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BEES BEHAVIOURAL PLASTICITY IN A GLOBAL CHANGE CONTEXT

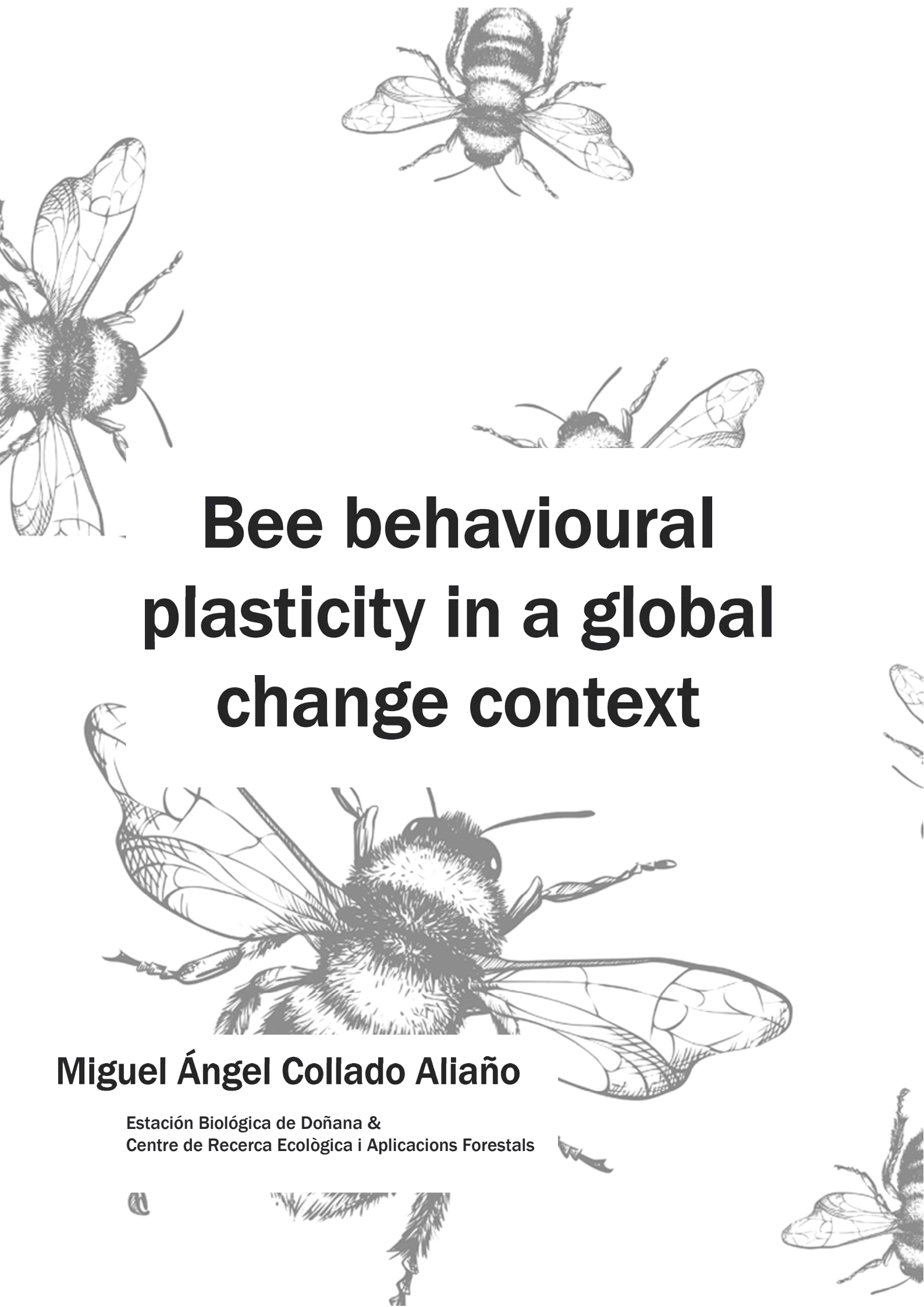
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Bee behavioural plasticity in a global change context

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Prólogo

"Ὁ δὲ ἀνεξέταστος βίος οὐ βιωτὸς ἀνθρώπῳ" *Apología*
"La vida inexplorada no merece la pena ser vivida"

Cita atribuida tradicionalmente a Sócrates, la cual aparece en *Apología* cuando se le dejó elegir entre el exilio y la muerte, y ante la imposibilidad de cumplimentar su cometido vital desde el exilio, eligió la muerte. Dejando un poco de lado las connotaciones clasicistas y de cierta forma anacrónicas que pueda llegar a tener, he decidido empezar mi tesis doctoral con esta cita. Esta tesis me ha llevado mucho tiempo y esfuerzo, pérdidas y ganancias, y en definitiva, exploración. Aún así, aunque esta frase podría servir de justificación para cualquier tesis doctoral, o cualquier investigación, para mi tiene un significado especial.

A lo largo de mi vida, me ha entusiasmado aprender los diferentes mecanismos del funcionamiento de las cosas. El conocimiento de diferentes cosas en su totalidad, ha llegado a obsesionarme en diversas ocasiones. Incluso a frustrarme por aquellas cosas de las que no he podido obtener respuesta, pese a la poca utilidad que pudieran llegar a tener algunas de ellas. Ni la ciencia, la filosofía, o incluso las religiones me han ayudado de forma absoluta a entender mi yo y el mundo que me rodea, pero han podido colaborar en ello. El camino al conocimiento está pre-destinado al fracaso, y este viaje ha sido en cierta parte decepcionante, pero a su vez motivador. A diferencia de lo que sorprendentemente se nos enseña en

las escuelas o templos, he podido llegar a saber y experimentar que el conocimiento humano tiene un límite, un límite en continuo movimiento del que debemos ser responsables de expandir aunque sea mínimamente. Sin embargo, es tremendamente frustrante no llegar a saber si el camino tomado es el correcto. Desde Platón con su mundo de las ideas, Kant con sus noúmenos, Schopenhauer con su voluntad, e infinidad más de científicos y filósofos posmodernos, no sabemos realmente si avanzamos en la dirección de la verdad, o si por el contrario estamos construyendo sobre nuestra percepción, construyendo sobre axiomas falsos castillos en el aire, o ayudándonos a interpretar la realidad mediante los instrumentos científicos, pero nunca llegando a conocer realmente la verdad, suponiendo que su existencia no sea nada más que algo en lo que nos hemos puesto de acuerdo unos pocos. Lo que sí deberíamos asumir, tal y como analizó Karl Popper del trabajo de Sócrates es que, aunque nuestro conocimiento sea finito y vaya en crecimiento, nuestra ignorancia siempre será por necesidad infinita.

Esta continua expansión del conocimiento nos ha llevado a una hiper-especialización, rozando para muchos niveles absurdos, tal y como son las tesis doctorales. Nos encontramos en las próximas páginas un ejemplo más de esta hiper-especialización. Para este caso concreto, estamos trabajando para un fin último "útil" para el conocimiento humano. Nuestro fin último es saber si las abejas, los polinizadores más importantes, pueden ser lo suficientemente fle-

xibles en su comportamiento como para afrontar los cambios rápidos inducidos por el hombre, y los mecanismos que explican y subyacen este comportamiento. Por supuesto, adelanto al atrevido lector que la pregunta no queda respondida a lo largo de esta tesis, pero sí conseguimos avanzar unos cuantos pasos en la evaluación del sistema, pues parece que todo apunta a que algunas especies puedan beneficiarse de los cambios rápidos inducidos por el hombre, aunque lo más común sea que salgan perjudicadas.

Definitivamente, he acabado la tesis con más dudas que cuando la empecé. La duda ante todo está siempre presente, cada paso está lleno de incertidumbre, cada afirmación que hago necesito que esté respaldada por alguna afirmación previa o estudio, y todo esto es muy alientante en la era de la posverdad. La ciencia con la que está trabajada esta tesis, y con ella muchísimas ramas del conocimiento está basada y validada por la estadística. El método científico que utilizamos, intenta encontrar la verdad basando en el análisis y modelaje de pequeños fragmentos de la realidad, con la esperanza de que nos apunten en la dirección correcta, la cual es, la que nosotros hemos hipotetizado.

Sin más divagaciones filosóficas, espero que esta tesis sea de ayuda al lector, o alguno de los capítulos contenidos en ella, en pro de avanzar hacia la verdad, y hacia la conservación del medio ambiente.●

Miguel Á. Collado

General Abstract

Despite bees are, in general, declining due to habitat loss and transformation, some bee species can adapt to live in human transformed habitats. However, we lack enough information on the mechanisms that allow them to survive in transformed habitats (e.g. using new resources, avoiding new threats, etc.). Behavioural plasticity could be a key factor for bees to survive in changing environments, as we expect better acclimation to rapid changes, but there is scarce knowledge on behavioural aspects for insects. To evaluate population stability of bees and their capacity to adapt to new habitats, I combined ecological aspects (habitat use, population trends), with cognitive aspects (learning, innovative-like behaviour), and detailed brain measurements (brain weight). I used both experimental and field data to study bees' plasticity, learning and innovation abilities and use of natural and urban habitats. I found that solitary bees can innovate to cope with new challenges. I also found that some bees are able to use or even prefer urban habitats, and that this preference and their learning abilities are related to their brain sizes.

Experimental approach

I first explored bees' behavioural plasticity through laboratory experiments in chapters one and two. While there have been several experiments with model species such as honeybees and bumblebees, testing complex behaviours like reverse learning and cultural trans-

mission, very few have been done using solitary species, even though 80% of the bee taxa are solitary. I predict that innovation and learning should be important to adapt to new environments, but these are aspects that have been poorly studied for solitary bees. In chapter one, I was interested in evaluating innovation abilities in solitary bees, but also in knowing which behaviours lead to better chances of innovation. To test innovation abilities in solitary bees I chose *Osmia cornuta* as a study system because they have been observed using human-made materials for nesting and they are easy to raise in laboratory conditions. I used a closed experimental arena to perform an assay designed to measure the individual capacities to solve a novel task (i.e. lifting a lid to reach a reward), after learning where the reward was located. I also related innovation to other behaviours: exploration, shyness, activity, and learning, through a battery of previous tests designed to record the selected behaviours. In particular, I measured shyness and exploration by placing the bee inside a cardboard refuge and letting the bee decide to stay or get out and explore the arena. I hypothesize that learning abilities can be important for innovation because to keep an innovative behaviour in their ethograms (inventory of behaviours), it must first be learned. I measured learning by training the bee to associate a colour to a reward and tested if they selected the same colour pattern when there was no reward. The results showed that solitary bees can innovate, as many individuals were able to solve the new task proposed. I found that slow explorers, bold

bees and more active bees were more likely to innovate. More explorative bees were also better learners. However, I could not find a correlation between innovation and learning as I initially suspected.

Learning is, in fact, a fundamental trait for surviving in natural environments and to adapt to new environments. It has been demonstrated already that common managed eusocial bees (i.e. honeybees and bumblebees), can learn in behavioural experiments, but little is known about learning abilities in solitary bees. Therefore, to do a broader study comparing several species, in chapter two, I tested individuals from several bee species captured directly from the field. I designed an experiment to test learning abilities from different bee species and compared learning performance with brain sizes. Brain sizes had already been demonstrated to be relevant for achieving higher cognitive abilities for other taxa, but its role is still unclear for insects, including bees. I built experimental enclosures to measure associative learning in a protocol where the individuals had to learn to associate a reward (food) with a conditioned stimulus (a colour) in a series of learning trials, and then test this association in a learning test. I evaluated the color-reward learning process by means of a conditioned stimulus test, where none of the colours had a reward. Immediately after the tests, brains were extracted to measure brain weight (a proxy of brain volume) and I compared brain sizes with the performance of the learning tests. Despite there were some species that did not react to the experiment as I expected, most bee species were able to learn and those with bigger brains were more likely to succeed in the learning test.

Macroecological approach

In addition to the experimental approach, I focused on documenting potential adaptation to new environments with empirical data in chapters three and four. Despite information about the conservation status of most bee species is actually scarce, we know that the main driver of pollinator diversity decline is habitat loss and transformation, mainly due to human activities. In order to survive habitat alterations, pollinators need to move from one habitat to another or locally adapt to the new conditions. However, little is known about bee habitat preference or avoidance, and habitat importance for pollinators. In chapter three I used a large sampling of more than 400 species along north eastern USA, including sampling from highly modified to more natural habitats, to evaluate habitat use, habitat diversity, rank habitat importance and extract habitat preference for the most abundant species. To that end, I extracted habitat information from GIS raster maps for each of our sampling points and used network analyses, null models comparisons, and beta diversity metrics. I found that habitat importance is negatively related to the level of human modification. Forests were the most important habitat type and urban areas the least important, with intermediate levels of importance for herbaceous, shrubs and cultivated crops. To study more in deep the contribution of habitats to bee diversity,

and to see if the same habitats in different locations have complementary or redundant bee fauna, I studied the diversity within habitats itself (beta-diversity). I found contrasting patterns for different forests types, as evergreen forests seemed to be the most heterogeneous forested habitat, and deciduous forests were more redundant in species composition. Surprisingly, I found a considerable amount of diversity in urban and crop areas. When evaluating the specific habitat preferences for the most abundant species I found that most bees can easily use most habitats, included those highly transformed. Moreover some opportunistic species preferred urbanized habitats. In conclusion, urbanization can have a light positive impact, but just for some species.

Because the reported importance of brain sizes for learning, I wanted to test if brain size is related to species habitat preferences, as I expect that to survive in urban environments the cognitive requirements are higher, because the complexity of these environment. At the same time, I wanted to test if species preferring certain habitats, or with bigger or smaller brains have contrasting population trends. We already know that some insect ecological traits are correlated with their population trends. For example, oligolectic (diet specialists) species have declined faster than polylectic (diet generalists) species, because the absence of their preferred sources of food. However, most species traits studied so far do not capture the behaviour flexibility of the species. It is known for some taxa that the establishment of new populations in new habitats may be related to its relative brain sizes, as it happens for example in birds and mammals. Therefore relative brain sizes may contribute to explain the population trends of a species. In chapter four, I used the data obtained from chapter three on habitat preferences, pre-existing data on bee population trends and a newly developed bee brain database to find a potential correlation between population trends, habitat preference and brain sizes. I found that smaller relative brains were related to forest preference, and bigger relative brain sizes have to urban preference. However, population trends were not correlated with habitat preference or relative brain sizes. Therefore, urban bees are just opportunistic species that can survive in transformed habitats, but not necessarily are increasing its populations. ●

General Introduction

Over the past few years, the concern about global environmental change and its negative consequences for biodiversity has grown among the general population. Science is studying the causes and effects of environmental global change from different perspectives and knowledge branches. Global change is a broad term that covers different phenomena in addition to just global warming – climate change, biodiversity loss, dwindling water resources, habitat transformation, etc. –. Ecology is one of the main branches of science that scientists are using to predict the fate of biodiversity across the globe, and the possible effects of the loss of species and their ecological functions. As a multidisciplinary science, ecology can easily integrate and coordinate the work of other knowledge branches to evaluate the status of ecosystems and predict possible future scenarios. However, this is not an easy task, as ecology, even having a highly intuitive theoretical basis, is in practice highly dependent on observational data, and can be deceptive when we try to find the causality of different ecological observations, with many signals pointing in different directions, making it in general, very difficult to build general rules (Lawton, 1999).

Ecology has been used classically to understand ecosystems, communities, and populations, but for that, it is also necessary to understand the animals' biology, and the mechanisms underlying animal behaviours. For example, understanding animal senses and subjective perception, how information is processed, the

functioning of the brain, innate and learned behaviors, and cognitive faculties are very relevant to ecology. The traditional neurobiological work is usually done in mammals and birds, but curiously, some senses and cognitive abilities that insects use are similar to what we humans use: vision, smell, taste, touch, a precise time sense, distance estimation, measures of direction related to the sun compass and landmarks, and memory for all these features (Kevan & Menzel, 2012). In this thesis, I integrate across disciplines to link neurobiology (the brain structure), cognitive aspects (learning, innovative-like behavior) and ecology (habitat use, population stability) and how all these different approaches converge to study and evaluate the flexibility of adaptation of bees in human-induced rapid changing environments.

The status of global change in pollinators

One of the main concerns in recent years motivated by global change is pollinator decline. Pollinator decline has been so relevant lately that it has even made an important impact on mainstream media (Fig. 1). Pollinators are a key group of species for terrestrial ecology, as they mediate the reproduction of most angiosperms, important primary producers of these systems. Pollinators can belong to a wide range of taxa, including bats and birds but fundamentally insects (Winfree et al., 2011). The most common pattern or image that comes to mind when talking about pollinators for the



Figure 1 | Bees are a trendy issue, and have been a focus of media lately. However the focus has been mainly in the honeybee, ignoring the rest of the species.

non-specialized public are honeybees (usually *Apis mellifera*). Due to its pollination services and honey production, *A. mellifera* is an essential species for socio-economical reasons. However, managed species are not a conservation concern but wild bees are. Most of the 20 000 species of the superfamily Apoidea are solitary and are not managed, suffering worldwide population declines (Michener, 2000; Goulson et al., 2015).

Global change itself another trendy issue in the media. It consists of a group of many events induced by human activities that are happening faster than the natural expectations. Habitat loss is the main driver of pollinator decline, due to land-use changes (Winfree et al., 2011). As a consequence of rapid changes in our landscapes by human activities, natural habitats are being reduced, fragmented and isolated. The original natural habitats are being transformed into cultivated crops and urban areas. Therefore, the loss of food sources and nesting places is drastically affecting the species that are not plastic enough to adapt to the rapid construction of new environments. In fact, over 40% of

Earth's terrestrial surface has already been altered by humans (Ellis et al., 2010). Under these new transformed habitats, their options are reduced to move to nearby natural habitats, adapt to the new environment or just die. We can see the consequences of these alterations through several studies in the last years, pointing out that we are reaching declining levels that should worry us. The IUCN considers that in Europe around 9% of bee species to be threatened and 5.2% are considered near threatened (Nieto et al., 2014). Besides, the population trends for 1,535 species in Europe remains unknown (Nieto et al., 2014), and there is not enough suitable data available to address global pollinator populations (Biesmeijer et al., 2006).

Animals facing new challenges

As a consequence of land use change and landscape transformation, bees need to learn and innovate in order to use new resources, avoid new predators and build nests using new materials. Acclimation to new environments need to be as fast as the landscape trans-

formation. Slow paced evolutionary adaptations driven by natural selection may not be fast enough, as specific traits would be needed to survive in new environments. Therefore, it is usually assumed that flexible, and more innovative species are more likely to adapt to new environments (Lee, 2011). Innovative behaviors have been long studied in animals, for example, the opening of milk bottles by tits in England (Sherry & Galef, 1984) and the discovering of potato and wheat washing by a Japanese macaque and its transmission of the new knowledge to their population (Kawai, 1965). These classic examples show the specific capacity of some species to identify new resources, and how to access to the consumption of those resources. More in deep, innovative behavior and decision making in animals are key processes on the individual level and will affect their survival and reproduction, leading to consequences at the population level. The traditional notion holds that insect behavior is inflexible and stereotypical and that insects are cause-effect mechanisms (i.e. reacting to stimulus in a pre-programmed way of acting), and lacks cognitive-affective components (i.e. use of reasoning and emotionally directed responses). However, insects to survive facing new threats if they were just stimulus-driven animals, as they could not find a way to solve new problems. Under this scenario, only species that are pre-adapted to similar conditions will survive facing human-induced rapid environmental changes. This does not seem to be the case, first because insects are obviously not extinct, and second because insect neurobiology and

cognitive ethology has advanced a lot pointing out that insects might display higher cognitive process than initially thought.

Animals, in general, have been overlooked in their behavior and cognitive capacities. However, complex behavioral traits have been observed in animals. This includes, for example, playing in non-human mammals or face recognition in wasps (Bekoff & Allen, 2009; Tibbetts et al., 2018). Bees specifically possess high levels of cognitive sophistication in the context of navigation, foraging, and social communication. Some examples are learning (Giurfa, 2007), numerosity (Chittka et al., 1995; Dacke & Srinivasan, 2008), novel shortcutting in navigation (Menzel et al., 2005), expectation (Cross & Jackson, 2016) or selective attention (Menzel & Giurfa 2001). However, despite these experiments are being more common nowadays, most of these experiments are done in very few managed and eusocial bees (*Apis mellifera*, *Bombus spp.*), usually bought and born in captivity. There are around 20 000 bee species worldwide from which we do not know much about, with contrasting life histories, such as solitary lifestyles, and we have scarce information about their behavior and cognition despite its relative abundance.

In contrast to task-specialized eusocial bee individuals, solitary bees have many tasks to complete during their lifespan. Eusocial bees are usually organized by castes which specializes in single functions, but in solitary bees, every single female has to do all the tasks: mate, find a suitable place to build a nest, use materials to build

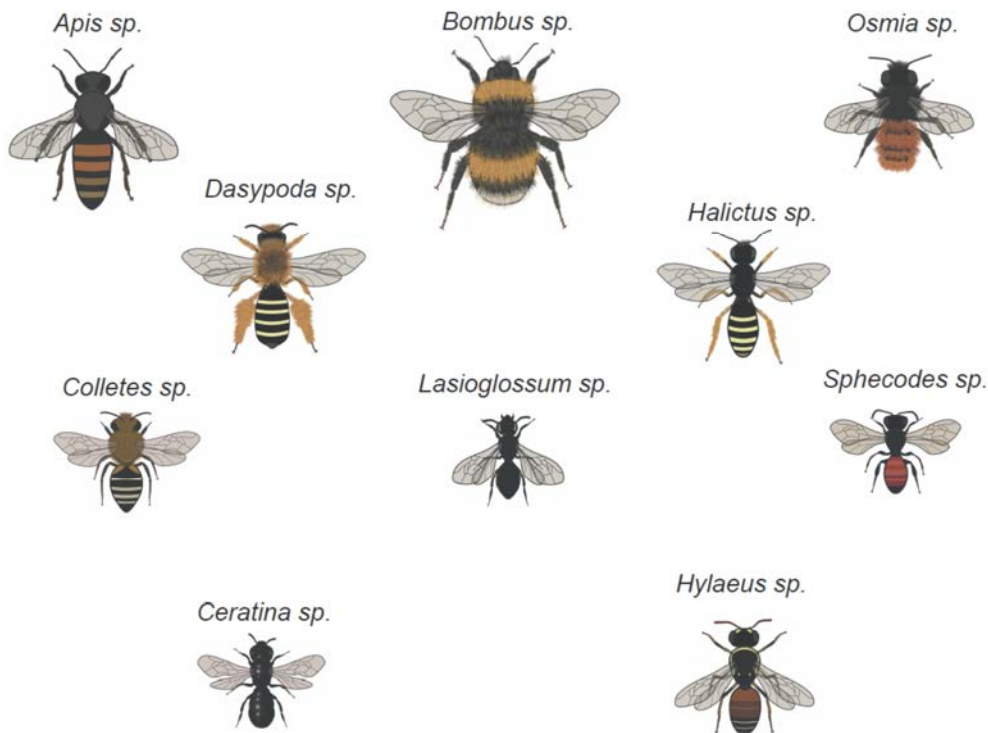


Figure 2 | Bees are very diverse in size, forms, colours, sociality, diet, etc. | Illustrations by Jose Luís Ordoñez

the nest, oviposit, forage for energy, provision the next generation and seal the nest to avoid external disturbance. In fact, bees show a wide spectrum of behaviors in natural conditions. For example, there is some evidence of them being able to use new materials for nest building (Allasino et al., 2019; Mac Ivor & Moore 2013). However, solitary bees had not been used much for experimentation, and innovation abilities are something that has not been tested or studied before. Therefore, we lack information about cognitive abilities in solitary bees to predict how well they will do in new environments.

Neurobiology in ecology

In addition to ecology, neurobiology is also essential to understand more in depth the ecological processes performed by bees. Many processes are connected ecologically concerning learning and memory: senses and processing of sensory information, integration, brain function, innate behaviour, and cognitive faculties that range from simple to complex. How pollinators perceive and interact with their world, like the flowers they pollinate, and how they react to human-induced stressors in their environments such as pathogens, parasites, pesticides, pollutants, and landscape changes are closely interlinked (Straub et al., 2019). The study in depth of bee cognitive abilities and their plasticity to face the new challenges imposed by global change need to integrate neurobiology knowledge and tools. Neurobiology usually study the brain as the focus of their research, and the study of the nervous system and the organization of neural cells into functional circuits that process information and mediate behaviour. Because the brain is the common neural center of most of the Animalia kingdom, we should in principle be able to compare between taxa, however, we find a great variability of neuropils, shapes, and functions.

A usual assumption in neurobiology is that larger brains generate more complex behaviours (Healy & Rowe, 2017). Craniometry and brain sizes have been a main focus of study, getting even some scientist obsessed during the s. XIX, and making these ideas transcending in the s. XX. The theoretical basis of this line of work could be set with Franz Joseph Gall (1758-1828) theories and first works on the size and shape of skulls, where he hypothesized that: (i) the brain is the organ of the mind, and (ii) the size of an organ is a measure of its power (Lamarck's *Recherches sur l'organisation des corps vivants* was already published, with his theories of heritable modification of organs through continued use and loss through disuse). Therefore, (iii) as the skull takes its shape from the brain, the surface of the skull can be used as an accurate index of psychological aptitudes and tendencies (Zola-Morgan, 1995; Lamarck, 1802). There has been a big effort put into the search for correlations between a not always well defined "intelligence" and brain size or cranial measurements. However, the absence of a clear correlation has frustrated several researchers to the point of "faking results". For example, S. G. Morton, guided by his belief that cranial capacity de-

termined intellectual ability, and with a clear racist bias (Mitchell, 2018), claimed in his *Crania Americana* (1839) that the Caucasians had the biggest brains, averaging 1 426 cc, Indians were in the middle with an average of 1 344 cc and black people had the smallest brains with an average of 1 278 cc. These kinds of works led later inevitably to the infamous phrenology (Morton, 1839). However, this work was reported to be done incorrectly, from sampling to statistical analysis (Gould, 1978; Weisberg, 2014).

Despite the focus on the human species, brains have been increasing its size over evolutionary time across birds and other mammals as well. On the other hand, fish and reptiles continue to thrive with brains of very modest size (Lefebvre et al., 2004). Not every brain general enlargement aggregates behavioral traits. Many increases in certain brain areas, especially those involved in sensory and motor processing, only produce quantitative improvements: more detail, finer resolution, higher sensitivity or greater precision. The main paradox of big brains, in a natural selection context, is that they should give notorious advantages, because of the high investment required in energy to make neural tissue.

Despite the focus on the human species, brains have been increasing its size over evolutionary time across birds and other mammals as well. On the other hand, fish and reptiles continue to thrive with brains of very modest size (Lefebvre et al., 2004). Not every brain general enlargement aggregates behavioral traits. Many increases in certain brain areas, especially those involved in sensory and motor processing, only produce quantitative improvements: more detail, finer resolution, higher sensitivity or greater precision. The main paradox of big brains, in a natural selection context, is that they should give notorious advantages, because of the high investment required in energy to make neural tissue.

Therefore, it is not well understood why species with very modest brain sizes are able to do similar, if not, the same tasks than those with bigger brains. Perhaps one of the biggest obstacles to correlate brain size with behavioural ability arises when one considers invertebrates. Many insects use small brains to produce sophisticated behaviors (Chittka & Niven, 2009). Darwin himself compared bees' skills at 'imitation' and 'understanding' with primates (Leadbeater & Chittka, 2007). Moreover, some species use very low neural power to do important tasks, for example, bees use only four neurons to count (Giurfa, 2013; Vasas & Chittka, 2019). Even the smallest nervous systems, such as that of the nematode *Caenorhabditis elegans* with just 302 neurons, are capable of associative learning (Zhang et al., 2005). So, if it is possible to generate a large behavioral repertoire with an insect brain size, then it makes poor sense to our current understanding the investment of bigger brains, given the cost of neural tissue (Attwell & Laughlin, 2001). It is suspected that an increase of neural tissue does not produce more types of neuronal operations or more advanced computations, just more "storage

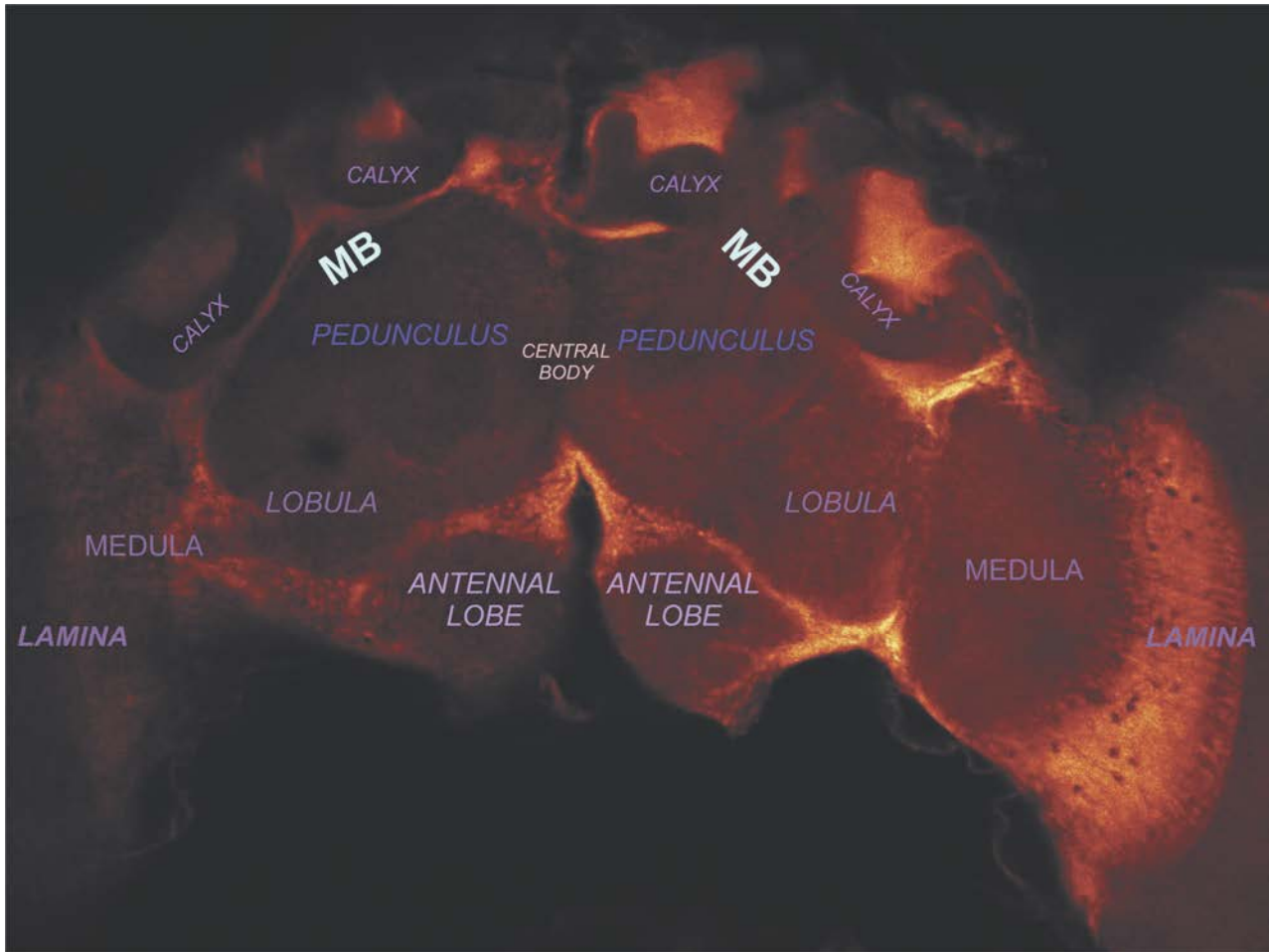


Figure 3 | Confocal microscope image of an *Osmia cornuta* brain. The mushroom bodies (MB), associated with most cognitive abilities are in the upper part of the brain, divided in the upper calyx, and the base, the pedunculus. The central body is placed between both mushroom bodies, and its function is to integrate the information coming from the sensorial lobes. The antennal lobes are in the lower part of the brain and receive sensory information from the antennae. At the lateral extremes we have the optic lobes, divided by the lobula and the medula, and at the end the medula that is in contact with the compound eye.

capacity" (Chittka & Niven, 2009). However, more information stored might enable an animal to generate more and better novel solutions to a problem. This raises the possibility that maybe insects can solve complex problems using simpler information processing principles than animals with bigger brains to face the same problems. Moreover, maybe it is not the brain but the density of neurological connections (Chittka & Niven, 2009). Physicists have even suggested that some functions of the brain do not work like a computational network, but like a quantum field (Hameroff & Penrose, 2014).

One possible solution about the problem of understanding the brain cognitive abilities is that maybe it is not having big brains what matters, but big brains relative to its body size (i.e. encephalization). In some taxa, cognitive abilities like learning or innovation frequency have been correlated with encephalization as well as with the size of neuropils involved (Lefebvre *et al.*, 1997; Reader & Laland, 2002; Clayton & Lee 1998; Clayton & Crebs, 1995). For example, in birds the innovation rate is correlated with the hyperstriatum ventrale and

neostriatum volumes and in primates with the isocortex (Lefebvre *et al.*, 2004; Emery & Clayton, 2004; Pearce, 2014). In insects, memory retention was found to be related to intraspecific brain enlargement in parasitic wasps (Van der Woude *et al.*, 2018). However, it is still unsolved whether bigger brains are related to better performance.

The bee brain

The insects' homonymous neuropil of the hyperstriatum ventrale and isocortex from avian and primates respectively are the mushroom bodies (MB), originally described by Dujardin for bees and ants and called "organs of intelligence" (Dujardin, 1850). The mosaic brain hypothesis (see below) assumes MB sizes to be important for better cognitive abilities. Mushroom bodies seem to be related to insects' complex behaviors, as they provide insects with basic and advanced cognitive capacities (Giurfa, 2013). The MB are a complex morphologically diverse sensory integration neuropil centers composed by a synaptically dense region but containing a relatively low

number of cells (Menzel & Müller, 2003; Strausfeld et al., 1998; Strausfeld et al., 2009; Zars, 2000; Gronenberg, 2001; Fahrbach, 2006). They are normally a pair of discernible neuropils separated visually from the rest of the brain but still interconnected with projections both inside and outside the brain (Zars, 2000; Fig. 2). These neuropils comprise thousands of densely packed parallel neurons called Kenyon cells. Kenyon cells often form cup-shaped protrusions called calyces, ending in two lobes pointing in orthogonal directions (Heisenberg, 1998). The calyces are structures of three vertically stacked rings: basal, collar and lip (Rein et al., 2002). Mushroom bodies are centers that participate in an array of higher-order functions including olfactory associative learning and processing, spatial learning, orientation, memory, sensory integration and attention (Heisenberg, 1998). However, they are not the only area of the brain to process behavioral inputs, as neural correlates of attentional processes have been found in other regions of the fly brain (Seelig & Jayaraman, 2015; Weir et al., 2014; Perry et al., 2017). Mushroom bodies are common for several invertebrate groups, as chelicerates, diplopods, chilopods, and some non-insect hexapods, as well as in three other invertebrate phyla, the Onychophora, the Annelida, and the Platyhelminthes. However, it seems that MB arose independently more than once (Strausfeld et al., 1998).

Honeybees have big MB and receive olfactory and visual information (in MB calyx lip and MB calyx collar respectively, Gronenberg, 2001; Groh et al., 2014). There is variability in size depending on the role of the individuals in social Hymenoptera. Honeybee drones have smaller MB but larger eyes and optic lobes, useful for catching fertile queens (Jonescu, 1909). Queens in large honeybee colonies have smaller MB than workers, maybe because of the limited functions they achieve, which are mainly reproductive tasks for the majority of their lives (Roat & da Cruz Landim, 2008). However, this is not maintained in other bees as queens had larger brains and MB calyces in *Megalopta genalis* (Jauman et al., 2019). In solitary bees, every single female needs to cope with several different tasks: nest building, defense, reproduction and foraging on its own. The relationship between the size in the brain centers and sociality is unclear in these lineages, the acquisition of large brain centers long before the behavioral innovation of sociality suggests that maybe is a pre-adaptation rather than a consequence of sociality (see social brain hypothesis below). It has been proposed that parasitoidism, not sociality, is associated with the evolution of elaborate mushroom bodies in the brains of Hymenoptera (Farris & Schulmeister, 2010).

The functioning of the mushroom bodies is not fully understood and their importance, for example, for visual tasks is still in discussion. According to some previous studies that inhibited them, they are not necessary (Heisenberg et al., 1985; Wolf et al., 1998; Tang and Guo, 2001). But other studies defend the visual information is processed in the MB. Clearer is the function in olfactory conditioning

and learning (Barth & Heisenberg, 1997; Liu et al., 1999; Brembs & Wiener, 2006; Van Swinderen et al., 2009), and also in gustatory conditioning (Masek & Scott, 2010; Keene & Masek, 2012). This connection is a synaptic complex called microglomeruli (Gronenberg, 2001), and moreover, it is known in ants and bees that a high level of plasticity of microglomeruli is associated with associative learning and long-term memory (Farris, 2016). Insect brains are complex organs that we still do not understand, but there are some theories that try to explain its size, functioning and composition.

Brain sizes theories

The mosaic brain hypothesis claims that not a general enlargement of the brain, but parts of the brain should improve the abilities associated with that specific part of the brain. In the case of MB sizes, they are suspected to be plastic and influenced by learning and the environment. An interesting experiment was done by Barth and Heisenberg, where they put during four days a group of flies in total darkness and another group in constant light. The ones reared in constant light increase the volume of their mushroom bodies up to 15% compared to the ones in total darkness (Barth & Heisenberg, 1997). In honeybees, worker bees have bigger mushroom bodies because of the big variability of tasks they do, as forage, construct, defend, etc, and bees calyces volume changes in association with behavioral development, foraging and visual experience (Withers et al., 1993; Withers et al., 1995; Fahrbachand & Robinson 1995). Another big factor affecting the individual size of MB is temperature during the larval development (Steigenga & Fischer, 2009). The temperature experienced during development might affect the final structure of the adult brain, but the temperature of their brood is effectively regulated, maintaining nests temperature in a range of 33-36°C (Himmer, 1927; Jones et al., 2004; Jones & Oldroyd, 2006). This is because different temperatures have negative effects like mortality, malformations, irregular brain structure (MB calyx neuroarchitecture or synaptic organization) and deviations in behaviour, learning and memory (Himmer, 1927; Radmacher, 2011; Heisenberg 1998; Strausfeld et al., 1998; Strausfeld et al., 2009). Therefore, under the mosaic brain consideration, bigger MB sizes should improve cognitive abilities. However, we have examples pointing opposite directions. The biggest MB are found in the living fossil horseshoe crabs, described by Pandazis (1930) as animals' with "low level of spiritual life". On the other hand, species with smaller MB size, like bees themselves, can do some sophisticated cognitive tasks in laboratory environments, like innovation and cultural transmission (Perry et al., 2017; Alem et al., 2016).

The social brain hypothesis is a established hypothesis articulated for vertebrated animals, studied mostly in apes, that assumes that greater brain investment is necessary for more complex social relationships (Dunbar, 1998). However, this hypothesis hardly holds for social insects (Farris, 2016). In social insects, especially in Hyme-

noptera, sociality does not mean always more individual behavioural repertoires and it is more associated with small variations in the size of the MB (O'Donnell et al., 2015; Farris, 2016). Adaptations of the insect MB are more reliably associated with sensory ecology (selection for novel visual behavior, spatial learning, etc) which led to the acquisition of novel visual inputs, than social behaviour. Sociality and eusociality in insects were selected for very different individual behavioral repertoires compared to primates (Gronenberg & Riveiros, 2009; Dunbar & Shultz, 2007). There is an alternative model for the social brain hypothesis. The distributed cognition models assume group members can rely on social communication instead of their own cognition, and this predicts reduced brain investment in social species. An example of testing this was done in Vespidae by Sean O'Donnell et al in 2015, where a large study of 29 species of wasps, ranging from solitary to highly eusocial, showed that the largest MB calyces were found in solitary species, so MB investment decreased from solitary to social species in this case.

The relationship between the structure of the brain and its functions in different species has always been one of neurobiology main questions. Comparative studies in vertebrate and invertebrate brain anatomy have been done to check differences in ecology and behaviour (Stöckl et al., 2016) but this has not been done for bees. Central nervous system and nervous tissue is one of the most metabolically expensive, so its size is limited by production and maintenance cost and evolutionary investments that enlarge brains must conclude in some type of improved behavioural performance (Navarrete et al., 2011; Niven & Laughlin 2008; Laughlin, 2001; Aiello & Wheeler 1995). This metabolic cost must have biological sense and ecological consequences, providing advantages. I am exploring some of these issues in this thesis. ●

Study system

In this thesis, we focused exclusively on bee pollinators (Anthophila). Bees are a wide and diverse group of insects (~ 20 000 species), with many different life-history traits and strategies. The most popular bees are social bees, which are organized as macro-organisms, with different castes within the colonies, but ~ 80% of bees are solitary, meaning that one single female has to face all the functions of foraging, nesting, mating, etc. Bees are a good study system to focus on because they contain some model species that have been studied extensively (*Apis mellifera*, *Bombus sp.*) but most species remain unexplored with a lot of potential research value. Bees are a focus of conservation studies because they are the main players of the pollination ecological game, therefore they are invaluable ecologically, and also economically in agricultural production. While a few social (and very few solitary) species are managed and sold commercially for agricultural production, a large fraction of bee species are in danger. ●

General objectives

To study the behavioural plasticity of bees and assess their responses to the present and future environmental changes. To that end, we combined an experimental and macroecological approach while taking into account species neurobiology. In particular I aim to:

- Describe and understand bee behavioural plasticity: First, we want to characterize which species can live in transformed habitats such as urban environments. Despite the importance of bee pollinators, little is known about their habitat preferences. Second, we aim to understand what drives new behaviours. Innovation is thought to be a fundamental plastic behaviour in a global change context, but why some individuals are more innovative than others is not well understood.
- Find the mechanisms that explain bee brain size. I focused on brain size as has been hypothesized to be linked to plastic behaviours. First, we aim to test if bigger relative brain sizes can explain species learning abilities. Second, we want to understand if species living in urban environments require from larger brains. ●

Innovation is driven by exploration, shyness and activity levels in a solitary bee

Abstract

Behavioural innovation is the process through which animals adopt a new behaviour or apply an already known behaviour in a novel situation, and is widely considered an important mechanism by which animals respond to rapid environmental changes, including those induced by human activities. Despite its functional and ecological relevance, much of our current understanding of the innovation process comes from studies in vertebrates. Instead, our understanding of the innovation process in insects has lagged behind, partly because they are not perceived to have the cognitive machinery required to innovate. This perception is however challenged by recent evidence demonstrating sophisticated cognitive capabilities in insects despite their small brains and reduced number of neurons. Here we explore the innovation capacity of a solitary bee (*Osmia cornuta*) in a laboratory setting. We used an experimental arena to assess the performance of naïve individuals in a battery of tests designed to measure innovation, learning, exploration, shyness and activity levels. We found that solitary bees can innovate, with 21 of 58 individuals (36.20%) being able to solve a new task consisting in lifting a lid to reach a reward. The propensity to innovate was uncorrelated with learning capacities, but increased with exploration, boldness and activity. These results provide solid evidence that non-social insects can innovate, and highlight the importance of interpreting innovation in the light of both cognitive and non-cognitive processes. ●

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Introduction

Animals exhibit an extraordinary wide repertoire of behaviours. Bees, for example, have developed a broad repertoire of sophisticated behaviours that facilitate foraging, nesting, navigation, and communication (Gould & Gould, 1988). Although the ecological and evolutionary importance of behaviour is widely recognised, our current understanding of how new behaviours emerge is insufficiently understood. Some simple behaviours have a clear genetic basis, and hence may have been acquired through mutation and natural selection. Studies in *Drosophila* show, for example, that a mutation in a single neuropeptide caused several abnormalities on their behavioural circadian rhythms (i.e. biological clocks, Renn et al., 1999). However, the accumulation of mutations seems insufficient to understand the emergence of complex behaviours arising from the combination of different types of behaviours. Rather, the emergence of these novel behaviours also require learning, that is, the process of acquiring new knowledge by means of experience to guide decision-making (Dukas, 2012). The emergence of new learnt behaviour is a process known as behavioural innovation (Ramsey et al., 2007; Lefebvre et al., 2004; Reader et al., 2003; Sol 2003; Lefebvre et al., 1997). Although initially learnt, innovative behaviours can later become genetically encoded through genetic assimilation (Price et al., 2003; Cripso et al., 2007).

The concept of innovation has attracted considerable interest of researchers for its broad implications for ecology and evolution (Ramsey et al., 2007; Lefebvre et al., 2004; Reader, 2003; Sol, 2003). Innovating designates the possibility of constructing plastic behavioural responses to novel ecological challenges, thereby potentially enhancing the fitness of animals when exposed to unusual or novel situations. For instance, evidence is accumulating that innovation abilities enhances the success of animals when introduced to novel environments (Sol et al., 2005). By changing the relationship of individuals with the environment, innovative behaviours also have a great potential to influence the evolutionary responses of the population to selective pressures (Lefebvre et al., 2004; Reader et al., 2016). Hence, in a global change context, innovative behaviours are considered central to understand how animals will respond to rapid changes induced by human activities.

While innovation is considered one of the main processes behind the emergence of novel behaviours in vertebrates (Reader, 2003; Ramsey et al., 2007), the relevance of innovation is currently insufficiently understood in insects. The traditional notion holds that insect behaviour tends to be relatively inflexible and stereotypical, a perception that partially arises from their small brains and reduced number of neurons (Dukas, 2008). Such a belief is however changing as evidence accumulates of unsuspected sophisticated capabilities that transcend basic forms of cognition, including rule

learning (Giurfa et al., 2001; Gil et al., 2007), numerosity (Chittka et al., 1995; Dacke & Srinivasan, 2008), development of novel routes and shortcuts while navigating (Menzel et al., 2005) or attentional learning (Menzel & Giurfa, 2001). The fact that insects exhibits sophisticated cognition (Dukas, 2008), suggests that new behaviours may also be commonly acquired through the process of innovation.

Here, we address the critical questions of whether insects are capable of innovate and how they achieve it. We used a solitary common bee - *Osmia cornuta* (Megachilidae) - as a model system to address these questions. While our current understanding of cognition in solitary bees is limited in comparison to that of eusocial species (Chittka & Thompson, 2005), they are also easy to rear and manipulate in captivity. An advantage of solitary bees is that they can be tested individually for innovative propensity without having to consider the pitfall of separating individuals from the social group. Importantly, solitary bees compose most of the bee fauna and are suffering worldwide population declines associated with rapid human-induced environmental changes (Goulson et al., 2015), posing at risk the essential pollination services that they provide for cultivated crops and wild plants. Thus, there is an urgent need for understanding whether and how they are capable of innovate to cope with rapid human-induced environmental changes.

The capacity to innovate is difficult to measure directly (Lefebvre et al., 2004), but one widely adopted approach is the use of problem-solving experiments motivated by a food reward (Bouchard et al., 2007; Griffin et al., 2014). In our experiments, we exposed naïve *Osmia* to a novel task consisting in lifting a lid to reach a food reward, an essay that mimics the encounter of a new complex flower. Whether or not individuals solve the task and the latency in doing so can be used as measures of innovation performance (Sol et al., 2011). Because some bees were capable of innovate, we investigated the underlying mechanisms. We first explored whether the propensity to innovate reflects a domain-general ability to learn. Hence, we related our measures of innovation performance to measures of performance in an associative learning test. Next, we tested the effect of a number of emotional and state-dependent intrinsic features that are suspected to either facilitate or inhibit innovation (Reader et al., 2003; Houston & McNamara 1999; Sol et al., 2012), including exploration, shyness and activity levels. We finally considered whether problem-solving ability might be explained by sex, an additional state-dependent intrinsic feature (Houston & McNamara, 1999). In *Osmia*, females are more involved in parental activities (e.g. are in charge of all nest provisioning activities) and are typically larger than males (Bosch, 1994). These fundamental differences in the biology and ecology between sexes are expected to affect how they deal with novel challenges, potentially affecting their problem-solving ability. ●

Material and methods

Study subjects

Osmia cornuta cocoons were bought from the company WAB-Mauerbienenzucht (Konstanz, Deutschland) and kept cold at 4°C. Prior to the experiments, cocoons were put in 15 ml falcon tubes in a pitch black environment and kept in an incubator at 26°C for 24-48 hours until the emergence of offspring. In total, 101 females and 42 males were born, and used in the experiments. In order to force bees to walk instead of flying, we anesthetized them with a cold shock treatment and cut their right wings (Crook, 2013).

Experimental device

We conducted the experiments in a controlled environment laboratory at the Institut für Biologie-Neurobiologie (Freie Universität Berlin) from February to April 2017. Behavioural essays were conducted in a composed experimental device with two parts, the “arena” (Fig. 1a) and the “dome” (Fig. 1b). The arena was a 30 x 30 x 10 cm empty methacrylate rectangular prism with no roof, containing a grey cardboard as floor and sustained over a wood structure. The dome was a dark brown upside-down plastic flowerpot, illuminated homogeneously with attached LED lamps. The dome covered the arena to create a controlled environment for the experiments. We attached different geometrical patterns in the inside walls to facilitate the orientation of the bees during the tests (Jin et al., 2014). The dome had a hole in

the roof to attach a video camera to record the tests. Citral odour was perfused evenly and restored regularly, as it is known to stimulate bumblebees, and probably other bees, during foraging (Lunau, 1991; Shearer & Boch, 1966).

Experimental protocol

Along three days, each individual passed a sequence of five behavioural essays (Fig. 1 c, d, e, f) of 15 minutes each designed to measure five different behaviours: exploration, shyness, activity, learning and innovation (see Table 1). We waited four hours between trials if the next trial was done the same day and around 16 if the next trial needed to be done the next day (Fig. 1 c, d, e, f). Activity, measured as the time in movement, was measured for every trial (Table S1). Individuals did not show any correlation in their activity levels along the trials (Fig. S1) and therefore, we did not estimate a single average activity value for each individual. Activity levels did not decrease along the trials (LM Activity ~ Trial, Estimate ± SE = 0.003 ± 0.008, p = 0.718). Note that not every bee survived to perform all the essays; only 45% of the individuals that started the experiment reached the final essay. Although individuals were not fed during the experimental process other than during the trials, the lack of correlation between eating events and moving activity in the leaning test (Pearson correlation = -0.09) or in the innovation test (Pearson correlation = -0.01) suggests that this high mortality is not attributable to starving.

The first essay aimed at measuring exploration and shyness. The arena included four coloured cardboard cues (two blue and two

Table 1 | This table contains all variables measured during the tests.

Behavioural component	Behavioural variable	Essay	Behavioural variable
Shyness	Time spent in the refuge	1	Initial time spent inside the cardboard refuge once the essay started
Exploration	Exploration success	1	Touching the four cardboards during the 15-min of the essay
	Exploration time success	1,5	Latency to touch the four cardboards in essay 1 and to touch the lid covering the reward in essay 5
	Re-exploring the refuge	1	Re-entering the refuge after exploring the arena, coded as yes or not
Activity	Activity time	1-5	Time moving or cleaning themselves, measured as the proportion of the time being active
Learning	Learning success	4	Choice of the correct cue
	Learning time success	4	Latency to make the correct choice. Bees that failed to achieve this were assigned the maximum time possible
Innovation	Innovation success	5	Success to lift the lid and reach the reward
	Innovation time success	5	Latency to open the lid and reach the reward. Bees that did not do resolve the problem were assigned the maximum time possible

yellow, Fig. 1c). The bee was placed in a little cardboard refuge and was kept inside for five minutes to allow habituation. Next, the refuge was opened and the individual was allowed to explore the arena. To quantify exploration, we recorded whether the bee explored all the cardboards during the essay and the time it took to do so. Shyness was measured as the time spent inside the refuge (Table 1). Re-exploring the refuge was originally thought to be a descriptor of shyness, however the analysis of the videos showed that bees did not re-enter the refuge to stay inside and hide, but rather did it as part of their arena exploration.

The second and third essays were the learning essays, where we trained bees to associate a colour with a reward (Fig. 1d). We displayed two cardboards cues with sprues on it, one rewarded with 50% sucrose solution and the other empty. We chose blue and yellow because they are easily discriminated by bees (Jin *et al.*, 2014). The reward for each individual was randomly assigned to

one of the two colours. The position of the reward was also randomly assigned for each individual.

In the fourth essay, the learning test, we tested if individuals had learned to associate colours with rewards as trained. The test consisted of both cues displayed as in the second and third essays, but this time with both sprues empty (Fig. 1e). We measured if the individuals approached to the formerly rewarded coloured cue and quantified the time spent until checking the right feeder. To ensure that bees had learned to associate colour and reward, we broke the association between colour and reward from the training essays for 67 randomly selected individuals from a total of 143 (control group, hereafter).

In the final essay, we measured the propensity for innovation by using the same coloured cue and reward combination as in essays 2 and 3, but this time the sprue containing the reward was covered with a cardboard lid (Fig. 1f). Bees had thus to innovate

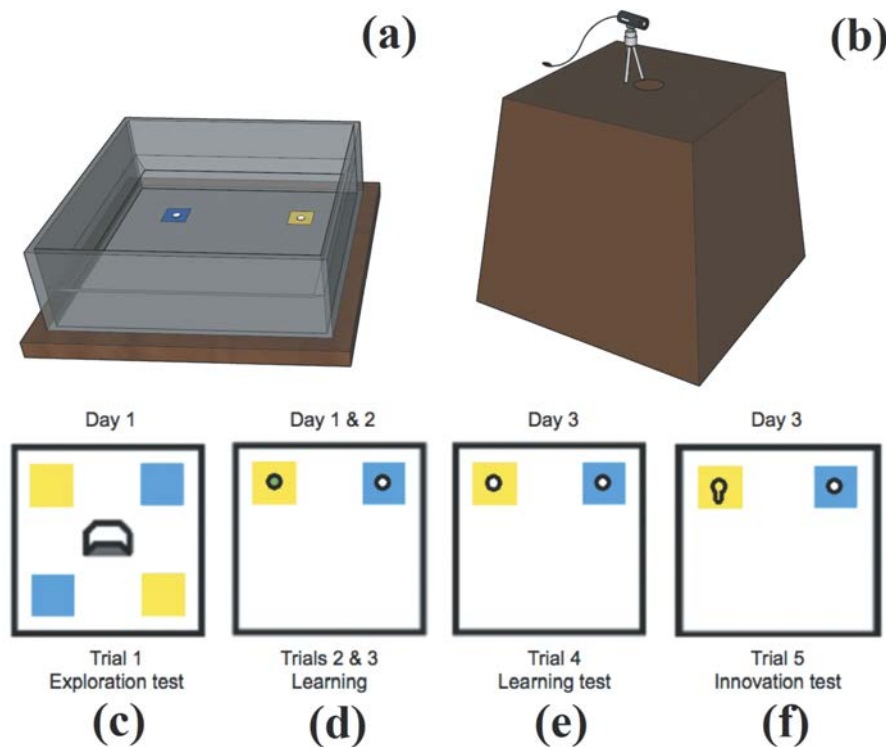


Figure 1 | The experimental arena (a) laying in a neutral grey ground, surrounded by plastic walls with plastic cornices attached to avoid escaping. It was covered by the dome (b) with a landscape pattern glued inside and citric odour, also a webcam was placed in the ceiling to record all the experiments. The experiment had four different displays. In essay 1 (c) we put the bee in a refuge, and start counting the time after opening the door. The aim of the essay was to see whether the bee stayed in the refuge (as a shyness proxy), and/or explored the color cues around. Touching all the cues was considered a success in exploration test. In essays 2 and 3 (d), we displayed the bee two sprues over cardboard cues, one rewarded and the other was empty. The colour was randomly selected but maintained along the essays. Essay 4 was the learning test (e), the display was the same than in essay 2 and 3, but this time we removed the reward and the sprues were empty. We checked if the individual remembered correctly where the reward was. Essay 5 was the innovation test (f), the display was the same than in essay 2 and 3 as well, but this time we covered the reward with a lid, forcing the bee to innovate and figure out how to lift the lid to get to the reward below. We only show treatment experiments, control experiments are defined in methods.

-i.e. lift the cardboard- to reach the reward. Innovation propensity was measured in terms of test success and latency to succeed (Table 1). Control bees used in the learning essays were not tested for innovation.

Data analysis

We modelled problem solving performance in the innovation essay as a function of learning, shyness, exploration, activity and sex (see Table S1 for definitions, and the supplementary material for the validation of the variables used as predictors). We modelled the success or failure in solving the task using a generalized linear model with a binomial error and logit link. To model the latency to solve the task, we instead used survival analysis based on cox proportional hazards regressions when the predictors were continuous (Cox, 1972; Table 2).

In order to avoid model over parametrization, we first explored each response variable against all predictors (shyness, exploration and learning) along with activity and sex as co-variables (See supplementary material). For innovation, time variables were the most explanatory along with activity, but sex was not explanatory for any model. Hence, we built a multivariate model with time to exit the refuge (shyness), time to explore (exploration), time to learn (learning) and activity. In contrast, for learning none of the behavioural variables were explanatory (but see results about re-exploring the refuge in supplementary material), but sex was. For consistency sake, for learning we built a multivariate model with

latency to exit the refuge (shyness), latency to explore (exploration) and sex. Activity was measured in all essays, we found no correlation between the bees activity along the different essays (Fig. S1). ●

Results

Our experiments showed that *Osmias* were able to innovate. Eleven out of the 29 bees we tested solved the innovation task, lifting the lid to reach the reward within the 15 minutes of the essay. Innovation time was correlated with time until touching the lid for the first time (GLM estimate \pm SE = $-3.51 \cdot 10^{-6} \pm 1.44 \cdot 10^{-6}$, $p = 0.01$), indicating that bees that passed the innovation test did not spend much time solving the new task.

Innovation test success was explained by other behavioural traits (Figure 2 , full model in Table 2a) and results were consistent both for the dichotomous response (success / no success) and the innovation success time. Below we detail the observed effect sizes reported in Table 2a. Shy individuals were worst innovators. The probability of innovating drops from 0.80 for bees that spent two seconds inside the refuge to 0.01 for bees that did not leave the refuge in the first essay (Table 2a, Fig. 2a). Shy individuals were also slower at resolving the innovation test (Table 2b). In fact, from the five bees that did not leave the refuge in the first test and did the innovation test, none of them passed the innovation test.

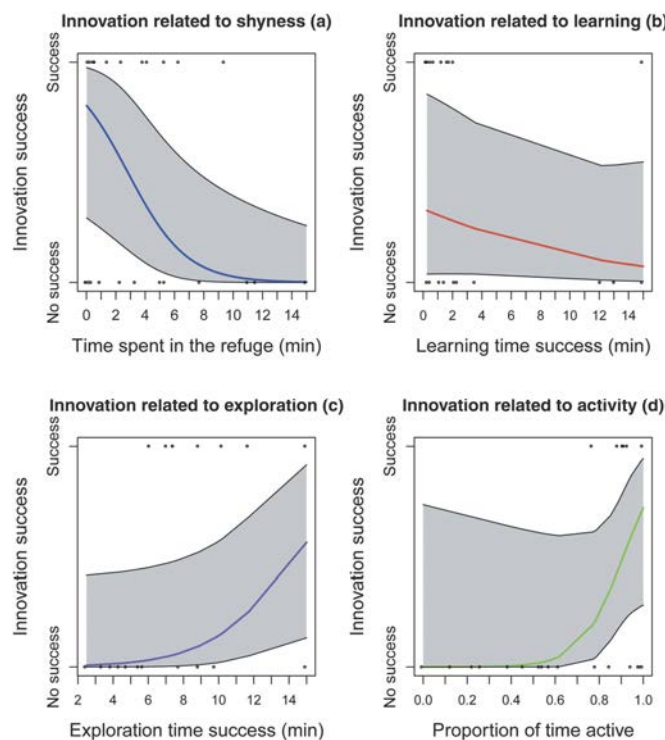


Figure 2 | Innovation related to each measured behavior. These graphs plot the estimates extracted from the multivariate model described in Table 2a measuring the dichotomous success or failure of the innovation test.

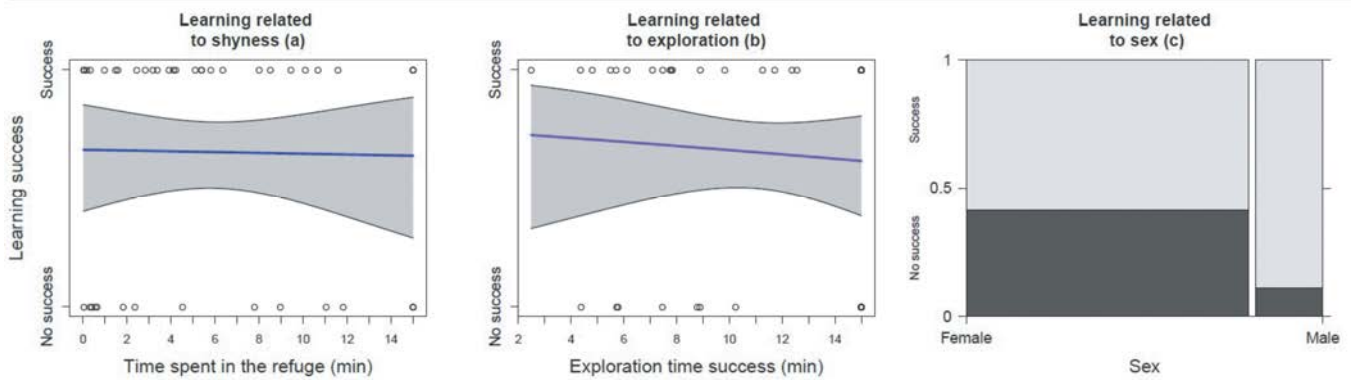


Figure 3 | Learning related to each measured behaviour. These graphs are extracted from the multivariate model described in Table 2c measuring the success or failure in the learning test.

Second, slow explorers were also better at the innovation test. Bees that spent more time solving the exploration test had more chances to succeed in the innovation test (Table 2a, Figure 2c). These individuals also solved the innovation test faster (Table 2b). Finally, active bees during the test had better chances of solving the innovation test (Table 2a, Figure 2d). The velocity at solving the test also correlated positively with the proportion of time active during the test (Table 2b).

Osmia cornuta bees were able to learn with 63% of individuals succeeding in the learning test (chi-squared = 3, df = 1, p-value = 0.08), while control bees had a success rate close to that expected by random (52% success, chi-squared = 0.11, df = 1, p = 0.74). Time spent in the refuge, exploration time success and activity time during the learning test were not correlated with neither learning success nor latency (Table 2c; Figure 3). Males nonetheless tended to learn better than females, showing slightly higher success rates (Table 2c) and learning faster (Table 2d).

Finally, learning was not a good predictor of innovation success and latency (Table 2, Figure 2b) and time to innovate showed no clear relationship with learning (Table 2b). ●

Discussion

Innovation-like behaviours have been previously reported in wild solitary bees. These include the use of new materials for nesting and anecdotal examples of bees nesting in new places, such as cardboard or wooden blocks (Bosch & Kemp, 2001). However, the innovative ability of solitary bees had never been demonstrated before in laboratory experiments. Ours is the first experimental demonstration that *Osmia cornuta* can develop innovative behaviours to resolve problems in the laboratory. This discovery reinforces thus the view that the capacity to innovate may be an important mechanism

through which solitary bees exploit novel resources in the wild. Our task presented was both challenging—only 36 % of the bees succeeded—and novel as it is a human constructed feeding system they never experienced before. However, we did not find evidence that the ability to innovate reflects domain-general learning.

We did find that bees were able to rapidly associate colours and rewards, a process that is expected to afford important advantages in the wild. We also found that individuals showed substantial variation in their propensity for associative learning. However, individuals that were better at solving the associative learning test were not more likely or faster to solve the innovation test. This finding is puzzling because learning is critically important in bees for vital tasks like foraging, the identification of high quality foraging sites, right mixtures of nectar and pollen, and navigation back to the nest for brood provisioning (Roulston & Goodell, 2011; Minckley et al., 2013).

The lack of evidence for domain-general cognition does not mean that innovation does not require learning. However, learning is not only necessary to fix the new behaviour in the individual repertoire (Ramsey et al., 2007; Lefebvre et al., 2004; Reader et al., 2003, Sol 2003; Lefebvre et al., 1997), but also essential to solve the task itself. Indeed, we found that bees that succeeded in the innovation test went directly towards the lid covering the reward, probably reflecting that they had learnt the rewarding colour during training essays. However, learning can be insufficient if solving the task is challenging because also requires motor flexibility to remove the obstacle hiding the reward.

The failure to relate innovation and associative learning does not simply reflect that we performed a short training phase because success in the learning test success rate was comparable to those found in previous similar experiments using more training days (e.g. Jin et al., 2014; Jin et al., 2015). A more likely explanation is that other factors are more relevant and can have obscured the effect of learning. Indeed, we found consistent differences between fast

Table 2 | Multivariate model coefficients for innovation success and learning. We ran parallel models for innovation and learning success, and also for time to innovate and learn.

(a) Innovation success (Binomial GLM) ~ Time spent in the refuge + Learning time success + Exploration time success + Activity innovation test.				
Variables	Estimate	SE	z-value	p-value
Time spent in the refuge	-8.34*10 ⁻⁶	3.54*10 ⁻⁶	3.54*10 ⁻⁶	3.54*10 ⁻⁶
Exploration time success	6.84*10 ⁻⁶	3.58*10 ⁻⁶	3.58*10 ⁻⁶	3.58*10 ⁻⁶
Proportion of the time active	10.04	5.98	5.98	5.98
Learning time success	-2.05*10 ⁻⁶	2.00*10 ⁻⁶	2.00*10 ⁻⁶	2.00*10 ⁻⁶
(b) Innovation time success (Cox) ~ Time spent in the refuge + Learning time success + Exploration time success + Activity in the test				
Variables	Estimate	SE	z-value	p-value
Time spent in the refuge	-2.70*10 ⁻⁶	1.22*10 ⁻⁶	-2.22	0.03
Exploration time success	2.15*10 ⁻⁷	1.22*10 ⁻⁶	1.77	0.08
Proportion of the time active	5.67	3.02	1.87	0.06
Learning time success	-1.57*10 ⁻⁷	1.11*10 ⁻⁶	-0.14	0.89
(c) Learning success (Binomial GLM) ~ Time spent in the refuge + Exploration time success + Sex				
Variables	Estimate	SE	z-value	p-value
Time spent in the refuge	-1.27*10 ⁻⁷	1.54	-0.10	0.92
Exploration time success	-6.62*10 ⁻⁷	1.54*10 ⁻⁶	-0.43	0.67
Sex (Male)	2.07	1.20	1.73	0.08
(d) Learning time success (Cox) ~ Time spent in the refuge + Learning time success + Sex				
Variables	Estimate	SE	z-value	p-value
Time spent in the refuge	-6.57*10 ⁻⁷	7.60*10 ⁻⁷	-0.87	0.39
Exploration time success	-1.45*10 ⁻⁷	9.58*10 ⁻⁷	-0.15	0.88
Sex (Male)	0.98	0.43	2.29	0.02

and slow innovators in their tendency to approach and explore the experimental apparatus. Specifically, individuals that were better able to lift the lid to access the food reward tended to be bolder and explored slower in the exploration test than those that failed to solve the task. Boldness and exploration have been previously identified as important determinants of innovation propensity in vertebrates and highlight that innovation propensity may largely reflect particular motivational states or emotional responses of individuals to novel situations rather than cognitive differences (Sol et al., 2012). In line with this conclusion, successful innovators also exhibited higher activity levels. Activity may reflect motivation to feed, which in other animals has been found to be a major determinant of innovation propensity (e.g. Sol et al., 2012). However, it may also increase the chances to solve the task accidentally by trial and error. Closed environmental spaces can also be stressful and what we defined as “high activity levels” and “fast exploring” (i.e. defined as touching the four cues in the exploration essay) can be a by-product of stereotyped stress behaviours.

We also found intriguing sex-related differences in learning. Males showed a tendency to perform better in the associative learning test than females. This is unexpected because females have to deal with more tasks during their lifetime, including for-

aging and nest provisioning, and may perhaps indicate that the cognitive demands for males to locate females are higher than suspected. Regardless of the exact mechanism, it may be that the underrepresentation of males in our experiments has reduced the power to detect the effect of learning on innovation propensity.

To sum up, we found that solitary bees were able to perform new cognitive demanding tasks, as they were able to innovate and learn in a closed environment experiment. However, we failed to find a link between the two. Instead, innovation propensity was better explained by exploration, boldness and activity levels. These results suggest that solitary bees can readily accommodate their behaviour to novel context with no need of sophisticated cognitive processes. In a context of global change, the ability to rapidly accommodate behaviour to novel contexts seems highly relevant. In novel environments, bees must for instance learn how to forage new plant species, which sometimes presents complex flowers with whom bees have not co-evolved (Bartomeus et al., 2012). Therefore, we should abandon the notion that insect behaviour is inflexible and stereotypical, and better appreciate that insects can readily accommodate their behaviour to changing conditions through innovation and learning. ●

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Supplementary material

Table S1 | *Behaviours observed and their interpretation*

Action interpreted	Behaviour
Exploration of the upper left cue in essay 1	Being at the upper left cue
Learning success / failure in essay 4	Learning success / Learning not success
Exploration of the upper right cue in essay 1	Being at the upper right cue
Learning success / failure in essay 4	Learning success / Learning not success
Exploration of the lower left cue in essay 1	Being at the lower left cue
Exploration of the lower right cue in essay 1	Being at the lower right cue
Activity in any essay	Being active, in movement or standing up but still moving antennas or legs
Inactivity in any essay	Being completely inactive, standing up with no movement
Escaping	Being trying to climb the walls of the arena
Eating	Being eating in an eppendorf full of sucrose solution
Lid exploring	Exploring the lid in the fifth essay that covers the sucrose reward
Success	Succeeding at the current test (exploration, learning, innovation)
Shyness	Being inside the refuge in the first essay

Table S2 | *Alternate multivariate models*

(a) Learning success ~ Refuge re-exploration + Sex				
Variables	Estimate	SE	z-value	p-value
Incept	1.38	1.11	1.24	0.22
Refuge re-exploration	1.83	0.77	2.36	0.02
Sex (Male)	-1.74	1.16	-1.49	0.13
(b) Learning time success (Cox) ~ Refuge re-exploration + Sex				
Variables	Estimate	SE	z-value	p-value
Refuge re-exploration	0.821	0.4042	2.032	0.042
Sex (Male)	0.789	0.4334	1.819	0.069

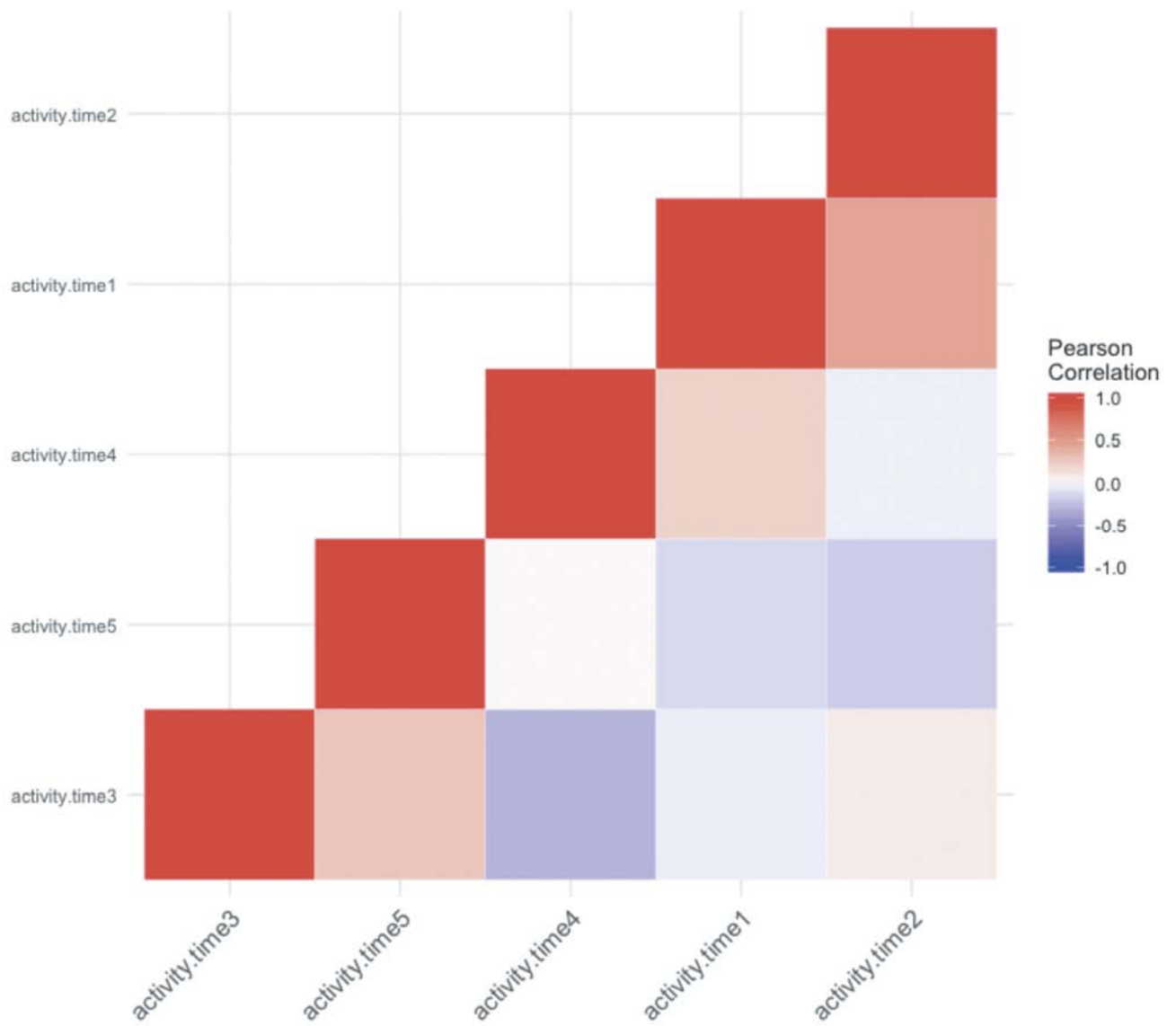


Figure S1 | *The lack of correlation for the time being active along the different assays for each individual*

Supplementary analysis

Alternate model

Outside the multivariate model, we found some univariate correlations worth commenting.

Re-exploring the refuge during the exploration essay increased from 0.24 to 0.58 the probability of success in the innovation test (GLM estimate \pm SE = 1.52 ± 0.82 , $p = 0.06$). Males tended to have a higher probability of innovation than females (GLM estimate \pm SE = 1.52 ± 0.977 , $p = 0.12$; Female = 30.4%, Male = 66.7%, Fig. 4a).

Males were also faster in innovating (Kaplan-Meier survival curves chi-squared = 4.1 on 1 df, $p = 0.04$). The probability of succeeding in the innovation test decreases with the number of resting times (no rest = 0.72, one resting = 0.24, resting two times = 0.04, GLM estimate \pm SE = -2.05 ± 0.82 , $p = 0.01$).

Interestingly, in an alternative multivariate model we found that bees re-exploring the refuge in the first essay, a proxy of exploration, have a higher probability of success in the learning test than bees not having re-entered the refuge (GLM estimate \pm SE = 1.83 ± 0.77 , $p = 0.02$, Table S3) and learned faster (Coxphr coef. \pm SE = 0.82 ± 0.40 , $p = 0.04$, Table S3).

Innovation treatment vs control

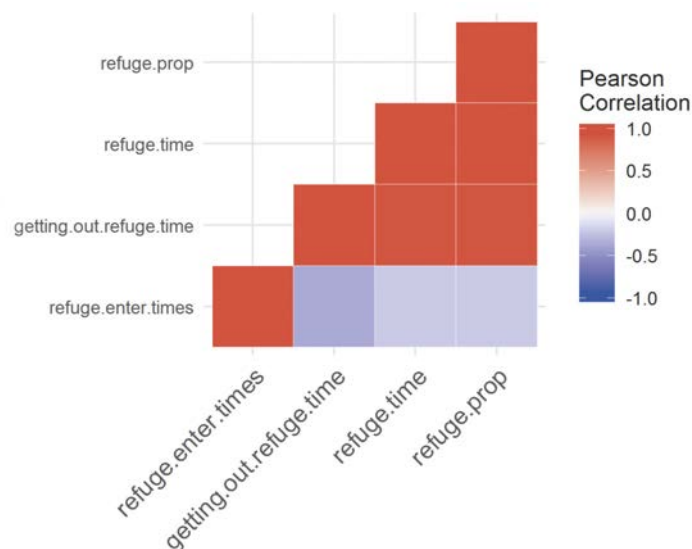
From the 153 individuals that started the experiment, only 58 reached and did the final test. The proportion of individuals that were able to innovate (13 of 34) was equal than in the control counterpart (8 of 24) (Chi-squared p -value = 0.997). And is consistent when using time to innovate (Survival analysis stats). We did not expect differences in innovation for treatment and control because the tests were similar. The lid was in the opposite learnt position, but bees were going to check it after going to the learnt position and cue.

Learning treatment vs control

From the total individuals starting the experiment, 84 reached the learning test (Figure 1e). Treatment individuals that passed the learning test (30 of 48), resolved the it very quickly (90% of them in less than three minutes and 35 seconds, of the 15 minutes they had to solve it), control bees that passed the test also did it in similar time, but they were less individuals passing the test (19 of 36, 52.77%) compared to treatment bees (30 of 48, 62.5%). We expected the control bees to have near 0.5 probabilities of succeeding and the treatment to have higher probabilities than that. We did a chi-squared goodness of fit to check out these probabilities. A p -value of 0.74 confirms that control bees acted randomly. A p -value of 0.08 for the treatment bees mean that they actually learnt and have more probabilities to success if they went through the treatment essays than through the control essays. ●

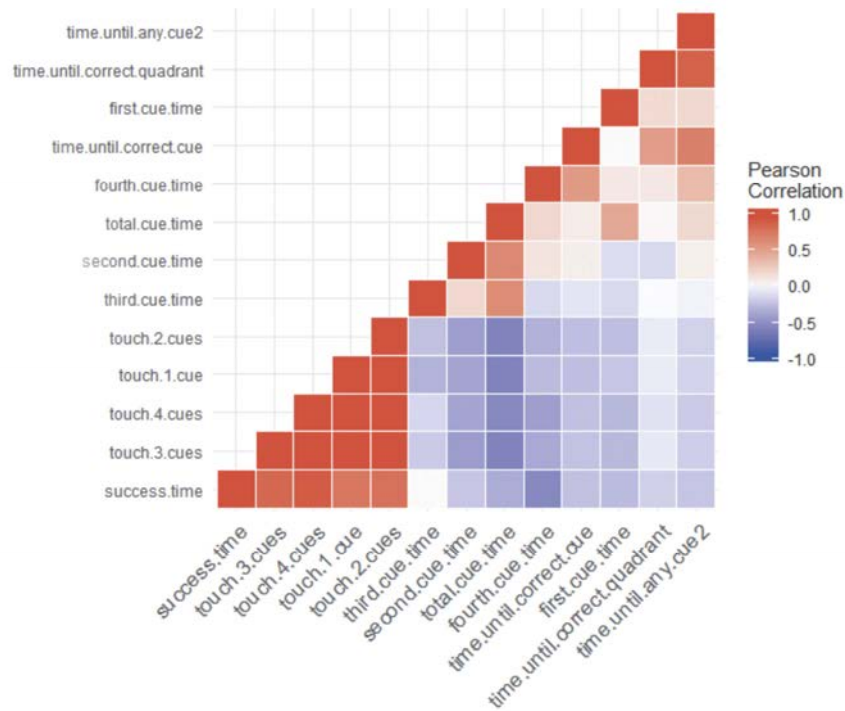
Variable selection:

Shyness



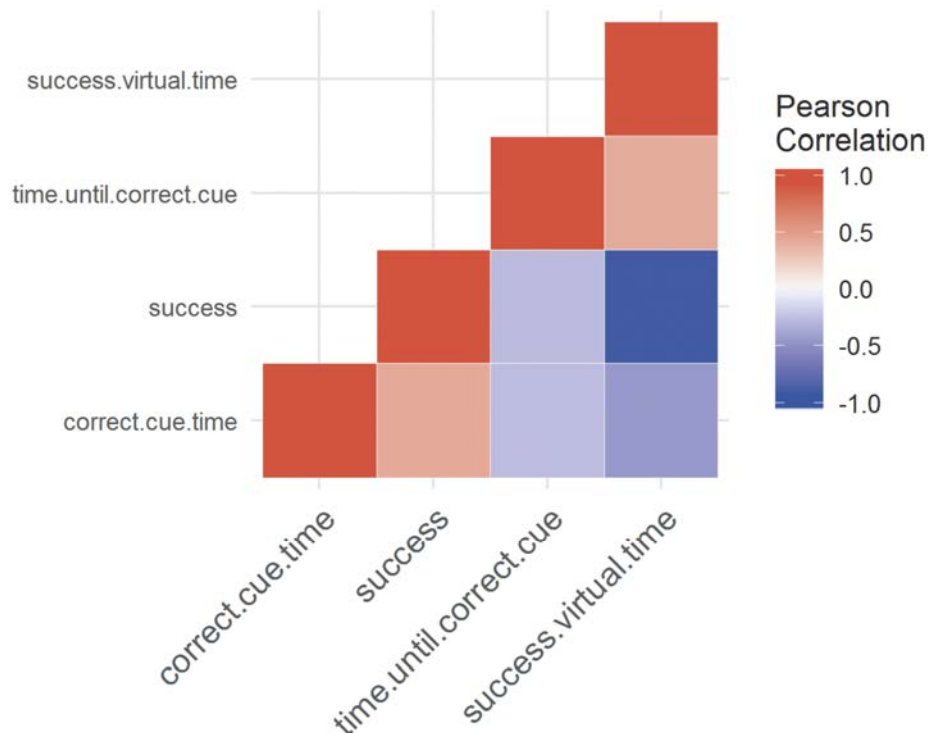
Heatmap exploring correlation between shyness variables we selected: success time in exploration test, re-entering in the refuge / refuge enter times, censored time until lid exploring, exploration test success. We chose time spent in the refuge.

Exploration



Heatmap exploring correlation between exploration variables we selected: success time in exploration test, re-entering in the refuge / refuge enter times, censored time until lid exploring, exploration test success. We chose exploration time success

Learning



Heatmap exploring correlation between learning variables we selected learning test success and censored learning time

Brain size predicts variation in learning speed in bees

Abstract

A large brain is widely considered a distinctive feature of intelligence, a notion that mostly derives from studies in mammals. However, studies in insects demonstrate that cognitively sophisticated processes, such as social learning and tool use, are still possible with very small brains. Even after accounting for the allometric effect of body size, substantial variation in brain size still remains unexplained. A plausible advantage of a disproportionately larger brain might be an enhanced ability to learn new behaviors to cope with novel or complex challenges. While this hypothesis has received ample support from studies in birds and mammals, similar evidence is not available for small-brained animals like insects. Our objective is to compare the learning abilities of different bee species with brain size investment. We conducted an experiment in which field-collected individuals had to associate an unconditioned stimulus (sucrose), with a conditioned stimulus (colored strip). We show that the probability of learning the reward-colour association was related to absolute and relative brain size. However, the time needed to solve the learning test was mainly explained by the phylogenetic position of the species. This study shows that other bees aside from the long studied *Apis mellifera* and *Bombus spp.*, can be used in cognitive experiments and opens the door to explore the importance of relative brain sizes in cognitive tasks for insects and its consequences for species survival in a changing world. ●

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Introduction

A large brain is widely considered a distinctive feature of intelligence, a notion that mostly derives from studies in mammals. However, studies in insects demonstrate that cognitively sophisticated processes, such as social learning and tool use, are still possible with very small brains (Chittka & Niven, 2009). In fact, the million-fold increase in a large mammal's brain compared to an insect brain allows mammals to have behavioral repertoires that are only just two to three times as big (Chittka & Niven, 2009). This is hardly the kind of difference expected to find between insects and mammals.

If a large behavioural repertoire is possible with a miniature brain, what benefits obtain animals by investing in larger brains? Because brain size scales allometrically with body size (Burger *et al.*, 2019), an explanation is that biophysical constraints forces larger animals to have more and/or larger neurons (Chittka & Niven, 2009). It is for instance easy to imagine that the bigger muscles of larger animals will require greater numbers of motor neurons and axons with larger diameters to cover longer distances (Chittka & Niven, 2009). More neurons may also allow greater replication of neuronal circuits, adding precision to sensory processes, detail to perception, more parallel processing and enlarged storage capacity (Chittka & Niven, 2009). These explanations are however insufficient because substantial variation in brain size remains when the allometric effect of body size is taken out (Gonda *et al.*, 2013). Given that neural tissue is extremely costly to maintain, what is the purpose of expanding the brain?

A plausible advantage of a disproportionately larger brain might be an enhanced ability to learn new behaviors to cope with novel or complex challenges (Sol *et al.*, 2008). While this hypothesis has received ample support from studies in birds and mammals, similar evidence is not available for small-brained animals like insects. Our insufficient understanding of the benefits of miniature brains remains thus a major obstacle for a general theory of brain evolution, and even cast doubts on whether variation in brain size is biologically meaningful (Chittka & Niven, 2009).

In this study, we try to address this gap with an experimental comparative analysis in bees. Bees have historically fascinated biologists because of their small nervous systems compared to the complexity and diversity of their behavior (Chittka & Niven, 2009; Vasas & Chittka, 2019). Numerous species, including bees, are reported to be able to create memories of rewarding experiences (Matsumoto & Mizunami 2000; Menzel, 1999; Daly & Smith, 2000) as well as of punishment (Vergoz *et al.*, 2007), and those memories can be retrieved at different times after learning, both in the short- and in the long-term (Giurfa, 2015). Species have also substantially diversified in brain size despite sharing the

same brain architecture. Bigger neuropils have been suggested to be related to diet generalism in beetles, as generalist need to discriminate and process a wider variety of resources (Farris & Roberts, 2005).

We measured learning abilities in wild individuals of 32 bee species captured in the wild, and then used a phylogenetic comparative framework to test whether species that performed better in the learning task had larger absolute and/or relative brains. Learning abilities were measured with a novel quick-to-perform experimental method proposed by Muth *et al.*, 2017 to assess speed at colour learning. Associative learning is a highly developed cognitive ability in bees with substantial ecological relevance. Importantly, associative learning essays are short enough to be suitable for highly stress-intolerant species and facilitate as well standardization across species with varying life histories and ecologies, two major obstacles hindering past progress in linking brain size and learning performance. The possibility to perform experiments directly from individuals captured in the field allows experimentation with non-model taxa, providing opportunities for broader comparative analyses of cognition. ●

Material and methods

Study subjects

We captured bees by hand netting ($n = 202$ individuals) from March to June 2018 in different open fields and urban parks from Andalusia, South of Spain. Bees were kept individually in vials in cold storages and transported to the laboratory, where they were transferred into separated transparent plastic enclosures for the behavioral essay (Fig. 1). After the essays, all individuals were identified at the species level by a taxonomist (F. P. Molina), yielding a sample of 32 unique species from 14 genera (Table 1).

Experimental essay

Before starting the learning essays, bees were left 30 minutes in the individual enclosures to allow them to awake from the cold and habituate to the experimental conditions. The experimental enclosures were built by the attachment of two 2.5 cm transparent PVC angles with ventilation holes, with removable perforated lids attached at both extremes (Fig. 1a). Associative learning was measured by a multi-choice free-moving proboscis extension protocol (FMPER, modified from Muth *et al.*, 2017), where the animal had to learn to associate a reward (50% sucrose) with an arbitrary stimulus (a color). Each experimental trial consisted in the presentation of a yellow and a blue strip easily distinguishable by bees vision (Chittka & Wells, 2004). The strips were presented always at the opposite extreme from

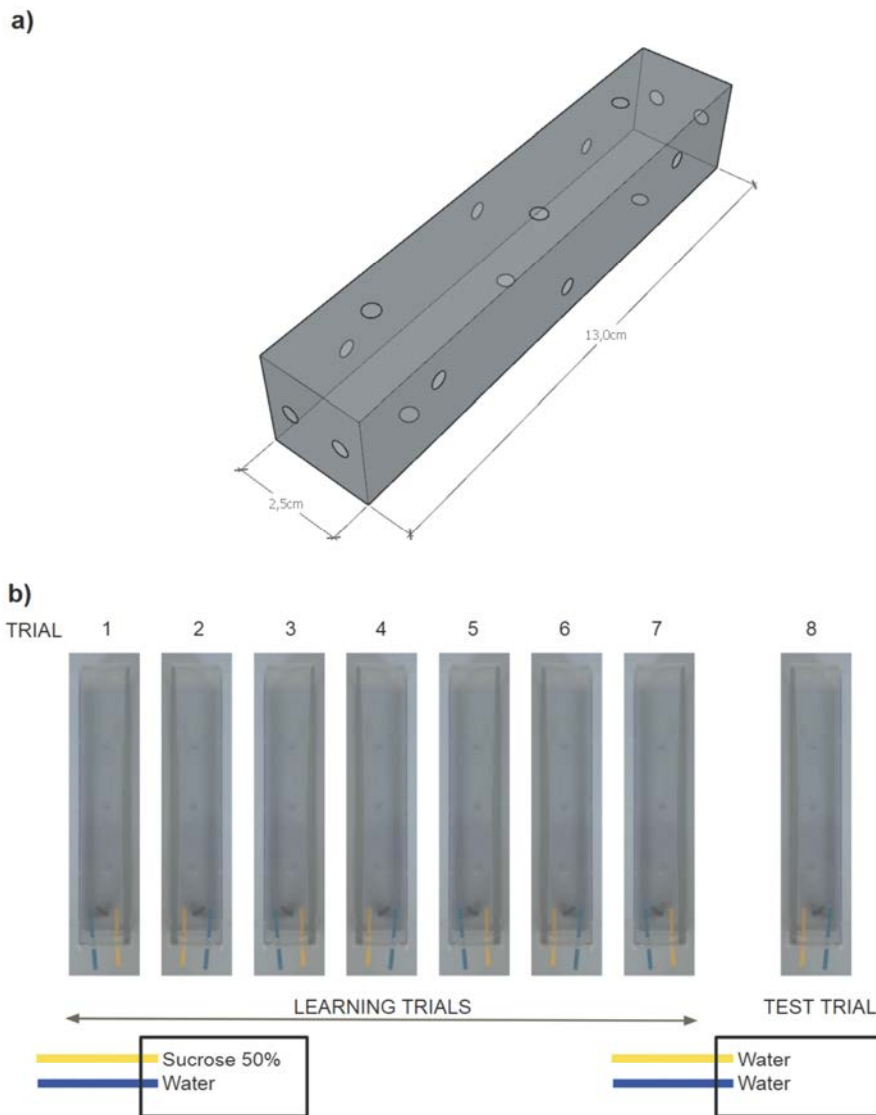


Figure 1 | The complete experimental display. a) Experimental PVC experimental enclosures used for the experiment (2.5 cm width and 13 cm long). Multiple holes were done for air circulation and strip offering from both extremes. b) An example of the sequence of one complete experiment for one single individual, where one color is associated with a reward and it is maintained until the test trial, where both strips are unrewarded.

where the individual was staying in the enclosure. One of the colored strips was dipped in sucrose and the other one in water. The color with the reward was randomly chosen but maintained during the whole experiment for each individual. The essay started when the individual reached the middle of the enclosure in its way towards the strips. We measured the time needed for the individual to reach the strips and extend its proboscis to start drinking on one of them, waited for three seconds and removed the strip (Fig. 1b). We allowed the bee to explore the remaining strip and again removed it after the individual had drunk for three seconds. Once this exploratory trial ended, the process was repeated every 10 minutes six more times to allow individuals to associate the stimuli (colour) with the reward (food) through operant conditioning (training trials), switching the strips position to avoid confounding color with position (Fig. 1b). We removed the originally included proposed acclimatization

trials in the FMPER protocol, and considered first trial as acclimatization/exploration.

A trial was considered successful when the bee chose first the strip with sucrose and unsuccessful otherwise. The trial was considered finished when the subject drank from both strips or otherwise capped after two minutes. After seven training trials, we tested whether the individual had learned to associate a color with a reward by means of a rewarding-unconditioned stimulus test, where both strips were dipped into water. Learning performance was defined in terms of success or failure in solving the test and if solved, we also quantified the time needed to start drinking from the right strip.

In general, bees responded well to the experimental procedure, especially those from *Andrena sp.*, *Apis mellifera*, *Bombus sp.*, *La-*

sioglossum sp., and *Rhodanthidium sticticum*. However, forty-five individuals from *Anthophora* sp., *Eucera* sp. and *Xylocopa* sp. either ignored or did not react to the full experimental procedure (Table 1). Consequently, these species were not used in the analyses.

Brain measurements

After the experiment, bees were anesthetized in cold chambers (Crook, 2013) and decapitated. The head was fixed in 4% paraformaldehyde with phosphate buffer saline (PBS). The fixative solution embedded the brain and dehydrated the tissue, preventing brain from degradation for a long period. Brains were extracted from the head capsule, separated from the tracheas and fat bodies to avoid weighting errors, and placed on a small piece of tared Parafilm®. Fixative solution was dried from the brain using Kimwipes® tissues and then the brain was weighed in a microbalance to microgram accuracy (Sartorius Cubis®). Brain weight was used as a proxy of brain size, as it is strongly correlated with brain volume of the mushroom bodies (correlation coefficient = 0.85; p-value < 0.001, Sayol et al. 2019, in prep.), which are the neuropil centers of most cognitive abilities (Dujardin, 1850). Body size was measured as the inter-tegular distance, that is, the distance between the wing bases, usually used as a proxy of body size in bees (Kendall et al., 2019).

Data analysis

To assess whether bees learned to associate color and reward we used Bayesian phylogenetic generalised linear mixed models (PGLMM), as implemented in the package *brms* (Bürkner, 2017). Specifically, we analysed whether the probability of success during the training trials (i.e. the correct strip is chosen) increased over time and whether for those trials that were successful the time to start feeding declined over time. To model success/failure we used models with Bernoulli error structures, while to model latencies we used Poisson error structures. In all models, species were treated as a random factor and we incorporate a phylogenetic covariance matrix to control for the influence of phylogeny. The phylogeny used was a maximum-likelihood phylogenetic tree of the superfamily Apoidea at the genera level (Hedtke et al., 2013). Due to the absence of infrageneric phylogenies for our genera, we simulated infrageneric polytomies within our phylogeny. Species tips were added to the phylogenetic tree genera nodes as polytomies of equal branch length relative to the genera branch length (Kendall et al., 2019) using the *phytools* package (version 0.6 44; Revell, 2012). Intra-class coefficients (ICC) were used to validate the assumption of comparative approach that variation in learning ability across species is higher than within species. ICC is a descriptive statistic, ranging from zero to one, that can be used when quantitative measurements are made on units that are organized into groups, in this case different measurements within species grouped by phylogenetical relationships. A value near zero is interpreted as the variable is not explained by the

phylogenetic relationships, and a value nearer one means that the variable is better explained by intrinsic characteristics of the phylogeny.

To test the prediction that large brains enhance learning abilities, we modelled the success and latency to succeed in the test trial as a function of brain size using the PGLMM framework described above. Following previous studies, we analyzed brain size both in absolute terms (brain weight) and relative to body mass, fitting separate models for each brain size metric: Absolute brain size (log-transformed) and relative brain size estimated as brain size residuals (Wurm & Fiscaro, 2014). These residuals are extracted from a log-log regression of brain size against body size (LM estimate \pm SE = 2.07 ± 0.08 , $p < 0.001$, $R^2 = 0.82$).

We investigated possible innate preferences in the multi-choice free-moving proboscis extension test in the two species with larger sample size: *Bombus terrestris* ($n = 43$) and *Rhodanthidium sticticum* ($n = 25$). *Bombus terrestris* showed a weak preference for the left side, that is, individuals that started the protocol with the rewarding strip (28 out of 43) at their left side showed a quicker response to sugar during the protocol (GLM estimate \pm SE = -0.18 ± 0.08 , $p = 0.03$). *Rhodanthidium sticticum* specimens trained with a yellow rewarding cue (17 out of 25) were more likely to succeed than those trained with a blue one (GLM estimate \pm SE = 2.14 ± 0.58 , $p < 0.001$) and took less time to approach to it (GLM estimate \pm SE = -0.13 ± 0.07 , $p = 0.06$). To avoid that innate preferences systematically bias the experiment, we randomized position and colour associations with the reward.

Finally, we also examined whether individuals that better learned to associate color and reward during the training period exhibited a higher probability of successfully solving the unconditioned stimulus test than those that did not learn it. ●

Results

Most bees learned to associate a color with a food reward. More than half of the individuals that reached the test drank from the correct strip (66%). This was higher than expected by chance (50%) (Chi-squared goodness of fit 10.8, $p = 0.001$). Latency to touch the correct strip decreased along the trials (PGLMM Negative Binomial, $\beta = -0.13 \pm 0.02$, IC = $-0.16 - -0.10$, ICC: 0.28, Fig. 2, Table 2) and bees had more chances of success in the later trials than in the earlier ones (PGLMM Negative Binomial $\beta = 0.07 \pm 0.02$, IC = $0.03 - 0.10$, ICC: 0.03). Removing the first trial, which can be merely exploratory, did not change the results. Finally, those with higher number of learning successes during the whole experimental process were more likely to pass the learning test (PGLMM bernoulli $\beta = 0.74 \pm 0.16$, IC = $0.45 - 1.06$, ICC = 0.17, Table 2).

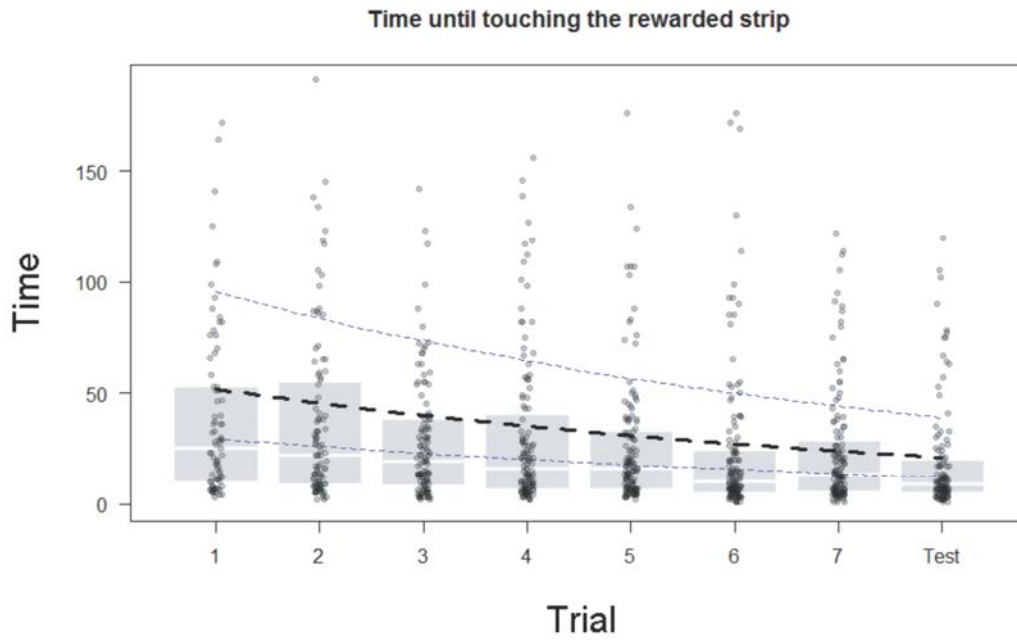


Figure 2 | Time (in seconds) until touching the rewarded strip decreased along trials. Dots represent each individual success. Boxplots represent from Q2 to Q3 of those success for each trial, with the median drawn in white. Overlapping the boxplot is the estimate and confidence intervals of the PGLMM negbinomial model ($\beta = -0.13 \pm 0.02$, IC = $-0.16 - -0.10$, ICC: 0.28). Last trial was considered the test as it was not rewarded.

Phylogenetic tree:
Body size - brain weight residuals

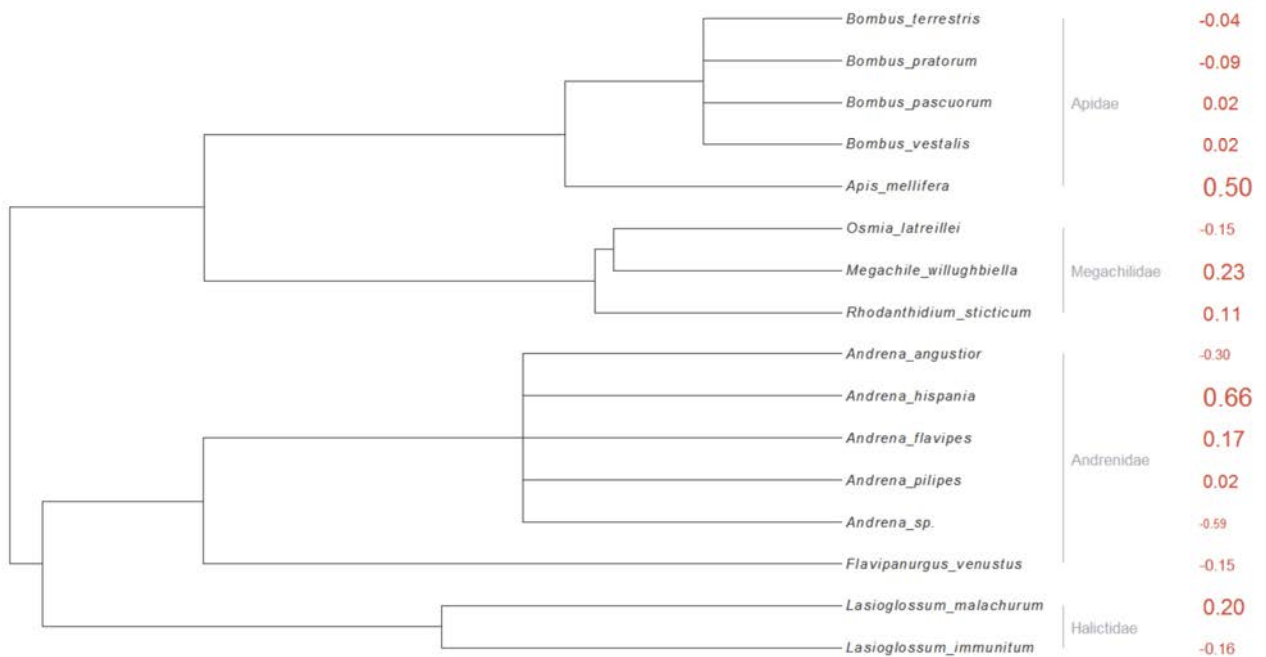


Figure 3 | Phylogenetic tree of the studied species used for the Bayesian analysis to control whether models were explained by the phylogenetical group heritage. Numbers in red represent the body size - brain size residuals for each species. Values above zero have bigger brains by expected by their body sizes, and values below zero have smaller brain sizes.

Table 1 | The list of the species used for the experiment, with the minimum time to respond to the first trial (the trial considered exploratory), the proportion of succeeders in the test compared to the number of individuals that made it to the test, and the number of captured individuals that started the test. Unidentified species were assigned to unique morphospecies.

Species	Minimum reaction time first trial (seconds)	Proportion of individuals that succeeded in the test	Captured individuals
<i>Bombus pratorum</i>	3	2/4	5
<i>Apis mellifera</i>	4	4/4	7
<i>Bombus terrestris</i>	4	35/41	43
<i>Andrena morpha1</i>	6	0/2	15
<i>Bombus pascuorum</i>	7	4/5	5
<i>Lasioglossum malachurum</i>	7	4/8	12
<i>Rhodanthidium sticticum</i>	7	13/21	26
<i>Andrena angustior</i>	10	1/3	3
<i>Andrena pilipes</i>	11	3/6	7
<i>Andrena flavipes</i>	18	2/4	6
<i>Andrena hispania</i>	22	1/1	1
<i>Lasioglossum immunitum</i>	29	7/15	18
<i>Psithyrus vestalis</i>	53	1/1	1
<i>Flavipanurgus venustus</i>	31	0/2	5
<i>Osmia latreillei</i>	NA	1/2	5
Species that did not respond to any complete set of trials			
<i>Xylocopa cantabrita</i>	47	0/0	6
<i>Andrena cinerea</i>	53	0/0	2
<i>Panurgus dargius</i>	60	0/0	1
<i>Lasioglossum morpha1.</i>	138	0/0	4
<i>Eucera morpha1.</i>	141	0/0	2
<i>Megachile willughbiella</i>	NA	0/0	1
<i>Andrena rhyssonota</i>	NA	0/0	7
<i>Andrena labialis</i>	NA	0/0	1
<i>Eucera elongatula</i>	NA	0/0	2
<i>Osmia caerulea</i>	NA	0/0	2
<i>Panurgus morpha1.</i>	NA	0/0	1
Species that did not respond to any trial			
<i>Eucera rufa</i>	NA	0/0	1
<i>Eucera notata</i>	NA	0/0	4
<i>Anthophora plumipes</i>	NA	0/0	1
<i>Anthophora dispar</i>	NA	0/0	1
<i>Xylocopa violacea</i>	NA	0/0	4
<i>Anthophora retusa</i>	NA	0/0	2

Learning performance and the probability of learning success were poorly explained by phylogeny as shown by the low ICC values reported above. However, the latency at solving the learning task had a high phylogenetic signal (ICC: 0.75; IC: 0.62 – 0.89 Fig. 4c, d), indicating that certain groups, such as the Apidae family are faster at reaching the strips.

Bees with bigger brains were more likely to succeed in the learning test. Success in the learning test was positively correlated with both absolute brain size (PGLMM Bernoulli $\beta = 0.79 \pm 0.27$, IC = 0.29 – 1.38, ICC: 0.10, Fig. 4a) and with brain residuals (PGLMM Bernoulli $\beta = 1.26 \pm 0.78$, IC = -0.26 – 2.80, ICC: 0.24, Figure 4b). In addition, for the subset of bees that succeed in the learning test, slower learners had slightly bigger brains, both in absolute terms (PGLMM Brain size $\beta = 0.19 \pm 0.06$, IC: 0.08 – 0.30, ICC: 0.73, Figure 4c, Table 2) and relative to body size (PGLMM Bernoulli, $\beta = 0.94 \pm 0.17$, IC: 0.63 – 1.28, ICC: 0.75, Figure 4d, Table 2). ●

Discussion

Highly controlled laboratory experiments usually allow testing only for a handful of species (e.g. Honeybees, Bumblebees, Cockroaches, Fruit flies), that can be raised in laboratory conditions. In ad-

dition, some of these experiments often involve stressful conditions, like individuals being fully harnessed in Proboscis Extension Reflex (PER) protocols (Takeda, 1961). Therefore, laboratory experiments are only suitable for highly stress-tolerant species. We chose a modified version of the multi-choice free-moving proboscis extension protocol (FMPER, modified from Muth et al., 2017) trying to test apoidea species never tested before. This novel quick-to-perform experimental method allowed us to conduct a comparative analysis including multiple bee species captured directly from the field. We found that most bee species, including solitary species never used before in cognitive experiments, can learn to associate a color with a reward. Interestingly, species differed in the speed at which they learned, and these differences were in part explained by brain size. Thus, probability to learn increased with both absolute and relative brain sizes. The speed to solve the learning test was also related to brain sizes, and was as well influenced by the species position in the phylogeny.

Expanding the range of species evaluated by applying quick-to-perform experimental protocols to wild species can provide important insight into the relationship between brain size and learning. Thus, although our experiment was not designed to analyze differences across social and solitary species, we observed that some solitary species can show similar learning abilities than social species. This

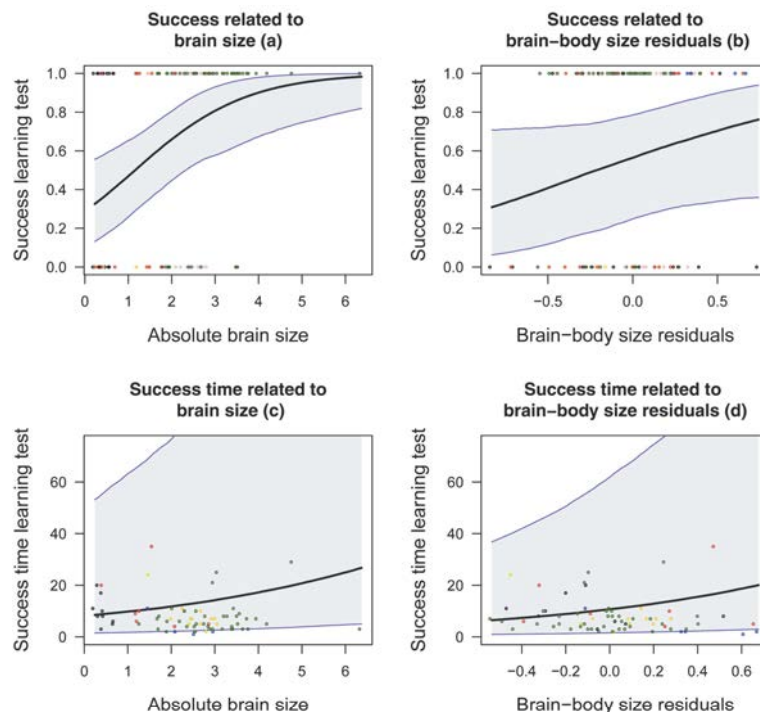


Figure 4 | Correlation between brain size and learning tests. a) Increasing probability (from zero to one) of success with bigger brain sizes (Brain weight in mg). b) Increasing probability of success with bigger relative brain sizes. c) The correlation between time until success and for absolute brain sizes. d) The correlation between time until success and relative brain sizes. Overlapping lines are the tendency lines extracted from the PGLMM models. The colours from the graph are assigned to the following genera: *Andrena* red; *Apis* blue; *Bombus* green; *Lasioglossum* black; *Megachile* dark blue; *Osmia* yellow; *Panurgus* khaki; *Rhodanthidium* pink.

Table 2 | Results of the Bayesian models of learning, and learning related to brain size.IC = Interval Confidence, ICC = Intra-class coefficient, β = Estimate \pm Standard Error.

Formula	β	IC	ICC (Species)	Notes
LEARNING				
Time until touching correct strip ~ Trial number	-0.13 \pm 0.02	-0.16 – -0.10	0.28	All trials considered
Learning success ~ Trial number	0.07 \pm 0.02	0.03 – 0.10	0.03	All trials considered
Number of successes ~ Trial number	0.74 \pm 0.16	0.45 – 1.06	0.17	First trial removed
LEARNING COMPARED WITH BRAIN SIZES				
Correct strip touching ~ Absolute brain size	0.79 \pm 0.27	0.29 – 1.38	0.10	Just learning test
Correct strip touching ~ Relative brain size	1.26 \pm 0.78	-0.26 – 2.80	0.24	Just learning test
Time until touching the correct strip ~ Absolute brain size	0.19 \pm 0.06	0.08 – 0.30	0.73	Just learning test
Time until touching the correct strip ~ Relative brain size	0.94 \pm 0.17	0.63 – 1.28	0.75	Just learning test

appears to contradict the traditional view that social bees have more complex learning abilities than solitary bees (Reader & Laland, 2002), although more research is needed to properly confirm it.

A handful of species did not react to the experimental settings, showing no interest for the colored stripes. Specifically, we found 11 species that did not react to any complete experimental protocol (i.e. were not active in enough trials to consider a valid test, etc.). Moreover, six species fully ignored the experimental setting. Despite the experiment was designed to isolate learning, there are other confounding variables that can affect the experimental responses including: stress management (Even et al., 2012), neophobia (Forrest & Thomson, 2009), motivation (Dyer et al., 2002) or colour perception (Chittka & Wells, 2004). Therefore, species that did not react were not necessarily “species unable to learn” and alternative explanations are possible. Using wild animals can also have caveats, as stress may make individuals to behave in strange ways. However, evidence from *Apis mellifera* does not suggest that using wild individuals change the results of learning tests compared to bees born in laboratory (Muth et al., 2017), but further analyses are needed for species that do not habituate so well to experimental settings.

Our results provide the first evidence that insects with larger brains, both in absolute and relative terms, perform better in an associative learning test than species with smaller brains, challenging previous claims that variation in brain size is not biologically meaningful (Chittka & Niven, 2009; Healy & Rowe 2007). However, it remains to be demonstrated whether similar patterns can be extended to other learning mechanisms. The underlying processes also warrant explanation. The challenge is to elucidate whether variation in learning speed across species reflects sensorial, cognitive, physical or emotional responses, and how these responses are associated with finer brain structures like mushroom bodies, neuron density or optimized neurons synapses. ●

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Supplementary material

Table S1 | Table S1 Names and coordinates from the sample sites where the individuals were captured.

Site	Latitude	Longitude
Alamillo Park	37,42	-5,99
El Arboreto, botanical garden	37,39	-6,04
Pablo de Olavide campus	37,35	-5,94
Aznalcázar, natural park	37,23	-6,17
La Rocina, Doñana natural park	37,12	-6,51
Niebla, plot	37,41	-6,68
Convento de la luz, plot	37,29	-6,75

Bees use anthropogenic habitats despite strong natural habitat preferences

Abstract

Aim

Habitat loss and alteration are widely considered one of the main drivers of current pollinator diversity loss. Yet little is known about habitat importance and preferences for major groups of pollinators, although this information is crucial to anticipate and mitigate the current decline of their populations. We aim to rank and assess the importance of different habitats for bees, to determine the preference for and avoidance of particular habitat types by different bees, and to quantify the diversity of bees within and among habitats.

Location

Northeastern US.

Time period

The sampling was done over 15 years (2001-2015).

Major taxa studied

Apoidea

Methods

We used an unprecedented extensive dataset of >15,000 bee specimens, comprising more than 400 species collected across northeast USA. We extracted habitat information from the points and used network analyses, null models comparisons and beta-diversity analysis to assess habitat importance, habitat preference, use and diversity.

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Results

We found that natural habitats sustain higher bee diversity and a different set of species than agricultural and urban areas. Although many bee species can use human-altered habitats, most species exhibited strong preferences for forested habitats and only a few preferred altered habitats over more natural habitats. In contrast to previous studies, landscape composition only had moderate buffer effects on diversity loss. The loss of biodiversity in human-altered environments could have been higher had not been partially compensated by the presence of human commensals and exotic species.

Main conclusions

Although human-altered environments may harbour a substantial number of species, our work reveals that preserving natural areas is still essential to guarantee the conservation of bee biodiversity.

Keywords

Habitat importance, Habitat preference, Habitat use, Landscape, Pollinators, Urban. ●

Introduction

Plant pollinators are considered to be of conservation concern worldwide (Goulson, Nicholls, Botías & Rotheray, 2015). In Europe, for example, more than 20% of bees assessed by the IUCN are threatened (Nieto et al., 2014), and in North America there is evidence that the populations of many bee species have drastically declined in the last decades (Cameron et al., 2011; Bartomeus et al., 2013). While the current loss of pollinators may have a variety of causes, the conversion of natural habitats into urban and agricultural systems is widely thought to be one of the main drivers (Winfree, Bartomeus & Cariveau, 2011). Currently, over 40% of Earth's terrestrial surface has already been altered by humans (Ellis, Goldewijk, Siebert, Lighthman & Ramankutty, 2010) and the surface is expected to continue increasing in the next decades (Tilman et al., 2001). Given that more than 80% of plants are pollinated by animals (Ollerton, Winfree & Tarrant, 2011), including 75% of crops species (Klein et al., 2007), the extinction of pollinator species is expected to have far-reaching impact on ecosystem functioning and human well-being.

Despite growing concern over the consequences of pollinators loss, it is currently uncertain how they are affected by changes in land use intensity. A major obstacle has been the paucity of large-scale datasets on species-habitat associations at large geographical scales. In fact, current knowledge on habitat importance for bees comes mainly from expert opinion and modelling efforts (Lonsdorf et al., 2009, Koh et al., 2016).

In the present study, we use an unusually large dataset from an extensive monitoring program for bees in the northeast and mid-west US to directly estimate habitat importance and species habitat-preference across the entire region. The dataset comprises 15,762 individual bees from 433 species recorded over 15 years, covering most of the habitats and species in the region. Based on this dataset, we first asked how bee communities differ in richness and composition within and among habitats at a regional scale. Although previous work has established that bee community richness and composition can strikingly differ between natural vegetation and altered habitats for particular regions (Brosi, Daily, & Ehrlich, 2007; Kleijn et al., 2015), ours is the most general and comprehensive assessment to date. We investigated habitat importance for bees using tools derived from network theory, which allow us to describe the complex web of habitat-species interactions. As pollinators are mobile species and the surrounding landscape often determines the presence of a species in the focal habitat (Kremen, Williams & Thorp, 2002), we also investigated the effect of the surrounding landscape in determining bee responses beyond the habitat where each bee was observed.

The assessment of relative habitat importance can provide general insights into species sensitivity to environmental change. However, the presence of a species in a particular habitat does not necessarily indicate that the species is doing well in that habitat, but may reflect that this is the only habitat available. So to better assess species sensitivities to habitat alteration, it is necessary to assess specific habitat preferences. Preference is defined as the tendency of a species to be non-randomly associated with certain environments (Rice, 1984). Therefore, a second goal of our study was to investigate such species-habitat associations. We used null models to assess habitat preference and avoidance for 45 bee species with a sufficiently large sample of occurrences (species with ≥ 100 independent records). We then characterised their sensitivity to human altered habitats by estimating the extent to which the species occurs in highly-altered environments or, instead, use multiple habitats that buffer them against destruction of their preferred habitat.

Although human-induced changes in the habitat are generally perceived to have a negative impact on pollinator species (Winfree et al., 2011), they can also offer ecological opportunities to some species (McFrederick & LeBuhn 2005, Matteson, Ascher & Langellotto, 2008). Pollinators are highly mobile animals, and some are capable of using multiple habitat types (Kremen et al., 2002). For example, some bee species nest in forested habitats while foraging in agricultural habitats (Klein, Steffan-Dewenter & Tscharrntke, 2003), and some even use highly transformed environments such as those altered by urbanisation and intensive agriculture (Saure, 1996, Baldock et

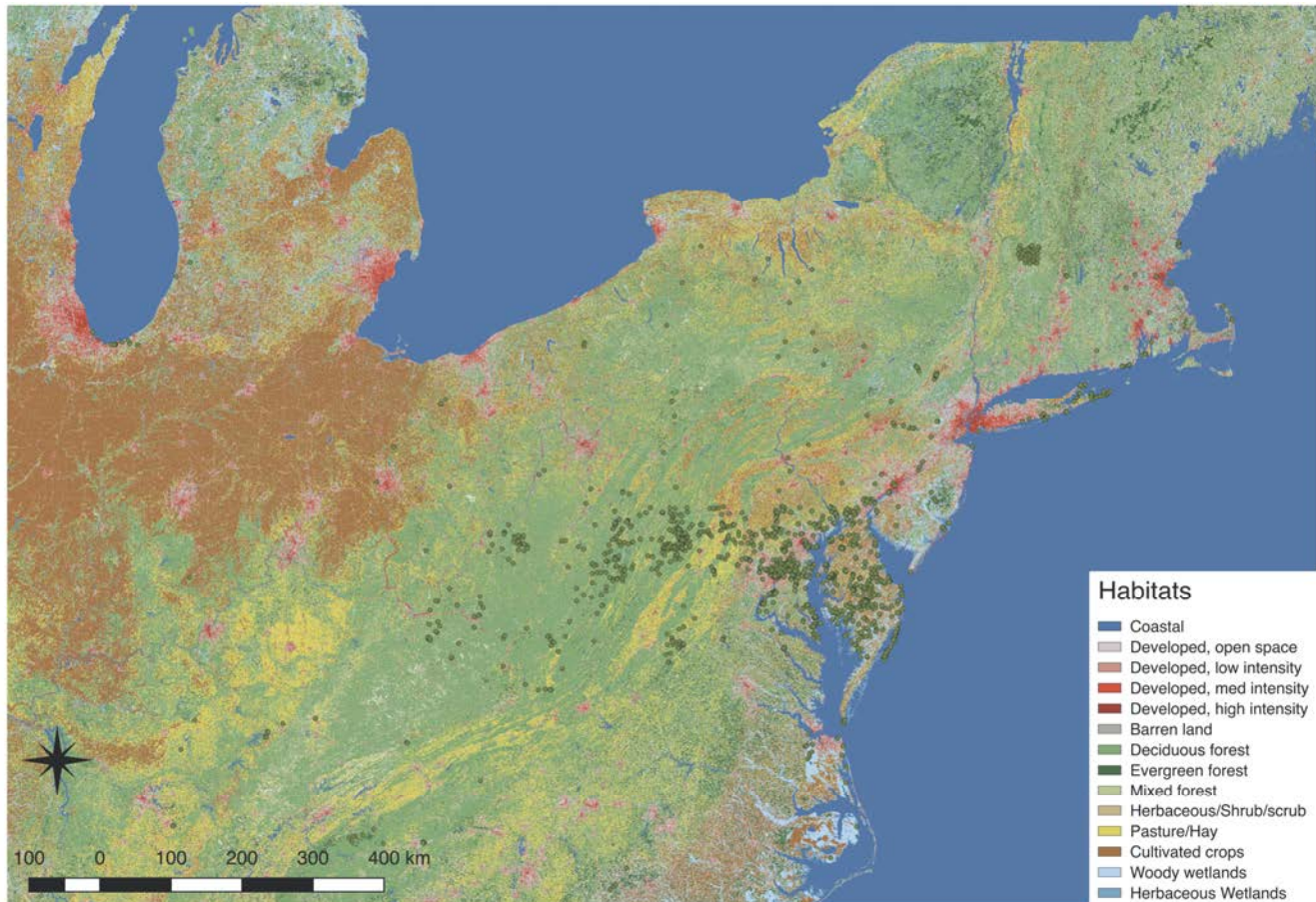


Figure 1 | Map of the sampling area. It covers the area from 35.01S to 42.79N and -87.54° W to -69.97° E, North East USA. This area was selected as it represents a large but homogeneous region.

al., 2015). If human altered habitats create new opportunities for some pollinators, this may reduce the impact of habitat loss and alteration on pollinator communities and associated ecosystem services (Kremen et al., 2002). Our final goal was to assess the extent to which human-altered habitats provide opportunities for native and exotic species. ●

Methods

Sampling design

Bees were intensively sampled from 2000 to 2015 by USGS Native Bee Inventory and Monitoring Laboratory, their collaborators, and volunteers using pan traps, (~75%) and hand netting (~25%) (Westphal et al., 2008). As the sampling was designed to maximise the study area to accurately cover all the main habitats of the region, a different location was selected for each sampling point. However, care was taken to cover the entire phenology and to avoid biases in the use of monitoring techniques among habitats (Figure S1, Table S1). Although sampling was carried out over a larger region, we restricted our analyses to

samples taken from the area with the highest sampling effort, covering latitude 35.01 S to 42.79 N and longitude -87.54° W to -69.97° E (Fig. 1).

After capture, the coordinates of the collection site were recorded using GPS and the specimen was identified to the species level by expert taxonomists. Unidentified individuals or extremely rare species (i.e. those collected only once) were removed from the dataset. Some species that were particularly difficult to separate taxonomically were pooled together for the analysis (See Table 2). Overall, we retained 291,195 individuals, which represent a 68% of the original data. To ensure the independence of the collection events, we excluded from analysis specimens belonging to the same species when collected at the same locality during the same day. After this last filtering, the final dataset comprised 15,762 individuals from 433 species collected from 1,452 different sites, all of which were used in subsequent analysis. All specimens were vouchered at USGS Native Bee Inventory and Monitoring Laboratory.

For each georeferenced sampling site, we extracted habitat information using the National Land Cover Database (NLCD) raster layer

(Homer et al., 2015) with the R packages raster, rgdal and stringr (Bivand, et al., 2018 a); Bivand et al., 2018 b); Hijmans, 2015). The 14 habitats considered in this study are described in Table 1 and the number of sampling points for each habitats in Table S1. We extracted the habitat type from the focal point based on the precise coordinates. To take into account the surrounding landscape, we also extracted the habitat composition in a buffer of 1,000 m radius around each focal point; 1,000 m is the maximum distance that most bees under 4 mm of intertegular span can forage (Greenleaf, Williams, Winfree & Kremen, 2007). While our dataset spans 15 different years, information on land cover was only available for 2001, 2006 and 2011. To account for this, bees sampled

before 2005 were assigned to habitats based on information from the 2001 layer (28 % of our data), those sampled between 2006-2010 were assigned to the 2006 layer (42%) and for the rest (2011-2015) we used the 2011 layer (29%). The ninety eight percent of the sites sampled maintained their land use classification along the three raster layers used. Field notes were taken during the sampling, and most of them matched with the NLCD raster layer, but there were too many note categories and only 51% of the data collected had field notes. To estimate availability of each habitat in our study region, we divided all the pixels of the habitat by the total pixels of the entire study area (Fig. S2 in Supporting information).

Table 1 | Description of the habitats used to assess importance, use and preference for bee pollinator species, as they are defined and contained in the National Land Cover Database 2011, which is a modified version of the Anderson Land Cover Classification system (Anderson, Hardy, & Roach, 1976)

Habitat	Description
Barren land	Areas of bedrock, desert pavement, scarps, talus, slides, volcanic material, glacial debris, sand dunes, strip mines, gravel pits and other accumulations of earthen material. Generally, vegetation accounts for less than 15% of total cover.
Coastal	Areas next to open water, generally with less than 25% cover of vegetation or soil.
Cultivated Crops	Areas used for the production of annual crops, such as corn, soybeans, vegetables, tobacco, and cotton, and also perennial woody crops such as orchards and vineyards. Crop vegetation accounts for greater than 20% of total vegetation. This class also includes all land being actively tilled.
Deciduous Forest	Areas dominated by trees generally greater than 5 m tall, and greater than 20% of total vegetation cover. More than 75% of the tree species shed foliage simultaneously in response to seasonal change.
Developed: Low Intensity	Areas with a mixture of constructed materials and vegetation. Impervious surfaces account for 20–49% of total cover. These areas most commonly include single-family housing units.
Developed: Medium Intensity	Areas with a mixture of constructed materials and vegetation. Impervious surfaces account for 50–79% of the total cover. These areas most commonly include single-family housing units.
Developed: Open Space	Highly developed areas where people reside or work in high numbers. Examples include apartment complexes, row houses, and commercial/industrial. Impervious surfaces account for 80–100% of the total cover.
Emergent Herbaceous Wetlands	Areas where perennial herbaceous vegetation accounts for greater than 80% of vegetative cover and the soil or substrate is periodically saturated with or covered with water.
Evergreen Forest	Areas dominated by trees generally greater than 5 m tall, and greater than 20% of total vegetation cover. More than 75% of the tree species maintain their leaves all year. Canopy is never without green foliage.
Herbaceous /Hay/Pasture*	Areas of grasses, legumes, or grass–legume mixtures planted for livestock grazing or the production of seed or hay crops, typically on a perennial cycle, also containing areas dominated by graminoid or herbaceous vegetation. Pasture/hay vegetation accounts for greater than 20% of total vegetation.
Mixed Forest	Areas dominated by trees generally greater than 5 m tall, and greater than 20% of total vegetation cover. Neither deciduous nor evergreen species are greater than 75% of total tree cover.
Shrub/Scrub	Areas dominated by shrubs less than 5 m tall with shrub canopy typically greater than 20% of total vegetation. This class includes true shrubs, young trees in an early successional stage or trees stunted from environmental conditions.
Woody Wetlands	Areas where forest or shrub-land vegetation accounts for greater than 20% of vegetative cover and the soil or substrate is periodically saturated with or covered with water.

*Herbaceous and Hay/Pasture are classified as two different habitats in NLCD. We merged them because herbaceous areas in our sampling region are always for livestock (Koh et al 2016).

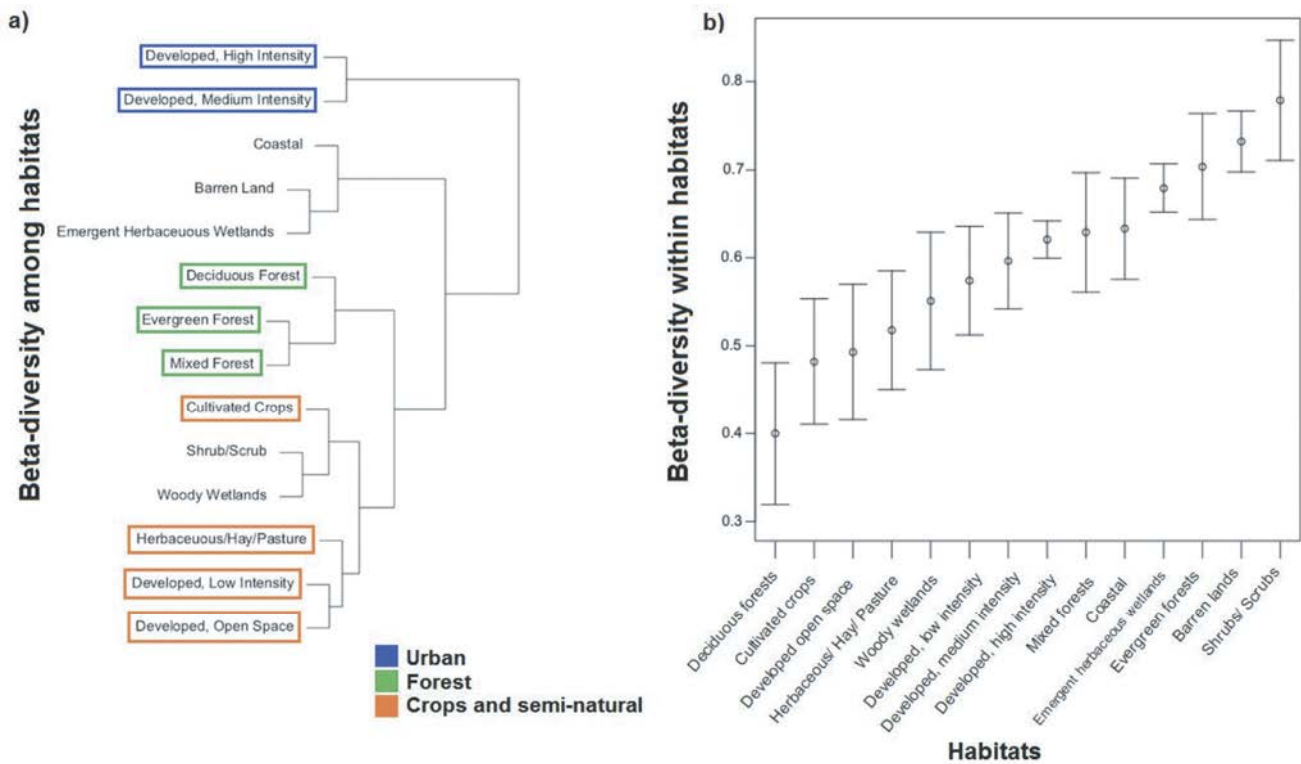


Figure 2 | Beta diversity analysis results: (a) using the beta diversity values among habitat types (Sørensen beta diversity dissimilarity matrix). We grouped the 14 habitats according to their similarity in community composition. Groups of habitats used for the preference analysis are highlighted. (b) Estimated beta diversity and standard deviation within habitats, calculated as the slopes of the species sample accumulation curves for each habitat, as an indicator of the rate new species for that habitat appear with increasing sample size. Larger values indicate more rapid gain of new species with increased sample size

We analysed the possible spatial autocorrelation in sampling events using Moran's Index and a multivariate homogeneity of groups dispersions analysis using the package *ape* (Paradis & Schliep 2018) and *vegan* (Oksanen et al., 2018). First, we tested autocorrelation for each habitat and we found that the autocorrelation values were very low (ranging from observed *I* values of 0.01 to 0.16). Second, we compared the dispersions of the habitat sampling points across habitats to assess the potential differences in the clustering of the sampled points in different habitats. Most of the habitats did not present different dispersions and have around the same distribution of distances to the centroid (Figure S5). Hence, and as our analysis are pooled by habitat type, we do not further correct for spatial autocorrelation, but acknowledge that the results are driven by the habitat configuration of the region and can not be extrapolated to other regions. ●

Data analysis

Habitat importance

We evaluated the importance of different habitats for bee species using the number of species detected in each habitat (i.e., species

richness). Although species richness is a widely used index of habitat importance (Chao & Jost 2012), it treats all species as equal, which may not be justified for conservation purposes. For instance, a habitat may have high species richness but primarily sustain common species that are widely present elsewhere, whereas another habitat with equal or lower species richness could mostly support rare species that are highly dependent on this particular habitat. To tackle this limitation, we also evaluated the importance of different habitats by means of a metric of habitat strength.

Habitat strength was calculated using a metric derived from network analysis. The strength of a node (i.e., a single element from a network, in this case the focal habitat) in a bipartite interaction network of species per habitats is defined as the sum of the dependencies of nodes corresponding to the other level in the bipartite network (in this case, the bee pollinator species) linked to that habitat (Bascompte, Jordano, & Olesen 2006). The dependence of a bee pollinator on a given habitat is calculated as its proportional use of this habitat relative to the other habitats and ranges from zero to one. For example, if a species node has a dependence value near one on a habitat node, we conclude that species depends strictly on that habitat. However, if the dependence is

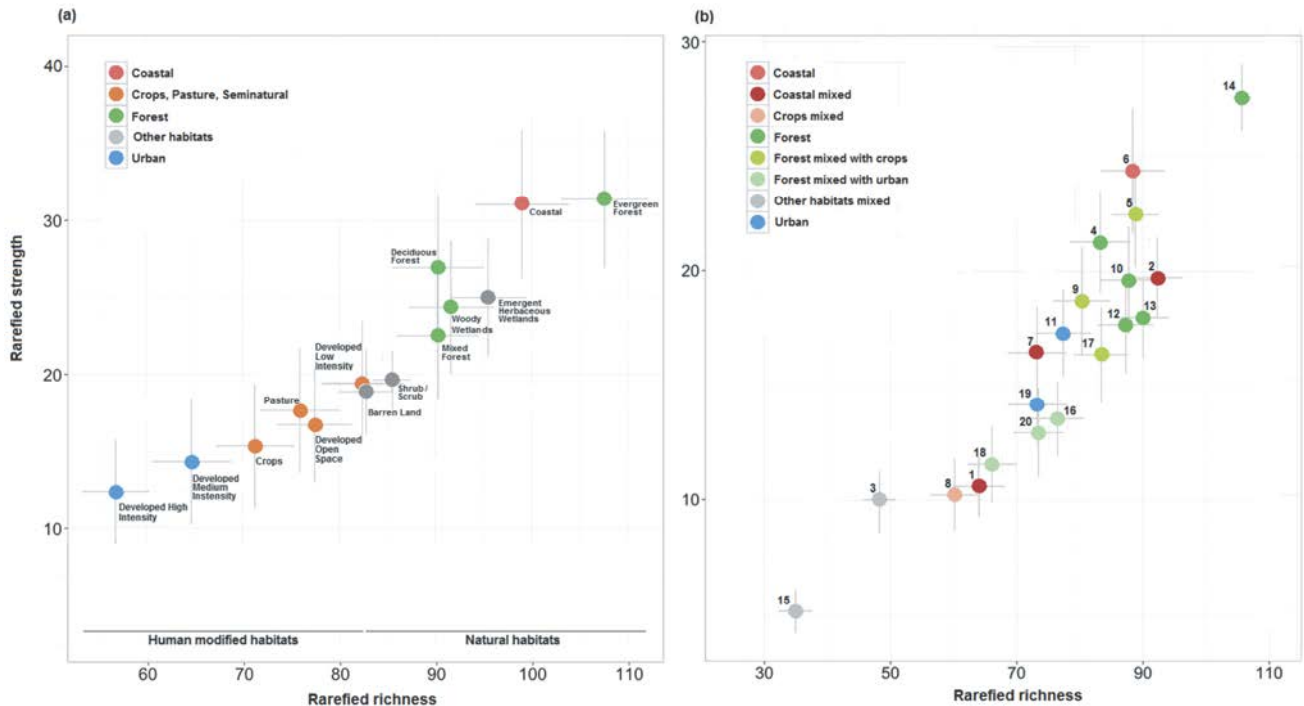


Figure 3 | Importance of each analysed habitat. We defined importance as a function of both strength and richness. Both metrics are correlated, but give different information (see text for details). Each point represents the mean of 100 rarefied strength and richness values for each habitat. Bars are the standard deviation across 100 runs for both strength and richness. (a) Shows habitat importance results considering only the habitat where bees were found. While human modified habitats are less important than the natural habitats, they still sustain a substantial amount of pollinator species. (b) Shows habitat importance considering landscape composition where species were collected. Similar landscapes were grouped by colour; detailed composition of each landscape can be found in Supporting Information Table S2

close to zero, the species does not depend on that single habitat and instead, uses other habitats.

To calculate richness and strength for each habitat, we first rarefied each habitat to equalise sampling effort to that of the least well-sampled habitat. To this purpose, we first calculated the coverage value (the percentage of the total species diversity) for each habitat and then rarefied to the number of individuals necessary for equal coverage of all habitats (Hsieh, Ma, & Chao, 2016). We chose the common coverage value to be 0.60, because shrub/scrub was the less covered habitat (0.62 of coverage), meaning that 60% of species richness from each habitat was sampled to calculate richness and strength. Coverage was calculated using the “iNEXT” package (Hsieh et al., 2016). By using the same coverage for every habitat we avoided that the most sampled habitats were over-represented. However, the total richness at the regional scale (i.e., gamma-diversity) is likely to depend on the area covered by each habitat, independent of the number of samples for each habitat, so we show the proportion of each land cover type (Fig. S2) to aid interpretation of gamma-diversity values. However, species diversity did not increase with the total area of each habitat ($p = 0.56$, $R^2 = -0.052$). Although sampling

date and trap/hours might also affect richness, this information was not available and hence we assume that these factors are not biasing the data.

Although the importance of a habitat can be characterised in terms of species richness and habitat strength, beta-diversity among and within habitats is fundamental to identify the habitats that are complementary in species composition and to determine the degree of species turnover within habitats across space (Whittaker, 1960). Among habitat beta-diversity was calculated using Sørensen beta-diversity dissimilarity index across all pairs of habitats (Sørensen, 1948). Habitats were then grouped according to their similarity using a hierarchical cluster analysis (Fig. 2a). Within habitat beta-diversity was calculated as the slope of the species-samples accumulation curves for each habitat (Fig. 2b). This metric represents the rate at which new species appear within that habitat as sample size increases. The species-samples relationship was almost linear and hence we did not log-transformed the data (Baselga & Orme, 2012), although log-transforming the data using natural logarithms produced similar results.

As bees are mobile organisms that likely depend on adjacent habitats in the landscape, we repeated the above analyses at a lands-

Table 3 | Species habitat preference or avoidance. The first column indicates the number of rarefied habitats used for each species listed, the other three columns show for every habitat (see Figure 4 for habitat grouping) the habitat preference (>0.95, marked in blue) or avoidance (>0.05, marked in red), calculated as the probability of having a higher or lower observed abundance than expected under the null model

Habitat	Habitats used	Pasture and Crops	Forests	Urban
<i>Agapostemon virescens</i>	12.47	0.66	0.1	0.89
<i>Andrena carlini</i>	11.38	0.43	0.78	0.01
<i>Andrena cressonii</i>	12.41	0.53	0.61	0.02
<i>Andrena erigeniae</i>	11.43	0.1	1	0.01
<i>Andrena nasonii</i>	12.32	0.09	0.99	0.03
<i>Andrena perplexa</i>	10.82	0.94	0.51	0.01
<i>Andrena violae</i>	11.85	0.39	0.92	0.01
<i>Apis mellifera</i>	12.46	0.5	0.61	0.69
<i>Augochlora pura</i>	11.34	0.29	0.96	0.21
<i>Augochlorella aurata</i>	12.3	0.05	0.75	0.5
<i>Bombus bimaculatus</i>	12.04	0.05	0.99	0.04
<i>Bombus fervidus</i>	12.93	0.87	0.03	0.73
<i>Bombus griseocollis</i>	12.28	0.19	0.43	0.82
<i>Calliopsis andreniformis</i>	12.68	0.76	0.14	1
<i>Ceratina calcarata/dupla/mikmaqi*</i>	12.53	0.53	0.24	0.27
<i>Ceratina strenua</i>	12.06	0.99	0.02	0.73
<i>Halictus confusus</i>	12.2	0.52	0.6	0.99
<i>Halictus ligatus/poeyi</i>	12.42	0.09	0.26	0.95
<i>Halictus rubicundus</i>	11.64	0.78	0.18	0.67
<i>Hylaeus affinis/modestus</i>	12.38	0.3	0.95	0.41
<i>Lasioglossum bruneri</i>	11.81	0.87	0	0.96
<i>Lasioglossum callidum</i>	12.35	0.99	0.01	0.11
<i>Lasioglossum coriaceum</i>	12.52	0	1	0.02
<i>Lasioglossum cressonii</i>	11.9	0.01	1	0.04
<i>Lasioglossum hitchensi</i>	12.13	0.88	0.17	0.55
<i>Lasioglossum illinoense</i>	11.59	0.79	0.77	0.89
<i>Lasioglossum imitatum</i>	12.18	0.11	0.99	0.9
<i>Lasioglossum near_admirandum</i>	11.29	0.85	0.44	0.83
<i>Lasioglossum oblongum</i>	12.33	0.34	0.01	0.15
<i>Lasioglossum pectorale</i>	11.59	0.58	0.11	0.15
<i>Lasioglossum pilosum</i>	12.59	0.79	0	0.99
<i>Lasioglossum tegulare</i>	12.51	0.76	0.01	0.25
<i>Lasioglossum versatum</i>	11.96	0.5	0.7	0.25
<i>Megachile brevis</i>	12	0.89	0.01	0.63
<i>Megachile mendica</i>	11.9	0.08	0.34	0.81
<i>Melissodes bimaculatus</i>	11.94	1	0	0.84
<i>Nomada bidentate_group</i>	12.52	0.42	0.98	0.02
<i>Nomada pygmaea</i>	11.65	0.21	0.96	0
<i>Osmia atriventris</i>	10.27	0.18	0.98	0
<i>Osmia bucephala</i>	11.94	0.29	1	0.11
<i>Osmia georgica</i>	12.93	0.22	0.95	0.12
<i>Osmia pumila</i>	12.45	0.19	0.97	0.02
<i>Osmia taurus</i>	11.64	0.57	0.59	0.01

*Continues

<i>Ptilothrix bombiformis</i>	11.53	0.78	0	0.99
<i>Xylocopa virginica</i>	11.65	0.28	0.6	0.79

* This group of species was merged because they are morphologically similar and very difficult to separate by classic taxonomy. Note grouping can mask specific habitat preferences, but give different information (see text for details). Each point represents the mean of 100 rarefied strength and richness

cape scale. We classified landscapes at a 1,000 m radius surrounding each sampling site into discrete groups using a k-mean algorithm. The total number of groups (k) was determined using “the elbow method”, where k is the number of clusters beyond which additional clusters no longer improve the model. In our analyses, k was estimated as 20 (Fig. S3) and hence 20 types of landscapes were defined. These 20 landscape categories range from mainly forested landscapes, to more complex landscapes that include a mix of agricultural and forested areas (Table S2).

Species habitat use and preference

Disentangling species habitat use and preference requires a large sample size for each species studied, thus we only used here species that had >100 independent collection events ($n = 45$ species). We considered that an habitat was used for a species when at least one individual of that species was sampled in that habitat. Although we cannot know if the species is nesting or foraging on the habitat where it was captured, we assume that the repeated capture of a species in the same habitat indicates that this is likely to happen. To normalise for differences in the number of species occurrences, we assessed habitat use on 100 rarefaction events for each species, i.e. performing 100 sub-sampling events of 100 occurrences, and extracting the mean number of habitats used by each species over 100 the sub-sample events.

We defined habitat preferences as the non-random association of a species with certain habitats. Therefore, a species was considered to exhibit habitat preference if it was sampled in a habitat more frequently than expected by chance. Species preferences can be confounded with species distributions if their geographic range only covers some of all available habitats. For example, species distributed only in the northern part of the sampling area may appear to prefer evergreen forests simply because this habitat is more common there. However, this limitation was negligible in our study because the geographic range of the almost all species studied covered the entire study area (Table S4, Bartomeus et al., 2013; Schuh, Hewson-Smith & Ascher, 2010.), implying that all sites could have been potentially occupied by any species if habitat choice was completely random. We compared a habitat-species matrix (i.e., the “observed” matrix) to 1,000 null matrices (i.e., the “null” matrices). These null matrices were created by means of the function “nullmodel” contained in the “bipartite” package (Dormann, Frund, Bluthgen & Gruber, 2009). This function generates random bipartite tables maintaining the sum of rows and columns using Patefield’s (Patefield, 2012) algorithm, so the proportional abundance of species and habitats is maintained, but their associations are re-shuffled. We considered that a species exhibited preference for a particular habitat if it was more abundant than the 0.95 quantile of expected abundances, species less abundant than the 0.05 quantile were considered to avoid that habitat (Sol, González-Lagos, Moreira, Maspons & Lapiedra, 2014). However, the degree of preference is better described as a continuum, and hence we also describe it as effect sizes. Note that for parasitic species, like No-

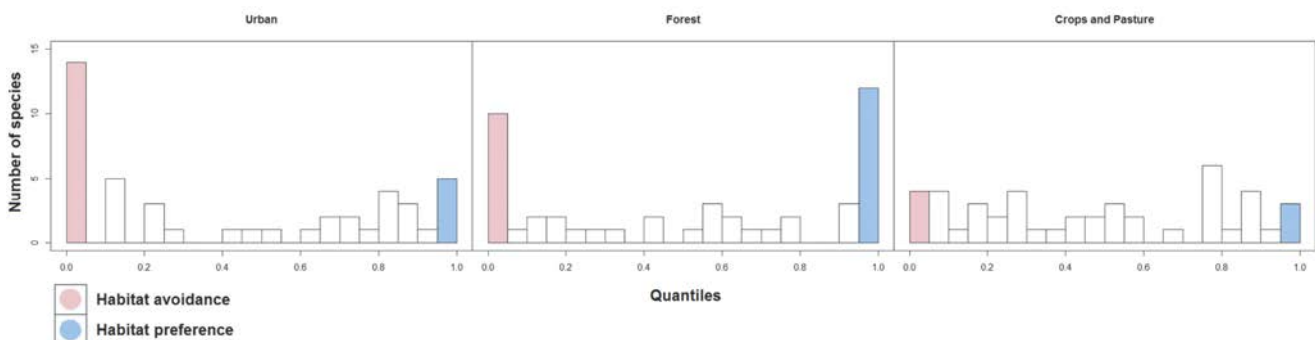


Figure 4 | The distribution of species habitat preferences (See also Supporting Information Table S4). Red bars are the number of species avoiding that habitat, and blue bars are the number of species preferring that habitat. Urban habitats have both avoiders (14 of 45) and exploiters (6 of 45). Forests also have avoiders (10 of 45) but have a higher proportion of exploiters (14 of 45). Crops and pasture are more equally distributed, with few species preferring or avoiding them.

mada spp., habitat preferences should correspond to those of their hosts. Preferences calculated for groups of species may mask each species preferences if they are not specialist and our results for this groups must be interpreted carefully, specially for those groups of non-specialist bees.

For the sake of clarity, we present in the results section habitat preferences grouped by three main habitat types: 1) urban: developed, high intensity and medium intensity; 2) crops and semi-natural areas: cultivated crops, herbaceous/hay/pasture, developed, low intensity and open space; and 3) forested: deciduous forest, evergreen forest and mixed forest, see Fig. 2 for details. ●

Discussion

Four main conclusions can be drawn from our comprehensive analysis of bee species across northeast USA. First, although no habitat appeared to be completely inhospitable to bees, many species showed a strong preference for natural habitats while consistently avoiding human-altered habitats. Second, the dominant habitat within the landscape was the strongest determinant of species diversity and, contrary to previous studies (Steffan-Dewenter and Tscharrntke 1999), landscape composition only had moderate buffer effects on diversity, but different communities could be found in the same habitat for different spots. Third, and as a consequence of the two previous findings, human-altered habitats supported significantly less diversity of species (and had less strength) than the surrounding natural environments. Fourth, the loss of biodiversity in human-altered environments could have been higher had not been partially compensated by the addition of human commensals and some exotic species.

Natural habitats were the most suitable for bees regarding both importance (richness and strength) and the number of species that preferred them. Evergreen forests in particular exhibited the highest habitat importance in the region, despite harbouring very few habitat specialists. These forests are widespread in the region and comprise flower rich areas like the coastal Pine Barrens that are fragmented and crossed by right of way infrastructures, which can increase their attractiveness for bees and help explain why they exhibited high spatial beta-diversity (Hill & Bartomeus, 2016). Unlike evergreen forests, bee communities associated with deciduous forests and other natural habitats had lower beta-diversity and lower overall species richness. However, they sustained a large number of habitat specialists, a possibility already advanced in previous studies (Burkle, Marlin & Knight, 2013).

As specialised adaptations to particular habitats may limit the success of bee species in other habitats, it is unsurprising that the majority of forest specialist species avoid urban habitats and/or crops and pastures. It follows that many of these species may become ex-

tinct, at least locally, if forested habitats disappear from the landscape (Burkle et al., 2013).

Much of the current risk of species loss comes from the replacement of natural forests by crops and pastures, the most frequent alteration of natural habitats (Newbold et al., 2015). Crops and pastures exhibited a significantly low species richness and strength values compared to natural forests. Thus, while some species used crops and pastures opportunistically, very few are plastic enough to exploit them (see also Kleijn et al., 2015). Moreover, within habitat beta-diversity for agricultural habitats was low, reinforcing the view that these habitats sustain a limited set of common habitat generalists. Cropping systems are however highly diverse, ranging from cereal monocultures to diverse flowering cropping systems (Donald, 2004) implying that the impact may vary depending on the intensity of the alterations. Although our dataset does not allow for finer scale analyses separating the effects of different crops, current evidence suggests that most modern crop managing practices (i.e., herbicide and insecticide application) are likely to negatively impact on bee populations (Goulson et al., 2015, Woodcock et al., 2016).

While the conversion of natural habitats to cities is not so widespread as the replacement by crops and pastures (Newbold et al., 2015), such conversion is considered the most drastic alteration of natural ecosystems. In line with previous studies (Chapin, 1997, Sol et al., 2014), urbanised habitats harboured substantially fewer species than the surrounding natural habitats. The persistence of bee populations in urban habitats may be limited by resource availability. Food resources are often dominated by exotic or ornamental species (Ellis, Antill, & Kreft, 2012), which few bee species are able to exploit (Bartomeus, Fründ & Williams, 2016). However, urban habitats also offer resource opportunities for some species. For example, *P. bombiformis* specialises in exploiting plants from the *Hibiscus* genus, a popular ornamental plant. New opportunities may also emerge for some bees that are able to use pre-existing cavities or holes in of human-made constructions (Cane, Griswold, & Parker, 2007). As natural enemies are often scarcer in cities (Sorace & Gustin, 2009), these human commensals may proliferate despite their little opportunity to adapt to the new environments. Likewise, we show that non-indigenous species, proliferate in urbanised environments, being most of the exotic bees collected only in urban areas. Although the presence of human commensals and non-indigenous species importantly contributed to increase species richness in urbanised environments, their diversity was low and hence did not fully compensate for the loss of diversity associated with urban avoiders (see also Sol, Bartomeus, González-Lagos & Pavoine, 2017).

Past work suggests that while undisturbed habitats are essential to preserve biodiversity, habitats that have experienced low intensity alterations may still help buffer against extreme diversity

loss (Frishkoff et al., 2014, Sol et al., 2017). Our results provide some support to this view, showing that species loss was not as accentuated in moderately altered habitats (Table 2). For example, large gardens within cities provide diverse food resources for pollinators, harbouring a higher bee diversity and abundance than city centres. As example, in Berlin, half of the total German bee fauna was recorded inside the city (Saure, 1996) and in San Francisco, USA, higher mean abundances of *Bombus* spp were found in urban gardens compared with natural parks beyond the city boundaries (McFrederick & LeBuhn 2006). Likewise, some flowering crop fields provide good foraging opportunities for generalist bee species (Magrach et al., 2018), despite low plant diversity and short bloom periods (Donald, 2004).

Although the analyses of single focal habitats are essential to establish habitat importance and assess the sensitivity of species to habitat alterations, species diversity typically depends on the mosaic of habitats present in a region (Steffan-Dewenter & Tschardt, 1999). We expected that species able to use multiple habitats would be less vulnerable to habitat modification than species with specific habitat requirements. However, at the landscape level, our results show that the dominant habitat within the landscape was the strongest determinant of species diversity and that more complex landscapes only had intermediate diversity levels. This is exemplified by the finding that forested habitats intermixed with human-altered habitats had lower species diversity than fully forested habitats.

Altogether, our results provide clear evidence that the loss and alteration of natural habitats caused by human activities leads to many “losers” and a few “winners”. Albeit the specific bee-habitat associations vary as a function of the intensity of the alterations and may change in other geographical regions, the observed diversity loss associated with land use changes might be general (Palma et al., 2017). Admittedly, our estimations of species sensitivity to habitat alterations are conservative, as these analyses were restricted to common species and hence some habitat specialists may have been missed. However, the analyses using the strength index, which did include rare species, consistently showed that the species dependency on a given habitat decreased with the degree of habitat modification. Although the loss of bee diversity may be in part compensated by the colonisation of native opportunists and exotic species, their diversity is insufficient to replace the species that are lost by land use changes. Moreover, the new species may differ from those they replace in functional traits, particularly those that provide environmental tolerance such as life history, body size, multivoltinism and dietary generalism (see Kitahara, Sei & Fujii, 1994; Bartomeus et al., 2013; Sol et al., 2014; Scheper et al., 2014), and hence there is little guarantee that they may play similar roles in the ecosystem (Bartomeus, Cariveau, Harrison &

Winfree, 2017). Thus, preserving natural habitats may provide the most effective strategy to guarantee in the long-term the ecosystem functions and services provided by bee biodiversity. ●

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Data accessibility

The data used for this research is archived in dryad <https://data-dryad.org/resource/doi:10.5061/dryad.8md5419>

Code used to reproduce the analysis can be consulted at GitHub <https://github.com/MiguelAngelCollado/habpref> ●

Supplementary material

Table S1| This tables shows that we used for each habitat the different sampling methods in similar proportion and the number of sites sampled for each habitat.

Habitat	Pan traps	Hand nets	Other	Sampling sites
Barren land	0.86	0.14	0	10
Coastal	0.67	0.20	0.13	98
Cultivated crops	0.80	0.15	0.05	148
Deciduous forest	0.72	0.23	0.05	457
Developed low intensity	0.80	0.20	0	70
Developed medium intensity	0.81	0.19	0	47
Developed high intensity	0.65	0.35	0	25
Developed open space	0.84	0.13	0.03	131
Emergent herbaceous wetlands	0.80	0.20	0	47
Evergreen forest	0.81	0.19	0	43
Herbaceous/Hay/Pasture	0.74	0.23	0.03	139
Mixed forest	0.68	0.30	0.02	52
Shrub/Scrub	0.87	0.13	0	19
Woody wetlands	0.73	0.24	0.03	121

Table S2| Contains the centroids of every group created with the k-means algorithm. grouping this way the 1452 sampled occurrences with different landscape composition in 20 groups. A centroid value equals to the percentage of each habitat contained within the group. For a better visualisation. the main habitats contained in every group (Those with more than a 0.1% under our criteria) are marked in bold letters. For example. group 1 has 0.40 proportion of coastal and 0.26 of cultivated crops. the rest are too small to be accounted. so we described this group as coastal-crops.

Habitat	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Coastal	0.40	0.33	0.14	0.01	0.04	0.78	0.42	0.02	0.01	0.02	0.07	0.03	0.01	0.00	0.09	0.02	0.04	0.00	0.02	0
D. Open Space	0.04	0.09	0.03	0.04	0.06	0.02	0.04	0.06	0.06	0.06	0.12	0.10	0.02	0.07	0.05	0.26	0.05	0.10	0.26	0.11
D. Low Intensity	0.01	0.03	0.02	0.00	0.02	0.02	0.04	0.06	0.06	0.06	0.12	0.10	0.02	0.07	0.05	0.26	0.05	0.10	0.26	0.11
D. Medium Intensity	0.00	0.16	0.01	0.00	0.01	0.01	0.03	0.01	0.00	0.00	0.34	0.01	0.00	0.00	0.01	0.06	0.01	0.01	0.16	0.01
D. High Intensity	0.00	0.01	0.00	0.00	0.00	0.01	0.01	0.00	0.00	0.00	0.25	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.05	0
Barren Land	0.00	0.01	0.00	0.00	0.01	0.54	0.09	0.00	0.00	0.00	0.01	0.00	0.01	0.03	0.00	0.00	0.00	0.00	0.00	0
Deciduous Forest	0.03	0.27	0.00	0.86	0.08	0.02	0.01	0.07	0.51	0.67	0.02	0.43	0.02	0.30	0.02	0.30	0.16	0.19	0.08	0.31
Evergreen Forest	0.02	0.01	0.01	0.20	0.31	0.00	0.01	0.02	0.03	0.42	0.00	0.05	0.05	0.14	0.03	0.15	0.03	0.04	0.01	0.01
Mixed Forest	0.01	0.04	0.00	0.02	0.07	0.01	0.00	0.02	0.03	0.05	0.00	0.05	0.01	0.32	0.01	0.03	0.03	0.02	0.02	0.02
Shrub Scrub	0.01	0.01	0.00	0.00	0.07	0.00	0.01	0.02	0.00	0.01	0.00	0.02	0.03	0.02	0.02	0.02	0.03	0.01	0.01	0.03
Herbaceous /Hay/Pasture	0.10	0.05	0.01	0.03	0.04	0.00	0.01	0.15	0.31	0.09	0.00	0.06	0.01	0.05	0.05	0.04	0.04	0.47	0.01	0.16
Cultivated Crops	0.26	0.03	0.04	0.00	0.11	0.01	0.02	0.48	0.01	0.02	0.00	0.06	0.07	0.03	0.16	0.03	0.14	0.07	0.02	0.27
Woody Wetlands	0.07	0.07	0.07	0.00	0.15	0.02	0.06	0.13	0.01	0.01	0.01	0.11	0.71	0.04	0.21	0.04	0.43	0.02	0.03	0.05
Emergent Herbaceous Wetlands	0.06	0.04	0.67	0.00	0.01	0.04	0.26	0.01	0.00	0.00	0.00	0.01	0.05	0.00	0.28	0.01	0.02	0.00	0.01	0.01

Table S3 | Number of habitats used and habitat preferences. The first column shows the rarefied number of habitats used by each species. following columns show for every habitat the quantile where the observed abundance falls in the distribution of expected abundance under the null model assumptions. Values over 0.95 are marked in blue (preference) and under 0.05 in red (avoidance).

Species	Habitats used	Barren land	Coastal	Cultivated crops	Deciduous forest	D. high intensity	D. low intensity	D. medium intensity	D. open space	Emergent herbaceous wetlands	Evergreen forest	Herbaceous/Hay/Pasture	Mixed forest	Shrub/Scrub	Woody wetlands
<i>Agapostemon virescens</i>	12.47	0.33	0.90	0.45	0.05	0.69	0.55	0.81	0.49	0.64	0.54	0.68	0.76	0.63	0.16
<i>Andrena carlini</i>	11.38	0.16	0.02	0.28	0.71	0.01	0.95	0.17	0.48	0.36	0.71	0.15	0.47	0.75	0.96
<i>Andrena cressonii</i>	12.41	0.21	0.49	0.50	0.63	0.04	0.54	0.06	0.75	0.76	0.48	0.20	0.36	0.27	0.6
<i>Andrena erigeniae</i>	11.43	0.22	0.07	0.01	1.00	0.01	0.65	0.04	0.72	0.14	0.67	0.17	0.51	0.25	0.27
<i>Andrena nasonii</i>	12.32	0.38	0.02	0.18	0.96	0.06	0.04	0.10	0.21	0.48	0.66	0.77	0.67	0.45	0.79
<i>Andrena perplexa</i>	10.82	0.00	0.24	0.98	0.53	0.01	0.80	0.04	0.60	0.30	0.60	0.10	0.24	0.57	0.58
<i>Andrena violae</i>	11.85	0.58	0.03	0.02	0.70	0.01	0.53	0.06	0.83	0.36	0.86	0.62	0.83	0.97	0.46
<i>Apis mellifera</i>	12.46	0.34	0.23	0.88	0.74	0.55	0.44	0.73	0.23	0.31	0.22	0.26	0.40	0.62	0.21
<i>Augochlora pura</i>	11.34	0.33	0.03	0.33	0.93	0.27	0.04	0.26	0.91	0.02	0.35	0.22	0.83	0.01	0.93
<i>Augochlorella aurata</i>	12.30	0.29	0.58	0.24	0.53	0.72	0.05	0.27	0.13	0.99	0.80	0.60	0.61	0.38	0.57
<i>Bombus bimaculatus</i>	12.04	0.13	0.62	0.84	0.98	0.06	0.05	0.12	0.01	0.51	0.81	0.30	0.70	0.42	0.26
<i>Bombus fervidus</i>	12.93	0.54	0.50	0.75	0.03	0.37	0.20	0.81	0.83	0.35	0.18	0.70	0.46	0.85	0.4
<i>Bombus griseocollis</i>	12.28	0.60	0.66	0.66	0.49	0.49	0.24	0.84	0.02	0.30	0.07	0.65	0.61	0.14	0.8
<i>Calliopsis andreniformis</i>	12.68	0.35	0.08	0.55	0.10	0.68	0.36	0.99	0.44	0.12	0.20	0.89	0.75	0.68	0.08
<i>Ceratina calcarata/ dupla/mikmaq</i>	12.53	0.93	0.97	0.41	0.37	0.15	0.55	0.49	0.49	0.66	0.08	0.51	0.50	0.53	0.17
<i>Ceratina strenua</i>	12.06	0.05	0.04	0.70	0.02	0.75	0.91	0.56	0.92	0.13	0.71	0.95	0.30	0.96	0.18
<i>Halictus confusus</i>	12.20	0.91	0.03	0.13	0.72	0.97	0.62	0.98	0.79	0.01	0.20	0.50	0.33	0.23	0.07
<i>Halictus ligatus/poeyi</i>	12.42	0.54	0.00	0.28	0.32	0.84	0.15	0.83	0.27	0.44	0.21	0.34	0.44	0.77	0.39
<i>Halictus rubicundus</i>	11.64	0.48	0.14	0.96	0.22	0.58	0.73	0.63	0.22	0.04	0.39	0.30	0.25	0.01	0.91
<i>Hylaeus affinis/modestus</i>	12.38	0.43	0.86	0.02	0.96	0.59	0.26	0.31	0.36	0.31	0.51	0.98	0.31	0.52	0
<i>Lasioglossum bruneri</i>	11.81	0.73	0.99	0.43	0.01	0.99	0.53	0.43	0.41	0.64	0.04	0.96	0.10	0.01	0.49
<i>Lasioglossum callidum</i>	12.35	0.67	0.34	0.99	0.01	0.22	0.25	0.10	0.61	0.85	0.10	0.81	0.43	0.18	0.84
<i>Lasioglossum coriaceum</i>	12.52	0.53	0.32	0.20	1.00	0.19	0.21	0.01	0.01	0.18	0.46	0.11	0.52	0.26	0.19

*Continues

Species	Habitats used	Barren land	Coastal	Cultivated crops	Deciduous forest	D. high intensity	D. low intensity	D. medium intensity	D. open space	Emergent herbaceous wetlands	Evergreen forest	Herbaceous/Hay/Pasture	Mixed forest	Shrub/Scrub	Woody wetlands
<i>Lasioglossum cressonii</i>	11.90	0.12	0.36	0.01	1.00	0.09	0.18	0.12	0.14	0.30	0.99	0.12	0.39	0.15	0.53
<i>Lasioglossum hitchensi</i>	12.13	0.09	0.12	0.48	0.29	0.26	0.55	0.67	0.95	0.38	0.16	0.59	0.24	0.38	0.73
<i>Lasioglossum illinoense</i>	11.59	0.51	0.02	0.48	0.72	0.96	0.44	0.46	0.99	0.01	0.18	0.12	0.59	0.23	0.03
<i>Lasioglossum imitatum</i>	12.18	0.10	0.05	0.00	0.99	0.59	0.97	0.89	0.47	0.10	0.16	0.59	0.26	0.40	0.01
<i>Lasioglossum near admirandum</i>	11.29	0.00	0.18	0.25	0.29	0.97	0.43	0.30	0.86	0.02	0.11	0.82	0.97	0.19	0.29
<i>Lasioglossum oblongum</i>	12.33	0.88	1.00	0.66	0.01	0.13	0.37	0.20	0.52	0.80	0.89	0.13	0.01	0.20	0.96
<i>Lasioglossum pectorale</i>	11.59	0.00	0.82	0.97	0.28	0.13	0.02	0.25	0.27	0.30	0.41	0.57	0.01	0.99	0.87
<i>Lasioglossum pilosum</i>	12.59	0.98	0.99	0.90	0.00	0.97	0.73	0.83	0.59	0.19	0.15	0.22	0.79	0.44	0.29
<i>Lasioglossum tegulare</i>	12.51	0.93	0.45	0.72	0.02	0.99	0.24	0.98	0.67	0.34	0.21	0.59	0.14	0.41	0.63
<i>Lasioglossum versatum</i>	11.96	0.24	0.90	0.61	0.61	0.69	0.04	0.06	0.93	0.83	0.24	0.22	0.87	0.14	0.03
<i>Megachile brevis</i>	12.00	0.98	0.30	0.36	0.01	0.63	0.65	0.49	0.61	0.99	0.22	0.89	0.50	0.01	0.44
<i>Megachile mendica</i>	11.90	0.69	0.81	0.18	0.56	0.88	0.38	0.42	0.04	0.73	0.02	0.59	0.33	0.01	0.87
<i>Melissodes bimaculatus</i>	11.94	0.00	0.70	1.00	0.00	0.53	0.54	0.86	0.34	0.81	0.18	0.60	0.08	0.55	0.87
<i>Nomada bidentate group</i>	12.52	0.54	0.21	0.18	0.90	0.05	0.94	0.07	0.30	0.36	0.85	0.12	0.69	0.27	0.47
<i>Nomada pygmaea</i>	11.65	0.26	0.05	0.57	0.87	0.01	0.79	0.01	0.36	0.66	0.89	0.02	0.44	0.29	0.94
<i>Osmia atriventris</i>	10.27	0.48	0.11	0.16	0.91	0.01	0.79	0.00	0.52	0.02	0.89	0.25	0.55	0.23	0.97
<i>Osmia bucephala</i>	11.94	0.29	0.01	0.01	1.00	0.26	0.69	0.01	0.38	0.01	0.57	0.51	0.46	0.70	0.05
<i>Osmia georgica</i>	12.93	0.30	0.05	0.10	0.94	0.08	0.23	0.28	0.44	0.24	0.79	0.68	0.12	0.35	0.69
<i>Osmia pumila</i>	12.45	0.52	0.02	0.17	0.93	0.09	0.72	0.03	0.20	0.63	0.60	0.33	0.83	0.96	0.66
<i>Osmia taurus</i>	11.64	0.53	0.16	0.96	0.62	0.05	0.80	0.05	0.11	0.35	0.85	0.17	0.07	0.01	0.96
<i>Ptilothrix bombiformis</i>	11.53	0.21	0.78	0.90	0.00	0.04	0.67	1.00	0.60	1.00	0.47	0.19	0.51	0.01	0.56
<i>Xylocopa virginica</i>	11.65	0.00	0.67	0.15	0.83	0.73	0.96	0.67	0.20	0.26	0.15	0.25	0.10	0.23	0.5

Table S4 | List of species whose habitat preference were extracted and their known distribution. Data extracted from Bartomeus et al. 2013 and Schuh, Hewson-Smith & Ascher 2010.

Species	Max Lat.	Min. Lat.
<i>Agapostemon virescens</i>	46.88	32.84
<i>Andrena carlini</i>	46.08	32.70
<i>Andrena cressonii</i>	46.89	29.70
<i>Andrena erigeniae</i>	42.54	32.60
<i>Andrena nasonii</i>	44.21	25.37
<i>Andrena perplexa</i>	44.20	29.64
<i>Augochlora pura</i>	46.93	25.10
<i>Augochlorella aurata</i>	47.47	27.19
<i>Bombus bimaculatus</i>	46.87	27.47
<i>Bombus fervidus</i>	47.54	28.42
<i>Bombus griseocollis</i>	48.27	26.01
<i>Calliopsis andreniformis</i>	46.88	30.84
<i>Ceratina calcarata</i>	44.90	31.19
<i>Ceratina dupla_sensu_lato</i>	50.20	26.46
<i>Ceratina strenua</i>	44.57	32.52
<i>Halictus confusus</i>	49.74	31.33
<i>Halictus ligatus</i>	48.37	25.90
<i>Halictus rubicundus</i>	49.55	31.33
<i>Hylaeus affinis</i>	49.74	26.10
<i>Hylaeus modestus</i>	43.39	26.10
<i>Lasioglossum bruneri</i>	48.50	29.21
<i>Lasioglossum coriaceum</i>	47.47	32.70
<i>Lasioglossum cressonii</i>	49.55	33.91
<i>Lasioglossum imitatum</i>	47.92	31.00
<i>Lasioglossum oblongum</i>	48.51	33.92
<i>Lasioglossum pectorale</i>	47.93	27.07
<i>Lasioglossum pilosum</i>	45.58	30.33
<i>Lasioglossum tegulare</i>	43.53	25.31
<i>Lasioglossum versatum</i>	46.31	31.32
<i>Megachile brevis</i>	48.37	27.19
<i>Megachile mendica</i>	46.88	25.31
<i>Melissodes bimaculatus</i>	46.88	25.31
<i>Nomada pygmaea</i>	43.53	31.66
<i>Osmia atriventris</i>	46.88	33.53
<i>Osmia bucephala</i>	46.30	32.70
<i>Osmia pumila</i>	44.86	31.33
<i>Xylocopa virginica</i>	43.39	28.04
<i>Andrena violae</i>	44.81	29.70
<i>Lasioglossum callidum</i>	44.36	28.10
<i>Lasioglossum hitchensi</i>	48.50	31.20
<i>Lasioglossum illinoense</i>	46.40	30.55
<i>Lasioglossum admirandum</i>	48.50	25.39
<i>Nomada bidentate_group</i>	NA	NA
<i>Osmia georgica</i>	44.80	29.59
<i>Osmia taurus</i>	48.50	35.62
<i>Ptilothrix bombiformis</i>	43.82	31.20

Table S5 | Richness and strength values comparisons when exotic species were removed and the list of exotic species extracted from Bar-
tomeus et al., 2013.

	Richness	Richness without exotic species	Strength	Strength without exotic species	List of species
Barren Land	82.70	77	18.99	17.55	<i>Andrena wilkella</i>
Coastal	99.81	94.21	30.69	29.05	<i>Anthidium manicatum</i>
Crops	71.42	70.07	15.27	14.85	<i>Anthidium oblongatum</i>
Deciduous forest	91.00	87.67	26.32	26.06	<i>Hylaeus leptocephalus</i>
Developed high	56.52	52.76	12.91	11.33	<i>Hylaeus punctatus</i>
Developed low	82.12	78.88	19.62	18.44	<i>Lasioglossum leucozonium</i>
Developed med.	64.96	60.86	14.32	12.75	<i>Megachile apicalis</i>
Developed open	77.36	73.64	17.28	16.67	<i>Megachile concinna</i>
E. Herb. Wetlands	95.27	89.43	25.03	24.46	<i>Megachile rotundata</i>
Evergreen forest	108.62	104.41	31.71	31.25	<i>Megachile sculpturalis</i>
Herb./Hay/Pasture	75.49	73.52	17.30	16.72	
Mixed forest	89.66	86.56	22.28	21.72	
Shrub/Scrub	85.72	84.03	19.67	19.62	
Woody Wetlands	91.02	89.35	24.60	24.04	

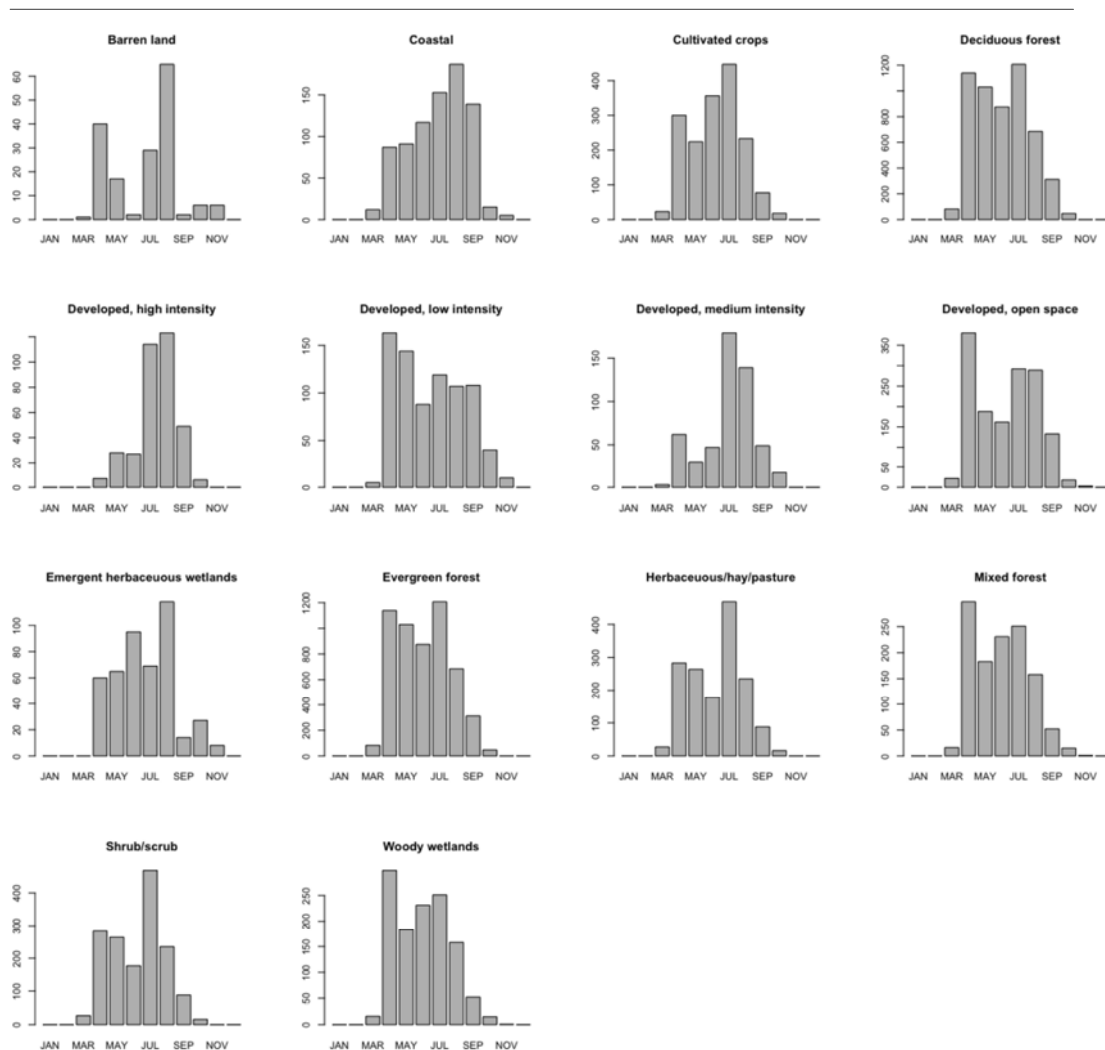


Figure S1 | Habitat sampling were sampled through the season. The histograms shows the amount of individuals captured each month for every habitat.

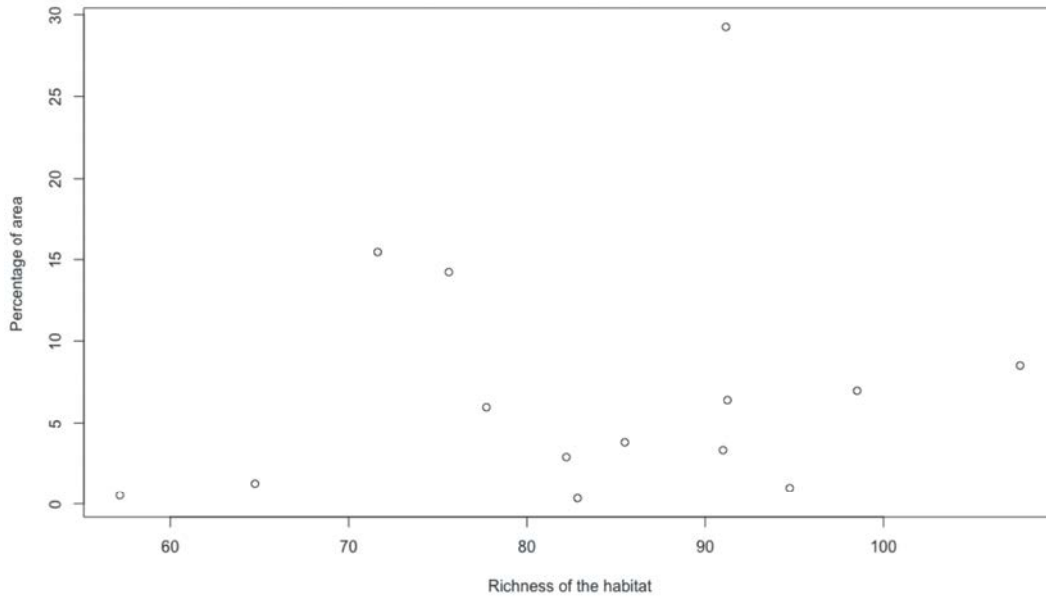


Figure S2 | Percentage of each habitat in the region and its respective rarefied richness.

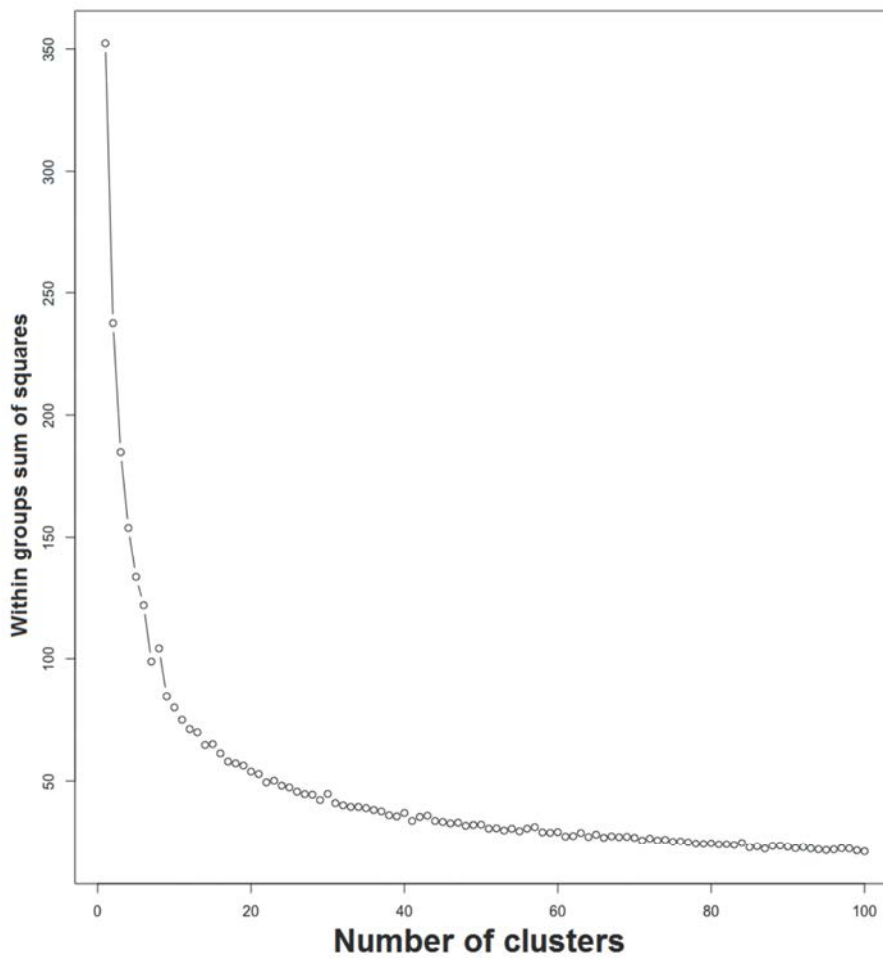


Figure S3 | Sedimentation curve showing the criteria we followed to select the number of mosaic-groups (k) using “the elbow method”. The habitat composition of the mosaic-groups is described in Table S1.

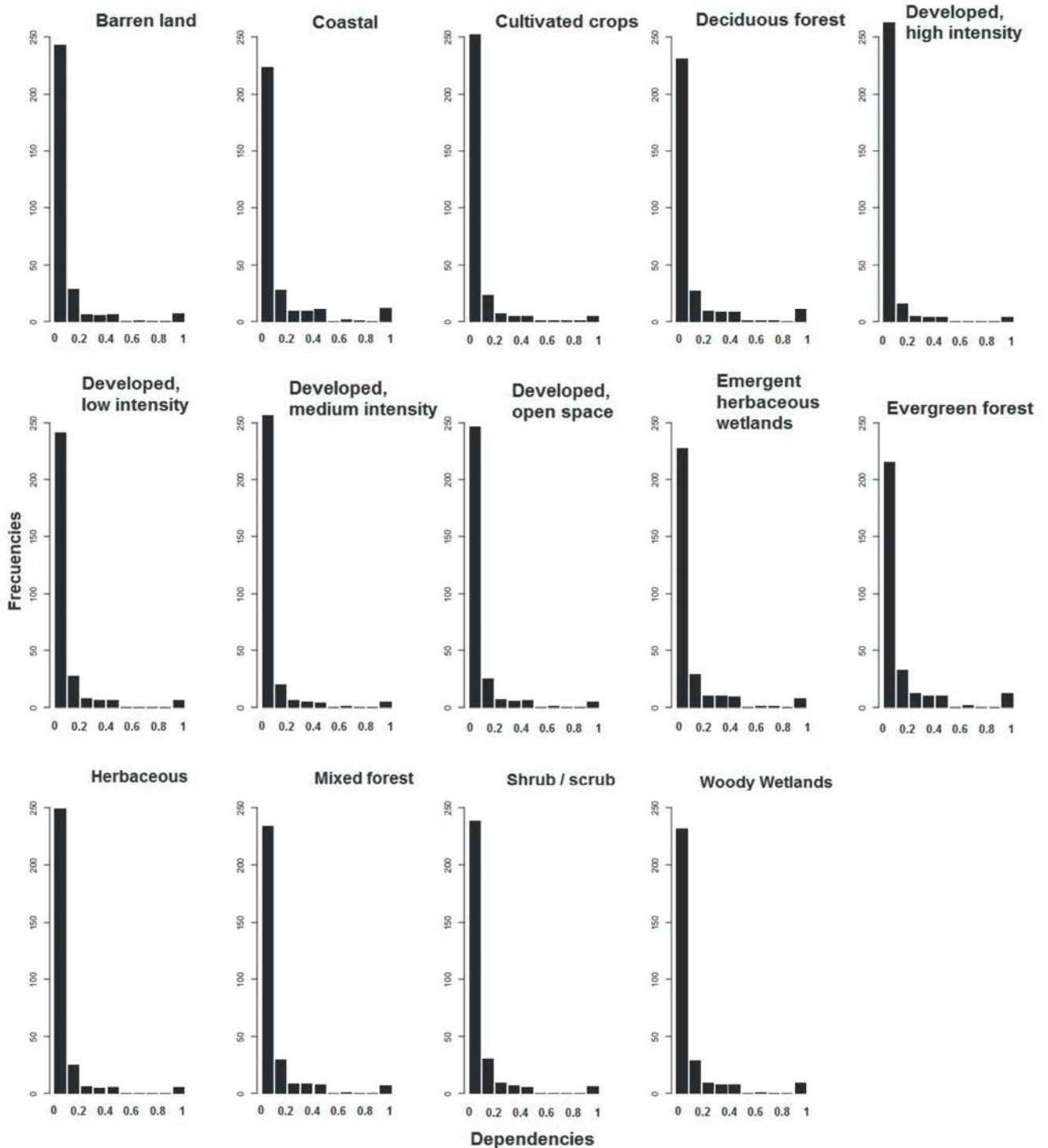


Figure S4 | Histograms of the dependences of the species for every habitat. the dependence is the proportional use that a species does of a single resource. ranging from no use to total dependence (from 0 to 1). Higher values near the “1” column mean high number of species fully dependent on that habitat. and the large number of species near zero are species using several habitats. and hence not depending strongly in any single habitat.

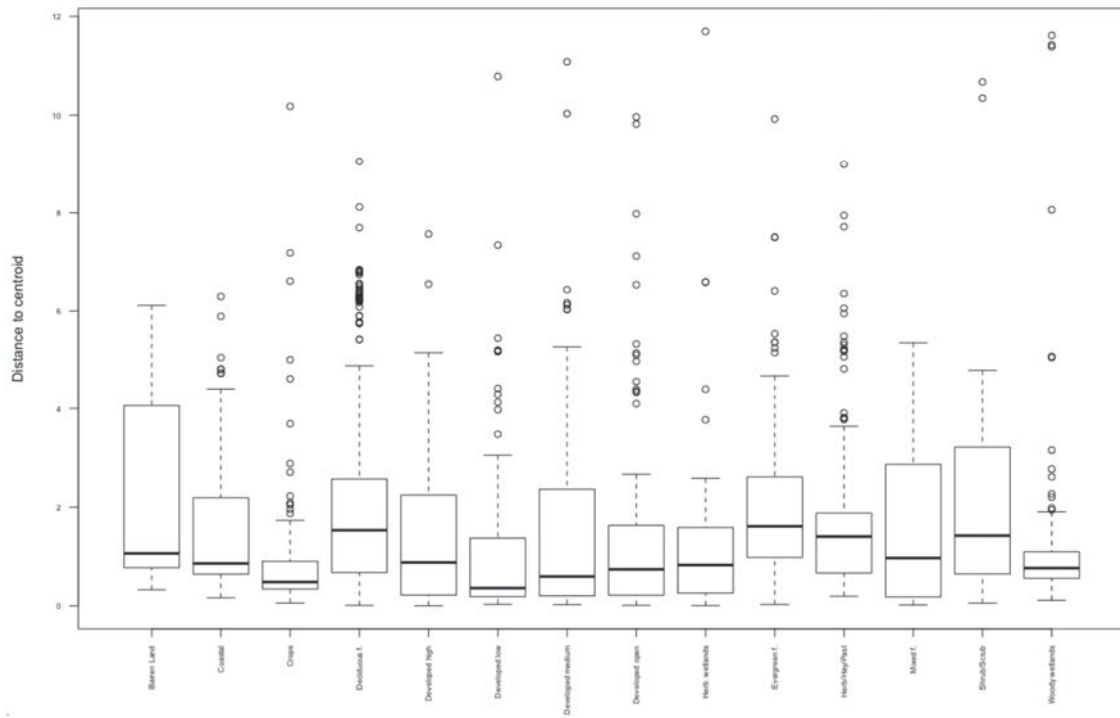


Figure S5 | *The results of the multivariate homogeneity of groups dispersions shows that most habitats do not have different dispersions and have around the same distribution of distances to the centroid, this means that different habitat sites are well distributed among the sampling area and not clustered.*

Bee brain size is positively associated with urban lifestyles, but not necessarily with positive population trends

Abstract

The rapid conversion of natural habitats to anthropogenic landscapes is threatening insect pollinators worldwide, raising concern on the consequences for their fundamental role in ecosystems for fruit and seed production. However, not all pollinators are negatively affected by habitat conversion, but some find in anthropogenic landscapes appropriate resources to survive and reproduce. Why some animals can thrive in anthropogenic environments that most species cannot tolerate remains insufficiently understood, but it might in part reflect enhanced behavioural plasticity to exploit new resource opportunities. While this hypothesis has received ample support in studies of vertebrates, it is less obvious whether the small brain of insects can offer enough behavioural plasticity to cope with the many challenges of anthropogenic habitats. To address this issue, we measured brains for 145 individuals from 44 species of bees—the most important group of pollinators—from North America, and compared them with detailed information on habitat preferences and population trends. Our analyses revealed that bees that prefer forest habitats had smaller brains relative to their body size, but those who prefer urban habitats tended to have larger brains compared to forest dwellers. However, we found no evidence that either larger brains or a preference for urban habitats are associated with positive population trends. These results suggest that a large brain can help maintaining bee urban populations, but also highlight that being tolerant to urbanisation is not enough to compensate for the worldwide decline of their populations. ●

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Introduction

Pollination is a fundamental ecosystem service that connects the earth's vegetation and human economy. Without pollinators, both wild plant communities and agricultural areas will suffer severe negative consequences (Ashman et al., 2004; Klein et al., 2007). Pollinators decline should be concerning as pollination itself is already limiting for some plant populations (Aguilar et al., 2006). In fact, most wild plant species (80%) and crop species (75%) are directly dependent on insect pollination for fruit and seed production (Klein et al., 2007; Ollerton et al., 2011). Pollination services are performed by both managed and wild pollinators, mostly bees (James & Pitts-Singer, 2008; Winfree et al., 2011), both of which might be affected by a range of current and projected environmental changes. In fact, there are clear shreds of evidence of recent declines in pollinator populations, and parallel declines in the plants that rely upon them (Potts et al., 2010; Scheper et al., 2014). So far, habitat loss is generally thought to be the most important factor driving bee declines both by destruction and fragmentation of habitats, especially through agricultural intensification but also due to increasing urbanization (Brown & Paxton, 2009; Natural Research Council, 2006). Hence, the ability to adapt to human-dominated new habitats and to exploit new opportunities have become important for species to survive (Manenti et al., 2013).

Long-term data on the status of bee species is limited (Bartomeus et al., 2018, Scheper et al., 2014), but the trends show general declines which are projected to increase in the future (Dormann et al., 2008). For example, in the UK, six of the 16 total non-parasitic bumblebees have declined considerably, including the extirpation of *B. subterraneus* (Williams & Osborne, 2009). Moreover, the third part of wild pollinator species has decreased from 1980 to 2013 in the same area (Powney et al., 2019). However, not all bee species are declining. There are also “winners” of this environmental change that thrive in human-altered environments, as well as species that can at least maintain its populations stable (Biesmeijer et al., 2006; Bartomeus et al., 2013; Reemer et al., 2012; Collado et al., 2019). For example, *Bombus terrestris*, which is also a managed species, is rapidly spreading (Rasmont et al., 2008). The opportunity of new nesting spots, the creation of new microhabitats with less predators, and the introduction of novel foraging plants can have positive effects in some guilds (Cane et al., 2006; Winfree et al., 2007; Collado et al., 2019) and several studies demonstrate some positive effects of urbanization or agriculture on a few bee species (Cane et al., 2006; Carré et al., 2009; Winfree et al., 2007).

In a rapid environmental change context, being successful in new or human-altered areas has been related to cognitive abilities and bigger relative brain sizes in some taxa. For example, birds and mammals that flourish after translocation to a new area tend to

have larger brains than unsuccessful invaders (Sol et al., 2016; Sol et al., 2005; Amiel et al., 2011). Across vertebrates, there is a clear relation between neuroanatomy and the type of habitat preferred by a species. In general, species occupying habitats classified as more structurally complex have relatively larger brains and exaggerated structures related to navigating and exploiting those habitats (Powell & Leal, 2014). In other groups such as cichlid fishes, total brain size, telencephalic, and cerebellar size are also positively correlated with habitat complexity (Shumway, 2008). However, this is not always the case as this pattern was not found in *Anolis* (Iguanas) species (Powell & Leal, 2014). Overall, we expect that relative bigger brain sizes, as a proxy of cognitive flexibility (e.g. see chapter two of this thesis for its relationship with learning), would be related to preferences for complex human-altered habitats such as urban habitats, and ultimately with population trends. Determining the flexibility of cognitive/behavioural traits across a range of external conditions would allow us to predict the adaptive capacity of a population when facing an unexpected change in the environment, such as habitat transformation (Nussey et al., 2007).

We used previously published data on bee forest preference and urban preference (Collado et al., 2019) as opposite points of habitat complexity, expecting to find bigger brain sizes for urban dwellers because a wider array of cognitive abilities are needed to inhabit these habitats. Species population trends were extracted from Bartomeus et al., 2013 and both datasets were crossed with our own collected brain weight database. We wanted to explore if there is a relationship between brain size, habitat preference, and population trends. Hence, our questions were the following:

- Are brain sizes, as a proxy of behavioural plasticity, related to habitat preferences?
- Is population stability related to brain size as it has been seen in other taxa?
- Are habitat preferences related to population stability? Or alternatively, are urban dwellers populations increasing? ●

Material and Methods

Brain data

Bees were captured by hand netting in different areas of New York State (USA) and Europe (Spain and the Netherlands). We captured 145 individuals from 44 species (Table S2), kept them in cold storage and sent them to the laboratory to be processed. Once in the laboratory, bees were identified by expert taxonomists (Parker Gambino and Ivo Raemakers) and anesthetized in cold to be decapitated (Crook, 2013). To prevent degradation, the head was fixed in 4% paraformaldehyde

Table 1 | Population trends estimates, brain relative and absolute size, urban and forest preference values and body size (IT). Species without information (i.e. NA) were not included in the model for that particular analysis.

Species	Pop. trend	Forests	Urban	Mean Brain	Mean IT	Residuals
<i>Agapostemon virescens</i>	Estimate	preference	preferences	weight (mg)	(mm)	
<i>Agapostemon sericeus</i>	0.005	NA	NA	0.855	2.477	-0.170
<i>Agapostemon virescens</i>	0.005	0.100	0.890	1.281	1.715	0.926
<i>Andrena barbilabris</i>	-0.010	NA	NA	0.983	2.280	0.125
<i>Andrena carlini</i>	-0.005	0.780	0.010	1.558	3.102	0.006
<i>Andrena crataegi</i>	-0.007	NA	NA	0.798	1.969	0.193
<i>Andrena dunningi</i>	-0.005	NA	NA	1.285	2.556	0.178
<i>Andrena fragilis</i>	-0.002	NA	NA	0.378	1.270	0.270
<i>Andrena frigida</i>	-3e-04	NA	NA	0.560	2.143	-0.321
<i>Andrena hippotes</i>	0.002	NA	NA	0.570	1.969	-0.144
<i>Andrena hirticincta</i>	-0.005	NA	NA	1.168	2.667	0.003
<i>Andrena milwaukeeensis</i>	-9e-04	NA	NA	1.003	2.286	0.140
<i>Andrena miserabilis</i>	-0.003	NA	NA	0.455	2.055	-0.451
<i>Andrena nasonii</i>	-0.002	0.990	0.030	0.331	1.270	0.136
<i>Andrena nubecula</i>	-0.007	NA	NA	0.416	1.334	0.273
<i>Andrena pruni</i>	0.022	NA	NA	0.661	1.762	0.212
<i>Andrena rugosa</i>	0.002	NA	NA	0.515	1.545	0.211
<i>Andrena simplex</i>	-0.003	NA	NA	0.587	2.751	-0.744
<i>Andrena vicina</i>	-0.003	NA	NA	1.560	2.826	0.184
<i>Augochlora pura</i>	0.006	0.960	0.210	0.586	1.938	-0.087
<i>Augochlorella aurata</i>	0.004	0.750	0.500	0.352	1.445	-0.043
<i>Augochloropsis metallica</i>	0.006	NA	NA	0.701	1.969	0.063
<i>Bombus griseocollis</i>	0.025	0.430	0.820	3.053	4.297	0.067
<i>Bombus impatiens</i>	0.007	NA	NA	2.594	4.142	-0.027
<i>Bombus ternarius</i>	-0.014	NA	NA	1.987	3.112	0.244
<i>Bombus vagans</i>	-0.008	NA	NA	1.179	2.794	-0.075
<i>Calliopsis andreniformis</i>	0.007	0.140	1.000	0.365	1.524	-0.108
<i>Ceratina calcarata</i>	0.019	NA	NA	0.332	1.706	-0.415
<i>Ceratina strenua</i>	0.013	0.020	0.730	0.153	0.889	0.036
<i>Colletes thoracicus</i>	0.022	NA	NA	1.521	3.281	-0.123
<i>Halictus confusus</i>	0.004	0.600	0.990	0.418	1.786	-0.269
<i>Halictus ligatus</i>	0.007	NA	NA	0.689	2.499	-0.403
<i>Halictus rubicundus</i>	-0.008	0.180	0.670	0.839	2.314	-0.061
<i>Hylaeus mesillae</i>	-0.003	NA	NA	0.340	1.760	-0.450
<i>Hylaeus modestus</i>	0.015	NA	NA	0.310	2.080	-0.856
<i>Lasioglossum coriaceum</i>	2*10 ⁻⁴	1.000	0.020	0.742	1.905	0.182
<i>Megachile campanulae</i>	0.011	NA	NA	0.822	2.286	-0.059
<i>Megachile centuncularis</i>	-0.007	NA	NA	1.382	3.330	-0.247
<i>Megachile gemula</i>	0.003	NA	NA	1.408	2.953	-0.002
<i>Megachile mendica</i>	-0.001	0.340	0.810	1.803	2.858	0.307
<i>Megachile pugnata</i>	-0.007	NA	NA	3.000	6.430	-0.709
<i>Megachile texana</i>	-0.001	NA	NA	2.351	3.112	0.412
<i>Melissodes bimaculata</i>	-0.004	0.000	0.840	1.842	2.572	0.526
<i>Nomada cressonii</i>	0.006	NA	NA	1.760	3.340	-0.010
<i>Nomada luteoloides</i>	0.021	NA	NA	1.700	3.530	-0.149
<i>Osmia atriventris</i>	-0.008	0.980	0.000	0.447	2.032	-0.446

*Continues

Species	Pop. trend Estimate	Forests preference	Urban preferences	Brain weight mean	IT mean	Residuals-
<i>Osmia bucephala</i>	0.002	1.000	0.110	1.421	3.048	0.052
<i>Osmia lignaria</i>	-0.023	NA	NA	1.174	3.461	-0.483
<i>Osmia pumila</i>	0.002	0.970	0.020	0.604	1.810	0.072
<i>Sphecodes ranunculi</i>	-0.003	NA	NA	0.364	1.651	-0.261
<i>Xylocopa virginica</i>	0.006	0.600	0.790	5.300	7.849	-0.515

with phosphate buffer saline (PBS). Brains were extracted from the head capsule, and tracheae and fat bodies were removed to maximize accuracy in neural tissue weight. Brains extracted were placed on a small piece of tared Parafilm® and exceeding fixative solution was dried from the brain using Kimwipes® tissues. Finally the brain was weighted in a microbalance. Brain weights can give errors due to dehydration or insufficient removal of fat bodies and trachea around the brain. We tackle this problem in two ways. First, we estimated a representative value of brain mass per species by averaging the values from all collected individuals (using

only workers for eusocial species). Second, we removed 24 individuals with brain weight values one standard deviation higher or lower than the species average (see sup mat analysis). Because some species have larger brains simply because their body is bigger, we also measured the inter-tegular distance of all individuals as a proxy of body size (Kendall et al., 2019) and obtained a measure of relative brain size as the residuals from a log-log regression of brain weight against body size (LM estimate \pm SE = 1.88 ± 0.06 , $p < 0.001$, $R^2 = 0.83$, $n = 163$ species) (see Wurm & Fiscaro, 2014).

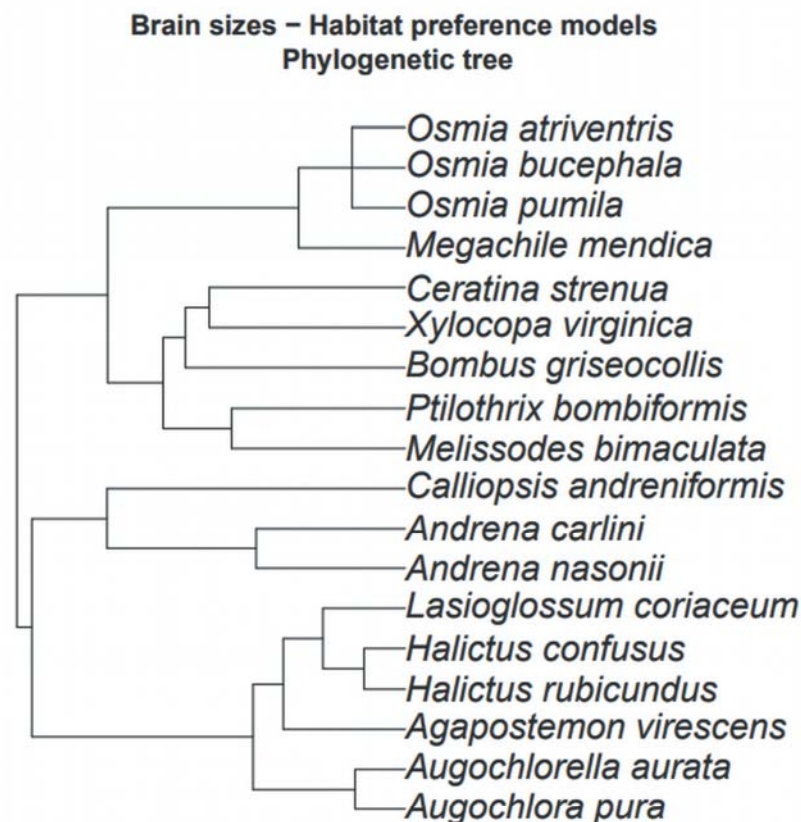


Figure 1 | Phylogenetic tree used for the Bayesian models used in order to analyze correlations between habitat preference and brain sizes. The trees used for the other models can be consulted in Fig. S2 and Fig. S3.

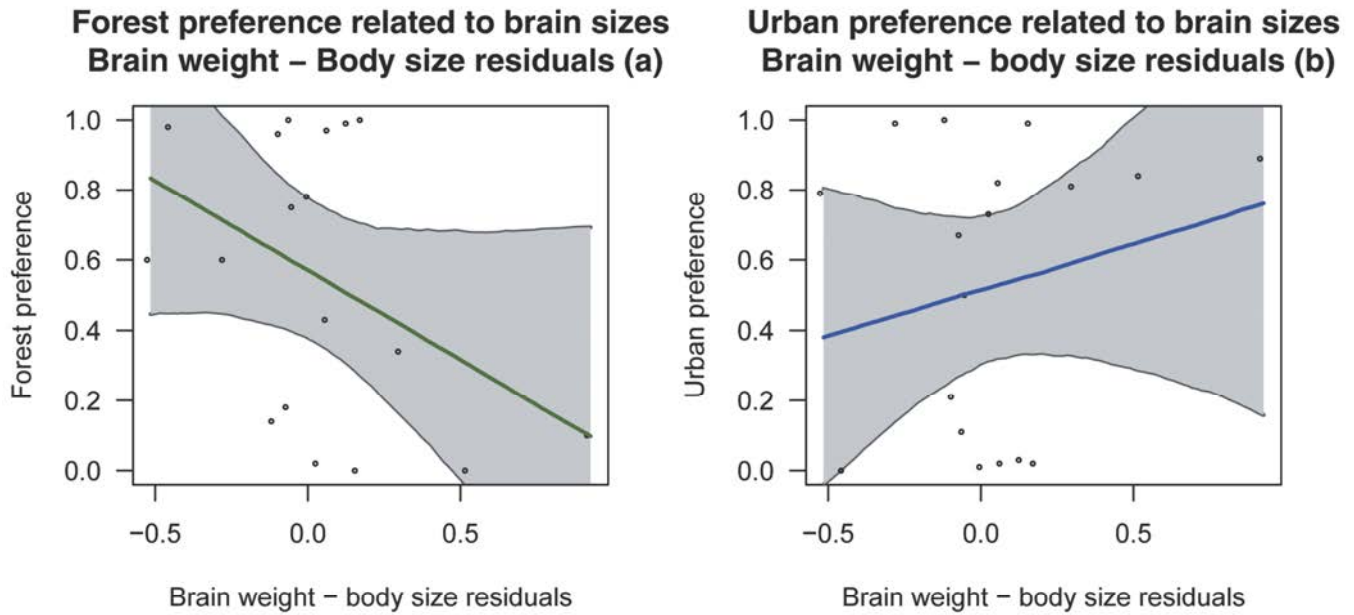


Figure 2 | PGLMs showing the correlation between habitat preference (urban and forest) and relative brain sizes. Habitat preferences for USA bee data were extracted from Collado et al., 2019, where habitat preference for forests and urban areas was calculated comparing species occurrence in each habitat to what would be expected if species occur at random using null models. This preference index ranges from zero (avoidance) to one (preference). A zero value for relative brain weight is an expected value for their body size, therefore negative values are species with smaller brain sizes and positive values are species with bigger brain sizes than expected by their body size. a) Positive correlation between relative brain weight size and urban preference. b) Negative correlation between encephalization and forest preference.

Ecological data

The quantitative data of population trends for North American bees were extracted from Bartomeus et al. (2013). Using information from pinned bees from different North American museums and universities, Bartomeus et al. (2013) estimated population trends (positive for increasing populations and negative for decreasing populations) as the relative change in abundance over time. Habitat preferences for USA bee data were extracted from Collado et al., 2019, where habitat preference for forests and urban areas was calculated comparing species occurrence in each habitat to what would be expected if species occur at random using null models. This preference index ranges from zero (avoidance) to one (preference).

Data analysis

We used Bayesian phylogenetic generalised linear models (PGLM), as implemented in the package brms (Bürkner, 2017), to assess whether brain size, habitat preference and population trends are associated across species. In all models, we incorporated a phylogenetic covariance matrix. The phylogeny used were maximum-likelihood phylogenetic trees of the superfamily Apoidea

at the genera level modified from (Hedtke et al., 2013, Fig. 1, Fig. S2, Fig. S3). Due to the absence of infrageneric phylogenies for all our genera, we simulated infrageneric polytomies within our phylogeny. Species tips were added to the phylogenetic tree genera nodes as polytomies of equal branch length relative to the genera branch length using the phytools package (version 0.6 44, Revell, 2012). We did not have full information of habitat preference and population trends for the 44 species for which brain size data was available. Therefore, analyses can differ in sample size (see below). ●

Results

Brain sizes and habitat preference

Smaller brains relative to body size were found for bee species with preferences for forested habitats (PGLM $\beta = -0.51 \pm 0.31$, IC = $-1.12 - 0.11$, $n = 18$ species, Fig. 2a) and bigger brain size relative to body size were related to urban preferences (PGLM $\beta = 0.26 \pm 0.33$, IC = $-0.39 - 0.92$, Fig. 2b), however the variability of the model estimates was high, especially for the urban preference relationship. Absolute brain weights also tended to be positively correlated with urban preference (PGLM $\beta = 0.10$

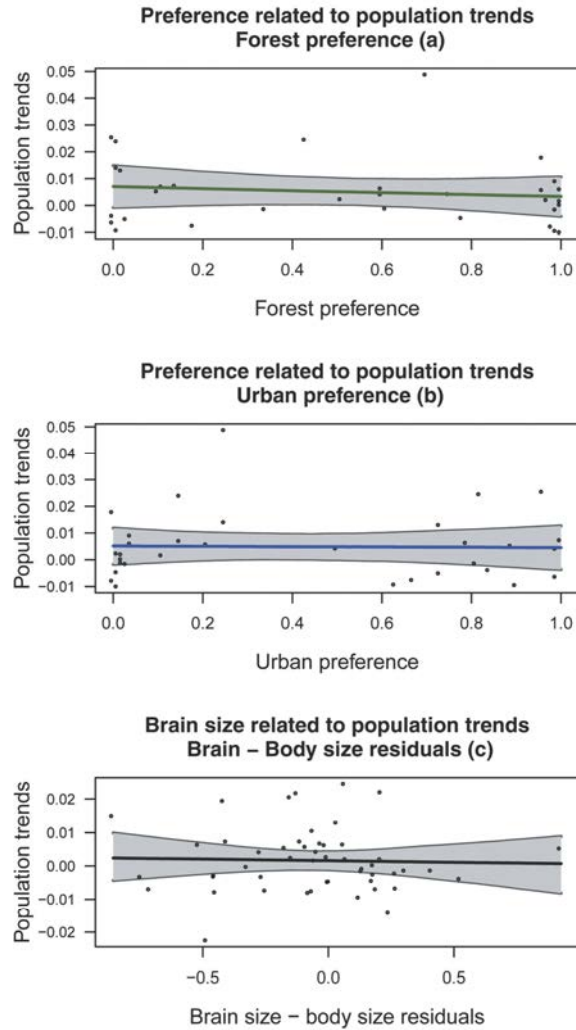


Figure 3 | PGLMs showing the correlation between population trends, habitat preference and brain sizes. a) No significant correlation between population trends and urban preference, as shown in b) with forest preference. In c) we do not detect any correlation between population trends and relative brain size.

± 0.08 , IC = -0.07 – 0.27, Fig. S1), however for forest preferences there was too much variability to detect a trend (PGLM $\beta = -0.05 \pm 0.09$, IC = -0.22 – 0.11). Given the known allometry between absolute brain and body size, it is not surprising that habitat preference correlations with body size show similar patterns (PGLM $\beta = 0.05 \pm 0.07$, IC = -0.09 \pm 0.19 for urban preference and PGLM $\beta = -0.01 \pm 0.07$, IC = -0.14 – 0.14 for forest preference).

Population trends

Population trends did not seem to be correlated with habitat preferences, for neither urban preference (PGLM $\beta = -0.00 \pm 0.01$, IC = -0.02 – 0.01, n = 32 species, Fig. 2a) nor forest preference (PGLM $\beta = -0.00 \pm 0.01$, IC = -0.01 – 0.01, Fig. 2b). In addition, population trends were not correlated with absolute brain weight (PGLM $\beta = 0.00 \pm 0.00$, IC = -0.00 – 0.00, n = 50 species, Fig. 2c), relative brain

weight (PGLM $\beta = 0.00 \pm 0.00$, IC = -0.01 – 0.01, Fig. 2d), or body size (PGLM $\beta = 0.00 \pm 0.00$, IC = -0.00 – 0.00).

Population trends were not correlated with habitat preferences, for neither urban preference (PGLM $\beta = -0.00 \pm 0.01$, IC = -0.02 – 0.01, n = 32 species, Fig. 3a) nor forest preference (PGLM $\beta = -0.00 \pm 0.01$, IC = -0.01 – 0.01, Fig. 3b). In addition, population trends were not correlated with relative brain weight (PGLM $\beta = 0.00 \pm 0.00$, IC = -0.01 – 0.01, Fig. 3c), absolute brain weight (PGLM $\beta = 0.00 \pm 0.00$, IC = -0.00 – 0.00, n = 50 species), or body size (PGLM $\beta = 0.00 \pm 0.00$, IC = -0.00 – 0.00).●

Discussion

Our analyses revealed that bees with preferences for urban habitats are characterized by bigger brains relative to their body size, while those with forest preferences have relatively smaller brains. Howe-

ver, neither brain sizes nor habitat preference explained population trends. Therefore, the demographic consequences of a high tolerance to urbanization, whether associated with enlarged brains or other adaptations, are not enough to reverse the trend of these species to be declining in numbers.

We hypothesized that bees with bigger brains, relative to their body size, should be more flexible in their behavior to deal with the challenges of urban life, and because a large brain may confer better cognitive abilities to learn (Chapter two of this thesis). Urban dwellers may need better cognitive abilities and flexibility in their behavior than forest dwellers because they are more frequently exposed to resources and threats that are new and can rapidly change over time (Wheater, 1999). Some examples of behaviours needed to survive in urban environments (see Table S2 for a full set of examples) are the use of human-made materials to nest (Allasino et al., 2019) or being able to collect pollen and nectar from exotic flowers from gardens (Lowenstein et al., 2014). We should not expect forested habitats to require such a plastic behaviour because resource distribution is more homogeneous and species are expected to have had more opportunities to adapt to the resources and threats (Smith et al., 2019). In accordance, we found smaller brain sizes than expected by their body sizes in bees that preferred forests.

The association between relative brain size and habitat preferences cannot be simply attributed to the confounding effect of body size. Although a larger relative brain can result from selection for a smaller body, thus not necessarily reflecting selection on larger brains, we found no evidence that body size was related to population trends. This contrasts with previous analyses (Bartomeus et al., 2013; Scheper et al., 2014). In North American bees, larger species are more likely to experience population declines than larger species (Bartomeus et al., 2013). In mammals, populations of larger species also more likely to be decreasing (Damuth, 1981; González-Voyer et al., 2016).

The absence of relationship between brain size and population trends is unexpected, however. Previous studies in birds suggest that enlarged brains buffer populations against environmental changes (Shultz, 2005; Fristoe et al., 2017), but our results indicate that this cannot be generalized to bees. An explanation is that our analyses lacked power to detect patterns. On one hand, we used brain mass in our analyses because it is relatively easy to measure and standardize, allowing to increase sample size. However, some structures within the brain, like mushroom bodies, may be more relevant because they are closer to the integration centers of cognitive processing (Barth & Heisenberg, 1997; Withers et al., 1995; Fahrbachand & Robinson 1995). On the other hand, we also note that population trends data are difficult to obtain for invertebrate taxa. Long-term information is generally lacking, forcing researchers to draw on data indirectly taken from museums which are harder to standardize

(Bartomeus et al., 2018). Our dataset also contains fewer species per genus and may be biased towards the most common genera. This may for instance explain why we did not find a correlation between body size and population trends, while a previous analysis with the complete dataset did detect it (Bartomeus et al., 2013).

Ducatez et al., recently suggested that species may thrive in cities because they have specific adaptations to urban conditions, and can cope with artificial habitats in general, or because they are generalists that can live in a wide range of conditions. In clear contrast with vertebrates (Ducatez et al., 2018), where urban dwellers tend to be generalist species capable of using a wide diversity of natural and artificial habitats, in bees we find evidence that some species are true urban specialists (Collado et al., 2019). This can imply that the cognitive demands of agricultural areas are different from those required to thrive in cities. It also helps explain the absence of a relationship between urban preferences and increasing population trends, despite previous evidence showing that population trends of bee species can be related to the availability of their preferred resources (Scheper et al., 2014). Because the current extension of urbanized areas is substantially smaller than that used for agriculture, doing well in cities but not in other artificial habitats is unlikely to have general effects on population trends. Clearly, more research is needed to fully understand how cognition alters the costs and benefits of living in human-altered environments. ●

Acknowledgements

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Supplementary material

Data filtering

Table S1 | Individuals that had a brain size >1 standard deviation were removed from the sampling, queens were therefore removed.

Species	Brain weight (mg)	Mean species brain weight (mg)	Standard deviation species brain weight
<i>Andrena barbilabris</i>	0.296	0.846	0.326
<i>Andrena dunningi</i>	0.600	1.148	0.349
<i>Augochlorella aurata</i>	0.705	0.423	0.170
<i>Bombus pascuorum</i>	3.932	2.800	0.923
<i>Bombus pascuorum</i>	4.937	2.800	0.923
<i>Bombus pascuorum</i>	3.871	2.800	0.923
<i>Bombus pratorum</i>	3.626	2.160	0.67
<i>Bombus pratorum</i>	3.278	2.160	0.67
<i>Bombus pratorum</i>	3.121	2.160	0.67
<i>Bombus pratorum</i>	1.070	2.160	0.67
<i>Bombus pratorum</i>	1.300	2.160	0.67
<i>Bombus terrestris</i>	5.838	3.207	0.92
<i>Bombus terrestris</i>	5.564	3.207	0.92
<i>Bombus terrestris</i>	6.370	3.207	0.92
<i>Bombus lapidarius</i>	4.258	NA	NA
<i>Bombus lapidarius</i>	5.075	NA	NA
<i>Bombus lapidarius</i>	4.474	NA	NA
<i>Bombus lapidarius</i>	4.655	NA	NA
<i>Bombus bimaculatus</i>	3.641	NA	NA
<i>Bombus bimaculatus</i>	3.633	NA	NA
<i>Halictus ligatus</i>	0.395	0.652	0.110
<i>Osmia caerulescens</i>	1.530	0.746	0.273
<i>Xylocopa virginica</i>	6.613	5.5	0.515
<i>Xylocopa virginica</i>	5.787	5.5	0.515

Table S2 | Description of human-made materials collected or used by bees in urban environments.

Species	Use	Material	Reference	Credit
<i>Megachile apicalis</i>	Brood cell building	Plastic	https://www.flickr.com/photos/habropoda/11642856344/	NA
<i>Osmia bicornis</i>	Nest material	PVC	http://beediverse.com/blog/?p=2010	Margriet Dogterom
<i>Osmia bicornis</i>	Nest material	Table	http://www.opalexplornature.org/bee-hotels#/2	OPAL
<i>Osmia bicornis</i>	Nest material	Brick	http://cuartodechismes.blogspot.com.es/2010/07/casas-rurales-de-la-subbetica.html	Antonio Manuel Jiménez Conejo
<i>Osmia bicornis</i>	Nest material	Cardboard	https://twitter.com/Rob_Fowler/status/474098475161092096/photo/1	Rob Fowler
<i>Bombus bimaculatus</i>	Nest material	PVC	Hongjamrussilp and Warrit (2014)	Hongjamrussilp and Warrit (2014)
<i>Megachile campanulae</i>	Brood cell	Plastic	Maclvor et al 2013	Scott Maclvor
<i>Osmia cornuta</i>	Nest material	Plastic table	http://www.naturamediterraneo.com/forum/to-pic.asp?TOPIC_ID=227020	NA
<i>Osmia cornuta</i>	Nest material	Polycarbonate	http://notasdecampoyjardin.blogspot.com.es/2009/03/abejas-albanilas-en-el-policarbonato.html	Jesús Dorda
<i>Anthidium florentinum</i>	Nest material	Latch hole	I. Bartomeus	I. Bartomeus
<i>Tetragonula hockingsi</i>	Collecting	Dried paint	(Medler 1966).	Medler
<i>Bombus hypnorum</i>	Nest material	Metal	https://twitter.com/Eucera/status/609294141990768640	Stuart Roberts
<i>Bombus hypnorum</i>	Nest material	Small mammal house	http://www.bwars.com/bee/apidae/bombus-hypnorum	M Edwards
<i>Bombus lucorum</i>	Nest material	Small mammal house	M A Collado	M A Collado
<i>Bombus melanopygus</i>	Nest material	Small mammal house	http://www.sciencedirect.com/science/article/pii/S0006320705004817	Quinn S McFrederick
<i>Bombus sp.</i>	Nest material	Rockwool	http://planetearth.nerc.ac.uk/news/story.aspx?id=975&cookieConsent=A	Colin Bluck
<i>Bombus sp.</i>	Nest material	Small mammal house	http://planetearth.nerc.ac.uk/news/story.aspx?id=975&cookieConsent=A	NA
<i>Bombus sp.</i>	Nest building	Fluff	https://twitter.com/BuzzLMK/status/472101682760146944/photo/1	Limerick Buzzing
<i>Osmia sp.</i>	Nest material	Plastic straw	http://beediverse.com/blog/?p=1493	Margriet Dogterom
<i>Osmia sp.</i>	Nest material	Plastic	http://beediverse.com/blog/?p=1493	Margriet Dogterom
<i>Rhodanthidium sp.</i>	Nest building	Paper	http://cuartodechismes.blogspot.com.es/2010/07/casas-rurales-de-la-subbetica.html	Antonio Manuel Jiménez Conejo
<i>Monodontomerus obscurus</i>	Parasitizing	Plastic	Maclvor et al 2013	Scott Maclvor
<i>Bombus pratorum</i>	Nest material	Small mammal house	http://www.bwars.com/bee/apidae/bombus-pratorum	M Edwards
<i>Megachile rotundata</i>	Brood cell	Plastic	Maclvor et al 2013	Scott Maclvor
<i>Bombus terrestris</i>	Nest material	Small mammal house	http://planetearth.nerc.ac.uk/news/story.aspx?id=975&cookieConsent=A	Norman Sellers
<i>Bombus terrestris</i>	Nest material	Brick	https://www.youtube.com/watch?v=1gysdWdZIBk	Dave Goulson
<i>Bombus terrestris</i>	Nest building	Small mammal house	http://www.bwars.com/bee/apidae/bombus-terrestris	M Edwards

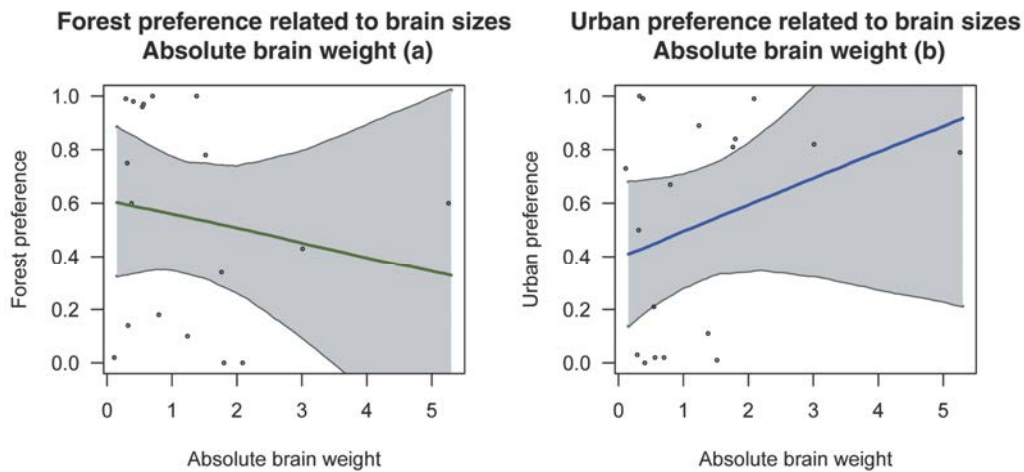


Figure S1 | PGLMs showing the correlation between habitat preference (urban and forest) and absolute brain sizes. Habitat preferences for USA bee data were extracted from Collado et al., 2019, where habitat preference for forests and urban areas was calculated comparing species occurrence in each habitat to what would be expected if species occur at random using null models. This preference index ranges from zero (avoidance) to one (preference). A zero value for relative brain weight is an expected value for their body size, therefore negative values are species with smaller brain sizes and positive values are species with bigger brain sizes than expected by their body size. a) Negative correlation between brain size and forest preference. b) Positive correlation between brain size and urban preference.

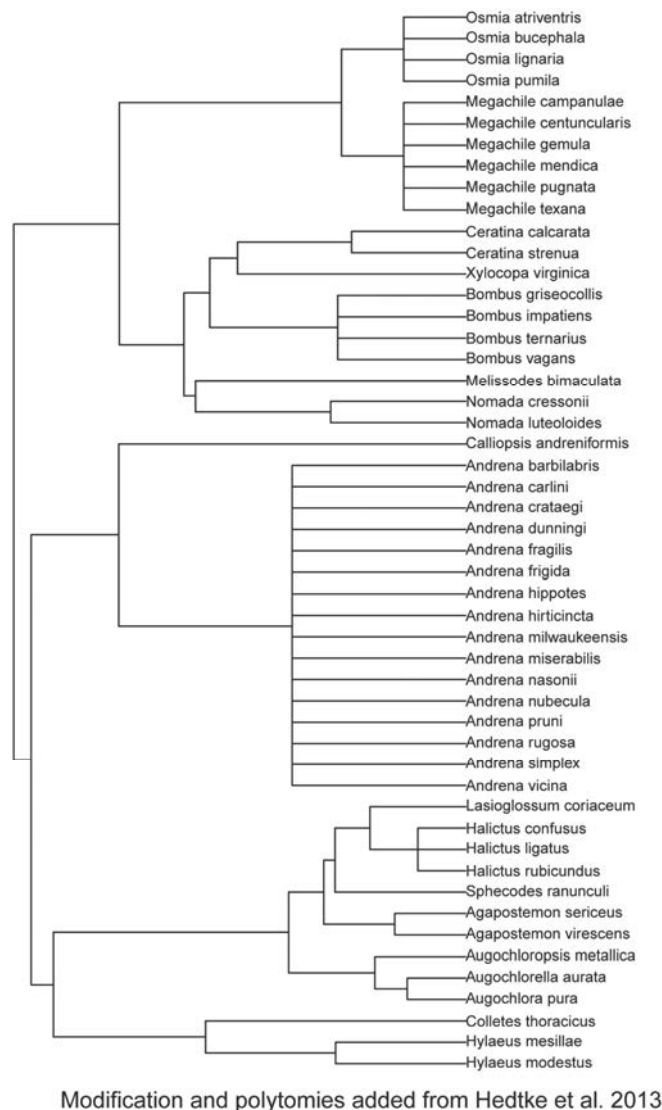
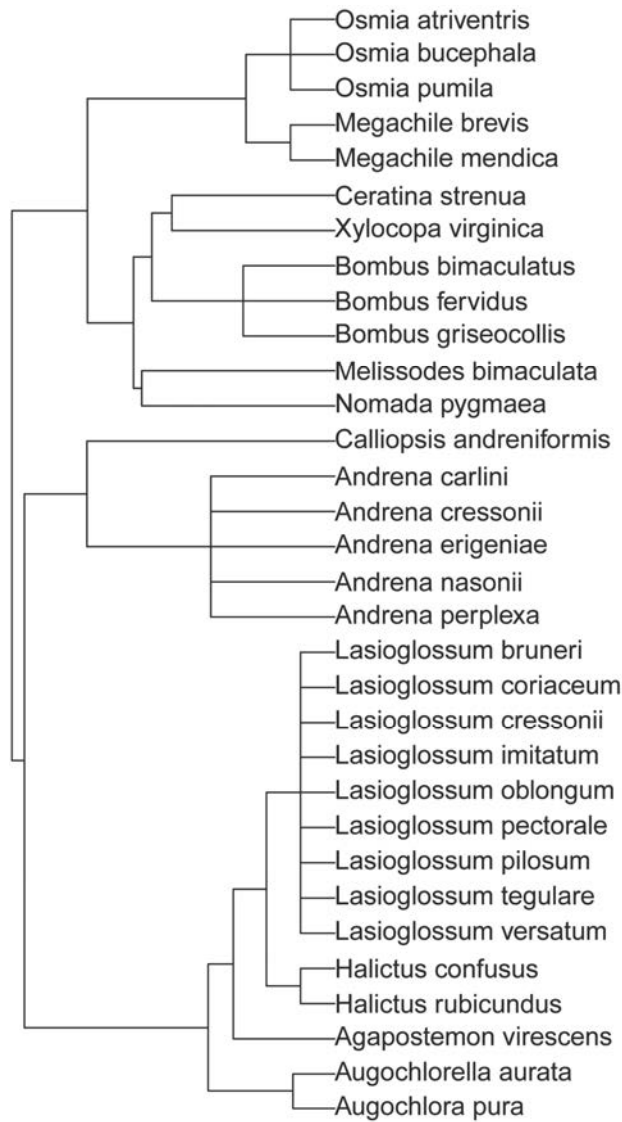


Figure S2 | Phylogenetic tree used for the Bayesian models used in order to analyze correlations between population trends and brain sizes.



Modification and polytomies added from Hedtke et al. 2013

Figure S3 | *Phylogenetic tree used for the Bayesian models used in order to analyze correlations between habitat preference and population trends.*

General conclusions and further research

In general, I found that bees can show plastic behaviours. *Osmia cornuta* bees were able to innovate by finding a previously learned rewarded sprue and lifting a cardboard lid to access the reward. It is important to test species other than model species, as model species do not cover all the spectrum of life-history traits present in related taxa. Therefore, I used species never tested before and captured directly from the field. Most bees with larger relative brains were better at learning in a closed experimental setting. Besides, I found that most bees can use, and some even prefer urban habitats. Interestingly, urban bees are characterized by having with larger relative brain sizes.

The results of this thesis suggest that at least some species are plastic enough to cope with rapid environmental changes, showing use and preference for urban habitats. Urban habitats present new challenges, which probably require bees to show innovative behaviours. In fact, I support the idea that urban bees require larger brains. However, I did not find urban bees showing positive population trends, indicating that these urban dwellers are just surviving, but not increasing its populations. With our current understanding and despite the lack of data from most pollinator species (Winfree et al., 2011), I suspect most bees will suffer strong population declines if the current habitat transformation into urban and agricultural areas continues (Nieto et al. 2014).

It is interesting that the investment in bigger brain sizes, which has a high metabolic cost, can be associated with better learning abilities. There is no scientific consensus about how brain size may improve cognitive abilities (see introduction), but the results of this thesis point out that relative bigger brains could matter for bees. However, it is important to note that the effect sizes of our results are small and there is still a lot of unexplained variability. Hence, in addition to brain size, it would be interesting to explore other factors that can be associated with learning abilities or preference for urban habitats. For example, better vision or olfactory senses can improve the chances of learning and/or finding resources in new environments. Besides, specific measurements of neuropils sizes, such as the mushroom bodies, can be more precise than the general brain size for explaining learning or behavioural plasticity (Sivinski, 1989; Zars, 2000, Durst et al., 1994). Measuring brain volume, number of neurons, or optimization of the neural network can also have an important role in explaining these variables (Chittka & Niven, 2009). Other factors such as diet breadth may also play a key role. Generalist species may be pre-adapted to learn to use new resources in urban habitats, however, it has been found recently that specialist bees tend to have bigger brain sizes (Sayol et al., 2019 on prep). Moreover, learning abilities may differ between social and solitary species due to the differences in their life requirements. Furthermore, despite there is no correlation between learning and innovation for *Osmia cor-*

nuta (Chapter one), it would be interesting to test this correlation on other species.

In this thesis, I have focused in a few key behaviours, but if I had a larger variety of behaviours, we could have a better understanding of plasticity in bees. Neophobia could condition plastic or new behaviours due to the fear of unknown new elements in the environment and can help to interpret other measured behaviours (Cohen et al., 2015). Adding a neophobia test could help to disentangle between lack of interest, lack of exploration capacities, or fear. In fact, I initially explored the possibilities of adding to the experimental tests a new object resembling a potential predator to measure neophobia. However, I decided not to add it to the experiments because it was needed to understand first innovation isolated from other stimuli. Personality tests could be also interesting to perform, as they are not well defined in insects (Kralj-Fišer & Schuett, 2014). However, defining consistent personality patterns and testing which perform better in laboratory conditions was not feasible. The only variable that I could measure in all tests of chapter one was activity, and bees do not show any consistency in their activity levels across trials. Other behaviours may show more constancy, and hence be part of the individual personality, such as a boldness. Unfortunately the experimental design did not allow to test for consistent personalities.

Hopefully, with this thesis I have put a stepping stone in the path to understand better behavioural plasticity in bees. This is an unexplored area, and as in all unexplored areas, the scarcity of data often difficults the progress. However, the joy of opening the door to test new hypothesis compensates any difficulty encountered. I provide new and interesting information about bee pollinators: ranking of habitat importance, brains and body size measurements for more than 50 species, learning abilities for species never tested before and the first test of innovation abilities on a solitary species. However, more work in studying non-model bees is needed to confirm the results of this thesis and to move forward on subsequent analysis. In conclusion, some bees may have the tools to overcome part of the global change pressures they are encountering, but others may prove unable to adapt to new environments. Unfortunately, even the ones able to occur in new environments may survive, but not thrive. ●

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