration might also be driven by unsuccessful trips. Therefore, when the demand for pollen by the colony is increased, either because of internal factors such as high brood production [21] or external factors such as a period of rain [19], the colony needs to increase pollen foraging. This burden may be carried principally by the more experienced foragers in the colony, who may have a limited capacity to respond to the additional demand. This situation could be dramatically compounded if the colony and/or its foraging force are impacted by environmental stressors. A range of environmental stressors is known to reduce the foraging life and performance of

bees [61,62]. An inference from our data is that, in a stressful environment, a high proportion of colony foragers may die before they have accumulated sufficient experience to begin foraging for pollen [63–66]. A bee colony would respond to the loss of foragers by having younger hive bees recruited into the foraging force and starting foraging precociously [47,67,68], but these new foragers would be expected to forage mostly for nectar initially and not help in addressing the colony pollen deficit.

As a consequence of how pollen and nectar collection are distributed across the foraging force, 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 [69,70] and stressed conditions [71]. 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 [44] so do immune-stressed bees (Célia Bordier *et al.* in prep, manuscript in annexe 1). Also, bumblebee colonies exposed to realistic doses of neonicotinoids collect less pollen than control colonies [72,73]. A pollen deficit in a colony could establish a vicious cycle of nutritional imbalance since pollen starved larvae become poor foragers themselves [74], 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 [20,75–79]. So far, however, few models have considered pollen and nectar fluxes separately through a colony [20,77]. Lihoreau et al. [16,17] proposed an approach to model the complex nutritional system of social 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 can be a good call for combining both approaches within a unique framework to better understand bee foraging ecology and resilience capacity.

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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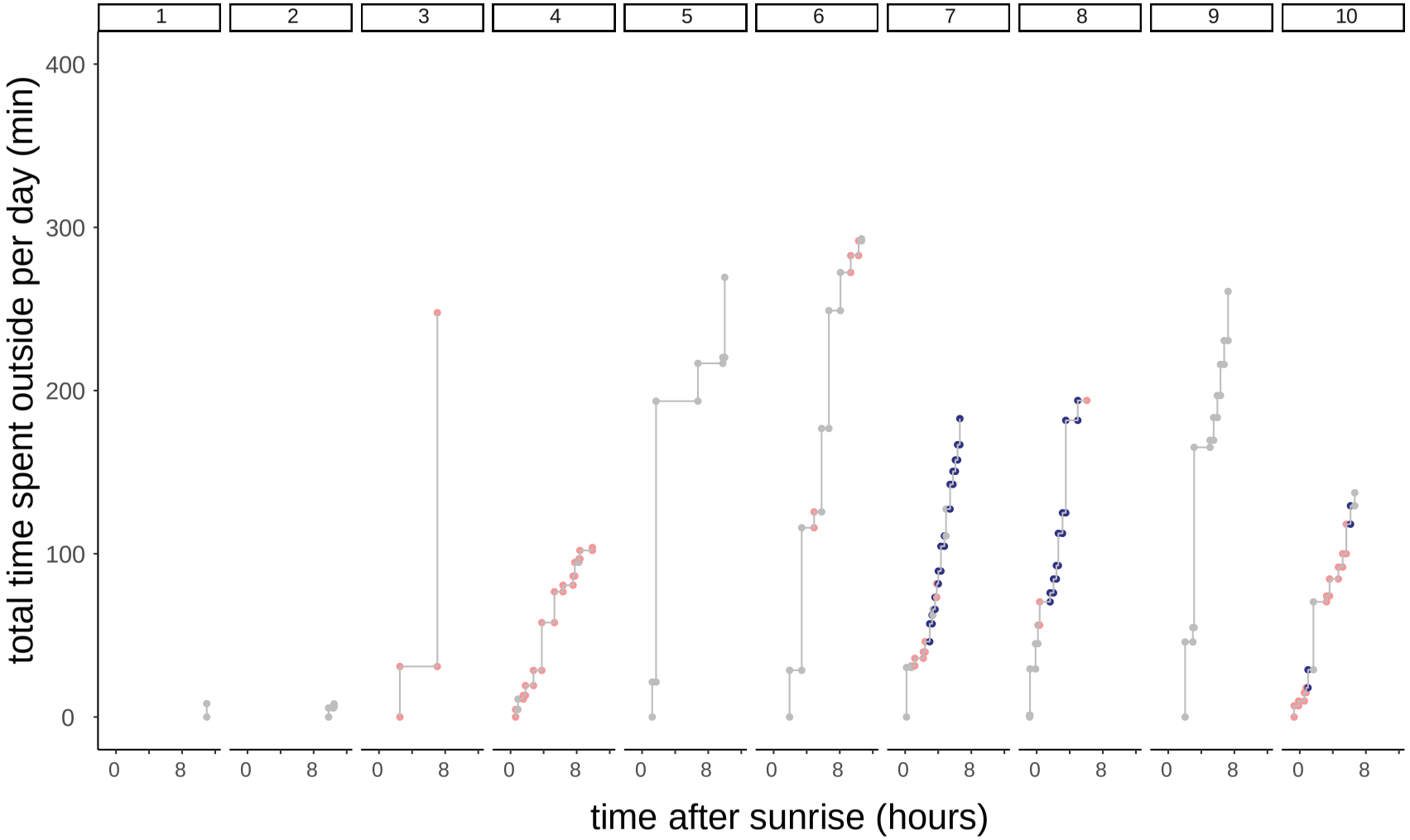
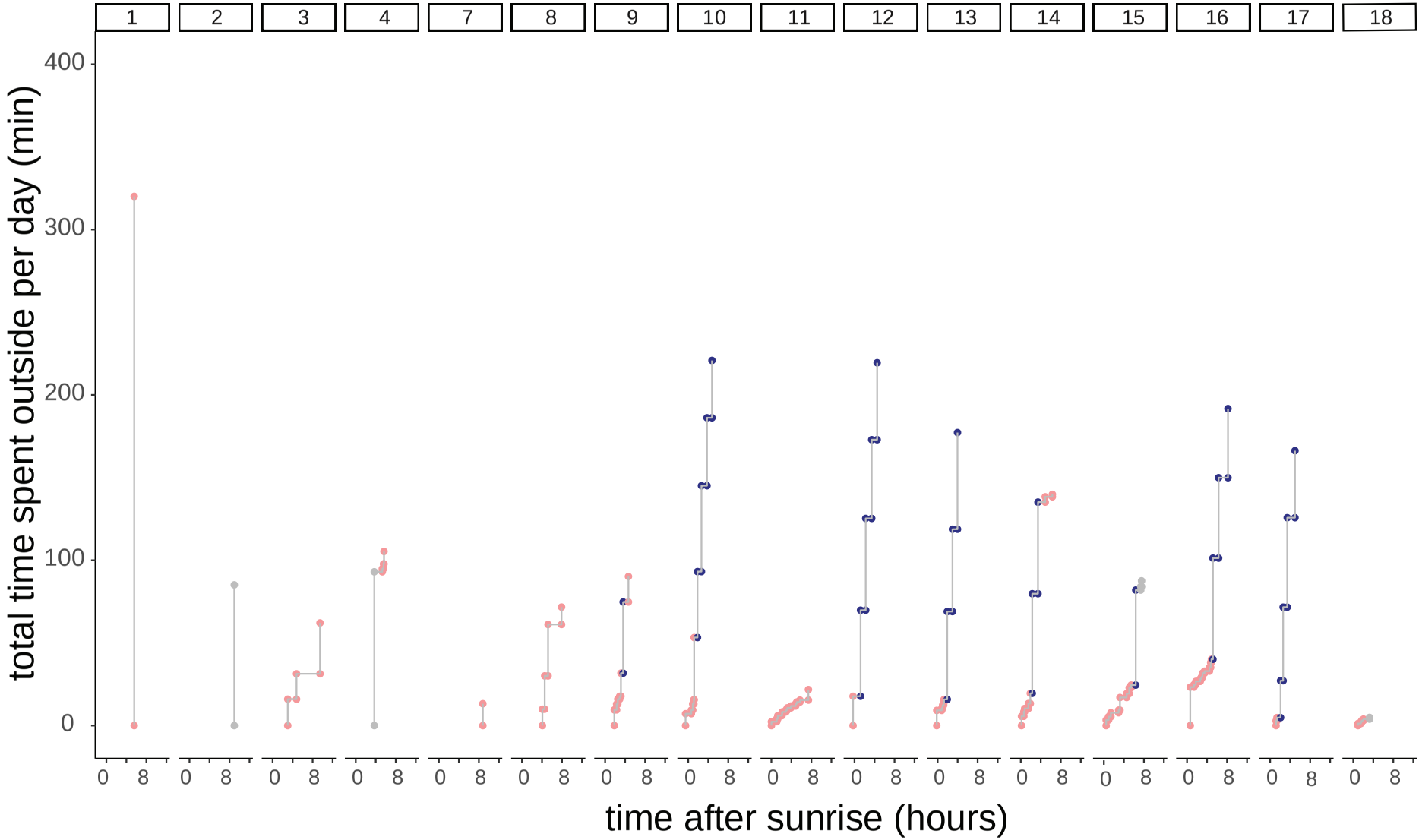
Supplementary material:

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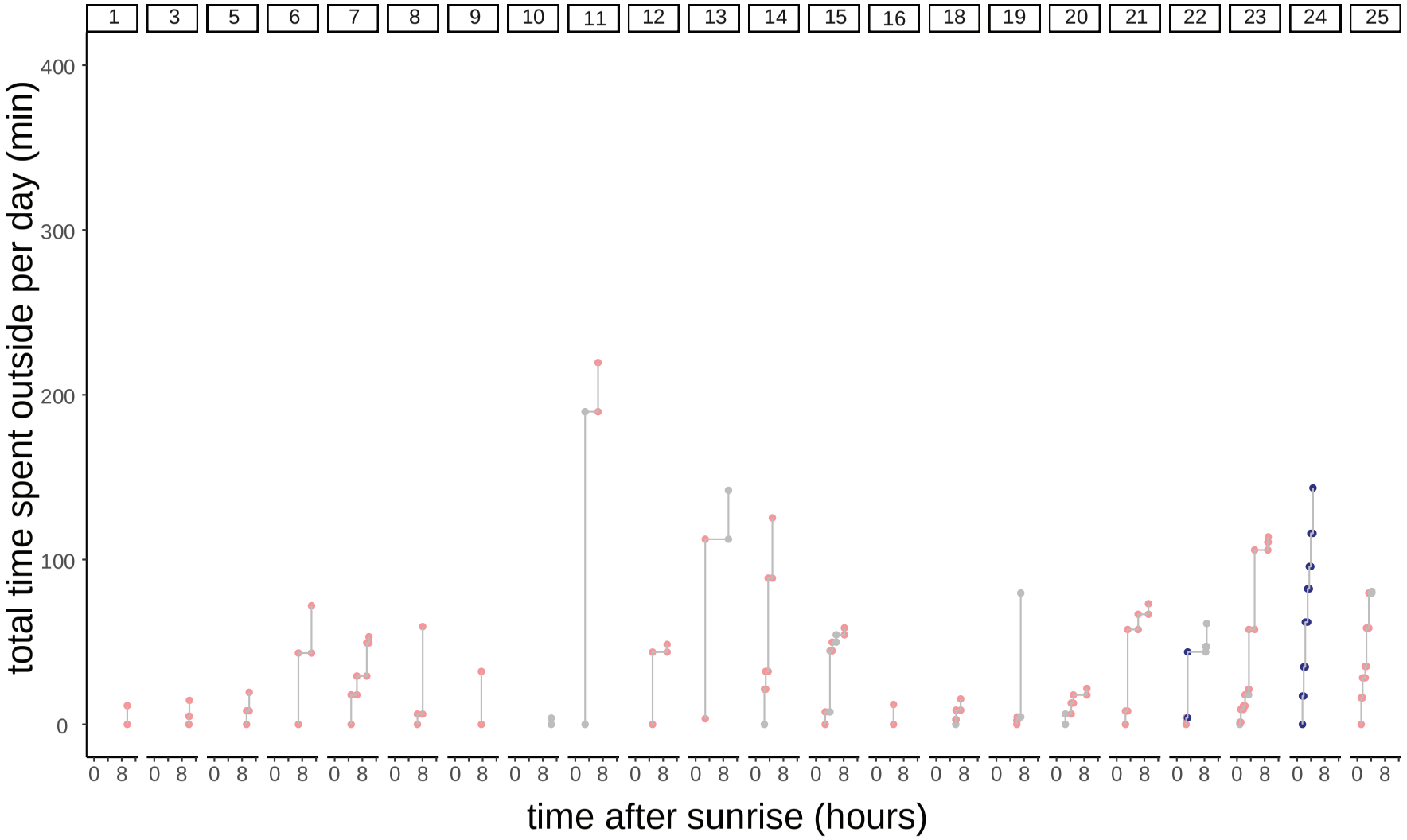
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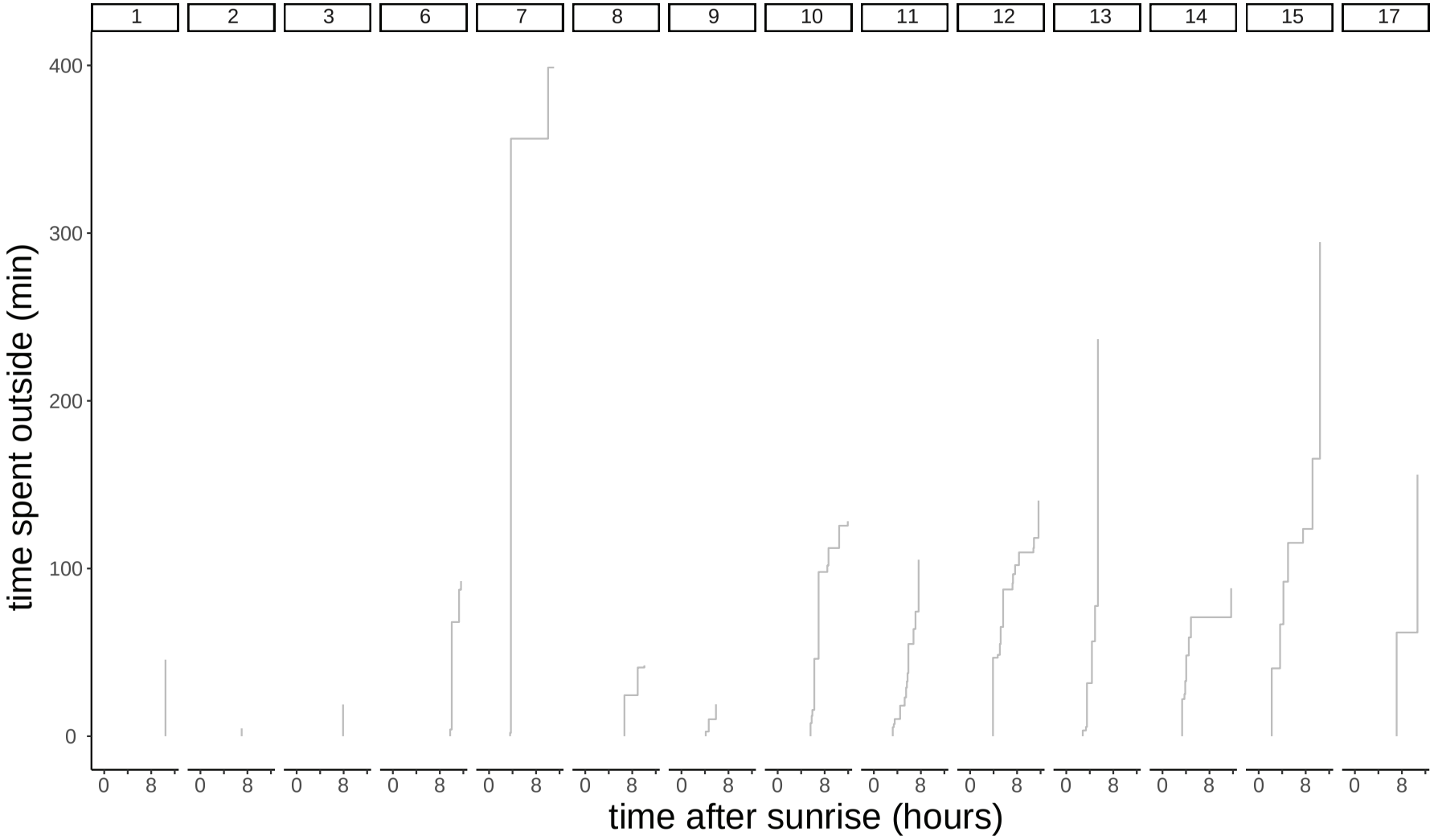
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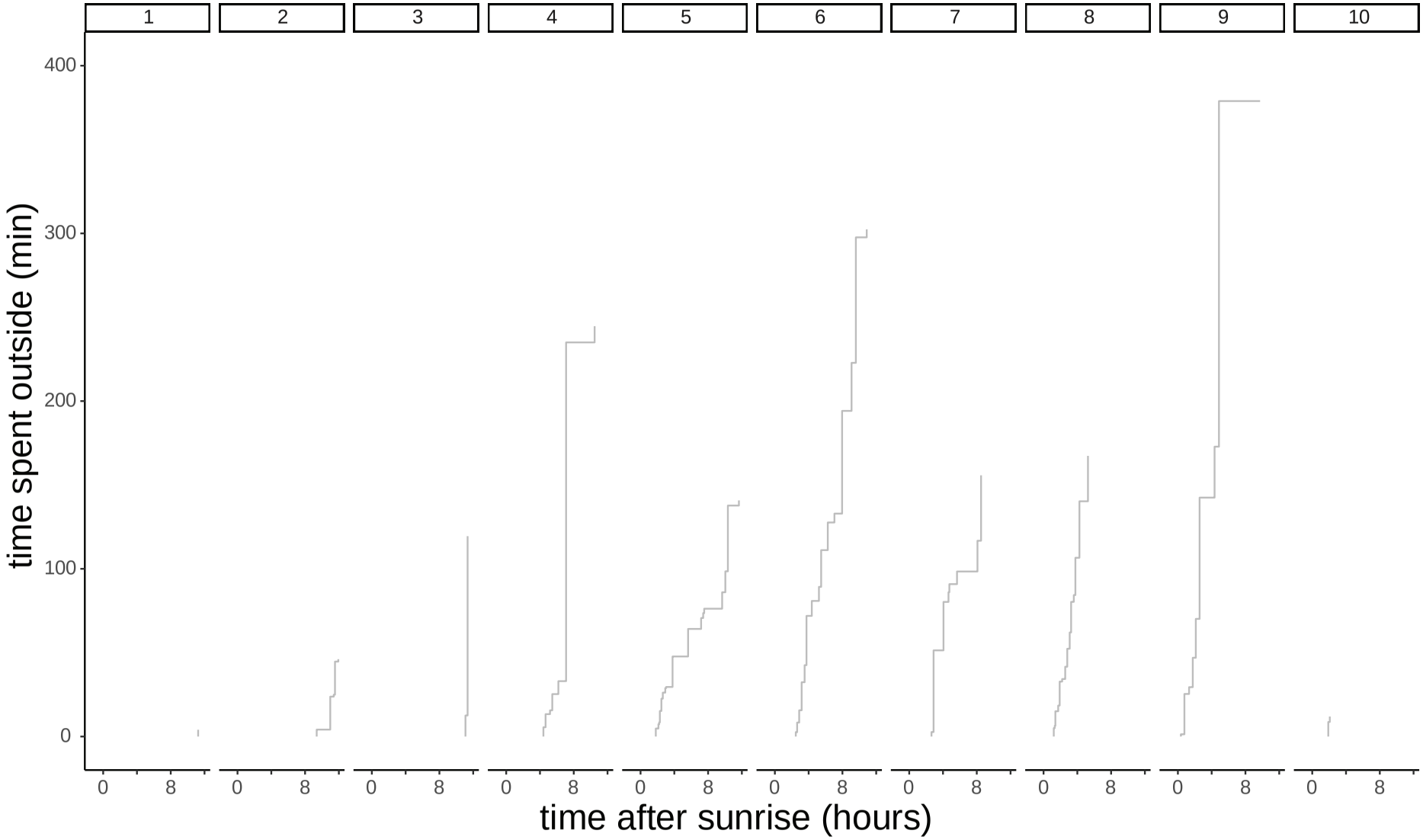
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3
4
5
6
7
8
9
10
400
300
200
100
0
time spent outside (min)
0
8
time after sunrise (hours)

Figure S1: Example of foraging trips patterns for four 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.

Table S1:
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**	**Chi^2**	**P**
Resource ~ experience	2	2433.3	-1214.6		
Resource ~ experience + experience|ID	**5**	**2320.3**	**-1155.2**	**118.9**	**<.0001**
Resource ~ experience + colony + experience|ID	6	2319.9	-1153.9	2.4	0.12
Resource ~ experience * colony + experience|ID	7	2321.8	-1153.9	0.1	0.72

Table S2:
Selection of the best linear model representing the trip duration when bees are foraging for pollen or nectar (resource). Best model is highlighted in bold.

	df	**AIC**	**Loglik**	**Chi^2**	**P**
Log(trip duration) ~ resource	3	20352	-10173		
Log(trip duration) ~ resource + resource|ID	6	20282	-10135	76	<.0001
Log(trip duration) ~ resource + colony + resource|ID	7	20282	-10134	2.5	0.11
Log(trip duration) ~ resource * colony + resource|ID	**8**	**20279**	**-10131**	**5.1**	**0.024**

Dataset S1:
Raw data of each trip for every bee.
Variables:
ID: identity of the bee based on its RFID tag number.

day_in: day of the bee entering the hive

trip_time: trip duration (min)

w_out: weight on departure (g)

w_in: weight on return (g)

w_diff: weight difference for the trip (g)

colony: colony origin

cumul_trip: experience (number of trips performed)

pollen: resource, either pollen ‘p’, non-pollen ‘n’, or unknown ‘na’.

Dataset S2:

Video records of bees entering the hive.

Video 1: bee that collected pollen.

Video 2: bee that return from an trip classified as ‘non-pollen’. It does not have any pollen on its legs.

CHAPTER 3:

The majority of honey bee (*Apis mellifera*) colony productivity is supplied by a minority of foragers

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Chapter 3: The majority of honey bee (*Apis mellifera*) colony productivity is supplied by a minority of foragers.

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Abstract:

In social groups of animals, sometimes a subset of individuals makes a disproportionately large contribution to the group's collective duties. Such high contributors have been identified in several social insect species. In a honey bee (*Apis mellifera*) colony, a small subset of very active foragers (so-called elite bees) performs the majority of the collective foraging activity. This skew in the distribution of foraging activity has not been assessed in terms of the nutritional benefit the elite bees actually provide to the colony. Here we use an automatic monitoring system of foraging activity, utilising RFID technology to follow foragers' activity and efficiency in two honey bee colonies. We confirm the existence of a skew in the foraging activity of bees. We also found that the most active foragers are also the most efficient, and that foraging efficiency is correlated with the experience of the worker. Our work provides a better understanding of collective foraging activity in a social insect, showing the importance of individual experience for developing foraging efficiency. These results also stress the vulnerability towards environmental stressors of the entire social group that is supported by a subset of very active individuals.

Key words: *Apis mellifera,* foraging efficiency, experience, RFID technology, inter-individual differences, intra-individual differences, keystone individuals

Introduction:

Honey bee colonies rely on a subset of active bees, the caste of foragers, to provide nutritional supply to the whole colony [1]. However, these individuals do not contribute equally to the common foraging effort: some bees are more active than others [2]. Nevertheless, colony performance is dependent on forager efficiency more so than forager activity. Whether the most active bees are also more efficient as foragers is not yet clear. To analyse the distribution of foraging activity and efficiency among the honey bee worker population, we used a radio frequency identification (RFID) device to follow different individual bees foraging activity and performance throughout their foraging life.

Inter-individual variability has been extensively observed in a wide range of animal species (e.g. mammals [3,4], birds [5] or insects [6–12]). In social animals, inter-individual variability can affect group structure and dynamics, and a subset of very influential individuals can have a disproportionally large impact on the group in general [13]. These individuals have been described as keystone individuals [13,14], following the terminology used in community ecology. Various group situations can be influenced by few individuals: such as social structure or foraging performance. For instance, hyper aggressive water striders affect group mating dynamic [15]; or the social structure of the group is stabilized by some keystone individuals, that actively link different individuals, in primates [16] or dolphins populations [17]. One way to define the importance of a keystone individual is to look at the impact of its removal on group activity and structure [13]. For example: few hyper active individuals are vital for group-foraging in groups of zebrafish [18], ants [19,20] or bees [2].

In honey bees, inter-individual differences provide a highly structured division of labour [21]. A honey bee colony is composed of one reproductive female (the queen), a few hundred reproductive males and up to 50,000 females workers that undertake different tasks according to their age, and thus belong to different behavioural castes: in-hive nurse bees, guards and foragers [22]. Differences between foragers have also been discussed in terms of different tasks or subcastes or even personality traits [8,10,11,23]. Some studies of social insects have argued that not all foragers make an equal contribution to the collective foraging effort [13,24,25], with observed skews in the distribution of foraging activity due to a few very active individuals [2]. In ants (e.g. *Temnothorax sp.* [20,26]), there is a large proportion of

inactive workers and a small subset of very active ones [25]. In wasps (*Vespa germanica* [27], *Polybia occidentalis* [28]) we also see a skew in foraging activity, due to the coexistence of active and inactive individuals.

In honey bees (*Apis mellifera*), Tenczar et al. [2] identified that around 20% of the foragers performed 50% of the colony's number of foraging trips. These keystone individuals were coined "elite bees". However, it is still not known how efficient the two groups of elite and non-elite bees are in their foraging task. More activity does not necessarily mean a more efficient supply of resources to the colony. Highly active but inefficient foragers would be of limited benefit to the colony. In colonies of temnothorax ants, the most active foragers are also more efficient, on average, than the rest of the forager population [19,20]. We may expect to find a similar correlation in honey bee colonies, even though foraging activity appears to narrow the cognitive repertoire of the bees [29]. To test this hypothesis, we used the radio frequency identification (RFID) technology combined with video analyses and measures of weight to assess whether the elite bees are also more efficient foragers and thus have a strong influence on colony nutritional balance. The RFID technology has been increasingly 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 [30–35], or individual foraging strategies [2,36]). Here we made the most of this high throughput behaviour quantification to analyse the relationship between activity and efficiency of foraging behaviour in honey bees.

Material and methods:

Experimental design:

We used two experimental colonies in autumn and spring 2014. We used RFID technology to record trip time and microbalances to record the net changes in weight across trips, in order to observe bee foraging behaviour. Experimental methods have been described in **Chapter 2** where the same equipment and methods were used as in this study. We conducted the analyses on the same dataset.

Data analyses:

Data were analysed and graphed with R software [37] (R studio version 1.0.136 [38]). We used the *nlme* package for the analyses [39]. 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. 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 mixt model (GLMM). In all the models bee identity was included as a random effect. All models were selected by comparing their Akaike Information Criterion (AIC) to null models [40] (electronic supplementary material table S1).

Results:

Identification of elite bees:

Foragers from two different colonies were observed throughout their lifetime foraging activity in term of number of foraging trips performed. Because we aimed to study only foraging behaviour, we excluded the first five trips of each bee, corresponding to an orientation phase ([41], see also chapter 2). We generated a dataset of 10 546 foraging trips (4,944 trips for the colony 1 and 5,602 for colony 2) by 565 individuals (N = 296 from colony 1 and N = 270 from colony 2).

From this dataset we examined the proportion of foraging trips performed by each bee relative to all the foraging trips of their colony The Lorenz curve [42] (Figure 1*a*), which represents the proportion of the total activity of the hive performed by a certain proportion of individuals, 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). Similar to Tenczar *et al* [2], we called elite bees the group of bees that cumulatively accounted for 50% of the total foraging trips performed by the colony.

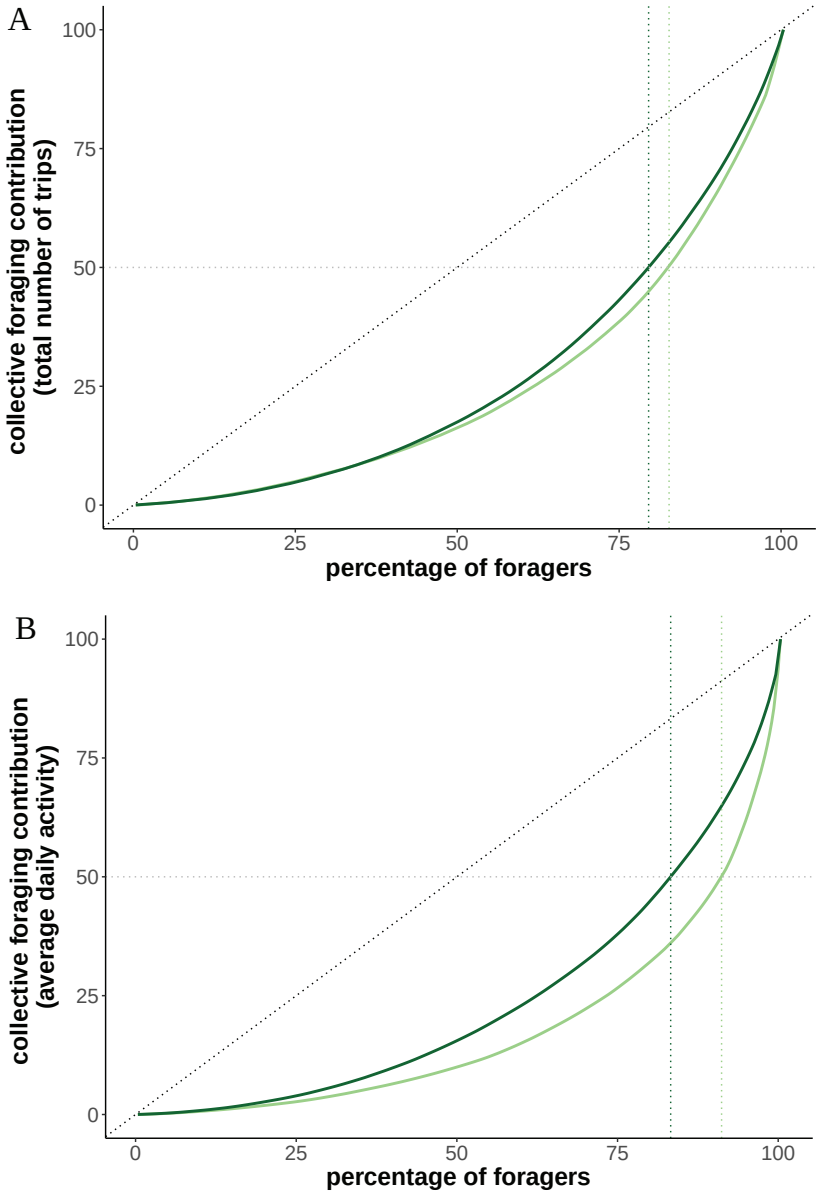

Figure 1: Lorenz curves of relative individual contributions to the colony foraging activity. Such curves present, for each colony, the proportion of foragers that contribute to a certain amount of the total number of trips (**A**) or to the relative activity (number of trips) of the bee per day compared to the total number of trips performed by the colony that day (**B)** [2]. For each colony (light green: colony 1, dark green: colony 2), bees were ranked by the number of trips (**A**) or relative daily activity (**B**) they performed in ascending order, and the fraction of each bee's contribution to the total foraging activity 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.

We then examined the skew of foraging activity between all the foragers by computing a Gini coefficient [43]. 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 1B). 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. This is comparable with the total number of foraging trips performed by an individual bee (which is of around 19%).

In the following analyses, we focused on the individual contributions to the total number of foraging trips performed by the colony. Similar to Tenczar *et al* [2], we considered as elite bees the group of most active bees that cumulatively accounted for 50% of the total foraging trips performed by the colony.

Activity of the foragers:

To assess bee activity we considered the number of foraging days, number of trips per day and total number of trips during their foraging lifetime. These three metrics were correlated and the more active bees (the elite bees) can be placed in a continuum between two behavioural patters: highly frequent daily trips performed over a few days, at one end, and few daily trips performed over a longer period at the other end of the continuum (electronic supplementary material figure S2). On average, bees performed a total of 19 trips (mean ± SE, colony 1: 17 ± 1 trips, N = 291; colony 2: 21 ± 1 trips, N = 263) and foraged for less than a week (colony 1: 4.20 ± 0.18 days, N = 291; colony 2: 4.85 ± 0.18 days, N = 263).

Differences in age at foraging onset and weight between elite and non elite bees:

Considering the age of first foraging trip (figure 2A) elite bees from colony 1 started foraging earlier than non-elite bee (mean ± SE, elite = 20.21 ± 0.71 days; non-elite = 25.1 ± 0.63 days; Wilcoxon rank test: W = 8,368, P = 0.0005). This effect was not significant in colony 2 (mean ± SE, elite = 18.43 ± 0.56 days; non-elite 18.92 ± 0.30 days; Wilcoxon rank test: W = 6,717, P = 0.194).

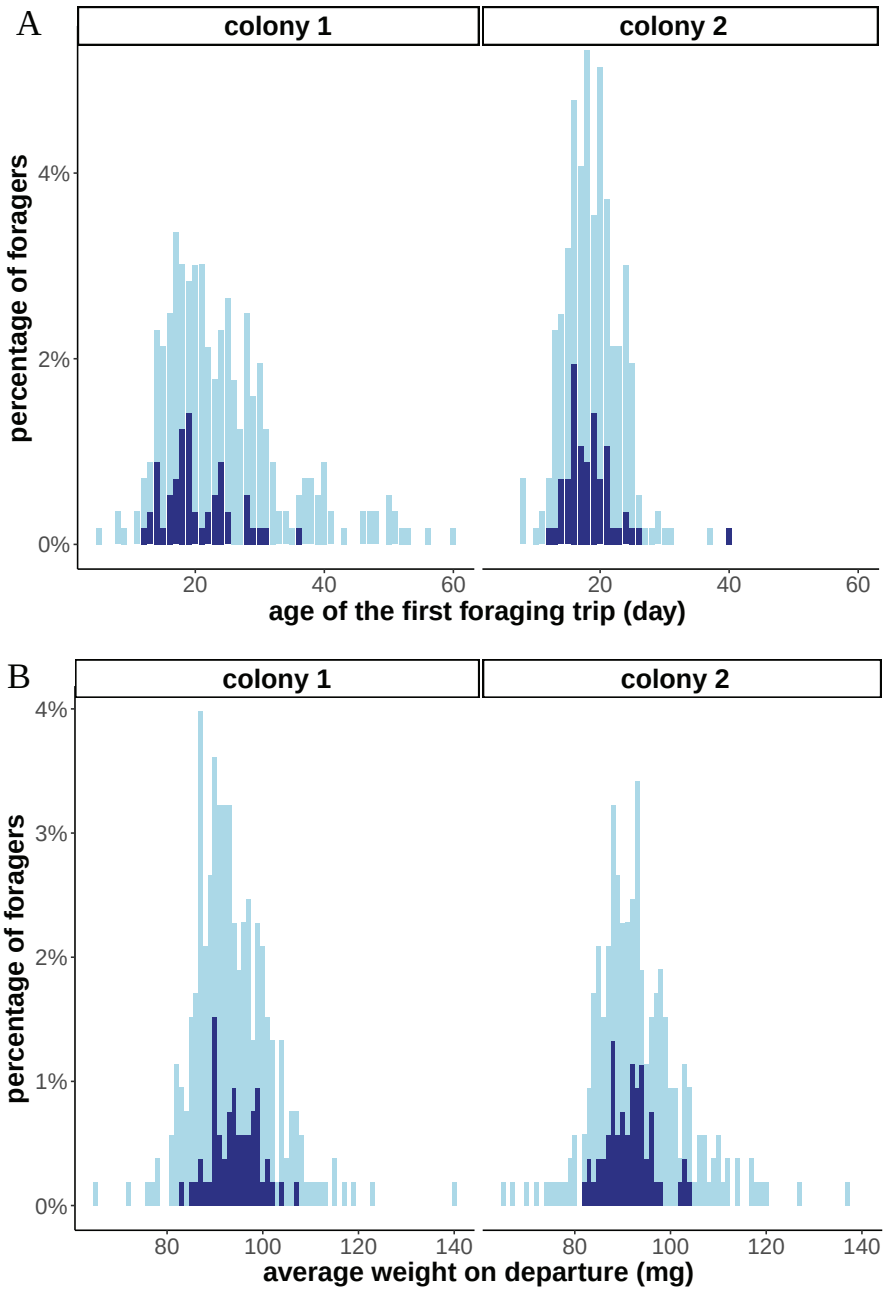

Figure 2: A. Proportion of bees of a given colony that started foraging at a given age (in days since emergence). Left: colony 1, right: colony 2. Dark blue represents the elite bees and light blue represents the rest of the foragers. Elite bees of colony 1 started to forage earlier than the other bees. 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 (GLMM, N = 1,870, $F_{(1,413)}$ = 0.74, P = 0.39, Table 1 for details and Supplementary material Table S1*a* for model selection).

Table 1:

Weight on departure difference for elite and non-elite and pollen and non-pollen trips for each of the two colonies. Models were selected using the AIC method (supplementary material table S1*a*). Elite|ID indicates that bee identity has been used as random factor. Significant values are in bold.

	Estimate (SE)	**DF**	**t**	**P**
*weight on departure ~ elite * colony * pollen + (elite |ID)*				
Non elite colony 1 non pollen	**93.30 (0.17)**	**1449**	**131.41**	**<.0001**
Elite colony 1 non pollen	93.74 (1.09)	413	0.41	0.68
Non elite colony 1 pollen	**96.30 (1.44)**	**1449**	**2.09**	**0.037**
Elite colony 1 pollen	92.40 (1.74)	1449	-0.41	0.683
Non elite colony 2 non pollen	92. (0.08)	147.1	10.91	<.0001
Elite colony 2 non pollen	89.07 (1.87)	413	-1.78	0.076
Non elite colony 2 pollen	87.01 (3.83)	1449	-1.65	0.100
Elite colony 2 pollen	93.67 (4.71)	1449	0.08	0.936

In terms of average weight on departure, there was no difference between the elite bees and the non-elite bees (GLMM, N = 1,870, $F_{(1, 413)} = 0.74$, P = 0.390, more details in table 1). There was no difference between average individual weight on departure for pollen or for non-pollen resource collection for elite or non-elite bees (GLMM interaction between elite and resource, $F_{(1, 1449)} = 0.08$, P = 0.777, more details in table 1).

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 3, 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). For non-pollen trips, the more active the bees were, the greater their average weight difference (between weight on return and

weight on departure for a given trip) (figure 4A). This resulted in a larger amount of nectar collected by the more active bees (figure 4B). Therefore, the more active bees tended to collect more of both resources (pollen and non-pollen, i.e. nectar or water) through their lifespan.

Foraging efficiency increases with experience:

Across the foragers active lifetime their foraging activity increased with bees progressively performed more trips per day (figure 5A, N = 2,539, DF = 1,974, F = 8.66, P = 0.003), and they progressively started foraging earlier (electronic supplementary material figure S3B). Bees also reduced their trip duration for non-pollen trips and increased the average trip duration for pollen trips (electronic supplementary material S3A).

Bees increased their efficiency trip after trip: there is an increase in weight difference for non-pollen trip with experience for both elite and non-elite foragers (figure 5B, GLMM, N = 925, DF = 647, F = 6.05, P = 0.014).

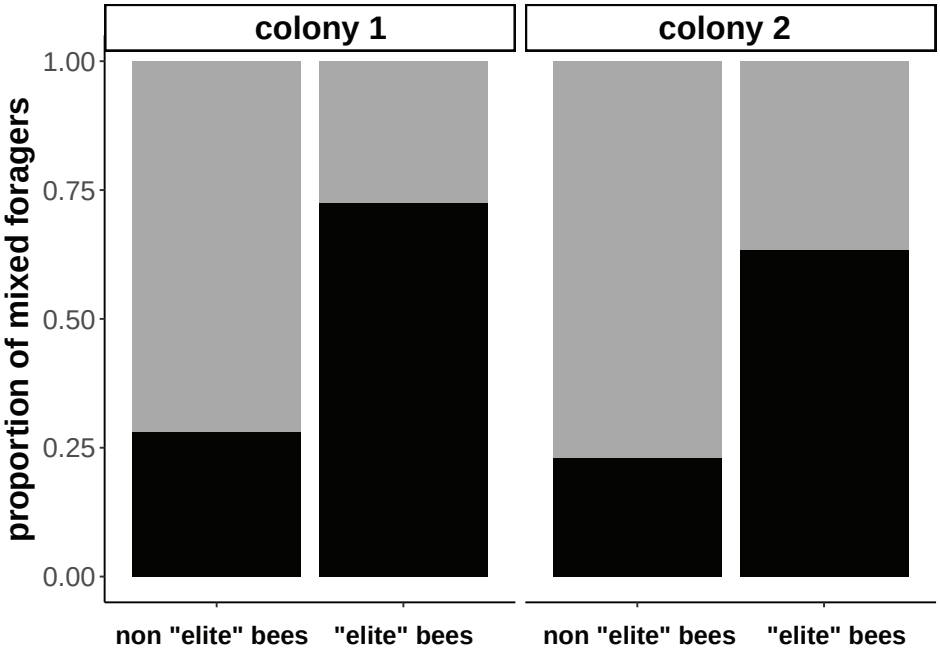

Figure 3: Relative proportions of mixed foragers among elite bees and other foragers. While non-pollen foragers (in grey) represent the majority of non-elite bees, elite bees are mostly mixed foragers (i.e. those that performed at least one trip collecting pollen, in black). (Left: colony 1: χ^2 = 55.68, DF = 1, P < .0001; right: colony 2: χ^2 = 46.99, DF = 1, P < .0001).

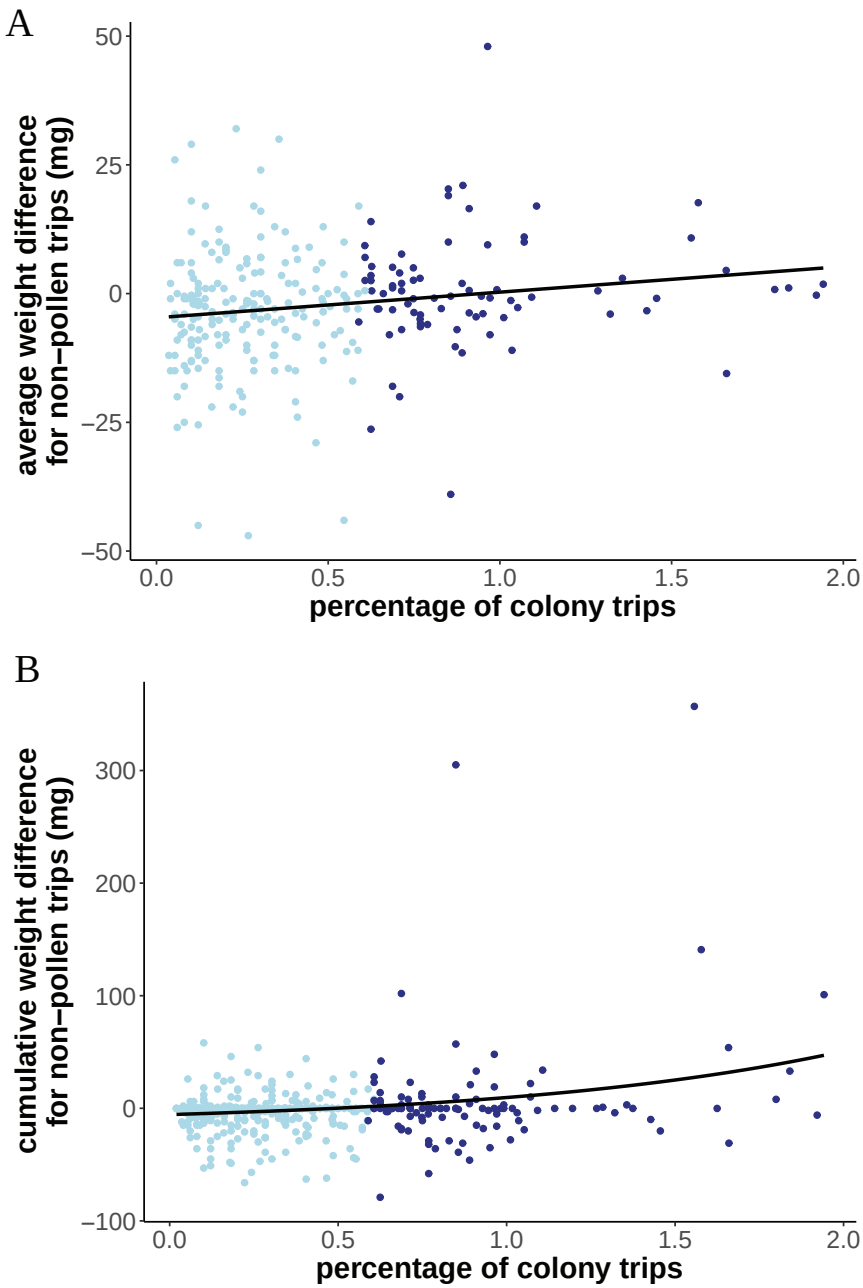

Figure 4: Comparative foraging efficiency of elite (*dark blue*) and other foraging bees (*light blue*). **A.** Average weight difference for trips identified as non-pollen trips for each individuals for both colonies according to their contribution to the total number of trips for their colony (N = 527 individuals, colony 1: 281, colony 2: 246). The most active bees are also, on average, the most efficient ones at nectar collecting. General linear model with individual as random effect: intercept: -4.62 ± 1.06, DF = 275, T = -4.36, P < 0.0001; slope: 4.85 ± 1.79, DF = 275, T = 2.70, P = 0.007. Model selection in electronic supplementary material table S1*b*. **B.** Accumulated weight difference for each of the bees. The most active bees accumulated larger amounts of food. General quadratic model with individual as random effect: $y \sim ax^2+bx+c$: c = 0.35 ± 1.70, DF = 561, T = 0.20, P = 0.840; a = 28.14 ± 5.52, DF = 561, T = 5.10, P < .0001; b = -21.82 ± 7.52, DF = 561, T = -2.90, P = 0.004. Model selection in electronic supplementary material table S1*c*.

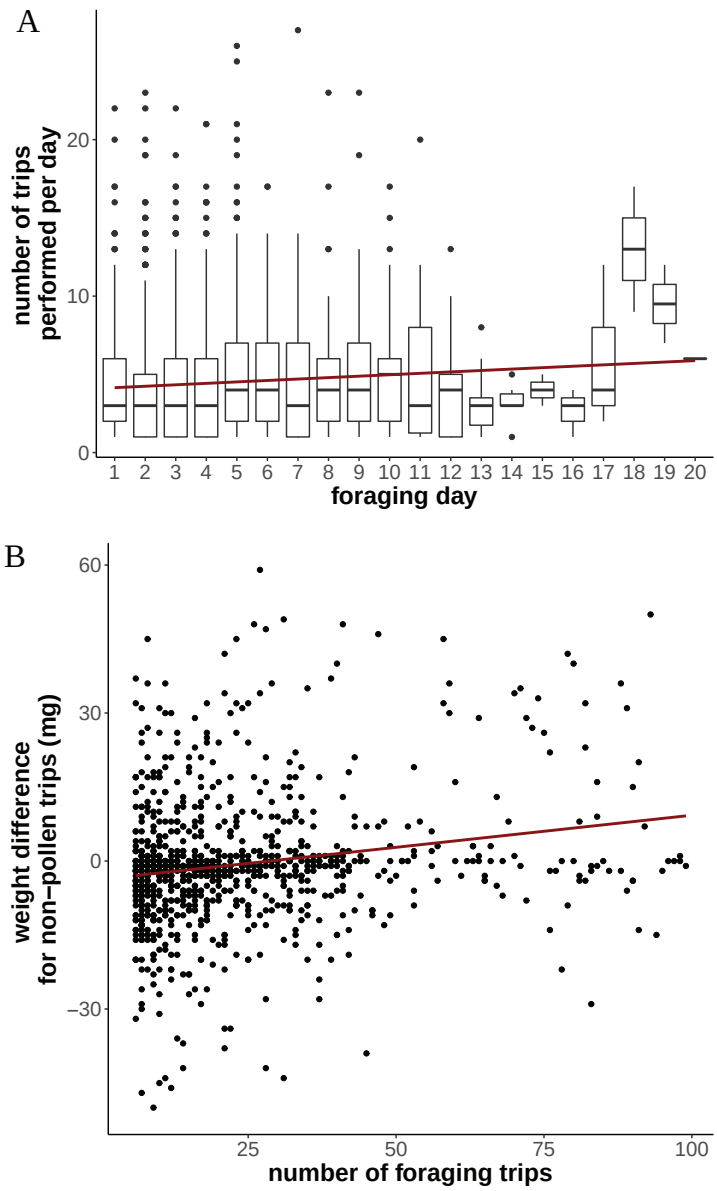

Figure 5: Analysis of the influence of foraging experience on foraging activity and efficiency. A. Changes in foraging frequency with experience: relation between the number of trips performed per day and the consecutive foraging days. Boxplot: the line shows the median, boxes and the whiskers represent 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 with individual as random effect: intercept = 4.03 ± 0.14, DF = 1946, T = 28.22, P < 0.0001; slope = 0.11±0.03, DF = 1946, T = 3.02, P = 0.0025. Model selection in electronic supplementary material table S1*d*. **B.** Foraging efficiency per trip according to the number of trip 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 with individual as random effect: intercept = -3.76 ± 0.93 DF = 648, T = -4.06, P = 0.0001; slope = 0.11 ± 0.04, DF = 648, T = 2.49, P = 0.0131. Model selection in electronic supplementary material table S1*c*.

Discussion:

We present an analysis of the foraging performance of elite individuals in honey bees colonies. Our results confirm that colony nutrient provision is largely driven by a subset of very active foragers (elite bees) (figure 1). These individuals are not only the most active, but they are also the most efficient at collecting pollen and other resources (figure 3 and 4). The link between activity and performance is explained by the fact that foragers increase their foraging efficiency with experience (figure 5).

In our study 19% of the foragers completed more than 50% of the total number of foraging trips in their colonies. This is comparable to the skew in foraging effort reported by Tenczar et al [2] in the same species, even if they used a different metric to us in order to overcome a limit in the accuracy of their RFID sensor detection. These authors compared individual activity with total colony activity on a day-by-day basis. Applying this particular metric to our data, we found an even greater skew in the foraging distribution: 12% of the workers performed more than 50% of the colony's daily activity (figure 1B) whereas Tenczar study, they found a skew of 20% [2]. Tenczar *et al.* [2] tagged between 100 and 500 bees with RFID chips as a single group, in small colonies of 1000-1500 bees, whereas we repeated the tagging process on a fortnight basis over the time of the experiment, and thus had more tagged bees. The differences we find between the skews of the distribution for the two colonies, for the same metric, could be explained by difference in the abundance of tagged bees in the colonies during the experiments.

Our results indicate that there is no clear separation between a group of elite bees and the remaining foragers, but rather that they correspond to the tail of a continuous distribution of levels of foraging activity, from poorly active foragers to very active ones. A gradual continuum of activity within foragers is a common feature of collective foraging activity patterns in social insects [2,13,19]. Our data supports the interpretation that there is no distinct sub-caste of super active foragers. This is confirmed by the absence of clear physiological (weight, onset of foraging) differences between elite and non-elite bees in our population. Previous studies that observed a skew in the foraging activity of social insects either failed to identify any particular link with higher foraging efficiency in the active individuals [2] or reported that the most active foragers where also the most efficient [19,20] but did not analyse the link between activity and efficiency. In our case, the more active foragers were

also the more efficient as they collected more nectar and were also more likely than others to be mixed (pollen and nectar) foragers. This can be explained by the fact that bees increase their efficiency with experience [44–47]. We demonstrated in Ch. 2 that experienced foragers are more prone to collect pollen. It is also true that bees increase their nectar intake with experience (figure 5B). Thus, bees that are the most active may also end up being the most efficient simply because their performance improves with experience that is gained faster with higher activity.

Individuals can improve their foraging efficiency with learning and memory [48]. For instance chimpanzees [49] or cockroaches [50] increase their foraging efficiency with learning about the food sources. Bees are efficient and well documented learners [51]. They learn the features of their environment and accordingly enhance their foraging skills [46]. With experience, bees improve their ability to navigate to and from their food sources [12,41,52,53], and their ability to discriminate for flower quality (based on colours of odours or shape discrimination [51]) and efficiency in handling flowers [54,55]. Moreover, individual foraging experience has been reported to influence foraging strategies used by the individual. For example, ants (*Cerapachys biroi*) that are successful at foraging during their first attempts are more likely to continue foraging later in life [56]. Bumblebee (*Bombus impatiens*) workers that start off by foraging for pollen collect more pollen during their lifetime than the ones that started collecting nectar [57]. Thus the early experience, during initial foraging trips, influences the rest of an individual's foraging life.

The survival of the whole colony depends on the quality of the foraging force. For more than a decade now, there have been concerns over increased rates of honey bee colony failure [58], due to diverse environmental stressors that impair the lifespan and the cognition of the foragers [59]. Knowing that there is a skew in the individual contribution to the collective foraging effort, we can expect the most active bees to be more frequently exposed to environmental stressors. Therefor, stressors that shorten the foragers' lifespan [35,60–70] will also be disproportionately impact the most active elite bees. Elite bees cannot be rapidly replaced, because of the foraging experience needed to develop higher foraging efficiency. Chronic stressors that reduce forager lifespan might doubly impact colony foraging performance by eliminating the keystone foragers.

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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Supplementary material:

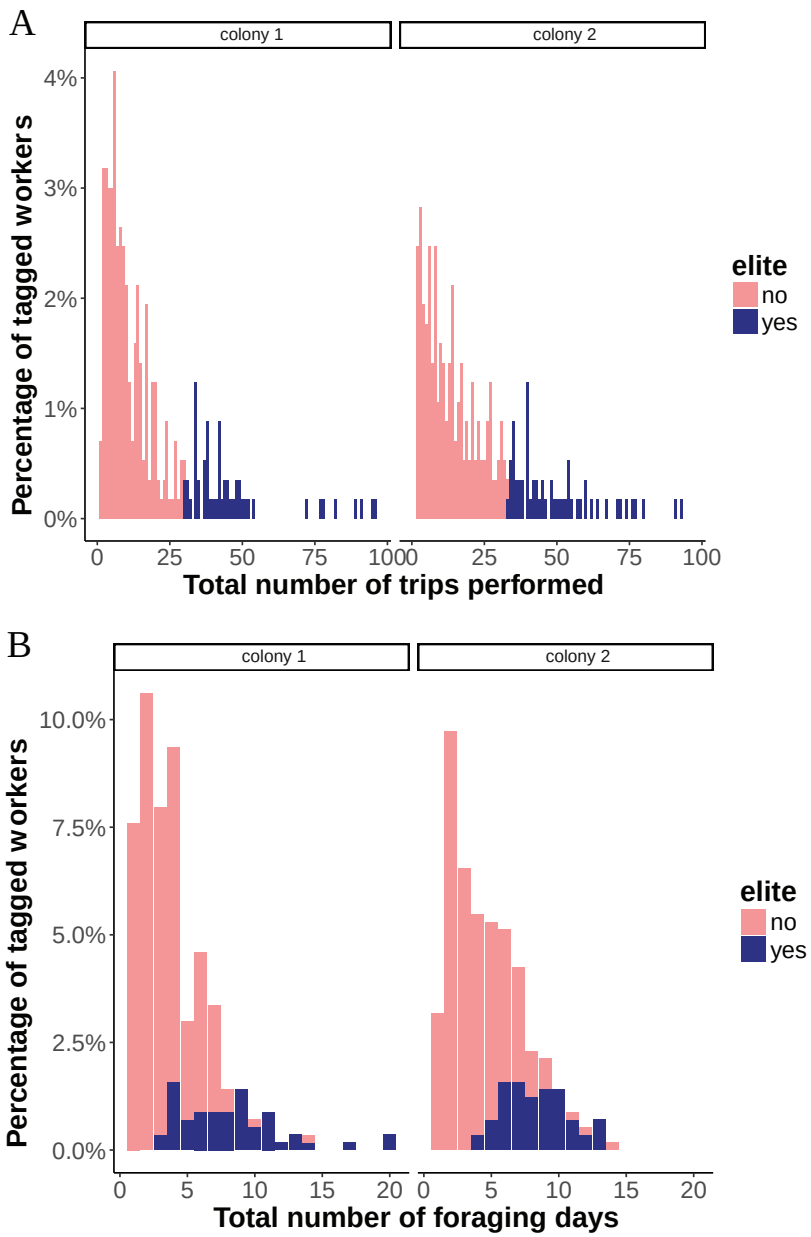

Figure S1: A. Distribution of lifespans of the bees (in number of trip performed). **B.** Distribution of the number of trips they have done. Colony 1 and two are plotted in each panel separately. Median foraging lifespan: 6days, median = 6.5 days. Details of the number of days foraged based on the age of the bees: bees started their first foraging flight in average at around 22 days (mean ± SE = 21.7 ± 0.33 days; colony 1: mean ± SE = 24.32 ± 0.54 days; colony 2: mean ± SE = 18.81 ± 0.27 days). Bees started to go out for the first time (for orientation flights) at around 15 days old (mean ± SE = 14.67 ± 0.22 days; colony 1: mean ± SE = 15.9 ± 0.33 days; colony 2: mean ± SE = 13.31 ± 0.26 days). Thus, bees spent on average 7 days between their first orientation flight and their first foraging flight (mean ± SE = 7.03 ± 0.30 days; colony 1: mean ± SE = 8.41 ± 0.51 days; colony 2: mean ± SE = 5.50 ± 0.23 days).

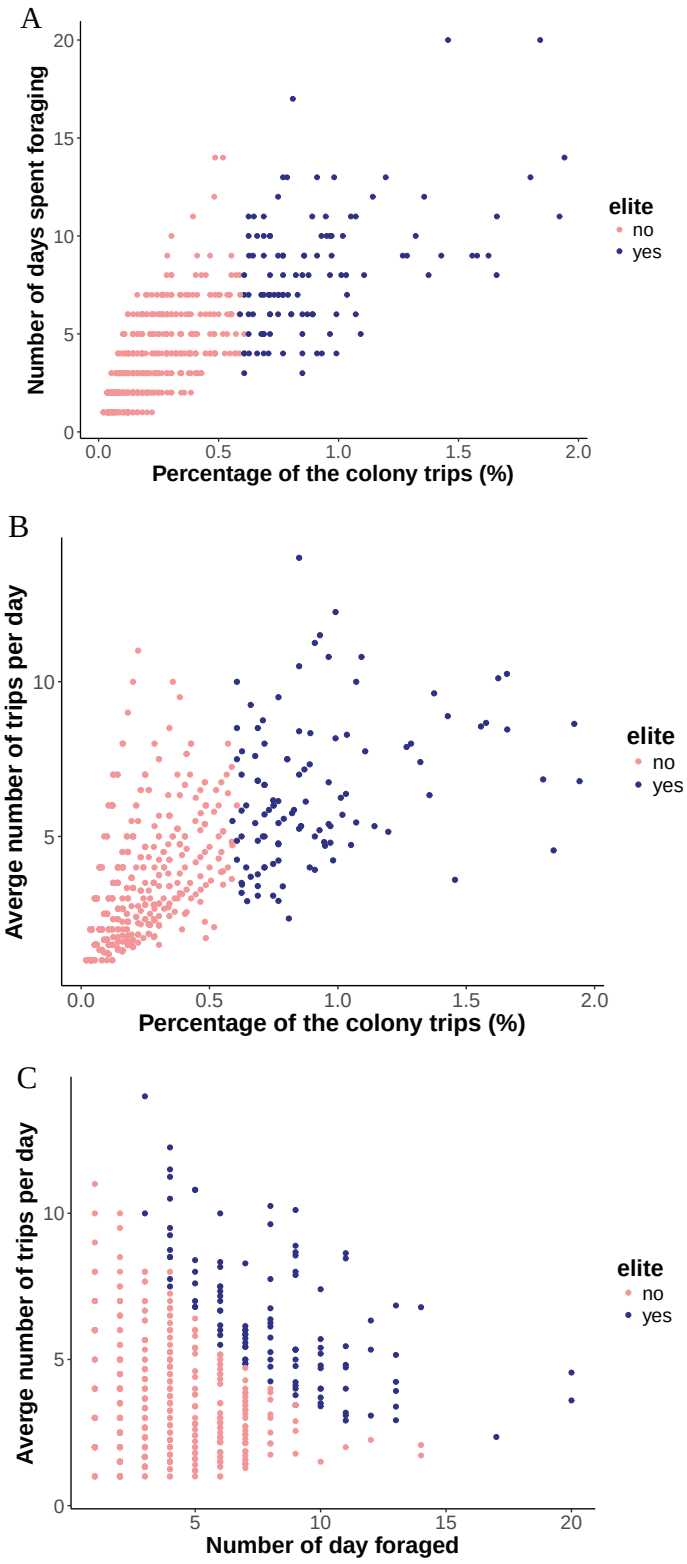

Figure S2: Positive correlation between the different components of activity: proportion of the total trips of a colony performed by a bee; total number of days spent foraging; number of trips performed per day. Pink dots are non-elite bees and blue dots are elite bees.

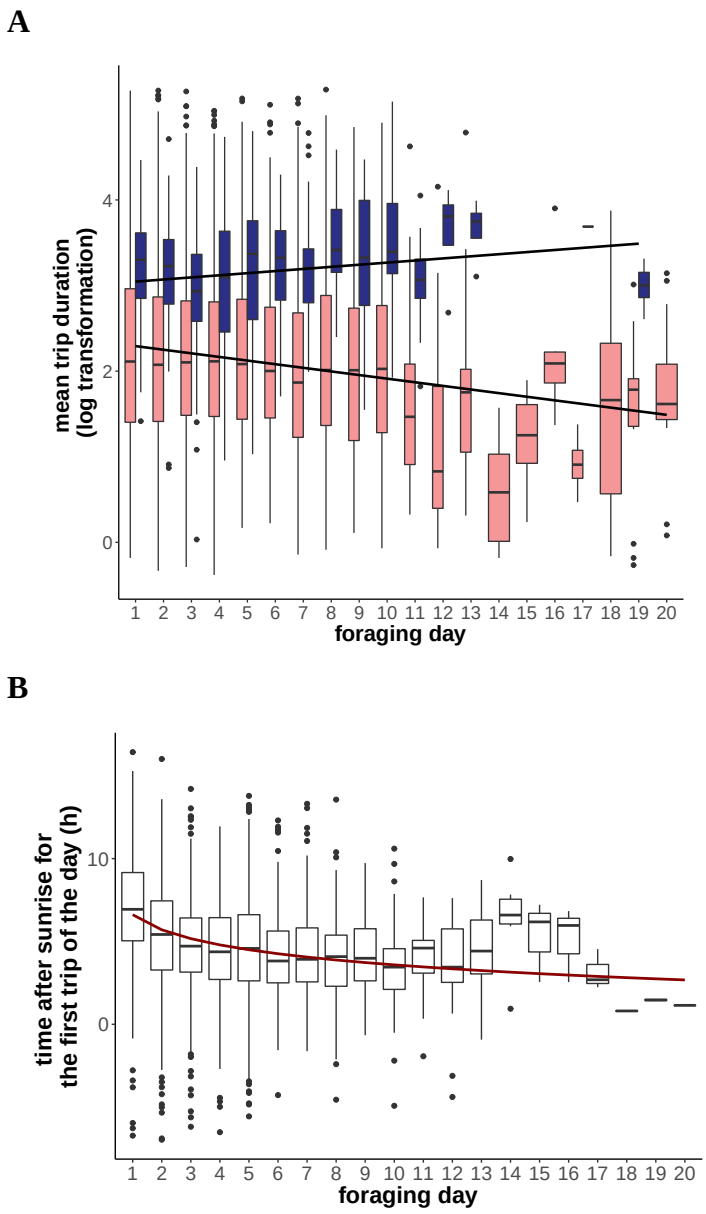

Figure S3: A. Average trip duration on successive foraging days. Pink is for non-pollen resources and blue for pollen collection trips. The duration of non-pollen trips decreases whereas pollen trip duration increases. (n = 554 bees). Linear mixed model with bee identity as random effect and interaction with day identity (proxy for environmental daily conditions): non-pollen trips: intercept: 2.27 ± 0.05, DF = 3122, T = 42.69, P < 0.0001; slope = -0.03 ± 0.01, DF = 3122, T = -3.24, P = 0.0012; pollen trips: intercept: 2.89 ± 0.14, DF = 3122, T = 6.61, P < 0.0001; slope = 0.041 ± 0.02, DF = 3122, T = 4.51, P < 0.0001; day identity intercept: 2.27 ± 0.05, DF = 3122, T = 3.01, P = 0.0026. **B.** Time of the first foraging trip of the foraging day (n = 554 bees). Bees started foraging earlier each day after the start of the experiment. Logarithmic mixed model, with bee identity as random factor and interaction with day identity (proxy for environmental daily conditions): intercept: 5.26 ± 0.16, DF = 1973, T = 33.26, P <0.0001; slope: -1.70 ± 0.09, DF = 1973, T = -18.52, P <0.0001; intercept of day identity: 5.29 ± 0.16, DF = 1973, T = 12.88, P <0.0001.

Table S1:

Selection of the best linear model representing the average weight difference for trips identified as non-pollen trips for each individual for both colonies according to their contribution to the total number of trips for their colony Best model is highlighted in bold.

	DF	**AIC**	**Loglik**	**Chi^2**	**P**
a. *weight according to elite bees*. Figure 2B					
weight ~ elite	3	14498.3	-7246.1		
weight ~ elite + elite|ID	6	14202.0	-7094.9	302.3	<.0001
weight ~ elite * colony + elite|ID	8	14195.2	-7089.6	313.1	<.0001
weight ~ elite * resource * colony + elite|ID	**12**	**14178.2**	**-7077.1**	**24.9**	**<.0001**
b. *average weight difference for non pollen trips according to total activity*. Figure 4A					
average weight difference ~ number of trips	3	2139.3	-1066.6		
average weight difference ~ number of trips + (number of trips)|ID	**4**	**2069.5**	**-1030.7**	**71.7**	**<.0001**
c. *total weight difference for non pollen trips according to total activity*. Figure 4B					
cumulative weight difference ~ number of trips	3	5286.2			
cumulative weight difference ~ number of trips + (number of trips)|ID	4	5142.9	-2567.4	113.5	<.0001
cumulative weight difference ~ (number of trips)^2 + number of trips + (number of trips)|ID	**5**	**5116.4**	**-2553.2**	**28.5**	**<.0001**
cumulative weight difference ~ exp(number of trips) + (number of trips)|ID	4	5174.6	-2583.3	60.2	<.0001

Following table S1.

d. *weigh difference for non pollen trip according to experience.* Figure 5A					
weight difference ~ trip number	3	7436.4	-3715.2		
weight difference ~ trip number + (trip number)|ID	**6**	**7400.9**	**-3694.2**	**38.4**	**<.0001**
weight difference ~ log(trip number) + log(trip number)|ID	6	7403.9	-3695.9	-	-
e. *number of trips performed per day according to experience.* Figure 5B					
trips per day ~ foraging day	3	13816.2	-6896.3		
trips per day ~ foraging day + (foraging day)|ID	**6**	**13548.2**	**-6768.1**	**256.5**	**<.0001**
f. *duration of the trip according to experience.* Figure S3A					
trips per day ~ foraging day	3	13816.2	-6896.3		
trips per day ~ foraging day + (foraging day)|ID	**6**	**13548.2**	**-6768.1**	**256.5**	**<.0001**
g. *time of the first foraging trip of the day accodring to experience.* Figure S3B					
Time of first trip ~ foraging day	3	12979.1	-6486.5		
Time of first trip ~ foraging day + (foraging day)|ID	6	12686.6	-6337.3	298.5	<.0001
Time of first trip ~ foraging day + day identity + (foraging day)|ID	7	12545.8	-6265.9	142.8	<.0001
Time of first trip ~ log(foraging day) + day identity + (log(foraging day))|ID	**7**	**12482.8**	**-6230.4**		
Time of first trip ~ $(\text{foraging day})^2$ + foraging day + day identity + $((\text{foraging day})^2$ + foraging day)|ID	11	12482.8	-6230.4	28.2	<.0001

Chapter 4: Inter-individual variability in the lifelong foraging activity of bumblebees

Chapter 4: Inter-individual variability in the lifelong foraging activity of bumblebees

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Abstract:

For bumblebee colonies, nutrition is provided by the foragers that collect nectar and pollen from flowers. Bumblebee foragers vary in size, with some individuals being up to three times larger than others from the same colony. Body size is an important factor influencing foraging behaviour, and we know that bumblebees exhibit strong inter-individual differences in various aspects of foraging behaviour. Whether all individuals contribute in the same way to the colony's foraging force has not yet been examined. Neither do we have a clear idea of the relationship between foraging activity and efficiency in bumblebees. Here we studied the inter-individual variability in the foraging strategies and performance of bumblebees using sensors to automatically monitor foraging behaviour. We found that the majority of foraging activity was undertaken by a small group of bumblebees (elite foragers) that did not differ morphologically from the remainder of the foragers. Unlike what has been reported for honey bees, elite forager bumblebees did not forage more for pollen. For bumble bees that performed both pollen and non-pollen collecting trips we found that they adapted their behaviour depending on the kind of resources they collected. Our work highlights the degree of inter-individual variability and behavioural plasticity in the collective foraging behaviour of social bees.

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 insects, inter-individual variability results in a division of labour where different individuals perform different tasks for their whole life, or a period of it [6–8]. Different groups are often called different behavioural castes. In bumblebees, for instance, the foragers (a minority of the whole hive population) must collect carbohydrate (nectar), protein, and lipids (pollen) from flowers to supply the nutritional demands of the entire colony [9–11]. These resources are temporally and spatially, patchily distributed in the environment [12] and the bumblebee colony nutritional needs can vary greatly [13]. It is presently unclear how the foraging force of a bumblebee colony collectively addresses such challenges.

Buffed-tailed bumblebee (*Bombus terrestris*) colonies are composed of a reproductive queen, up to three hundred non-reproductive worker females, and up to twenty reproductive males, with new queens in the population by the end of the summer [9]. Only a handful of the workers (typically less than 10) engage in foraging tasks each day. Non-forager workers (nurses) stay inside the colony to take care of the larvae and nest maintenance [7,9]. The distinction between nurses and foragers is based on body size: only the largest individuals tend to forage [7,9,14].

Bumblebee foragers show some degree of inter-individual behavioural variability. For instance, several studies report the existence of exclusive nectar foragers and mixed foragers that collect both pollen and nectar [14–18]. Variability in different aspects of foraging behaviour have been quantified: individuals show consistent differences in their navigation skills [19] and consistent differences in the degree of success to an olfactory learning test [20]. Inter-individual variability has also been reported for foraging activity. For example, a large proportion of the foragers show little activity or are completely inactive [21,22].

In other social insect species, we observe a skewed distribution of the collective foraging effort: some individuals perform disproportionately more of the total foraging task than others (e.g. bees: [23] and chapter 3; ants: [24]). For honey bees, the subset of very active foragers has been described as a group of “keystone” or “elite” individuals that make a disproportional contribution to the colony foraging effort [25]. Elite individuals are not only the most active [23] but also the most efficient foragers (chapter 3).

Whether *B. terrestris* workers show individual differences in foraging activity and efficiency, and thus contribute differently to the colony nutritional supply is not well known. Moreover, whether inter-individual variability in foraging behaviour relates to the substantial morphological differences between *B. terrestris* foragers is not clear.

To address these questions, 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 in natural conditions. Over recent years, RFID systems have been used to access the foraging activity of honey bees [23,26] and bumblebees [17,27–29]. This approach has often been used either to record bee foraging behaviour in laboratory conditions [17,30] or to test the effects of environmental stressors, such as pesticides, on colony dynamics [27–29,31,32]. Here we used this technology to analyse the complete foraging history of all individuals in a bumblebee colony exploiting natural resources.

In our study, we investigated: (1) variation in foraging activity between individuals; (2) whether foragers segregate according to collection of pollen and other resources, and; (3) whether pollen and non-pollen collection trips differ in duration or time performed.

Material and methods:

Bee colony:

The experiment was performed from 20/09/2016 to 22/11/2016 at the experimental apiary of the University Paul Sabatier (Toulouse, France). Two bumblebee colonies (*Bombus terrestris*) were purchased from Biobest (Westerlo, Belgium). Upon arrival, the queen and the workers (N = 50 bees) were transferred to a two-chamber wooden nest box. The original brood and honey pots were transferred to the first chamber of the nest box. The second chamber was filled with cat litter to reduce moisture and odour build-up. 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 (figure 1).

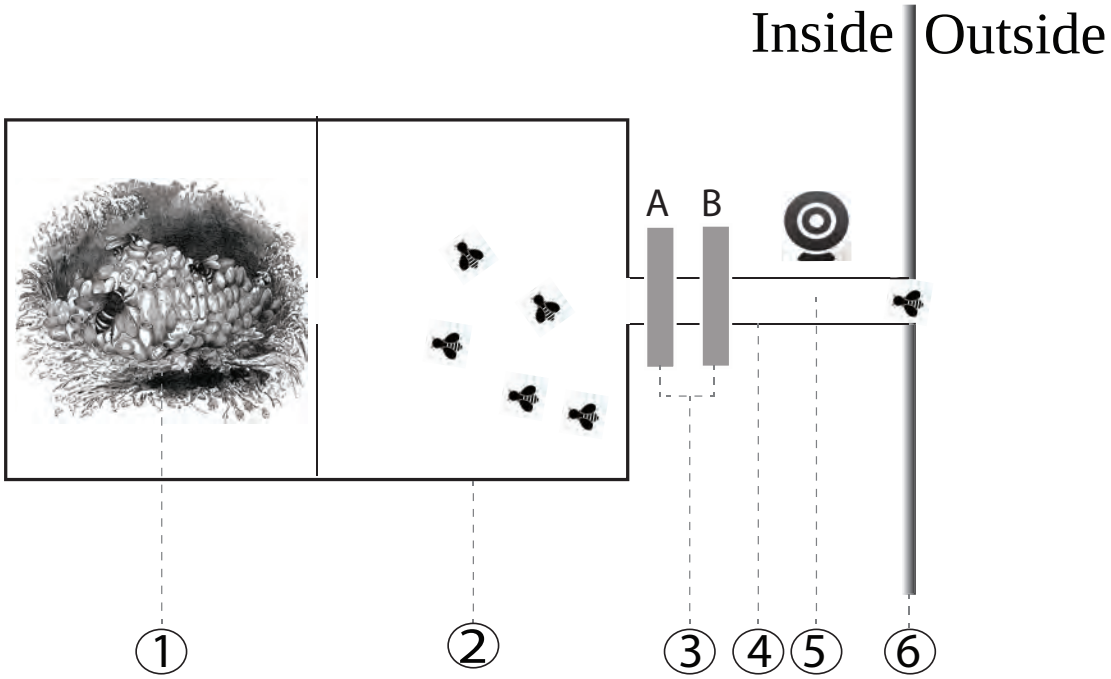

Figure 1: Experimental nest box. 1&2. Two-chambers wooden nest-box (15x28x11 cm). 1. Bumblebee nest. 2. Litter compartment. 3. RFID emitter/receptor gates. The two gates A and B indicated the direction of the bee in the colony entrance tube: AB means that a bee departed from the colony, BA means that a bee arrived at the colony. 4. Transparent plexiglass tunnel (⌀ 3 cm). 5. Webcam. 6. Wall.

Radio Frequency Identification (RFID) detection:

Bees could access the outside environment via a transparent entrance tube equipped with two RFID emitter/recorder gates (MAJA system, Microsensys GmbH, Erfurt, Germany) connected to a RFID reader. RFID chips were obtained from Microsensys (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 emitter/recorder on exiting and entering the hive. Each RFID emitter/recorder recorded the time and the ID of a bee crossing the gate. Data were stored in a .xml file on a SD card in the RFID reader.

A webcam (Logitech, Lausanne, Swiss) was placed on a retort stand (30cm tall) midway in the transparent tunnel, and a desk lamp (lamp from Ikea, France, light bulb, 15 W, 1250 lm, Ilight, Italy) was placed beside the tube to guarantee enough light for video caption. Motion detection video recording software (ZoneTriger, Omega Unfold Inc. Canada) was used to capture video footage 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). During this time, the colony was checked every day. The bees transferred on colony arrival (section *bee colony*) and 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, supplementary material Figure S1). We then fixed a RFID tag on the thorax of the bee using a toothpick and super-glue (Loctite, Henkel AG&Co. KGaA, Düsseldorf, Germany).

A second colony was set up with the same experimental design in an adjacent room. RFID data from the second colony are not included in the present manuscript, but some bees drifted from the second colony to the focal colony during the experiment. Individuals were thus classified as coming from colony 1 or colony 2. Bees foraged on the university campus and its surrounding flowering areas.

Collating data on bee trips:

For every arrival and departure at the colony entrance, the bees had to pass through two emitter/received RFID gates (figure 1). Gates were labelled A and B to allow us to reconstruct foraging trips from the RFID record file. A sequence AB was interpreted as a bee leaving the colony, whereas a sequence BA was interpreted as a bee returning to the colony. Thus 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 [17]. We removed them 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), no 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 (see example in Electronic Supplementary Material Videos S1). We were not able to discriminate if a bee, returning to the hive with no

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 in colony 1. When looking at the foragers only (bees that made at least one foraging trip), we excluded from the dataset bees that had only 'NA' as load type over all their trips (N= 124 bees). The final dataset contained 13 243 trips by 99 bees (92 bees from colony 1, and 7 bees from colony 2 that drifted to colony 1).

The date of emergence from the pupa of each bee was recorded in order to keep track of their age (except the 50 bees that were originally in the colony). The picture of each bee was analysed with imageJ (Mac OS X version, Wayne Rasband, Maryland, USA) and metrics of: total body length (from the head to the end of the abdomen); thorax width; inter-wing space (length of the space between the two wing bases on the thorax); and the length of the flagellum of the antenna (example showed in supplementary material Figure S1). For each of the body size metrics we used an average of three measurements.

Data analyses:

Data were analysed in R version 3.2.3 [33] (operating via Rstudio, version 1.0.136 [34]) using the packages lme4 [35] and lmerTest [36]. Differences in body size between groups of bees (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 certain proportion of individuals, in term of number of trips, was examined using a Lorenz curve [37]. The Gini coefficient [38] 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 the majority of the task). The difference in trip duration and in time of the day when trip 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 identity as random factor.

Results:

Inter-individual variability in foraging activity:

A total of 335 bees were tagged during the 63 days of the experiment. Among these bees, 99 were seen foraging at least once (30% of the tagged bees). These foragers did not differ in size from the non-foragers (table 1A).

We found strong variation in the activity of foragers. On average bees foraged for 10 days, and performed a total of 134 trips (9.95 ± 0.96 (SE) foraging days, for 134 ± 19.32 foraging trips, *i.e.* 8.97 ± 0.73 trips per day, N=99 bees). This activity was not equally distributed among foragers. A subset of 11.11% of the bees (hereafter called 'elite' bees) completed more than 50% of the total number of the foraging trips in the colony (figure 2*a* and supplementary material figure 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 (figure 2*b*, Gini index = 0.93).

We found no difference in the proportion of pollen trips (as a proportion of the total of foraging trips) by elite and non-elite foragers (elite: N = 12, proportion of pollen trips 34.24 ± 5.86 (SE) %; non-elite: N = 87, proportion of pollen trips = 24.81 ± 3.07 %, Wilcoxon rank test: W = 374, P = 0.105).

We found no difference in size between the elite and the non-elite foragers (table 1B and supplementary figure S3). 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.

Specialisation for pollen and/or non-pollen trips:

Two thirds of the foragers completed both pollen and non-pollen collection 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 (supplementary material figure S4). Mixed foragers were smaller than non-pollen foragers when considering thorax lengths and inter-wing base length, but not when considering the total body length or the antenna length (table 1C). Therefore, the specialisation of foragers for

one type of resource was incomplete and partially explained by morphological differences.

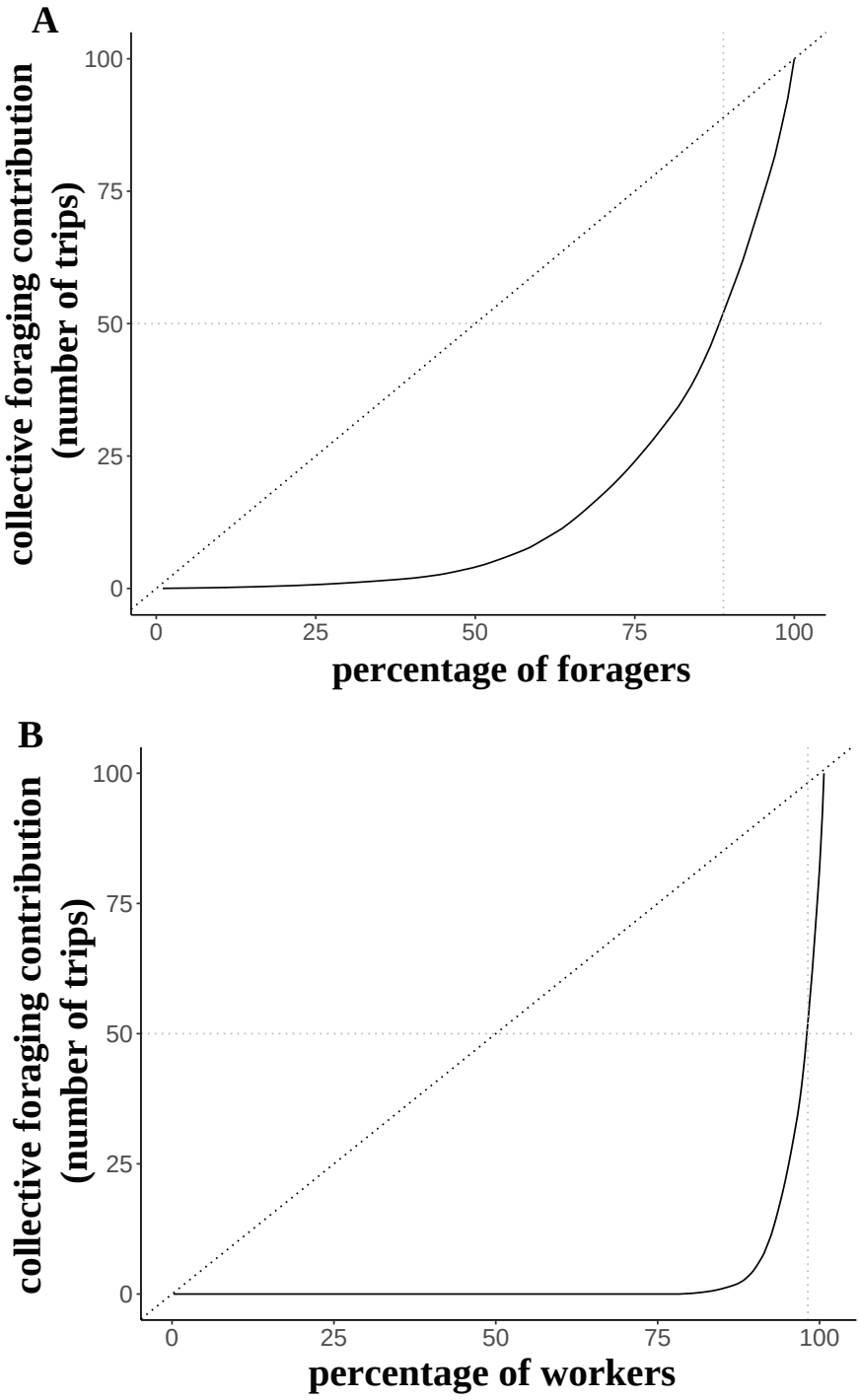

Figure 2: A. Lorenz curve representing the relative contribution of the foragers (X axis) to the collective effort of all foragers (Y axis). 11.11% of the foragers performed up to 50% of the total number of trips of the colony. **B.** Lorenz curve representing the relative contribution of the foragers (X axis) to the collective foraging effort of all workers (Y axis). 1.81% of the bees performed up to 50% of the total number of trips of the colony.

Table 1: Statistical analyses of the influence of different individual measurements on different groups. Significant results are highlighted in bold.

Body part	**Length in mm (SE)**		**W**	**P**
A	*Nurses (N = 267)*	*Foragers (N = 77)*		
Total length	17.78 (0.17)	18.47 (0.46)	8983.5	0.900
Thorax length	7.40 (0.07)	7.75 (0.18)	12884	0.652
Inter-wing length	5.22 (0.05)	5.01 (0.16)	12749	0.608
Antenna length	3.94 (0.05)	3.90 (0.08)	10646	0.836
B	*Non elite foragers (N = 87)*	*Elite foragers (N = 12)*		
Total length	18.27 (0.49)	17.5 (0.43)	348	0.833
Thorax length	7.74 (0.19)	7.15 (0.18)	425.5	0.367
Inter-wing length	5.26 (0.16)	4.61 (0.21)	448	0.219
Antenna length	3.79 (0.09)	3.82 (0.09)	213.5	0.671
C	*Non-pollen foragers (N = 30)*	*Mixed foragers (N = 47)*		
Total length	19.60 (0.91)	17.25 (0.32)	848	0.137
Thorax length	**8.37 (0.35)**	**7.20 (0.11)**	**944**	**0.013**
Inter-wing length	**5.78 (0.28)**	**4.78 (0.13)**	**935.5**	**0.016**
Antenna length	3.89 (0.17)	3.72 (0.07)	597.5	0.219

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

Mixed foragers made longer pollen trips than non-pollen trips (figure 2*a*, Wilcoxon paired rank test W = 4999100, P < 0.0001). These bees completed more pollen trips during the afternoon with a peak time around 3pm (figure 2*b* and figure S5, Wilcoxon paired rank test: W = 9373500, P < 0.0001). Before 12am, bees completed only 19% of pollen trips (figure 2*b* and figure S5, non-pollen trips = 2 943, pollen trips = 710, N

= 99 bees) whereas this proportion increased to 45% after 12am (non-pollen trips = 3,867, pollen trips = 3 163, N = 99 bees).

Mixed foragers completed a similar frequency of pollen trips throughout their foraging lifetime (binomial GLMM with bee identity as random factor: intercept: P = 0.181, slope P = 0.065, table S2), indicating that they did not specialise for one type of resource with experience. While some bees (N = 28) specialised on pollen or non-pollen resources on different days, others (35 bees) foraged on both resources during the same day (see supplementary material figure S2 for example of active bees). During these days bees 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 and 3,298 pollen trip, Wilcoxon paired rank test: W = 1124700, P < 0.0001, supplementary material Figures S4 and S5).

Discussion:

We monitored the foraging activity of all foragers of a bumblebee colony for two months. We found that a small subset of foragers (11%) performed most of the foraging trips. Most of the bees collected both pollen and nectar, fewer bees specialised on nectar only, and only one bee specialised on pollen. Mixed foragers performed longer trips for pollen, and collected pollen later in the day.

What makes an elite bumblebee? Elite bumblebees are, in our study, the very active foragers that, as a group, provided more than 50% of the total number of trips performed by the colony. Previous studies showed that the foraging performance of bumblebees increases with body size [14,39,40]. For instance, large bumblebees develop foraging routes faster and more efficiently than smaller ones (chapter 5, [19]). Surprisingly, here we did not find any effect of body size in the activity level of bees. In our data therefore, elite bees occupy one end of a continuous distribution of individual foraging activity in the colony (see supplementary material figure S1), and do not form a sub-caste of foragers based on morphological differences.

In chapter 3 we also discussed that elite honey bees did not differ morphologically from non-elite ones. Our results seem to indicate a similarity in the nature of elite foragers in honey bees and *B. terrestris*.

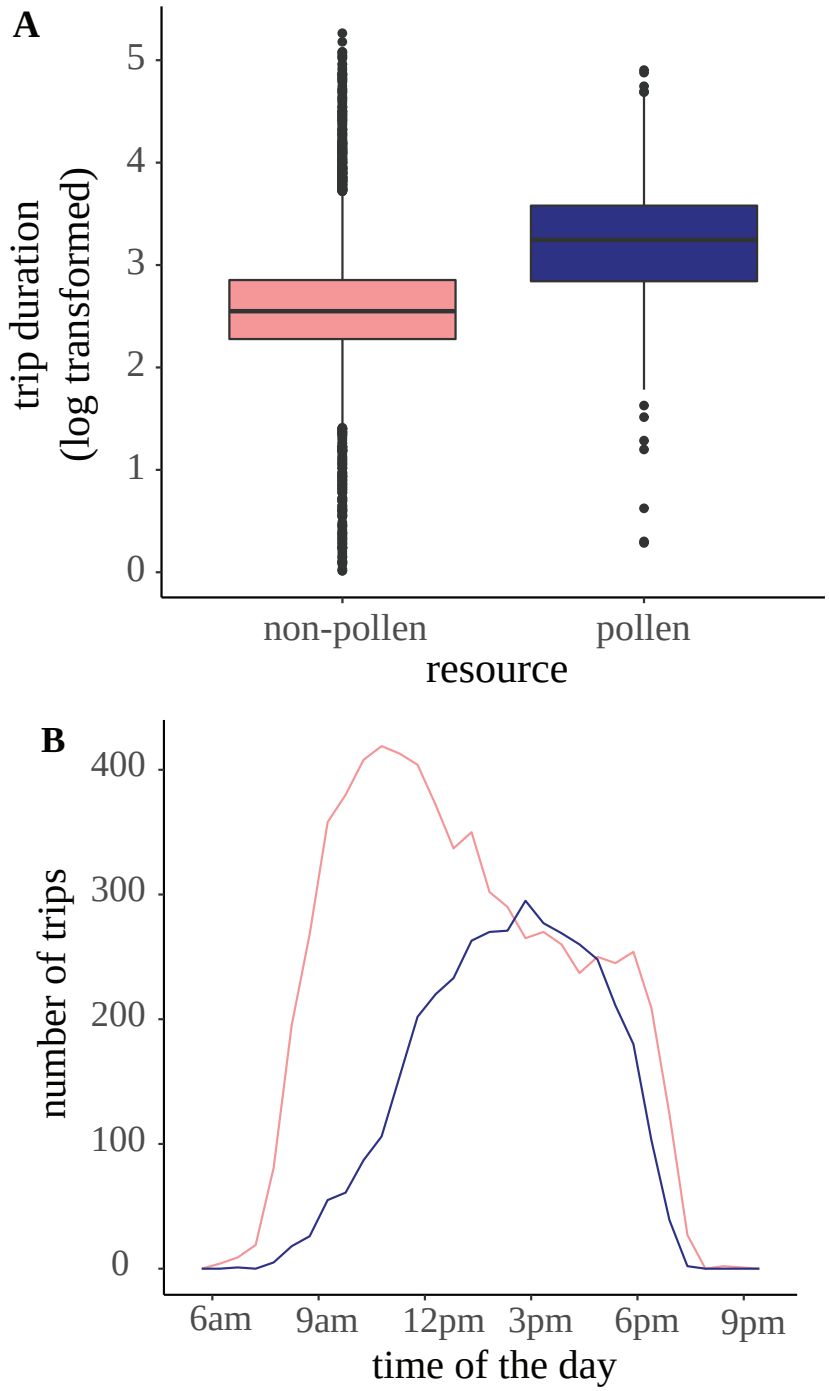

Figure 3: A. Trip duration for non-pollen (pink) and pollen trips (blue). Only trips shorter than 200 min were kept in the analyses (98.72% of the total number of trips). The duration of the trips was log transformed for normalisation of the data. Boxplot: the line shows the median, boxes and the whiskers 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 = 99 bees. Non-pollen trips = 7,063. Pollen trips = 3,858. Wilcoxon paired test: W = 4999100, P < 0.0001. **B.** Daily distribution of non-pollen (pink line) and pollen (blue line) trips. N = 99 bees. Non-pollen trips = 7,120. Pollen trips = 3,874. Wilcoxon paired test: W = 9373500, P < 0.0001.

But, honey bees have a monomorphic forager force and do not exhibit any morphological differences between the behavioural castes of the workers, whereas bumblebees show high morphological variation between the workers, which explains some aspects of the division of labour between nurse and forager bumble bees as well as some of the behavioural differences between foragers [14,41]. Thus, in the present study, we showed that body size does not explain variation in foraging activity in *B. terrestris*, but some morphological differences explain variation in foraging strategies: bees with smaller thorax forage more often for pollen.

Elite bumblebees did not differ from other foragers in the type of resources they collected. Contrary to what was found in honey bees (chapter 3), very active bumblebees did not collect more pollen. Further, in our population, bumblebees did not increase their probability of collecting pollen with foraging experience. In fact, our bees specialised on pollen collection on certain days, as has been suggested by previous studies in laboratory conditions [17], and in the field [42,43]. One of the proposed reasons for this is that pollen collection is dependent on temperature, with pollen collection occurring mostly on dry days [43]. We did not include meteorological conditions in our present models. In the honey bees colonies tested in chapter 3, we found that elite bees were also the most efficient at collecting nectar, and that this was due to the greater foraging experience of the elites. In the present study, we are not able to measure foraging efficiency because we have no weight measures for the forager bumblebees.

Pollen foraging may involve different challenges to nectar foraging, since bumblebees primarily collected pollen in the afternoon and took more time to perform pollen trips than non-pollen trips. Our results are consistent with the study of Peat and Goulson [43], who found that bees preferentially collect pollen during the warmest hours of the day. We also found that honey bees take more time to collect pollen than non-pollen resources (Chapter 2). Bumblebees exhibit interesting strategies to access food from the flowers such as robbing nectar [44] or buzzing behaviour to unload pollen from flower anthers [45]. Foragers load nectar in their crops [9]. Pollen, however, is packed as pellets of pollen on their legs that they carry back to the colony [9,45]. These behavioural differences may explain the observed difference of trip duration between the two [45].

Our new findings have some interesting implications for how a bumblebee colony might be able to respond to environmental stressors. Stressors such as pesticides reduce the proportion of pollen collected by bumblebee foragers [27,46]. Stressors also affect foragers' lifespan [46–57], which would impact the most active bees most severely. Interestingly, for bumblebees the many inactive and less active foragers may serve as a backup pool of individuals to rapidly allocate to the forager workforce in case elite foragers die. Indeed, when the very active foragers are artificially removed from a honey bee colony, the reserve of less active foragers allows a quick replacement of elite individuals [23]. The same phenomenon has been observed in ants [23,58]. These inactive foragers may therefore improve the resilience of the group or superorganism [8,59].

Since elite bumblebees do not tend to specialise for particular resources (nectar or pollen) and did not differ morphologically, their replacement may be facilitated, potentially providing a degree of flexibility and resilience at the colony level.

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Author contributions:

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

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Supplementary materials:

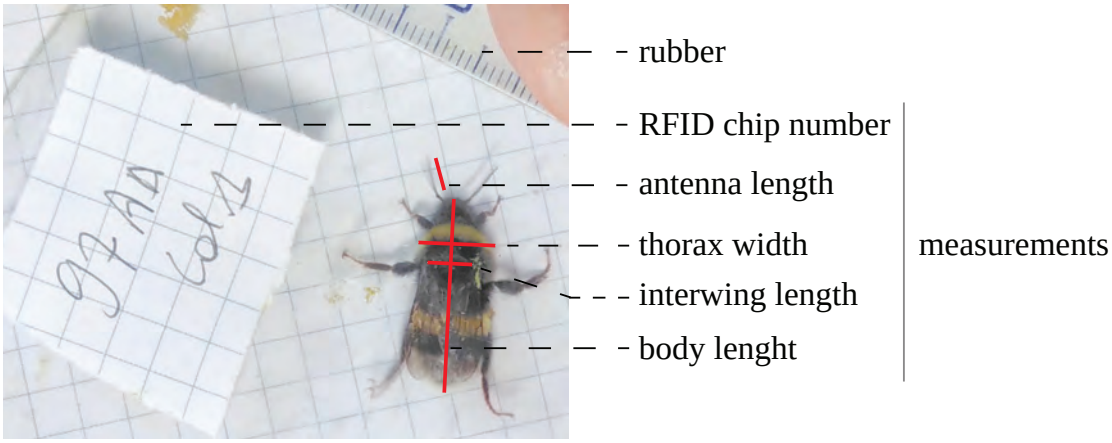


Figure S1: Measurements of bumblebee workers dimensions from individual pictures before the bee is tagged.

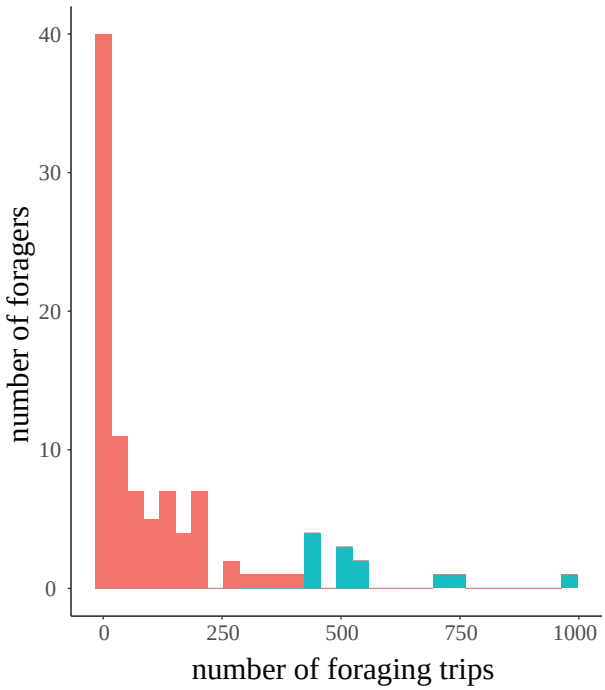


Figure S2: Distribution of non-elite (red) and elite (blue) bumblebees in term of foraging activity.

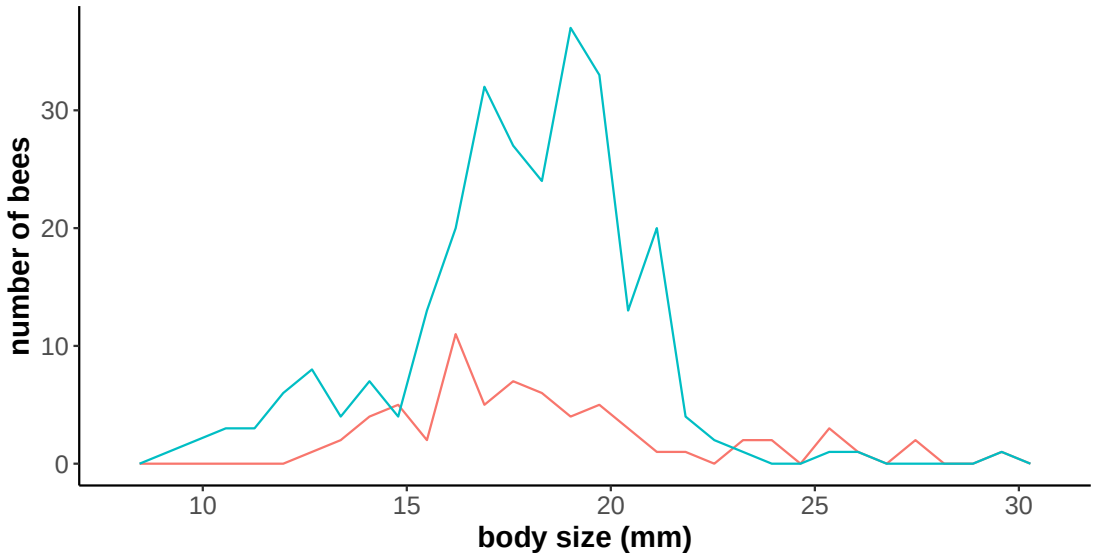
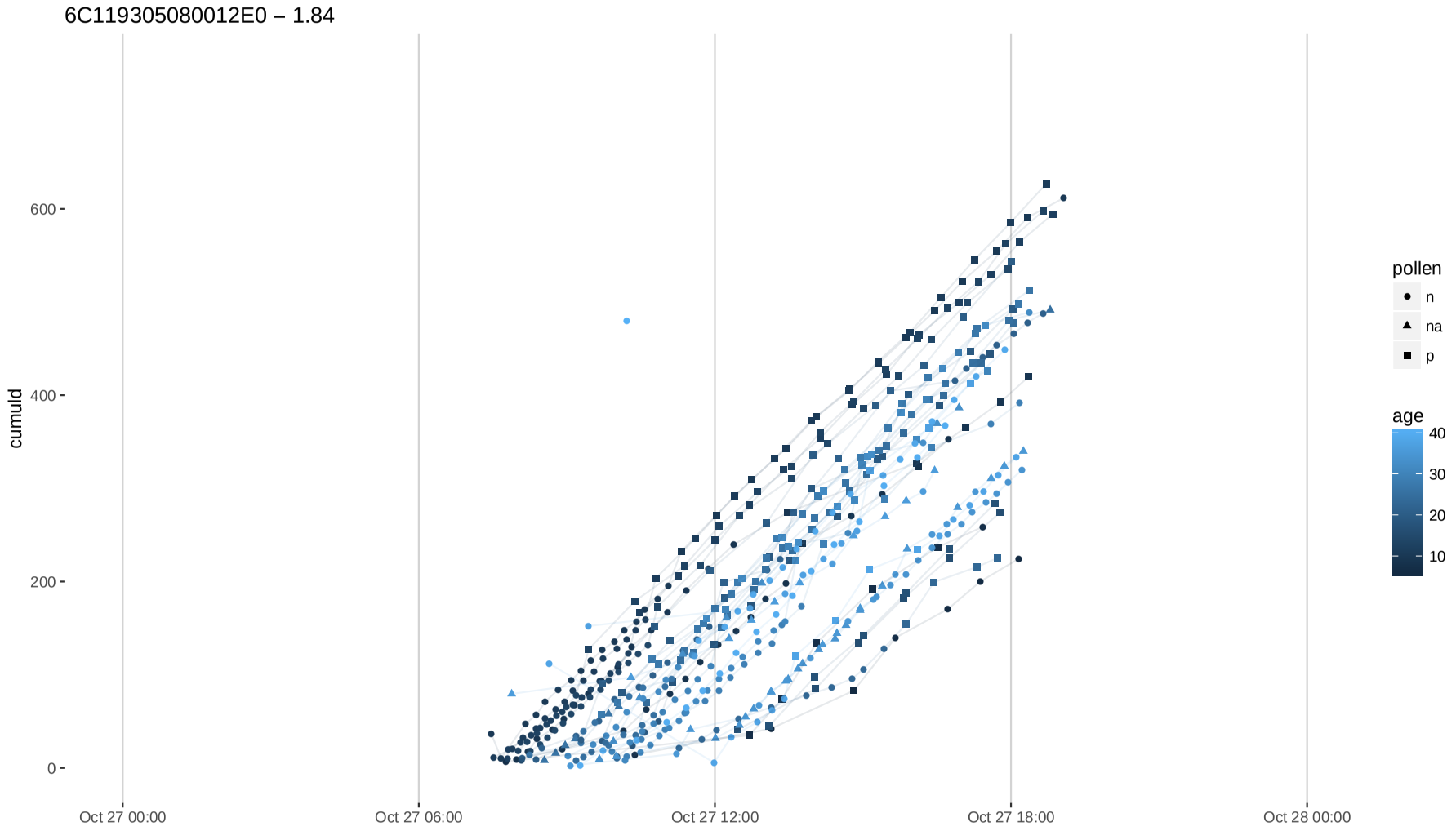


Figure S3: Distribution of the total body length for bumblebees that never foraged (blue) and for foragers (red).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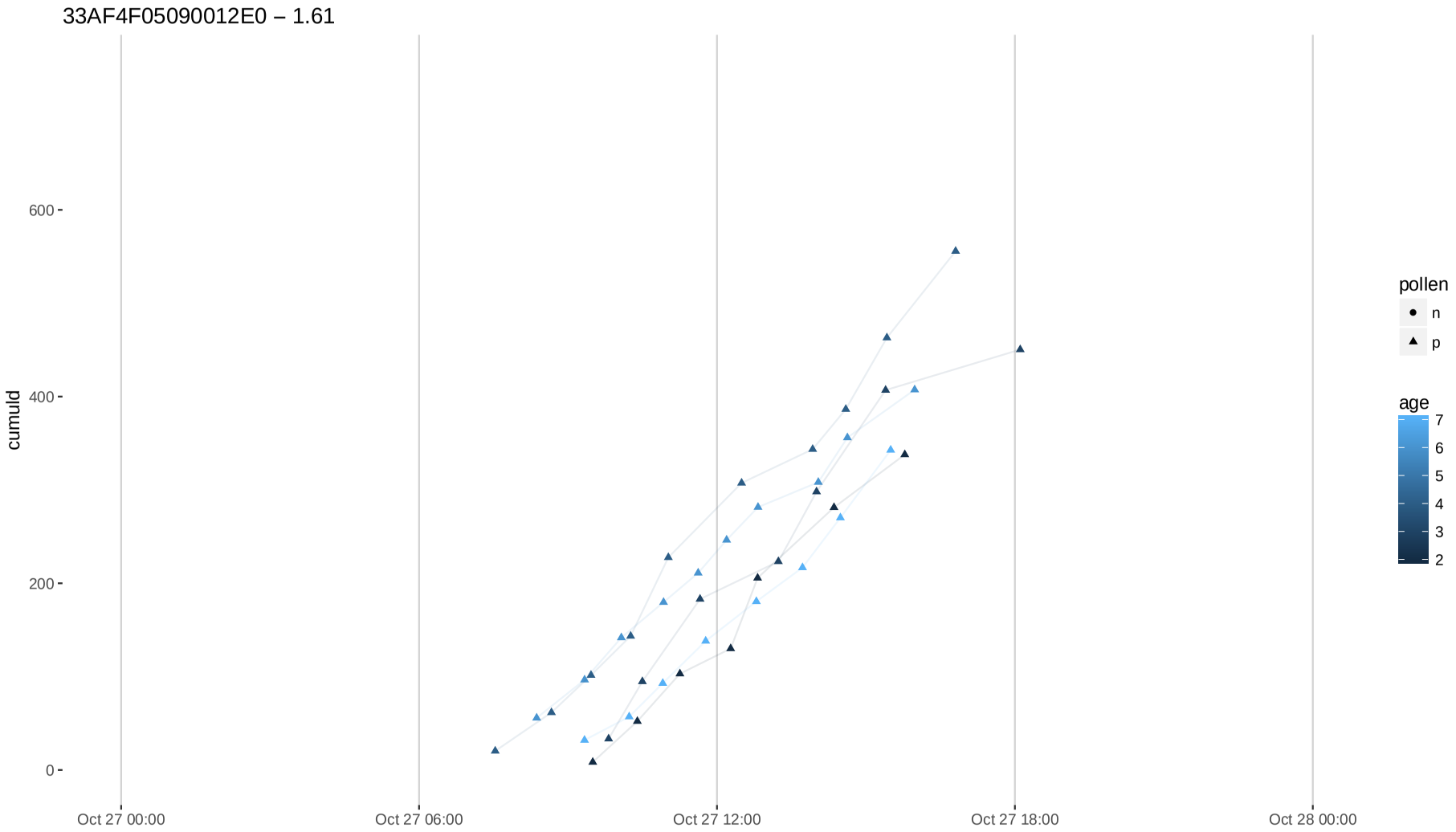
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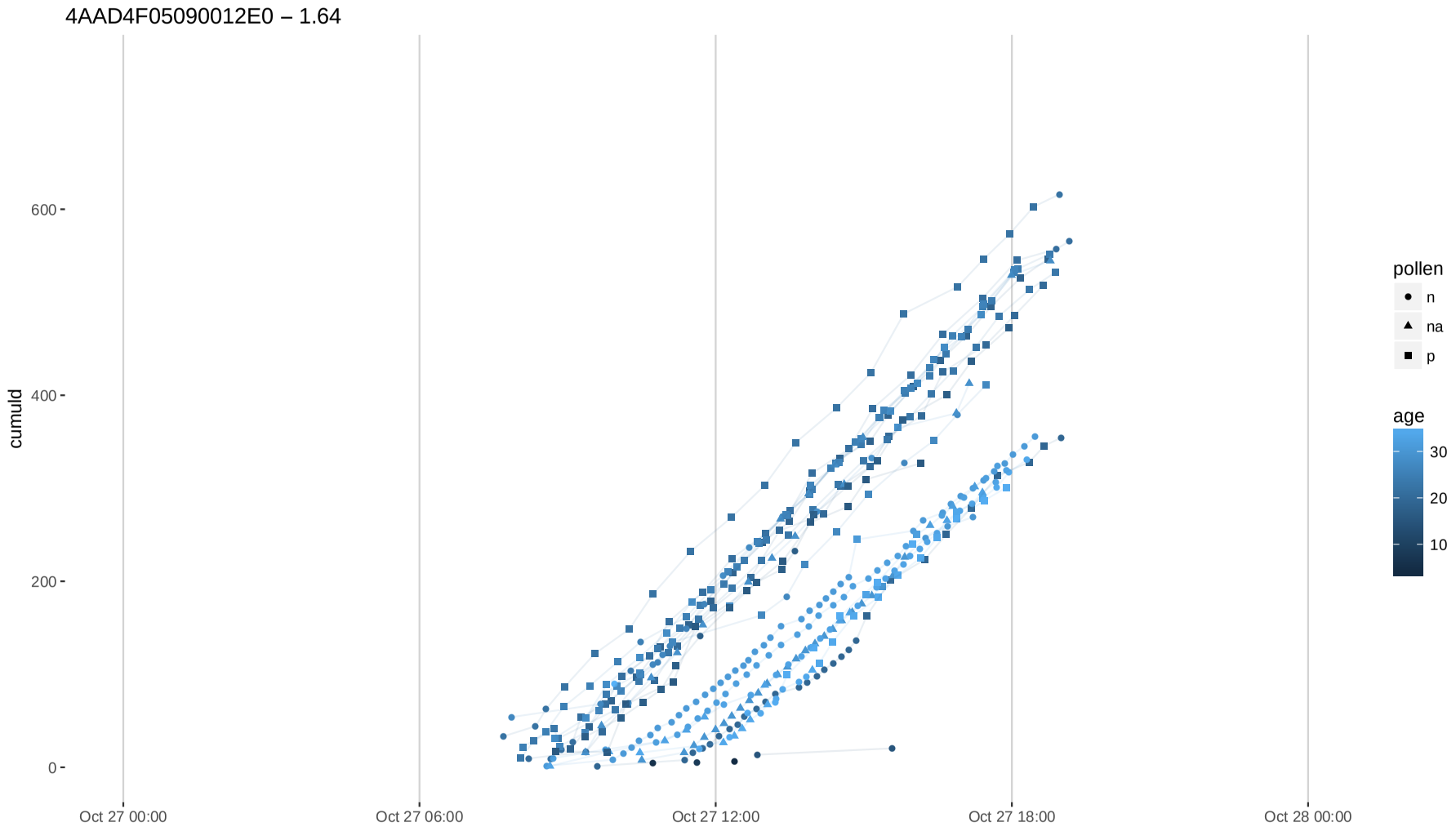
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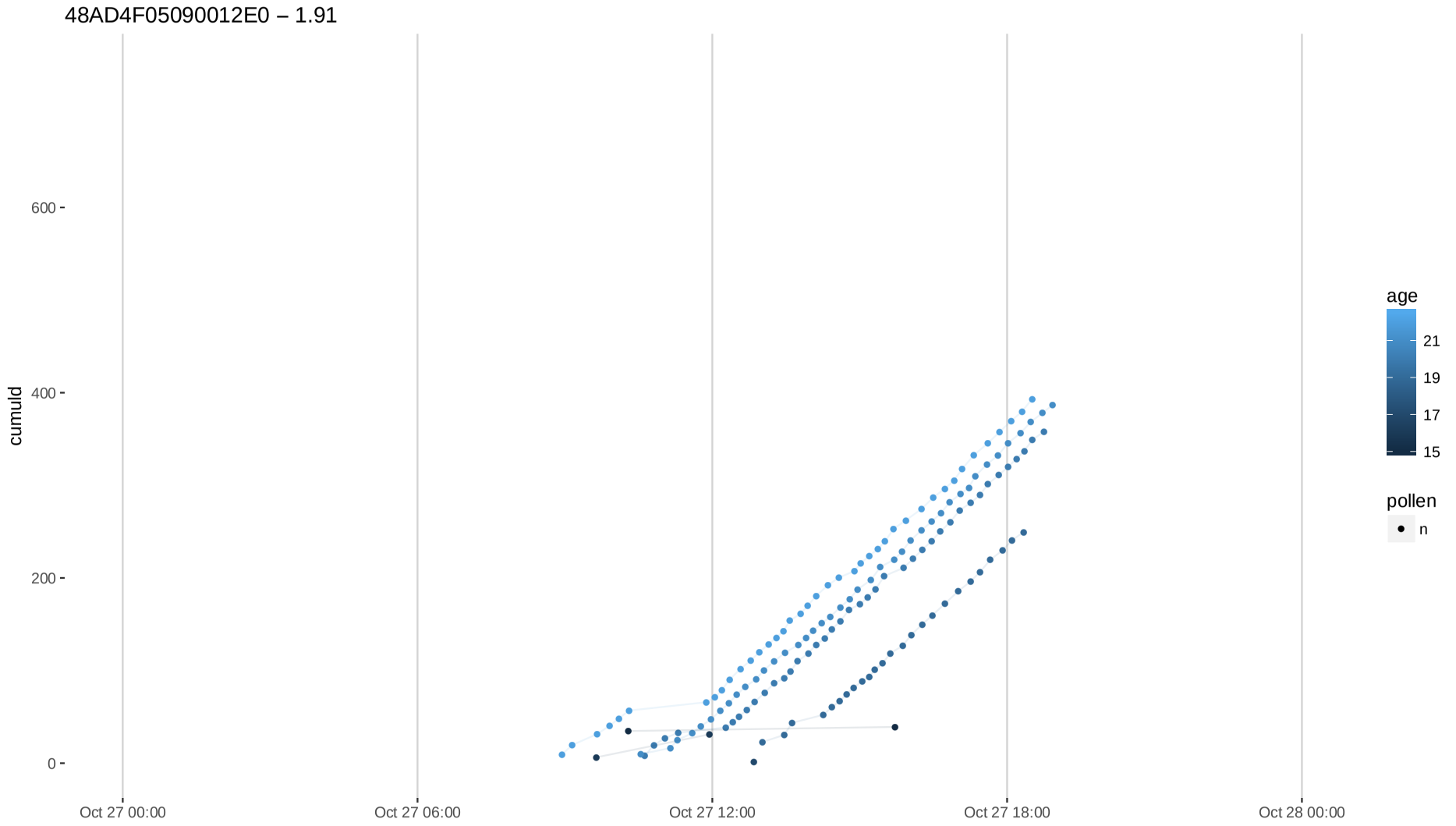
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<- **Figure S2**. Examples of foraging activity for some active bees. Plots show the cumulative time spent outside the colony-nest on a given day according to the time of the day. Shades of blue refer to the age of the individual on a given day. The 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.

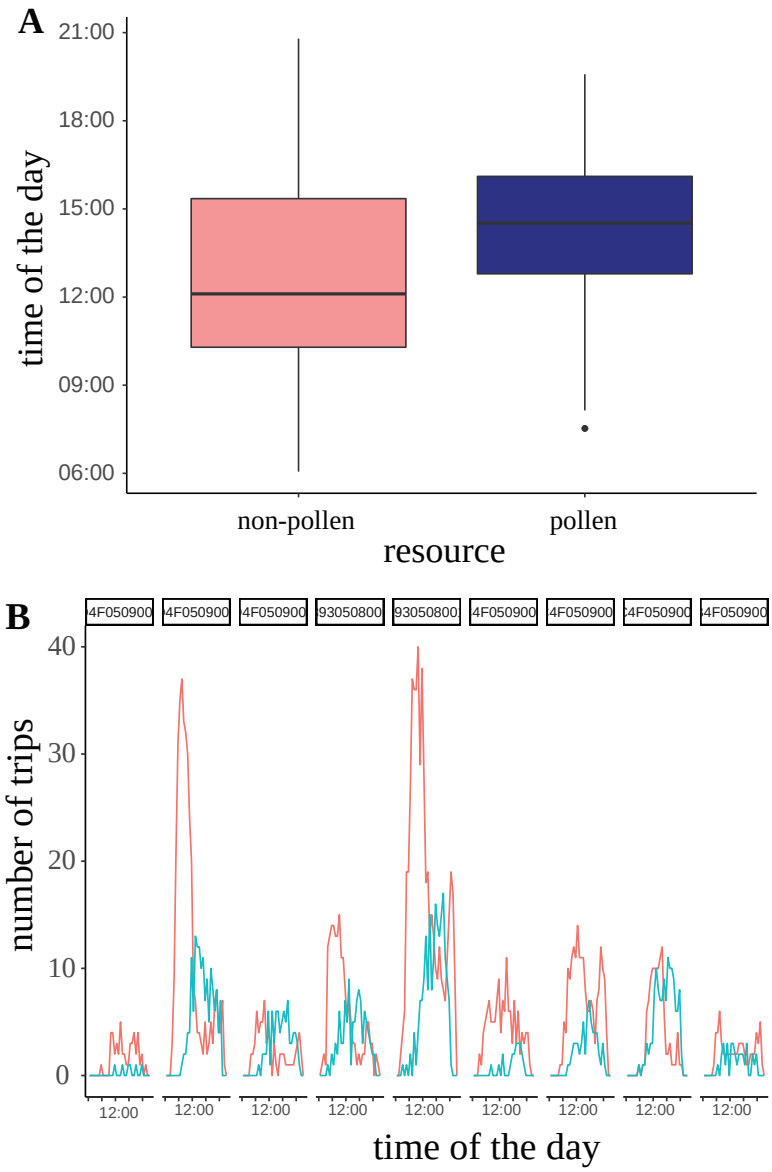

Figure S3: A. Difference in time of the day between non-pollen and pollen trips for days when individual bumblebees foraged for both resources. N = 35 bees, for 2,432 non-pollen trips and 1,3298 pollen trip. Wilcoxon paired rank test: W = 1124700, P < 0.0001. Pink is for non-pollen and blue for pollen 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 a few active bumblebees are represented (foraged more than 200 trips).

Table S2: binomial GLMM with logit linked function of the probability of collecting pollen with experience (as the number of foraging trips).

	Estimate (SE)	**DF**	**t**	**P**
a. *resource ~ number of trips performed + (number of trips performed |ID)*				
Intercept	**-1.63 (0.17)**	**2210**	**-9.70**	**<.0001**
Experience	**0.021 (0.006)**	**2210**	**3.69**	**<.0001**

CHAPTER 5:

Inter-individual variability in the foraging behaviour of traplining bumblebees

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Chapter published in Scientific Reports

Chapter 5: 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 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 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).

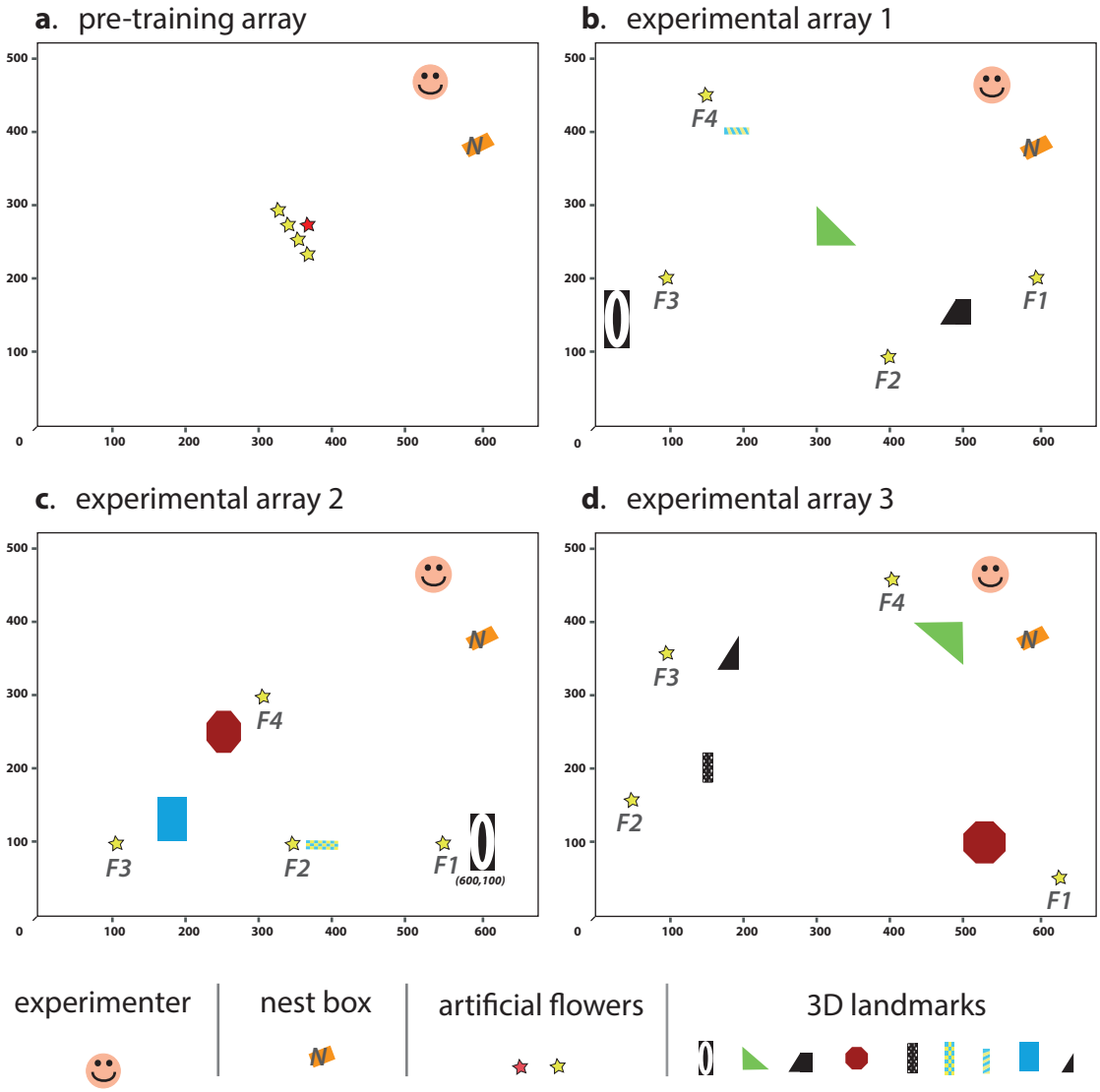


Figure 1: Experimental arrays of flowers and landmarks. **a.** Pre-training array. Bees were allowed to forage on a pre-training flower (red star) in a landmark-free environment for one hour. A selected bee was then observed foraging on four training flowers (yellow stars) during five foraging bouts 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 (± 0.01mm). 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 ± 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 ± 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 ± 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 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 × 3 × 2 × 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:

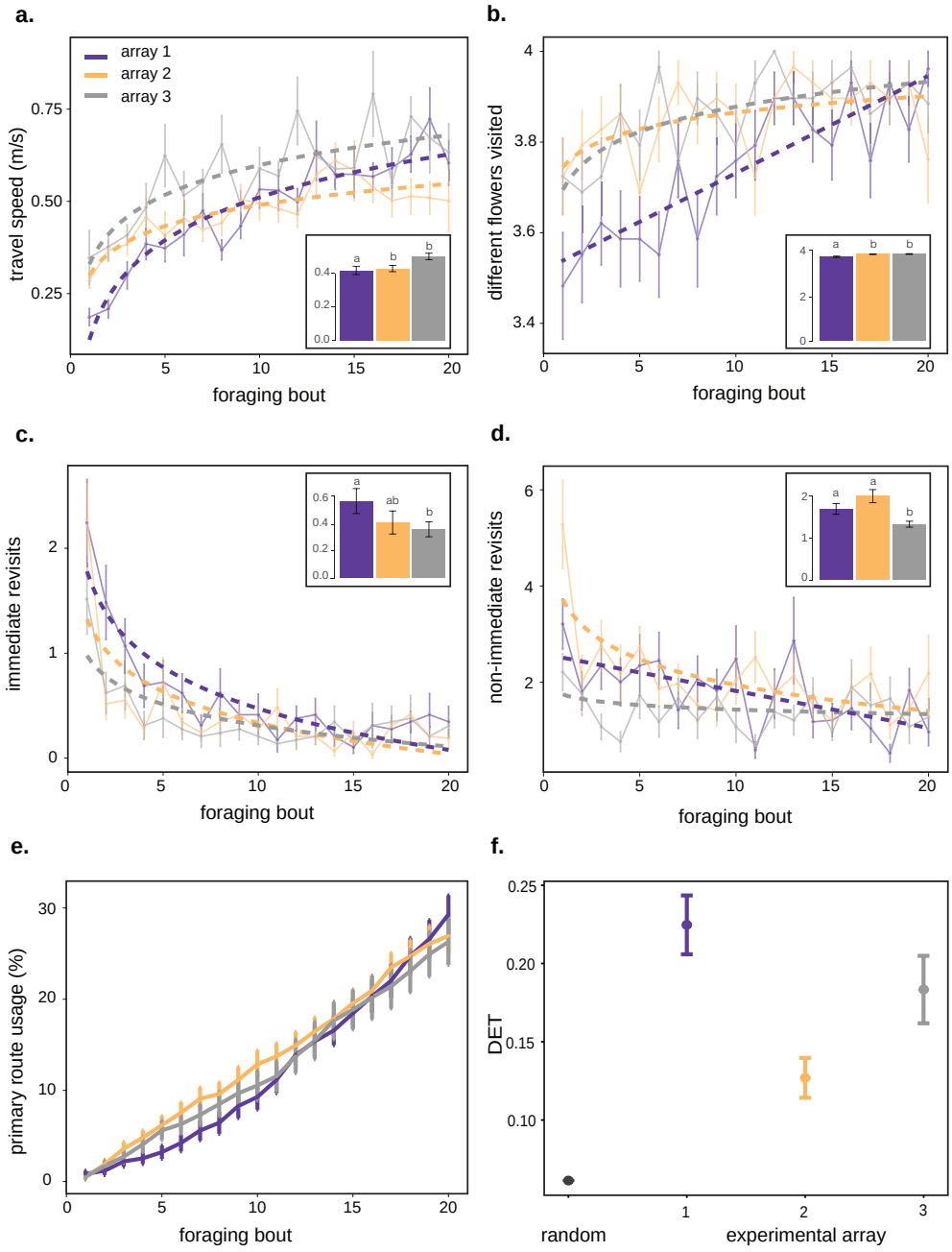
We tested 29 bees (N = 15 from colony 1, N = 14 from colony 2). Each bee was successively observed for 20 consecutive foraging bouts (flower visits followed by returns to the colony nest box) in three experimental arrays each characterised by four flower locations and four different landmarks (Figure 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 ± 48.3% (mean ± 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 ± 3.8% (mean ± SE) the average number of different flower locations they visited per bout (Figure 2B, Table 1), decreased by 85.3 ± 3.5% (mean ± 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 ± 8.0% (mean ± 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 ± 2.2 % (mean ± 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).



se **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 ± (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 ± SE). **a** to **d**: Bar plots show means ± 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.

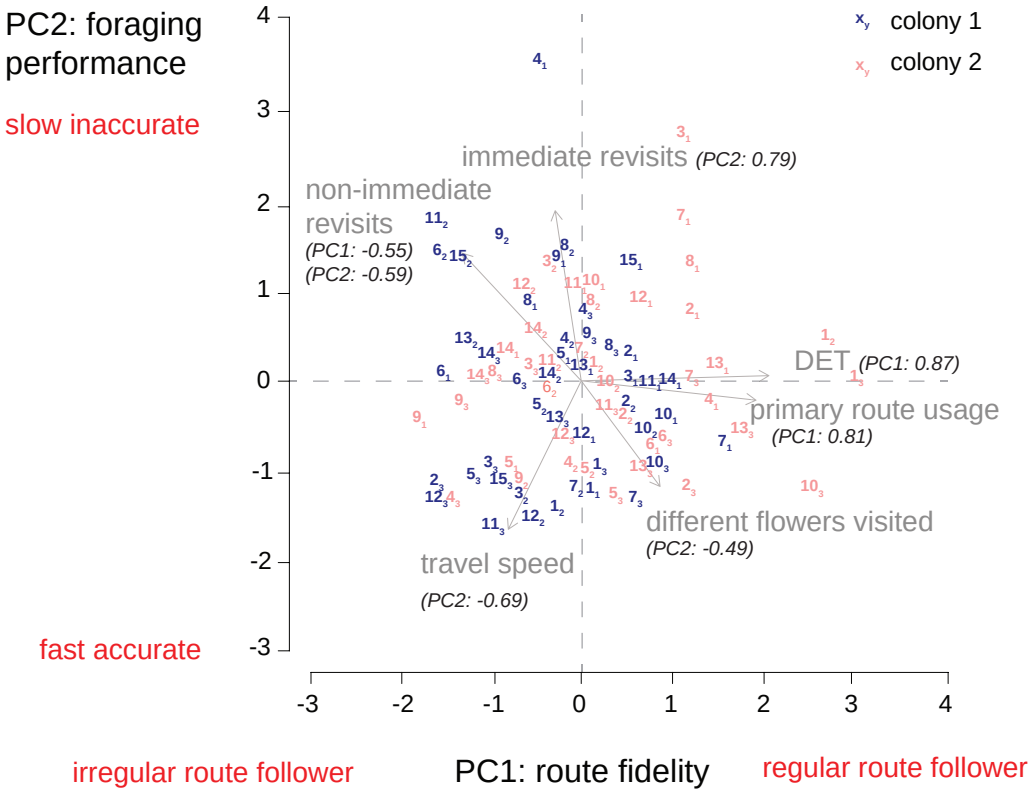


Figure 3: Correlations between the two first components (PCs) of the principal component analysis (PCA). Grey arrows represent the six behavioural measures on PC1 (route fidelity) and PC2 (foraging performance). PC loadings are in brackets. Only loadings > |0.4| were retained (see Supplementary Table 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.					
Random intercept model PC1					
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**
Random intercept model PC2					
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.					
Random slope model PC1					
LME_1|colony/ID	7	242.57	-114.29		
LME_0+array|colony/ID	**6**	**235.93**	**-111.96**	**4.64**	**0.031**
Random slope model PC2					
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.

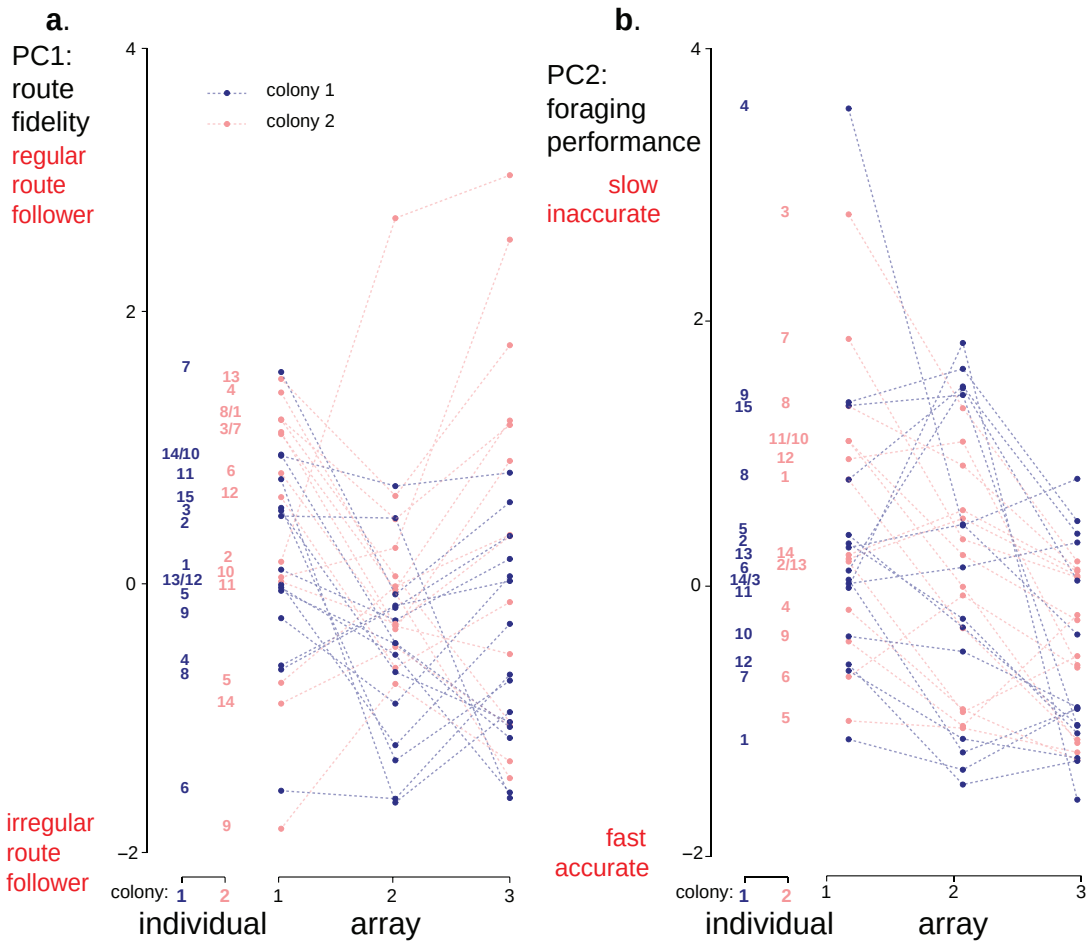


Figure 4: Intra- and inter-individual behavioural variance across experimental arrays. **a**. Route fidelity (PC1). **b**. Foraging performance (PC2). Data points connected by a dashed-line represent the scores of the same individual over the three arrays. Blue: colony 1 (N=15 bees), red: colony 2 (N=14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

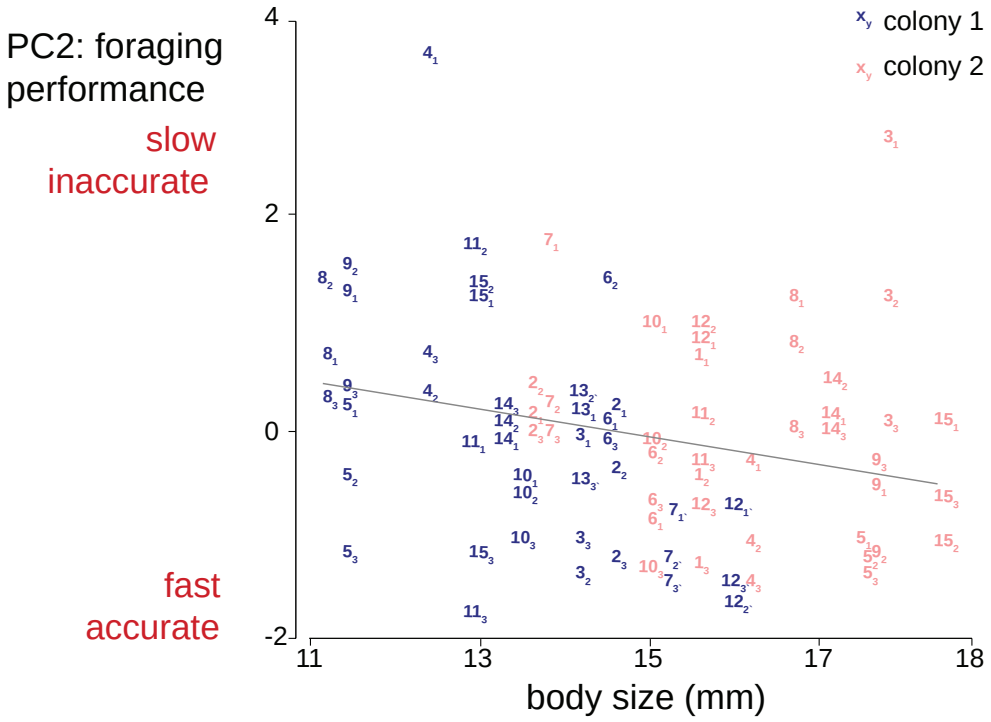


Figure 5: Inter-individual variance in foraging performance (PC2) is partly explained by body size (length from top of head to end of abdomen). Each data point represents the average score of an individual in an experimental array (three values per individual). Blue: colony 1 (N=15 bees), red: colony 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**
Route fidelity (PC1)				
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
Foraging performance (PC2)				
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**	
Model type	*linear*	*logarithmic*	*linear*	*logarithmic*	*linear*	*logarithmic*
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
Travel speed			
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
Different flowers visited			
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
Immediate revisits			
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
Non-immediate revisits			
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

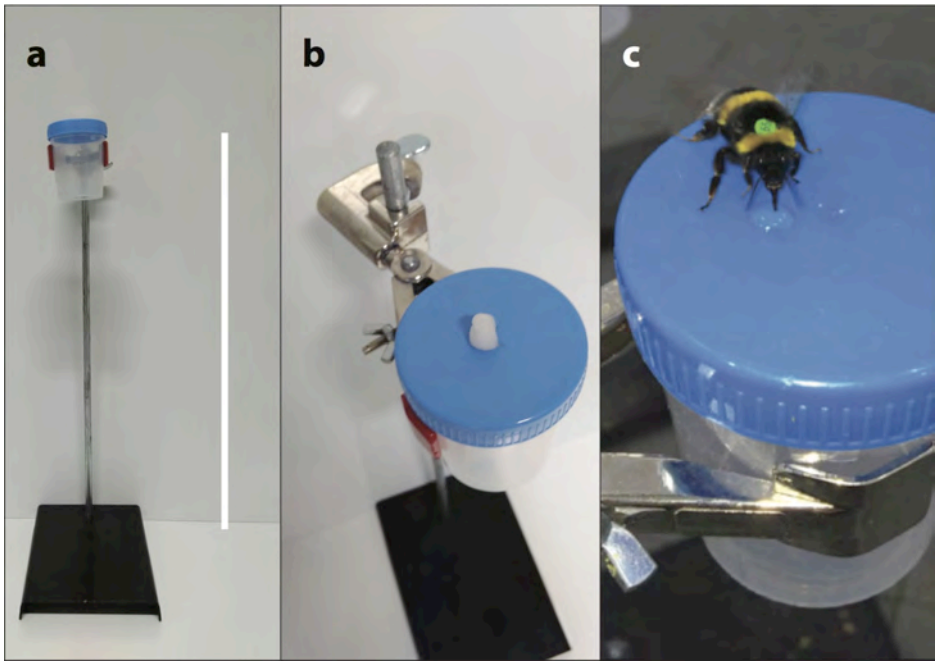

Figure S1: Photos of the artificial flowers. **a.** General flower design. The flower is made of a blue circular landing platform on top of a transparent, colourless, cylindrical reservoir of sucrose solution held by a stand clamp. White bar = 30 cm. **b**. Pre-training flower. Bees can drink *ad libitum* 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.

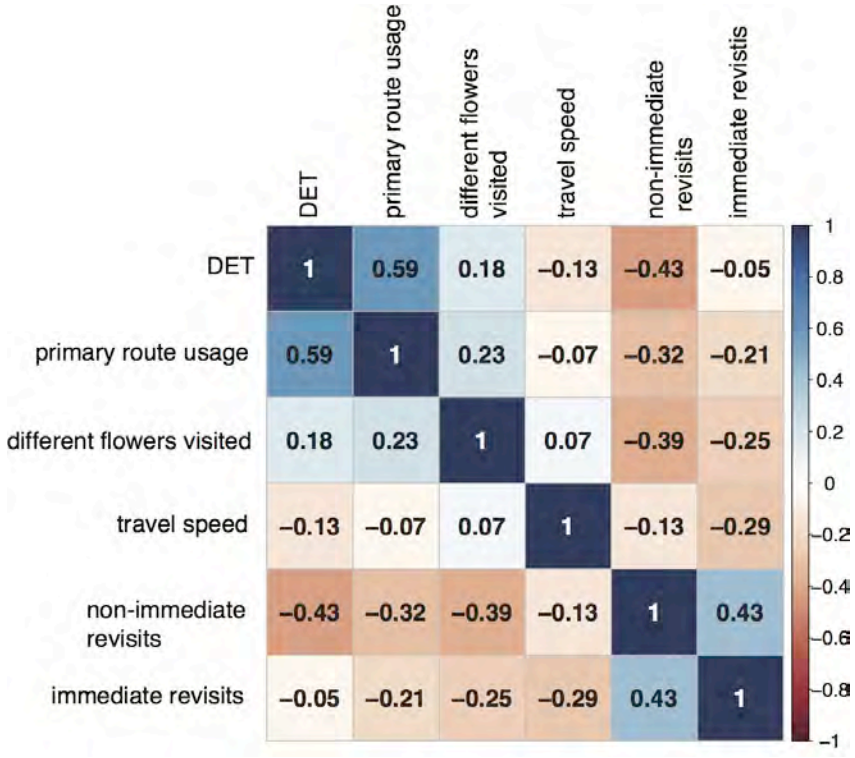

Figure S3: Correlation matrix of the six behavioural variables included in the principal component analysis. Travel speed per foraging bout (flight duration divided by the Euclidian distance between all successively visited flowers); number of different flowers visited per foraging bout; number of immediate 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.

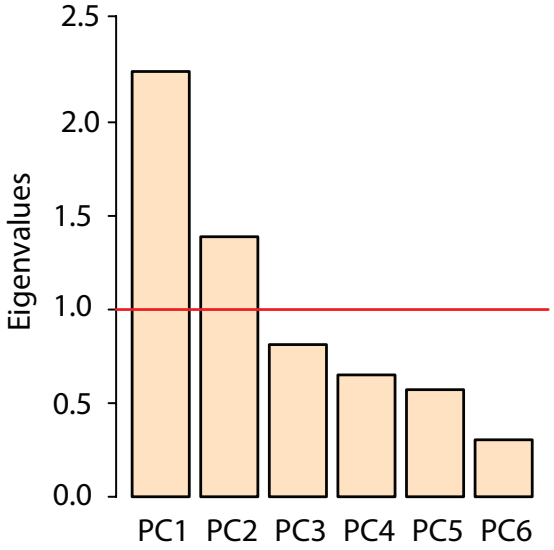

Figure S4: Selection of the principal components (PCs) based on the Kaiser-Guttman criterion. Two PCs with an eigenvalue higher than the average (red line) were retained to construct the principal component analysis.

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 6:

Sublethal effects of miticides on honey bee visual learning

Chapter 6: Sublethal effects of miticides on honey bee visual learning

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Abstract:

The varroa mite has been pointed to be one of the major causes of honey bee colony failure. In order to control this parasite, beekeepers use miticides to treat hives. The effect of a 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 miticides 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 importance to consider harmful effects of in-hive chemicals on bee cognition that could result in impaired foraging performance and altered colony dynamics.

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

Introduction:

Bees exhibit high learning capacity, which they use to find food and their way home in complex environments [1,2]. However a current increase in environmental stressors impacting bees is damaging bee cognitive capacities [3] and affecting bee populations [4]. One of the main stressors of the honey bees is the parasitic mite *Varroa destructor* [5,6]. To control this parasite, beekeepers use different kinds of miticides, applied directly in the honey bee hive [7]. While sublethal effects of various plant pesticides on bees' learning abilities have been studied in detail using learning and memory assays [8–11], effects of chronic exposure of in-hive chemicals is poorly understood, even though the presence of chemicals in the hive environment is an increasingly common situation for honey bee colonies [12–16]. Here, we propose to study the effect of miticides on bee visual learning capacity.

Varroa, an ectoparasite that feeds on the haemolymph of larvae and adult bees [7], is a worldwide-distributed honey bee pest that has been recognised as a major driver of honey bee colony losses [5,6]. It causes cognitive impairments in adult bees [17] such as reduced olfactory non-associative and associative learning and memory [18] or homing behaviour [19]. It is also a vector of viruses (such as the wing deformed virus [20,21]), that causes learning and memory impairments possibly through effect on bee immune system [22] and gene expression [23].

Miticides have been developed to control varroa levels in hives [7,24,25]. These chemicals are used directly in the hive so that their active compounds are in contact with the brood and the adult bees. We focus here on two common miticides worldwide: tau-fluvalinate and thymol. Tau-fluvalinate (hereafter fluvalinate, commercialized as *Apistan*®) is a synthetic pyrethroid compound [26]. This class of compounds acts as an arthropod neuron excitoxin which prevents the closure of voltage-gated sodium channels of axonal membranes, leading to prolonged membrane depolarization and thus to paralysis or death [26–28]. Fluvalinate suppresses neuron excitability in the adult bee brain structures [29] and impairs olfactory memory [9]. Thymol is a monoterpenoid extracted from essential oil of thyme (*Thymus vulgaris*) and has been used for varroa control under the commercial product *Apiguard*® [24]. Even if it is considered less harmful for the environment as it is based on plant products [30], thymol may have negative effects on bees. Topical applications of high doses (10 – 100ng / bee) of thymol reduce long-term memory of the association

between an odour and a food reward [31]. Although effects of chronic exposure of thymol on honey bee cognition have been poorly studied so far [24,31–34], it has been shown to impair phototactism [32].

In-hive pest-control products are detected in the wax, honey and pollen of a hive for a long time after treatment [12–16]; therefore we expect developing larvae and in-hive bees to undergo chronic exposure [14]. Presently it is not clear what effect chronic exposure to contaminants from miticide treatments during in hive development could have on foragers' behaviour and cognition. An additional difficulty is that all studies looking at the effects of miticides on bee cognition did not control for potential cofounding effects as they have been conducted on populations also exposed to the varroa mite [9,29]. It is then highly valuable to address such question with bee populations not exposed to varroa, such as in Australia [35], in order to specifically test the effect of the miticide on bee cognition.

Here we analysed the effect of in-hive chronic exposure to fluvalinate or thymol on bee forager learning. For this, we used a recently developed visual aversive learning assay called Automatic Performance Index System (APIS, [36,37]). The APIS assay consists of a chamber that automatically tracks movements of a walking individual that has to learn to associate one of two different coloured light environments (provided by LEDs in the chamber's walls), with electric shocks (provided by a metal grid on the floor of the chamber). Using this system, we were able to capture cognitive deficits in adult foragers from hives treated with miticides.

Material and methods:

Animals:

We used six European honey bee (*Apis mellifera*) colonies from the Macquarie University experimental apiary, North Ryde, Australia. The colonies of around 50,000 workers and a queen 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 the last two hives were non-treated (control colonies, 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 top-box frames of each of the thymol-treated hives for two weeks. After two weeks, the treatment containers were replaced by another thymol gel container in each of the two hives for another three weeks. Treatments started on the 1st of February 2017. 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) [38] and the transition from hive bees to foragers (around two weeks) [38], we confidently were able to test foragers that had been passively exposed to the chemicals during their development in the hive. The experiment lasted for a month (14th of March 2016 to 16th of April 2016).

A total of 146 bees have been tested in two APIS chambers in parallel (48 bees from non-treated hives, 48 bees from thymol treated hives and 50 bees from fluvalinate-treated hives, see details in supplementary material table S1). The sequence of bees selected from treatment hives was randomised 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 bee vacuum (BioQuip Products, Inc., CA, USA). Bees were captured no greater than 15 minutes prior to testing and stored for a minimum of 10 minutes in the dark, at approximately 24ºC in 50mL Falcon™ conical centrifuge tubes. Since foragers were selected randomly, information about age and foraging experience is unknown and also random.

Testing apparatus (APIS box):

We used an aversive visual conditioning apparatus (figure 1A) developed and manufactured at Konstanz University, Germany [36,37,39]. The apparatus is a conditioning box of 148mm long, 20mm wide, and 6mm deep. Bee motion is tracked with 26 infra-red LED sensors lining the walls. It automatically tracks the movements of a bee walking in the chamber. Bee movements are registered by position, direction and distance only, and any turn made by the animal is only registered by the sensors if it is a complete reversal of direction. The program uses sensor feedback to determine the bee's location and initiates visual stimuli according to the bee location in the chamber. Visual stimuli are provided by coloured light environments generated on tricolour (RGB) LEDs lined up the walls of the box.

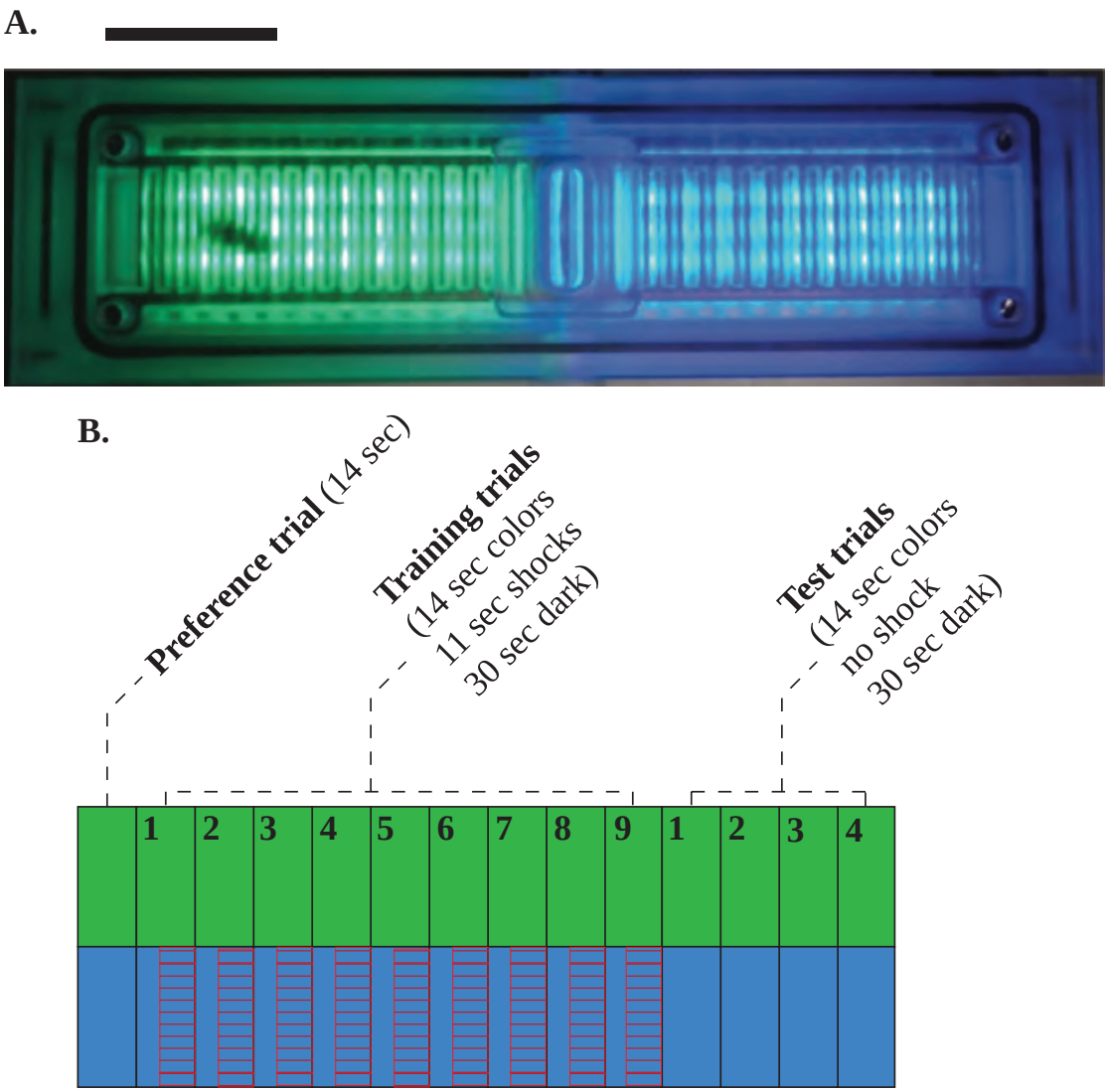


Figure 1: A. APIS chamber (black bar = 20 mm). Either side of the chamber can be illuminated with two different light fields thanks to LEDs: light appearing green (λ = 525 nm) to humans and light appearing blue (λ = 465 nm) to humans. The chamber is equipped with an electrifiable grid to deliver 10 V shocks to the bee's tarsa and with infrared sensors to automatically track the bee's movement. **B.** Protocol of training and testing the visual learning assay. After an acclimatization period of 7 min with no light, the bee was exposed to 14s of both green and blue illumination as a preference test. 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 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 [36], and left to air dry, in order to avoid any remaining alarm pheromones, which are known to impair learning [40].

Conditioning protocol:

Almost all individual bees transferred themselves into the aversive conditioning chamber (figure 1A) by walking from the falcon tube (in dark conditions) 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, as this was interpreted as a sign of stress. Following testing, bees were euthanized by freezing.

Bees were conditioned to avoid a blue light environment (conditioned stimulus (CS)) associated with electric shocks (unconditioned stimulus (US)) by moving towards a safe zone (green light environment, for which bees exhibit a spontaneous preference [39]). This protocol has been successfully applied in previous studies [37,39]. The conditioning protocol consisted of one unreinforced preference test followed by nine reinforced training trials and ended with four unreinforced test trials (figure 1B). In each trial, blue light LEDs (λ = 465 nm, luminous intensity: 105 mcd) were switched on in the half of the chamber where the bee was located and green light LEDs (λ = 525 nm, Luminous intensity: 119 mcd) illuminated the opposite half. All trials lasted 14 s, with an inter-trial interval of 30 s. For the training trials, electric shock pulses (10 V, 4Hz, 100ms) were activated 3 s after light onset. These shock pulses were delivered to the tarsa of the bee through the metal grid as long as movement sensors on the blue side were triggered. Trials were discontinued if it rained, even if bees were still active, as any water in the chamber may have altered the electrical conduction.

Statistical analysis:

Analyses were adapted from Plath et al. [37]. We used a Performance Index (PI) as a metrics showing the learning performance of the bees:

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

Where *t*(green) is the time spent on the green (safe) side of the chamber, and *t*(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 that on the shocked side, 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 spend equal amounts of time on each side or more time on the blue side, and thus have low PI-values.

We also recorded the number of electric pulses delivered to each bee, during each training trial, which was highly correlated with the PI values (correlation: Pearson's $\boldsymbol{\rho}$ [CI]= -0.84 [-0.85,-0.82], df = 1378, t = -56.82, P < .0001). These metrics were used to assess the degree of learning during the training phase.

We also 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.

Data were analysed and graphed using R version 3.2.3 [41] (operating via Rstudio, version 1.0.136 [42]). Statistical analyses of PI, number of shocks, crossing latency and speed were run with General Linear Mixed Models (GLMMs) with bee identity as a random factor to correct for repeated measurements (lme function from nlme package [43], supplementary material table S1). Minimum adequate models were identified among other models following the Akaike Index Criterion (AIC) selection method [44]. For all metrics, we found that hive origin did not have any significant effect on variance (supplementary material table S2). Differences between the treatments groups were then assessed with Tukey post-hoc tests, using the R multcomb package [45]. Since bees moving slowly could not perform well in this assay in which performance is based on movement, animals with average speeds lower than 2.1 cm/s were excluded from the analysis [37,39] (8 of the 146 tested bees, supplementary material Table S1).

Results:

138 bees were kept for our analyses, consisting of 44 bees from non-treated hives (NT), 49 from tau-fluvalinate treated hives (FL) and 45 from thymol treated hives (TH) (details available in supplementary material table S1). 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.

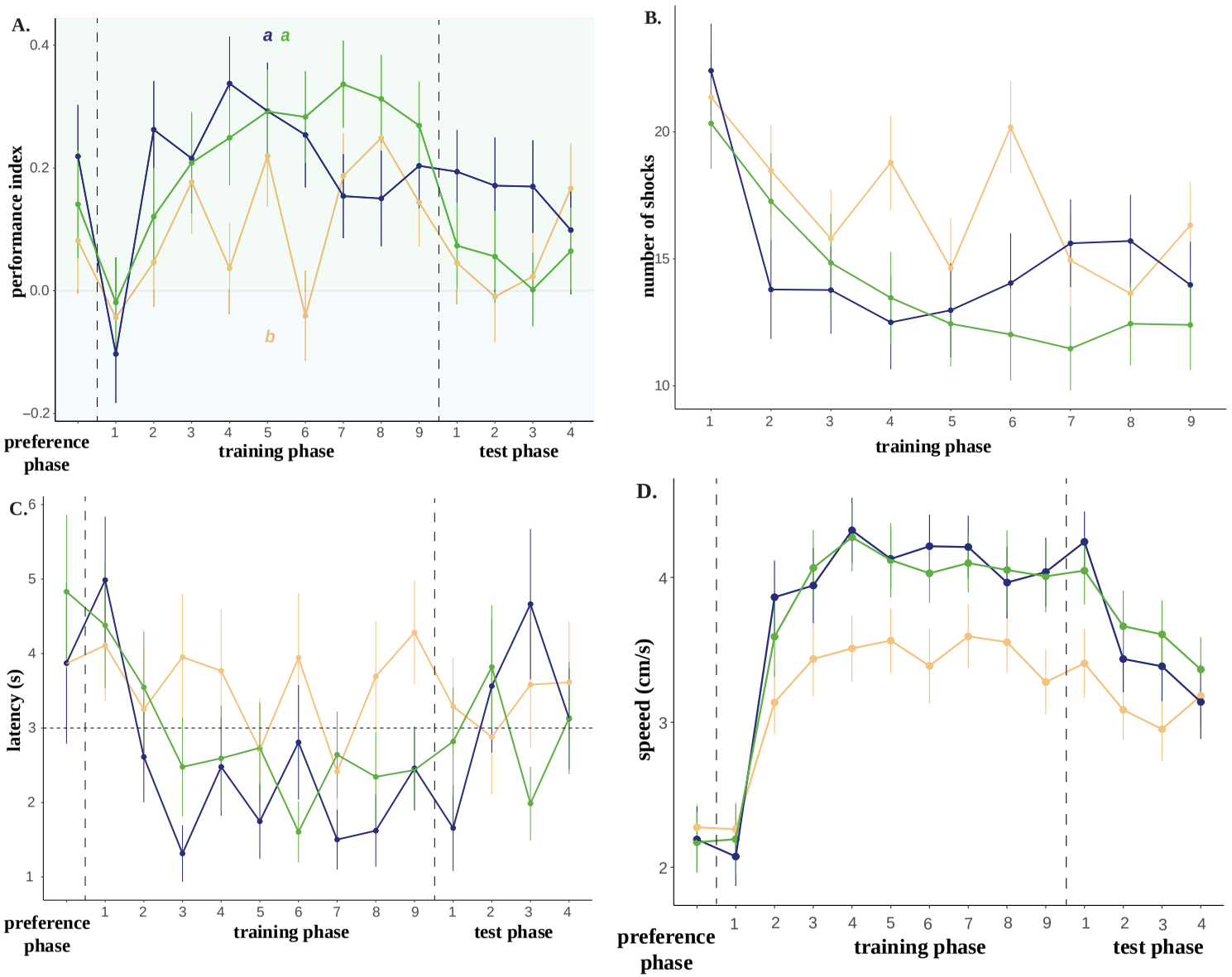
Fluvalinate-treated bees showed reduced learning performances:

Considering the overall changes in performance index (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 conditioning (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 value with trials was accompanied by a decrease of the number of electric shocks received by the bees (figure 1B, 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).

FL bees had overall inconsistent and lower PI values than NT bees (figure 2A, GLMM, N = 138, df = 135, F = 3.64, P = 0.029, HDS Tuckey post-hoc test, NT – FL = 0.096 ± 0.038, Z = 2.50, P = 0.037). FL bees spent similar amounts of time in the green zone during the first (48%) and the last (57%) training trials (PI difference *t*-test: df = 48, t = -1.78, P = 0.08, difference in shock received: df = 48, t = 2.03, P = 0.048).

No effect of treatments on short-term memory:

NT bees learned to avoid the blue side and remembered it during the test phase. During the first test trial, on average these bees crossed to the green side before the onset of shocks during the training phase (3 seconds after light onset) (mean ± SE, crossing latency = 1.66 ± 0.58 s, figure 2C). TH bees showed a longer latency, however, in average still under the 3 s (2.42 ± 0.72 s, figure 2C). FL bees showed an even longer latency, crossing, in average, after the 3 s of light onset (3.29 ± 0.65 s, figure 2C). Nevertheless, there is no significant difference between groups for PI values (figure *2*A, GLMM, N = 138, $F_{(2,135)}$ = 2.32, P = 0.10) or crossing latency during test trials (figure 2C, GLMM, N = 138, df = 135, $F_{(2,135)}$ = 0.15, P = 0.86).


A.
a a
b
performance index
0.4
0.2
0.0
−0.2
preference phase
training phase
test phase
B.
number of shocks
20
15
10
training phase
C.
latency (s)
6
5
4
3
2
1
preference phase
training phase
test phase
D.
speeed (cm/s)
4
3
2
preference phase
training phase
test phase

←**Figure 2:** Learning performances. Means ± SE are plotted for all variables. Blue: non-treated, N = 44. Green: thymol, N = 45. Yellow: fluvalinate, N = 49. Bees were subjected to one preference trial, nine training (reinforced) trials and four (unreinforced). test trials. **A.** Preference Index. There was a significant difference between the treatments (GLMM, supplementary material table S3). Treatment comparison with a Tukey HSD post-hoc test showed differences in PIs of control and fluvalinate 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 (electronic supplementary material table S3). **B**. Number of shocks received during the training phase. There was no significant difference between the treatments (GLMM, supplementary material table S3). **C.** Crossing latency to move towards the safe side (green side) after the light onset. During the 3 first seconds there was no shock. There was no significant difference between the treatments (GLMM, supplementary material table S3). **D.** Speed (cm/s) of displacement during the first 3 seconds after onset of each trial (before bees start potentially receiving shocks). GLMM shows a significant different between the treatments for the overall assay (supplementary material table S3). Tukey HSD test shows a trend for fluvalinate treated bees to be slower (NT - FL: 0.46 ± 0.21, Z = 2.57, P = 0.060; TH - FL: 0.48 ± 0.20, Z = 2.32, P = 0.060). After the first training trial, fluvalinate treated bees were slower than the two other groups (Tukey HSD, NT – FL, Est = 0.56 ± 0.23, Z = 2.50, P = 0.033, TH – FL, Est = 0.60 ± 0.22, Z = 2.54, P = 0.033)

Discussion:

We compared the effect of two miticides frequently used to reduce varroa loads in honey bee colonies, fluvalinate and thymol, on foragers' cognition. We found that whereas thymol did not affect bee visual aversive learning abilities, bees that treated with fluvalinate were worse learners than the non-treated bees in this particular task. None of the treatments affected short-term memory.

Our assay was able to capture visual learning behaviour in honey bee foragers: non-treated bees started with a low performance index that increased during the training phase as bees associated blue light with electric shocks (figure 2A). Our result for the non-treated bees was consistent with findings for control bees in Plath et al. [37]. Bees from the thymol-treated hives showed a very similar pattern to that of non-treated bees (figure 2). Thus, our study does not reveal any clear impact of thymol on bee visual cognition but further experiments, such as foraging performance monitoring (chapter 2 & 3), should be conducted to analyse better the foraging behaviour of bees exposed to the substance.

On the other hand, fluvalinate-treated bees performed less well in the learning assay than non-treated bees (figure 2A). They also performed irregularly during the training phase, with no clear increase of PI with trials (figure 2A). Fluvalinate has

previously been reported to have negative effects on bee olfactory learning and memory [9], but also to reduce larval survival [9,46]. Acute exposure to fluvalinate also affects bee locomotion, since it increases the time spent near a food source and decreases mobility in a test arena [47,48]. Our results confirm that fluvalinate-treated bees were slower than the non-treated and the thymol-treated group after the second training trial (supplementary material figure S1). In our case, Speed and Performance Index were correlated (Spearman ρ [CI] = 0.09 [0.05,0.13], df = 1928, t = 4.04, P <.0001), thus we cannot conclude whether the observed variations in visual learning performance for fluvalinate-treated bees were due to a locomotor impairment alone or to a more complex interplay between a locomotor impairment and a visual cognition impairment. More studies, using other visual learning paradigms requiring no locomotor response [49], are needed at this point to address such question.

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. Previous studies suggest that fluvalinate increases brood and larval mortality [46,50] and is involved in cell mortality in midgut, salivary glands and the ovaries during larval stage [51]. Our observations at the cognitive level point towards an effect of fluvalinate on brain development, possibly by reducing neuronal excitability [29] and leading to malfunction of the visual pathway and/or learning centres of the forager brain [52], thus calling for future neurobiological investigation.

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.

Together with other studies, our work provides element to assess the potential drawbacks of varroa treatments for bees themselves, which have not been explored much for now. In addition, our results suggest a more negative impact of tau-fluvanilate than thymol on aversive learning. Nevertheless, further work is needed to assess other possible behavioural or cognitive impacts, particularly in free-flying bees foraging on food sources and navigating to the hive.

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: Number of bees kept in each group after removing individuals that were too slow (<2cm.s^{1}).[37]

treatment	**Non-treated (NT)**		**Fluvalinate (FL)**		**Thymol (TH)**	
hive	**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. Best model is in bold. **A.** Model selection for PI index. Hives origin seems to have no effect. **B.** Number of shocks received during the training phase.

	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. *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

Table S3: GLMMs for the different learning variables. **A.** Preference Index. **B.** number of shocks received by bees per treatment. **C.** Crossing latency. **D.** Speed.

	DF	**F**	**P**
A. *PI ~ treatments + trials + 1|ID*			
Intercept	**1793**	**89.02**	**<.0001**
Treatments	**135**	**3.64**	**0.029**
Trials	1793	0.13	0.721
B. *shocks ~ treatments + trials + 1|ID*			
Intercept	**1793**	**814.72**	**<.0001**
Treatments	135	2.88	0.060
Trials	1793	285.17	**<.0001**
C. *crossing latency ~ treatments + trials + 1|ID*			
Intercept	1793	0.19	0.665
Treatments	135	1.30	0.276
Trials	1793	3.07	0.080
D. *speed ~ treatments + trials + 1|ID*			
Intercept	**3723**	**1815.24**	**<.0001**
Treatments	135	2.09	0.127
Trials	**3723**	**12.13**	**0.0005**

General discussion:

In light of my results (summed up in table 1), here I discuss the causes and consequences of behavioural diversity amongst foragers for the European honey bee, *Apis mellifera* and the buff-tailed bumblebee, *Bombus terrestris,* in the context of an increased exposure to environmental stressors. First, I consider the causes of variability in foraging behaviour in honey bees and bumblebees. Second I discuss our findings in terms of costs and benefits for the colony and the interaction with environmental stress. Finally, I develop some potential future research directions inspired by my work.

How do foragers vary in behaviour? And what makes a good forager?

Several factors influence bee foraging performance. My detailed data on the foraging activity of hundreds of bees suggest the major influences on foraging performance are: foraging experience, morphology, age and exposure to stressors (table 1).

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 5 and 6**, when I looked at the behaviour of bees in the same task, I observed an improvement in performance in successive trials as bees mastered the task. In **chapter 5**, bees were trained to find the most efficient route between artificial flowers; they sped up and made fewer navigation errors with experience (**chap. 5., Fig. 2**). In **chapter 6** control and thymol-treated bees learned to avoid the colour associated with electric shocks, by repeated exposure to the colour associated with shock (**chap 6., Fig 2**).

This beneficial effect of experience on task performance also translated to foraging behaviour. I also found, in **chapter 3**, that consistent with existing literature (Dukas, 2008; Dukas & Visscher, 1994), foraging efficiency for nectar increases with experience (**chap. 3, Fig. 5B**) as bees get faster and better at identifying flowers. I showed in **chapter 2** that pollen foraging is more likely to be performed by more experienced honey bees (**chap. 2. Fig 2**). Thus, differences in foraging efficiency in honey bees seem to be driven by forager experience.

In contrast, I did not find any effect of experience on bumblebee foraging performance for pollen (**chapter 4**). But similar to honey bee foragers, bumblebees increase their nectar foraging intake with experience (Durisko, Shipp, & Dukas, 2011; Peat & Goulson, 2005).

Age

Division of labour in honey bees is linked to the age of the bees (Huang & Robinson, 1996). Each individual starts foraging typically around two weeks after hatching following a period working inside the hive as a nurse bee (Huang & Robinson, 1996). But variability in age of onset of foraging occurs naturally (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 example of precocious foraging induced by miticide in **appendix 2**). Precocious foragers are poorer navigators (Ushitani, Perry, Cheng, & Barron, 2015) and have poor olfactory learning and memory (Cabirol, 2017) than normal age foragers. Precocious foraging can also have dramatic consequences for the whole colony as mathematical models of colony dynamics show that high numbers of precocious bees, as they are short-lived and are less active foragers than normal age foragers, could lead to a colony collapse (Perry, Søvik, Myerscough, & Barron, 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 **chapter 3**. Also, I did not find any effect of age of the first foraging on bumblebees' performance in the navigation assay (**chapter 5**).

Morphology

Honey bee foragers are considered monomorphic and I did not find any indication that a weight difference between foragers affected honey bee foraging activity and performance (**chapters 2 and 3**). By contrast, bumblebee workers vary in size and within a colony of *Bombus terrestris*, some individuals can be three times bigger than others (Goulson, 2010). Size seems to influence foraging behaviour as I found that larger foragers were more likely to perform better at the traplining task (**chapter 5**). I also found that bigger foragers were less likely to collect pollen (**chapter 4**). These results are consistent with many studies showing that larger individuals are more

efficient at foraging for nectar (Goulson et al., 2002; Spaethe & Weidenmüller, 2002; Worden, Skemp, & Papaj, 2005).

Impact of stressors

Exposure to different stressors at different stages of development may shape adult performance. I show that chronic exposure to a miticide during larval development and during the time adult bees spent inside the hive can impact directly honey bee forager's visual learning in **chapter 6**. Rearing environment during young adulthood also influences honey bee cognitive ability (Cabirol, Brooks, Groh, Barron, & Devaud, 2017). Honey bees deprived from the sensory and social stimuli of the hive environment during the early weeks lives are poor performers at a learning task that relies on the mushroom body.

I also demonstrated that, when a bee is stressed with a non-pathogenic stressor at its nurse stage; it is less likely to collect pollen when it becomes forager (**appendix 1**). In the same way, 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 neonicotinoids insecticides collect less pollen (Gill & Raine, 2014).

Consequences for the colony of behavioural variability among foragers

By focusing on foragers' individual behaviour, I asked the question of the role of the individual in the colony entity and what are the costs and benefits of variation in individual actions for the group.

Importance of individual variation for different social groups

It has been argued that social groups become more efficient with a reduction in the variety of tasks performed by any individual and that specialization for few actions profits the whole society (Anderson & McShea, 2001).

When comparing the specialization of honey bee (**chapter 2**) and bumblebee **(chapter 4**) foragers, it appears that *B. terrestris* colonies display a higher proportion of foragers that ever foraged for pollen. This is in line with theories of specialisation with sociality: the more socially advanced (in terms of level of division of labour) the species, the more specialised the individuals (Anderson & McShea, 2001; Fewell, Holbrook, & Kukuk, 2013).

However, because insect societies produce many individuals, several individuals can collectively achieve the flexibility required for a collective task that might be too complex to be achieved by a single individual, such as colony food regulation (Jeanson, Dussutour, & Fourcassié, 2012). Thus, instead of having a range of generalists that might not be as efficient as a specialist for a given task, the colony might benefit from having a diversity of specialists for different tasks, such as pollen *vs*. nectar foragers (**chapter 2 and 4)**, or foragers with variable traplining performance (individuals that are more able to respond to novelty as they change trapline routes often) (**chapter 5**).

Achieving colony nutritional balance

This thesis highlights the importance of individual variability in the foragers as a collective strategy for optimising colony macronutrient collection. In both honey bees and bumblebees, I found two levels of variability in the foraging behaviour, regarding pollen *vs.* non-pollen foraging (**chapters 2 and 4**): at the collective level (inter-individual) and at the individual level (behavioural plasticity). At the collective level, I noticed in both species an uneven distribution of the mixed foragers (foragers collecting both pollen and non-pollen resources) compare to the non-pollen foragers in the colonies. Having different specialisations increases the efficiency of the collective foraging effort.

At the individual level, the mixed foragers displayed a high behavioural plasticity regarding the choice of collected resources. This translates into differences in foraging duration and a specific time of collection between pollen and non-pollen (**chapters 2 and 4**). The plasticity of such foragers towards one or the other resource is beneficial for regulating the nutritional needs of the colony.

Thus, our results, even if they did not address this question *per se*, suggest that diversity and plasticity in foraging behaviour are key elements for the collective regulation of the superorganism's food intake, theorised by collective nutritional ecology concepts (Lihoreau et al., 2014). The colony's needs, such as lack of pollen, are driving the foragers' choice for the resource to collect (Dreller & Tarpy, 2000). Such needs are changeable, and so is the flowering environment bees are feeding on, so behavioural plasticity is needed.

Coping with complex environment

The diversity of foraging behaviours and the individual plasticity that I revealed on several occasions (**chapters 2, 3, 4, 5**) can be interpreted as an adaptation of the superorganism to challenging environments. I discussed in **chapter 1** that, at an individual level, bees show impressive cognitive abilities that allow them to develop plastic foraging activity. The cognitive capacities include memory and learning skills that can lead to potential adaptive behaviour. This is illustrated here in the quantified differences in foraging according to the resource collected: honey bee foragers adapted their weight on departure according to the resource collected and the length of the trip (**chapter 2),** and both honey bees and bumblebees spent more time collecting pollen (**chapters 2 and 4**).

The rest of the thesis addressed this question at a group level. For instance, the existence of several strategies in traplining is an illustration that inter-individual diversity is maintained in a group of *B. terrestris* workers (**chapter 5**). Some individuals will be constantly good at finding a particular efficient route to visit patches of flowers, but then would not be efficient at adapting to a new route if there is a change in the environment (**chapter 5)**. Having several strategies within the same colony might help, at the collective level, to cope with a changing environment.

Behavioural variability as a cause of vulnerability for the colony

I described huge concerns about the impacts the global increase of environmental stressors have on bee cognition and bee species survival and evoked some points of vulnerability (**chapters 1 and 6**).

In the rest of the thesis, I provided insights which indicate that several points of vulnerability can arise from inter-individual variability. First of all, because most of the foraging activity in both species is undertaken by a subset of elite foragers (the keystone individuals, **chapters 3 and 4**), the colony dynamics and nutritional balance can be quickly impaired if this particularly active individuals are lost. The vulnerability due to the risk of losing elite individuals can be balanced out by the fact that the remaining inactive foragers can quickly replace the active ones (Tenczar, Lutz, Rao, Goldenfeld, & Robinson, 2014). But, I demonstrated that the more active, (and thus more experienced) foragers are also those most efficient pollen and nectar collectors. Then, even if elite foragers are quickly replaced, they need time to gain efficiency. Thus chronic stressors can heavily impact colony dynamics.

Because *A. mellifera* colonies have a larger forager population and a larger proportion of active elite bees than those of *B. terrestris*, I suggest that colony resilience in the face of environmental stressors is higher in honey bees than it is in bumblebees. This has been illustrated in field conditions in a study examining colony resilience when exposed to neonicotinoid insecticides. For the same level of exposure to pesticides, bumblebee colonies had a lower survival rate than honey bee colonies (Rundlöf et al., 2015).

Perspectives

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

Integrating environmental and social conditions in the 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, based on individual behaviour and weather conditions. By modelling colony dynamics and fitness, based on the diversity of individual actions, we can potentially create a powerful predictive model of colony dynamics and nutritional regulation. With it, we can have a fine understanding of the dynamics of the superorganism in its environment, which can lead to better conservation strategies and outcomes for social bees.

Importance of variability for the resilience towards stressors

My thesis shows that individual behavioural variation is naturally present in colonies. I suggest that this variation is a way to cope with changing and/or stressful environments. To validate such a statement, I firstly would need to address the importance of foraging diversity regarding stressful environments. Experimentally it would be interesting to artificially build up less diverse colonies (looking at monogenetic honey bee colonies, or monomorphic bumblebee colonies, or excluding elite foragers for instance), and compare their resilience to more diverse colonies.

Then it would be interesting to test whether, under stressful conditions, the superorganism displays of foraging diversity are changed. This could be tested quite

easily by using some experimental stressor (such as the pathogen free stress condition, used in **appendix 1**) on a beehive and measuring the impact of the stressors on the degree of foraging variability. Does a stressed colony adopt more inter-individual foraging profiles 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 dynamic of such plasticity of the superorganism?

Finally, following a similar method, I could examine individual level and see how stressors differentially affect the diverse individual behavioural profiles. What are the different susceptibilities to a given stressors of one or the other behavioural trait? For instance, are the most active bees more vulnerable to stressors, potentially due to the high energetic cost of such an active foraging life?

Importance of foraging diversity for pollination services:

I perceived fine changes in terms of pollen or non-pollen foraging strategies that can influence the flower distribution or be influenced by it. Flowers are spatially and temporally patchily distributed in the environment. The interplay between behavioural variation of individual pollinators and plant distribution can be tested with simple experimental designs. By experimentally increasing or decreasing the patchiness of the environment, we could test the effect of plant distribution on the degree of behavioural variability in social bees.

In the field, the patchiness, incompleteness or idiosyncrasy in pollination are still puzzling growers and pollination biologists (Cunningham, Fournier, Neave, & Le Feuvre, 2016). It is crucial to understand how we can change human practices that influence the floral environment (such as farming) (Garibaldi et al., 2017), in order to get better-pollinated yields. For instance, as we pointed out, only a few individuals concentrate the majority of the foraging effort for the colony. 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. We also recognised a variation of navigation skills in bumblebee foragers that can have different impacts on pollen transportation. Thus, by analysing the foraging dynamics and the individual variability in farming systems of the two most commercially important pollinating bees we can better address recommendations for landscape management (Klein et al., 2007).

Finally, I believe that this thesis, 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 collect more pollen when more experienced (**Chap. 2**)
		Honey bees foragers are more active and collect more nectar with experience (**Chap. 3**)
		Bumblebee foragers are better at traplining with experience (**Chap. 5**)
		Non-treated and thymol treated honey bees are better at associate colour with shock with experience (**Chap. 6**)
	Environmental stressors affect bee cognition	Environmental stressors can directly affect brain functions involved in different bee cognition skills (**Chap. 1**)
		Miticide tau-fluvalinate impairs visual learning (**Chap. 6**)
		Immune-challenged honey bee foragers show changes in biogenic amines concentration in their central brain (**Appendix 1**)
	Environmental stressors affect bee foraging behaviour	Environmental stressors can affect bee homing behaviour and navigation (**Chap. 1**)
		Immune-challenged honey bee foragers collect less pollen than non stressed bees (**Appendix 1**)
	High inter-individual variability in foraging behaviour	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. 4**)
		Some honey bees are more active and more efficient than others (**Chap. 3**)
		Some bumblebees are more active than other (**Chap. 4**)
		Some bumblebees foragers are better at traplining than other (**Chap. 5**)
	Behavioural plasticity	Honey bees take more time for pollen foraging, they also adapt their weight on departure according to the resource collected (**Chap. 2**)
		Bumblebees take more time for pollen foraging and forage more for pollen in the afternoon (**Chap. 4**)
	Difference in morphology	Bigger bumblebees are better at traplining (**Chap. 5**)
		Slightly biger bumblebees forage less for pollen (**Chap. 4**)

Collective level	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. 3**)
		A few individuals (elite bees) performed more trips in bumblebees. (**Chap. 4**)
	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. 4**)
	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**)
		If the elite honey bees are hit by stressors, the whole colony can be impacted (**Chap. 3**)
		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. 5**)

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

article submitted at Journal of Experimental Biology

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Stress decreases pollen forager performance in honeybees

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Abstract

For honeybees foraging is energetically demanding. Here we examined whether stressors which increase metabolic demands can impair foraging performance. A controlled non-pathogenic stressor, which triggers an immune reaction in bees, resulted in a change in the bees' foraging preferences. It reduced pollen foraging, and increased the duration of trips in pollen foragers. Stress also reduced the amount of octopamine in brain of pollen foragers (a biogenic amine involved in the regulation of foraging and flight behaviour in insects). Flight metabolic rate is higher during pollen foraging than nectar foraging, and nectar gives a higher energetic return relative to the foraging effort when compared to pollen. We 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 on both bee foraging behaviour and thereby the nutritional balance of the colony.

Introduction

For honeybees, which are central-place foragers, foraging behaviour places demands on both cognitive capacities (Klein et al., 2017), and metabolic capacity: indeed, insect flight is known to be among the most intense and energy-demanding physiological process in the animal kingdom (Dudley, 2000). The metabolic rates of flying insects, mainly fuelled by carbohydrates, can be up to 170 times higher than resting individuals (Bartholomew and Casey, 1978). As a consequence, it is expected that environmental stressors, 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 higher in nectar foragers than 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, since the majority of larva protein intake comes from the hypopharyngeal gland secretions of nurse bees, and the development of these glands depends on their pollen diet and adequate provision of pollen to the colony (Brodschneider 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), here 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 behavior throughout their life with a Radio Frequency Identification Device (RFID), and a camera at the colony entrance to identify if they carried pollen loads. Finally, we assessed the influence of stress on brain biogenic amine levels, which is known to be involved in the regulation of bee behaviour (Schulz and Robinson, 2001; Schulz et al., 2002).

Materials and methods

Experiments were performed with honeybees (*Apis mellifera*) obtained from the research apiary at Macquarie University (Sydney, 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 3 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 already shown that the bee's immune system is activated by this wounding, 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 release back into their colony. This experiment was repeated 3 times.

Experiment 1: Impact of immune challenge on foraging performance

Following the stress treatment at 7 days, 380 control and 370 stressed bees (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 inside the hive. Each path was made of transparent 1cm of diameter plastic tubing (BUNNINGS ®, Gordon, Australia). To avoid bees using the wrong path, a small plastic gate with plastic bristles, that opened in one direction only, 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. Each time a bee broke the

infrared beam, the linked gates were closed behind the bee for 10 seconds, 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 entering and exiting channels. Each RFID tag (diameter 4 mm; weight 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 and a white LED light enclosed in a plastic box. Motion detection video recording software (Netcam Studio X, Moonware Studios ® and ZoneTriger, Omega Unfold Inc. 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 (in or out of the hive) and time (day, hours, minutes and seconds) were recorded in .csv files. From this 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 seconds before RFID detection were inspected to identify the resource of 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 seconds were not considered as foraging flights and were excluded. As in Perry et al. (2015), trip longer than 8h were also removed. Of the 380 control and 370 stressed bees, a completed 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 the death during its first flight. In total, 979 flights identified as pollen or non-pollen foraging flights were recorded.

Experiment 2: Impact of immune challenge on brain biogenic amines levels

After the stress treatment at day 7, 637 control and 695 immune challenged bees 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 into 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 (VirTis Benchtop^{TM}) 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 Clare, CA, USA), coupled to an electrochemical detector (ESA Coulochem III) connected to an analytical cell (ESA 3011A, Chelmsford, MA, USA). A 100 mm Hypersil BDS octadecylsilane column was used to separate samples (Thermo Fisher Scientific Waltham, MA, USA). Signals were integrated using the Chemstation software with reference to a standard curve obtained from perchloric acid solutions containing 10 pg/µl of dihydroxybenzylamine and varying amount of OA, DA, TYR, 5-HT, (Sigma-Aldric, St. Louis, MO, USA).

In total, we obtained information on brain levels of biogenic amines for 94 control bees (32 with pollen and 62 without pollen) and 50 stressed bees (12 with pollen and 38 without pollen). Tyramine 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 (R Core Team, 2015). For the RFID experiment, the last day any individual bee was detected 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 on 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 assuming a poisson, binomial and gaussian distribution, respectively. To analyse the number of trips and the collected resource, the treatment (stressed 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.

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 the trial was analysed as random factors.

Results

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

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

The best-fit model explaining the variability in the number of trip 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. 2A 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. 2B), 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. 2B), 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 amines levels

Brain DA and 5-HT levels did not differ significantly between treatment groups (ANOVA: $P = 0.67$; $P = 0.14$, respectively) or the collected resource (ANOVA: $P = 0.75$; $P = 0.27$, respectively; Fig. 3A and B). However, we found a significant treatment by resource interaction on brain OA levels (ANOVA: $P = 0.02$; Fig. 3C). No difference in brain OA levels was found in non-pollen foraging bees (Tukey's post-hoc tests: $P = 1$), however when sampled returning to the hive carrying pollen, immune stressed bees had significantly less OA in the brain than control bees (around 27% less, Tukey's post-hoc tests: $P = 0.032$; Fig. 3C).

Discussion

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 empty bees) was observed at the expense of pollen foragers. Since more than 90% of non-pollen foragers are nectar foragers and empty bees (Bordier et al., 2017b) and those 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 bees (Lach et al., 2015). Indeed, compare to pollen, nectar gives a higher energetic return relative to the foraging effort (8:1 gain pollen vs 10:1 gain nectar; Winston, 1987).

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 deficiency may have detrimental effects on brood care, resulting in undernourished larvae (Blaschon et al., 1999). Consequently, nurse bees may also reduce the number of larvae to feed and cannibalize eggs and young larvae (Schmickl and Crailsheim, 2001). Furthermore, pollen feeding during the larva development has an important impact on emerging adults as it may determine its size and life expectancy (Roulston and Cane, 2000; Schmidt et al., 1987), and during adult stage it is essential for stress tolerance (DeGrandi-Hoffman et al., 2010; Di Pasquale et al., 2013; Wahl and Ulm, 1983).

Pollen foraging trips were also 30% longer for stressed bees, suggesting a significant affect of the stressor on foraging capacity. It has been found that the thorax temperature differs between different classes of foragers and ranks pollen > nectar > water foragers (Feuerbacher et al., 2003). Those differences were linked to flight metabolic rate, with pollen foragers exhibiting a 10% higher hovering metabolic rate than nectar foragers, regardless 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, since it is the most energetically demanding resource to collect (Feuerbacher et al., 2003) and the stressor likely decrease the energy budget of bees. 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. These results indicate that OA is as an important mediator of the stress response in honeybees. A previous study reported a rapid decrease in OA and DA but not 5-HT levels in response to stress exposure (chilling anesthesia and vertical spin, Chen et al., 2008). However, we did not find variation in DA levels after this stress exposure.

In conclusion, our study suggests that the highly energy-demanding foraging activity of pollen foragers make them susceptible to stress, even at low levels, which could potentially

impact the colony nutrient balance (pollen vs nectar). Therefore, future studies on whether stress might narrow the colony foraging flexibility to environmental changes might help to better understand colony decline.

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Figure legends

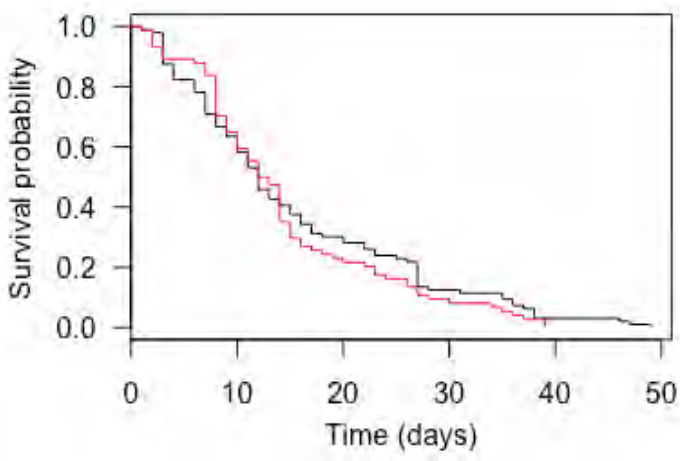
Figure 1. Effect of immune challenge on bee survival probability. Data show survival over 49 days for control bee (black line) and immune challenged bees (red line); day 0 was the day of stress exposure. Bees from the two treatment groups did not differ in survival probability (Kaplan-Meier test, P = 0.42).

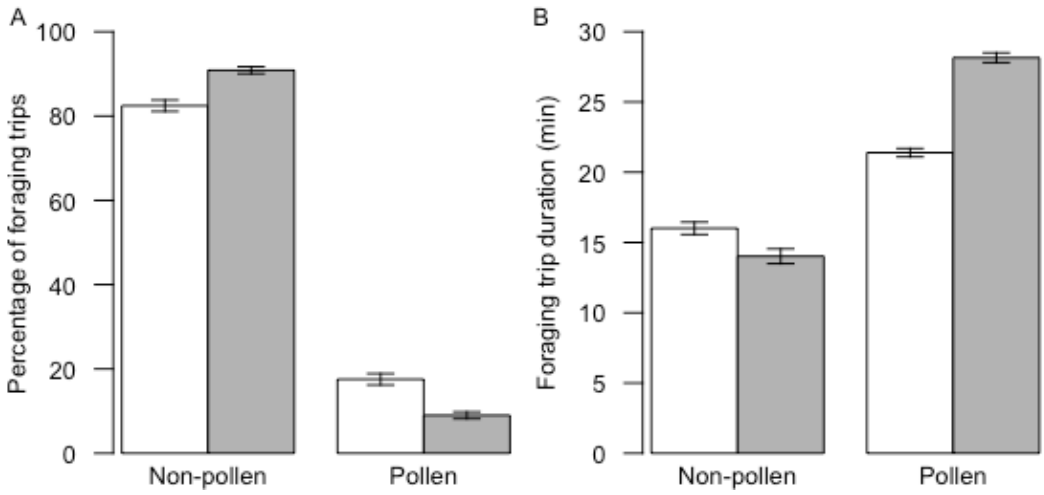
Figure 2. Foraging trip characteristics according to the treatment. Data shown the percentage of pollen and non-pollen foraging trip (A) and the foraging trip duration (B). Mean and 95 % confidence intervals predicted by the model (Table 1) are shown according to the collected resource and the treatment: control (white bars) and immune challenge (grey bars).

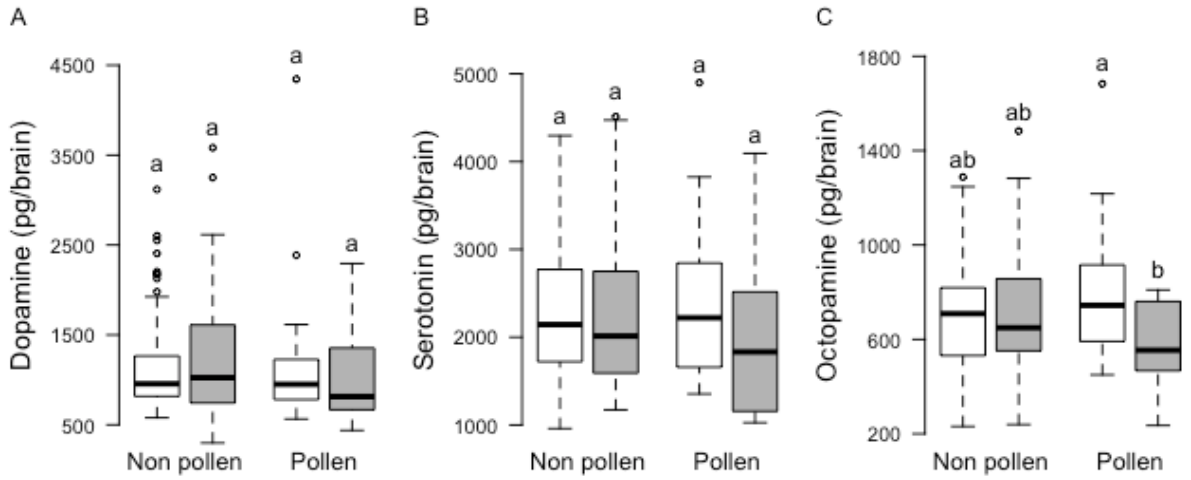
Figure 3. Brain biogenic amines levels in response to immune challenge in different forager groups. The amount of (A) dopamine, (B) serotonin and (C) octopamine is shown for control (white boxes) and immune challenged (grey boxes) bees, and according to the collected resource. Boxes show 1^{st} and 3^{rd} interquartile range with line denoting median. Whiskers encompass 90 % of the individuals, beyond which each outlier is represented by a point. Plots that do not share a common superscript are statistically different (ANOVA followed by Tukey's post hoc comparisons).

Table 1. Summary of best-fit mixed models to analyse the impact of immune challenge on foraging behaviour. 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, number of statistical units, degree of freedom (df) and corrected Akaike's information criterion (AICc) are detailed. For each dependent variable, the selected model, i.e., with the lowest AICc, is indicated in bold.

Dependent variables	Fixed explanatory variables	Random explanatory variables	Number of statistical units	df	AICc
Number of foraging	**Treatment**	Trial	170 bees	**3**	**1594.7**
	Null		belonging 3 trials	2	1606.1
Foraging trip duration	**Treatment * Resource**	Trial/Bee	979 observations	**6**	**9120.3**
	Treatment + Resource		of 170 bees	5	9129.0
	Treatment		belonging 3 trials	4	9148.3
	Resource			4	9130.9
	Null			3	9150.2
Foraging preference	**Treatment**	Trial	170 bees	3	**379.2**
	Null		belonging 3 trials	2	391.3


Survival probability
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
Time (days)


A
Percentage of foraging trips
100
80
60
40
20
0
Non-pollen
Pollen
B
Foraging trip duration (min)
30
25
20
15
10
5
0
Non-pollen
Pollen


A
Dopamine (pg/brain)
4500
3500
2500
1500
500
a
a
a
a
Non pollen
Pollen
B
Serotonin (pg/brain)
5000
4000
3000
2000
1000
a
a
a
a
Non pollen
Pollen
C
Octopamine (pg/brain)
1800
1400
1000
600
200
ab
ab
a
b
Non pollen
Pollen

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).***

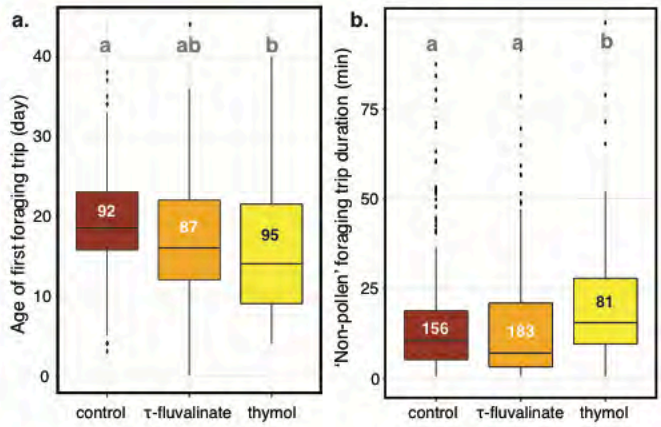

Fig. 1a: Age of the first foraging trip. Kruskal-Wallis test: $\chi^2 = 8.6421$, p-value = 0.013. Post-hoc Conover test: ctl-τflu = 0.124, ctl-thy = 0.011, thy-τflu = 0.314.
b: 'Non-pollen' foraging trip duration. Kruskal-Wallis test: $\chi^2 = 16.8705$, p-value = 0.0002. Post-hoc Conover test: ctl-τflu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

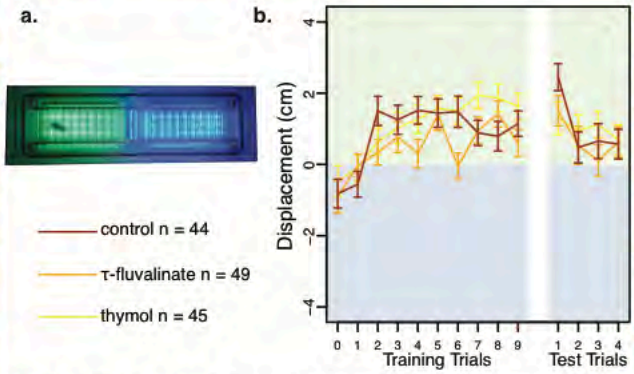

Fig. 2a: APIS chamber. ***b:*** Relative displacement of the bees during the first three sec. after the light is on. Bars indicate ±1 SEs. Mean of displacement: Kruskal-Wallis $\chi^2 = 8.859$, p-value = 0.02. Post-hoc Conover test: ctl-τflu = 0.032, ctl-thy = 0.929, thy-τflu = 0.032.

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.

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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.

Appendix 3 of this thesis has been removed as it contains published material. Please refer to the following citation for details of the article contained in these pages.

Klein, S., Cabirol, A., Devaud, J.-M., Barron, A. B. & Lihoreau, M. (2017). Why bees are so vulnerable to environmental stressors. *Trends in Ecology and Evolution*, 32(4), p. 268-278.

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

Published version of Chapter 5 in Scientific Reports.

SCIENTIFIC REPORTS

OPEN

Inter-individual variability in the foraging behaviour of traplining bumblebees

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Simon Klein[1,2], Cristian Pasquaretta[1], Andrew B. Barron[2], Jean-Marc Devaud[1] & Mathieu Lihoreau[1]

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 (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]), age (e.g. honey bees[17]), physiology and genetics (e.g. honey bees[18,19]), 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]. 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 inaccurate[24]. Foragers also show inter-individual variability in learning performance[22,26] and colonies containing foragers with high visual learning speeds have a higher foraging efficiency[27]. These differences are independent of body size or any other measurable morphological attributes[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

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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]. 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 parasites[40]. 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 ± 48.3% (mean ± 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 ± 3.8% (mean ± SE) the average number of different flower locations they visited per bout (Fig. 2B, Table 1), decreased by 85.3 ± 3.5% (mean ± 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 ± 8.0% (mean ± 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 ± 2.2% (mean ± 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 dataset[44]. 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

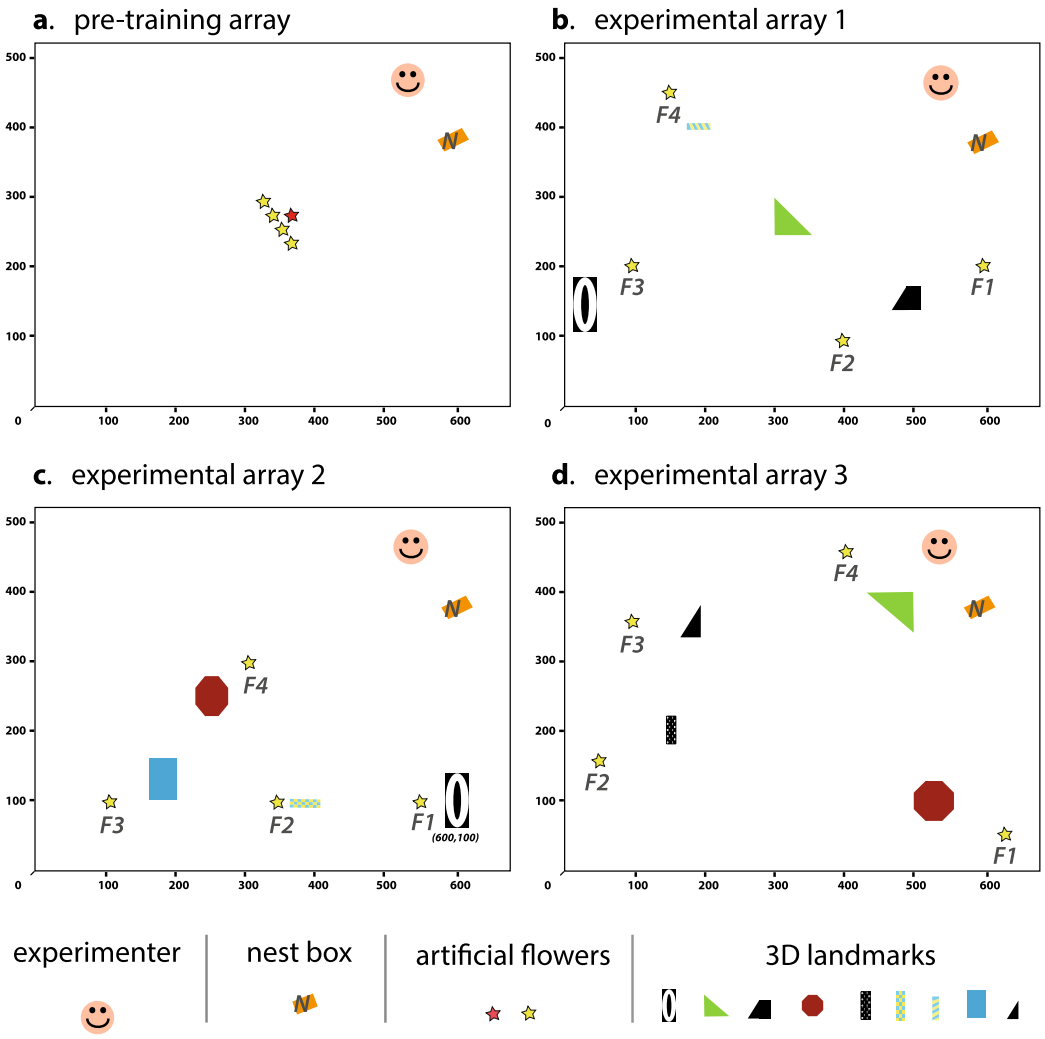

Figure 1. Experimental arrays of flowers and landmarks. (**a**) Pre-training array. Bees were allowed to forage on a pre-training flower (red star) in a landmark-free environment for one hour. A selected bee was then observed foraging on four training flowers (yellow stars) during five foraging bouts 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

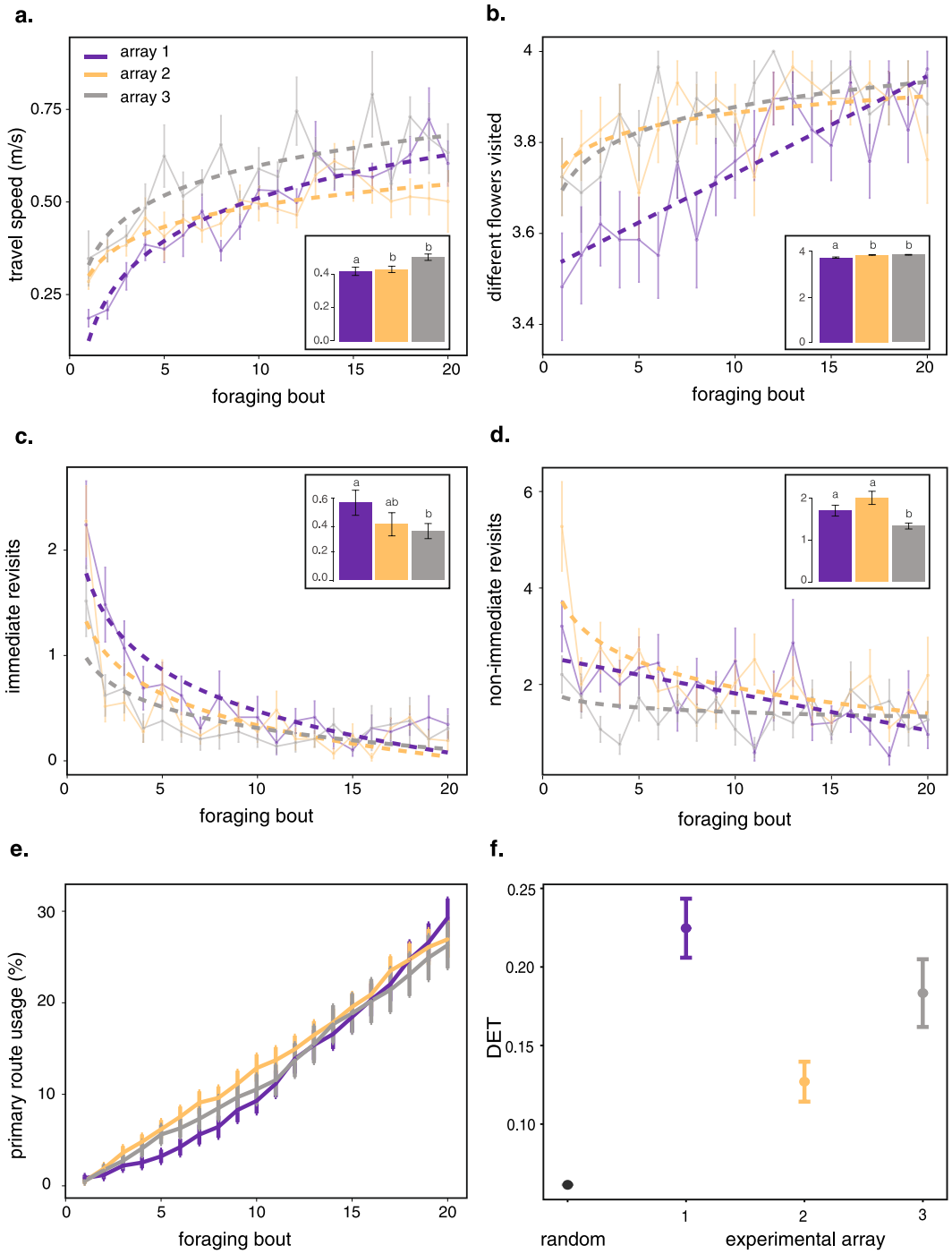

Figure 2. Average behavioural measures in the three experimental arrays (array 1: purple, array 2: orange, array 3: grey, see details of flower and landmark configurations in Fig. 1). (**a**) Travel speed per foraging bout (flight duration divided by the Euclidian distance between all successively visited 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 ± 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 ± SE). (**a**–**d**) Bar plots show means ± 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.

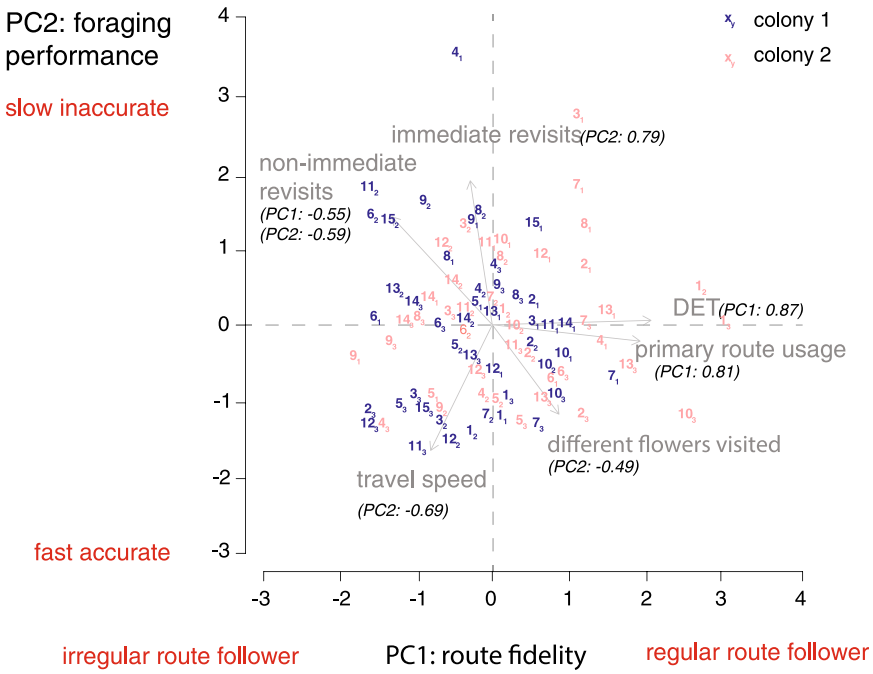

Figure 3. Correlations between the two first components (PCs) of the principal component analysis (PCA). Grey arrows represent the six behavioural measures on PC1 (route fidelity) and PC2 (foraging performance). PC loadings are in brackets. Only loadings >|0.4| were retained (see Supplementary Table 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

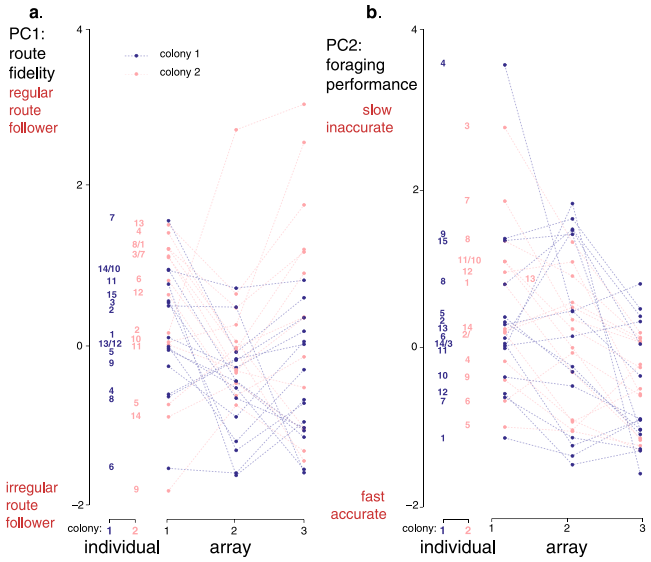

Figure 4. Intra- and inter-individual behavioural variance across experimental arrays. (**a**) Route fidelity (PC1). (**b**) Foraging performance (PC2). Data points connected by a dashed-line represent the scores of the same individual over the three arrays. Blue: colony 1 (N = 15 bees), red: colony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

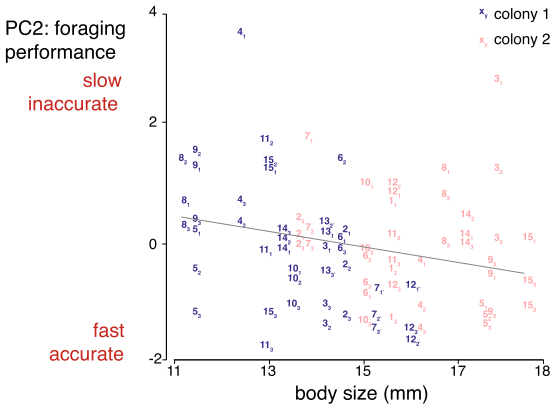

Figure 5. Inter-individual variance in foraging performance (PC2) is partly explained by body size (length from top of head to end of abdomen). Each data point represents the average score of an individual in an experimental array (three values per individual). Blue: colony 1 (N = 15 bees), red: colony 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**)					
Random intercept model PC1					
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**
Random intercept model PC2					
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**)					
Random slope model PC1					
LME_1|colony/ID	7	242.57	−114.29		
LME_0+array|colony/ID	**6**	**235.93**	**−111.96**	**4.64**	**0.031**
Random slope model PC2					
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
Route fidelity (PC1)				
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
Foraging performance (PC2)				
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 animals[45], including insects[21,46–48]. Inter-colonial behavioural variability has been reported previously in bees, (e.g. aggression in honey bees[49] or for both vision- and olfaction-related cognitive tasks in bumblebees[27]) 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 temperature[51] and nutrition[52] 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 adults[53]. Size polymorphism is considered a main factor of caste determinism in bumblebees, such that only the largest individuals tend to undertake foraging the tasks[54]. 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 trips[55], take less time[16] and collect more nectar in natural conditions[16]. Large bumblebees also tend to learn faster in visual discrimination tasks[56]. 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[57]. 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[53]. 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 thermoregulation[59]. 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[60]. 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 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.

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; Fig. 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 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.1.0[64] (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[65]. 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 ± 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 ± 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 package[66]).

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[44]. 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 ± 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 package[66]). To compare the three observed DETs of the same bee (1 per experimental array), we applied a least-square means test (≪lsmeans≫ R package[67]) on a linear mixed effect model (LMM) including the experimental array as fixed effect and individual identity as random effect (≪nlme≫ R package[68]).

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[69]. We then run the PCA function from the ≪psych≫ R package[70] 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[71]. 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.

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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
Academic rigour, journalistic flair

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

Authors

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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

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 loosetrife 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.

Pollution Bees Pollination Honey Pollinator Colony Collapse Disorder Varroa destructor Honeybees

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.
https://theconversation.com/vingt-ans-apres-le-debut-de-leffondrement-des-colonies-comment-se-portent-les-abeilles-78807

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

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C'était il y a vingt ans déjà : les apiculteurs français alertaient sur l'utilisation de pesticides comme le Gaucho, 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 Gaucho, 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.

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 ?

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.

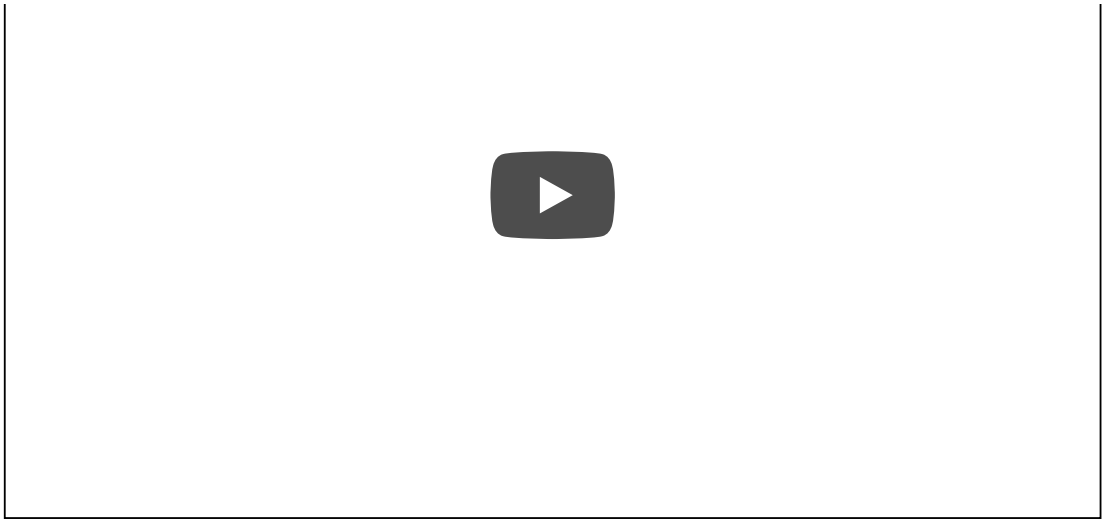
Butiner le pollen, une activité exigeante au niveau cognitif. Simon Klein, CC BY-NC-ND

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 neonicotinoides, 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**